Influence of different co-cultivation temperatures, periods and media on *Agrobacterium tumefaciens*-mediated gene transfer

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Abstract

Tobacco leaf disc explants were inoculated with *Agrobacterum tumefaciens* strain GV2260 carrying p35S GUS-INT to determine the influence of different co-cultivation temperatures (18 - 26 °C), periods (24 - 96 h) and media (solid and liquid) on transformation efficiency. Kanamycin-resistant shoots developed on leaf discs inoculated with *Agrobacterium* after 4 weeks of culture initiation. Regenerated shoots were excised and rooted in the basal medium supplemented with 100 mg dm^{-3} kanamycin and 250 mg dm^{-3} augmentin. The rooted plantlets were finally transferred to compost and confirmed by GUS assay and PCR analysis. The highest transformation frequency was achieved from the explants co-cultivated with *A. tumefaciens* in liquid medium for 48 h at 22 or 24 °C.

Additional key words: GUS activity, leaf disc, PCR, shoot regeneration, tobacco, transformation.

Introduction

Plant transformation mediated by the soil plant pathogen *Agrobacterium tumefaciens* has become the most used method. Many factors such as plant genotype, explants type, pH, co-cultivation media, temperature and period influence the gene transfer efficiency from *Agrobacterium* to plant cells. Dillen *et al.* (1997) reported that co-cultivation temperature plays a critical role in T-DNA transfer mechanism. The 30 kb virulence (*vir*) region of *A. tumefaciens* contains six main operons (*vir*A, *vir*B, *v*irC, *vir*D, *vir*G and *vir*E) that are essential for the T-DNA transfer to plant cells (Hooykaas and Schilperoort 1992, Zupan and Zambryski 1995, Jeon *et al.* 1998). The activation of *vir* genes depends on external factors such as temperature. Alt-Mörbe *et al.* (1989) reported that high temperatures inactivated *vir*D operon and expression of *vir*G gene required temperatures below 28 °C. *Vir* genes are not expressed at temperatures greater than 32 °C because of a conformational change in the folding of *vir*A that induce the inactivation of its properties (Jin *et al.* 1993).

Effects of co-cultivation period and media on T-DNA transfer have also been studied previously. Transient GUS gene expression in soybean cotyledons and garlic callus tissue was affected by both temperature and length of co-culture period (Santarem *et al.* 1998, Kondo *et al.* 2000). Solid co-cultivation media has been used routinely in most reported protocols (Dong and McHughen 1991, Holford *et al.* 1992, Muthukumar *et al.* 1996, Cervera *et al.* 1998); whereas, liquid co-cultivation medium has also been preferred in some studies (Herman *et al.* 1989, Paula *et al.* 1993). These studies suggested that co-cultivation media may also influence the T-DNA transfer. In the present work, we investigated the effect of different co-cultivation temperatures, periods and media on gene transfer efficiency from *A. tumefaciens* to tobacco leaf disc explants.

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Abbreviations: BAP - 6-benzylaminopurine; GUS - β-glucuronidase; MS - Murashige and Skoog medium; NAA - α-naphthalene-acetic acid; PCR - polymerase chain reaction.
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Materials and methods

Plant and *Agrobacterium* strain: Seeds of tobacco (*Nicotiana tabacum* L.) cv. Samsun were surface-sterilized in 30 % commercial bleach (*Axion*) for 20 min, followed by 3 washes in sterile distilled water and germinated in Petri dishes containing 35 cm^3 of basal medium. The basal medium consisted of Murashige and Skoog (1962; MS) salts and vitamins, 3 % sucrose and 0.8 % agar. The medium pH was adjusted to 5.7 with 1 M NaOH or 1 M HCl prior to autoclaving at 121 ºC, 1.4 kg cm^{-2} for 20 min. After 14 d of culture seedlings were transferred to jars containing basal medium and subcultured onto fresh medium every 5 weeks using single nodes. All cultures were maintained under 16-h photoperiod with irradiance of 35 µmol m^{-2} s^{-1} and temperature of 24 ± 2 °C. Leaf discs explants obtained from *in vitro* growing plantlets were used in transformation studies.

