

H. Wu · C. Sparks · B. Amoah · H. D. Jones

Factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat

Received: 11 September 2002 / Revised: 14 November 2002 / Accepted: 15 November 2002 / Published online: 16 January 2003
© Springer-Verlag 2003

Abstract The development of a robust *Agrobacterium*-mediated transformation protocol for a recalcitrant species like bread wheat requires the identification and optimisation of the factors affecting T-DNA delivery and plant regeneration. We have used immature embryos from range of wheat varieties and the *Agrobacterium* strain AGL1 harbouring the pGreen-based plasmid pAL156, which contains a T-DNA incorporating the *bar* gene and a modified *uidA* (β -glucuronidase) gene, to investigate and optimise major T-DNA delivery and tissue culture variables. Factors that produced significant differences in T-DNA delivery and regeneration included embryo size, duration of pre-culture, inoculation and co-cultivation, and the presence of acetosyringone and Silwet-L77 in the media. We fully describe a protocol that allowed efficient T-DNA delivery and gave rise to 44 morphologically normal, and fully fertile, stable transgenic plants in two wheat varieties. The transformation frequency ranged from 0.3% to 3.3%. Marker-gene expression and molecular analysis demonstrated that transgenes were integrated into the wheat genome and subsequently transmitted into progeny at Mendelian ratios.

Keywords *Agrobacterium tumefaciens* · Genetic modification · Immature embryo · Transformation · Wheat

Introduction

Plant genetic transformation has become an important tool for functional genomics and as an adjunct to conventional breeding programmes. At the present time

gene transfer by *Agrobacterium* is the established method of choice for the genetic transformation of most plant species. It is perceived to have several advantages over other forms of transformation (such as biolistics), including the ability to transfer large segments of DNA with minimal rearrangement and with fewer copies of inserted genes at higher efficiencies with lower cost (see reviews by Hansen and Wright 1999; Hiei et al. 1997; Gheysen et al. 1998; Shibata and Liu 2000). In addition, *Agrobacterium* transformation may facilitate the removal of plant selectable marker genes by segregation (Komari et al. 1996; Matthews et al. 2001; Miller et al. 2002). These are important considerations, particularly when creating genetically manipulated lines in crop species for field testing, when the presence of unnecessary DNA and transgene arrangement/copy number are scrutinised as part of the regulatory processes. With the notable exception of Arabidopsis, in which a variety of effective germ-line transformation methods have been developed (e.g. Clough and Bent 1998), *Agrobacterium* transformation in other plant species relies on a tissue-culture phase. Cereal species, particularly wheat, have lagged behind dicots in their ability to be transformed by *Agrobacterium* and remain relatively genotype-dependent. The technology is the most efficient and shows the least genotype limitation in rice, with transformation possible in Japonica (Hiei et al. 1994), Javanica (Dong et al. 1996) and Indica varieties (Rashid et al. 1996). In maize and barley, *Agrobacterium* transformation has been achieved mainly in genotypes selected for their good response in tissue culture [for example, the maize inbred line A188 (Ishida et al. 1996) and in barley cvs. Golden Promise (Tingay et al. 1997), Dissa (Wu et al. 1998, 1999) and Schooner (Wang et al. 2001)]. Although there has also been progress in wheat (Cheng et al. 1997; Weir et al. 2001), it has been confined mainly to a few responsive varieties, and published methods have proved difficult to follow. In the investigation reported here, we examined six major variables influencing T-DNA delivery and the regeneration of fertile adult plants, and investigated each in one or more wheat varieties. The resulting consolidated

Communicated by W. Harwood

H. Wu · C. Sparks · B. Amoah · H.D. Jones (✉)
CPI Division, Rothamsted, Harpenden, AL5 2JQ UK
e-mail: huw.jones@bbsrc.ac.uk
Tel.: +44-158-2763133

protocol was used to generate 44 transgenic wheat plants in two winter wheat varieties.

Materials and methods

Donor material and tissue culture

A range of *Triticum aestivum* (L.) germplasm (spring wheats: *Bob-white*, *Canon*; winter wheats: *Florida*, *Cadenza*) was grown in a controlled environment room at 18–20/10–14°C (day/night) under a 16/8-h (light/dark) photoperiod with light supplied by 400 W sodium lamps at an irradiance of approximately 750 $\mu\text{E s}^{-1} \text{m}^{-2}$, with 50–70% relative air humidity. When appropriate, vernalisation was carried out for 8 weeks at 4°C for the winter varieties. Spikes were collected at 12–14 days post-anthesis. Immature caryopses were removed from the spikelets, surface-sterilised first with 70% ethanol for 1 min, followed by 10% (v/v) Domestos (Lever) for 15 min and then rinsed five times with copious amounts of sterilised distilled water. Immature embryos (IEs) were isolated under aseptic conditions, either intact or after removal of the entire axis. Immature embryos ranged in length from 0.8 mm to 2.0 mm, or as otherwise indicated. The media used for inoculation, co-cultivation and induction were based on Cheng et al. (1997) (Table 1). Silwet L-77 (Lehle seeds, USA) was added during inoculation at a concentration of 0.01% unless otherwise specified. Cultures were incubated at 24–25°C in the dark.

Agrobacterium tumefaciens strain and plasmid vectors

A. tumefaciens strain AGL1 was used for all experiments. The plasmid combination pAL154 and pAL156 was based on the pSoup/pGreen series (Hellens et al. 2000). The pSoup-derived plasmid (pAL154) contained the 15-kb Komari fragment and functioned as a helper plasmid providing replication functions in *trans* for pAL156. The pGreen-based plasmid (pAL156) contained T-DNA incorporating the bar gene and a modified *uidA* gene (coding for β -glucuronidase, GUS) with an intron within the open reading frame (to prevent expression in *Agrobacterium*). Both genes were driven by the maize Ubiquitin1 promoter plus ubiquitin1 intron (Christensen and Quail 1996). The *bar* gene was adjacent to the left border and *uidA* to the right (Fig 1). *A. tumefaciens* was grown from glycerol stock in MGL liquid medium (Tingay et al. 1997) supplemented with 1 $\mu\text{g/l}$ biotin, 100 mg/l kanamycin, 200 mg/l carbenicillin. The culture was incubated overnight at 27–29°C with shaking (250 rpm), and when the culture was at log phase or immediately thereafter, cells were pelleted by centrifugation at 4.5 g for 10 min and then resuspended in inoculation medium (Table 1) with or without 200 μM acetosyringone. The inoculation density of *Agrobacterium* (OD_{600}) ranged from 1.0 to 2.0.

