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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_1 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 foreigns 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 (Wordagon and Dons, 1992; Fisk and Dandekar, 1993; Ishida *et al*., 1996; Hiei *et al*., 1994).

A. tumefaciens-mediated transformation in many recalcitrant 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

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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*: Chowrira *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 has reported that the transgenes were transmitted to the progeny in a mendelian fashion eventhough 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 CL_2 (10 min) and commercial sodium hypochloride (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 $m^{-2}s^{-1}$. 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. Jefferson) was used for transformation. The pCAMBIA 1301 plasmid contained a *gusA* reporter gene and hygromycin phosphotranferase (*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 OD_{600} 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 $ms^{-2}s^{-1}$ 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 T_1 progenies. After 2 days of co-cultivation (after treating with *A. tumefaciens* suspension ; OD_{600}= 0.55) the explants were transferred to MS medium containing hygromicin 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 T_1 progeny.

Segregation analysis of T_1 progenies: To test the functional expression of the *hpt* gene in the T_1 progenies, a germination test was performed. At least 180 seeds collected from To 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.

ß-Glucuronidase (GUS) histochemical analysis): Histochemical assay of GUS was performed on hygromycin resistant seedlings of To and T_1 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 Na_2HPO_4, 10 mM K_4 Fe (CN), 03% (v/v) Triton X-1, 0.5 mM $K_3Fe(CN)6$, 0.5 mM sodium ascorbate (pH 7.0). In order to facilitate the penetration of the substract, 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 To and T_1 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' – CGCCGATGCAGATATTCGGTA -3' and 5'-ATTAATGCGTGGTCGTGCAC3' for the 789 bp fragment of the *gus* gene; 5'- ACAGCGTCTCCGACCTGATGCA3' and 5' – AGTCAATGACCGCTGTTATGCG -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 $MgCl_2$, 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 90 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 scapel. 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.

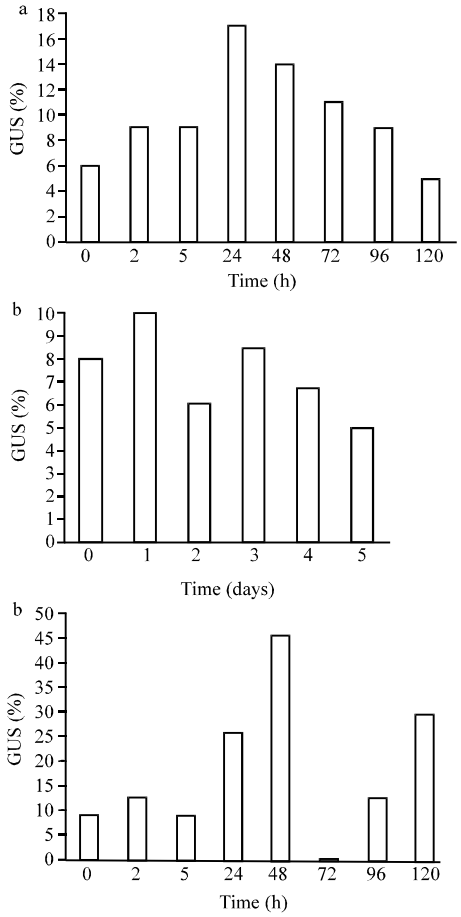

Fig. 1: Percentage of explants with GUS expression following different precultured treatments; a) Incubation on MS liquid media at 25°C and gently shake at 120 rpm, b) Incubation on wet filter paper and kept at 4°C, c) Incubation on wet filter paper and kept in the culture room at 25°C under a 16 h day photoperiod

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

Seeds treatments	Number of seeds for germination(3 replicates)	Percentage of seeds germinated
1	300	40.3±12.5
2	300	77.0±16.7
3	300	96.3±8.9

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 I, seeds were cultured in MS media at 25°C and shake at 120 rpm for 0-7 days. Based on Sunilkumar

