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Optimisation of procedures for microprojectile bombardment of microspore-derived embryos in wheat

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Abstract

Using the PDS-1000/He Biolistic® Particle Delivery System, the microprojectile travel distance, rupture disk pressure and DNA/gold particle concentrations were assessed in order to optimise short and longer-term β-glucuronidase reporter gene expression in microspore-derived embryos of wheat. The effects were also evaluated of using sterile filter paper to support explants and treatment with a high osmoticum medium (0.2 M mannitol/0.2 M sorbitol or 0.4 M maltose). In the optimised procedure, wheat microspore-derived embryos (MDEs), were placed on filter paper and incubated on medium containing 0.4 M maltose, for 4 h pre- and 45 h post-bombardment. Five μl pAHC25 (0.75 mg ml^{-1} in TE buffer) was precipitated onto 25 μl gold particles (60 mg ml^{-1} in sterile water), using 20 μl spermidine (0.1 M) and 50 μl $CaCl_2$ (2.5 M). The particles were centrifuged and resuspended in 75 μl absolute ethanol prior to the preparation of 6 macrocarriers. A microprojectile travel distance of 70 mm, a rupture pressure of 1300 p.s.i., and a vacuum of 29″ Hg were employed. Maltose at 0.4 M in the support medium was the most important factor influencing GUS activity in bombarded tissues. GUS activity, 1 day post-bombardment, reached 52 $\pm$ 17 GUS-positive foci/MDE (mean $\pm$ s.e.m, n=3), with 17 $\pm$ 4 foci/MDE at 15 days, giving a 3.0-fold increase ($p<0.05$) compared to expression in MDEs bombarded on medium without a high osmoticum treatment.

Abbreviations: *bar* – phosphinothricin acetyl transferase gene; *gus* – β-glucuronidase gene; GUS – β-glucuronidase activity; LRR – medium after Loeb and Reynolds, 1994; MDEs – microspore-derived embryos

Introduction

Microspores of wheat can be induced to develop into somatic embryos through anther or microspore culture, producing either haploid or spontaneously doubled-haploid (dihaploid) plants. Spontaneously doubled-haploids are fertile and homozygous, allowing their exploitation in plant breeding without further treatment (Jähne & Lörz 1995). Haploid plants can also be utilised in breeding following exposure to a chromosome doubling agent, such as colchicine, to create fertile doubled-haploids. The *in vitro* production of haploid plants avoids the need for selfing of hybrids over several generations to produce pure lines, permitting rapid phenotypic analysis and acceleration of breeding programmes. Bombardment of MDEs integrates transformation procedures with haploid plant production. Since the production of chimaeric plants could occur following the germination of bombarded MDEs, the preferred method would incorporate an intervening callus induction stage, as with immature embryo transformation systems (Altpeter et al. 1996), leading to the development of somatic embryos from transformed cells. Thus, the present study was designed to optimise transient gene expression in MDEs of wheat.

Methods and materials

Donor plant growth conditions and anther culture followed earlier procedures (Triggs et al. 1998). Fifty

MDEs from 5–9 week-old cultured anthers, were placed in a 2.5 cm diameter circle on a 7 cm Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK) overlaying 10 ml of shoot regeneration medium (Loeb & Reynolds 1994; designated LRR) in a 9-cm Petri dish. Explants were distributed randomly across treatments to reduce variation within experiments, with a minimum of 3 replicates for each experiment.

Plasmid AHC25 (*ubi1-uidA*; *ubi1-bar*) was multiplied in and isolated from *Escherichia coli* (provided by Dr. P. Lazzeri, IACR-Rothamsted, Harpenden, UK) and purified using Wizard^{TM} Megapreps DNA purification system (Promega Corporation, Southampton, UK), according to the manufacturer's instructions. Plasmid AHC25 contained the *ubi1* promoter, which was used to drive both the *gus* reporter gene (*uidA*) and the selectable marker gene, *bar*. The maize *ubi1* promoter first exon and intron (Christensen et al. 1992) was chosen since this was known to be an effective promoter for wheat (Loeb & Reynolds 1994).

Gold particles (0.4–1.2 μm diam.; ChemPur GmbH, Karlsruhe, Germany) were prepared as described previously (Marchant et al. 1998). A microprojectile suspension was prepared using 25–50 μl gold particles (60 mg ml^{-1}), 5–10 μl pAHC25 [0.75 mg ml^{-1} in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0)], 50 μl 2.5 M $CaCl_2$ (pH 5.0), and 20 μl 0.1 M spermidine free-base (Sigma Chemical Co., Poole, UK; stored as a frozen stock solution for a maximum of 2 months) (Marchant et al. 1998). Ten μl aliquots of microprojectile suspension were pipetted onto each of 6 macrocarriers and dried in a vibration-free, laminar flow hood. Macrocarriers were checked microscopically for uneven particle distribution or agglutination; such preparations were discarded.

