Acta Physiol Plant (2009) 31:1313–1317
DOI 10.1007/s11738-009-0365-5

SHORT COMMUNICATION

An efficient method for transformation of pre-androgenic, isolated *Brassica napus* microspores involving microprojectile bombardment and *Agrobacterium*-mediated transformation

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Received: 22 February 2009 / Revised: 26 June 2009 / Accepted: 28 July 2009 / Published online: 4 September 2009

Abstract The physical barrier imposed by the thick microspore wall constitutes an obstacle for an efficient *Agrobacterium*-mediated transformation of vacuolate microspores prior to androgenic induction and haploid embryogenic commitment. It is thus necessary to implement additional methods to overcome this drawback. In this study, we focused on the optimization of a protocol to allow for the exogenous DNA to enter the microspore in an efficient manner. We tested different options, based on microprojectile bombardment, to be applied prior to agroinfiltration. From them, the best results were obtained through co-transformation by microspore bombardment with DNA-coated microprojectile particles, followed by *Agrobacterium tumefaciens* infection. This method provides an efficient means to integrate extraneous DNA into rapeseed microspores prior to androgenesis induction.

Keywords *Agrobacterium*-mediated transformation · Agroinfiltration · Biolistics · Rapeseed · GUS expression · Microprojectile bombardment

Communicated by L.A. Kleczkowski.

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Introduction

In doubled haploid technology, microspores are particularly attractive as targets for genetic transformation, due to their haploid and single-cell nature. Through the application of a wide range of stressing agents, a haploid microspore can be deviated to become a haploid androgenic embryo and, upon chromosome doubling, eventually a doubled haploid plant (Zhou et al. 2002a, b; Gu et al. 2003, 2004; Zhang et al. 2006a, b; Seguí-Simarro and Nuez 2008a, b). Transformation of microspores prior to androgenesis induction has the potential to avoid not only hemizygosis but also chimerism, generating embryos and plants where all of their cells express the introduced transgene in homozygosis (Dormann et al. 2001). However, the different attempts to produce transformed microspores have yielded variable results in terms of transformation efficiency. Some authors have reported a relative success for transient and stable integration of introduced marker genes into the microspore/pollen grain genome via different methods such as electroporation (Obert et al. 2004), microinjection (Jones-Villeneuve et al. 1995), microprojectile bombardment (Jardinaud et al. 1995; Stoger et al. 1995; Fukuoka et al. 1998; Nehlian et al. 2000), and infection with *Agrobacterium* (Pechan 1989; Dormann et al. 1998, 2001), but a reliable, efficient, and general protocol is still to be presented.

Among the different procedures used for transformation, the most efficient and widely used is infection with *Agrobacterium* (agroinfiltration). Agroinfiltration has been extensively used to transform a variety of plant tissues and species (Sharma et al. 2005). Additional treatments for physical wounding by sonication, silicon carbide, sand (Singh and Chawla, 1999; reviewed in Sharma et al. 2005), or microprojectile bombardment (Bidney et al. 1992) prior

to agroinfiltration have proven to enhance the efficiency of *Agrobacterium*-mediated transformation. However, agroinfiltration of microspores or pollen grains has been for long reported as extremely difficult if not impossible (Potrykus 1991; Sangwan et al. 1993). The thick cell wall of the microspores has been traditionally considered as the main obstacle for *Agrobacterium* to penetrate the cell, and published alternatives to overcome this physical barrier are limited (Dormann et al. 2001). Therefore, the development of additional or alternative strategies to effectively introduce transgenes still deserves attention.

Direct gene transfer by microprojectile bombardment (biolistics) seems the best method to surpass the microspore wall barrier and reach the cell nucleus (Klein and Fitzpatrick-Mcelligott 1993; Sharma et al. 2005). However, biolistics is known to be less efficient than *Agrobacterium*-mediated gene transfer (Sharma et al. 2005). Thus, it would be desirable to combine the advantages of both techniques into an integrated protocol for microspore transformation. In this study, we focus on the development of a strategy to overcome the difficulties imposed by the microspore wall to the entry of exogenous DNA for microspore transformation. We report on an efficient method to transform *Brassica napus* microspores using an integrated bombardment and agroinfiltration system that combines the advantages of both methods in terms of delivery and expression of exogenous DNA, and generation of microwounds to enhance the efficiency of bacterial infection.

Materials and methods

Plant material

Brassica napus cv. PF_{704} plants were used as donor of microspores, grown in a growth chamber with a 16 h photoperiod (300 μE m^{-2} s^{-1}) and 15/10°C day/night temperature.