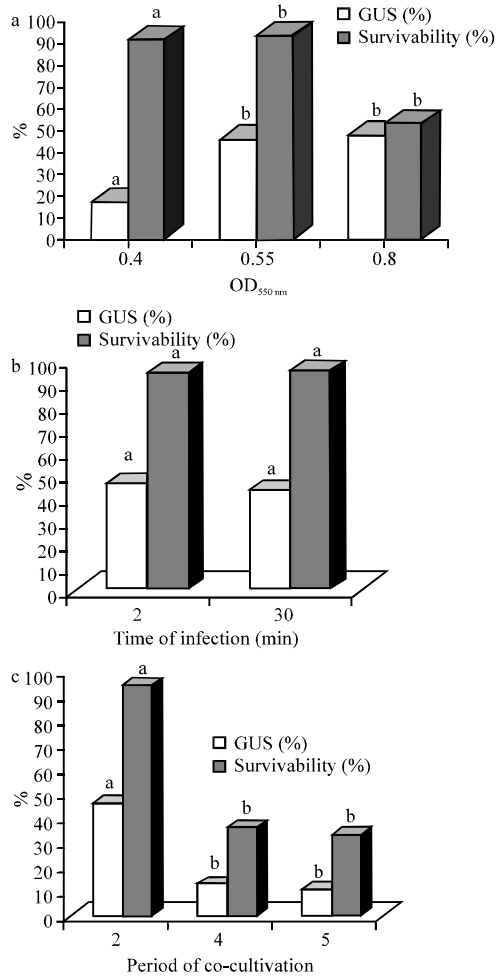

Fig. 2: The effect of a) *Agrobacterium* concentration, b) infection time and c) co-cultivation period on survival rate and gus expression of chili. Data represents the average number of explants expressing GUS on three independent experiments. Means bearing the same letter are not significantly different (p = 0.05)

(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

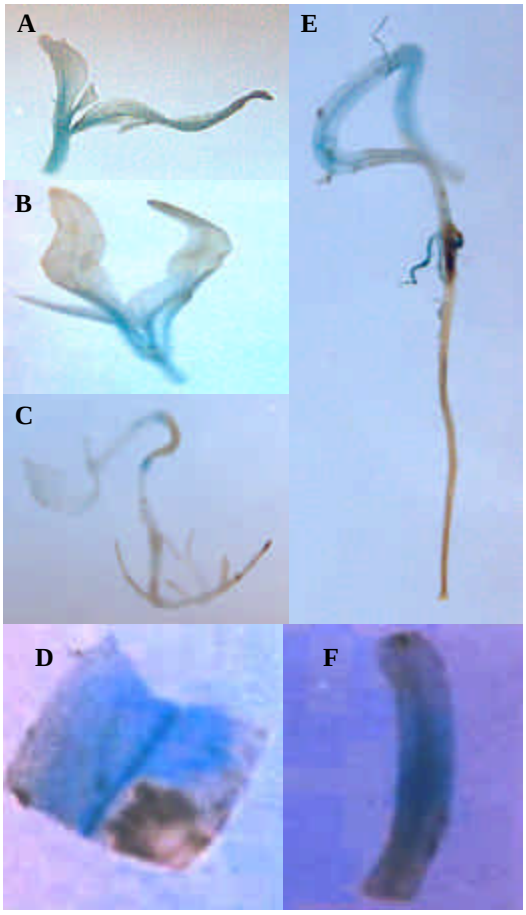

Fig. 3: Transgenic seedlings of *C. annum* plants showing gus expression in different part of explants: A, D and F: T_0 plants; B, c and E: T_1 plants

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 (OD_{600}= 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 OD_{600} = 0.55 was the most suitable for infection of seeds with an average of 45% explants expressing the GUS activity. Eventhough higher number of explants (48%) with GUS activity was obtained with *Agrobacterium* concentration at OD_{600}=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 eventhough 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).

