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Factors influencing *Agrobacterium*-mediated transient expression of *uid*A in wheat inflorescence tissue

B.K. Amoah, H. Wu, C. Sparks and H.D. Jones[1]

Biochemistry and Physiology Department, IACR-Rothamsted, Harpenden AL5 2JQ, UK

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Abstract

A critical step in the development of *Agrobacterium tumifaciens*-mediated transformation is the establishment of optimal conditions for T-DNA delivery into tissue from which whole plants can be regenerated. The efficient transformation of inflorescence tissue from 'Baldus', a commercial wheat variety, using the *Agrobacterium* strain AGLI harbouring the binary vector pAL156 is reported here. The effects of various factors on delivery and the transient expression of the *uid*A gene were studied including the duration of preculture, vacuum infiltration, the effect of sonication treatments, and *Agrobacterium* cell density. Optimal T-DNA delivery (as measured by *uid*A activity) was obtained from inflorescence tissues precultured for 21 d and sonicated. Increasing *Agrobacterium* cell density, the duration of inoculation/co-cultivation, and vacuum pressure, up to a threshold, increased *uid*A expression. The investigation of factors that influence T-DNA delivery is an important first step in the utilization of *Agrobacterium* in the transformation of immature wheat inflorescence tissue.

Key words: *Agrobacterium tumefaciens*, wheat inflorescence, GUS, transformation, commercial variety.

Introduction

The notion that defined genes could be transferred between organisms was born out of the discovery that bacteria of the genus *Agrobacterium* possessed the ability to transfer a segment of their DNA into plant species that were susceptible to the bacteria, and that the integration of this DNA into the plant genome and the expression thereof was responsible for the crown gall phenotype (see review by Zupan and Zambryski, 1997). The implications for crop improvement and also as a tool for studies in basic plant biology have since been noted (Potrykus, 1990). Indeed, successful gene transfer techniques that exploit this natural gene transfer system of *Agrobacterium* sp. soon became widely available for the routine transformation of crops such as cotton, lettuce, celery, flax, and potato (Grant *et al*., 1991). However, it was also noted that the graminaceous monocots, which include such major food crops as wheat, maize and rice, were recalcitrant in this system because this group of crop plants was not naturally susceptible to *Agrobacterium* sp. Other methods were, therefore, sought by which this group of crops could be transformed. Several have been investigated, with varying levels of success, including the electrical induction of cells to aid their reception of foreign DNA (Rhodes *et al*., 1989), electroporation of tissues with DNA (Shimamoto *et al*., 1989), microinjection of DNA into floral tillers (De la Pena *et al*., 1987), DNA uptake by germinating pollen (Luo and Wu, 1988), and by the bombardment of cells and tissues with DNA-coated particles (Vasil *et al*., 1992). However, research to make the *Agrobacterium*-based transformation method amenable to this group of crops has continued as the system is perceived to possess several advantages over other forms of transformation including (i) the ability to transfer large segments of DNA with minimal rearrangement; (ii) the precise insertion of transgenes resulting in fewer copies of inserted genes; and (iii) simple technology with lower cost. One of the simplest available plant transformation systems involves the infiltration of *Agrobacterium* cells into *Arabidopsis* plants before flowering, and direct selection for rare transformants in the resulting seedling population (Chang *et al*., 1994). However, the features which make this possible, including the small plant size, the rapid generation time and the high seed

[1] To whom correspondence should be addressed. Fax: +44 1582 763010. E-mail: huw.jones@bbsrc.ac.uk

yield are not shared by any economically important plant species. Thus nearly all of the current practical transformation systems, particularly those for cereals, involve the use of tissue cultures at some stage. In recent years the image that monocotyledonous plants are recalcitrant to such approaches, has gradually been shed as one member after another joins the list of plants amenable to *Agrobacterium*-based transformation systems: rice (Raineri *et al*., 1990), maize (Ishida *et al*., 1996), barley (Tingay *et al*., 1997), and wheat (Cheng *et al*., 1997*a*). Success in each case seems to have followed the identification of a model tissue culture system with a high capacity for producing regenerable cells, the optimization of parameters for gene transfer into those cells and tailoring selection and regeneration procedures to recover transgenic plants. To date, success in wheat has been confined to culture-responsive but commercially less relevant model genotypes such as Bobwhite. There is a lack of effective and reproducible protocols for the *Agrobacterium*-mediated transformation and regeneration of commercial wheat varieties.

