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Full Length Research Paper

Gene transformation potential of commercial canola (*Brassica napus* L.) cultivars using cotyledon and hypocotyl explants

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Canola (*Brassica napus* L.) is one of oil crops cultivated in many areas of Iran. Its molecular breeding and production of varieties with new characteristics using genetic engineering needs the establishment of efficient transformation methods in commercial varieties. In this research transformation potential of 8 commercial cultivars; Licord, SLM046, RGS003, Zarfam, Okapi, Sarigol, Modena and Opera adapted to different regions of Iran was studied using cotyledon and hypocotyl explants. *Agrobacterium tumifaciens* strain AGL0 containing the plasmid pCAMBIA3301 was used for transformation. Cotyledon and hypocotyl explants after inoculation with *Agrobacterium* were co-cultivated on MS medium containing 1 mg/l 2,4-D and 4.5 mg/l BAP, respectively. Cotyledonary explants after co-cultivation were transferred on selection MS medium, containing 4.5 mg/l BAP and 3 mg/l phosphinothricin. Hypocotyl explants were transferred to selection MS medium containing 4 mg/l BAP, 2 mg/l Zeatin and 5 mg/l phosphinotricin. The regenerated plants were analyzed by PCR and histochemical GUS assay for gene transformation. The results showed that all of genotypes had gene transformation potential using hypocotyl segments, while this potential was limited to some cultivars using cotyledonary explants. Among transgenic plants regenerated from hypocotyls, Licord cultivar had the most transformation rate (15.26%) and Sarigol the least (0.2%). Also among regenerated plans from cotyledon, SLM046 cultivars had the most transformation frequency (4.7%), and Modena, Opera and Zarfam cultivars did not respond to transformation.

Key words: Canola, gene transformation, *Agrobacterium*, hypocotyls, cotyledon, GUS assay.

INTRODUCTION

Improvement of canola and extension of its cultivation areas has recently received priority in Iran. Genetic engineering techniques, in addition to classic methods, have made it possible to produce new varieties of canola. There are reports of gene transformation in canola in order to produce plants with new characteristics such as oil composition, tolerance to herbicides (De Block, 1989), protein composition and resistance to insects (Stewart, 1996). *In vitro* regeneration and transgenic plants selection efficiency are two important elements in molecular breeding (Cardoza and Stewart, 2003). Regeneration and gene transfer rate to canola varieties are very variable and is highly dependent on genotype (Keara and Mathias, 1992; Cardoza and Stewart, 2004; Poulsen, 1996). Although different transformation methods have been used in canola, *Agrobacterium* method is generally the method of choice.

Different plant tissues such as stem internodes (Fry et al., 1987), stem segments (Pua et al., 1987), cotyledon (Sharma et al., 1990; Hachey et al., 1991; Ono 1994; Moloney 1989), hypocotyls segments (Radke et al., 1988; De Block et al., 1989 Dunwell, 1981; Stewart et al., 1996; Yang et al., 1991), pedancle segments (Eapen and George, 1997), epithermal and subepidermal thin layer cells (Klimaszewska and Keller, 1985), roots (Sharma and Thorpe, 1989; Chi et al., 1990) and protoplast (Hu et al., 1999), have been used as explants for gene transfer

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in canola. Cotyledon and hypocotyl has been suggested as the best explants in regeneration and tansformation experiments (Zihang and Bhalla, 2004).

In an experiment with 100 varieties, the regeneration rate was from 0 to 91% (Ono, 1994). *Agrobacterium*–mediated transformation needs high frequency regeneration (Zihang and Bhalla, 2004). Most canola transformation protocols which use Westar variety are not repeatable for most spring and winter varieties. However, this variety is susceptible to Black leg disease and is defective for other agronomic characteristics (Cardoza and Stewart, 2004).

In this research gene transformation potential of 8 commercially used canola cultivars was studied using two different explants.