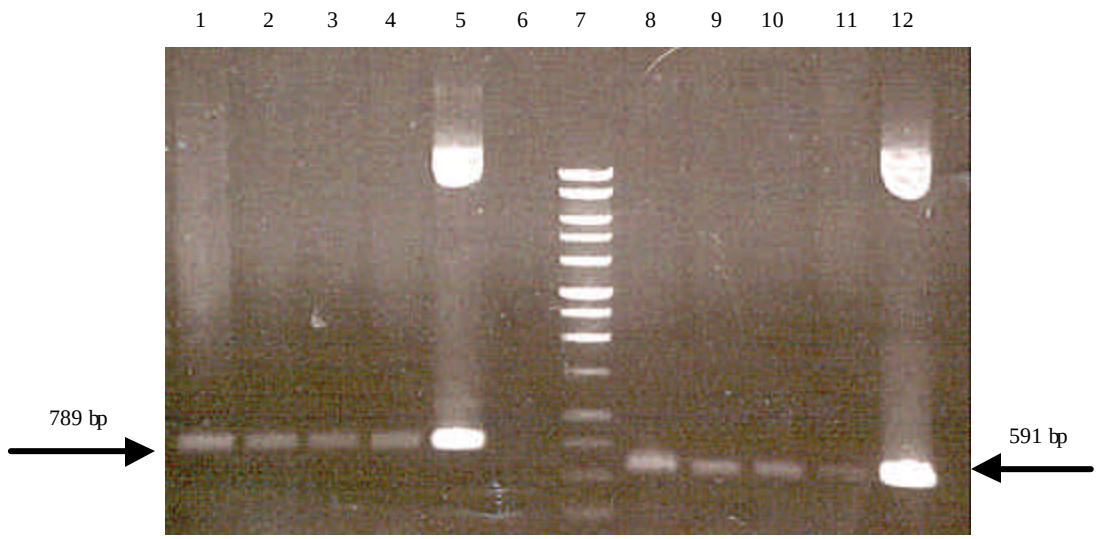

Fig. 4: Identification of transgenic plants based on PCR detection fragments of hus (789 bp) and *hpt* (591 bp) in T_0 and T_1 plants by PCR. Lane 1-4: PCR products using primers for gus and template DNA from T_0 plants (Lane 1-2) and T_1 plants (Lane 3-4); Lane 5: pCAMBIA 1301 DNA; Lane 6: Blank; Lane 7: Molecular marker (1 kb ladder); Lane 8: PCR products using primers for *hpt* and template from T_0 plants (Lane 8-9) and T_1 plants (Lane 10-11); Lane 12: pCAMBIA 1301 DNA

Although numerical differences were observed between the two infection times (2 and 30 min) in which the shorter time has a higher survival rate and higher number of explant with GUS activity, these were not found to be significantly different (Fig. 2b). It may be that additional experiments are needed to establish if there are significant differences due to the infection time period since there was such a high variability in survival rate and GUS-positive explants in the present experiment. However, it may be simply be that the infection time period, in the range tested, do not have a significant effect on the survival of the explants and GUS activity. A comparison of the co-culture times showed that the shorter the co-culture period, the higher the survival rate of the explants. Of the surviving explants, those that were co-cultured for 2 days exhibited the highest percentage of explants with GUS expression. The differences were not statistically significant between the 4- and 5- day co-culture periods. The longest co-culture period (5 days) significantly reduced the number of surviving explants and these subsequently produced lowest percentage of GUS activity (Fig. 2c). Thus, the longest co-culture time with *A. tumefaciens* appears to be detrimental to the survival and subsequent GUS expression of the explants.

Regeneration of To Transgenic Chili and its T_1 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 T_1 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 T_1 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

To Lines	No. of T1 seeds germinated	*Hpt*$^+$	*Hpt*	Ratio	$\div^2$	Number of Explants with GUS positive
1	200	148	52	3:1	0.11	16/20
2	200	154	46	3:1	0.43	17/20
3	200	189	11	15:1	0.19	17/20
4	180	138	40	3:1	0.27	15/20
5	189	135	54	3:1	1.28	13/20
6	175	136	39	3:1	0.69	17/20
7	200	158	42	3:1	1.71	18/20
8	190	169	21	15:1	7.47	15/20
9	200	160	40	3:1	2.67	17/20
10	185	142	43	3:1	0.31	12/20
11	190	62	128	-	-	3/20
12	180	0	180	-	-	0/20
13	200	0	160	-	-	0/20
14	200	38	162	-	-	3/20
15	150	17	133	-	-	0/20
Control K2a	200	34	186	-	-	0/20