Agrobacterium tumefaciens strain GV2260 (Deblaere *et al.* 1985) harboring plasmid p35S GUS-INT (Vancanneyt *et al.* 1990) was kindly provided by Prof. Lothar Willmitzer (Institut für Genbiologische Forschung, Berlin, Germany). The binary plasmid p35S GUS-INT contains neomycin phosphotransferase II (*npt-II*) gene driven by nopaline synthase (NOS) promoter and β-glucuronidase (GUS) gene controlled by cauliflower mosaic virus (CaMV35S) promoter. GV2260 strain carrying p35 GUS-INT strain was grown overnight in a liquid NB medium containing 50 mg dm^{-3} kanamycin and 100 mg dm^{-3} rifampicin at 28 °C in a rotary shaker and used for transformation of tobacco leaf discs.

Plant transformation: Tobacco leaves were cut into 0.5 - 1 cm squares and inoculated with an overnight culture of bacteria diluted to 1×10^8 for 30 min in a liquid regeneration medium consisting of basal medium, 1 mg dm^{-3} 6-benzylaminopurine (BA) and 0.1 dm^{-3} α-naphthaleneacetic acid (NAA). Thereafter, explants were co-cultivated using different temperatures, periods and media. In the first experiment, leaf disc explants were co-cultivated with GV2260 p35S GUS-INT strain for 2 d at 18, 20, 22, 24 and 26 °C on solid regeneration medium in a growth chamber. In the second experiment, they were co-cultivated with the bacteria on solid regeneration medium for 24, 48 and 72 h at 24 °C. In the last experiment, liquid and solid media were used for co-cultivation of leaf discs for 48 h at 24 °C. Co-cultivation in liquid medium was carried out in a thermal shaker at 120 rpm. Leaf discs without bacterial inoculation were used as control in all experiments. Following co-cultivation, leaf discs were rinsed in liquid regeneration medium supplemented with 1 000 mg dm^{-3} augmentin for 10 min to prevent bacterial over-growth and transferred to solid regeneration medium supplemented with 75 mg dm^{-3} kanamycin and 500 mg dm^{-3} augmentin. After 4 weeks of inoculation, shoots developed on kanamycin resistance callus were rooted on basal medium containing 100 mg dm^{-3} kanamycin and 250 mg dm^{-3} augmentin. Rooted plantlets were acclimatized to ambient conditions and later established under greenhouse conditions.

Histochemical assay for GUS activity: Histochemical GUS assay was carried out as reported previously (Jefferson 1987, Özcan *et al.* 1993). Leaves from each putative transgenic plant were incubated at 37 °C for 4 h to overnight in histochemical reagent consisting of 1 mM 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc), 10 mM EDTA, 0.1 % Triton X-100 and 100 mM sodium phosphate pH 7.0. The samples were then fixed in 70 % ethanol. A sample was described as GUS positive if there was at least one discrete dark-blue region on the tissue.

Analysis of transgenic plants by PCR: Plant genomic DNA isolation and PCR amplification were performed as described by Özcan (1997). Genomic DNA was analyzed by polymerase chain reaction (PCR) with oligonucleotide primers for the *npt-II* gene, resulting in fragment of 1.0 kb. Primer sequences were 5'-ACA AGA TGG ATT GCA CGC AGG-3' and 5'- AAC TCG TCA AGA AGG CGA TAG-3'. The PCR parameters of 31 cycles were as follows: 95 °C for 1 min to denature the DNA, 60 °C for 1 min to hybridize primers and 72 °C for 1 min for polymerase reaction. The samples were left at 72 °C for 5 min at the last cycle. The samples were then run in a 1.5 % (m/v) agarose gel at 80 V to observe amplified DNA.

Observations and statistical analysis: The number of shoots per explant was scored after 4 weeks of culture. Each treatment had four replicates consisting of Petri dishes each containing 10 explants on regeneration medium. All experiments were repeated twice. Significance was determined by analysis of variance (*ANOVA*) and the differences between the means were compared by Duncan's multiple range test using *MSTAT-C* computer program (Michigan State University).

Results and discussion

Kanamycin-resistant callus clusters developed on leaf discs inoculated with GV2260 p35S GUS-INT after 10 - 12 d of co-cultivation in all experiments. At later stages of transformation, shoot initials were visible on these callus clusters as reported earlier (Özcan *et al.* 1993, Firek *et al.* 1993). However, no kanamycin-resistant calli or shoots were observed on uninoculated control explants. After 4 weeks of culture, the number of shoots per explant was scored. Kanamycin-resistant shoots were excised and rooted in basal medium supplemented with 100 mg dm^{-3} kanamycin and 250 mg dm^{-3} augmentin. The rooted plantlets were then successfully transferred to compost and later established under greenhouse conditions.

