http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (18): 3141-3145, 2007
ISSN 1028-8880

Effect of Integrated Bombardment and *Agrobacterium* Transformation System on Transient GUS Expression in Hypocotyls of Rapeseed (*Brassica napus* L. cv. PF_{704}) Microspore-Derived Embryos

[1]M.R. Abdollahi, [1]A. Moieni, [2]A. Mousavi, [2]A.H. Salmanian, [1]M. Jalali Javaran and [1]M. Majdi
[1]Department of Plant Breeding, Faculty of Agriculture, Tarbiat Modarres University,
P.O. Box 14115-336, Tehran, Iran
[2]National Institute for Genetic Engineering and Biotechnology,
P.O. Box 14155-6343, Tehran, Iran

Abstract: A new method for transformation of rapeseed microspore-derived embryos (MDEs), based on microwounding of MDEs by particle bombardment prior to inoculation with an *Agrobacterium* suspension was reported. In this study, effects of two transformation systems (integrated bombardment and *Agrobacterium* transformation system and the singular bombardment) on transient GUS expression in hypocotyls of rapeseed (*Brassica napus* L. cv. PF_{704}) MDEs were studied. Bombardment parameters were: helium pressure, 1350 psi; distance between stopping screen and target tissue, 9 cm, gold particles size of 1.0 μm and chamber vacuum pressure, 24 in Hg. In integrated system *A. tumefaciens* strain AGL1 carrying the binary vector pCAMBIA3301 was used. Bombarded hypocotyls of MDEs were inoculated with *Agrobacteria* at OD_{600} = 1.0 for 10 min or OD_{600} = 0.25 for 24 h. Integrated transformation system increased the mean number of blue spots about 2-2.5 fold compared to singular bombardment and inoculated hypocotyls with OD_{600} = 1.0 for 10 min produced the highest number of blue spots per bombardment (743±23.67).

Key words: *Brassica napus* L., microspore-derived embryos, transient GUS expression, particle bombardment, *Agrobacterium*, integrated transformation system

INTRODUCTION

Microspore culture system has been reported for various higher plant species that are of commercial importance (Khush and Virmani, 1996). In rapeseed, isolated uninucleate microspores undergo the first cell division within a day and develop into fully differentiated globular embryos in about 8 days (Keller *et al*., 1987). In addition, more than 10,000 independent embryos are easily induced from 10 mL of culture suspension containing 4×10^5 microspores (Fukuoka *et al*., 1998). 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. Therefore, microspore-derived embryos (MDEs) are ideal material for genetic engineering (Sangwan *et al*., 1993, 1995). When these characteristics are taken into consideration, MDEs become ideal targets for production of rapeseed transgenic plants. The secondary embryos obtained from primary MDEs are mainly haploid and can lead, after chromosome doubling, to the regeneration of homozygous plants (Loh and Ingram, 1982; Nehlin *et al*., 1995). The Particle bombardment has been successfully used for transformation of rapeseed haploid tissues, including microspore-derived embryos (Chen and Beversdorf, 1994) and microspores (Fukuoka *et al*., 1998; Nehlin *et al*., 2000). However, the frequency of transgenic haploids production was usually very low. In order to increase the efficiency of rapeseed haploid transformation using particle bombardment, we used an integrated bombardment and *Agrobacterium* system compared to singular bombardment based on transient GUS expression. This method combines the advantages of the *Agrobacterium* with the ability of particle bombardment to generate microwounds, thus enhancing the attachment of bacteria and subsequent gene transfer (Droste *et al*., 2000). Particle bombardment method using *Agrobacterium* has been previously used to transform tobacco leaves and sunflower meristems (Bidney *et al*., 1992), banana meristems (May *et al*., 1995), common and tepary bean meristems (Brasileiro *et al*., 1996) and soybean embryogenic cultures (Droste *et al*., 2000).

Corresponding Author: A. Moieni, Department of Plant Breeding, Faculty of Agriculture, Tarbiat Modarres University, P.O. Box 14115-336, Tehran, Iran

MATERIALS AND METHODS

Plant growth conditions and microspore culture: Seeds of a spring oilseed *Brassica napus* cv. PF_{704} (kindly provided by Oilseed Research and Development Co. Tehran, Iran) were grown in the growth chamber with a 16 h photoperiod (300 μE m^{-2} s^{-1}), 15/10°C day/night temperature. Plants were fertilized every second week with 3 g granular fertilizer (12:5:14 NPK and microelements). MDEs were produced by the procedure of Fletcher *et al.* (1998). Microspores isolated from the donor plants were resuspended at a density of 40000 microspores/mL, in modified NLN-13 liquid medium (Lichter, 1982) supplemented with 13% sucrose in 100×15 mm Petri dishes, each containing 12.5 mL of the liquid medium. Cultures were incubated in darkness at 30°C for 14 days.

