BIOLOGIA PLANTARUM 53 (3): 573-577, 2009

BRIEF COMMUNICATION

Secondary embryogenesis and transient expression of the β-glucuronidase gene in hypocotyls of rapeseed microspore-derived embryos

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Abstract

Secondary embryogenesis from rapeseed microspore-derived embryos (MDEs) was studied in three *Brassica napus* L. cultivars Global, PF_{704} and Option. The best results in terms of secondary embryogenesis percentage obtained in cultures of Global and PF_{704} MDEs (75.88 and 65.97 %, respectively) and PF_{704} produced the highest number of secondary embryos per each primary embryo (14.91 ± 2.18). After optimization of physical parameters, rapeseed hypocotyls of MDEs were bombarded with microcarriers coated with a plasmid containing GUS reporter gene. The highest levels of transient GUS expression were obtained using bombardment with gold particles of 1.6 μm, at helium pressure of 9.3 MPa, a bombardment distance of 9 cm, chamber vacuum pressure of 7.1×10^{-6} kPa and single bombardment in bombardment medium containing 0.4 M mannitol.

Additional key words: anther culture, biolistic transformation, doubled haploid, gene transfer.

Thousands of embryos from microspore culture of rapeseed (*Brassica napus* L.) can be regenerated in a single experiment (Pechan *et al*. 1988, Telmer *et al*. 1992). Besides having the capacity to regenerate into plants, these embryos contain embryogenic or pre-embryogenic cells, which in response to induction signals have the capacity to develop directly into secondary embryos, avoiding a callus phase. Secondary embryos obtained from primary microspore-derived embryos (MDEs) are mainly haploid, and can lead, after chromosome doubling, to the regeneration of homozygotic plants (Nehlin *et al*. 1995). Therefore, MDEs are ideal material for genetic engineering (Sangwan *et al*. 1995). A fundamental problem in the production of fertile transgenic plants is not the delivery or integration of the introduced gene, but rather the regeneration of plants from transformed cells (Sangwan *et al*. 1995). Manipulation of rapeseed MDEs culture conditions combined with the intrinsic capacity of these embryos to develop high frequencies of secondary embryogenesis provides us with an desirable and efficient transformation system (Nehlin *et al*. 1995). In addition, the transfer of genes to such explant can lead to haploid transgenic plants from which homozygotic diploid transformants can be produced after chromosome doubling (Folling and Olesen 2001). In recent years, many studies have reported the transient and stable expression of introduced marker genes into rapeseed microspores and MDEs. The first report is the publication of Pechan (1989) on microspores co-cultivated with *Agrobacterium* from which the antibiotic resistant plants were produced but the DNA integration or the sexual transmissibility of the transgene have not been proved (Huang 1992). Microinjection has also been attempted unsuccessfully in *B. napus* microspores (Jones-Villeneuve *et al*. 1995). Particle bombardment has been reported for transformation of rapeseed haploid tissues including MDEs (Chen and Beversdorf 1994) and microspores (Nehlin *et al.* 2000). Microspores are single cells and can be stimulated to embryos. The transformation of microspores usually leads to non-chimeric plants, but due to their thick cell walls, pollens are rather inaccessible to many transformation techniques and the frequency of transgenic haploid production is usually very low. On the other

Received 12 April 2007, *accepted* 15 December 2007.
Abbreviations: GUS - β-glucuronidase; MDE - microspore-derived embryo.
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hand, MDEs are multi-celled tissues and transformation of these segments may lead to chimeric sectors. Transformation of MDEs using biolistic method in combination with secondary embryogenesis property can increase the efficiency of production of rapeseed transgenic plants. In order to increase the haploid transformation efficiency using particle bombardment protocols, many critical parameters are needed to be optimized.

In this study, three cultivars of rapeseed (*Brassica napus* L.) Global, PF_{704} and Option (provided by *Oilseed Research and Development Co*., Tehran, Iran) were grown in the growth chamber with a 16-h photoperiod (300 μmol m^{-2} s^{-1}) and day/night temperature 15/10 °C. Microspore derived embryos were produced as described by Fletcher *et al.* (1998). Microspores isolated from the donor plants were resuspended at a density of 40 000 microspores cm^{-3}, in modified NLN-13 liquid medium (Lichter 1982) supplemented with 13 % sucrose in 100 × 15 mm Petri dishes, each containing 12.5 cm^3 of the liquid medium. Cultures were incubated in darkness at 30 °C for 14 d. When globular embryos became visible, they were diluted to approximately 10 embryos per 12.5 cm^3 of the NLN-13 culture medium and allowed to further develop on a shaker (40 rpm) until most embryos had formed two large cotyledons. After 2 weeks, secondary embryos became visible on the hypocotyls of primary embryos and after 30 d from transferring of embryos to secondary embryogenesis medium, percentage of secondary embryogenesis for each cultivar was investigated.

