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Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola (*Brassica napus* L.) from hypocotyl segment explants

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Abstract An efficient protocol for the production of transgenic *Brassica napus* cv. Westar plants was developed by optimizing two important parameters: preconditioning time and co-cultivation time. *Agrobacterium tumefaciens*-mediated transformation was performed using hypocotyls as explant tissue. Two variants of a green fluorescent protein (GFP)-encoding gene – mGFP5-ER and eGFP – both under the constitutive expression of the cauliflower mosaic virus 35S promoter, were used for the experiments. Optimizing the preconditioning time to 72 h and co-cultivation time with *Agrobacterium* to 48 h provided the increase in the transformation efficiency from a baseline of 4% to 25%. With mGFP5-ER, the transformation rate was 17% and with eGFP it was 25%. Transgenic shoots were selected on 200 mg/l kanamycin. Rooting efficiency was 100% on half-strength Murashige and Skoog medium with 10 g/l sucrose and 0.5 mg/l indole butyric acid in the presence of kanamycin.

Keywords Green fluorescent protein · *Agrobacterium tumefaciens* · *Brassica napus* · Preconditioning · Co-cultivation

Introduction

Canola (*Brassica napus* L.) is an important oil crop, ranking third only to soybean and palm oil in global production. Canola oil is widely used as a cooking oil, salad oil, and for the production of margarine. Of all the edible vegetable oils widely available today, it has the lowest saturated fat content, making it appealing to health-conscious consumers. Canola oil is also used in lubricants and hydraulic fluids, especially when there is a significant risk of oil leaking to waterways or into ground water (Sovero 1993).

There are several reports on canola transformation with respect to the introduction of various new traits such as modified oil composition (Knutzon et al. 1992), herbicide tolerance (De Block et al. 1989), altered protein composition (Altenbach et al. 1992) and insect resistance (Stewart et al. 1996). Transformation has been carried out using various explants, such as stem internodes (Fry et al. 1987), stem segments (Pua et al. 1987), cotyledonary petioles (Moloney et al. 1989) and hypocotyl segments (Radke et al. 1988; De Block et al. 1989; Stewart et al. 1996). Stewart et al. (1996) and Halfhill et al. (2001) have reported transformation efficiencies of only 4% in *B. napus* cv. Westar using hypocotyls as the explant source. An increase in the transformation efficiencies is desirable in order to decrease the amount of resources needed to produce transgenic plants, and to potentially provide a higher baseline for subsequent transformation of other canola varieties. Two important factors that govern the efficiency of transgenic plant recovery are obtaining healthy shoots that are not hyperhydrated and having a good rooting efficiency.

In this communication we report an increase in the transformation efficiency of canola and 100% rooting of the transformed shoots. Improved efficiency was achieved through altering the preconditioning and co-cultivation times, circumventing hyperhydration and improving the rooting efficiencies.

Materials and methods

Vectors

Two gene constructs were used for transformation. The genes used were those encoding mGFP5-ER and eGFP (green fluorescent protein).

The pBin mGFP5-ER vector was made available by J. Haseloff and is described in (Haseloff et al. 1997). The pBin plasmid contained the *nptII* gene coding for neomycin phosphotransferase,

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which allows for the selection of plants resistant to kanamycin. The *nptII* gene was under the control of the NOS (nopaline synthase) promoter and terminator. The mGFP5-ER gene was under the control of the cauliflower mosaic virus (CaMV) 35S promoter and a NOS terminator.

The eGFP vector was constructed by cutting the eGFP from peGFP (Clontech, Palo Alto, Calif.) with *Stu*I and partially with *Xba*1 and subsequently cloning it in pSam 15; a derivative of pCambia 2301 (Cambia, Canberra, Australia). The *nptII* gene was placed under the control of the CaMV 35S promoter and the CaMV poly A terminator. The eGFP was under the control of the CaMV promoter and the NOS terminator. The plasmids were transformed into *Agrobacterium* strain GV3850 by electroporation.

