Barley Transformation using *Agrobacterium*-Mediated Techniques

Wendy A Harwood, Joanne G Bartlett, Silvia C Alves, Matthew Perry, Mark A Smedley, Nicola Leyland and John W Snape

Abstract

Methods for the transformation of barley using *Agrobacterium*-mediated techniques have been available for the past ten years. *Agrobacterium* offers a number of advantages over Biolistic-mediated techniques in terms of efficiency and the quality of the transformed plants produced. This chapter describes a simple system for the transformation of barley based on the infection of immature embryos with *Agrobacterium tumefaciens* followed by the selection of transgenic tissue on media containing the antibiotic hygromycin. The method can lead to the production of large numbers of fertile, independent transgenic lines. It is therefore ideal for studies of gene function in a cereal crop system.

Key Words: Barley transformation, *Agrobacterium tumefaciens*, transgenic plants, hygromycin, immature embryo.

1. Introduction

The first reports of successful barley transformation (***1***) used biolistic-based techniques to introduce DNA to immature embryos. Immature embryos were also the target tissue used in the first reports of the generation of transgenic barley plants using *Agrobacterium* (***2***). Although alternative target tissues have been examined for use in barley transformation systems, immature embryos remain the target tissue of choice for obtaining high transformation efficiencies. An alternative *Agrobacterium*-mediated barley transformation system uses microspore cultures as the target tissue (***3***). A comparison of biolistic and *Agrobacterium*-based methods for barley transformation highlighted some of the advantages of the *Agrobacterium* system (***4***). These included higher transformation efficiencies, lower transgene copy number and more stable inheritance of the transgenes with less transgene silencing.

Barley transformation is still very genotype dependent. The most responsive genotype is the spring cultivar Golden Promise. There have been reports of the transformation of other genotypes using *Agrobacterium*-mediated techniques but at lower frequencies (***5***). As well as the choice of target tissue and genotype, there are a number of other important variables in the *Agrobacterium*-mediated transformation system. These include *Agrobacterium* strain, co-cultivation conditions and timing, selection system and plant regeneration system. For barley, *Agrobacterium* strains AGL1 or AGL0 are commonly used (***6***) but LBA 4404 has also been successfully used (***7***). In many reports of barley transformation, the *bar* gene conferring resistance to the glufosinate group of herbicides was used for selection together with bialaphos or PPT as the selective agent (***4***). This has now been largely replaced with the *hpt* gene conferring resistance to the antibiotic hygromycin as the selectable marker of choice. The hygromycin resistance gene can be driven by a CaMV 35s promoter for selection in barley leaving stronger promoters such as the maize ubiquitin promoter (*ubi1*) available to drive a gene or cassette of interest. Many constructs have been developed that can be used for barley transformation. Here we describe the use of constructs from the pBract series (www.bract.org) which give high transformation frequencies and are simple to work with as they are Gateway compatible for easy introduction of genes or sequences of interest.

The method described below details the *Agrobacterium*-mediated barley transformation procedure from growth of donor plants to provide immature embryos through to confirmation of the transgenic status of the regenerated plants.

2. Materials

2.1 Plant Material and Growth Conditions

1. Seed of the spring barley genotype Golden Promise is sown at two weekly intervals in a barley growth mix consisting of a 2:2:1 mix of Levington M3 compost: Perlite: Grit. The mix also contains the slow release fertilizer, Osmocote at the manufacturers recommended concentration.
2. Seed is initially sown in 5 cm diameter pots and after approximately 30 days germinated plants are potted into 13 cm diameter round pots in the same growth mix for continued development.
3. Plants are grown in a controlled environment room at 15^{o}C day and 12^{o}C night temperatures, 80% relative humidity and with light levels of 500 µmol/m^{2}/s^{1} at the mature plant canopy level provided by metal halide lamps (HQI) supplemented with tungsten bulbs (***8***) (*see* **Note 1**).