The plasmid pCAMBIA3301 (*CAMBIA*, Canberra, Australia) contained the cauliflower mosaic virus (CaMV) 35S promoter-bar (bialaphos resistance gene)-35S terminator and the 35S promoter-gus first exon-catalase intron-gus second exon-nos (nopaline synthase) terminator located between the left and right borders of the T-DNA was used for gene transfer experiments. The *Bio-Rad* (Hercules, USA) helium driven PDS-1000/He was used for gene transfer. For precipitation of DNA onto particles (gold or tungsten), 50 mm^3 of a particle solution (60 μg cm^{-3}, suspended in sterile 50 % glycerol) was constantly vortexed while adding 5 mm^3 DNA (1 μg mm^{-3}), 50 mm^3 2.5 M $CaCl_2$ and 20 mm^3 0.1 M spermidine. This solution was vortexed for 3 min, then briefly spined and the supernatant fluid removed. The pellets were washed with 140 mm^3 70 and 100 % ethanol, respectively and finally resuspended in 48 mm^3 100 % ethanol. Aliquots (8 mm^3) of this solution were spotted onto the center of macrocarriers. The physical and biological parameters analyzed were as follows: helium pressures (7584 and 9300 kPa); stopping screen to target tissue distance (6, 9 and 12 cm); chamber vacuum pressure (5.9, 6.5, 7.1 and 7.7×10^{-3} Pa); particles type (gold and tungsten); gold particles size (1.0 μm and 1.6 μm), number of bombardment (single and double bombardment) and application of mannitol in bombardment culture medium as an osmotic agent. The dissected hypocotyls (10 explants) from MDEs of PF_{704} cultivar were placed at the center of each of the 100 × 15 mm Petri dishes containing 12.5 cm^3 B_5 medium (Gamborg *et al*. 1968) supplemented with 0.8 % agar, 0.1 mg dm^{-3} gibberellic acid, 2 % sucrose and subjected to bombardment. 30 h after bombardment, histochemical staining of GUS activity was performed. The assay solution containing 0.5 M Na-phosphate buffer (pH 8.0), 0.1 % Triton X-100, 10 mM EDTA, 2 mM X-gluc, 0.78 mm^3 2-mercapto-ethanol and 28 % methanol (v/v) in the reaction buffer was used. After staining, blue spots were scored optically under stereoscopic microscope (*Nikon*, Tokyo, Japan) and photographed using the *Olympus SZX12* (Tokyo, Japan) system.

Petri dishes in each experiment were completely randomized with three replications. Normality was tested for data obtained from each variable using the univariate procedure of *SPSS version 11.5*. The data were then subjected to analysis of variance (*ANOVA*) and Duncan's Multiple-Range Test (DMRT) using *SPSS* statistical software.

The percentage of primary embryos producing secondary embryos and the mean number of secondary embryos in each primary embryo were calculated for cvs. Global, PF_{704} and Option. Global and PF_{704} showed significantly higher percentage of secondary embryogenesis (75.88 and 65.97 %, respectively) than cv. Option (Table 1) and PF_{704} produced the highest number of secondary embryos on each primary embryo (14.91 ± 2.18). The majority of secondary embryos were induced on the hypocotyls of primary embryos and a few number of embryos showed secondary embryogenesis in their cotyledons. Secondary embryos could be dissected from the parental tissue and develop further into plantlets (Fig. 1*A*). Production of secondary embryos from hypocotyls of MDEs has been reported for *Brassica napus* cv. Topas (Nehlin *et al*. 1995) and *Medicago sativa* (Uzelac *et al*. 2007).

Table 1. Secondary embryogenesis percentage and mean number of secondary embryos (SB) per each primary embryo in different cultivars of rapeseed. Means ± SE, $n = 3$, values with different letters are significantly different at $P < 0.05$.