Inoculation and co-cultivation

Freshly isolated IEs were pre-cultured on co-cultivation medium (Table 1) for 0.5–1 h (unless otherwise specified), then immersed in *Agrobacterium* suspension for 3 h (unless otherwise specified) in the dark. Excess bacteria were removed and the explants transferred, scutella-side-up, without blotting, to fresh co-cultivation medium. Co-cultivation was carried out in darkness at 24–25°C.

Agrobacterium control, plant regeneration and selection

After 3 days (unless otherwise indicated) of co-cultivation in darkness, explants were transferred to induction medium containing 160 mg/l of the antibiotic Timentin [Smithkline Beecham, UK; Ticarcillin:clavulanic acid (15:1)]. All subsequent media plates contained Timentin at this concentration (Timentin was added just before pouring the medium). Explants were maintained intact on induction medium for 3–4 weeks, after which they were transferred to R_{DZ} medium (R medium Table 1 plus 5 mg/l zeatin and 0.1 mg/l 2,4-dichlorophenoxyacetic acid), and moved to light. After a further 3–4 weeks, regenerating tissues were transferred to R_{PPT} medium [R medium containing 2–4 mg/l L-phosphinothricin (PPT)]. Approximately 3–4 weeks later, shoots or plantlets which survived the first round of selection were transferred to a second round and, if necessary, a third round of selection. Plants showing resistance to PPT were subjected to a polymerase chain reaction (PCR) analysis and/or a GUS activity screen. Positive plants were transferred to soil in a containment glasshouse after 8 weeks vernalisation at 4°C if appropriate.

Assay for GUS activity

Transient GUS expression was determined on explants sampled after 2 days (or as indicated otherwise) on induction medium containing Timentin, using the histochemical GUS assay (Jefferson 1987). Explants were incubated overnight at 37°C in buffer containing 1 mM X-Gluc, 100 mM sodium phosphate buffer pH 7.0, potassium 0.5 mM ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100. Blue foci were counted with the aid of a microscope. T-DNA delivery was assessed by counting embryos that had at least one GUS focus and then counting the number of foci per embryo (where appropriate, error bars are given as 1 standard deviation of the mean). To assay for stable expression, we incubated calli, shoots and leaf fragments from regenerating plantlets overnight at 37°C and, if necessary, for a further 1–2 days at 25°C.

Herbicide leaf painting assay

For each plant tested, three approximately equal-sized, healthy-looking leaves from separate tillers were selected for leaf painting. PPT (at 0.2 g/l and 2 g/l) was applied in the form of Challenge

Table 1 Composition of media

Medium type	Composition
Inoculation	MS basal, 500 mg/l glutamine, 100 mg/l casein hydrolysate, 1.95 g/l MES, 10 g/l glucose, 40 g/l maltose, pH 5.8, autoclaved. Picloram (2.2 mg/l), 2 mg/l 2,4-dichlorophenoxyacetic acid, $\pm 200 \mu\text{M}$ acetosyringone and 100 mg/l ascorbic acid added after autoclaving
Co-cultivation	Same as inoculation medium, but solidified with 2 g/l Phytigel
Induction	Same as inoculation medium, but no acetosyringone or glucose and 0.5 mg/l 2,4-D. Add 160 mg/l timentin and 2 g/l Phytigel
Regeneration (R)	L7 macrosalts (250 mg/l NH_4NO_3 , 1.5 g/l KNO_3 , 200 mg/l KH_2PO_4 , 350 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 450 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$); L microsals (13.4 mg/l $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg/l H_3BO_3 , 7.5 mg/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 mg/l $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.025 mg/l $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.025 mg/l $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$); vitamins (10 mg/l thiamine-HCl, 1 mg/l pyridoxine-HCl, 1 mg/l nicotinic acid, 1 mg/l Ca-pantothenate, 1 mg/l ascorbic acid); plus 10 ml/l FeNaEDTA (Sigma), 200 mg/l inositol, 30 g/l maltose (pH 5.7), filter-sterilised, solidified by 5 g/l agar gel

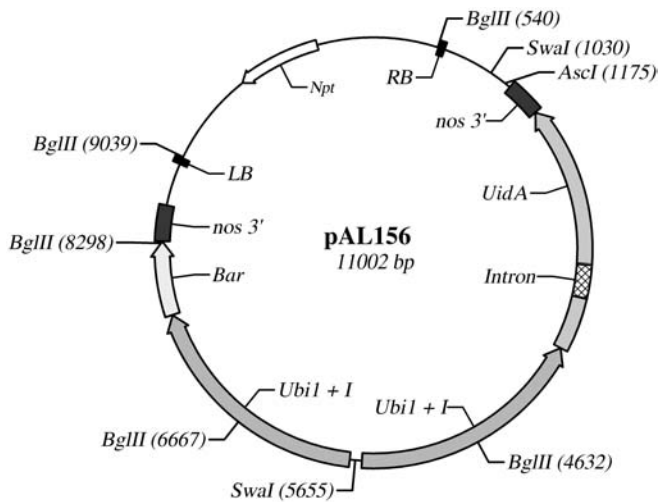


Fig. 1 Map of pAL156. The T-DNA contains the *bar* gene and a modified *uidA* (GUS) gene with an intron within the open reading frame (to prevent expression in *Agrobacterium*). Both genes are driven by the maize Ubiquitin1 (*Ubi1*) promoter plus *Ubi1* intron. The positions of the restriction sites used for the Southern analyses are also marked

herbicide (Unilever, UK) with Tween-20 (0.1%), using a cotton bud to paint the upper surface of the distal half of the selected leaves. Tween-20 (0.1%) alone was used as a control. After 7 days PPT resistance was determined according to the percentage of necrosis suffered over the area painted with the herbicide solution.

Statistical analysis

To compare the number of GUS expression foci per embryo, we used GENSTAT (Payne et al. 1993) to perform an analysis of variance between treatment means (log transformations were carried out to normalise data). For the comparison of embryo size (Table 2), we calculated the standard error of differences of means (sed) and least significant differences of means (lsd) at the 5% level for all pairs of size classes within each variety; the maximum calculated value for each variety is quoted. In the study of the effect of acetosyringone on DNA delivery (Table 4), analysis of variation was performed on the two data sets for each variety and the calculated sed and lsd at the 5% level quoted.