Source of vectors and constructs: A binary vector pCAMBIA3301 (Curtis and Nam, 2000) 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.

DNA-particle preparation and bombardment: The Bio-Rad helium driven PDS-1000/He was used for gene transfer. For precipitation of DNA onto gold particles, after three times washing the particles with 1 mL of sterile water, 50 μL of a particle solution (60 μg mL^{-1}, suspended in sterile 50% glycerol) was constantly vortexed while adding 5 μL DNA (1 μg μL^{-1}), 50 μL 2.5 M $CaCl_2$ and 20 μL 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 μL 70 and 100% ethanol, respectively and finally resuspended in 48 μL 100% ethanol. Aliquots (8 μL) of this solution were spotted onto the center of macrocarriers. The dissected hypocotyls (about 30 explants) from MDEs were plated around the center of each of the 100×15 mm Petri dishes containing 12.5 mL B5 medium (Gamborg *et al.*, 1968) supplemented with 0.8% agar 0.1 mg L^{-1} gibberellic acid and 2% sucrose.

Bacteria preperation: *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991) carrying the binary vector pCAMBIA3301 was used. *Agrobacterium* was grown 24 h in LB medium containing 50 mg L^{-1} rifampicin, 50 mg L^{-1} kanamycin under continuous shaking at 28°C. Cells were centrifuged and resuspended in NLN-13 liquid medium to an OD_{600} of 1.0 and 0.25.

Integrated transformation system: One day prior to particle bombardment, about 30 dissected hypocotyls of rapeseed MDEs were placed in the center of a plate containing B5 medium. Bombardment were performed. Bombardment conditions were: helium pressure, 1350 psi; distance between stopping screen and target tissue, 9 cm, gold particles size of 1.0 μm and chamber vacuum pressure, 24 in Hg. Following bombardment, the half of bombarded embryos were inoculated and incubated for 10 min into the bacterial suspension (OD_{600} = 1.0) or 24 h (OD_{600} = 0.25). Inoculated hypocotyls were co-cultured for 48 h in NLN-13 liquid medium. After co-cultivation, explants were washed in distilled water and transferred to NLN-13 liquid medium supplemented with 200 mg L^{-1} cefotaxime. The explants were maintained for 2 h in this medium to remove the excess of bacteria from their surface. After this period, histochemical staining of GUS activity was performed on inoculated embryos. In this experiment, three transformation systems of singular bombardment, singular *Agrobacterium* mediated transformation and the integrated systems were used.

Histochemical GUS assay: Thirty hours after bombardment, histochemical staining of GUS activity was performed according to Jefferson (1987) with some modifications. The assay solution containing 0.5 M Na-phosphate buffer (pH 8), 0.1% Triton X-100, 10 mM EDTA, 2 mM X-gluc, 10 mM 2-mercapto ethanol and 28% methanol (v/v) in the reaction buffer to inhibit endogenous *B. napus* β-glucuronidase activity which may mask the activity originating from the introduced GUS gene (Kosugi *et al.*, 1990) was used. The hypocotyls of MDEs were assayed in 1.5 mL microfuge tubes with 500 μL of X-gluc solution. Tubes were incubated in the dark at 38°C overnight.

Analysis of data: The univariate procedure showed that the data were normally distributed. Each treatment was carried out in three replications. Data were analyzed using SPSS statistical software. The numbers of GUS positive spots (blue spots) were counted in each treatment under using a binocular microscope.

RESULTS

Results of evaluation for transient GUS expression in integrated transformation system are presented in Fig. 1. Inoculation of bombarded hypocotyls of rapeseed MDEs with of bacterial suspension OD_{600} = 1.0 for 10 min and OD_{600} = 0.25 for 24 h, increased number of blue

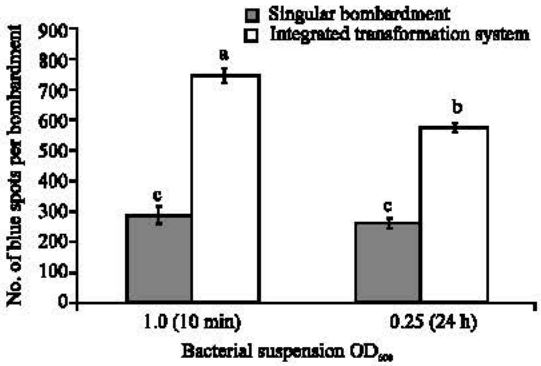

Fig. 1: Effect of integrated transformation system (Inoculation with bacterial suspension OD_{600} = 1.0 for 10 min and OD_{600} = 0.25 for 24 h) on transient GUS expression compared to singular bombardment in hypocotyls of rapeseed microspore-derived embryos, PF_{704} cultivar. Bars indicate standard error (n = 3). Means with the same letter are not significantly different at p = 0.05