Histochemical GUS assay and PCR analysis were carried out to confirm the presence of T-DNA in putative transgenic plants. The presence of GUS gene in these plants was confirmed with discrete dark-blue regions on leaf tissue incubated in histochemical reagent (Fig. 1). For PCR analysis, genomic DNA was extracted from leaf material of the independent putative transformants as well as from that of wild-type. Using two *npt-II* primers, PCR amplification was performed on the genomic DNA. PCR reactions showed that 11 out of 13 putative transformants carried 1 kb *npt-II* gene; whereas, wild-type plant did not have it (Fig. 2).

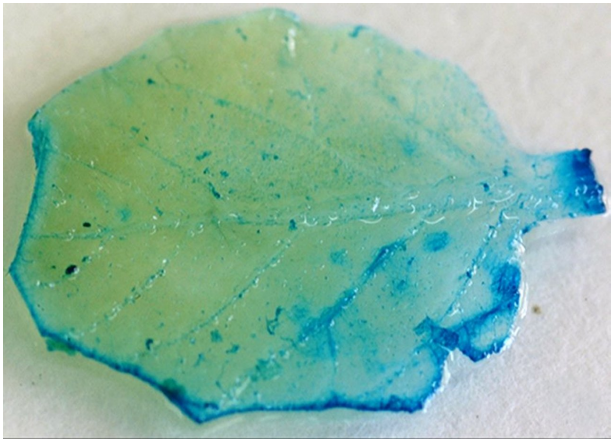

Fig. 1. Localization of GUS activity in leaf of transgenic tobacco plant.

The number of kanamycin resistant shoots per explant and the percentage of GUS and PCR positive plants varied with the different co-cultivation temperatures (Table 1). Analysis of variance showed that main effects of co-cultivation temperatures were significant ($P < 0.01$). The highest number of kanamycin resistant shoots per explant was obtained at 22 and 24 °C (Table 1, $P < 0.05$). Percentage of GUS and PCR positive plants were also highest at these temperatures. Co-cultivation temperatures below and above 22 and 24 °C reduced the transformation frequency significantly (Table 1). From these results, we concluded that 22 and 24 °C were the optimal co-cultivation temperatures for *A. tumefaciens*-mediated transformation of tobacco.

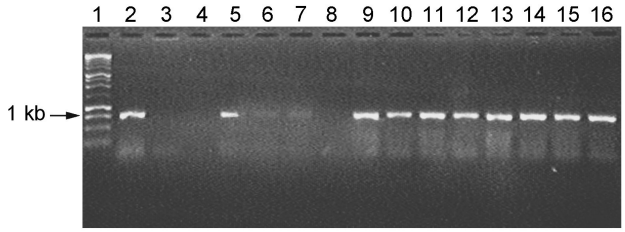

Fig. 2. PCR analysis to detect the presence of *npt-II* gene in independent tobacco transformants. *Lane* 1 is molecular mass marker, lane 2 is positive control (plasmid DNA) and lane 3 is negative control (wild-type). *Lanes 4 - 16* represent the independent tobacco transformants. The expected 1 kb amplified fragment of *npt-II* gene is indicated with an arrow. Note that 11 of 13 putative transformants carry 1 kb *npt-II* gene.

Table 1. Number of kanamycin resistant shoots per tobacco leaf disc explant inoculated with GV2260 p35S GUS-INT using different co-cultivation temperatures and percentage of GUS and PCR positive plants. Values within a column followed by the different letters are significantly different at the 0.05 level using Duncan's multiple range test.

Temperature [°C]	Number of kanamycin-resistant shoots [explant^{-1}]	GUS and PCR positive plants [%]
18	2.2 c[1]	78
20	5.2 bc	75
22	9.9 ab	80
24	12.5 a	80
26	7.2 b	78

There is much evidence that only a minority of cells re-entering cell division at wound sites are competent for both stable transformation by *Agrobacterium* and shoot regeneration (Lipetz 1966, Firek *et al.* 1993, Özcan *et al.* 1993). There may be an optimal temperature at which the plant cells are most receptive to bacterial infection, possibly related to the cell division (Kudirka *et al.* 1986). Fullner and Nester (1996) showed that development of tumors on plants following infection by *A. tumefaciens* was optimal at temperatures around 22 °C and tumor formation did not occur at temperatures above 29 °C. In tobacco, Dillen *et al.* (1997) found no difference between 19 and 22 °C, and there was a considerable decrease in GUS expression when leaf discs were co-cultivated at 25 °C. Srivatanakul *et al.* (2001) reported that shoots did not survive at 16 and 19 °C co-cultivation temperatures; however, 25 and 28 °C yielded a 4 and 3 % shoot

survival rate, respectively. Salas *et al*. (2001) stated that 19 °C may be the best temperature for the *Agrobacterium* transfer machinery; whereas, co-culture at 25 °C appears beneficial for plant cell susceptibility to infection and for stable T-DNA insertion into the plant chromosomes.