MATERIALS AND METHODS

Plant materials and transformation vector

Seeds of eight commercial canola (*Brassica napus* L.) cultivars; Licord, Sarigol, Okapi, Zarfam, RGS003, SLM046 and Opera, were obtained from the Seed and Plant Improvement Research Institute, Oilseeds Research Department. They were surface sterilized using 20% commercial sodium hypochlorite, washed and germinated on MS medium. Cotyledon and hypocotyl explants were excised from 4 day old and 6 day old seedlings, respectively. *Agrobacterium tumi-faciens* strain AGL0 carrying transformation vector pCAMBIA3301 containing *uidA* gene and phosphinothricin resistance gene (*bar*) as a selectable marker was used for transformation.

Inoculation, co-cultivation and selection

The *Agrobacterium* was grown O/N to an OD_{600} = 0.8 in liquid LB medium containing 100 mg/l rifampicin and 50 mg/l kanamycin sulfate. The culture was centrifuged and the cells were suspended in half-MS medium (pH 5.2) plus 0.05 mM acetosiringon and 1 mg/l 2,4-D. Hypocotyl explants were precultured on callus-inducing medium including MS medium with 1 mg/l 2,4-D for 3 days and then inoculated with *Agrobacterium* suspension for 5 min, blotted on sterile filter paper and transferred onto fresh callus-inducing medium for co-cultivation at 25°C for 2 days. They were transferred on callus-inducing medium containing 500 mg/l carbenicilin and 3 mg/l phosphinothricin. After 10 - 14 days, they were transferred to organogenesis MS medium with 4 mg//l BAP, 2 mg/l zeatin, 5 mg/l AgNO3, 500 mg/l carbenicilin and 3 mg/l phosphinothricin. For shoot regeneration, the cultured tissues were placed on MS medium containing 3 mg/l BAP, 2 mg/l zeatin, 8 mg/l phosphino-thricin and 500 mg/l carbenicilin. After 2 weeks, they were transferred to MS medium plus 0.05 mg/l BAP, 8 mg/l phosphino-thricin and 500 mg/l carbenicilin. Regenerated shoots were rooted on MS root inducing medium containing 5 mg/l IBA, 3 mg/l phosphinothricin and 500 mg/l carbenicilin.

Cotyledon segments were inoculated with *Agrobacterium* sus-pension for 10 s (only the cut surface of cotyledonary petiole was inoculated with *Agrobacterium*). Tissues were co-cultured on MS medium containing 4.5 mg/l BAP for 2 days. They were transferred on shoot induction medium containing 4.5 mg/l BAP, 3 mg/l phosphinothricin and 500 g/l carbenicilin, and subcultured every 2 weeks on fresh medium. After 4 - 6 weeks, green regenerated shoots were excised from explants and placed on shoot growing medium containing 0.05 mg/l BAP, 3 mg/l phosphinothricin and 500 mg/l carbenicilin. After 2 weeks, the elongated green shoots were rooted on root inducing medium (mentioned above). All culture media had pH 5.8 and cultures were incubated at 25°C under a photoperiod of 16/8 h light/dark conditions.

Rooted plants on the selection media were transferred to mixture of pith, perlite and soil (1:1:1), maintained at greenhouse conditions (25°C and 16 h light). Regenerated plants of winter cultivars first were cold treated (4°C for 8 weeks) and then transferred to greenhouse.

Confirmation of transformation

Transformation was confirmed using histochemical GUS assay and PCR analysis. Activity of β-glucuronidase in transgenic plants was analyzed based on Jefferson et al. (1987). Leaf segments from putative transgenic plants were immersed in GUS staining solution, incubated at 37°C for 24 h and after discoloring in ethanol were examined using a microscope. For PCR analysis, genomic DNA was extracted from leaves using CTAB method (Murray and Thompson, 1980). PCR reactions were performed using specific primers; GF: 5´-CCGTTTGTGTGAACAACG-3´ and GR: 5´-GCACAGCACATCAAAGAG-3´ for the *gus* gene and PF: 5´-ATCTCGGTGACGGGCAGGAC-3´ and PR: 5´-CGCAGGACCCGCAGGAGTG-3´ for the *bar* gene.

