Project AR1003

Title of Project

Reproducible *Agrobacterium*-mediated transformation systems for wheat and barley

Proposer

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Policy Relevance

The development of routine and facile transformation and regeneration systems for cereals, other major arable and horticultural food and non-food crops (MAFF CTD9401, 1994/5).

Abstract

Transformation systems for wheat and barley, based on particle bombardment, are now routine. However, this methodology has a number of disadvantages such as multiple copy gene integration events, leading to subsequent expression instability. Consequently, improved transformation techniques such as *Agrobacterium*-mediated systems are desirable. Firstly, the transgenic plants produced using *Agrobacterium*-mediated approaches tend to have simpler integration patterns and lower copy numbers. Secondly, the *Agrobacterium* approach allows the possibility of removing marker genes by segregation in the progeny; "Clean Gene Technology".

Therefore, the main objective of this proposal was to develop routine and reproducible *Agrobacterium*-mediated transformation procedures for spring wheat and barley varieties, Bobwhite and Golden Promise, respectively.

The development of reproducible and transferable *Agrobacterium*-mediated techniques for

the major UK cereal crops has been identified as a key priority. This report details such a method for the *Agrobacterium*-mediated transformation of barley, thus enabling a significant technological advance for U.K. agriculture.

Scientific Objectives and Milestones

1. To carry out large-scale *Agrobacterium*-mediated transformation experiments in wheat and barley that will lead to methodology that allows the reproducible production of transgenic plants.

2. Subject to regulatory approval, confirm the value of the experimental transformation systems in field trials.

Milestone 01-01: Complete the first set of large-scale transformation experiments with wheat and barley, and report on the transformation efficiencies obtained.

Milestone 01-02: Confirm the transgenic nature of plants from the first set of experiments using standard molecular techniques.

Milestone 01-03: Finalise methodology that allows the reproducible production of transgenic wheat and barley using *Agrobacterium*-mediated approaches.

Milestone 01-04: Report on the effect of using recycled material[1] in the *Agrobacterium* transformation system.

Milestone 02-01: Plan and conduct barley field trials.

Milestone 02-02: Report initial results on the field performance of *Agrobacterium*-derived lines.

[1] Recycled material; donor plant material which has been through one round of tissue culture and regeneration.

Results

Milestone 01 – 01

Initial investigations, utilising up to 800 embryos at a time, yielded no transformed plants of either barley or wheat. However, stable gene integration was observed in (non-regenerable) callus lines of barley, whereas only transient expression was observed in wheat (Figures 1 & 2). Subsequent experiments therefore focused on barley, the results from which could be utilised in optimising ensuing wheat studies.

Adoption and modification of the Tingay *et al*. (1997) protocol for the *Agrobacterium*-mediated transformation of barley resulted in our first transgenic barley plants (Figure 3). These plants were transformed with pDM805, as described in the original paper. Subsequent investigations with modified protocols and a variety of plasmids, produced additional transformed lines (Table 1). At the time of writing, 80 plants from 14 independent transformed lines have been established, with an average transformation frequency of 2.2% per successful experiment. It is anticipated that a further 19 independent lines could be generated from additional experiments in progress (Table 2).

The variable nature of the plant source material means that further repetitions are required to establish the significance of many of the results observed in the course of this investigation. However, the following points address a number of issues crucial to the *Agrobacterium*-mediated transformation of a variety of commercial monocotyledonous species.

? Wounding of the tissue by bombardment with naked gold particles, as described by Tingay *et al*. (1997) is not required.
? The presence of Acetosyringone is not an essential pre-requisite for transformation.
? The removal of all antibiotics from the *Agrobacterium* broth, prior to inoculation is strongly advised.
? The period of inoculation should be relatively brief.

The above points are incorporated in the *Agrobacterium*-mediated barley transformation protocol described below, under Milestone 01 - 03.