2.2 Agrobacterium *Strains and Vectors*

1. *Agrobacterium* strain AGL1 is used together with appropriate pBract vectors. In this example we use vector pBract 204 which contains the *hpt* gene conferring hygromycin resistance under a 35s promoter at the left border (LB) and a *gus* gene encoding β-glucuronidase under the control of the maize ubiquitin promoter at the right border (RB) (**Fig. 1**) (*see* **Note 2**).
2. The pBRACT vectors are based on pGreen, which is a small, versatile vector designed for easy manipulation in *E. coli* with a high copy number (***9***). To enable the small size of pGreen, the pSa origin of replication required for replication in *Agrobacterium*, is separated into its' two distinct functions. The replication origin (ori) is present on pGreen, and the trans-acting replicase gene (RepA) is present on an additional vector, named pSoup. Both vectors are required in *Agrobacterium* for pGreen to replicate.

2.3 Bacterial Culture Medium

The basic bacterial culture medium is MG/L (***10***) which contains: 5.0 g/l tryptone, 5.0 g/l mannitol, 2.5 g/l yeast extract, 1.0 g/l L-glutamic acid, 250 mg/l KH_2PO_4, 100 mg/l NaCl, 100 mg/l $MgSO_4.7H_2O$ and 10 µl Biotin (0.1 mg/l stock). The media is adjusted to pH 7.2 with NaOH. For the preparation of plates 15 g/l agar is added.

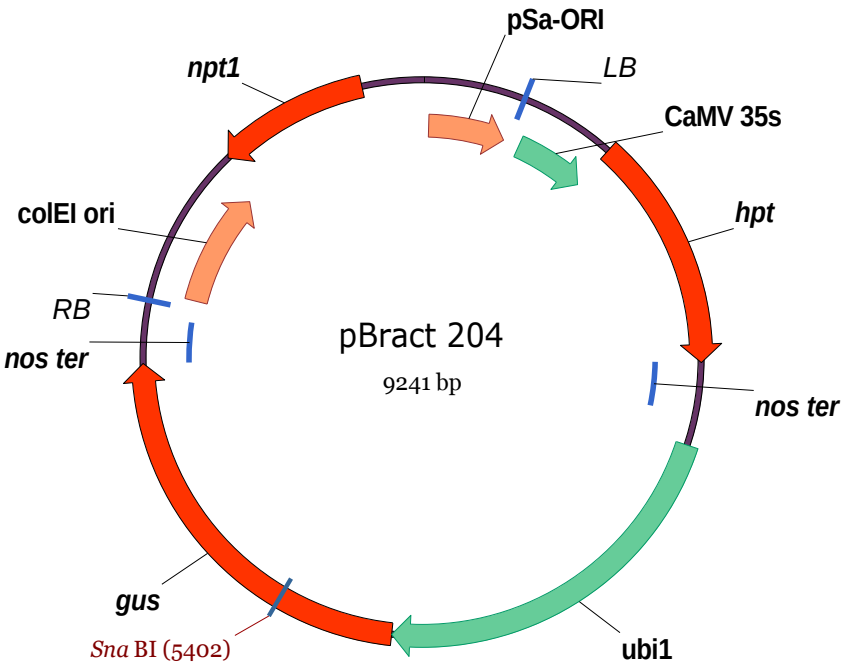

Figure 1.
pBract 204, a pGreen based plasmid containing the *hpt* (*Hyg*) gene conferring resistance to the antibiotic hygromycin under the control of a CaMV (cauliflower mosaic virus) 35s promoter at the left border and a β-glucuronidase (*gus*) gene under the control of a maize ubiquitin promoter (Ubi1) at the right border.

2.4 Plant Tissue Culture Media

Three different basic plant tissue culture media are used during the transformation and regeneration process; callus induction, transition and regeneration media. During all selection stages hygromycin is added to the media at 50 mg/l (Hygromycin B supplied as a sterile 50 mg/l stock from Roche). The antibiotic Ticarcillin with Clavulanic acid (Duchefa T0190) (otherwise known as Timentin) is also added at 160 mg/l to all selection stages. Additional copper (1.25 mg/l

$CuSO_4.5H_2O$), is added during callus induction and transition stages. Unless otherwise stated, all media components are supplied by Sigma-Aldrich and all media and stocks made up using water from an Elga water purifier.