RESULTS AND DISCUSSION

Callus formation was observed on both cut ends of hypocotyl segments cultured on callus induction medium. At later stages, non-transformed calli gradually showed necrosis (Figure 1). Regeneration of green shoots of all cultivars was obtained from 5 to 6 old-week explants. Regeneration from cotyledon segments were selected from 5 cultivars (unless Zarfam, Opera and Modena). Green shoots were excised and rooted on root induction medium. They were transferred to soil and grown under greenhouse conditions. PCR analysis on putative transgenic plants showed the expected band of 1000 bp and 420 bp for the *gus* gene and *bar* gene, respectively (Figures 2 and 3). Histochemical assays confirmed the expression of the *gus* gene in transgenic plants showing blue color. No blue color was observed in tissues of control plants (Figure 4). The highest frequency of gene transformation (15.26%) was obtained from hypocotyl explants of Licord and the least (0.2%) recorded for Sarigol cultivar. While using cotyledon as explant, the highest transformation rate (4.7%) was obtained for SLM046 cultivar (Table 1). Among the commercial cultivars that used in this experiment, Licord showed a better response to transformation using hypocotyl explant. But for cotyledonary explants Modena, SLAM046 and Zarfam cultivars are candidate cultivars for genetic transformation experiments.

Based on obtained results (Table 1), there is gene transformation potential for all cultivars, although diffe-rences were observed among genotypes and explant type. The highest transformation frequency was observed when hypocotyl explants were used. Licord cultivar showed superiority to other cultivars. Using cotyledon, the best transformation frequency was obtained for Sarigol and RGS003. These results emphasizes that

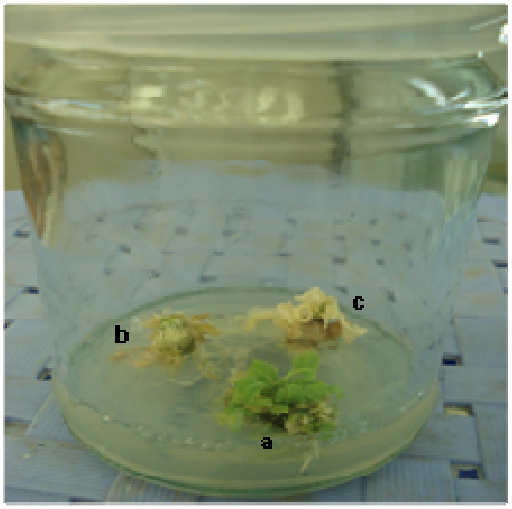

Figure 1. Transformed (**a**) and non-transformed (**b** and **c**) shoot regeneration on the selection medium supplemented with phosphinothricin.

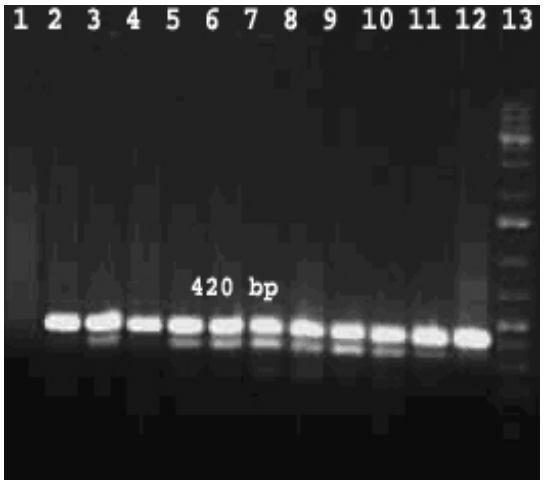

Figure 3. PCR analysis of transgenic canola plants using *bar* specific primers. Lane 1: negative control, lanes 2 - 11: amplification of a 420 bp band in transgenic plants, lane 12: positive control plasmid DNA, lane 13: 1 kb DNA Ladder plus size marker.