Cultivar	Embryogenesis [%]	Number of SB [embryo^{-1}]
Global	75.88 ± 4.94a	5.89 ± 0.56b
PF_{704}	65.97 ± 4.78a	14.91 ± 2.18a
Option	18.95 ± 4.00b	0.87 ± 0.12c

We showed that the hypocotyl parts have a higher capacity for secondary embryos differentiation than the cotyledons which is consistent with the report of Shu and Loh (1991). Apparently, single epidermal pre-determined embryogenic cells of the hypocotyls can be triggered by

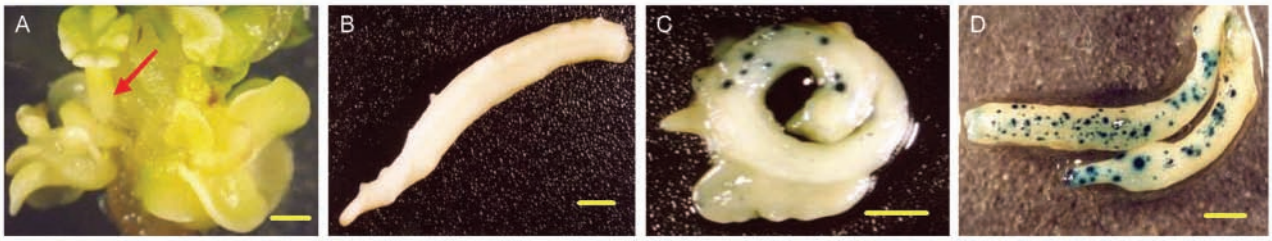

Fig. 1. Induction of secondary embryogenesis in microspore-derived embryos of rapeseed. *A* - dissectable secondary embryo from parental tissue indicated by the *arrow*. *B*, *C*, *D* - Transient GUS expression in hypocotyls of rapeseed microspore-derived embryos after bombardment with plasmid pCAMBIA3301. Control (bombarded without DNA) (*B*), low efficiency expression (*C*), high efficiency expression (*D*). *Bar* 1000 μm.

Table 2. The effects of the bombardment physical parameters and mannitol concentrations for transient GUS expression in hypocotyls of rapeseed microspore-derived embryos. Means ± SE, n = 3, values with different letter are significantly different at $P < 0.05$.

Exp.	Treatments		Number of spots [embryo^{-1}]
1	He pressure [kPa]	gold particle size [μm]	
	7584	1.0	150.00 ± 23.29b
	7584	1.6	132.33 ± 10.10b
	9300	1.0	108.00 ± 9.71b
	9300	1.6	266.67 ± 18.19a
2	He pressure [kPa]	distance [cm]	
	7584	6	168.67 ± 18.77c
	7584	9	171.67 ± 8.95c
	7584	12	90.67 ± 2.28d
	9300	6	66.00 ± 6.56d
	9300	9	270.67 ± 14.43b
	9300	12	305.33 ± 7.31a
3	particle type		
	gold		273.33 ± 14.15b
	tungsten		156.00 ± 22.11a
4	vacuum pressure [× 10^{-3} Pa]		
	5.9		10.00 ± 5.77c
	6.5		55.18 ± 2.89b
	7.1		93.94 ± 6.06a
	7.7		96.67 ± 3.33a
5	mannitol conc. [M]		
	0		233.00 ± 9.54d
	0.1		375.33 ± 16.56d
	0.2		587.00 ± 73.91c
	0.3		862.67 ± 2.60b
	0.4		1120.33 ± 104.3a
	0.5		846.33 ± 46.84b
6	bombard. number		
	1 ×		150.00 ± 23.29a
	2 ×		71.67 ± 4.41b

wounding stimuli to develop secondary embryos. However, extensive chopping of the hypocotyl part results in loss of this capacity and necrosis of the tissues (Nehlin *et al*. 1995). The use of hypocotyls as targets for transformation is assumed to be advantageous over the use of intact embryos, because hypocotyls, once detached from cotyledons, starts multiple secondary embryogenesis (Kott and Coventry 1988). The occurrence of many secondary embryogenic apices increases the frequency of plantlet regeneration.

Bombardment data showed that the microprojectile apparatus was capable of delivering DNA into hypocotyls of rapeseed MDEs (Fig. 1*B-D*). To determine whether the size of the gold particles and He pressure has any effect on transient expression, the efficiency of 1.0 and 1.6 μm gold particles at two different He pressures (7 584 and 9 300 kPa) was analyzed. The 1.6 μm gold particles and 9 300 kPa He pressure provided the highest number of blue spots (Table 2). No significant differences were observed between two He pressures for 1.0 μm gold particles. Further experiment compared the effect of different distances of stopping screen from target tissue on transient GUS expression at the same He pressures. For 7 584 kPa, the highest GUS gene expression was obtained at 9 and 6 cm, while at 12 cm the gene expression level was significantly reduced. This may be due to the reduction in particle acceleration. Other studies in embryo axes of cotton (Banerjee *et al*. 2002) and sonia protocorm-like body (Janna *et al*. 2006), indicated similar results using 7584 kPa pressure with a target distance of 6 or 9 cm. For 9 300 kPa, the highest gene expression level was observed at 12 cm and by decreasing distance the GUS expression was reduced (Table 2).