Vectors and preparation of bacteria

A pBGWFS7-64 binary vector (kindly provided by Dr. Kim Boutilier, Plant Research International, Wageningen, The Netherlands) carrying the GUS reporter gene under the control of the pollen-specific p28640 promoter (AT2G28640, Exo70 gene family), was used for both microprojectile bombardment and infiltration with *Agrobacterium tumefaciens* (strain LBA4404).

Agrobacterium tumefaciens strain LBA4404 harboring the binary vector pBGWFS7-64 was taken from a single colony and grown at 28°C for 24 h in LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.5) supplemented with 50 mg/l rifampicin and 100 mg/l streptomycin, under continuous shaking. Finally, bacteria were centrifuged and suspended in liquid NLN-13 medium at different concentrations depending on the experiment (see results).

Preparation of microspores and transformation procedure

Prior to transformation experiments, microspores were isolated, centrifuged, and resuspended in NLN-13 medium according to Seguí-Simarro et al. (2003). For bombardment, 100 μl culture droplets were deposited onto polyester filters over solid NLN medium with different concentrations of sucrose and mannitol. Bombardment was performed either with DNA-free or DNA-coated gold particles of different sizes, using a Bio-Rad PDS-1000/He biolistic particle delivery system. After bombardment, microspores were transferred to liquid NLN medium with the same sugar and mannitol concentration used for bombardment, and 0.5 ml of a suspension of *A. tumefaciens* at different concentrations was added. Then, they were incubated for 24 h at 28°C in darkness, treated with carbenicilin, washed, and analyzed for GUS expression.

GUS histochemical assay

Microspores were analyzed for GUS expression using a procedure modified from Jefferson et al. (1987). The substrate buffer contained 200 mM $NaPO_4$ buffer (pH 7.0), 1 mM $K_3[Fe(CN)_6]$, 1 mM $K_4[Fe(CN)]_6$, 20 mM EDTA, 13% sucrose. and 0.1% (w/v) X-GLUC (5-Bromo-4-Chloro-3-Indolyl-D-gIucuronic acid). For each sample, 0.5 ml of the substrate buffer was added to 3 ml of solid NLN-13 medium in 10 × 35 mm Petri dishes. Samples were then placed on top and incubated for 4 or 24 h at 37°C. Finally, samples were placed at 4°C until counting and analysis.

Results and discussion

First, we set up the conditions for microprojectile bombardment over rapeseed microspores. We tested three critical parameters previously described as strongly influencing the efficiency of transformation in other systems (Vain et al. 1993; Jardinaud et al. 1995; Folling and Olesen 2001), including the amount of microspores to be bombarded in each shot, the size of the gold microprojectile particles, and the effect of different concentrations of osmoticum in the bombardment medium (Table 1). Our results showed that transformation efficiency is strongly dependent upon microspore density in the bombardment

Table 1 Optimization of parameters for particle bombardment

Parameters	GUS-positive microspores
No. of bombarded microspores	
10^5	530.0 ± 36.56*
2×10^5	328.3 ± 34.04
3×10^5	325.6 ± 23.81
Particle size (μm)	
0.6	390.3 ± 18.70
1	468.0 ± 42.25
1.6	144.7 ± 31.69
0.6 + 1	576.7 ± 35.18*
0.6 + 1.6	266.3 ± 37.12
1 + 1.6	321.3 ± 25.98
Bombardment medium	
13% Sucrose	514 ± 11.3
6.5% Sucrose + 0.2 M mannitol	672 ± 27.5*
13% Sucrose + 0.2 M mannitol	653 ± 38.6*
13% Sucrose + 0.4 M mannitol	340 ± 42.2

* Significance at $P < 0.001$

medium, with an optimal density of 10^5 microspores in the 100 μl of the bombarded sample. It is easy to conceive that the density of microspores must have a critical influence on the rate of transformation, since only those microspores of the top layer would be exposed to the gold particles. Thus, it is essential to maximize the number of exposed microspores, but always keeping them distributed as a monolayer (Figs. 1b, c). Increasing microspore density beyond 10^5 microspores/100 μl had a negative effect, and similar for the assays with 2×10^5 microspores/100 μl and those with 3×10^5 microspores/100 μl. This suggests that higher densities have an additional detrimental influence on microspore viability after bombardment.

Particle size was found to have an effect on the efficiency of transformation. The best results were achieved with a combination of particles of 0.6 and 1.0 μm. This combination clearly overperformed other particle sizes, either combined or alone. The combined use of two gold particle sizes has been proven useful for microspore transformation in wheat (Folling and Olesen 2001). As for wheat, it is likely that in rapeseed highly-sized particles may carry more DNA but could irreversibly damage the microspore, whereas smaller particles are capable of transferring the vector into the nucleus without compromising cell viability.