Transformation and plant regeneration

The transformation and regeneration protocol was modified from Mehra-Palta et al. (1991) and Stewart et al. (1996). The experiments were carried out with *Brassica napus* L. cv. Westar. Seeds were surface-sterilized for 5 min with 10% sodium hypochlorite with 0.1% Tween added as a surfactant. The sterilization was followed by a 1-min rinse with 95% ethanol. The seeds were then washed thoroughly with sterile distilled water and germinated on MS (Murashige and Skoog 1962) basal medium with 20 g/l sucrose solidified with 2 g/l Gelrite. Hypocotyls were excised from 8-to 10-day-old seedlings, cut into 1-cm pieces and preconditioned for 24, 48, or 72 h on MS medium supplemented with 1 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) and 30 g/l sucrose, solidified with 2 g/l Gelrite. The preconditioned hypocotyl segments were then inoculated with *Agrobacterium.* The *Agrobacterium* was grown overnight to an OD_{600}=0.8 in liquid LB medium, pelleted and re-suspended in liquid callus induction medium to which acetosyringone was added to a final concentration of 0.05 m*M*.

Co-cultivation with *Agrobacterium* was performed on explants pre-conditioned for 24, 48 and 72 h. They were co-cultivated for 24, 48 or 72 h on MS medium with 1 mg/l 2,4-D. Following co-cultivation, the explants were transferred to the same medium with 400 mg/l timentin and 200 mg/l kanamycin to select for transformed cells. After 2 weeks, the explants were transferred to MS medium with 4 mg/l BAP (6-benzylaminopurine), 2 mg/l zeatin and 5 mg/l silver nitrate, antibiotics as mentioned above and 30 g/l sucrose, solidified with 2 g/l Gelrite, to promote organogenesis. After a further 2 weeks, the tissue was transferred to MS medium containing 3 mg/l BAP, 2 mg/l zeatin, antibiotics and 30 g/l sucrose and 2 g/l Gelrite for shoot development. The shoots that developed were transferred to MS medium with 0.05 mg/l BAP, 30 g/l sucrose, antibiotics as above, solidified with 2 g/l Gelrite or 3 g/l Gelrite, for shoot elongation. The elongated shoots were transferred to rooting medium consisting of half-strength MS salts, 10 mg/l sucrose, 3 g/l Gelrite, 5 mg/l IBA and antibiotics as above. All the cultures were maintained at 25±2°C under a 16/8-h (light/dark) photoperiod with light supplied by cool-white daylight fluorescent lights. The rooted shoots were transferred to soil and grown under a photoperiod of 16/8 h (light/dark) at 20°C in a plant growth chamber.

Polymerase chain reaction

The putative transgenic plants and the T_1 transgenic plants were analyzed by the polymerase chain reaction (PCR) to confirm the presence of the transgenes. DNA extraction was carried out according to Stewart (1997). The primer pairs used for DNA amplification were 5′-ACCCAGATCATATGAAGAGG-3′ and 5′-TTGGGATCTTTCGAAAGGGC-3′ for mGFP5-ER and 5′-CCACAAGTTCAGCGTG-3′ and 5′-CAGGACCATGTGATC-3′ for eGFP. PCR was carried out using the Promega PCR Mastermix (Promega, Madison, Wis.). The initial denaturation of DNA was done at 94°C for 5 min; this was followed by 35 cycles of amplification of 1 min at 94°C (denaturing), 1 min at 55°C for mgfp5e*r* and 60°C for eGFP (annealing) and 1 min at 72°C (extension). Amplicons were visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Western blot analysis