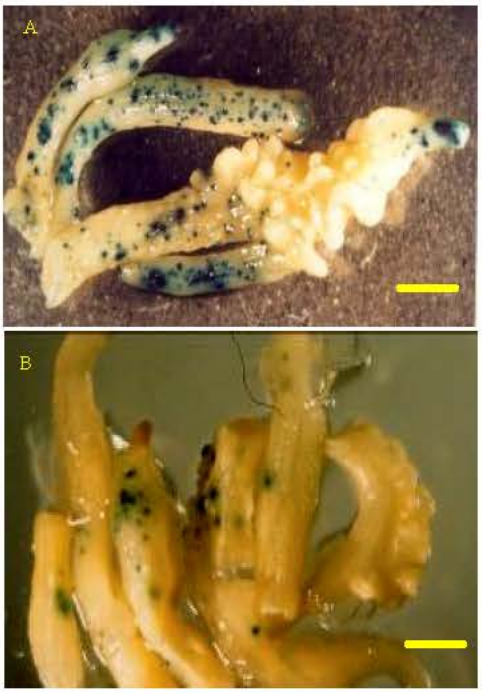

Fig. 2: Transient GUS expression in hypocotyls of rapeseed microspore-derived embryos in two different transformation systems. A) Integrated transformation system B) Singular bombardment. Bar: 1000 µm, Magnification: 10X

spots about 2-2.5 fold (Fig. 2A) compared to singular bombardment (Fig. 2B) and inoculation with OD_{600} = 1.0 for 10 min produced the highest number of blue spots (743±23.67) per bombardment (Fig. 1).

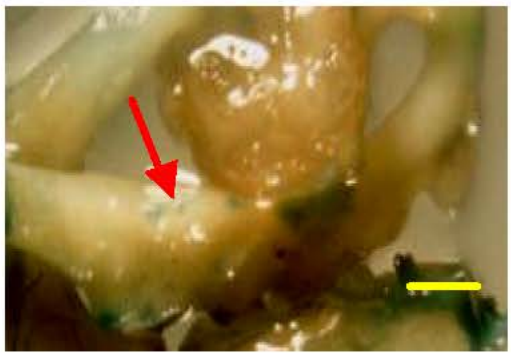

Fig. 3: Transient GUS expression in inoculated hypocotyls after bombardment with uncoated particles. The produced blue spots on hypocotyls indicated by arrow that were very weak in staining and were not countable. Bar: 1000 µm, Magnification: 10X

In order to investigate the effect of DNA-coated microcrriers in the integrated system, hypocotyls were treated with particles that were not coated with DNA and then inoculated with *Agrobacterium*. The results showed that, although this treatment was able yield in transient expression, it had a lower efficiency than the results obtained following singular bombardment with DNA coating (data not shown) and produced very weak blue spots in staining that were not countable (Fig. 3).

DISCUSSION

In this study, the effect of different transformation strategies were investigated on transient GUS expression in hypocotyls of rapeseed MDEs. The integrated system of bombardment and *Agrobacterium* greatly improved transient GUS expression. Particles coated with plasmid DNA are believed to carry DNA through the cell wall and membranes, thus entering the cell, inside which the coated DNA was released from the particles (Klein *et al*., 1988). During this process, the particles create many tiny holes in the cell barriers. Membrane wounding seems to be a crucial factor influencing the successful introduction of foreign gene into plant cells (Chen and Beveresdorf, 1994) and without wounding, DNA transferring by *Agrobacterium* is difficult, as illustrateted by our lack of success in obtaining of blue spots in singular inoculation treatments. These data are in agreement with the results obtained previously by Droste *et al*. (2000). In order to ascertain the effect of *Agrobacterium*, experiments consisting of biolistic treatment using particles without DNA coating followed by inoculation with

Agrobacterium were carried out. They resulted in a lower frequency of blue spots with weak staning than that obtained with singular particle bombardment using DNA-coated particles. These observations indicated that the increased number of blue spots via integrated transformation system was partly due to inoculation treatments only if the hypocotyls were first damaged to some extent. Besides a higher number of blue foci obtained by the integrated method, the areas of blue staining are not more extensive than those produced by bombardment. The rates of transient expression are likely to be increased when other more effective *Agrobacterium* strains are used. Other researchers showed that wounding of tobacco leaves and sunflower meristems (Bidney *et al*., 1992) and soybean embryogenic cultures (Droste *et al*., 2000) by bombardment prior to *Agrobacterium* treatment increases the transformation frequencies. May *et al*. (1995) obtained transgenic banana plants by *Agrobacterium* inoculation of previously bombarded meristems. The results of these transformation protocols are difficult to compare due to differences in plant species, physiological status of the source tissue, type of explant and culture system. In spite of these differences, it is obvious that the microwounds caused by particle bombardment can significantly enhance the *Agrobacterium* mediated transformation frequency in different target tissues (Droste *et al*., 2000). In this study, although the positive GUS assays only demonstrate transient expression of the introduced gene, the described method holds much promise to obtain stable transformation of rapeseed MDEs.