Immature scutella have been used as the starter explant for most cereals: maize (Schlappi and Hohn, 1992; Ishida *et al*., 1996), barley (Tingay *et al*., 1997) and wheat (Cheng *et al*., 1997*a*; Guang-Min *et al*., 1999; Singh and Chawla, 1999). However, immature inflorescences have also been shown to provide a practical source of young tissues for somatic embryogenesis and plant regeneration in maize, rice, barley, wheat, rye, and tritordeum (Maddock *et al*., 1983; Pareddy and Petolino, 1990; Barcelo *et al*., 1989; Rout and Lucas, 1996). The authors believe this is the first report of transformation in inflorescence tissue from a commercial wheat variety using *Agrobacterium tumefaciens*.

Materials and methods

Plant material

'Baldus' (breeder Cebeco), a commercial spring variety of wheat (*Triticum aestivum*) was used in these studies because of its high regeneration capacity in preliminary experiments (Barro *et al*., 1999). Baldus has hard endosperm and good breadmaking characteristics, and was on the UK recommended list for cereal varieties (NIAB Council) from 1992 to 1997. Cultures were established according to the general procedures outlined previously (Barcelo *et al*., 1994; Barcelo and Lazzeri, 1995; Rasco-Gaunt and Barcelo, 1999). Donor plants were grown in growth chambers where conditions were maintained at 18–20 °C during the day and 16 °C at night and a 16 h photoperiod provided by 400 W HQI lamps (Osram). Tillers were harvested when inflorescences were generally between 0.5–1.0 cm in length. Surface sterilization was performed by sequentially washing the tillers in 70% ethanol for 5 min and then 10% Domestos for 20–25 min, followed by three rinses in sterile distilled water. All procedures that followed the Domestos treatment were conducted under the conditions of a sterile lamina flow hood to ensure tissue sterility. Inflorescences were isolated (Fig. 1a) and then cut transversely into approximately 1 mm sections (Fig. 1b) which were plated on callus induction medium (L7P4-V medium containing 4 mg l^{-1} picloram, 10 mg l^{-1} $AgNO_3$, 5 g l^{-1} agar gel and, unless otherwise stated, 4% maltose).

