Seed Transformation System using Hygromycin-B Selection for Malaysian Chili Varieties via Agrobacterium tumefaciens

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Abstract
An improved transformation system for transgenic chilli in three Malaysian varieties was developed by combining strategies to enhance Agrobacterium tumefaciens-mediated T-DNA delivery by seeds infection with the development of a rapid, efficient selection protocol based on hygromycin-B. Seeds of chili cultivars were precultured and infected with Agrobacterium tumefaciens strain LBA 4404 carrying the pCAMBIA 1301 binary vector. This plasmid contains β-glucuronidase (GUS) as a reporter gene and hygromycin phosphotransferase (hpt) gene which confer resistance to hygromycin-B. Direct transformation approach was used and callus phase was omitted. The optimal hygromycin concentration for selection was shown to be at 15 mg/l based on its effect on germination, plantlet formation and necrosis. Seeds were cultured on Murashige and Skoog (MS) medium containing hygromycin-B for selection. Transformants were confirmed by GUS histochemical analysis and polymerase chain reaction (PCR). GUS activity was exhibited in the individual plantlet as indicated by blue color. In PCR analysis using specific primers for gus and hpt genes, DNA fragments of 789 and 591 bp in length were amplified respectively from the total DNA of young leaves of mature transgenic plants. Seeds of To plants were then grown in greenhouse, left to mature and seeds collected to produce T1 regenerants. Molecular analysis were carried out in the T1 generation to study the integration and expression stability of transformed genes. Polymerase chain reaction showed that both gus and hpt genes were present in T1 generation and expression confirmed by GUS histochemical analysis.

Keywords: Seed transformation, Agrobacterium tumefaciens, C. annuum, direct shoot regeneration.

Introduction
Chilli (Capsicum annuum) is an important crop grown worldwide for its use as spices and vegetables [1]. However, chilli is highly susceptible to fungal and viral infection which cause considerable damage to the crop [2]. Although it is a Solanaceous member, the culture development in plant cell, tissue and organ culture as well as on plant genetic transformation, have lagged far behind compared to those achieved for other members of the same family, such as tobacco, tomato and potato. These latter species have been frequently used as model systems because of their ability to regenerate in vitro, and are able to be genetically engineered by currently available transformation methods. In contrast, members of the Capsicum family have been reported to be recalcitrant to differentiation and plant regeneration under in vitro conditions. Thus genetic improvement efforts against pests and disease through recombinant DNA technology via genetic transformation is difficult [3,4]. The demand for chilli is expanding, therefore, there is widespread interest in incorporating novel genetic traits, which can often be introduced only via genetic transformation technology, in order to improve the quality and also quantity of the plants. The most well established plant transformation method relies on the intricate mechanism adapted by the soil bacterium, Agrobacterium spp., a member of the Rhizobiaceae family. The preference for Agrobacterium-mediated transformation gene delivery is not only for economic reason, but also for the fact that insertion of transgene/s into the host by Agrobacterium is a very precise event in which DNA between two defined border sequences is transferred exclusively. The use of Agrobacterium transformation gene delivery system however requires the identification of competent to be transformed as well as the development of a tissue culture system. However, generalities for chilli regeneration are difficult to formulate. The different genotypes and/or explant materials may be contributory to the divergent results reported on regeneration response [4]. Despite several recent articles describing established systems for regeneration of chilli cultivars, plant regeneration, especially elongation of buds produced by cultured explants, is seemingly a formidable challenge [5]. Feldman and Marks [6] were the first to report the success of utilizing dry seeds as material for transformation and produced transgenic plants rapidly without an intermediate callus phase. Phenotypically normal, these fertile transgenic plant contained functional transgenes which were inherited in a Mendelian fashion. Transgenic plants were also successfully generated utilizing dry seeds for transformation in Pea [7], peanut [8] and soy bean [9].

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The objective of this study was to develop cultivar and culture-independent method of transformation for chilli using dry mature seeds as starting material. The suitability of using hpt gene for *Capsicum annuum* transformation was also investigated. We conclude that hygromycin can be used as an alternative to kanamycin as an effective selectable marker in combination with *Agrobacterium* infection of mature seeds.