The presence of additional osmotic agents was also proven positive for transformation efficiency. Whereas different sucrose concentrations did not affect the efficiency, a moderate concentration (0.2 M) of mannitol increased the percentage of GUS-expressing microspores independently of the concentration of sucrose used. Several reports support the notion of a beneficial effect of increased osmolarity before and during bombardment, in the enhancement of transgene expression (Vain et al. 1993). It was previously hypothesized that a mild plasmolysis of the bombarded cells may prevent the cell contents from escaping through the microholes created by bombardment. In our rapeseed microspore system, this effect would be achieved by a 0.2 M concentration of mannitol, while higher concentrations would negatively affect cell viability.

After setting up the best conditions for bombardment, we compared the efficiency, in terms of transient GUS expression, of the optimized bombardment protocol with respect to agroinfiltration alone, agroinfiltration after bombardment with naked (DNA-free) gold particles, and agroinflitration with DNA-coated particles (Table 2). For all of the assays involving *Agrobacterium*-mediated transformation, we tested different bacterial concentrations. Figure 1 and Table 2 show the experiments using concentrations of 0 (control with no bacteria), 4×10^{-3}/100 μl ($OD_{600nm} = 0.004$), 8×10^{-3}/100 μl ($OD_{600nm} = 0.008$), and 16×10^{-3}/100 μl ($OD_{600nm} = 0.016$). For concentrations higher than 16×10^{-3}/100 μl ($OD_{600nm} = 0.016$; data not shown), the liquid

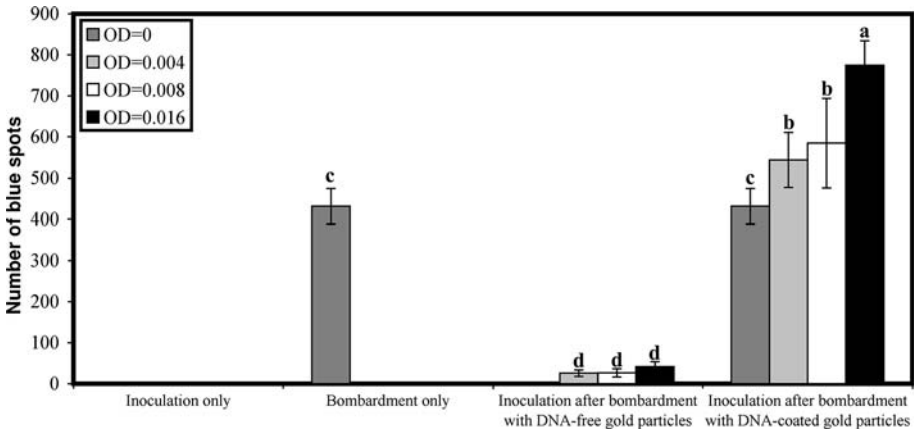

Fig. 1 Effect on transient GUS expression of the use of agroinfiltration only, bombardment only with DNA-coated particles, integrated bombardment with DNA-free particles and agroinfiltration, and integrated bombardment with DNA-coated particles and agroinfiltration, at different *Agrobacterium* concentrations. Means with the same letter are not significantly different at $P = 0.001$

Table 2 Assessment of different strategies for microspore transformation

Transformation method	Density of Agrobacterium ($OD_{600\ nm}$)	GUS-positive microspores
Agrobacterium only	0	0
	4×10^{-3}	0
	8×10^{-3}	0
	1.6×10^{-2}	0
Bombardment only, with DNA-coated particles	0	431.0 ± 43.03
Bombardment (DNA-free particles) + *Agrobacterium*	0	0
	4×10^{-3}	25.7 ± 7.77
	8×10^{-3}	26.3 ± 10.21
	1.6×10^{-2}	41.7 ± 12.05
Integrated system: Bombardment (DNA-coated particles) + *Agrobacterium*	0	431.0 ± 43.03
	4×10^{-3}	543.7 ± 67.00
	8×10^{-3}	584.7 ± 109.26
	1.6×10^{-2}	774.3 ± 59.70*