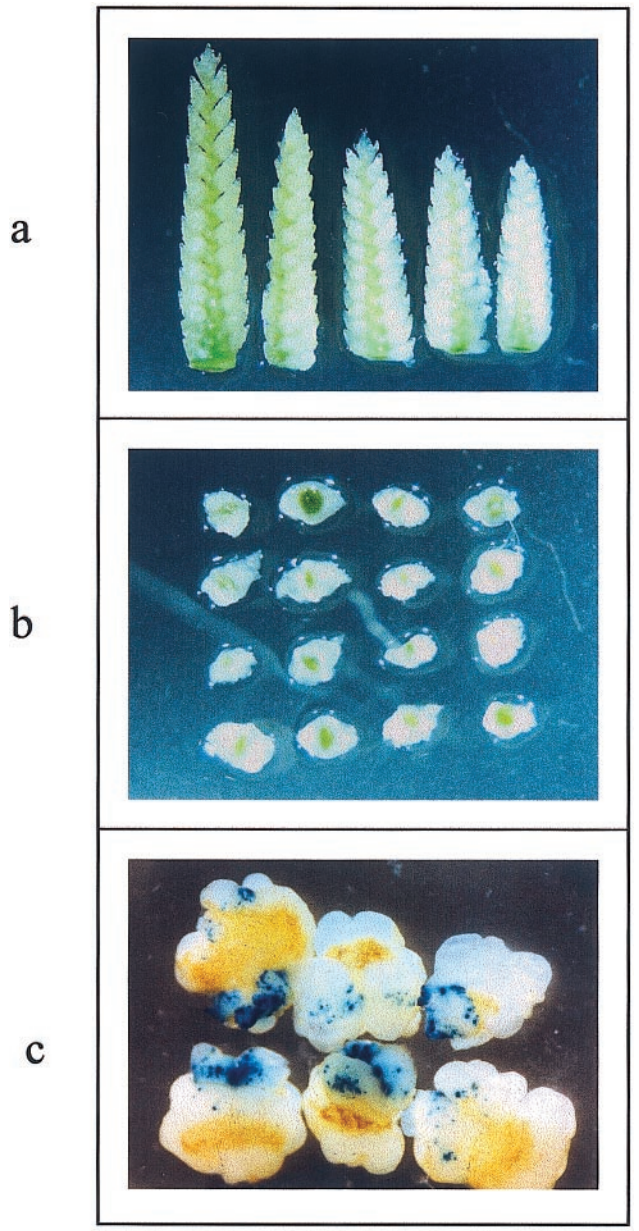

Fig. 1. (a) Isolated wheat inflorescences ranging in length from 0.5–0.8 cm. (b) Approximately 1 mm sections of wheat inflorescence. (c) Transient GUS expression in wheat inflorescence tissue.

Bacterial strains, plasmids and culture conditions

The *Agrobacterium tumefaciens* strain AGL1 containing one of several binary vectors based on the pGreen/pSoup system were supplied by Alison Harvey (JIC). The pGreen plasmid and its derivatives can replicate in *Agrobacterium* only if pSoup, which provides replication factors *in trans* for pGreen, is co-resident in the same strain (Hellens *et al*., 2000) [further information may also be obtained at: http://www.pgreen.ac.uk/plasmid.htm]. The

plasmid pSoup carries a gene that confers resistance to tetracycline in bacteria. pAL155 contains $VirG^{542}$ and pAL154 contains the 15 kb 'Komari' fragment (Komari, 1990). The plasmid pAL156 is a binary vector derived from pGreen. It contains the modified *uid*A gene (with an intron at nt 385 designed to prevent expression in *Agrobacterium*), and *bar*. Both genes are driven by the ubiquitin promoter (plus intron) (Christensen *et al*., 1992). The plasmid pAL186 is a derivative of pAL156 also containing the 15 kb 'Komari' fragment (Table 1). *Agrobacterium* cultures, initiated from glycerol stocks, were grown overnight in MG/L medium (Garfinkel and Nester, 1980) containing 1 μg l^{-1} biotin, 100 mg l^{-1} kanamycin and 200 mg l^{-1} carbenicillin, at 26–27 °C, in the dark, shaking at 250 rpm. Cultures were centrifuged at 5000 rpm for 6 min and the *Agrobacterium* cells resuspended in L7P4-V medium containing 10 g l^{-1} glucose to give OD_{600} of up to 2.0. Acetosyringone was added just prior to inoculation, to a final concentration of 200 μM.

Inoculation, co-cultivation and regeneration

Explants were transferred into 6 cm Petri dishes or 60 ml plastic containers (Sterilin) containing inoculum, usually allowing 4 ml of inoculum per 40 explants, ensuring that all explants were completely submerged. Inoculation was carried out in the dark at 26 °C, for up to 3 h. Inoculum was then pipetted out and infected explants were blotted on sterile Whatman filter paper (grade 1) and then plated onto callus induction medium containing 10 g l^{-1} glucose and acetosyringone at 200 μM. Co-cultivation was carried out in the dark at 26 °C for 2–3 d. After co-cultivation, a proportion of the explants were transferred to regeneration medium with or without selection and induced to form shoots and roots following methods described earlier (Barcelo *et al*., 1994; Barcelo and Lazzeri, 1995; Rasco-Gaunt and Barcelo, 1999).

Sonication

For sonication treatments, explants were transferred into 60 ml plastic bottles containing inoculum at the ratio of 5 ml to 50 explants. The bottles were capped and sonication applied for up to 6 s using a Sonomatic 175D, power output 120 W, frequency 40 kHz (Jencons Scientific Limited, Leighton Buzzard, UK). Inoculation was then allowed to continue in the dark, with or without vacuum infiltration, depending on the design of the experiment.

Table 1. *The properties of the plasmids used in the study (supplied by Alison Harvey JIC)*

pAL154 and pAL155 are both derived from pSoup which functions as a helper plasmid providing replication functions *in trans* for pGreen. pAL156 and pAL186 are derived from pGreen, a T-DNA cloning vector. How these plasmids differ from the parental plasmids are noted. pSoup and pGreen have been described previously (Hellens *et al*., 2000).