Extraction of DNA, PCR and Southern blot analysis

Genomic DNA was isolated by homogenising 100–200 mg of leaf tissue and extracting essentially according to Stacey and Issac (1994). PCR was used to confirm the presence or the absence of transgenes in the primary transformants and their progeny (Pastori et al. 2001). The primer sequences were: *bar* – 5'-GTCTGCACC-ATCGTCAACC-3' and 5'-GAAGTCCAGCTGCCAGAAAC-3'; *uidA* – 5'-AGTGTACGTATCACCGTTTGTGTGAAC-3' and 5'-ATCGCCGCTTTGGACATACCATCCGTA-3'. Annealing temperatures and the approximate product length were 57°C, 444 bp for *bar*, and 62°C, 1051 bp for *uidA*. At least two replicates were carried out for each PCR analysis. Integration of transforming DNA from the plasmid pAL156 was analysed using Southern hybridisation to probe for the presence of the *uidA* gene. Genomic DNA and pAL156 were each digested with two enzymes (in separate reactions) – *SwaI* and *BglIII* – which cut the plasmid as shown in Fig 1. When integrated into genomic DNA, *BglIII* cuts within the plasmid and again in the genomic DNA beyond the T-DNA right border (>4,092 bp) and *SwaI* excises a 4,625-bp fragment contain-

ing the *uidA* gene. Genomic digests (5–20 µg) or pAL156 plasmid digests (5 µg) were separated by electrophoresis on a 0.9% (w/v) agarose gel run at 20 V for approximately 40 h and transferred by capillary blotting onto positively charged nylon membrane (Roche Diagnostics, Indianapolis, Ind.) following the method of Sambrook et al. (1989). A Digoxigenin-labelled probe was generated using a PCR DIG probe synthesis kit (Roche Diagnostics) with plasmid pAL156 as template DNA and *uidA* primers as detailed above. Hybridisation and detection of the probe was carried out using a non-radioactive, DIG Luminescent Detection Kit for Nucleic Acids (Roche Diagnostics) according to the manufacturer's instructions.

Results

Effect of embryo size on survival, DNA delivery and regeneration

Four size classes of intact immature embryos (i.e. with axis not removed), isolated from four wheat varieties, were used to assess the effect of size on survival, DNA delivery and regeneration. After co-cultivation with *Agrobacterium*, approximately one-third of the small (0.4–0.8 mm) embryos died, while the remainder stopped growing; the larger (>1.5 mm) embryos survived well. This result was consistent across the four varieties tested (Table 2). Transient expression of the *uidA* (GUS) marker gene was used to assess *Agrobacterium*-mediated DNA delivery in the various size groups. Relative to the smaller embryos, more of the larger ones had at least one foci of GUS staining, and the larger embryos also had a disproportionately higher number of foci per embryo (Fig. 3A–D). With two exceptions, the mean number of foci per embryo for any particular size class was significantly different (at the 95% level) from any other size class in the same variety (Table 2). The two exceptions were between the size classes M and L in var. *Canon* and between L and XL in var. *Florida*. No blue foci were found on explants of any size that had been treated with inoculation medium instead of *Agrobacterium* suspension, which indicated an absence of endogenous GUS activity. The response in tissue culture showed the opposite trend, with the smaller-sized classes showing significantly higher regeneration frequencies. Although this was tested in only one variety (*Florida*), it was significant that all 14 transformed plants from this experiment originated from a single size class (0.8–1.5 mm). The effect of *Agrobacterium* co-cultivation on regeneration was tested, and for each size class a slight reduction was observed relative to the minus-*Agrobacterium* controls (Table 3).

Effect of acetosyringone on DNA delivery

Intact immature embryos of mixed sizes (but typically between 0.8–2.0 mm), isolated from the four wheat varieties, were cultured for 3 days in the presence of *Agrobacterium* cells in media either with or without 200 µM acetosyringone. The addition of acetosyringone to the in-

Table 2 Effect of embryo size on survival and DNA delivery (as indicated by transient GUS expression) in four wheat varieties (*Nd* not determined)

Variety	Size of Embryo ^a	Number of embryos tested ^b	Survival rate (%)	Embryos with GUS foci (%)	Mean GUS foci/ embryo	Mean log GUS foci/ embryo ^c
<i>Bobwhite</i>	S	52	67.3	82.6	12.5	2.306
	M	32	87.5	92.9	23.4	2.902
	L	27	100	100	50.8	3.746
	XL	30	100	100	97.0	4.473
						sed=0.2010 lsd=0.4123
<i>Cadenza</i>	S	65	66.2	21.1	5.9	1.656
	M	103	92.2	88.6	16.2	2.533
	L	73	97.3	94.4	30.4	3.078
	XL	35	100	91.4	50.7	3.691
						sed=0.2125 lsd=0.4385
<i>Canon</i>	S	59	69.5	78.6	14.5	2.388
	M	84	96.4	90.5	40.4	3.472
	L	54	100	100	50.9	3.750
	XL	47	100	100	109.2	4.568
						sed=0.2278 lsd=0.4739
<i>Florida</i>	S	65	Nd	81.5	12.0	2.252
	M	59	Nd	96.6	31.1	3.261
	L	44	Nd	100	78.6	4.343
	XL	33	Nd	100	101.4	4.491
						sed=0.1244 lsd=0.2566

^a Freshly isolated, intact immature embryos were grouped into four size classes: S, 0.4–0.8 mm; M, 0.8–1.5 mm; L, 1.5–2.2 mm; XL, larger than 2.2 mm

^b Between 30 and 100 embryos for each size class were used. Those that survived *Agrobacterium* co-cultivation were assayed for GUS activity

^c Analysis of variance was performed on log-transformed data of GUS foci per embryo. For each variety, the standard error of differences (sed) and least significant differences (5% level) (lsd) were calculated for all pairs of size classes and the maximum calculated figure quoted

Table 3 Effect of embryo size (see Table 2 on embryogenesis, regeneration and stable transformation efficiency in wheat var. *Florida* after *Agrobacterium* co-cultivation (*NA* not applicable)

	Size of embryo	Number of embryos tested	Frequency of regeneration (%)	Number of transformed plants
After <i>Agrobacterium</i> co-incubation	S	286	73.1	0
	M	497	62.4	14
	L	300	24.0	0
	XL	100	1.0	0
Controls with no <i>Agrobacterium</i>	S	74	87.8	NA
	M	67	74.6	NA
	L	60	40.0	NA
	XL	58	15.5	NA

oculation and co-cultivation media increased the efficiency of T-DNA delivery, both in terms of the number of embryos that displayed at least one focus of GUS staining on the scutellum surface and the average number of GUS foci per explant (Table 4). The effect of acetosyringone in the medium on the number of GUS foci per explant was statistically significant at the 95% level in all varieties, but was particularly marked in *Cadenza* and *Florida*. This effect was less obvious in vars. *Canon* and *Bobwhite*, which showed higher levels of T-DNA delivery, even in the absence of acetosyringone. The presence of acetosyringone also enhanced GUS expression in vars. *Imp*, *Riband*, *Buster* and *L88-31* (data not shown). In the absence of acetosyringone, some GUS expression was still evident in all varieties.