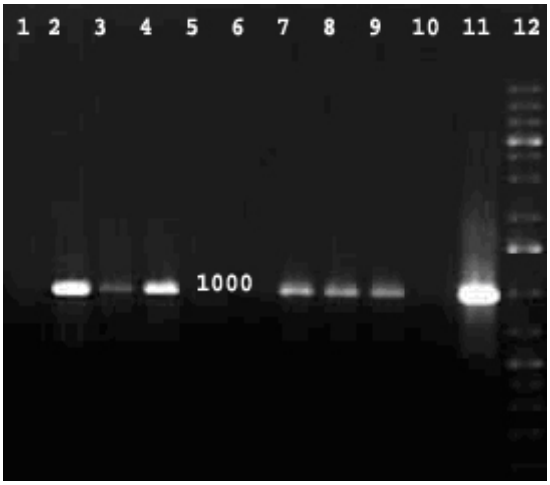

Figure 2. PCR analysis of transgenic canola plants using *gus* specific primers. Lane 1: Negative control, lanes 2 - 4 and 7 - 9: amplification of a 1000 bp band in transgenic plants, lane 11: positive control plasmid DNA, lane 12: 1 kb DNA Ladder plus size marker.

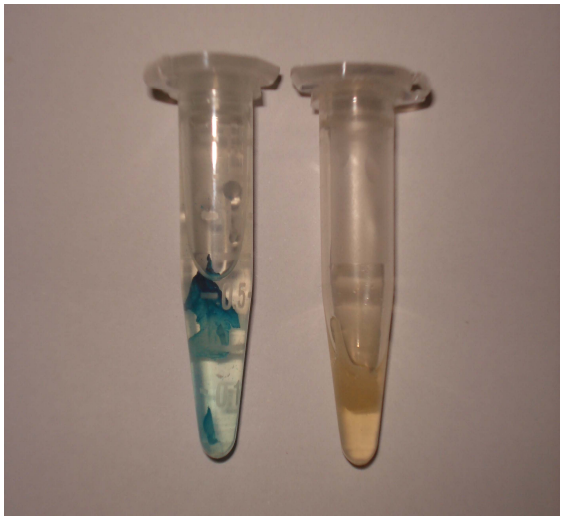

Figure 4. Histochemical GUS assay showing blue color in transgenic tissues.

genetic transformation in canola is highly dependent on genotype and explant type. This is consistent with results obtained in other investigations. High transformation frequency using hypocotyl explants was reported by Jonoubi et al. (2005) for PF7045/91 cultivar (11.4%) and by Zebarjadi et al. (2006) for Maplus and PF cultivars. Cardoza and Stewart (2004) reported 17 - 25% transformation rate for hypocotyl explants. Zhang et al. (2005) produced gene transformation in Rainbow, Oskar, Westar, RI25 and RK7 with 28.3, 5.3, 21.9%, 50.1 and 32.8%, respectively. Moghaieb et al. (2006) reported 9.5 and 1.5% transgenic plants production in Sarow-4 and Semu-249 cultivars, respectively. Wang et al. (2005) reported 25% regeneration rate for Maplus cultivar. However, we cannot compare gene transformation rates reported in different researches due to different genotypes and explant types used. Hypocotyl explant, compared to cotyledon, is suggested as a more suitable tissue for tissue culture because of its good regeneration potential, ease of preparation and also because many explants can be cultured in concurrently (Cardoza and Stewart, 2003).

This efficient method is being used in current projects aimed at improving genetic transformation of canola in Iran.

Table 1. Regeneration frequency of GUS positive plants using hypocotyl and cotyledon explants in different canola cultivars.

Genotype	Explant type	No. of explants used	No. GUS positive transgenic plants	Transformation frequency (%)
Licord	Cotyledon	182	1	0.50
	Hypocotyl	511	78	15.26
Sarigol	Cotyledon	137	5	3.60
	Hypocotyls	494	1	0.20
SLM046	Cotyledon	167	8	4.70
	Hypocotyl	502	45	8.96
Zarfam	Cotyledon	140	0	0.00
	Hypocotyls	417	34	8.15
Opera	Cotyledon	127	0	0.00
	Hypocotyls	426	29	6.80
RGS003	Cotyledon	132	4	3.03
	Hypocotyls	495	11	2.20
Okapi	Cotyledon	193	1	0.51
	Hypocotyls	477	5	1.04
Modena	Cotyledon	114	0	0.00
	Hypocotyl	497	51	10.30

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