1. Callus induction: 4.3 g/l Murashige & Skoog plant salt base (Duchefa M0221), 30 g/l maltose, 1.0 g/l casein hydrolysate, 350 mg/l myo-inositol, 690 mg/l proline, 1.0 mg/l thiamine HCl, 2.5 mg/l Dicamba (Sigma-Aldrich D5417), 3.5 g/l Phytagel. The media is adjusted to pH 5.8 with NaOH.
2. Transition: 2.7 g/l Murashige & Skoog modified plant salt base (without NH_4NO_3) (Duchefa M0238), 20 g/l maltose, 165 mg/l NH_4NO_3, 750 mg/l glutamine, 100 mg/l myo-inositol, 0.4 mg/l thiamine HCl, 2.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) (Duchefa), 0.1 mg/l 6-benzylaminopurine (BAP) (Duchefa), 3.5 g/l Phytagel. The pH is adjusted to 5.8.
3. Regeneration: 2.7 g/l Murashige & Skoog modified plant salt base (without NH_4NO_3) (M0238 Duchefa), 20 g/l maltose, 165 mg/l NH_4NO_3, 750 mg/l glutamine, 100 mg/l myo-inositol, 0.4 mg/l thiamine HCl, 3.5 g/l Phytagel. The pH is adjusted to 5.8.
4. Media stocks:
    a. Callus induction 100x vitamin stock: 100 mg/l thiamine HCl, 35 g/l myo-inositol and 690 mg/l proline. This stock should be filter sterilized ready for use and stored at 4^oC.
    b. Transition and regeneration 100x vitamin stock: 40 mg/l thiamine HCl and 10 g/l myo-inositol. This stock should be filter sterilised ready for use and stored at 4^oC.
    c. Dicamba: 2.5 mg/l stock made up in water, filter sterilised, divided into 1ml aliquots and stored frozen.
    d. 2,4-D: 2.5 mg/ml stock made up in 100% ethanol and stored at -20^oC.
    e. BAP: 1 mg/ml stock made up in water with a few drops of 1 *M* NaOH and stored at -20^oC.
    f. $CuSO_4$ stock: 125mg of $CuSO_4.5H_2O$ dissolved in a total volume of 100 ml water, filter sterilised and store at 4^oC.
    g. Hygromycin: purchased as a sterile 50 mg/ml stock, divided into 1 ml aliquots and stored frozen.
    h. Timentin: 160 mg/ml stock made up in water, divided into 1 ml aliquots and stored frozen.

2.5 Other Equipment

1. Fine forceps No. 5 type (TAAB Laboratories Equipment Ltd. Ref. T083)
2. Binocular microscope (e.g. Leica MZ6).

3. Methods

3.1 Tissue Culture Media Preparation

Phytagel is prepared in advance at 2x the required concentration and sterilised by autoclaving (*see* **Note 3**). All tissue culture media are filter sterilised therefore all media components, accept those stored as sterile stocks, are added at 2x the required concentration, dissolved, the pH adjusted and the media filter sterilised (Steritop 0.22 µm filters, Millipore). Both the 2x phytagel and the 2x media are warmed to 60^{o}C in a waterbath before use. The required stocks are added under sterile conditions to the warm media, the media and the phytagel mixed and poured into 9 cm Petri dishes (Sterilin). For regeneration, media is poured into deeper dishes (tissue culture dish, 100 x 20 mm, Falcon).

3.2 Preparation of Agrobacterium *Standard Inoculum*

1. Standard inoculums are prepared using a slightly modified method described in (***2***).
2. A single colony of *Agrobacterium* AGL1, containing the appropriate pBract vector, is used to inoculate 10 ml of MG/L medium with 25 µg/ml Rifampicin and 50 µg/ml Kanamycin. This is incubated at 28^{o}C and shaken at 180 rpm for 40 h.
3. 10 ml of sterile 30% aqueous glycerol is added to the bacterial culture and mixed by inverting several times.
4. Aliquots of 400 µl of the standard inoculum are placed into 0.5 ml Eppendoff tubes and maintained at room temperature for 2 h mixing by inversion every 30 min.
5. Standard inoculums are then stored at -80^{o}C ready for use.