As the case in co-cultivation temperatures, main affects of co-cultivation periods were significant ($P < 0.05$). The number of kanamycin resistant shoots per explant and the percentage of GUS and PCR positive plants were influenced by the co-cultivation periods tested ($P < 0.05$; Table 2). Co-cultivation period of 48 h resulted in the highest number of kanamycin resistant shoots per leaf disc; whereas, the highest percentage of GUS and PCR positive plants was achieved from 72 h co-cultivation. Although leaf disc explants were rinsed in a medium containing 1000 mg dm^{-3} augmentin for 10 min after co-cultivation, over-growth of *Agrobacterium* occurred on explants co-cultivated for 72 and 96 h. Bacterial over-growth caused explant death and decreased the number of shoots.

Table 2. Number of kanamycin-resistant shoots per tobacco leaf disc explant inoculated with GV2260 p35S GUS-INT using different co-cultivation periods and percentage of GUS and PCR positive plants. Values within a column followed by the different letters are significantly different at the 0.05 level using Duncan's multiple range test.

Co-cultivation period [h]	Number of kanamycin-resistant shoots [explant^{-1}]	GUS and PCR positive plants [%]
24	1.3 b	80
48	6.8 a	80
72	1.8 b	90
96	2.6 b	70

Influence of co-cultivation period on *Agrobacterium*-mediated transformation has also been reported in a number of plant species (Holford *et al.* 1992, Muthukumar *et al.* 1996, Banerjee *et al.* 2002, Mohan and Krishnamurthy 2003). Similar to our results, in these studies co-cultivation of explants for 24 or 48 h generally provided the best transformation frequency. However, prolonged co-cultivation periods of 6 - 7 d increased transformation efficiency in flax (Dong and McHughen 1991) and a 5-d co-cultivation was the most effective for transient GUS expression in citrange plants (Cervera *et al.* 1998). The effectiveness of prolonged co-cultivation in some plant species might be due to the increased number of induced bacteria attaching to plant cells and/or the increased number of plant cells competent for *Agrobacterium*-mediated transformation (Dong and McHughen 1991, Dong *et al*. 1991, De Bondt *et al.* 1994, Cervera *et al.* 1998, Sakae *et al.* 2001).

Table 3. Number of kanamycin-resistant shoots per tobacco leaf disc explant inoculated with GV2260 p35S GUS-INT using different co-cultivation media and percentage of GUS and PCR positive plants. Values within a column followed by the different letters are significantly different at the 0.05 level using Duncan's multiple range test.

Temperature [°C]	Number of kanamycin-resistant shoots [explant^{-1}]	GUS and PCR positive plants [%]
Liquid	7.3 a	100
Solid	3.7 b	100

As compared to agar-solidified co-cultivation medium used commonly in *Agrobacterium*-mediated transformation studies, it was observed that liquid co-cultivation medium resulted in higher transformation frequency than solid medium (Table 3, $P < 0.05$), in agreement with Herman *et al.* (1989) and Paula *et al.* (1993) who obtained similar results from potato and *Gerbera hybrida* explants co-cultivated in liquid medium. Annie *et al.* (1995) also reported 7 % transformation frequency in liquid and 3 % on solid co-cultivation medium. The results obtained in previous studies and the current work confirm the potential use of liquid co-cultivation medium for successful transformation of crop plants. In general, the number of kanamycin-resistant shoots per explant was lower; whereas, percentage of GUS and PCR positive plants was higher in this experiment compared to experiments on co-cultivation temperatures and periods. These results could be related to developmental stage of explant and bacterial source.

In conclusion, the best transformation frequency in this work was achieved from the explants co-cultivated by *A. tumefaciens* in liquid medium for 48 h at 22 or 24 °C. These results could help to increase the gene transfer events in plant species difficult to transform by *A. tumefaciens*.

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