**Materials and Methods**

**Plant Material**

Dried mature seeds of *Capsicum annuum* var MC11 and Bangi 4 were obtained from the Malaysian Agricultural Research and Development Institute (MARDI) and Plant Biotechnology Laboratory, Universiti Kebangsaan Malaysia respectively. Seeds were surface sterilised with 1.05% (v/v) sodium hypochlorite (20% Clorox bleach) for 20 min, rinsed with sterile distilled water five times and precultured for 2 days prior to transformation.

**Bacterial strain and vector**

*Agrobacterium tumefaciens* strain LBA 4404, harboring the binary vector pCAMBIA 1301 (kindly supplied by Dr. Jefferson, CSIRO) was used. The vector has a T-DNA region containing the hpt gene, which confers resistance to hygromycin. In addition, an intron-containing B-glucuronidase (GUS) gene is also present to prevent bacterial gus gene expression, thus making it a useful tool for early detection in transformation. Bacteria were grown on solid Luria burtani (LB) medium at 28°C in the presence of appropriate antibiotics (50 mg l⁻¹ rifampicin, 300 mg l⁻¹ streptomycin and 50 mg l⁻¹ kanamycin).

**Establishing the selection conditions**

The internal resistance of *C. annuum* to hygromycin was tested by growing the seeds in Murashige and Skoog (MS) medium supplemented with different concentration (2.5, 5, 7.5, 10, 15, 20, 25, and 30 mg l⁻¹) of hygromycin B. The plants were left for one month at 25°C with 16 h photoperiod under a photon flux of 80 umol ms⁻². They were then transferred to fresh MS medium without hygromycin-B. The effect of hygromycin-B on the rate of seed germination, seedling formation and symptoms of necrosis were analysed during the period of 1 month.

**Plant transformation and regeneration**

*A. tumefaciens* LBA 4404 containing pCAMBIA 1301 were grown overnight at 28°C on a rotary shaker at 250 rpm until the OD₆₀₀ reached 0.5-0.6 in 25 ml liquid LB media supplemented with 50 mg l⁻¹ kanamycin sulfate (Sigma-Aldrich Co). The cells were collected by centrifugation at 1,000 g for 10 min at 4°C, and the bacterial pellet was resuspended in an equal volume of MS resuspension medium. Precultured seeds were immersed in the 25 ml *Agrobacterium* suspension for 30 min, blotted dry on filter paper and cultured on MS medium (solidified with 0.8% bacto agar). Co-cultivation was carried out for two days in the dark at 25°C. After co-cultivation, explants were washed with sterile water containing 500 mg l⁻¹ cefotaxime (Duchefa) in 125 ml flask and shaken at 125 rpm. The explants were then transferred to MS medium containing appropriate concentrations of hygromycin B for selection. All cultures were placed in growth chamber at 25°C with 16 h photoperiod under a photon flux of 80 umol ms⁻² provided by a cool white fluorescent tube. One month old putative transgenic plantlets which were resistant to hygromycin-B were transferred to a greenhouse. The plants were allowed to mature and produce seeds for the regeneration of T₁ plants.

**Histochemical GUS assay**

β-Glucuronidase (GUS) histochemical assay was performed on hygromycin resistant seedlings of To and T₁ generation according to Jefferson [11] with modifications. The explants were incubated at 37°C overnight in 100 µl to 300 µl in X-GlcA (Sigma-Aldrich) solution with a composition modified to 50mM Na₂HPO₄, 10 mM K₃Fe(CN)₆, 0.3% (v/v) Triton X-1, 0.5 mM K₄Fe(CN)₆, 0.5 mM sodium ascorbate (pH 7.0). In order to facilitate the penetration of the substrate, before reaction, the tissues were incubated in 90% acetone for 20 min at -20°C. After the development of the reaction, tissues were then immersed in 70% (v/v) ethanol to bleach the chlorophyll in the case of cotyledons to reduce background and then rehydrated before observation under a dissecting microscope.

**Polymerase chain reaction (PCR analysis)**

Molecular analysis on the selected To and T₁ regenerants was performed using polymerase chain reaction (PCR). Genomic DNA was extracted from young leaves of mature transgenic plants using 'Genomic PrepTM Cells and Tissues DNA isolation kit' (Amersham, United Kingdom) following the protocol provided by the manufacturer. The primer sequences created for amplification were as follows: 5'-CGCCGATGCAGATATTCGTA-3' and 5'-ATTAATTCCGGTTGCTGAC-3' for the 789 bp internal fragment of the gus gene; 5'-ACAGGCTCTCCGACCTGTAGAC-3' and 5'-AGTCAAAGCGCTGTTAATGCG-3' for the 591 bp internal fragment of the hpt gene. The final volume of PCR reaction mixture was 50 µl containing approximately 50-100 ng 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 and 10 mM dNTPs. The temperatures were programmed as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30s, 60°C for 90 s and 72°C for 2 min with a final 10 min extension at 72°C. The PCR product was then subjected to 1.2% agarose gel electrophoresis.
Analysis of T1 plants