The Western blot analysis was carried out according to Stewart et al. (1996). Fresh leaf tissue (0.2 g) was ground in liquid nitrogen with a hand drill-driven micro-pestle in a microcentrifuge tube. The ground tissue was left on ice for 30 min after the addition of 0.1 *N* NaOH. The homogenate was neutralized with 1 *M* Tris-HCl and centrifuged at 10,000 rpm for 7 min. The supernatant was decanted to a fresh tube, and the total protein in each sample was determined by Bradford total protein analysis using BSA (bovine serum albumin) as a standard. For the blot, 20 μg of the sample was loaded onto a 10% polyacrylamide gel. Purified GFP protein was used as a standard. The protein was transferred to a nitrocellulose membrane and immunostained. Immunostaining was carried out according to Pratt et al. (1986). The primary antibody wash was done with the rabbit anti-GFP (Clontech) serum, followed by goat anti-rabbit antibody (Sigma, St. Louis, Mo.). A rabbit anti-goat alkaline phosphatase conjugate (Sigma) was used as the tertiary antibody. GFP was detected on blots by exposure to nitroblue tetrazolium/bromochloroindolyl phosphate.

Statistical analysis

For each experiment, 100 explants (hypocotyls) were used and each experiment was repeated five times. The data were analyzed by ANOVA (analysis of variance). The means were compared using the Student-Newman-Keuls multiple comparison test at $P<0.05$.

Results

Improved transformation efficiency

Callus was induced from the cut ends of the hypocotyl segments within 10 days on the callus induction medium. The GFP-transformed callus showed bright green fluorescence when excited with UV (385 nm) or blue light (475 nm). For the blue-light excited eGFP, a green notch emission filter was used as described in Stewart (2001). After callus was transferred to the organogenesis medium, the transformed callus sectors were green and healthy, while the non-transformed sectors turned white or necrotic. Ninety-five percent of the callus sectors produced shoots. Preconditioning of the callus played a critical role in increasing the transformation efficiency. Those explants preconditioned for 72 h before co-cultivation for 48 h produced a higher number of transgenic plants than explants preconditioned for 24 or 48 h (Fig. 1). Control experiments with a preconditioning time of 24 h and co-cultivation for 72 h as described in earlier reports on canola transformation yielded a transformation rate of only 4%. Co-cultivation time with *Agrobacterium* was another important factor in increasing the transformation efficiency. Explants co-cultivated for 48 h yielded optimal efficiencies than those co-cultivated for 24 h and 72 h (Fig. 2). Taking into account the preconditioning time versus co-cultivation, we found that explants co-cultivated for 48 h after a 72-h preconditioning time gave significantly better results than explants preconditioned for 24 and 48 h (Fig. 2) at $P<0.05$.

Table 1 Transformation efficiency of canola cv. Westar transformed with mGFP5-ER and eGFP using *Agrobacterium*after preconditioning for 72 h and co-cultivation for 48 h

GFP gene	Number of explants	Callus	Number of shoots	Number of shoots lost due to hyperhydration	Number of rooted shoots	Number of transgenic plants
mGFP5-ER	732	422	171	47	124	124
EGFP	511	476	165	37	128	128

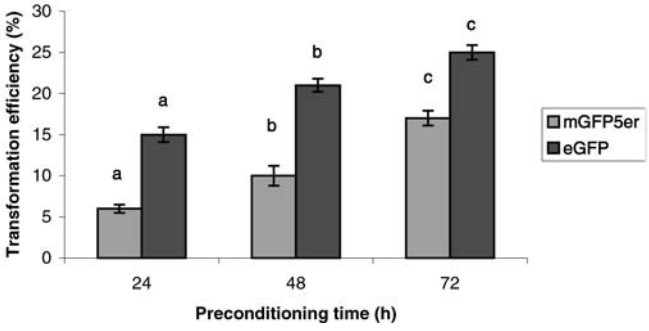

Fig. 1 The effect of explant preconditioning time on transformation efficiency. Columns denoted by different *letters* are significantly different at $P<0.05$ according to the Student-Newman-Keuls multiple comparison test. Each mean represents five replications with a sample size of 100 per replication. Multiple comparisons of mGFP5-ER and eGFP are independent of each other. *Vertical bars* represent the standard error