Indicator: --→ Data is not applicable for Mendelian ratio
[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 *hyg* genes in T_1 population

To Lines	No. of progeny analysed	*Gus*$^+$*Hpt*$^+$	*Hpt*$^+$*Gus*$^-$	*Hpt*$^-$*Gus*$^+$
1	10	10		
2	10	9	1	
3	10	10		
4	10	9	1	
5	10	10		
6	10	10		
7	10	10		
8	10	8	1	1
9	10	10		
10	10	9	1	
Control K2a	5	-	-	-

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 T_1 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 parental 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 T_1 progenies obtained after self pollination of randomly selected 15 T_o plants were studied for inheritance of hygromycin resistance. 10 of the 15 T_o 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 T_o plants(T_3 and T_8) have their progenies with 15:1 ratios. Progenies from 2 independent transgenic plant (T_{12} and T_{13}), however, were all hygromycin sensitive. From all the lines that showed mendelian segregation, 65% of their resistance T_1 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 T_1 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 T_1 plants thus suggesting stabily inheritance of the genes between generation (Fig. 4). In another experiment, 10 hygromycin resistance T_1 plantlets were selected at random from each of To plants for PCR analysis to determine the pattern of transgenes distribution. Most of all the To lines which followed the mendelian segregation (T_1 to T_{10}) have shown the presence of both genes in their progenies (Table 3). About 5 progenies of 4 T_o plants (T_2, T_4, T_8 and T_{10}) 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 Simmond, 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 eventhough 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 consistence 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 consistence with the optimum time required for T-DNA transfer and tumor formation in *Agrobacterium* infection which actually lies between 24 to 48 h (Binns, 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-supression and phenolic compound (Serres *et al*., 1997). In this study such a variation was observed in both To 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 (Serres *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 (Schnurr and Guerra, 2000).

We have shown the transformation procedure yields reproducible result eventhough 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 T_1 families while 2 lines showed the segregation at 2 locus in which the 15:1 ratio was observed. Probably because selection was performed on hygromycin-containing medium, the internal *hpt* sequence was detected in all 15 To plants and expression was confirmed for majority of the plants. From PCR anaylsis, most of the To 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.

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REFERENCES

Alejo, N.O. and R.R. Malagon, 2001. Invited review: *In vitro* chilli pepper biotechnology. *In vitro* Cell Dev. Biol., 37: 701-729.

Bean, S.J., P.S. Gooding, P.M. Mullineaux and D.R. Davies, 1997. A simple system for pea transformation. Plant Cell Rep., 16: 513-519.

Binns, 2002. T-DNA of *Agrobacterium tumefaciens*: 25 years and conting. Trends in Plant Sci., 7: 5.

Birch, R.G., 1997. Transformation: Problems and strategies for practical application. Annu. Rev. Plant Physiol. Plant Mol. Biol., 48: 297-326.

Chee, P.P., K.A. Fober and J.L. Slightom, 1989. Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefaciens*. Plant Physiol., 91: 1212-1218.

Chee, P.P. and J.L. Slightom, 1995. Tranformation of soybean (*Glycine max*) via *Agrobacterium tumefaciens* and analysis of transformed plants. Methods in Mol. Biol., 44: 101-119.

Chowrira, G.M., V. Akella and P.F. Lurquin, 1995. Electroporation mediated transformation gene transfer into intact nodal meristem in planta. Mol. Biotechnol., 3: 17-23.

Chowrira, G.M., V. Akella, P.E. Fuerst and P.F. Lurquin, 1996. Transgenic grain legumes obtained by in planta electroporation mediated gene transfer. Mol. Biotechnol., 5: 85-96.

Christopher, T. and M.V. Rajam, 1997. *In vitro* plant regeneration and *Agrobacterium*-mediated transformation in red pepper. *In vitro*, 33: 73A.

Cote, C. and R.G. Rutledge, 2003. An improved MUG fluorescent assay for the determination of GUS activity within transgenic tissue of woody plants. Plant Cell Rep., 21: 619-624.