Gold particles (1 μm) were generally found to result in significantly higher transient GUS expression than tungsten particles (Table 2). Gold particles are sometimes considered more suitable than tungsten because they are much more round and uniform in size. It is biologically inert, non-toxic and dose not degrade DNA bands. Tungsten is highly heterogonous in size and shapes, potentially toxic to some cell type and is subject to surface oxidation and may acidifies and degrades DNA bands (Sanford *et al*. 1993).

Vacuum pressure plays an important role in the acceleration of particles from the stopping screen into the target tissues. However, there is a drawback of holding a

high vacuum pressure in the chamber because a high vacuum pressure will cause the tissue to lose moisture rapidly and thereby reduced cell viability (McCabe and Christou 1993). Our results showed significant differences between chamber vacuum pressures for the mean number of blue spots per bombarded embryo and the best vacuum pressures were 7.1 and 7.7×10^{-3} Pa, which was in agreement with observations of other researchers (Parveez *et al*. 1997, Janna *et al*. 2006).

The results also showed significant differences between numbers of bombardments (single and double bombardment) for mean number of blue spots per embryo. Double bombardment decreased transient GUS expression (Table 2). This result is in agreement with observation of Janna and co-workers (2006) in *Dendrobium sonia* protocorm-like-body. Increasing the number of bombardments would un-doubtedly increase the injuries capacity of the targets and reduce the number of surviving cells expressing the transgene. It is accepted that the number of bombardment for a specific tissue is dependent on other factors like tissue type, particle velocity and particle size (Clemente *et al*. 1992).

The penetration of particles by high acceleration can damage the lipid membrane structure causing cell destruction and ethylene accumulation (Takahashi 1986). Mannitol as an osmotic agent can stabilize cell membrane for better healing of the lesion caused by the particle penetration and reduce pressure potential of cells thus reducing leakage and cell rupture. The effect of different concentrations of mannitol (0.1, 0.2, 0.3, 0.4 and 0.5 M) was investigated by transferring hypocotyls of rapeseed MDEs to B_5 medium containing the mannitol 1 day prior to and 1 day post bombardment. Increasing the mannitol concentration from 0.1 to 0.4 M was found to increase transient GUS expression approximately 5.5-fold compared to control (B_5 without mannitol) (Table 2). The highest transient GUS expression was obtained with 0.4 M mannitol in bombardment medium. However, a higher concentration of mannitol (0.5 M) significantly reduced the GUS gene expression level.

The efficiency of gene delivery by particle bombardment is largely affected by physical and biological factors (Nehlin *et al*. 2000). Our results are in agreement with most of the published data on embryogenic cultures including wheat (Leob and Reynolds 1994) and grape (Hebert *et al*. 1993) although optimum He pressure is believed to be cell and tissue type dependent. In addition, particle size plays an important role in determining the optimum He pressure. Bigger particles will need a higher force to travel and penetrate cells while the smaller particles need a lower force. Increasing the force for the small particles will result in lower expression as consequences of tissue damage (Parveez *et al*. 1997). However, highest transient expression is usually obtained with violent treatment because most of the injured cells will still be able to transiently express the gene but chances of obtaining stable transformants are low, because the cell division and growth are usually impaired in the injured tissues. Therefore, a lower pressure than the pressure giving the optimum transient expression should be used for obtaining stable transformation (Sanford *et al*. 1993).

At present, by choosing the suitable bombardment parameters in combination with secondary embryogenesis system we are able to transform the haploid embryos with interested genes for producing of homozygous rapeseed transgenic plants.

References

Banerjee, A.K., Agrawal, D.C., Nalawade S.M., Krishnamurthy, K.V.: Transient expression of β-glucuronidase in embryo axes of cotton by *Agrobacterium* and particle bombardment methods. - Biol. Plant. **45**: 359-365, 2002.

Chen, J.L., Beversdorf, W.D.: A combined use of microprojectile bombardment and DNA imbibition enhances transformation frequency of canola. - Theor. appl. Genet. **88**: 187-192, 1994.

Clemente, T.E., Robertson, D., Isleib, T.G., Beute, M.K., Weissinger, A.K.: Evaluation of peanut (*Arachis hypogaea* L.) leaflets from mature zygotic embryos as recipient tissue for biolistic gene transfer. - Transgenic Res. **1**: 275-284, 1992.