* Significance at $P < 0.001$

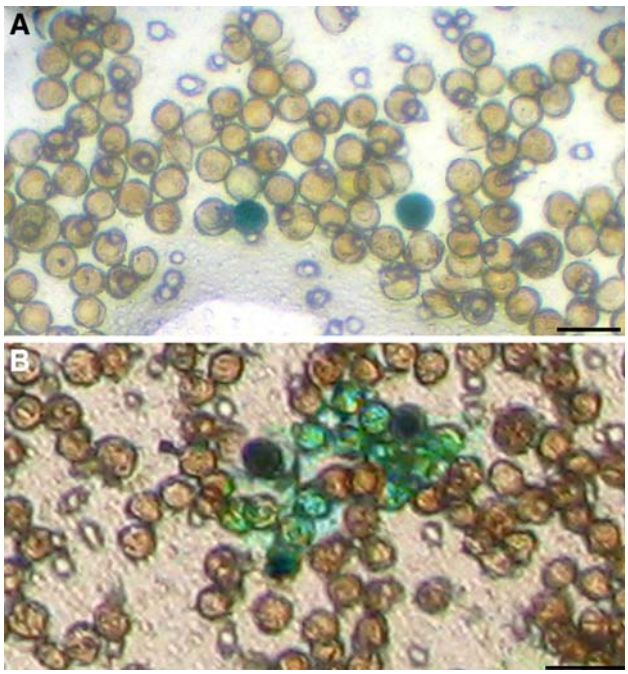

Fig. 2 **a** GUS-expressing microspores transformed by DNA-free bombardment + agroinfiltration. **b** Microspores transformed by DNA-coated bombardment + agroinfiltration. Bars: 40 μm

nature of the medium for microspore and bacterial coculture, and the presence of high sucrose levels gave rise to excessive *Agrobacterium* proliferation and contamination of cultures. At these levels of bacterial concentration, contamination was impossible to control even with high concentrations of carbenicilin. Therefore, we decided to focus on a range up to $OD_{600nm} = 0.016$. In terms of GUS expression, bombardment with DNA-coated particles was clearly more effective than single agroinfiltration or agroinfiltration after bombardment with DNA-free particles, for all of the *Agrobacterium* densities tested (Table 2). Bombardment with DNA-bearing microprojectiles seems to be critical for a high rate of GUS expression. This indicates that just perforation of the microspore wall to allow for bacteria to easily access the microspore may not be enough for efficient transgene expression. The ability of gold particles to directly carry DNA fragments into the cell nucleus would allow for the observed high level of GUS expression. This notion can be confirmed by the comparison of the results of transformation with agroinfiltration after bombardment (Fig. 1). When DNA-coated particles were used (Fig. 2a), GUS-expressing microspores were more abundant than with DNA-free particles (Fig. 2b). Although microperforation of the microspore coat is equivalent in both methods, particle coating provided a GUS expression level ~10-fold higher.

Despite of the fact that *Agrobacterium*-mediated transformation is a widely used method to transform a variety of plant tissues (Sharma et al. 2005), including *Brassica* tissues (Cardoza and Stewart 2004), its efficiency for microspore transformation is limited (this study; Stoger et al. 1995). In parallel, it is known that mechanical wounding may increase the efficiency of *Agrobacterium*-mediated transformation (Singh and Chawla 1999; Hoshi et al. 2004). In this study, we demonstrate that the generation of microholes with DNA-free particles is sufficient to allow for *Agrobacterium* penetration and to achieve an effective transformation of rapeseed microspores, otherwise very difficult if not impossible. Similar results were observed by Bidney et al. (1992) in sunflower meristems and tobacco leaves, and by Droste et al. (2000) in soybean. However, it must be noted that in our case, the difficulties imposed by the exine coat are higher than in somatic tissues. In this scenario, the generation of physical channels through the exine seems critical. Nevertheless, among all of our experiments, the best results were obtained with the combination of bombardment with DNA-coated particles, followed by infiltration with *Agrobacterium* (Figs. 1, 2a), in a manner dependent on the bacterial density used (Table 2). This method has the potential to combine the advantages of the *Agrobacterium* transformation system with the high efficiency of biolistic DNA delivery. Besides, the addition of a controlled bacterial coculture step after bombardment does not seem to contribute any additional constraint to the viability of microspores. Although the combined transformation method reported hereby only shows transient transgene expression, we show that this approach is a promising method to incorporate into the experimental protocols for eventually obtaining transgenic, non-chimeric androgenic embryos, and double haploid plants. In summary, this study sets up the conditions for an efficient

transformation protocol. Based on this protocol, further improvements in both the viability, survival and development of microspores/embryos and the stability of transformation, can now be addressed.

Acknowledgments Authors are grateful to Dr. Kim Boutilier for providing the pBGWFS7-64 plasmid with the p28640 promoter. This study was supported by grant AGL2006-06678 from the Spanish Ministry of Education and Science (MEC) to JMSS.