To test the functional expression of the \textit{hpt} gene in the To progeny, a germination test was performed. At least 180 seeds were collected from To plants of the two chilli varieties (MC II and Bangi 4) were germinated on medium containing 15 mg/l hygromycin. Untransformed plants were also germinated as controls or for comparison. PCR analysis were carried out to check for the presence of transgenes in the germinated seedlings selected randomly among those that survived on hygromycin-containing medium.

Results and Discussion

Hygromycin as a selective agent for transgenic \textit{C. annuum}

The basis of the promotion of \textit{Agrobacterium}-mediated transformation for \textit{Capsicum annuum} using hygromycin-B as selectable marker has not been completely established. In previous investigation on \textit{C. annuum} transformation, NPT II which encodes for kanamycin resistance was widely used as the selection marker but the levels and the method applied were very different. Different concentration of kanamycin was added to different medium [12]. In other experiment, Lim \textit{et al.} [13] added 100 mg/l kanamycin for selecting transformants. Totally different concentrations of kanamycin was also used by Ye \textit{et al.} [4] for induction, shooting and rooting medium. Most recently, Li \textit{et al.} [15] used 50 mg/l as selection level. The difference might be due to different genotype and explants used to investigate the optimal concentration of kanamycin. In contradiction to this data, Mihalka \textit{et al.} [16] has pointed out that kanamycin resistance is not an optimal selection marker for producing transgenic \textit{C. annuum} plants, as non-transformed \textit{C. annuum} cotyledons could survive in the medium supplemented with as high as 150 mg/l kanamycin. It was then concluded that the ineffectiveness of the kanamycin as a selective agent as one of the main problems of \textit{C. annuum} transformation. Since the kanamycin tolerance of explants from different genotype and explants is apparently different, it would be necessary to search an alternative selection agent and marker that give better results in pepper transformation. In this present study, the effectiveness of hygromycin-B was evaluated as a selective agent in combination with \textit{Agrobacterium}-mediated DNA delivery method into seeds of \textit{Capsicum annuum}. Since the effective concentration of a selective agent needs to be determined for each species or explant, the sensitivity of \textit{C. annuum} to hygromycin-B was also studied. The effect of hygromycin-B on \textit{C. annuum} was carried out specifically for seed transformation method employed and can be categorized under three sections. This experiment was carried in three replicates for each section.

Effect of hygromycin-B on seed germination

For this analysis, seed germination was defined as seeds that were only able to root with no further growth taking place but survive in the medium and also complete plantlets. Seed of \textit{C. annuum} were germinated on MS medium in the presence of different concentrations of hygromycin-B. As shown in Table 1, 86% of the seeds were able to germinate in the medium supplemented with 15 mg/l hygromycin-B. In the presence of \( \geq 15 \) mg/l, only formation of roots were observed. Most of these rooting seeds did not grow into plantlets following transfer to fresh medium without hygromycin. In contrast, seed germinated in the presence of \( \leq 10 \) mg/l formed complete plantlets when transferred to fresh medium without hygromycin-B.

<table>
<thead>
<tr>
<th>[Hygromycin-B] (mg/l)</th>
<th>% seed germinated</th>
<th>% plantlet</th>
<th>% necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>86</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>5.00</td>
<td>85</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>7.50</td>
<td>84</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>10.00</td>
<td>90</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>15.00</td>
<td>70</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>20.00</td>
<td>70</td>
<td>34.5</td>
<td>70</td>
</tr>
<tr>
<td>25.00</td>
<td>62.5</td>
<td>32</td>
<td>100</td>
</tr>
<tr>
<td>30.00</td>
<td>60</td>
<td></td>
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Table 1: Percentage of \textit{C. annuum} seeds germinated, plantlets and necrosis in MS media containing different concentrations of hygromycin-B.