3.3 Isolation of Barley Immature Embryos

1. Collection and sterilisation of immature seeds
    1. Barley spikes are collected when the immature embryos are 1.5-2 mm in diameter (*see* **Note 4**).
    2. Before cutting the spikes, a single immature seed from the middle of each spike is checked to make sure the size of the immature embryos is correct (**Fig. 2 A, B** and **C**).
    3. The immature seeds are removed from the spike and the awns broken off without damaging the seed coat.
    4. The immature seeds are then sterilised by firstly washing in 70% ethanol for 30 sec followed by three washes in sterile distilled water.
    5. This is followed by sterilisation in a solution of sodium hypochlorite (sodium hypochlorite solution, Fluka 71696) diluted 50:50 with water for 4 min.
    6. The sterilisation is followed by four washes in sterile distilled water after which the immature seeds are drained but left wet in a screw top sterile jar.

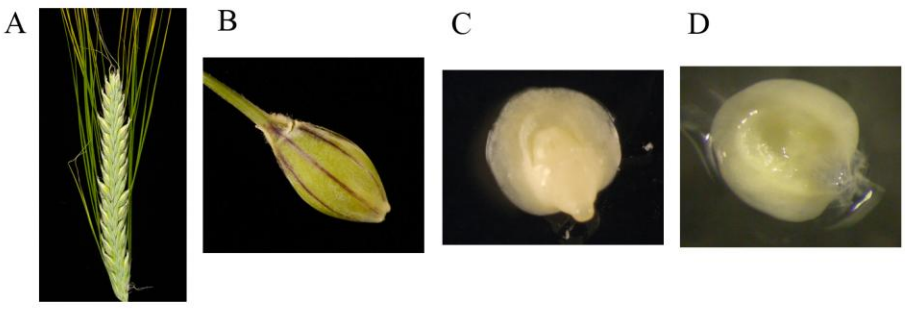

Figure 2.
Selection of barley spikes and immature embryos at the correct stage. A. A barley spike containing immature embryos at the correct stage. B. Isolated immature seed

with immature embryo at the correct stage. C. Intact immature embryo isolated from immature seed. D. Immature embryo with the embryonic axis removed ready for *Agrobacterium* inoculation.

2. Isolation of immature embryos and removal of embryonic axis.

1. All operations are performed in a laminar flow hood under sterile conditions.
2. Sterile seeds, approximately 20 at a time, are tipped onto a sterile blue or black tile under a dissecting microscope.
3. The seed is held firm with fine forceps and a second pair of fine forceps used to expose the immature embryo and remove the embryonic axis (*see* **Note 5**) (**Fig. 2 C** and **D**).
4. The embryo is then plated scutellem side up on callus induction medium.
5. Twenty five embryos are placed on each 9 cm plate ready for *Agrobacterium* inoculation and stored at 23-24^{o}C in the dark.

3.4 Agrobacterium *Preparation, Inoculation and Co-cultivation*

1. An overnight *Agrobacterium* culture is prepared by adding a standard inoculum to 10 ml of liquid MG/L medium without any antibiotics. This is incubated on a shaker at 180 rpm at 28^{o}C overnight (approximately 20 h).
2. The full strength *Agrobacterium* culture is used to inoculate the embryos.
3. Using a 200 µl pipette, a small amount of *Agrobacterium* is dropped onto each embryo so that the surface is just covered.
4. Once all 25 embryos on a plate have been treated the plate is tilted to allow any excess *Agrobacterium* culture to run off the embryos.
5. A maximum of two plates are treated with *Agrobacterium* before the embryos from the first plate are gently removed and transferred to a fresh callus induction plate, scutellum side down.
6. Care is taken not to transfer any excess culture medium or *Agrobacterium* culture with the embryos and if necessary the embryos are gently dragged across the surface of the medium to remove excess *Agrobacterium* before transferring to a fresh plate.
7. Plate are sealed with Micropore^{TM} surgical tape and incubated at 23-24^{o}C for three days. It is convenient to isolate immature embryos one day and to

inoculate with *Agrobacterium* the following day. However it is also possible to inoculate the embryos on the same day as they are isolated (*see* **Note 6**).

