Agrobacterium-mediated transformation of barley immature embryos

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Summary

Barley transformation is an essential tool for a range of functional genomics studies as well as for future crop improvement. The demand for efficient crop transformation systems continues to grow, with new genome editing technologies adding to that demand. Here we describe an efficient and routine transformation protocol for the spring barley Golden Promise, based on Agrobacterium-mediated inoculation of immature embryos. This protocol has been widely used for over-expression and RNAi applications and more recently for CRISPR/Cas9 mediated genome editing. Average transformation efficiencies of 25% can be easily achieved.

Key words:

Barley transformation, *Agrobacterium tumefaciens*, immature embryos, genome editing, genome engineering.

1. Introduction

Over the last few years enormous progress has been made in the development of new, more precise tools for the engineering of plant genomes. Genome editing using CRISPR / Cas9 is the highest profile of these new techniques [1, 2]. Transformation is often the bottleneck in the application of new techniques as it is usually required to deliver the components for genome editing. It also remains essential for a range of gene validation studies. For these reasons efficient transformation is vital to allow progress in a wide range of research areas.

Transformation of barley has been reported using a range of techniques including biolistic methods [3] and Agrobacterium-mediated protocols [4,5]. Currently the most efficient methods are Agrobacterium-mediated and these also tend to give higher quality transgenic events with lower numbers of inserted transgene copies [6]. A range of target tissues have been employed for barley transformation however by far the most popular are immature embryos. Immature embryos collected from high quality donor plants of the spring barley Golden Promise yield highly embryogenic callus that easily regenerates.

The preparation of constructs for transformation used to be time consuming but with new assembly techniques, in particular Golden Gate assembly, this is no longer a limiting factor as most steps can be automated [7]. The choice of selectable marker is important in any transformation protocol. In barley the most efficient selectable marker is the *hpt* gene

conferring resistance to the antibiotic hygromycin [5]. Other selectable marker genes such as *bar* or *nptII* can also be used [8]. The availability of a range of selectable markers gives the opportunity for re-transformation of existing transgenic lines if required.

The principal remaining challenge in barley transformation is genotype dependence. Whereas the spring barley Golden Promise transforms with high efficiency, many modern varieties are recalcitrant to transformation. For successful transformation a variety needs both efficient regeneration and good susceptibility to Agrobacterium.

In this chapter we describe a high-throughput method for the transformation of Golden Promise using Agrobacterium inoculation of immature embryos and selection on hygromycin. This protocol has been successfully used to introduce over 1,000 constructs to barley. These constructs include those for over-expression, for RNAi mediated silencing, for promoter testing and for CRISPR / Cas9 based genome editing. Large T-DNAs up to 40kb in size can be introduced. The protocol has been transferred to several laboratories worldwide and routinely gives efficiencies of 25%.

2. Materials

2.1 Plant material

Seeds of Golden Promise are sown every 2 weeks to provide a constant supply of immature embryos at the correct stage. The barley compost mix contains a 2:2:1 mix of Levington M3 compost: Perlite: Grit. The mix also contains a slow release fertilizer, Osmocote at the manufacturers recommended concentration. Seed is sown directly into trays with 24 5cm compartments and then plants are transplanted into 12cm pots once they reach a height of approximately 20cm. Plants are grown in controlled environment rooms with a day temperature of 15°C and a night temperature of 12°C. Light intensity is at least 400 µmol/m^{2}/s^{1} at the mature plant canopy level and is provided by metal halide lamps (HQI) supplemented with tungsten bulbs. Humidity is kept at 70%. (*see* **Note 1**)

2.2 Stock solutions

1. 100X Vitamin stock for callus induction (CI) medium: 100 mg/l thiamine HCl, 35 g/l myo-inositol, and 69g/l proline. This stock should be filter sterilized and stored at 4 °C.

2. 100X Vitamin stock for transition (T) and regeneration media (R): 40 mg/l thiamine HCl and 10 g/l myo-inositol. This stock should be filter sterilized and stored at 4 °C.

3. $CuSO_4$ stock: 125mg of $CuSO_4$. $5H_2O$ dissolved in a total volume of 100 ml water, filter sterilised and stored frozen.

4. Hygromycin: purchased as a sterile 50 mg/ml stock, divided into 1 ml aliquots and stored frozen.

5. Timentin: 160 mg/ml stock made up in water, divided into 1 ml aliquots and stored frozen.

6. Dicamba: 2.5 mg/l stock made up in water, filter sterilised, divided into 1ml aliquots and stored frozen.

7. 2,4-D: 2.5 mg/ml stock made up in 100% ethanol and stored at -20°C.

8. BAP: 1 mg/ml stock made up in water with a few drops of 1 *M* NaOH and stored frozen (*see* **Note 2**).

2.3 Bacterial medium

For culture of Agrobacterium, bacterial culture medium MG/L [9] is used, this 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 solid media 15 g/l agar is added.