References

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

Cardoza V, Stewart CN (2004) *Brassica* biotechnology: progress in cellular and molecular biology in vitro. Cell Dev Biol Plant 40:542–551

Dormann M, Datla N, Hayden A, Puttick D, Quandt J (1998) Non-destructive screening of haploid embryos for glufosinate ammonium resistance four weeks after microspore transformation in *Brassica*. Acta Horticulturae 459:91–197

Dormann M, Wang HM, Oelck M (2001) Transformed embryogenic microspores for the generation of fertile homozygous plants. USA Patent US 6,316,694 B1

Droste A, Pasquali G, Helena M, Zanettini B (2000) Integrated bombardment and Agrobacterium transformation system: an alternative method for soybean transformation. Plant Mol Biol Report 18:51–59

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

Fukuoka H, Ogawa T, Matsuoka M, Ohkawa Y, Yano H (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

Gu HH, Zhou WJ, Hagberg P (2003) High frequency spontaneous production of doubled haploid plants in microspore cultures of *Brassica rapa* ssp chinensis. Euphytica 134:239–245

Gu HH, Hagberg P, Zhou WJ (2004) Cold pretreatment enhances microspore embryogenesis in oilseed rape (*Brassica napus* L.). Plant Growth Regul 42:137–143

Hoshi Y, Kondo M, Mori S, Adachi Y, Nakano M, Kobayashi H (2004) Production of transgenic lily plants by *Agrobacterium*-mediated transformation. Plant Cell Rep 22:359–364

Jardinaud MF, Souvre A, Alibert G, Beckert M (1995) uidA Gene transfer and expression in maize microspores using the biolistic method. Protoplasma 187:138–143

Jefferson RA, Kavanagh TA, Bevan MV (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

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

Klein TM, Fitzpatrick-Mcelligott S (1993) Particle bombardment: a universal approach for gene transfer to cells and tissues. Curr Opin Biotechnol 4:583–590

Nehlian L, Mollers C, Bergman P, Glimelius K (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

Obert B, Ponya Z, Pretova A, Barnabas B (2004) Optimization of electroporation conditions for maize microspores. Maydica 49:15–19

Pechan PM (1989) Successful cocultivation of *Brassica napus* microspores and proembryos with *Agrobacterium*. Plant Cell Rep 8:387–390

Potrykus I (1991) Gene transfer to plants—assessment of published approaches and results. Annu Rev Plant Physiol Plant Mol Biol 42:205–225

Sangwan RS, Ducrocq C, Sangwan-Norreel B (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

Seguí-Simarro JM, Nuez F (2008a) Pathways to doubled haploidy: chromosome doubling during androgenesis. Cytogenet Genome Res 120:358–369

Seguí-Simarro JM, Nuez F (2008b) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiologia Plantarum 134:1–12

Seguí-Simarro JM, Testillano PS, Risueño MC (2003) Hsp70 and Hsp90 change their expression and subcellular localization after microspore embryogenesis induction in *Brassica napus* L. cv Topas. J Struct Biol 142:379–391

Sharma KK, Bhatnagar-Mathur P, Thorpe TA (2005) Genetic transformation technology: status and problems. In Vitro Cell Dev Biol-Plant 41:102–112

Singh N, Chawla HS (1999) Use of silicon carbide fibers for *Agrobacterium*-mediated transformation in wheat. Curr Sci 76:1483–1485

Stoger E, Fink C, Pfosser M, Heberle-Bors E (1995) Plant transformation by particle bombardment of embryogenic pollen. Plant Cell Rep 14:273–278

Vain P, McMullen M, Finer J (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep 12:84–88

Zhang GQ, He Y, Xu L, Tang GX, Zhou WJ (2006a) Genetic analyses of agronomic and seed quality traits of doubled haploid population in *Brassica napus* through microspore culture. Euphytica 149:169–177

Zhang GQ, Zhang DQ, Tang GX, He Y, Zhou WJ (2006b) Plant development from microspore-derived embryos in oilseed rape as affected by chilling, desiccation and cotyledon excision. Biol Plant 50:180–186

Zhou WJ, Hagberg P, Tang GX (2002a) Increasing embryogenesis and doubling efficiency by immediate colchicine treatment of isolated microspores in spring *Brassica napus*. Euphytica 128:27–34

Zhou WJ, Tang GX, Hagberg P (2002b) Efficient production of doubled haploid plants by immediate colchicine treatment of isolated microspores in winter *Brassica napus*. Plant Growth Regul 37:185–192