Donaldson, P.A. and D.H. Simmonds, 2000. Susceptibility to *Agrobacterium tumefaciens* and cotyledons node transformation in short-season soybean. Plant Cell Rep., 19: 478-484.

Dong, C., Ch. Jiang, L. Feng and J. Guo, 1992. Transgenic pepper plants (*Capsicum annuum* L.) containing CMV sat-RNA cDNA. Acta Hortic. Sin. (in Chinese), 19: 184-186.

Ezura, H., S. Nishimiya and M. Kasumi, 1993. Efficient regeneration of plants independent of exogenous growth regulators in bell pepper (*Capsicum annuum* L.) Plant Cell Rep., 12: 676-680.

Feldmann, K.A. and M.D. Marks, 1987. *Agrobacterium*-mediated transformation of germinating seeds of Arabidopsis thaliana: a non-tissue culture approach. Mol. Gen. Genet., 208: 1-9.

Fish, H.J. and A.M. Dandekar, 1993. The introduction and expression of transgenes in plant. Sci. Hortic., 55: 5-36.

Graves, H. and M. Goldman, 1986. Transformation of *Zea mays* seedlings with *Agrobacterium tumefaciens*: Detection of T-1 specific enzymes activities. Plant Mol. Biol., 47: 43-50.

Hiei, Y., S. Ohta, T. Komari and T. Kumashiro, 1994. Efficient transformation mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J., 6: 271-282.

Hoekema, A., P.R. Hirsch, P.J. Hooykas and R.A. Schilperoort, 1983. A binary vector strategy based on separation of *vir* and T-region of the *A. tumefaciens* Ti plasmid. Nature, 303: 179-180.

Horsch, R.B., J.E. Fry, N.L. Hoffman, D. Eicholtz, S.G. Rogers and R.T. Fraley, 1985. A simple and general method for transferring genes into plants. Science, 227: 1229-1231.

Ishida, Y., H. Saito, S. Ohta, Y. Hiei, T. Komari and T. Kumashiro, 1996. High efficiency transformation of maize (Zea mays L.) mediated by *Agrobacterium tumefaciens*. Natl. Biotechnol., 14: 745-750.

Ismail, I., Z. Zainal, S.H. Zainal Ariffin, S.K. Tzu Hui and A.S. Sued, 2005. Seed transformation system using hygromycin-B selection for Malaysian Chili varieties. Mal. J. Biochem. Mol. Biol., 12: 1-7.

Jayashankar, S., S. Bagga and G.C. Phillips, 1997. Sweet pepper (*Capsicum annuum*) transformation using *Agrobacterium rhizogenes*. Hortscience, 32: 454.

Jefferson, R.A., T.A. Kavanagh and M.W. Bevan, 1987. GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., 6: 3901-3907.

Kim, S.J., S.J. Lee, B.D. Kim and K.H. Paek, 1997. Satellite-RNA-mediated resistance to Cucumber mosaic virus in transgenic plants of hot pepper. Plant Cell Rep., 16: 825-830.

Kohl, A., D. Gahakwa, P. Vain, D.A. Laurie and P. Cristou, 1999. Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. Planta, 208: 88-97.

Li, D., K. Zhao, B. Xie, B. Zhang and K. Luo, 2003. Establishment of a highly efficient transformation system for pepper (*Capsicum annuum* L.). Plant Cell Rep., 21: 785-788.

Liu, W., W.A. Parrot, D.F. Hildebrand, G.B. Collins and E.G. William, 1990. *Agrobacterium* induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot-like structures expressing introduced genes. Plant Cell Rep., 9: 360-364.

Manoharan, M., C.S.S. Sree Vidya. and G.L. Sita, 1998. *Agrobacterium*-mediated genetic transformation in hot chili (*Capsicum annuum* L. var. Pusa jwala). Plant Sci., 131: 77-83.

McKently, A.H., G.A. Moore, H. Doostdar and P.R. Niedz, 1995. *Agrobacterium* mediated transformation of peanut (*Arachis hypogaea* L.) embryo axes and the development of transgenic plants. Plant Cell Rep., 14: 699-703.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant, 15: 473-497.