Effect of hygromycin-B on seedlings formation

Seedling formation ability was tested in the presence of hygromycin-B. Numerous seedlings appeared on the medium with 5 mg/l hygromycin-B. Seedlings formed on the medium supplemented with 10 mg/l or less continued to grow healthily when transferred to fresh medium without antibiotic. The growing capacity of seedlings was inhibited by hygromycin at 15 mg/l.

Effect of hygromycin-B on tissue necrosis

In our experiment, some of the roots turned brown including plantlets with retarded roots. Some of the plantlets showed necrotic meristem tissues which led to the lost of cotyledons. As shown in Table 1, no necrotic tissues were detected in the MS medium supplemented with 2.5 - 10 mg/l hygromycin. In contrast, plantlets cultured in medium containing 15 - 25 mg/l hygromycin-
B, lost of cotyledons or shoots were observed. However, no necrotic tissues symptoms were observed in these samples. In the presence of 30 mg/l hygromycin-B, all plantlets became necrotic.

The data obtained from the effects of different concentrations of hygromycin-B on seeds growth patterns suggest that 15 mg/l as antibiotic optimal concentration for selecting transformants in subsequent experiments. The hygromycin concentration used for *C. annuum* in these study is rather low compared to some other systems studied such as in rice [17], Rubus anticus [18] and Eucalyptus camaldulensi [19] where concentration of 30-50 mg/l, 50 mg/l and 40 mg/l of hygromycin-B were used respectively. This lower hygromycin-B concentration necessary for transformation in *C. annuum* suggest that the plant has lower internal resistance to hygromycin-B as compared to other species.

Transformation and selection of transgenic plants

A potential limitation of current *Capsicum annuum* transformation is the requirement for effective *in vitro* genotype-independent regeneration protocols. Minimizing the role of tissue culture in transformation procedure would therefore be advantageous under such circumstances. The present study provides an Agrobacterium tumefaciens-based transformation protocol that does not involve a callus phase for converting the transformant seeds into transgenic plants. In our protocol, based on a procedure described by Feldman and Marks [6], *C. annum* plants obtained from seeds subjected to *Agrobacterium* infection and allowed to germinate and grow into plant *ex vitro* were found to express the transgenes. Chee et al. [9] using soy bean, Feldman and Marks (6) using Arabidopsis and Rohini and Rao [8] using peanuts have earlier demonstrated in planta methods of similar nature. The growth of the transgenic lines in the presence of 15 ug/ml hygromycin-B following infection by *Agrobacterium* was delayed for 1-2 weeks compared to those in the medium without hygromycin-B (Figure 1). From our experience, the transgenic seedlings

![Figure 1: Germination of *C. annuum* seedlings in 15 mg/l hygromycin-B. a). Transgenic seedlings b). Non-transgenic seedlings](image)

however, need to be transferred into the medium without hygromycin after 3 weeks to avoid possible death due to high toxicity of the antibiotic. The percentage of hygromycin-resistant seedlings was between 33-36% and the transformation efficiency based on transformant lines with positive GUS activity was estimated at 30-33% (Table 2). Although the antibiotic resistance of transformed *C. annuum* was indicative of gene transfer, GUS expression was also measured to further confirm the T-DNA transfer.

Table 2: Percentage of transformation efficiency of MC II and Cili Bangi 4 varieties from *Agrobacterium*-infected seeds in the MS media containing 15 mg/l hygromycin-B.

<table>
<thead>
<tr>
<th>Variety</th>
<th># of seeds germinated (%)</th>
<th># of transformed lines (%)</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC II</td>
<td>36±12.1</td>
<td>79±20.1</td>
<td>30±15.2</td>
</tr>
<tr>
<td>Cili Bangi 4</td>
<td>33±9.8</td>
<td>78±21.6</td>
<td>33±13.4</td>
</tr>
</tbody>
</table>

Transformation efficiency is calculated as the number of GUS-positive lines divided by the number of seeds infected. Data represent the means values ± standard error (SE) of three independent experiments.

GUS expression and PCR analysis

The assessment of transformation by this method was based largely on uid A gene expression. Since the uid A gene construct used in these experiment precludes GUS expression in the bacteria, this feature was taken as a direct measure of transformation. Plant were considered to be GUS-positive only when a blue stain was present almost all over the tissue. The susceptibility response of the seeds of *Capsicum annum* to *Agrobacterium* infection was determine initially by scoring the GUS activity, resulting in blue colour development throughout the hygromycin-resistant seedlings (Figure 2a). The advantage of eliminating the background GUS activity resulting from bacterial presence in the tissue was conferred by the binary vector pCAMBIA 1301. The *Agrobacterium* LBA 4404 was found to be the most effective strain on *C. annum*. GUS activity was also expressed in T, plantlet suggesting that the integration of the GUS gene was stable (Figure 2d). None of the control plants expressed GUS (Figure 2b).