Plasmid	Size (bp)	Basic difference(s) from parent plasmid
pSoup	9275	–
pAL154	24332	+ 15 kb Komari fragment*
pAL155	10477	+ $VirG^{542}$
pGreen	3232	–
pAL156	11002	+ [(Gus + Bar)Ubiq + intron]
pAL186	26002	+ [(Gus + Bar)Ubiq + intron] + 15 kb Komari fragment*

*Komari, 1990.

Vacuum infiltration

The vacuum system consisted of a Speedivac 2 (Edwards High Vacuum, Crawley, England) vacuum pump to which a desiccator was attached. Sterile plastic bottles (Sterilin) containing explants immersed in inoculum were placed in the desiccator and the vacuum applied at 762 mm Hg (30 in Hg), for periods up to 3 h. The pump was then switched off and air was slowly let into the desiccator (over a period of 90 s), so as to minimize the damage to the tissues. Inoculum was removed, the explants blotted and plated on callus induction medium as described above. Co-cultivation was in the dark at 26 °C.

Antibiotic washes and GUS assays

Following co-cultivation, explants were washed twice in liquid L7P4-V medium containing 320 mg l^{-1} timentin, blotted on sterile Whatman filter paper and plated onto L7P4-V solid medium containing 160 mg l^{-1} timentin (resting medium), to suppress the growth of the *Agrobacterium*. Dishes were sealed and cultured in the dark at 26 °C for 2–3 d.

T-DNA delivery into inflorescence tissues was determined by histochemical assays for GUS (according to Jefferson, 1987). Explants from the resting stage were transferred into the wells of ELISA plates containing GUS-buffer (1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide [X-Gluc], 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100), allowing up to 700 μl of buffer per 25 explants. The plates were incubated overnight at 37 °C, and at 26 °C for a further 24 h. Explants were then washed once with sterile distilled water and steeped in 70% ethanol overnight to extract any chlorophyll that may be present in the tissues. Explants were then examined under a dissecting microscope and scored for the number of explants producing blue spots (Fig. 1c) per treatment as well as the number of spots per explant.

Results

The transfer of T-DNA is known to be influenced by several factors including plant genotype, type of explant, tissue culture medium, *Agrobacterium* strain and plasmid vector combination, cell density in inoculation medium, and the conditions of inoculation and co-culture. The optimal level of each of these elements needs to be determined for every transformation system. Thus in the absence of any report of the successful transformation of wheat inflorescence tissue using *Agrobacterium*, it was considered important to investigate the effect of some of these factors on T-DNA delivery.

The effect of different vectors on T-DNA delivery

The ability of different vectors, in the same *Agrobacterium* strain, to transfer T-DNA into inflorescence tissues was investigated. GUS activity assays, 2–3 d after co-cultivation, indicated that the pAL155/pAL156 combination, was by far the most efficient at delivering

T-DNA into inflorescence tissues (Table 2). Furthermore, pAL155/pAL156 produced more spots per explant than either pAL154/pAL156 or pSoup/pAL186, and was thus selected for use in all further experimentation.

To facilitate the standardization of the protocols, the effects of some of the basic elements of the culture medium, particularly the concentration of sugar and acetosyringone, on T-DNA delivery were determined in preliminary experiments. In general, higher levels of transient *uid*A expression were obtained from tissues that were cultured on media containing 4% maltose than on media containing 9% maltose. In subsequent experimentation, all media for preculture, co-cultivation and the resting stage contained 4% maltose.

No *uid*A expression was observed when acetosyringone was excluded from the inoculation and co-cultivation media. Discrete blue spots began to be observed at acetosyringone concentrations of 100 μM. Increasing acetosyringone concentration to 200 μM resulted in an increased number of explants producing blue spots as well as increases in the number of spots per explant and the intensity of the spots. When the concentration was increased to 400 μM, however, there were fewer responding explants and fewer, although more intense, spots per explant, indicating a possible harmful effect on T-DNA transfer. This may reflect the observation that whilst acetosyringone is effective at low concentrations in inducing T-DNA transfer, it may act as a bacteriostatic at higher concentrations (Sheng and Citovsky, 1996). Acetosyringone was thus used at 200 μM in all subsequent experiments.