Oger, P., A. Petit and Y. Dessaux, 1996. A simple techniques for direct transformation and regeneration of the diploid legume species *Lotus japonicus*. Plant Sci., 116: 159-168.

Polowick, E.A., H.L. Lenny and H.D. Franklin, 2000. The ability of pea transformation technology to transfer genes into peas adapted to Western Canadian growth conditions. Plant Cell, 153: 161-170.

Popelka, J.C. and Altpeter, 2003. *Agrobacterium tumefaciens*-mediated genetic transformation of rye (*Secale cereale* L.). Mol. Breed., 11: 203-211.

Potrykus, I., 1991. Gene transfer to plants: Assessment of published approaches and results. Annu. Rev. Plant Physiol. Plant Mol. Biol., 42: 205-254.

Rohini, V.K. and K.S. Rao, 2000. Transformation of peanut (*Arachis hypogaea* L): A non-tissue cultured based approach for generating transgenic plants. Plant Sci., 150: 41-49.

Salas, M.G., S.H. Park, M. Srivatanakul and R.H. Smith, 2001. Temperature influence on stable T-DNA integration in plant cells. Plant Cell Rep., 20: 701-705.

Satyavathi, V.V., V. Prasad, B.G. Lakshmi and G.L. Sita, 2002. High efficiency transformation protocol for three Indian Cotton varieties via *Agrobacterium tumefaciens*. Plant Sci., 162: 215-223.

Schnurr, J.A. and D.J. Guerra, 2000. The CaMV 35S promoter is sensitive to shorterned photoperiod in transgenic tobacco. Plant Cell Rep., 19: 279-282.

Serres, R., B. McCown and E. Zeldin, 1997. Detectable β-glucuronidase activity in transgenic cranberry is affected by endogenous inhibitors and plant development. Plant Cell Rep., 16: 641-646.

Subhash, K. and T. Christopher, 1997. Organogenesis and transformation in *Capsicum baccatum*. *In vitro*, 33: 57A.

Sunilkumar, G., K. Vijayachandra and K. Veluthambi, 1999. Preincubation of cut tobacco leaf explants promotes *Agrobacterium*-mediated transformation by increasing *vir* gene induction. Plant Sci., 141: 51-58.

Trifonova, A., M. Sten and A. Olesen, 2001. *Agrobacterium*-mediated transgenes delivery and integration into barley under a range of *in vitro* culture conditions. Plant Sci., 161: 871-880.

Tsukazaki, H., Y. Kuginuki, R. Aida and T. Suzuki, 2002. *Agrobacterium*-mediated transformation ofa double haploid line cabbage. Plant Cell Rep., 21: 257-262.

Wang, Y., M. Yang, N. Pan and Z.H. Chen, 1991. Plant regeneration and transformation of sweet pepper (*Capsicum frustescens*) (in Chinese). Acta Bot. Sin, 33: 780-786.

Weber, S., W. Friedt, N. Landes, J. Molinier, C. Himber, P. Rousselin, G. Hahne and R. Shorn, 2003. Improved *Agrobacterium*-mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzyme and sonication. Plant Cell Rep., 21: 475-482.

Wordagon, M.F. and H.J.M. Dons, 1992. *Agrobacterium tumefaciens* mediated transformation of recalcitrant crops. Plant Mol. Biol. Rep., 10: 12-36.

Ye, Z., H. Li, J. Zhang and Y. Jing, 1993. Genetic transformation and plant regeneration in pepper (in Chinese). Acta Bot. Sin, 35 [supplement]: 88-93.

Zhou, Zh., M. Su., D. Chen, R. Liu, L. Song and J. Yang, 1991. Preliminary study on regeneration induction and gene transformation of CMVcp in pepper (in Chinese). Acta Agri. Boreali-Sin., 6: 69-72.

Zhu, Y.X., W.J. Ou-Yang, Y.F. Zhang and Z.L. Chen, 1996. Transgenic sweet pepper plants from *Agrobacterium* mediated transformation. Plant Cell Rep., 16: 71-75.