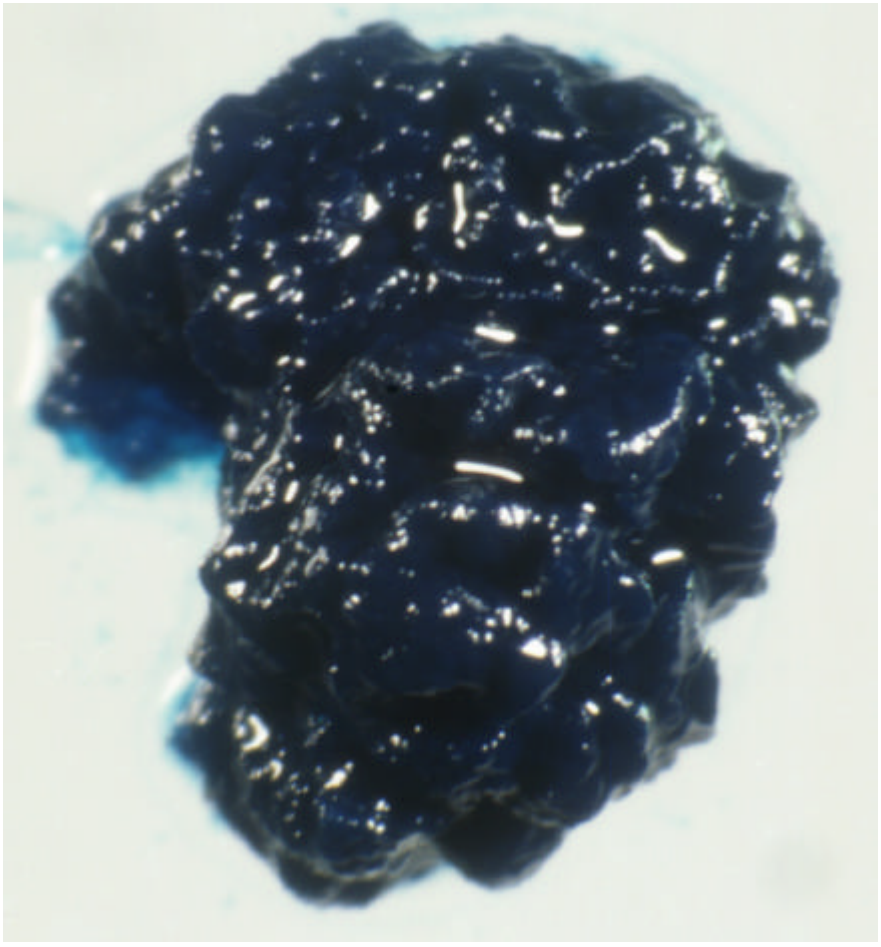

Figure 1 Stable *gus* expression in barley callus

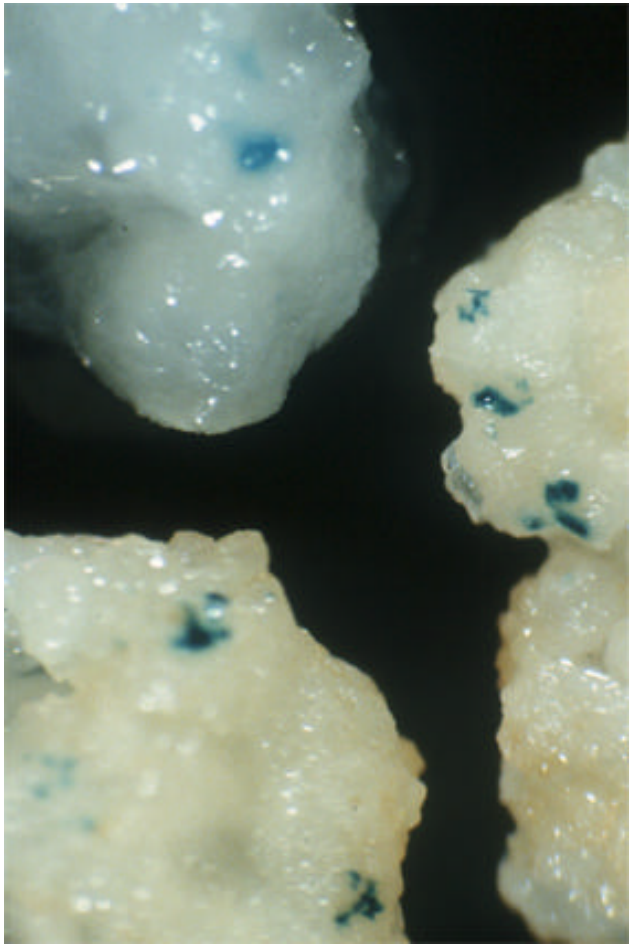

Figure 2 Transient *gus* expression in wheat callus

Figure 3 Transformed barley lines

Table 1 ***Agrobacterium*** **transformed barley lines**

Experiment	Number of embryos	Plasmid	Independent lines	Number of individuals	Transformation frequency (%)	Notes
B22	150	pDM805	a	4	2.0	7 day old embryo derived callus
			b	5		
			c	1		
B25	300	pDM805	a	1	0.3	
B27	252	pAL 135	a	31	1.2	pAL 135 carries barnase and luciferase genes - refer to MAFF project CE0127/160
			b	16		
			c	4		
B29	300	pDM805	a	1	1.0	
			b	3		
			c	1		
B32	36	pDM805	a	8	8.3	
			b	1		
			c	1		
B36	160	pDM805	a	3	0.6	
Total	**1198**		**14**	**80**	**Mean = 2.2%**	