3.5 Selection of Transformed Material

1. After three days co-cultivation, the embryos are transferred to fresh callus induction plates containing hygromycin as the selective agent and timentin to remove *Agrobacterium* from the cultures (*see* **Note 7**).
2. Embryos are cultured scutellum side down at 23-24^{o}C in the dark. This transfer is referred to as selection 1.
3. After two weeks, embryos are transferred to fresh selection plates as above (selection 2). The entire embryo and callus derived from it is transferred as a single unit and not split up.
4. After a further two weeks, each embryo is transferred to a third selection plate (selection 3) (*see* **Note 8**). At this stage the callus from each embryo may break up and if so, it should be placed in a marked area of the plate so that all material derived from a single embryo can be tracked (**Fig. 3 B**).
5. It is necessary to reduce the number of embryos per plate as the transformed embryo-derived callus starts to grow rapidly.
6. After six weeks selection on callus induction medium, the embryo derived callus is transferred to transition medium, again containing hygromycin and timentin, for two weeks at 24^{o}C under low light. The low light is achieved by placing the plates under light conditions in a tissue culture room but covering the plates with a thin sheet of paper. During this two week culture period transformed lines should become obvious and start to produce green areas and small shoots. Non-transformed callus rarely shows signs of regeneration on hygromycin containing medium.

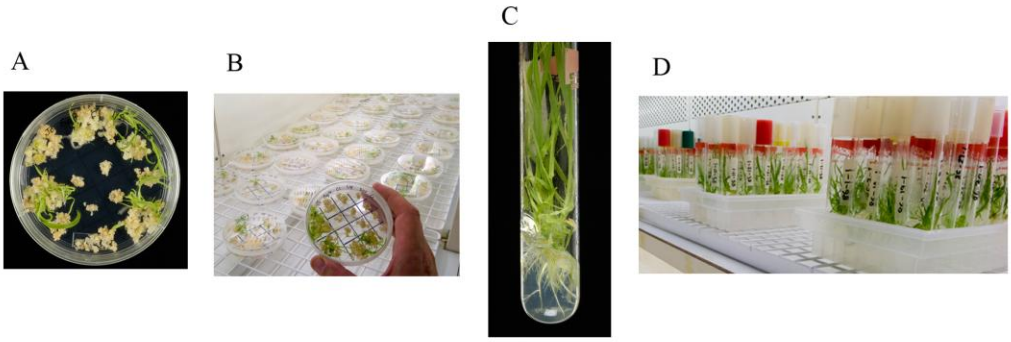

Figure 3.
Selection of transformed barley on hygromycin containing media. A. Regenerating transformed barley lines around the edge of a plate of regeneration medium with non-transformed lines in the middle. B. Plates of regenerating transformed barley. Plate in the foreground showing four transformed lines together with non-transformed lines and also showing the method of marking the plate to distinguish material from individual embryos. C. Transgenic barley plant transferred to culture tube showing good root system in hygromycin containing medium. D. Transgenic barley plantlets in the culture room just before transfer to soil.

3.6 Regeneration of Transgenic Plants

1. After the two weeks on transition medium, the embryo derived material is transferred one final time to regeneration medium, in deep Petri dishes, without any growth regulators but still with the same levels of hygromycin and Timentin.
2. As transformed lines will grow very vigorously, the number of lines per plate must be further reduced.
3. The appearance of typical regeneration plates can be seen in **Fig. 3 A** and **B**. It is important to keep all regenerating callus derived from a single embryo together.

4. Once shoots are 2-3 cm in length and roots have formed, carefully remove the small plantlets from the plates and transfer to glass culture tubes (Sigma C-5916) containing 12 ml of callus induction medium but without any dicamba or other growth regulators. This media should still contain hygromycin and timentin at the same concentrations.
5. The appearance of the plantlets in tubes can be seen in **Fig. 3 C** and **D**. The plantlets should quickly form a strong root system in the hygromycin containing medium.
6. Plants which form strong roots in hygromycin are always found to be transformed and this selection system does not give any 'escapes' or non-transformed plants that survive selection (*see* **Note 9**).
7. Once rooted plants reach the top of the tubes they can be transferred to soil.
8. Plants are gently removed from tubes using long forceps and all tissue culture medium washed from the roots under a running tap. They are then planted in the same barley growth mix described above in 5 cm diameter pots.
9. Plants are covered either with small individual plastic jars with holes in the top as individual propagators or entire trays are covered with propagators for a few days to maintain humidity until the plants are well established in soil.
10. Once plants are established in soil, leaf samples can be collected for further analysis to confirm the presence of the introduced genes. **Fig. 4** shows the expression of the *gus* gene in leaf samples from plants transformed with pBract 204.