Effect of Silwet L-77 on embryo survival, callus induction and DNA delivery

The surfactant Silwet L-77 has been shown to improve the *Agrobacterium*-mediated transformation efficiency of floral-dip methods in *Arabidopsis* (Clough and Bent 1998) and spring wheat var. *Bobwhite*, (Cheng et al. 1997). The effect of adding Silwet L-77 during the inoculation of winter wheat embryos (var. *Florida*) with *Agrobacterium* as well as the mortality, T-DNA delivery and subsequent callus induction were measured. Intact IEs 0.8–2.0 mm in size were inoculated in the presence of 0–0.1% Silwet L-77 (Fig. 2). At low concentrations of Silwet L-77, embryos swelled normally and developed a creamy colour (these embryos were described as ‘survivors’). At higher concentrations, embryos failed to swell, turned pale white and did not produce callus during subsequent tissue culture. At Silwet L-77 concentrations greater than 0.06% there was no embryo survival, while

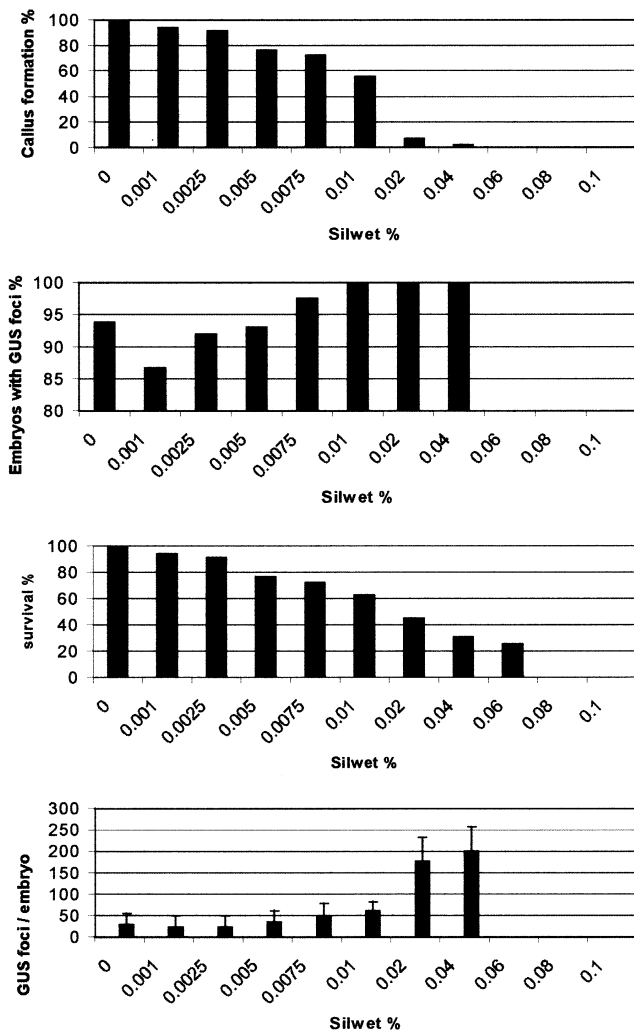


Fig. 2 Effect of Silwet L-77 concentration on embryo survival, callus induction and DNA delivery in wheat var. *Florida*. The survival of 40–90 embryos per Silwet L-77 concentration was assessed 3 days after *Agrobacterium* inoculation. Callus-forming potential was assessed after a further 2 weeks on induction medium. T-DNA delivery was assessed by counting embryos that had at least one GUS focus and by counting the foci per embryo (*error bars* represent one standard deviation of mean)

callus formation was severely compromised at 0.02%. DNA delivery was improved at higher Silwet L-77 concentrations, presumably due to the reduction in surface tension allowing better penetration of bacteria into the plant tissues. However, at those Silwet L-77 concentrations where this effect was most marked (0.02–0.04%), phytotoxicity of the surfactant killed embryos and/or prevented callus induction. In subsequent experiments Silwet L-77 was used at a concentration of 0.01%.

Effect of pre-culture, inoculation time and length of co-cultivation on explant survival, DNA delivery and regeneration

The duration of IE pre-culture and *Agrobacterium* inoculation/co-cultivation periods were all found to affect explant survival, transient GUS expression and response in tissue culture (Table 5). Prior to inoculation with *Agrobacterium*, IEs were left to pre-culture on co-cultivation medium for 0 h to 72 h. Although the IEs that were pre-cultured for less than 1 h had a slightly lower survival rate, the shorter pre-culture times resulted in higher transient GUS expression. After pre-culture, IEs were immersed in *Agrobacterium* suspension between 0 h and 5 h. Transient GUS expression increased with length of inoculation time, but both survival rate and regeneration capacity were dramatically reduced at the longer immersion periods. IEs were cultured for 1–5 days on a co-cultivation medium, then *Agrobacterium* cells were killed or inhibited with antibiotic. The longer co-cultivation times reduced the capacity of the IEs to form embryogenic callus and regenerate. In subsequent experiments in which transgenic plants were generated, the pre-culture time was between 0.5 h and 1 h, the inoculation time was 3 h and the co-cultivation period was 3 days.

Plant regeneration and selection

At the outset of this investigation, the length of time after *Agrobacterium* inoculation necessary for *bar* gene expression to confer an ability to grow on PPT was unknown. Initially, embryogenic callus, shoots and other regenerating tissues were sacrificed at 4–8 weeks after *Agrobacterium* inoculation in order to test for GUS activity. The frequency of GUS-stained structures in the four varieties tested ranged from 2.2% to 16.7% (Table 6, Fig. 3), thereby confirming successful *uidA* gene integration and the development of some regenerative tissue. In subsequent experiments, shoot production was allowed to proceed on regeneration medium with no selection agent present. After 3–4 weeks, plantlets were then transferred onto R medium supplemented with PPT (Table 1). Selection was carried out with 2–4 mg/l PPT for one to three rounds of 3–4 weeks each (Fig. 3J). Different varieties showed different levels of tolerance to late selection on PPT, with *Cadenza* showing more tolerance than *Florida*. Larger shoots survived very well during the first round of selection by rooting vigorously but were unable to last longer than 4 weeks if they were not transformed. Eventually the first leaf of these plantlets stopped growing and started to show signs of bleaching and desiccation, which then extended to the lower leaves. The roots turned brown, then stopped growing. To eliminate escapes and to gain insight into the efficacy of PPT selection, we carried out GUS assays on leaf pieces and root tips of surviving plantlets during the second round of selection. GUS-positive plantlets were considered to be putative positive transgenics and were

