Bulgarian Journal of Agricultural Science, 19 (No 2) 2013, 222-227
Agricultural Academy

AGROBACTERIUM MEDIATED GENETIC TRANSFORMATION OF *PAPAVER SOMNIFERUM* L. USING SEMI SOLID AGAR GELLED PRIMED SEEDS AS EXPLANT

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Abstract

ROYANDEZAGH, S. D., K. M. KHAWAR, A. R. OSALOU and S. OZCAN, 2013. *Agrobacterium* mediated genetic transformation of *Papaver somniferum* L. using semi solid agar gelled primed seeds as explant. *Bulg. J. Agric. Sci.,* 19: 222-227

The opium poppy *Papaver somniferum* is self-pollinating herbaceous annual plant with various rates of out-crossing and is cultivated since prehistoric times. It is used to obtain number of medicinally important alkaloids, rarely to obtain edible oil and is used as an ornamental plant. There is need to improve poppy by developing objective oriented superior cultivars. Traditional breeding methods for the development of improved new opium cultivars is very slow, due to high labor and lengthy breeding procedures. This makes it necessarry to look alternatives like developing easy and repeatable genetic transformation protocols. Previous reports in poppy suggest that it is very recalcitrant to *in vitro* regeneration, rooting and is difficult to acclimatize. Moreover, all regeneration protocols are variety and genotype dependent. The study aimed to develop an efficient genetic transformation protocol in poppy. To achieve the objective various explants including hypocotyl-cotyledon explant and poppy cultivar Ofis 96 seeds primed on agar solidified MS medium for 7 days were treated with with *Agrobacterium tumefaciens* strain GV 2260 harbouring p35 GUS INT plasmid for 30 minutes. Cocultivation was on MS medium for 48 hours. These seeds were selected on MS selection medium containing 50 mg/l Kanamycin and 500 mg/l Augmentin. After achieving length of 5-6 cm, the putative transgenic seedlings were transferred to pots for growth, development and acclimatisation. The leaves from acclimatised plants were subjected to GUS histochemical analysis. Gus positive plants were further confirmed by polymerase chain reaction (PCR). Putatve Transgenic plants were grown in glasshouse to flower and set seeds.

Key words: Agrobacterium tumefaciens, PCR, GUS analysis, opium poppy, *Papaver somniferum,* transgenic

Introduction

The opium poppy *Papaver somniferum* (2*n*=22) is a member of the genus *Papaver,* which includes some 100 species. It is self-pollinating species with various rates of self and out-crossing. The plant is an herbaceous annual with a distinct vegetative phase, characterized by numerous large pinnatisect leaves spreading horizontally (Krikorian and Ledbetter, 1975). It is used as a medicinal or ornamental plant as well and as a source for seeds and seed oil (Patra et al., 1992). Poppy seems to be one of the few species which was utilized, and cultivated even in prehistoric times (Bernath 1999).

Morphine, codeine, thebaine, narcotine and papaverine are the most important alkaloids produced by the plant and are exploited by the pharmaceutical industry as analgesics, antitussives and anti-spasmodics (Levy and Milo 1999).

Varietal, mass and pure line selections have been applied by several breeders of opium poppy for the development of improved cultivars (Sharma and Singh, 1983; Singh et al., 1995). However, progress in the development of new varieties and incorporating new characters in newly developed varieties is very slow, laborious and time consuming. This makes it necessarry to look alternatives for accelerating the work, reducing the labour and time. In recent years, plant biotechnologists for the production and improvement of prospective lines have favoured plant cell cultures. Plant cell cultures have generally proved beneficial for biochemical and physiological studies, but prog-

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ress is rather slow and difficult to predict when considering the economically important compounds (Drapeau et al., 1987). Plant biotechnology is also used for incorporating of desired genes into many plants employing various genetic transformation techniques (Park and Fachinni, 2000; Park et al., 2002; Park and Facchini, 2002; Khawar et al., 2004; Dogan et al., 2005).

Genetic transformation of poppy is very difficult owing to the presence of number of alkaloids that make difficulties in differentiation of plant cells for regeneration and rooting. There are only 4 reports on genetic transformation of this plant (Park and Facchini, 2002; Chitty et al., 2003; Frick et al., 2004; Facchini et al., 2008). The researchers have used difficult procedures to transform the plants and most of these protocols are difficult to repeat. Therefore, the study aimed to develop a simple, easy, repeatable protocol for genetic transformation of commercially important *P. somniferum* cv. Ofis 96 using (i) hypocotyl, (ii) cotyledon leaves, (iii) cotyledon nodes and (iv) hypocotyl-cotyledon explant from 7 days old germinated seedlings and (v) 1 day primed seeds (vi) 3 days primed seeds (vii) 5 days old primed seeds (viii) 7 days old primed seeds on agar solidified MS medium.