The PDS-1000/He device was used with a vacuum of 29″ Hg, 25 mm distance from rupture disk to macrocarrier and a 10 mm macrocarrier flight distance. Three rupture disk pressures (650, 1100 and 1300 p.s.i.) and two microprojectile travel distances (70 mm or 100 mm, using the fourth and fifth shelf positions from the top of the chamber of the instrument respectively), were evaluated. Additional assessments included the effects of using a filter paper over the semi-solidified medium to support and to remove excess moisture from the target MDEs, varying the DNA and gold particle concentrations (0.5 μg DNA with 200 μg gold particles or 1.0 μg DNA with 400 μg gold particles/bombardment) and a comparison of pre- and post-bombardment treatments with LRR medium, LRR medium containing 0.2 M mannitol, or 0.2 M sorbitol, or 0.4 M maltose. Subsequently, the most efficient osmoticum treatment (maltose) was optimised with respect to the lengths of pre- and post-bombardment treatments (0 h, 4 h, 8 h, 24 h and 0 h, 24 h, 45 h, 7 days, respectively).

Following bombardment, plates were sealed and cultured for 24 h at 28 $\pm$ 2°C in the dark. Bombarded explants from each plate were transferred to new LRR medium after 1 day. GUS activity was assessed histochemically at 1 day and longer-term GUS activity at 15 days post-bombardment, using 10–15 explants selected randomly from each plate. The histochemical assay solution (modified from Jefferson et al. 1987) contained 500 μl ml^{-1} 0.2 M phosphate buffer [pH 7.0, prepared by mixing autoclaved solutions of 0.2 M NaH_2PO_4 (39 ml) and 0.2 M Na_2HPO_4 (61 ml)], 369 μl ml^{-1} sterile reverse-osmosis water, 50 μl ml^{-1} 10 mM potassium ferricyanide, 50 μl ml^{-1} 10 mM potassium ferrocyanide, 20 μl ml^{-1} 0.5M Na_2EDTA, 1 μl ml^{-1} Triton X-100 and 0.5 mg ml^{-1} X-gluc (Gold Biotechnology Inc., St. Louis, US) dissolved in dimethyl formamide. The number of blue GUS-positive foci on explants were counted to assess the expression frequency. Control MDEs were bombarded using gold particles without DNA, or were not bombarded.

Results and discussion

Control MDEs did not exhibit endogenous GUS activity. A 1300 p.s.i. rupture disk pressure (Figure 1) resulted in the highest GUS activity in MDEs bombarded with pAHC25, compared to 650 or 1100 p.s.i. disks. The distance between the stopping screen and the plant tissue was critical, with GUS activity not being recorded in MDEs bombarded using shelf 5 (100 mm microprojectile flight distance) compared to shelf 4 (70 mm) of the PDS-1000/He instrument. This variability was a reflection of particle velocity, with impact velocities being inadequate to ensure particle penetration when MDEs were placed on shelf 5.

MDEs were placed on filter paper over semi-solidified LRR medium during bombardment to ensure removal of any anther culture medium from their surfaces, whilst providing a suitable medium to enable manipulation of high osmoticum treatments. The bombardment of MDEs on LRR medium without a filter paper on the surface of the medium, led to a 45% reduction in the mean number of GUS foci/MDE at 1 day post-bombardment (Table 1). The presence

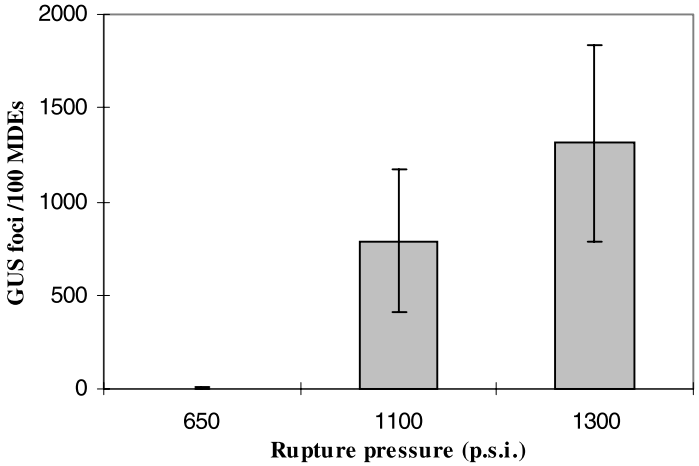

Figure 1. Mean (± s.e.m., n = 3) GUS foci/MDE, 1 day after bombardment, using 3 rupture disc pressures. MDE – microspore-derived embryos.