Table 4 The effect of acetosyringone on DNA delivery as measured by transient GUS expression. Intact immature embryos of mixed size (but typically between 0.8–2.0 mm) isolated from the four wheat varieties were cultured for 3 days in the presence of *Agrobacterium* cells, in media either with (+) or without (–) 200 μ M acetosyringone

Variety	Aceto-syringone	Number of embryos tested	Embryos with GUS foci (%)	Mean GUS foci/embryo	Mean log GUS foci/embryo ^a
<i>Bobwhite</i>	–	73	52.1	13.9	2.415
	+	88	76.1	28.8	3.050 sed=0.1737 lsd=0.3450
<i>Cadenza</i>	–	38	28.9	6.1	1.666
	+	31	93.5	26.7	3.006 sed=0.2674 lsd=0.5419
<i>Canon</i>	–	59	62.8	22.0	2.600
	+	132	92.4	46.0	3.654 sed=0.3214 lsd=0.6474
<i>Florida</i>	–	88	33.0	6.7	1.857
	+	72	91.7	39.0	3.477 sed=0.2296 lsd=0.4665

^a Analysis of variance was performed on log-transformed GUS foci per embryo data. For each variety, the standard error of differences of means (sed) and least significant difference of means (lsd) (5% level) were calculated

Table 5 Effect of pre-culture, inoculation time and length of co-cultivation on explant survival, DNA delivery and regeneration. Intact immature embryos of mixed size (but typically between 0.8–2.0 mm) isolated from var. *Florida* were cultured in the presence of *Agrobacterium* cells, with 200 μ M acetosyringone. When not the experimental variable, pre-culture time was less than 0.5 h, inoculation time was 3 h and the co-cultivation period was 3 days (NA not applicable)

	Time	Number of Explants	Survival rate (%)	Percentage immature embryos with GUS foci	Frequency of regeneration (%)
Pre-culture time (h)	No <i>Agrobacterium</i>	14	100	NA	71.4
	0	240	71.6	94.4	16.7
	0.5	74	87.8	90.0	23.3
	1.0	83	96.4	91.9	33.3
	1.5	73	93.2	90.0	36.1
	2.0	72	98.6	87.1	18.5
	3.0	167	98.8	87.3	17.2
	24	160	100	32.6	15.3
	72	123	100	8.3	14.1
	Inoculation time (h)	0	14	100	0
0.25		108	100	69.4	43.3
0.5		116	97.4	72.2	45.6
1		124	94.4	76.7	44.7
2		108	91.7	88.6	26.6
3		92	77.2	94.1	25.0
5		93	55.9	100.0	19.2
Co-cultivation time (days)	1	102	76.5	80.0	52.8
	2	112	70.5	89.1	26.5
	3	107	78.5	89.7	23.2
	4	131	84.7	96.9	21.4
	5	73	75.3	96.6	11.1

transferred to soil when a good root system had been established. Other plantlets surviving PPT selection were transferred after the third round of selection. Although labour-intensive, the combination of PPT selection and GUS assay seemed to work very well with minimum escapes and a shortened selection period for some plants.

Analysis of transgenic plants

Molecular analysis of the 44 plants that were identified by GUS expression or had survived selection and were transferred to soil revealed that 38 were PCR-positive for both *bar* and *uidA*, three were positive for *bar* only and another three were PCR-positive for *uidA* only

(Table 7). The plant genomic DNA preparations were tested and confirmed negative for contaminating DNA originating from *Agrobacterium* by PCR using the universal primer set based on the *VirC* operon as described by Sawada et al. (1995). All 41 plants that were PCR-positive for *bar* were also positive in the PPT leaf painting assay for *bar* expression. Of the 41 plants that were PCR-positive for the *uidA* transgene, GUS expression (by histochemical assay) was detectable in only 35. Studies of transgene inheritance and segregation are currently underway. Of those lines already tested, the majority (9) displayed a clear 3:1 inheritance ratio of the *uidA* trait in the T₁ generation. The remaining lines (4) showed a skewed ratio with a lower number of nulls than would be expected from a 3:1 ratio. Southern analysis of