Table 2 Putative *Agrobacterium* transformed barley lines

Experiment	Number of embryos	Plasmid	Independent lines	Number of individuals	Transformation frequency (%)	Notes
B38	100	pDM805	1	8	1.0	
B41	125	pDM805	1	8	0.8	
B43	358	pVec8Gus	8	32	8.9	pVec8Gus carries the hygromycin gene
B44	153	pDM805	2	2	1.3	
B46	50	pDM805	4	8	8.0	7 day old embryo derived callus (Repeat of B22)
B47	100	pVec8Gus	3	3	3.0	
Total	**886**		**19**	**61**	**Mean = 3.8%**	

For clarity, Tables 1 & 2 include only data from experiments which generated transformed lines. Over the duration of the project a total of 5614 barley and 1374 wheat embryos were transformed. The number of barley embryos, in particular, considerably exceeds the objectives in the original application.

Milestone 01 – 02

All transgenic lines have been painted with herbicide (0.5 % (v/v) Challenge) and shown to be resistant. Additional samples of either callus and/ or leaf material have been tested for either GUS or LUC expression (Figures 3 & 4).

Molecular confirmation of the presence of the transgene has been undertaken on several individuals, typical results of which are illustrated in Figure 5. To date, Southern Hybridisation to confirm copy number has not been undertaken. However, as mature plants express both phenotypes encoded on the T-DNA, it is anticipated that Southern Hybridisation will demonstrate the integration of at least one copy of the T-DNA within the plant genome.

Figure 3 GUS expression in transformed juvenile barley shoot

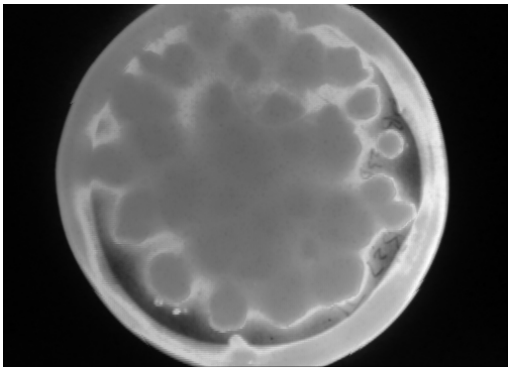

Figure 4 Callus from experiment B27 expressing the luciferase gene

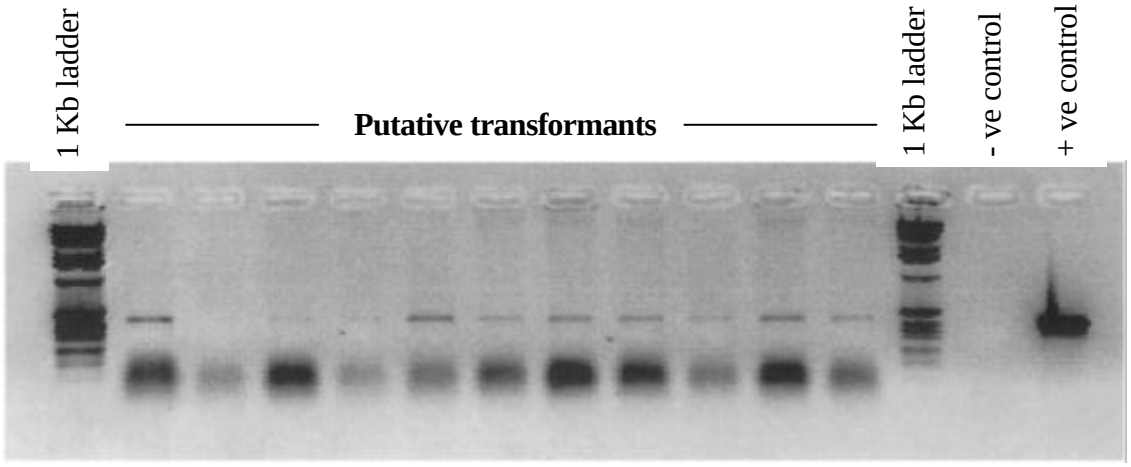

Figure 5 PCR amplification of the *bar* gene from selected transformants

Milestone 01 - 03

Protocol for the *Agrobacterium*-mediated transformation of barley (*Hordeum vulgare; cv.* Golden Promise)

Day 1 Prepare an overnight *Agrobacterium* broth by adding a standard inoculum (Tingay *et al*., 1997) of *Agrobacterium* to 10 ml of liquid MG/L medium - no antibiotics. Incubate for 24 h at 28?C, on a shaker (120 rpm).