Table 2. *The effect of different vectors in the same* Agrobacterium *strain (AGL1), bacterium cell density and sonication on GUS expression in 21 d precultured wheat inflorescence tissues*

(A) For this experiment, the inoculum OD_{600} was between 1.273 and 1.341, inoculation was by vacuum infiltration (762 mm Hg) for 60 min. (B) AGL1p155/p156 was used for these experiments; sonication was done with explants immersed in inoculum ($OD_{600} = 1.25$), after which inoculation continued for a further 2 h. (C) AGL1p155/p156 was used and inoculation was by vacuum infiltration.

Treatment	Proportion of explants with GUS stain (%)	GUS spots/explant
(A) Vectors		
p154/p156	36/140 (25.7)	704/36 (19.6)
p155/p156	31/60 (51.7)	1047/31 (33.8)
pSoup/p186	13/90 (14.4)	261/13 (20.1)
(B) Duration of sonication (s)		
0	11/40 (27.5)	590/11 (53.7)
2	17/40 (42.5)	782/17 (46.0)
4	19/40 (47.5)	604/19 (31.8)
6	25/40 (62.5)	721/25 (28.8)
(C) OD_{600}		
<1.0	54/145 (37.2)	793/54 (14.7)
1–1.5	36/70 (51.4)	600/36 (16.7)
$>1.5–2.0$	16/70 (22.9)	123/16 (7.7)

The effect of preculture duration on uid*A expression*

Preculture period is defined as the time between when inflorescences are first isolated and cultured, and when the explants are inoculated with *Agrobacterium*. Treatments ranged from freshly isolated explants, to those precultured for up to 39 d. No GUS activity was obtained from freshly isolated inflorescence tissue. Activity was first observed in 7 d precultured material (at less than 2%). However, increasing length of preculture resulted in a dramatic rise (to a peak of 76% at 21 d) in the number of explants showing GUS activity (Fig. 2). Indeed the increase in preculture length from 15 d to 21 d elicited a more than 100% increase in response. Preculture lengths beyond 21 d resulted in decreases in the number of explants showing *uid*A expression (decreasing to 16.7% at 39 d of preculture).

The number of spots per responding explant appeared to follow the same trend as the response frequency (Fig. 2), increasing with the length of preculture to a maximum at 21 d and generally decreasing with further increases in length of preculture till about 29 d, from where there was a slight increase in the number of spots per explant to 39 d.

The effect of vacuum infiltration on GUS expression

Vacuum treatments have been used as an aid in inoculation to infiltrate tissues with *Agrobacterium* in *Arabidopsis* (Clough and Bent, 1998) and other plant species. It has, apparently, been used in the transformation of immature scutellar tissues in wheat (Cheng *et al.*, 1997*b*), but the results were not reported. Experiments were thus designed to investigate the effects of the duration of vacuum treatments on T-DNA delivery into inflorescence tissues. The results (Fig. 3) showed that whilst some *uid*A expression was obtainable without vacuum infiltration, this treatment increased the levels of expression, both in terms of the numbers of explants producing blue spots and the numbers of spots per explant, but only up to 60 min of vacuum treatment. Vacuum in excess of 60 min duration, resulted in fewer numbers of responding explants and also fewer numbers of blue spots per explant, although these spots were usually more discrete, better defined and more intense. It was also observed that vacuum treatments of 2 h and longer resulted in *Agrobacterium* growth around the explants after co-cultivation, and that this growth was more difficult to control at the resting and subsequent stages.