Materials and Methods

Seed material

The seeds of poppy cv. Ofis 96 used in the study were obtained from the Bolvadin Alkaloid Factory, Afyon Turkey.

Preparation of seeds

The seeds of poppy cv. Ofis 96 were surface sterilised using 50% commercial bleach (Axion–Turkey containing 5-6% NaOCl) for 5 min. The sterilisation time and concentration of commercial bleach were optimised after series of experiments (not reported).

Bacterial material

Agrobacterium tumefaciens strain, GV2260 (McBride and Summerfelt, 1990), harbouring the binary vector pGUS-INT (Vancanneyt et al., 1990) was used for genetic transformation and was obtained from the Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey.

Preparation and inoculation of *Agrobacterium tumefaciens*

A loop of the *A. tumefaciens* strain GV2260; 35GUS-INT was obtained from cultures stored at -85°C and inoculated with 10 ml of nutrient broth solution containing 50 mg/l Rifampisin and Kanamycin each and left in shaker overnight to log phase (OD600≅ 0.8) set at180 rpm at 28°C. Thereafter, the multiplied *A. tumefaciens* cells were diluted in the ratio of 1:25 for treatment with the explants and selection on MS medium containing 0.1, 0.2, 0.3 and 0.4 mg/l TDZ with 500 mg/l Augmentin and 50 mg/l Kanamycin (Duchefa, Holland).

Co cultivation, selection, and culture conditions

After inoculation using (i) hypocotyl, (ii) cotyledon leaves, (iii) cotyledon nodes and (iv) hypocotyl-cotyledon explant from seven days old germinated seedlings and (v) 1 day primed seeds (vi) 3 days primed seeds (vii) 5 days old hydroprimed seeds (viii) 7 days old primed seeds on agar solidified MS medium inoculated with *A. tumefaciens* strain GV 2260; they were co cultivated on MS medium in Petri plates (100 x 10 mm) for 2 days in Sanyo Versatile Environmental Test Chamber at temperature of 24 ±2 °C and 16 h light photoperiod using Grolux fluorescent tubes (40 μmol m^{-2} s^{-1}).

Following cocultivation the explants were selected on MS medium containg 50 mg/l kanamycin monosulfate (Duchefa Biochemie, The Netherlands) and 500 mg/l bacteriostatic Augmentin (SmithKline Beecham, Istanbul, Turkey) in magenta GA7 vessels. They were again incubated in Sanyo Versatile Environmental Test Chamber at above-mentioned temperature and photoperiod conditions. The seed germination was followed closely; once the plantlets reached height of ≈ 5 cm, when they were transferred to pots containg peat for an inhibited growth and acclimatisation.

Acclimatisation

After removing agar from the roots of putative transgenic plants under tap water, they were cultured in pots containing sterilised peat. Each of the plants was covered with transparent bags and was transferred to growth room for acclimatisation under ambient conditions of temperature (≈ 20-24°C) for two weeks. Once the plantlets showed sign of growth, the transparent polythene bags were removed gradually by making holes of 1cm diameter in the bags. They were completely removed after about one week and the plants were allowed to grow in the growth chamber. The acclimatised plants were transferred to glasshouse for growth development, flowering and seed set.

Histochemical GUS assay

The leaves taken from < 15 cm long plants and seeds of the putative transgenic plants were subjected to histochemical GUS assay based on the method described by Jeffer-

son (1987) and Khawar et al. (2004). The leaf samples of putative transgenic plants were incubated at 38°C for 24 h. in 100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1 % Triton X - 100 and 1 mM 5-bromo-4-chloro-3-indole glucuronide (X-GLUC). Therafter, the putative transgenic tissues were rinsed in 96% ethanol for 2 days for digestion of chlorophyl. Blue staining in the tissues monitored presence of GUS enzyme activity.

PCR analysis

DNA was isolated from both seeds and leaves of putative transformed plants growing in the glasshouse according to Sangwan (2000) and Boyaci (2007) respectively. The leaf and seed samples were frozen with liquid Nitrogen before mashing with a disposable mortar. Standard PCR techniques were used to detect *uidA* sequence in leaf and seeds samples from the selected plants. The *uidA* primers were tested with primers having sequence of F- CTC GAC GGC CTG TGG GAC TTC, R- CTT TCG GCT TGT TGC CCG C primers *and nptII* primers were tested with primers sequence of F- ACA AGA TGG ATT GCA CGA AGG, R- AAC TCG TCA AGA AGG CGA TAG

PCR assay

Polymerase chain reaction was conducted in 0.05 cm reaction volumes using the following recipe: 0.005 cm^3 10X PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl and 0.1 % Triton X-100), 0.04 cm^3 of 10 mM dNTP, 0.003 cm^3 of 25 mM $MgCl_2$, 0.002 cm^3**.**

PCR reaction for *Gus*, and *npt II* genes was run as per following programme on Biometra thermocycler (Germany).