2.4 Plant culture media

1. Callus induction (CI): 4.3 g/l Murashige & Skoog plant salt base (Duchefa M0221), 30 g/l maltose, 1.0 g/l casein hydrolysate, 10ml/l of CI vitamin stock, 2.5 mg/l Dicamba, 1.25 mg/l $CuSO_4.5H_2O$, 3.5 g/l Phytagel. The media is adjusted to pH 5.8 with NaOH. Additional copper is omitted during co-cultivation. (*see* **Note 3**)

2. Transition (T): 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, 10ml/l T/R vitamin stock, 2.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 0.1 mg/l 6-benzylaminopurine (BAP), 1.25 mg/l $CuSO_4.5H_2O$, 3.5 g/l Phytagel. The pH is adjusted to 5.8.

3. Regeneration (R): 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, 10ml/l T/R vitamin stock, 3.5 g/l Phytagel. The pH is adjusted to 5.8.

2.5 Agrobacterium strain

Agrobacterium strain AGL1 is used for all transformation experiments.

2.6 Constructs

pBRACT vectors are appropriate for barley transformation [10]. Vectors should contain the *hpt* gene conferring hygromycin resistance under a 35s promoter at the left border (LB) of the T-DNA. Vectors suitable for over-expression or RNAi applications are described at www.bract.org. New vectors for CRISPR/Cas9 based gene knock-out are described in Chapter 14.

2.7 Equipment

1. Very fine, sharp forceps for embryo isolation.

2. Binocular dissecting microscope for embryo isolation.

3. 500ml disposable filter units with 0.22µm membrane.

4. Vacuum pump for filter sterilisation.

5. Standard and deep 9cm petri plates.

6. Glass culture tubes for plantlet rooting or disposable plastic rooting tubes.

7. Micropore tape for sealing petri plates.

8. Oven set to 60°C for warming media.

3. Methods

3.1 Plant culture media preparation

1. All plant culture media are solidified with phytagel. Prepare phytagel in Elga purified water at double the final concentration and sterilise by autoclaving. Store at room temperature until required.

2. Weigh all other media components, except for those listed as stock solutions above, to give double the final required concentration (*see* **Note 4**). Adjust pH to 5.8 and sterilise by filter sterilisation inside a laminar flow hood. Connect disposable filter units to a vacuum pump to speed up filter sterilisation.

3. Heat the filter sterilised media and the phytagel to 60°C in an oven.

4. Add all the required sterile stock components to the warm media and mix well. During all tissue culture steps following co-cultivation, Timentin is added at 160mg/l to control Agrobacterium growth and Hygromycin is added at 50mg/l for selection of transgenic material.

5. Pour the warm, double concentration medium into the bottle containing the warm double concentration phytagel.

6. Mix well and pour plates as quickly as possible. 500ml of CI medium will give 20 standard 9cm plates. 500ml of T or R medium will give 15 deep 9cm plates.

7. Leave plates with lids tilted to dry before packing for storage at 10°C (*see* **Note 5**).

3.2 Preparation of Agrobacterium

1. Select a single colony of Agrobacterium AGL1, containing the required vector, and inoculate 10 mL of liquid MG/L medium containing 25 µg/ml rifampicin and 50 µg/ml kanamycin.

2. Incubate at 28 °C with shaking at 180 rpm for 40 h.

3. Add 10 ml of sterile 30 % aqueous glycerol to the bacterial culture and mix by inverting several times.

4. Prepare 400 µl aliquots of the glycerol / bacterial mix in 0.5 ml tubes and maintain at room temperature for 2 h mixing by inversion every 30 min.

5. Store the prepared standard inoculums at −80 °C ready for use.

3.3 Collection and sterilization of immature seed

1. Barley spikes containing immature seed should be collected when the embryos are approximately 1.5mm in diameter. The spikes will have awns that are just beginning to splay out (Fig. 1A) and look yellow at the tips.

2. A single seed from the middle of the spike should be removed and the size of the embryo checked before cutting the spike.

3. Remove seeds from the spike and remove the awns taking care not to damage the seed coat. Place into a screw topped jar (Fig. 1B).

4. Wash the seed with 70% ethanol for 2 minutes with occasional shaking. All subsequent steps are carried out in a laminar flow hood under sterile conditions.

5. Tip off the ethanol and wash twice with sterile water.

6. Sterilise the seed for 4 min in a 10% sodium hypochlorite solution after diluting 1 to 1 with sterile water.

7. Remove the sodium hypochlorite and wash 4 times with sterile water. Drain off all water after the final wash and leave sterile seeds in the screw top jar (Fig. 1B).