Effect of sonication on transient GUS expression

Sonication has been reported to enhance the efficiency of *Agrobacterium*-mediated transformation substantially (Trick and Finer, 1997). To determine the effect of sonication on T-DNA transfer, 21 d precultured inflorescence tissues were sonicated for 0, 2, 4, and 6 s. The results

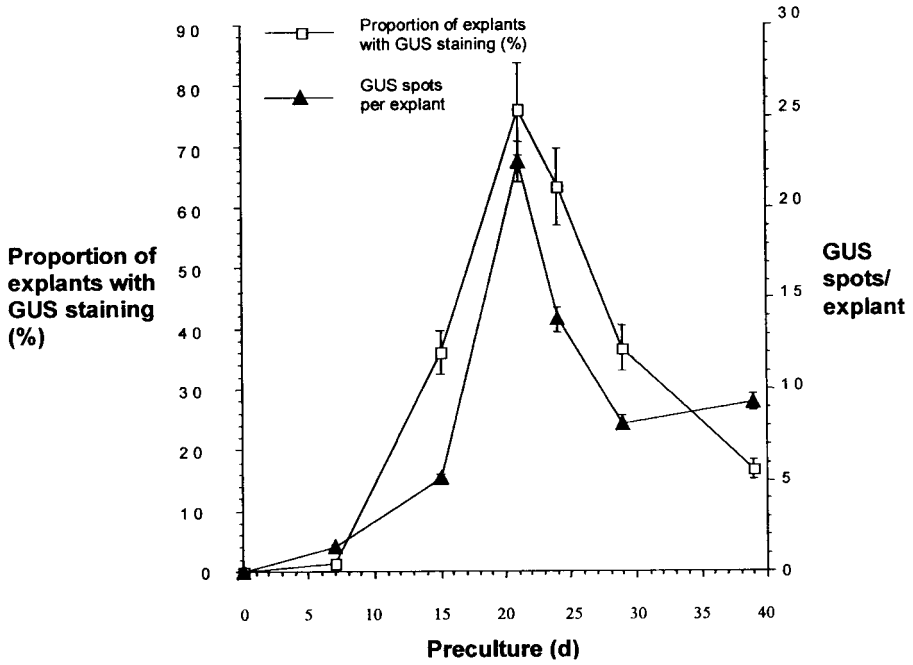

Fig. 2. The effect of length of pre-culture on transient GUS expression. The number of explants showing GUS staining was calculated as a percentage of the total number explants co-incubated for each treatment. The average number of blue spots per explant was also calculated. Data represents mean ± SE of between 25–360 inflorescence explants at each time point.

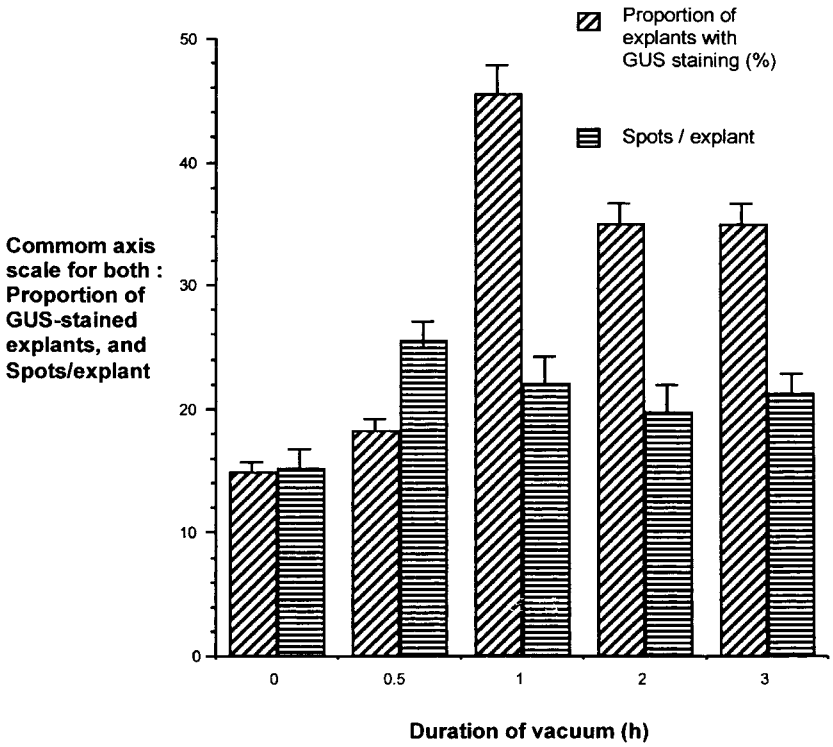

Fig. 3. The effect of duration of vacuum infiltration on transient GUS expression in inflorescence tissue. The number of explants showing GUS staining was calculated as a percentage of the total number of explants co-incubated for each treatment. The average number of blue spots per explant was also calculated. Data represents mean ± SE of between 2–4 replicates per treatment, each of at least 10 explants.