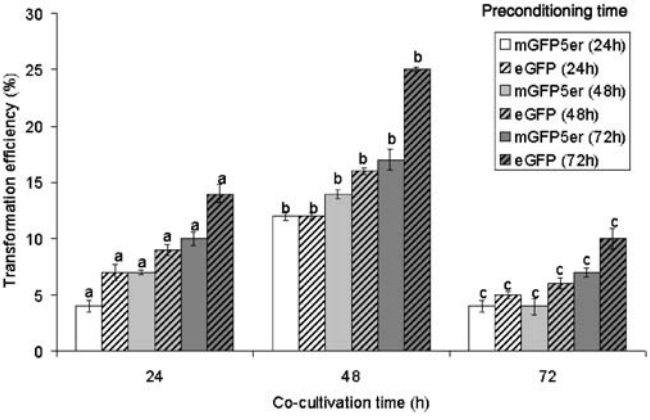

Fig. 2 The effect of co-cultivation time on the transformation efficiency of explants preconditioned for 24, 48 and 72 h. Columns denoted by different *letters* are significantly different at the $P<0.05$ level according to the Student-Newman-Keuls multiple comparison test. Each mean represents five replications with a sample size of 100 per replication. Multiple comparisons of mGFP5-ER and eGFP are independent of each other. *Vertical bars* represent the standard error

The optimal transformation efficiency was obtained with a preconditioning time of 72 h followed by a co-cultivation time of 48 h; the transformation rate was 25% for eGFP and 17% for mGFP5-ER, the latter value being significantly higher. There were no escapes since a high amount of kanamycin (200 mg/l) was used for selection.

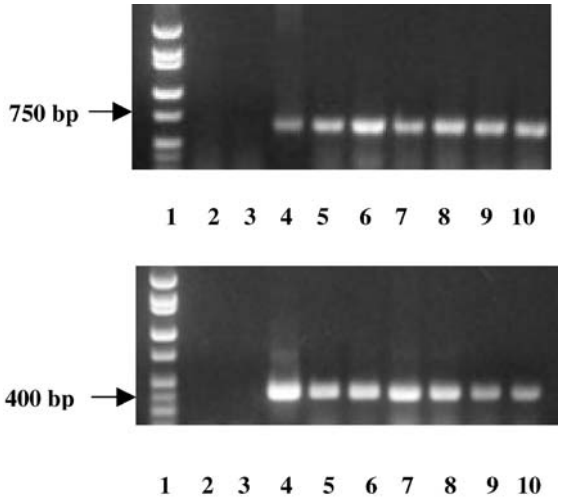

Fig. 3 PCR analysis of transgenic canola with eGFP (*top*) and mGFP5-ER (*bottom*). *Lanes*: *1* DNA marker. *2* purified water, *3* non-transgenic control canola DNA, *4* a positive control of eGFP (*top*) and mGFP5-ER (*bottom*) plasmid DNA, *5–10* DNA from eGFP (*top*) and mGFP5-ER (*bottom*) transgenic canola

All of the potential escapes were bleached and discarded at an early stage of growth.

Overcoming hyperhydration and improved rooting

A major problem faced during this and other canola transformation studies is the hyperhydration of the transgenic shoots, which results in the loss of a large number of transgenic shoots. This problem was addressed by increasing the percentage of Gelrite in the shoot elongation medium from 2 mg/l to 3 mg/l. The hyperhydrated shoots resumed normal growth within 2 weeks following transfer to this medium. Rooting was 100% on the root induction medium containing 3 g/l Gelrite and half-strength MS with 0.5 mg/l IBA and antibiotics. Reducing the strength of the medium to half and decreasing the sucrose concentration to 10 mg/l from the usual 30 mg/l that was used throughout the regeneration process facilitated rooting.