REFERENCES

Bidney, D., C. Scelonge, J. Martich, M. Burrus, L. Sims and G. Huffman, 1992. Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Mol. Biol., 18: 301-313.

Brasileiro, A.C.M., F.J.L. Aragão, S. Rossi, D.M.A. Dusi, L.M.G. Barros and E. Rech, 1996. Susceptibility of common and tepary beans to *Agrobacterium* sp. strains and improvement of *Agrobacterium*-mediated transformation using microprojectile bombardment. J. Am. Soc. Hortic. Sci., 121: 810-815.

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

Curtis, I.S. and H.G. Nam, 2000. Transgenic radish (*Raphanus sativus* L. *longipinnatus* Bailey) by floral-dip method-plant development and surfactant are important in optimizing transformation efficiency. Transgenic Res., 10: 363-371.

Droste, A., G. Pasquali, M. Helena and B. Zanettini, 2000. Integrated bombardment and *Agrobacterium* transformation system: An alternative method for soybean transformation. Plant Mol. Biol. Rep., 18: 51-59.

Fletcher, R., J. Coventry and L.S. Kott, 1998. Double Haploid Technology for Spring and Winter *Brassica napus*, Technical Bulletin, OAC Publication, Canada.

Fukuoka, H., T. Ogawa, M. Matsuoka, Y. Ohkawa and H. Yano, 1998. Direct gene delivery into isolated microspores of rapeseed (*Brassica napus* L.) and the production of fertile transgenic plants. Plant Cell Rep., 17: 323- 328.

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

Jefferson, R.A., 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep., 5: 387-405.

Keller, W.A., Z. Fan, P. Pechan, N. Long and J. Grainger, 1987. An efficient method for culture of isolated microspores of *Brassica napus*. Proc. 7th Int. Rapeseed Congr. Plant Breeding and Acclimatization Institute, Poznan, Poland, pp: 152-157.

Khush, G.S. and S.S. Virmani, 1996. Haploids in Plant Breeding. In: *In vitro* Haploid Production in Higher Plants. Mohan Jain, S., S.K. Sopory and R.E. Veilleux (Eds.), Kluwer Academic Publ, Dordrecht. The Netherlands, 1: 11-34.

Klein, T.M., E.C. Harper, Z. Svab, J.C. Sanford, M.E. Fromm and P. Maliga, 1988. Stable genetic transformation of intact Nicotiana cells by particle bombardment projectiles. Proc. Nat. Acad. Sci. USA., 85: 8502-8508.

Kosugi, S., Y. Ohashi, K. Nakajima and Y. Arai, 1990. An improved assay for β-glucuronidase in transformed cells: Methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci., 70: 133-140.

Lazo, G.R., P.A. Stein and R.A. Ludwig, 1991. A DNA transformation competent *Arabidopsis* genomic library in *Agrobacterium*. Biotechnology, 9: 963-967.

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

Loh, C.S. and D.S. Ingram, 1982. Production of haploid plants from anther cultures and secondary embryoids of winter oilseed rape, *Brassica napus* sp. *oleifera*. New Phytol., 95: 359-366.

May, G.D., R. Afza, H.S. Mason, A. Wiecko, F.J. Novak and C.J. Arntzen, 1995. Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. Bio/Technol., 13: 486-492.

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

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

Sangwan, R.S., C. Ducrocq and B.S. Sangwan-Norreel, 1993. Agrobacterium-mediated transformation of pollen embryos in *Datura innoxia* and *Nicotiana tabacum*: Production of transgenic haploid and fertile homozygous dihaploid plants. Plant Sci., 95: 99-115.

Sangwan, R.S., F. Dubois, C. Ducrocq, Y, Bourgeois, B. Vilcot, N. Pawlicki and B.S. Sangwan-Norreel, 1995. The Embryo as a Tool for Genetic Engineering in Higher Plants. In: Proceedings of the VIIIth International Congress on Plant Tissue and Cell Culture. Terzi, M., R. Cella and A. Falavigna (Eds.), Kluwer Academic Publishers. The Netherlands, pp: 271-277.