Isolate immature barley embryos (1.5 – 2 mm diameter), remove axis and transfer to callus induction medium (Wan and Lemaux, 1994; 25 embryos per plate), scutellum side up and incubate in the dark at 24?C.

Day 2 Using a pipette, drip full strength *Agrobacterium* suspension onto each embryo. Drag the embryo (gently!) across the surface of the medium to remove any excess *Agrobacterium* and transfer to fresh callus induction medium, scutellum side down. Incubate in the dark at 24?C and co-cultivate for 3 days. Discard any damaged embryos.

Day 5 Transfer embryos to callus induction medium + 150 mg l^{1} Timentin + selective agent (see below) and incubate in the dark at 24?C. Subculture the developing calli every 14 days, for a period of 12 - 24 weeks, following a suitable shoot regeneration programme (*e.g*. Harwood *et al*., 2000). Only transfer resistant embryogenic lines to regeneration medium and discard any material stained with oxidised polyphenols. Immediately discard any explants which become overgrown with *Agrobacterium*.

This protocol is based on Tingay *et al*. (1997) and was developed with the assistance of Peter Matthews at CSIRO (Australia), where the original barley transformation methodology was devised. Bialaphos (5 mg l^{-1}) was initially the selectable agent of choice, however, we are presently investigating hygromycin (150 mg l^{1}), which is widely used in rice transformation protocols and in subsequent barley transformation methods from CSIRO (Matthews *et al*., 2001). Preliminary results are encouraging, with a more rapid and clear-cut selection, and a subsequent reduction in the time required for subculture (Table 2).

The 12 month duration of this project has proved insufficient to effectively transfer this

technology into wheat. Details of the wheat experiments undertaken, may be found in Table 3. No putative transgenic wheat plants have been regenerated to date.

Table 3 Wheat transformation data

Experiment	Number of embryos	Plasmid [1]	Experiment	Number of embryos	Plasmid
W9	113	pAL 156	W15	99	pAL 135
W10	132	pAL 156	W16	200	pAL 135
W11	169	pAL 156	W17	133	pAL 135
W12	61	pAL 135	W18	106	pDM805
W13	104	pAL 135	W19	150	pDM805
W14	107	pAL 135	**Total**	**1374**	

[1] pAL 156 contains the *gus* marker gene - refer to MAFF project CE0127/160

Milestone 01 – 04

Recycled material was found to be unsatisfactory in a preliminary tissue culture trial involving an assessment of 300 embryos. The regeneration frequency of recycled embryos was significantly lower than that observed with wild type embryos (Table 4). Furthermore, phenotypic aberrations such as defective ear formation and poor seed set, were observed on a number of tissue culture derived donor plants, possibly due to somaclonal variation. Consequently, this material was not included in any transformation experiments.

Table 4 Shoot production/ regeneration capacity of recycled barley embryos

Embryo source	Mean number of regenerated shoots per embryo
Recycled barley	55.0
Control (non- recycled)	200.8

Milestone 02 – 01

An application for consent to release GM barley was submitted to DETR and approval for small-scale field trials of transgenic barley over the three years 2001-2003 was obtained from DETR on 11th April 2001 (consent reference 01/R29/3). The transgenic plants within the trial were sown on 12th April 2001. Due to the fact that the trial was sown late in the year because of the timing of the consent being received, it was decided to include only three transgenic lines in the 2001 trial together with the appropriate null-segregant populations and controls. Unfortunately, during the weekend of 15-17th June 2001 trespassers destroyed the trial. At the time that the trial was destroyed the GM plants were a few days away from flowering. Only a very small number of transgenic plants were left standing in one corner of the trial.

Although we had received consent to include up to seven lines in the 2001-2003 trials, two of which were produced using *Agrobacterium*-mediated techniques, these lines produced using *Agrobacterium*-mediated methods were not included in the 2001 trial.

We plan to include lines produced using *Agrobacterium*-mediated techniques in the 2002-2003 trials. The lines to be included were both produced using pGreen plasmid pAL135, the T-DNA of which contains the *bar* and luciferase genes both under the control of maize ubiquitin promoters. For the trials over the next two years, we plan to introduce a range of additional security measures. We therefore hope that we will be able to compare lines produced using *Agrobacterium*-based methods to lines produced using particle bombardment over two generations under field conditions.

Milestone 02 – 02

These results should be available after completion of the 2002 field trial.

References

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