Transgenic plants

A total of 124 transgenic plants with mGFP5-ER and 128 plants with eGFP were recovered following the 72-h

Table 2 Segregation of kanamycin-resistant and -sensitive plants in the selfed T_1 progeny of transformed canola plants

Transformation event designation	Number of germinated seeds	Seedling type:		χ^2 value[a]
		Resistant	Sensitive	
mGFP5-ER 1	42	30	12	0.311
mGFP5-ER 2	40	31	9	0.130
mGFP5-ER 3	38	28	10	0.035
mGFP5-ER 4	17	14	3	0.489
mGFP5-ER 5	45	32	13	0.363
mGFP5-ER 6	42	31	11	0.031
mGFP5-ER 7	50	35	15	0.660
mGFP5-ER 8	39	29	10	0.008
mGFP5-ER 9	25	16	9	1.600
mGFP5-ER 10	41	30	11	0.068
EGFP 1	40	31	9	0.140
EGFP 2	48	40	8	1.700
EGFP 3	40	32	8	0.533
EGFP 4	36	27	9	0.000
EGFP 5	45	34	11	0.007
EGFP 6	46	33	13	0.260
EGFP 7	35	27	8	0.085
EGFP 8	41	32	9	0.202
EGFP 9	36	26	10	0.137
EGFP 10	40	29	11	0.130

[a] In accordance with the expected Mendelian ratio of 3:1 at P=0.05

Fig. 4 Western blot analysis of GFP transgenic canola. *Top*: *Lanes 1–6* mGFP5-ER transgenic plant protein extracts, *lane 7* protein extract from non-transgenic canola, *lane 8* GFP protein standard (27 kDa). *Bottom: Lanes 1–6* eGFP transgenic plant protein extracts, *lane 7* protein from non-transgenic canola, *lane 8* GFP protein standard (27 kDa)

preconditioning and 48-h co-cultivation regime (Table 1). The putative transgenic plants were confirmed to be PCR-positive with GFP-specific primers (Fig. 3). Transgene expression was confirmed by Western blot analysis for T_0 (Fig. 4) and T_1 plants. The T_1 progeny of ten randomly chosen independent events each of mGFP5-ER- and eGFP-containing plants were analyzed for kanamycin resistance. They segregated according to the Mendelian ratio of 3:1 expected for single-locus-insertion inheritance (Table 2). The plants of the T_1 generation that segregated positively on kanamycin selection were also all found to be PCR-positive for GFP. This evidence, taken all together indicates that stable integration and expression of the transgenes occurred.

Discussion

Hypocotyl explants are very sensitive to co-cultivation with *Agrobacterium* and turn necrotic very easily. In the present study, we observed that the explants turned necrotic within a week in culture after transformation. Necrosis was overcome by preconditioning the explant on callus induction medium. This preconditioning of the explants before co-cultivation helped in inhibiting necrosis and increased the transformation efficiency. Improvement in transformation frequency upon preconditioning of the explants has been reported in *Arabidopsis thaliana* (Schmidt and Willmitzer 1988; Sangwan et al. 1992); sugarbeet (Jacq et al. 1993), tobacco (Sunilkumar et al. 1999); watermelon (Choi et al. 1994) and *Populus nigra* (Confalonieri et al. 1994). The preconditioning of explants and the postponing of selection has been reported in *Brassica napus* (Ovesná et al. 1993), who preconditioned the explants for 7 days on induction medium supplemented with 2,4-D. However, application of a 2,4-D pretreatment prior to co-cultivation has been reported to induce excessive callus growth during the subsequent shoot regeneration phase and also to increase the frequency of multiple-copy transformants (Janssens et al. 1995). In our study, we optimized the preconditioning time to 3 days so that there was no overgrowth of the callus, and our segregation data showed single-locus T-DNA inserts for all of the plants analyzed. Thus, a potential added benefit of improving transformation efficiency may be increasing the frequency of single-locus transgene insertion, a typically desired outcome.