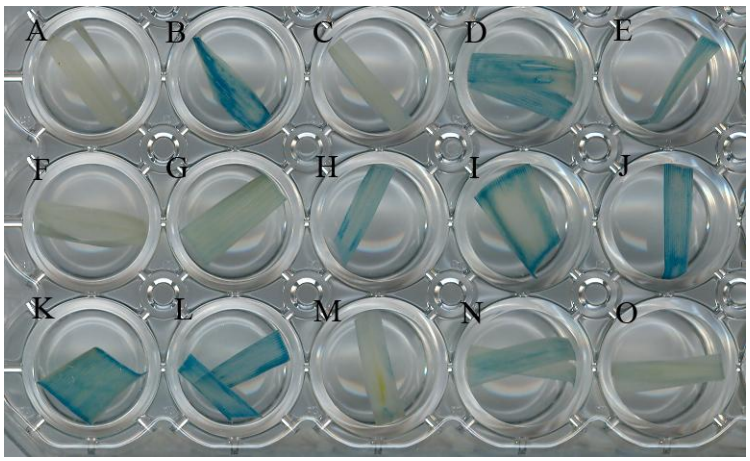

Figure 4.
The results of staining leaf samples for GUS activity following transformation with pBract 204. Wells A and F do not show any GUS activity. The other wells show varying levels of GUS expression.

4. Notes

1. The plants used to provide the immature embryos for transformation should not be sprayed with any pesticides. Spraying the plants as they approach flowering is a particular problem as it can reduce the response of the immature embryos in tissue culture.
2. T-DNA transfer starts at the right border and proceeds to the left border. Therefore it is advisable to have the selectable marker at the left border and the gene of interest at the right border so that the gene of interest is transferred first and the selectable marker second. This means that selected transformed plants have a greater chance of containing the gene of interest.
3. Phytagel can be prepared in advance at 2x concentration in water, autoclaved and stored at room temperature because it will not solidify until the other culture medium components are added.
4. The optimum size of immature embryos is 1.5 mm in diameter. Smaller embryos of 1 mm diameter are difficult to handle and more likely to suffer from overgrowth of *Agrobacterium.* Embryos of 2 mm diameter are at the upper limit of those that will respond well in culture. At the correct stage, the endosperm of the immature seeds should still be soft and quite liquid in appearance. If the endosperm is starting to look flowery then the embryos will be too old.
5. There are several different techniques for removing the immature embryos and removing the embryonic axis. The most efficient way is to carry out this operation in one step holding the immature seed by puncturing it with one pair of fine forceps and then exposing the immature embryo by peeling back the seed coat from the awn end of the seed. Once the embryo is exposed the embryonic axis can be removed by first pinching off the shoot axis with the fine forceps and secondly removing the root axis with a second pinching action. This should leave only the undamaged scutellum. If preferred, it is possible to carry out this operation in two steps. Firstly the immature embryos can be removed and placed intact onto callus induction medium. Once the required number have been isolated they can be

picked up in groups of around 10 together with a small amount of culture medium, placed on the sterile tile and the axis removed from each using the fine forceps. They are then immediately plated scutellum side up on callus induction medium.

6. Transformation efficiencies of 25% have been obtained by infecting immature embryos either on the same day as isolation or on the day following isolation..Transformation efficiency is defined as the number of independent transformed plants as a percentage of the original number of immature embryos treated.
7. Using the procedure described, excessive overgrowth of the immature embryos with *Agrobacterium* should be avoided. However, if any embryos are completely overgrown with *Agrobacterium* following the co-cultivation then they should be discarded. This can be a problem if very small embryos are used.
8. If callus growth looks good after four weeks of selection then it is possible to omit the final two weeks selection on callus induction medium (selection 3) and transfer to transition medium at this stage. If in any doubt continue with selection 3 as this is likely to yield better results.
9. Although non-transformed plantlets do not normally survive and root on hygromycin containing medium, it is occasionally possible to have two shoots close together, one transformed and one non-transformed. These shoots may be transferred together to a culture tube. This is not normally obvious until the plants are removed from the tube and the roots rinsed ready for planting in soil. In this case, it is possible for a transformed shoot to maintain the growth of a non-transformed shoot.

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