3.4 Isolation of immature embryos

1. Isolate immature embryos from the sterilised seed in a laminar flow hood under a dissecting microscope. It is convenient to place a dark coloured tile on the microscope base, clean well with 70% ethanol and to carry out all manipulation on the sterile tile.

2. Tip 10-20 sterile seeds onto the sterile tile. Position the first seed embryo side up (*see* **Note 6**), (Fig. 2A).

3. Hold the seed firm by piercing it with one pair of fine forceps (Fig. 2B).

4. With a second pair of fine forceps, place one blade under the seed coat close to where the seed is being held (Fig 2C).

5. Pinch the seed coat and peel back over the position of the immature embryo (Fig. 2D)

6. Further expose the immature embryo by gently pushing back the seed coat to the right of the embryo (Fig. 2E).

7. Position the fine forceps over the embryonic axis of the immature embryo with one blade just under the root axis and one blade under the tip of the shoot axis (Fig. 2F).

8. Pinch off the embryonic axis and discard (Fig. 2G).

9. Squeeze the seed gently to release the immature embryo (scutellum) and flip it onto the forceps so the it is positioned scutellum side up (Fig. 2H).

10. Place the embryo onto a prepared plate of CI medium without any antibiotics. Continue until 25 immature embryos have been isolated, place the embryos in a small block to one side of the 9cm petri plate (Fig. 3A). (*see* **Note 7**)

11. Continue until the required number of embryos have been isolated (*see* **Note 8**). Store plates of embryos at 23-24°C in the dark over-night.

3.5 Preparation of Agrobacterium for inoculation

1. Agrobacterium for inoculation of immature embryos is grown up over-night in the absence of antibiotics.

2. Remove one prepared Agrobacterium standard inoculum from the freezer and add to 10ml sterile MG/L medium. Incubate overnight (approx. 18 hours) at 28°C with shaking at 200rpm.

3. Use this full-strength culture for inoculation of immature embryos.

3.6 Agrobacterium inoculation and immature embryo co-cultivation

1. Use 200µl of the prepared Agrobacterium inoculum to inoculate each plate of 25 immature embryos.

2. Using a 200µl pipette, drop one small drop of inoculum onto the surface of each of the 25 immature embryos. When all 25 embryos have been treated, tilt the plate so that excess Agrobacterium runs to the side.

3. Leave the plate for 5-10 minutes to dry (*see* **Note 9**).

4. After drying, transfer the immature embryos to a clean CI plate for co-cultivation, placing embryos scutellum side down. For co-cultivation the CI medium should not contain

antibiotics or additional copper. Spread the immature embryos over the full 9cm petri plate (Fig. 3B) (*see* **Note 10**). Take care to place the embryos gently on the medium surface and do not touch the surface with the forceps as this may lead to excessive Agrobacterium growth.

5. Seal each plate with micropore tape and incubate at 23-24°C for 3 days. (*see* **Note 11**).

3.7 Callus induction

1. After 3 days co-cultivation transfer embryos to CI medium with the antibiotics Timentin and Hygromycin and with additional copper (selection plates). Seal plates with micropore tape and culture at 23-24°C in the dark for 2 weeks. This step is referred to as selection 1 (Fig. 3C).

2. It is possible to check for expression of a reporter gene such as the *gus* gene at this stage. It is common to see relatively little GUS expression after co-cultivation (Fig. 5A).

3. After 2 weeks transfer the immature embryos to a fresh CI selection plate (selection 2) Fig. 3D) and culture under the same conditions.

4. After a further 2 weeks transfer again to a fresh selection plate (selection 3) (Fig. 3E). At this stage it will be necessary to reduce the number of embryo-derived calli per plate. At each subculture the entire callus should be transferred and all callus from each individual immature embryo kept together.

5. Expression of the gus reporter gene can be checked after 4 weeks callus induction to monitor stable transformation events (Fig. 5B).

3.8 Regeneration and rooting

1. After a total of 6 weeks callus induction the immature-embryo derived calli are ready to start regeneration. Gus reporter gene expression at this stage will show intense blue embryogenetic callus (Fig. 5C).

2. Transfer calli to transition medium (T) keeping the level of hygromycin and Timentin the same as during callus induction (Fig. 4A). Culture in the light at 24°C. For the first week after transfer to the light, cover the plates with a thin sheet of paper to reduce the light levels. The first green areas, indicating the initiation of transgenic shoots, should be visible after a few days in the light. At this stage the calli will grow rapidly so further reduce the number of calli / plate during the transfer to T medium.

2. After 2 weeks on transition medium transfer all calli with green regions or developing shoots to regeneration medium (Fig. 4B). At this stage all growth hormones are omitted but hygromycin and Timentin remain at the same concentrations.

3. Leave regenerating calli on the same regeneration plates until small plantlets have shoots 2-3 cm in length and are forming roots (Fig. 4C). Regeneration shoots and roots can also be checked to monitor reporter gene expression (Fig. 5D).