Fletcher, R., Coventry, J., Kott, L.S.: Double Haploid Technology for Spring and Winter *Brassica napus*. - OAC Publication, Ontario 1998.

Folling, L., Olesen, A.: Transformation of wheat (*Triticum aestivum* L.) microspore-derived callus and microspores by particle bombardment. - Plant Cell Rep. **20**: 629-636, 2001.

Gamborg, O.L., Miller, R.A., Ojiwa, K.: Nutrient requirements of suspension culture of soybean root callus. - Exp. Cell Res. **50**: 151-158, 1968.

Hebert, D., Kikkert, J.R., Smith, F.D., Reisch, B.I.: Optimization of biolistic transformation of embryogenic grape cell suspensions. - Plant Cell Rep. **12**: 585-589, 1993.

Huang, B.: Genetic manipulation of microspores and microspore-derived embryos. - *In vitro* cell dev. Biol. Plant **28**: 53-58, 1992.

Janna, O.A., Maziah, M., Ahmad Parveez, G.K.A., Saleh, K.: Factors affecting delivery and transient expression of β-glucuronidase gene in *Dendrobium Sonia* protocorm-like-body. - Afr. J. Biotechnol. **5**: 88-94, 2006.

Jones-Villeneuve, E., Huang, B., Prudhomme, I., Bird, S., Kemble, R., Hattori, J., Miki, B.: Assessment of microinjection for introducing DNA into uninuclear microspores of rapeseed. - Plant Cell Tissue Organ Cult. **40**: 97-100, 1995.

Kott, L., Coventry, J.: Manual for Microspore Culture Technique for *Brassica napus*. - Crop Science Department, University of Guelph, Guelph 1988.

Leob, T.A., Reynolds, T.L.: Transient expression of the *uid A* gene in pollen embryoids of wheat following

microprojectile bombardment. - Plant Sci. **104**: 81-91, 1994.

Lichter, R.: Efficient yield of embryoids by culture of isolated microspore of different Brassicaceae species. - Plant Breed. **103**: 119-123, 1982.

McCabe, D., Christou, P.: Direct DNA transfer using electric discharge particle acceleration (ACCELL^{TM} technology). - Plant Cell Tissue Organ Cult. **33**: 227-236, 1993.

Nehlin, L., Mollers, C., Bergman, P., Glimelius, K.: Transient β-glucuronidase and gfp gene expression and viability analysis of microprojectile bombarded microspores of *Brassica napus* L. - J. Plant Physiol. **156**:175-183, 2000.

Nehlin, L., Mollers, C., Glimelius, K.: Induction of secondary embryogenesis in MDEs of *Brassica napus* L. - Plant Sci. **111**: 219-227, 1995.

Parveez, G.K.A., Chowdhury, M.K.U., Saleh, N.M.: Physical parameters affecting transient GUS gene expression in oil palm (*Elaeis guineesis* Jacq.) using the biolistic device. - Ind. Crops Products **6**: 41-50, 1997.

Pechan, P.M., Keller, W.A., Mandy, F., Bergeron, M.: Selection of *Brassica napus* L. embryogenic microspores by flow sorting. - Plant Cell Rep. **7**: 396-398, 1988.

Pechan, P.M.: Successful cocultivation of *Brassica napus* microspores and proembryos with *Agrobacterium*. - Plant Cell Rep. **8**: 387-390, 1989.

Sanford, J., Smith, F.D., Russel, J.A.: Optimizing the biolistic process for different biological applications. - Methods Enzymol. **217**: 483-505, 1993.

Sangwan, R.S., Dubois, F., Ducrocq, C., Bourgeois, Y., Vilcot, B., Pawlicki, N., Sangwan-Norreel, B.S.: The embryo as a tool for genetic engineering in higher plants. - In: Terzi, M., Cella, R., Falavigna, A. (ed.): Proceedings of the VIIIth International Congress on Plant Tissue And Cell Culture. Pp. 271-277. Kluwer Academic Publishers, Dordrecht 1995.

Shu, W., Loh, C.S.: Secondary embryogenesis from thin cell layers of *Brassica napus* spp. *oleifera*. - New Phytol. **119**: 427-432, 1991.

Takahashi, N. (ed.): Chemistry of Plant Hormones. - CRC Press, Boca Raton 1986.

Telmer, C.A., Simmonds, D.H., Newcomb, W.: Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. - Physiol. Plant. **84**: 417-424, 1992.

Uzelac, B., Ninković, S., Smigocki, A., Budimir, S.: Origin and development of secondary somatic embryos in transformed embryogenic cultures of *Medicago sativa*. - Biol. Plant. **51**: 1-6, 2007.