GUS* (*uidA*) *gene

94°C 5 min (Denaturation of DNA)
95°C 1 min (Denaturation of DNA) 40 cycle
58°C 1 min (annealing)
72°C 2 min (extention)
72°C 7 min.

NPTII gene

94°C 7 min (Denaturation of DNA)
95°C 1 min (Denaturation of DNA) 40 cycle
58°C 1 min (annealing)
72°C 2 min (extention)
72°C 10 min.

PCR for GUS and npt II gene was performed as described by Indraneel et al. (2003).

Observations and Statistical Analysis: Both regeneration and transformation experiments contained twelve replications containing 5 explants each (12 replications x 5 explants =60 explants). Data was analyzed with the help of SPSS 16.00 for windows statistical software using one way ANOVA and the post hoc tests were performed using Tukey's b test. All experimental data given in percentages were subjected to arcsine transformation before statistical analysis (Snedecor and Cocharan, 1968).

Results

***In vitro* plant regeneration**

The (i) hypocotyl, (ii) cotyledon leaves and (iii) cotyledon nodes failed to regenerate. Hypocotyl-cotyledon explant from seven days old germinated seedlings (Figure 1) cultured on MS medium containing 0.1, 0.2, 0.3 and 0.4 mg/l TDZ began to swell after 5 days and induced 0.57-0.80 cm long shoots (Figure 2a). Developing shoots transformed into well-developed shoots after 4 weeks of culture (Figure 2b). The results (Table 1) showed that shoot regeneration, number of shoots per explant and shoot length varied between 30.00-63.33%, 4.67-5.33, 0.57-0.80 cm respectively. The results showed that each increase in the concentration of TDZ was associated with corresponding decrease in the frequency of shoot regeneration. Number of shoots per explant on all regeneration media increased inconsistantly with maximum shoots per explant on MS medium containing 0.1 and 0.3 mg/l TDZ. The shoot length increased relatively to increased concentration of TDZ in the regeneration medium. The longest shoots were recorded on MS medium containing 0.4 mg/l TDZ (Table 1).

The developing shoots were rooted on MS medium containing 0.5 mg/l IBA. However, no shoot could be rooted, as each of the shoot-induced necrosis starting from the top to bottom and died.

Genetic transformation of cv. Ofis 96 using Hypocotyl cotyledon explant

A. tumefaciens treated explants were observed periodically. Hypocotyl-cotyledon explants developed shoot apice after 7-9 days; which gradually developed into shoots on MS medium containing kanamycin. The data pertaining to putative transgenic shoots was obtained after 4 weeks of culture. Kanamycin resistant shoots were dark green and healthy; whereas, none transformed shoots were under either developed, underwent necrosis, died (Figure 2) or grew albino plantlets. The results (Table 2) showed that each increase in the concentration of TDZ was associated with corresponding decrease in the frequency of kanamy-

cin resistant shoots. Shoot regeneration frequency ranged 23.33 -66.67%. Number of kanamycin resistant shoots per explant on all selection media increased inconsistantly with maximum kanamycin resistant shoots per explant on MS medium containing 0.1 mg/l TDZ followed closely by MS medium contaning 0.4 mg/l TDZ with 5.66 kanamycin resistant shoots per explant. MS medium containing each of 0.2 and 0.3 mg/l TDZ induced 3.67 kanamycin resistant shoots per explant. The kanamycin resistant shoot length decreased proportionately to the increased concentrations of TDZ in MS medium in range of 0.1-0.67 cm. Maximum kanamycin resistant shoot regeneration percentage, number of shoots per explant and shoot length was recorded on MS medium containing 0.1 mg/l TDZ.

None of the shoots could be rooted, as all shoots developed necrosis starting from the base, which gradually covered the whole shoots leading to the death of all putative transgenic shoots.