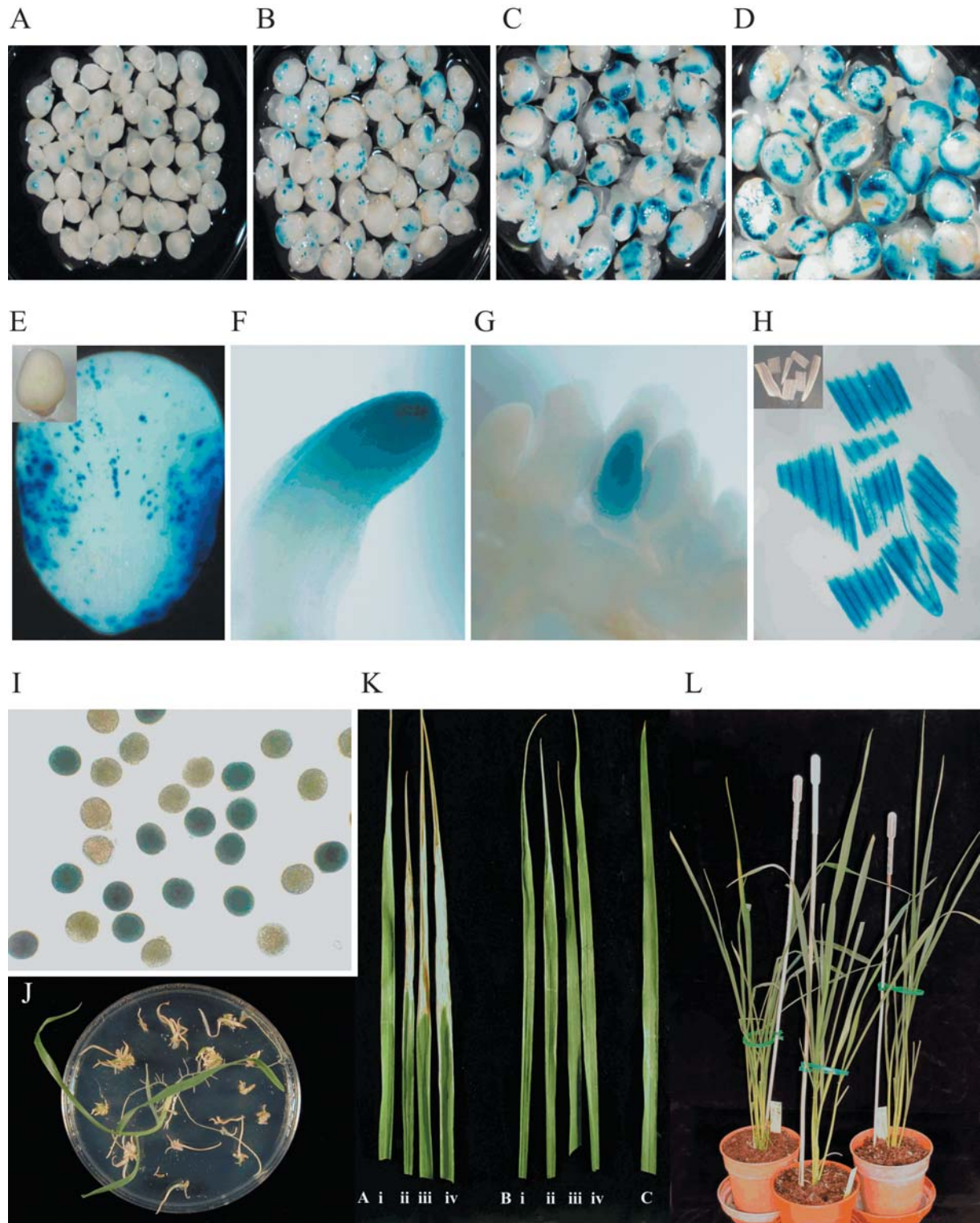


Fig. 3 A–D *Agrobacterium*-mediated transient expression of GUS in four size classes of immature wheat embryos (var. *Florida*): A small (0.4–0.8 mm), B medium (0.8–1.5 mm), C large (1.5–2.2 mm), D extra-large (>2.2 mm). E Close-up of scutellum surface showing individual foci of transient GUS expression (*inset* shows negative control). F, G Evidence of stable transformation in developing shoots in tissue culture, expressing GUS after inoculation and co-cultivation with *Agrobacterium*. H GUS assays of leaf samples from a transgenic wheat plant (*inset* shows negative control). I Pollen from a transgenic plant showing a 1:1 ratio of GUS expression. J Plantlet transformed with the *bar* gene surviving late

selection on PPT while non-transformed shoots died. K Expression of *bar* was confirmed by leaf-painting. PPT (2 g/l) was applied in the form of Challenge herbicide with Tween-20 surfactant (0.1%) to the terminal 7–10 cm of selected leaves: A leaves from non-transformed plants – *i* Tween-only (no PPT), *ii–iv* typical leaves from three non-transformed plants; B leaves from *Agrobacterium*-transformed plants – *i* Tween-only (no PPT), *ii–iv* typical leaves from three transformed plants; C non-transformed control with no application. L Morphologically normal transformed plants growing in soil

Table 6 Stable GUS expression events in regenerating tissue culture samples from four wheat varieties after *Agrobacterium* co-cultivation

Variety	Replicate	Number of immature embryos tested	Number of immature embryos giving GUS-positive tissues	Frequency (%)
<i>Bobwhite</i>	Ag92	38	1	2.6
	Ag102	36	6	16.7
<i>Cadenza</i>	Ag121	132	8	6.1
<i>Canon</i>	Ag102	44	3	6.8
<i>Florida</i>	Ag77	194	5	2.6
	Ag88	275	9	3.3
	Ag90	181	21	11.6
	Ag91	26	1	3.8
	Ag92	183	4	2.2
	Ag93	140	5	3.6
	Ag94	183	5	2.7

genomic DNA isolated from three primary transformants (Fig 4) showed simple, low-copy-number integration of the *uidA* gene. *SwaI* released a fragment of the expected size (4,625 bp) in the pAL156 control and all of the plants tested. *BglIII*, which cuts within the T-DNA and again in the plant genomic DNA, gave bands of approximately 4.9 kb in plants C2.3 and C2.8, and approximately 5.1 kb in plant F3.1. Plants C2.3 and C2.8 originated from the same experiment, and the size similarity of the *BglIII*-released bands indicates that these two may be clones.

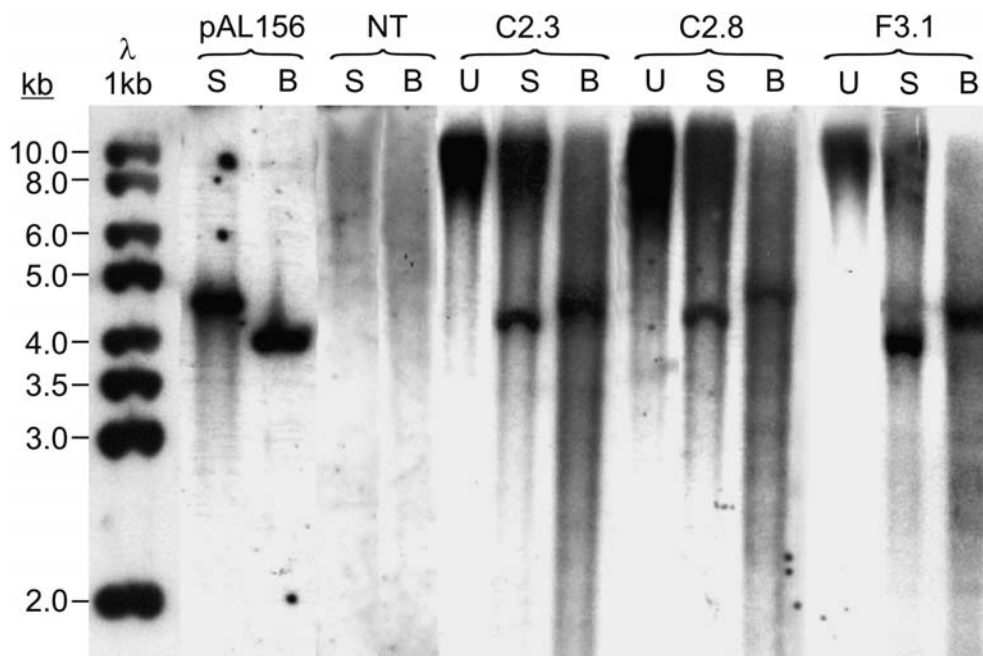
Discussion

The development of reliable transformation protocols for recalcitrant species depends on the ability to deliver intact DNA molecules into the nuclear genome of regenerable cells and to recover fertile adult plants from tissue