4. Carefully separate out individual plantlets using fine forceps and transfer them to rooting tubes (Fig. 4D). When transferring to rooting tubes take care not to transfer callus as well. Rooting tubes contain CI medium without growth regulators or additional copper but still with the same levels of hygromycin and Timentin (*see* **Note 12**).

3.9 Transfer to soil

1. Once plant shoots reach the top of the rooting tubes they can be transferred to soil. The same compost mix is used as described for the Golden Promise donor plants.

2. Gently remove plants from the culture tubes using a long spatula and the roots washed under a running tap to remove all culture medium. Plants are then potted into moist compost in 5cm pots. For the first week of growth they are covered with a propagator to maintain high humidity.

3. Grow plants to maturity under the same conditions as the Golden Promise donor plants (*see* **Note 13**).

4. The presence of the introduced genes can be confirmed by PCR and transgene copy number determined by qPCR (*see* **Note 14**).

4. Notes

1. The quality of the plants from which immature seed is collected is one of the most important considerations for success. If possible, plants should be grown under controlled conditions and should be kept free of pests and disease. Plants should not be sprayed, particularly when they are flowering.

2. All hormone, antibiotic and copper stocks are kept frozen in small aliquots and 1 tube is removed from the freezer each time media is prepared. This reduces the risk of contamination and ensure that only fresh stocks are used.

3. Adding additional copper during callus induction and the early stages of regeneration leads to a significant increase in the amount of embryogenetic callus and subsequent levels of regeneration. It should not however be included during co-cultivation with Agrobacterium.

4. If preparing a total volume of 500ml of medium then you will need 250ml of double concentration phytagel and 250ml of double concentration medium components.

5. It is convenient to wrap poured plates in cling film and then to place back into the original petri dish bags for storage. All plates should be used within 2 weeks of preparation.

6. The embryo is located at the opposite end of the seed to the awn and on the side with the pigmented stripes, not the side with the groove.

7. Generally 100 immature embryos are isolated for a stable transformation experiment. If only a small number of plants are required this number can be reduced to as few as 25.

8. A skilled operator can isolate 25 immature embryos in 10 minutes.

9. A maximum of 3 plates of immature embryos should be inoculated at once and left to dry before the first embryos are moved to a clean plate for co-cultivation.

10. During transfer to the clean plate for co-cultivation it is important to blot the immature embryos on a clean region of the original plate to avoid transferring too much Agrobacterium culture. However, do not over-clean the embryos so that there is insufficient Agrobacterium left during co-cultivation. This step may take practise so that there is very little Agrobacterium overgrowth after co-cultivation but also good transformation efficiencies.

11. Immature embryos are usually isolated one day and inoculated the following day. However, it is possible to isolate and inoculate on the same day or even to store isolated immature embryos for 2 days before inoculation.

12. We have found that young plantlets grow well on CI medium. However, at this stage transgenic plantlets are robust and will also be fine rooting on regeneration medium without any growth hormones. Long forceps are useful for transferring plantlets to rooting tubes. Disposable plastic culture tubes can also be used for rooting instead of glass.

13. We prefer to grow transgenic barley in controlled environment rooms until it has set seed at which point we move plants to the glasshouse to mature for harvest.

14. Quantitative Real-time PCR analysis of large numbers of transgenic plants has revealed an average of 50% single copy events with another 25% of plants carrying 2 copies of the introduced gene.

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Figure legends

1. Selection of immature seeds

   A: Spikes at the correct stage for immature embryo isolation

   B: Immature seeds following sterilisation

2. Isolation of immature embryos

   A: Immature seed positioned for embryo isolation

   B: Seed held firm with forceps

   C: Second pair of fine forceps positioned to remove seed coat

   D: Seed coat peeled back to expose the embryo

   E: Seed coat pushed back on the right hand side to further expose the embryo and embryo axis

   F: Fine forceps positioned to remove the embryonic axis

   G: Embryonic axis removed

   H: Embryo removed and flipped scutellum side up ready to transfer to CI medium

3. Transformation and callus induction

   A: Immature embryos on CI medium after isolation arranged scutellum side up ready for Agrobacterium inoculation

   B: Embryo co-cultivation with Agrobacterium.

   C: Callus induction - selection 1

   D: Callus induction - selection 2

   E. Callus induction - selection 3

4. Regeneration and rooting

   A: Callus on transition medium

   B. Early regeneration stage

   C: Late regeneration stage

   D: Plantlet rooting

5. GUS reporter gene expression

   A: Transient GUS expression following co-cultivation with Agrobacterium

   B: GUS positive sectors after 4 weeks callus induction

   C: Large GUS positive region following 6 weeks callus induction

   D: GUS expression in regenerating shoots and roots.