(Table 2) showed that sonication significantly increased the number of explants producing GUS expression and, up to the maximum period tested (6 s), expression increased with increasing duration of sonication. However, sonication also reduced the number of spots produced per explant, compared to the control.

Effect of Agrobacterium *cell density in inoculum on GUS expression*

Experiments were set up to determine the effects of variations in *Agrobacterium* cell density on transient GUS expression in inflorescence tissue. The results (Table 2) showed that whilst *Agrobacterium* cell densities of OD_{600} less than unity produced discrete blue spots, increasing the OD_{600} to between 1 and 1.5 caused substantial increases in the number of explants producing spots. Further increases in the cell density beyond this, however, resulted in very significant reduction in the number of explants producing blue spots and more than a halving of the number of spots per explant.

Further experiments showed that the effects of *Agrobacterium* cell density on GUS expression was influenced by the length of inoculation (Table 3). The higher cell density ($OD_{600} = 1.5$) produced the highest GUS expression frequency (64%), but this was obtained with a 1 h inoculation. This treatment also produced the highest number of spots per explant (25.4). When inoculation time was increased for this cell density, GUS expression decreased significantly both in terms of the frequency of responding explants (to 36%), as well as the number of spots per explant (10.9). On the other hand, the lower cell density ($OD_{600} = 0.7$) produced the lowest GUS expression frequency (32%) with 1 h inoculation. However, increasing the duration of inoculation to 3 h, increased both frequency of GUS expression, and number of spots per explant. A proportion of the calli from selected co-cultivation experiments were allowed to regenerate, either with or in the absence of selection. The plants that survived appeared phenotypically normal in all growth phases and produce viable seed that germinated normally. PCR analysis of genomic DNA prepared from these plants revealed that none were transformed.

Discussion

A basic requirement for a successful gene transfer system for producing transgenic plants is the availability of a target tissue, made up of a large number of regenerable cells that are accessible to the gene transfer treatment, and that will retain the capacity for regeneration for the duration of the necessary target preparation, cell proliferation, and selection treatments (Birch, 1997). In cereals, cells appear to lose their competence at an early stage in development and immature tissues, including inflorescences, have proved to be the most suitable sources of morphogenic structures (Maddock *et al*., 1983; Rout and Lucas, 1996). In wheat, the capability of inflorescence tissue to be a good source of embryogenic calli has been demonstrated in several cultivars (Barro *et al*., 1999). The advantages that inflorescence cultures offer over immature embryo cultures have been outlined previously (Rasco-Gaunt and Barcelo, 1999), and include, among others, the ease of isolation, a more efficient use of growth cabinet space, less variability among explants in regeneration, and better ability to withstand physical damage.

The objective of the work reported here was to develop an *Agrobacterium*-mediated transformation system for wheat using inflorescence tissue. Towards this end, the immediate priority was to establish the conditions for gene transfer. It has been demonstrated that wheat inflorescence tissue is transformable using *Agrobacterium tumefaciens* and, in the process, the effects of varying some of the factors known to influence *Agrobacterium*-mediated gene transfer have been reported.

The variation in the ability of different vectors, in the same *Agrobacterium* strain, to deliver T-DNA into inflorescence tissues have been reported. The vectors used here are based on the pGreen/pSoup system (Hellens *et al*., 2000) and differ essentially in their sizes and in the presence or otherwise of *vir* genes. Available evidence suggests that T-DNA processing may be affected by the size and/or organization of the T-DNA region, leading to the production of intact double-stranded or single-stranded forms of T-DNA (Steck, 1997), which may have some effect on T-DNA mobility and delivery into plant tissues. It is not clear what is responsible for this variation, but the results stress the need to screen a number of vectors in order to obtain one that will be optimal for a given transformation system. It is also important to note that there is a tendency for certain vectors to be more efficient in delivering T-DNA into specific explant types. The experiments also demonstrate the critical importance of length of preculture to T-DNA transfer. GUS expression was highest in explants cultured up to 3 weeks before co-cultivating with *Agrobacterium*, whilst no expression was observed in freshly isolated explants. Practically, the freshly isolated explants are more difficult to work with as cut sections often clump together and it takes considerable time and effort to separate them. Also, these are often completely colonized by the *Agrobacterium* and thus the elimination of bacteria at the resting and subsequent stages becomes more difficult. Perhaps the use of lower concentrations of *Agrobacterium* during inoculation coupled with shorter inoculation and/or co-cultivation times would help reduce