Table 7 Summary of transformation efficiencies and marker gene expression in adult transgenic plants after co-cultivation with *Agrobacterium* in two winter wheat varieties – *Florida* (F) and *Cadenza* (C)

Variety/ experiment no.	Number of immature embryos	Frequency of regeneration (%)	T ₀ -positive ^a plants	Transformation efficiency (%)	PCR of marker genes		Expression of marker genes	
					<i>uidA</i> +	<i>Bar</i> +	GUS	PPT ^R
F/96	89	14.6	1	1.1	1	0	1	0
F/105	157	45.9	4	2.5	4	4	4	4
F/107	359	17.0	1	0.3	1	1	1	1
F/108	353	63.7	2	0.6	2	2	2	2
F/110	605	49.1	7	1.2	7	5	5	5
F/111	323	62.5	9	2.8	7	9	3	9
F/113	197	68.0	5	2.5	4	5	4	5
F/117	300	57.0	10	3.3	10	10	10	10
C/112	204	42.6	5	2.5	5	5	5	5

^a Plants containing at least one transgene as determined by PCR

Fig. 4 Southern blot of one untransformed and three T₀ transformed plants (C2.3 and C2.8: wheat var. *Cadenza* and F_{3.1}: var. *Florida*). A DIG-labelled *uidA* probe was hybridised to 5 pg of plasmid or 10 µg plant genomic DNA, uncut or cut with either *SwaI* (S) or *BglIII* (B)

culture. The choice of starting material (explant) has proved to be crucial in successful *Agrobacterium*-mediated wheat transformation. There have been attempts with limited success using embryogenic calli (Guo et al. 1998; McCormac et al. 1998), aged calli (Xia et al. 1999), suspension cells (Weir et al. 2001) and immature inflorescences (Amoah et al. 2001) as starting material, but they all failed to produce stable, fertile transgenic plants. The scutellum of cultured immature seed embryos has long been known to be a good regenerable explant source for wheat (Ozias-Akins and Vasil 1982), and there are effective protocols using biolistics for transformation and regeneration of this tissue (reviewed by Barcelo et al. 2001). However, despite considerable interest there are very few publications describing successful *Agrobacterium*-mediated transformation of wheat, and the multiple factors involved have not been fully characterised in the literature. We found several variables that had a marked effect on DNA delivery and regeneration, including embryo size, the length of pre-culture, inoculation and co-cultivation, presence of acetosyringone, concentration of Silwet L-77 and the timing and strength of selection. The effects of particular parameters on survival, T-DNA delivery and regeneration did not always follow the same trend. For example, the ability of IEs to survive the rigors of *Agrobacterium* co-cultivation increased with increasing size, longer pre-culture times, shorter inoculation times and lower Silwet L-77 concentrations, whereas T-DNA delivery markedly increased with shorter pre-culture times, longer inoculation times and higher Silwet L-77 concentrations. The regeneration capacity of IEs generally followed the same trend as survival ability, except for embryo size where it was the opposite. These data emphasise the limitations of transient expression in non-regenerable tissues. It is clear that conditions favouring T-DNA delivery are not necessarily the same as those favouring the recovery of stable transformation events.

The effects of increasing pre-culture time on survival and GUS expression that we observed support the observations of Cheng et al. (1997), who found highly efficient T-DNA delivery in both freshly isolated and 1- to 6-day-old pre-cultured IEs. However, they run counter to those of Weir et al. (2001) who found that a minimum of 3 days of pre-culture was required to see transient GFP expression in IEs. The effect of embryo size on *Agrobacterium*-mediated transient expression, regeneration and transformation efficiency has not been reported previously. However, the data presented here concur with recent results obtained by Rasco-Gaunt et al. (2001) who demonstrated that, after bombardment with submicron gold, embryos 0.5–1.5 mm in size showed better embryogenesis and shoot regeneration than those smaller or larger than that size class. Our conclusion that 0.01% Silwet-L77 is a good compromise between increased T-DNA delivery and reduced survival and callus induction supports the observations of Cheng et al. (1997), who compared GUS expression at four concentrations of Silwet L-77 (0.01–0.5%) and concluded that 0.01–0.02%

was the most effective concentration for stable transformation in wheat var. *Bobwhite*. This is also reflected in floral dip methods of *Arabidopsis* transformation where Silwet L-77 concentrations of 0.005%, 0.05% and 0.1% produced higher transformation rates than did a vacuum, but the higher levels caused necrosis of plant tissues; therefore, a final concentration of 0.05% was chosen (Clough and Bent 1998). Phenolic inducers such as acetosyringone appear to act alongside specific temperature requirements and an acidic environment to promote the expression of *Agrobacterium vir* genes, the products of which mediate T-DNA delivery. We have shown that the presence of 200 μ M acetosyringone in the medium markedly increased T-DNA delivery without losing the regeneration potential of the IEs in the four wheat varieties tested. This supports earlier works in a number of species showing that the addition of acetosyringone during pre-culture and co-cultivation increases the number of transformed cells in the target tissues (carrot, Guivarch et al. 1993; apple, James et al. 1993; tomato, Joao and Brown 1993; wheat, Weir et al. 2001). In wheat inflorescence tissue, 200 μ M acetosyringone gave optimum transient gene expression, with higher concentrations being toxic and lower concentrations less effective (Amoah et al. 2001). The application of late PPT selection during the second round of regeneration combined with leaf GUS expression screening proved to be effective in identifying transgenic plants. Our transformation efficiencies ranged between 0.3% and 3.3%, which was comparable with those reported elsewhere (1.6%. Cheng et al. 1997; 1.8%, Weir et al. 2001). Complete analyses of transgene insertion and segregation patterns are underway and may reveal differences between biolistic and *Agrobacterium*-mediated transformations.

Acknowledgements We are grateful to Alan Todd (Biomathematics unit, AEN, Rothamsted) for statistical assistance and to Alison Harvey (John Innes Centre) for supplying plasmids pAL156 and pAL154. Rothamsted receives grant-aided support from the Biotechnological and Biological Sciences Research Council UK. HW was sponsored by the UK Ministry of Agriculture, Fisheries and Food (now DEFRA). BKA was a Rothamsted International Fellow.