Table 1. Mean (± s.e.m., n=3) GUS activity at 1 day, with or without filter paper

Parameters	% GUS-positive MDEs	GUS foci/MDE[1]
Filter paper	75 ± 11	20 ± 10
No filter paper	62 ± 16	11 ± 5

400 μg gold particles and 1.0 μg pAHC25/bombardment. MDEs were bombarded at 1300 p.s.i.
[1]MDE – microspore-derived embryos.

Table 2. Mean (± s.e.m., n=3) GUS activity at 1 day, using two concentrations of gold and pAHC25

μg/bombardment	% GUS-positive MDEs	GUS foci/MDE[1]
400 μg gold, 1.0 μg pAHC25	69 ± 7	9 ± 5
200 μg gold, 0.5 μg pAHC25	83 ± 13	28 ± 2

MDEs were placed on filter paper over LRR medium and bombarded at 1300 p.s.i.
[1]MDE – microspore-derived embryos.

of liquid medium over MDEs could have acted as a protective film, with its surface tension reducing the impact of gold particles. Supporting this, Klein et al. (1988) reported that transient expression in *Zea mays* cell suspensions was 30 times higher, using filter paper supports, than for the bombardment of cells in liquid medium.

When 200 μg gold particles were used with 0.5 μg pAHC25 per bombardment, the mean number of GUS foci/MDE increased (Table 2) by a factor of 3, compared to the use of 400 μg gold particles and 0.5 μg pAHC25 per bombardment. This was possibly due to reduced cell damage as a result of the lower concentration of gold particles. Therefore, for subsequent experiments to assess osmoticum treatments, bombardments were performed using 200 μg gold and 0.5 μg pAHC25 per bombardment, in combination with a 70 mm microprojectile flight distance, and with MDEs being placed on filter paper over LRR medium.

The most effective osmoticum treatment involved a 4 h pre- followed by a 45 h post-bombardment treatment on LRR medium containing 0.4 M maltose (Figure 2). The latter was superior to LRR medium alone, or with LRR medium containing 0.2 M mannitol combined with 0.2 M sorbitol. Using maltose treatment, the mean number of blue GUS foci/bombarded MDE, at 1 day post-bombardment, was 3-fold higher compared to the number for MDEs treated with LRR medium alone, reaching 52 ± 17 foci/MDE. Longer-term (15 days) GUS activity was 3 times higher, using maltose treatment (17 ± 4 foci/MDE). For wheat, this is the first report of the use of 0.4 M maltose as a high osmoticum treatment to enhance short- and longer-term *gus* gene expression. High osmoticum medium is thought to protect tissues during bombardment by reducing cell turgor, causing plasmolysis. This leads to reduced leakage of cell contents following bombardment (Vain et al. 1993). High osmoticum medium may also induce membrane changes, leading to increased cell tolerance to microprojectile impact (Clapham et al. 1995).

Using the optimised parameters, levels of short-term expression (85 ± 10% GUS-positive MDEs and 52 ± 17 GUS foci/MDE) were higher than those reported by Loeb and Reynolds (1994), using MDEs of wheat cv. Pavon, and by Shimada et al. (1991) us-

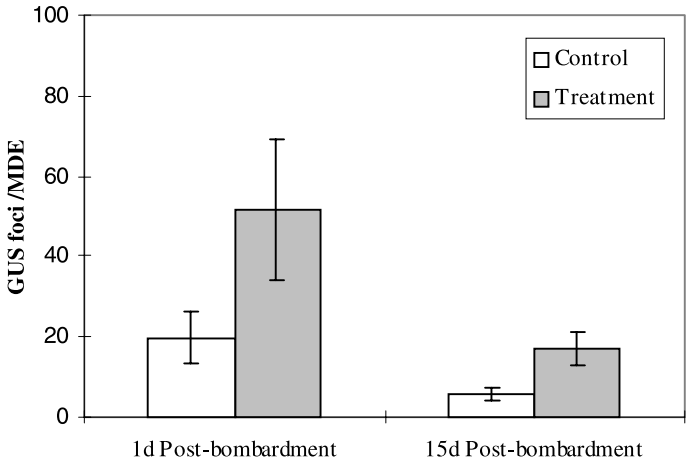

Figure 2. Mean (± s.e.m., n = 4) GUS foci/MDE with or without osmoticum treatment (0.4 M maltose). MDEs were placed on LRR medium (Control) or LRR medium containing 0.4 M maltose (Treatment) for 4 h pre- and 45 h post-bombardment. MDE – microspore-derived embryos.

ing MDEs of cvs. Haruyutaka, BW2589, Glennson 81 and Seri 82, but comparable to data presented by Altpeter et al. (1996) for the bombardment of immature embryos of the wheat cv. Bobwhite. The present study provides a foundation for the development of a procedure for stable transformation, based on the bombardment of haploid, microspore-derived embryos, for the rapid production of homozygous, transformed wheat plants.

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