This improvement in transformation efficiency as the result of preconditioning can be attributed to the initiation of active cell division upon wounding (Sangwan et al. 1992), the improved binding of *Agrobacterium* to the newly synthesized cell wall at the wound sites (Binns

1991) and the production of *vir*-inducing compounds by the metabolically active cells (Stachel et al. 1985; Spencer and Towers 1991). Sunilkumar et al. (1999) reported that the production of *vir* gene inducers by the explant during the preconditioning period is an important factor that contributes to increased transformation efficiency. We have also used acetosyringone routinely in our experiments since it is known to be a potent vir gene inducer and has been shown to enhance the transformation efficiency by *Agrobacterium* in *Arabidopsis thaliana* (Sheikholeslam and Weeks 1987) and *Glycine max* (Owens and Smigocki 1988).

Another interesting result of this investigation was that the time of co-cultivation also made a significant difference in the transformation efficiency. A co-cultivation time of 48 h gave the best transformation efficiency. Co-cultivation with *Agrobacterium* for 4 h was sub-optimal compared with co-cultivation for 48 h, but a longer period (72 h) proved to be detrimental, as the plant tissue died, which resulted in a very low number of transgenic plants. Zhang et al. (2000) reported that in Chinese cabbage, co-cultivation for 72 h yielded the highest transformation frequency.

In our study on canola, it follows that the production of *vir* inducers during preconditioning plays an important role in increasing the transformation efficiency along with the optimization of preconditioning and co-cultivation time.

A major constraint that we had to overcome was hyperhydration of the transgenic shoots. Hyperhydration is a physiological disorder of plants in tissue culture where the shoots appear translucent and thick, with retarded growth. Hyperhydration may result from various factors such as high cytokinin level, low light, high temperature, the type of culture vessels and the concentration of gelling agents in the media. When cultures are stressed and there is more water retention, plants take up excess water and hence become hyperhydrated. We attributed hyperhydration in our cultures to the concentration of Gelrite. Hyperhydration has been overcome by using a higher concentration of the gelling agent (Debergh et al. 1981; Ziv et al. 1983; Von Arnold and Eriksson 1984). We were apparently able to circumvent hyperhydration by increasing the Gelrite concentration from 2 g/l to 3 g/l.

Rooting of transformed shoots is one of the problems encountered in canola transformation studies. When we used full-strength MS medium supplemented with 0.1 mg/l IBAP (Halfhill et al. 2001), we observed that the shoots rooted after 2 months on rooting medium and that the rooting efficiency was only 25%. George (1996) reported being able to facilitate rooting by using a media with reduced ionic strength. The rooting of in vitro shoots using half-strength medium has been reported by several authors (Samantaray et al. 1995; Upreti and Dhar1996; Kooi et al. 1999). Decreasing the sucrose concentration in the rooting medium has proven to be beneficial for root induction (Kooi et al. 1999; Figueiredo et al. 2001). In our study, we used half-strength MS medium supplemented with indole-3-butyric acid and a lower concentration of sucrose (10 g/l), which gave us 100% success with rooting experiments. Rooting was also faster on this medium, with roots being induced within 1 week following transfer to the rooting medium.

We used a relatively high concentration of kanamycin (200 mg/l) relative to levels used in other *B. napus* experiments in which the range of kanamycin used was between 25 mg/l and100 mg/l as reviewed by Poulsen (1996). This high selection pressure helped us to obtain stable transformants without escapes. We also observed that transformation with eGFP gave a higher number of transgenic plants than did mGFP5-ER. The variation in transformation efficiencies could be due to the different plasmids we used. GFP is not cytotoxic, and previous results preclude targeting GFP to the endoplasmic reticulum (mGFP5-ER) as a contributing factor in plant fitness (Harper et al. 1999; Stewart 2001).

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