In addition to the ability to grow on the medium containing hygromycin-B and to express the transferred gus gene, PCR analysis was used to confirm at the DNA
level the presence of foreign genes in the genome of the
*C. annuum* plants. The presence of *gus* and *hpt* genes in
the plant genome was detected by PCR analysis of total
DNA extracted from young leaves of GUS positive To
plants. The absence of *vir A* gene in these putative
transgenic plants (PCR data not shown) suggest that
bacterial contamination was not present in the putative
transformants and that *gus* and *hpt* genes detected
originated from the plant genome. PCR analysis using
primer sets for the *gus* and *hpt* genes showed amplification
of the predicted 789 and 591 bp internal fragment of both
genes respectively (Figure 3a and 3b). Both gene
fragments are present in the pCAMBIA plasmid but not
detected in the uninfected plants.

The inheritance of the introduced genes in the *T*₁
generation of the two varieties was studied using a
germination test. None of the seeds from non-transformed
plants germinated on the medium containing 15 mg/l
hygromycin. For the transformed lines tested, the ratio of
resistance to sensitive was 3:1, the expected ratio for a

Table 3: Segregation analysis in progeny of the primary transformants (To) of MC II and CiliBangi 4 chili varieties as assessed by the hygromycin sensitivity test

<table>
<thead>
<tr>
<th>Variety</th>
<th>Number of seeds tested</th>
<th>Number of seeds germinated</th>
<th>$\chi^2$- value</th>
<th>GUS + (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hyg (+) Hyg (-)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCII</td>
<td>200</td>
<td>148 52</td>
<td>0.11 80</td>
<td></td>
</tr>
<tr>
<td>CiliBangi 4</td>
<td>180</td>
<td>138 40</td>
<td>0.27 75</td>
<td></td>
</tr>
</tbody>
</table>

| a | Hygromycin concentration of 15 mgL⁻¹ was used in the medium
| b | All $\chi^2$- values indicate significant fit to 3:1 ratio tested at $P = 0.05$ level
single dominant gene in a self-pollinated population (Table 3). Progeny seeds of To plants were germinated in the greenhouse and genomic DNA was prepared from young leaves of the T1 plants. PCR amplification produced the expected 789 and 591 bp fragments product of both gus and hpt genes (Figure 3a and 3b). These PCR results also indicated that both the linked marker gus and hpt genes were present in both To and T1 plants thus suggesting stable inheritance of the genes between generations. GUS histochemical analysis carried out on some of the T1 generation seedlings of the three varieties that survived on hygromycin-B containing medium showed that not all of the T1 hygromycin-B resistance lines were GUS positive (Table 3). It showed that MC II gives higher percentage (80%) of T1 lines that responded to GUS histochemical analysis.

Conclusions

Genetic transformation of Capsicum annuum through seed transformation using Agrobacterium tumefaciens was accomplished in this current study. The optimal concentration of hygromycin-B used for transgenic C. annuum selection was defined by looking at different effects of hygromycin-B on seed germination, plantlet formation and necrotic events. In our previous experiment, it was rather difficult to determine the rate of transformation due to the presence of some ‘escape’ plants when a suboptimal concentration of hygromycin-B was used for selection. Using 15 µg/ml of hygromycin-B for selection, the transformants lines which tested positive for GUS expression were further confirmed by PCR of both the marker linked gus and hpt genes. It is obvious that hygromycin is a better option for selection in C. annuum transformation as compared to kanamycin which requires different concentrations for different C. annuum genotype tested. Li et al. [15] reported 40.8% transgenic pepper production using kanamycin as selection agent at 50 mg/l.

Data from the present study indicate that hygromycin may also be used in genetic transformation of Capsicum annuum, thereby providing an alternative or additional selectable marker. In additions, while Li and his co-researchers showed the presence of internal gus gene in the hygromycin-resistant plantlets. Further work is necessary to optimize the transformation and regeneration protocol in some other cultivars. The result presented here could allow potential genetic modification of this important crop and facilitate the future goal of producing transgenic C. annuum with specific agronomical traits.

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