References

- Amoah BK, Wu H, Sparks C, Jones HD (2001) Factors influencing *Agrobacterium*-mediated transient expression of uidA in wheat inflorescence tissue. *J Exp Bot* 52:1135–1142
- Barcelo P, Rasco-Gaunt S, Thorpe C, Lazzeri PA (2001) Transformation and gene expression. In: Callow JS (ed) *Advances in botanical research incorporating advances in plant pathology*, vol 34, pp. 59–126
- Cheng M, Fry JE, Pang SZ, Zhou HP, Hironaka CM, Duncan DR, Conner TW, Wan YC (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol* 115: 971–980
- Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res* 5:213–218
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16:735–743

- Dong JJ, Teng WM, Buchholz WG, Hall TC (1996) Agrobacterium-mediated transformation of Javanica rice. *Mol Breed* 2: 267–276
- Gheysen G, Angenon G, Van Montague M (1998) Agrobacterium-mediated plant transformation: a scientifically intriguing story with significant application. In: Lindsey K (ed) *Transgenic plant research*. Harwood Academic Press, The Netherlands, pp 1–33
- Guivarch A, Caissard JC, Brown S, Marie D, Dewitte W, Vanonckelen H, Chriqui D (1993) Localization of target-cells and improvement of Agrobacterium-mediated transformation efficiency by direct acetosyringone pretreatment of carrot root disks. *Protoplasma* 174:10–18
- Guo G, Maiwald F, Lorenzen P, Steinbiss H (1998) Factors influencing T-DNA delivery into wheat and barley cells by Agrobacterium tumefaciens. *Cereal Res Commun* 26:15–21
- Hansen G, Wright MS (1999) Recent advances in the transformation of plants. *Trends Plant Sci* 4:226–231
- Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Mol Biol* 42:819–832
- Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza-sativa* L.) mediated by Agrobacterium and sequence-analysis of the boundaries of the T-DNA. *Plant J* 6:271–282
- Hiei Y, Komari T, Kubo T (1997) Transformation of rice mediated by Agrobacterium tumefaciens. *Plant Mol Biol* 35:205–218
- Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by Agrobacterium tumefaciens. *Nat Biotechnol* 14:745–750
- James DJ, Uratsu S, Cheng JS, Negri P, Viss P, Dandekar AM (1993) Acetosyringone and osmoprotectants like betaine or proline synergistically enhance Agrobacterium-mediated transformation of apple. *Plant Cell Rep* 12:559–563
- Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5:387–405
- Joao KHL, Brown TA (1993) Enhanced transformation of tomato co-cultivated with Agrobacterium-tumefaciens c58c1rifr/pgsfr1161 in the presence of acetosyringone. *Plant Cell Rep* 12:422–425
- Komari T, Hiei Y, Saito Y, Murai N, Kumashiro T (1996) Vectors carrying two separate T-DNAs for co-transformation of higher plants mediated by Agrobacterium tumefaciens and segregation of transformants free from selection markers. *Plant J* 10:165–174
- Matthews PR, Wang MB, Waterhouse PM, Thornton S, Fieg SJ, Gubler F, Jacobsen JV (2001) Marker gene elimination from transgenic barley, using co-transformation with adjacent 'twin T-DNAs' on a standard Agrobacterium transformation vector. *Mol Breed* 7:195–202
- McCormac AC, Wu H, Bao M, Wang Y, Xu R, Elliott MC, Chen DF (1998) The use of visual marker genes as cell-specific reporters of Agrobacterium-mediated T-DNA delivery to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). *Euphytica* 99:17–25
- Miller M, Tagliani L, Wang N, Berka B, Bidney D, Zhao ZY (2002) High-efficiency transgene segregation in co-transformed maize plants using an Agrobacterium tumefaciens 2 T-DNA binary system. *Transgenic Res* 11:381–396
- Ozias-Akins P, Vasil IK (1982) Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat – evidence for somatic embryogenesis. *Protoplasma* 110:95–105
- Pastori GM, Wilkinson MD, Steele SH, Sparks CA, Jones HD, Parry MAJ (2001) Age-dependent transformation frequency in elite wheat varieties. *J Exp Bot* 52:857–863
- Payne RW, Lane PW, Digby PGN, Harding SA, Leech PK, Morgan GW, Todd AD, Thompson R, Tunnicliffe Wilson G, Welham SJ, White RP (1993) *genstat 5*, release 3. Reference manual. Clarendon Press, Oxford, UK
- Rasco-Gaunt S, Riley A, Cannell M, Barcelo P, Lazzeri PA (2001) Procedures allowing the transformation of a range of European elite wheat (*Triticum aestivum* L.) varieties via particle bombardment. *J Exp Bot* 52:865–874
- Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by Agrobacterium in indica rice. *Plant Cell Rep* 15:727–730
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Sawada H, Ieki H, Matsuda I (1995) PCR detection of Ti and Ri plasmids from phytopathogenic Agrobacterium strains. *Appl Environ Microbiol* 61:828–831
- Shibata D, Liu YG (2000) Agrobacterium-mediated plant transformation with large DNA fragments. *Trends Plant Sci* 5:354–357
- Stacey J, Issac P (1994) Isolation of DNA from plants. In: Issac P (ed) *Methods in molecular biology – protocols for nucleic acid analysis by non-radioactive probes*. Humana Press, Totowa, pp 9–15
- Tingay S, McElroy D, Kalla R, Fieg S, Wang MB, Thornton S, Brettell R (1997) Agrobacterium tumefaciens-mediated barley transformation. *Plant J* 11:1369–1376
- Wang M, Abbott DC, Upadhyaya NM, Jacobsen JV, Waterhouse PM (2001) Agrobacterium tumefaciens-mediated transformation of an elite Australian barley cultivar with virus resistance and reporter genes. *Aust J Plant Physiol* 28:149–156
- Weir B, Gu X, Wang MB, Upadhyaya N, Elliott AR, Brettell RIS (2001) Agrobacterium tumefaciens-mediated transformation of wheat using suspension cells as a model system and green fluorescent protein as a visual marker. *Aust J Plant Physiol* 28: 807–818
- Wu H, McCormac AC, Elliott MC, Chen DF (1998) Agrobacterium-mediated stable transformation of suspension cultures of barley (*Hordeum vulgare* L.). *Plant Cell Tissue Organ Cult* 54: 161–167
- Wu H, McCormac AC, Elliott MC, Chen DF (1999) Agrobacterium-mediated stable transformation of barley (*Hordeum vulgare* L.). In: Altman A, Ziv M, Izhar S (eds) *Plant biotechnology and in vitro biology in the 21st century*. Proc 9th Int Assoc Plant Tissue Cult Biotechnol. Kluwer, Dordrecht, pp 231–234
- Xia G, Li Z, He C, Chen H, Brettell R (1999) Transgenic plant regeneration from wheat (*Triticum aestivum* L.) mediated by Agrobacterium tumefaciens. *Acta Phytophysiol Sin* 25:22–28