Genetic transformation of cv. Ofis 96 using primed seeds of cv. 96 on agar solidified MS medium

The seeds treated with *A. tumefaciens* soon after sterilizatıon failed to produce transgenic lines. The results showed that (i) 1 day primed seeds (ii) 3 days hydro primed seeds and (iii) 5 days primed seeds co cultivated with *A. tumefaciens* strain GV 2260; produced scanty and weak or albino plants (on selection medium); that died after one to two weeks and did not produce transgenic lines. Seven days primed seeds treated with *A. tumefaciens* were seemed more reactive to *A. tumefaciens* and resulted in large number of kanamycin resistant plants (Figure 3a, b). After 3 weeks of culture, number of deformed, necrotic, under sized, week and poor physiqued plantlets was recorded on the culture media, which died with the passage of time. Selected plants were transferred to pots for acclimatiaation (Figure 3c) followed by transfer of the putative transgenic plants to glasshouse (Figure 3d, e). The glasshouse temperature was maintained at 24°C, where these plants developed flower buds (Figure 3f) flowered (Figure 3g) and set seeds. Gus analysis of leaves taken from < 15cm long plants in the glasshouse showed GUS activity in all of the leaves (Figure3h) irrespective of their place and position on the plants. However, some rare examples of chimeras were also recorded. This suggests that GUS activity may be inhibited in some cells due to position effect at transcriptional or posttranscriptional level or as result of truncated transgenes as suggested by Kohli et al. (1999), resulting in chimeras or this activity may also be due to methylation of foreign genes as suggested by Rezmer et al. (1999). Moreover, it was felt that 35 S CaMV promoter could not be fully relied for localisation of GUS expression. It also suggests that histochemical GUS assay does not enable the localisation of transformed cells because the GUS expression was consideared disturbed in

Table 1
Effects of various doses of TDZ on shoot regeneration of papaver cv. Ofis 96

TDZ, mg/l	Frequency of shoot regeneration, %	Number of shoots per explant	Shoots length, cm
0.1	63.33a	5.33a	0.57b
0.2	40.00b	4.67c	0.70a
0.3	46.67b	5.33a	0.73a
0.4	30.00c	5.00ab	0.80a

Means with the same letter are not significantly different using Duncans Multiple Range Test at P<0.05

Table 2
Effects of various doses of TDZ on kanamycine resistant shoot regeneration of papaver cv. Ofis 96

TDZ, mg/l	Frequency (%) of kanamycine resistant shoots*	Kanamycine resitant number of shoots per explant	Length of kanamycine resistant shoots, cm
0.1	66.67a	6.00a**	0.67a*
0.2	46.67b	3.67b	0.33b
0.3	33.33c	3.67c	0.10c
0.4	23.33d	5.66ab	0.67a

* Means with the same letter are not significantly different using duncans multiple range test at P<0.05
** Means with the same letter are not significantly different using duncans multiple range test at P<0.01

Fig. 1. Hypocotyl-cotyledon explant from seven days old germinated seedlings (bar = 0.5 cm)

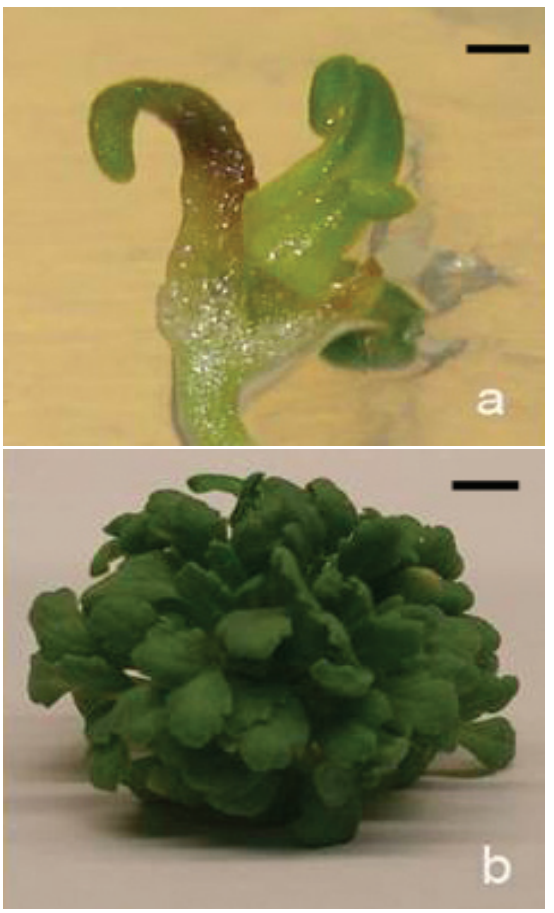

Fig. 2. *In vitro* **shoot regeneration of poopy from (a) hypocotyl-cotyledon explant from seven days old germinated seedlings cultured on MS medium containing 0.1 mg/l TDZ after 5 days and (b) induced shoots of variable length (bar of Fig. 2a,b = 0.5 cm)**