Table 3. *The effect of* Agrobacterium *cell density (OD_{600}) and length of inoculation on GUS expression in wheat inflorescence tissues*

OD_{600}	Length of inoculation (h)	Proportion of explants with GUS stain (%)	GUS spots/ explant (%)
1.5	1	16/25 (64.0)	406/16 (25.4)
1.5	3	9/25 (36.0)	98/9 (10.9)
0.7	1	8/25 (32.0)	78/8 (9.8)
0.7	3	14/25 (56.0)	172/13 (12.5)

this problem. Furthermore, in explants that had been cultured for a week or more before co-cultivation with *Agrobacterium*, GUS expression was often found in callus rather than on the original explant tissue itself. Thus for transient GUS expression, freshly isolated inflorescence tissues may not be the best target.

Sonication is thought to enhance T-DNA delivery through the microwounding of the tissues/cells which permit the *Agrobacterium* to travel deeper and more completely throughout the tissue than normal co-cultivation will permit (Trick and Finer, 1997), thus enhancing the *Agrobacterium* colonization and infection of the tissues. These are the initial events upon which gene transfer between bacteria and plant cells ultimately depends (Finer and Finer, 2000). The microwounding may also result in the active division of the cells and the accompanying DNA synthesis may enhance the incorporation of the T-DNA into the plant genome. In the experiments reported here, increased GUS expression frequencies were observed when explants were sonicated. Vacuum infiltration has been used in transformation protocols of several plant species including *Arabidopsis* (Clough and Bent, 1998) and wheat (Cheng *et al*., 1997*b*). Also called germ-line transformation, it has been used to transform *Arabidopsis* ecotypes and mutants that were recalcitrant to *Agrobacterium* transformation (Mysore *et al*., 2000). In the experiments described here, vacuum infiltration enhanced the frequency of GUS expression, both in terms of the number of responding explants and the number spots per explant. However, vacuum infiltration in excess of 60 min resulted in reduced frequency of GUS expression, even though the spots obtained were better defined and more intense. Vacuum treatments in excess of 2 h also resulted in explant tissues being completely colonized by the *Agrobacterium* that was then more difficult to eliminate in the resting and subsequent stages, resulting in the loss of callus.

Inoculation intensity which may be defined to include, either individually or in various combinations, the factors: cell density of the inoculum, the duration of inoculation and/or co-cultivation, the vacuum pressure, and the duration of the vacuum treatment, plays a critical role in determining the GUS response obtained. Increasing the inoculation intensity either by increasing the cell density of the inoculum, by longer inoculation and/or co-cultivation times, by higher vacuum pressure or prolonged vacuum infiltration, increases the response obtained in the form of transient GUS expression, including the number of spots per explant. Beyond a threshold value however, further increases in the inoculation intensity tends to cause reductions in the GUS response, perhaps as a result of a decrease in cell viability. These observations appear to lend credence to the hypothesis that each plant cell binds to a finite number of bacteria (Gutlitz *et al*., 1987). Beyond this threshold, it appears that cell viability may be compromised, resulting in lower proportion of GUS positive explants.

Determining the optimum inoculation intensity is important also because at much higher levels, the explant tissues are almost wholly colonized by the bacteria, elimination of which becomes more difficult during the resting and, subsequently, selection and regeneration stages. Usually this would necessitate the use of antibiotics at higher levels which, in itself, has detrimental effects on plant tissue development.

A critical step in the development of *Agrobacterium tumifaciens*-mediated transformation is the establishment of optimal conditions for T-DNA delivery into tissue from which whole plants can be regenerated. A range of factors that influence T-DNA delivery into wheat inflorescence tissue have been investigated. This is an important first step to utilizing *Agrobacterium* in the transformation of immature wheat inflorescence tissue.

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