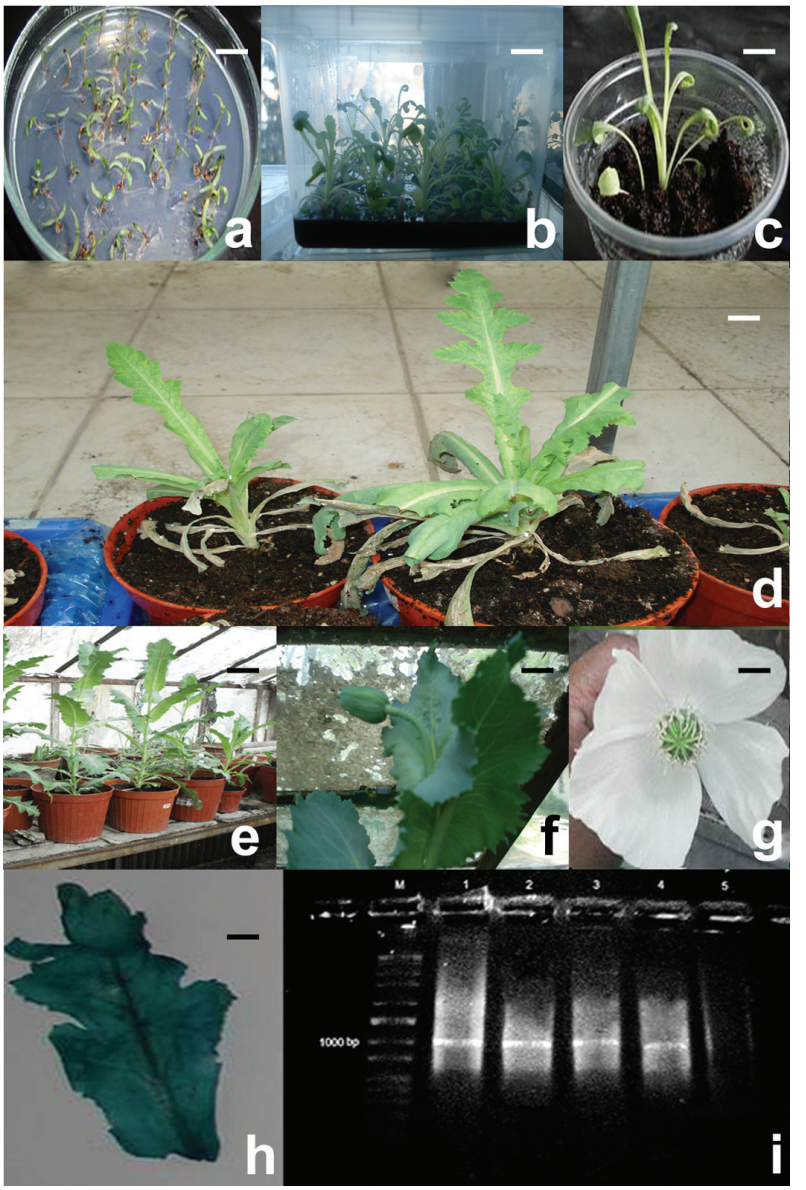

Fig. 3. Transformation of seven days old hydroprimed seeds with *A. tumefaciens* strain GV2260::35 GUS INT.
(a) selection on kanamycin;
(b) selected plants growing in the Magenta vessels;
(c) putative transgenic plants transferred to pots for acclimatisation;
(d,e) putative transgenics growing in the glasshouse;
(f) flower bud;
(g) flowering;
(h) gus positive leaves;
(i) pcr positve plants
(bar of Fig. 3a,b,c = 0.5 cm, Fig. 3d = 1.25 cm. Fig. 3e = 7 cm, Fig. 3f,g,h = 3 cm)

some areas (Khawar et al., 2004). However, PCR anlaysis showed that all cells were transformed irrespective of the position or expression of the *GUS* or *uidA* activity in the leaves (Figure 3 i). 20 transgenic plants were developed and the seeds were obtained from 11 plants. The seeds of these plants (T_1) were tested against gus expression, which proved gus positive in Mendellian fashion 9:3:3:1.

Conclusion

Genetic transformation of poppy is very difficult and has been reported by few workers only. It may be concluded that 7 days primed seeds could be easily used to transform the poppy. However, the process of transformation in poppy is not very simple as the explants other than seed failed to produce transgenics. The priming period of seeds must be selected carefully for fruitful transformation. The method could be benificially used for transformation of poppy with other strains *of A. tumefaciens* for development of transgenic plants for improving or suppressing alkaloid production and increased edible oil production.

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Received May, 2, 2012; accepted for printing December, 2, 2012.