Chapter 2

Brachypodium distachyon

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Abstract

The small grass *Brachypodium distachyon* has attributes that make it an excellent model for the development and improvement of cereal crops and bioenergy feedstocks. To realize the potential of this system, many tools have been developed (e.g., the complete genome sequence, a large collection of natural accessions, a high density genetic map, BAC libraries, EST sequences, microarrays, etc.). In this chapter, we describe a high-efficiency transformation system, an essential tool for a modern model system. Our method utilizes the natural ability of *Agrobacterium tumefaciens* to transfer a well-defined region of DNA from its tumor-inducing (Ti) plasmid DNA into the genome of a host plant cell. Immature embryos dissected out of developing *B. distachyon* seeds generate an embryogenic callus that serves as the source material for transformation and regeneration of transgenic plants. Embryogenic callus is cocultivated with *A. tumefaciens* carrying a recombinant plasmid containing the desired transformation sequence. Following cocultivation, callus is transferred to selective media to identify and amplify the transgenic tissue. After 2–5 weeks on selection media, transgenic callus is moved onto regeneration media for 2–4 weeks until plantlets emerge. Plantlets are grown in tissue culture until they develop roots and are transplanted into soil. Transgenic plants can be transferred to soil 6–10 weeks after cocultivation. Using this method with hygromycin selection, transformation efficiencies average 42 %, and it is routinely observed that 50–75 % of cocultivated calluses produce transgenic plants. The time from dissecting out embryos to having the first transgenic plants in soil is 14–18 weeks, and the time to harvesting transgenic seeds is 20–31 weeks.

Key words *Agrobacterium*, Biofuel, *Brachypodium*, Embryogenic callus, Grass, Model system, T-DNA, Tissue culture, Transformation

1 Introduction

The biological, physical, and genomic attributes of the small, inbreeding grass *Brachypodium distachyon* make it a good choice to serve as a model for studies designed to accelerate the acquisition of the basic knowledge necessary to improve cereal crops and grasses poised to serve as bioenergy feedstocks [1–3]. The small size (15–25 cm) and rapid generation time (as short as 8 weeks) of *B. distachyon* permit its use in high-throughput studies in controlled environments such as growth chambers and greenhouses. The sequenced 272 Mbp *B. distachyon* genome is one of the smallest of

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any grass, and the rapid development of genomic resources (including cDNA libraries, BAC libraries, a large EST collection, a high-resolution genetic linkage map, physical maps, extensive germplasm collections, microarrays, and SSR markers) has advanced both the utility and acceptance of *B. distachyon* as a modern model grass species. High-efficiency transformation is required for *B. distachyon* to reach its full potential. In the first steps toward this goal, conditions for inducing embryogenic callus were developed [4], and a successful biolistic transformation system was demonstrated [5]. However, biolistic transformation typically results in complex insertions containing many copies of the inserted DNA, often along with rearrangements of the native DNA [6–8]. Therefore, biolistic transformation is not suitable for many applications that require stable expression or sequencing of genomic regions flanking the transgene insertion site.

Agrobacterium tumefaciens-mediated transformation is currently the predominant technology used to generate transgenic plants [9]. This method typically results in simpler insertions and has been used extensively to create collections of *Arabidopsis* and rice insertional mutants [10–14]. Several reports of *B. distachyon* transformation by *Agrobacterium* also have been published [15–18]. *A. tumefaciens*-mediated transformation utilizes the natural ability of the bacteria to transform plant cells through transfer of a well-defined region of its tumor-inducing (Ti) plasmid into the host genome. For laboratory applications, binary vectors have been designed that contain transfer DNA (T-DNA) border sequences, sequences permitting replication in *E. coli* and *A. tumefaciens*, selectable marker genes, and multiple cloning sites (MCS). The MCS permits placement of genes of interest between the right and left border sequences of the T-DNA in place of the originally encoded set of oncogenes and opine biosynthetic genes. Many *Agrobacterium* strains, plasmids, and protocols have been developed to optimize transformations in various plant species [19].

This chapter describes an optimized, high-efficiency method for the transformation of *B. distachyon* embryogenic callus using *Agrobacterium* and is a substantial improvement over our previously described methods [15, 16]. The first step is the dissection of embryos from immature seeds for the production of embryogenic callus. Dissection of embryos is labor intensive, and therefore calluses are subjected to two rounds of subculture before transformation to yield 50–100 pieces of callus from each embryo. The callus initiation media (CIM) is designed for optimal growth of embryogenic callus, and this growth is substantially improved by the addition of $CuSO_4$ to the media. Next, the pieces of callus are inoculated with *Agrobacterium* carrying the desired transgene sequences. Cocultivation is carried out for 3 days under desiccating conditions that are critical to maintain viability of the calluses. Following cocultivation, the calluses are transferred directly to media designed

both to select for transgenic callus cells and to kill the remaining *Agrobacterium*. After growing to a sufficient size, transgenic calluses are transferred to regeneration media and moved into lighted growth conditions for the regeneration of transgenic plants. When plantlets are large enough to handle without damage, they are moved into tissue culture boxes until they root and are finally transferred to soil. Using this method with hygromycin selection, transformation efficiencies average 42 % and efficiencies of 50–75 % are often observed for individual experiments. We define efficiency as the percentage of calluses cocultivated with *Agrobacterium* that go on to produce transgenic plants. These efficiencies were achieved in a production setting where calluses were transferred on a set timetable and discarded after a set time to minimize labor and space required per transgenic line produced. Based on our experience in creating a population of >20,000 T-DNA lines ([20], http://*Brachypodium*.pw.usda.gov/), we estimate that one trained individual solely focused on transformation can produce and care for 100–150 plants/week. Transgenic plants can be moved into soil 14–18 weeks after dissecting embryos or 6–10 weeks after cocultivations.

2 Materials

2.1 Plant Materials

The tissue used in this transformation protocol is embryogenic callus derived from immature *B. distachyon* seeds. Inbred lines Bd21-3 [16] and Bd21 [15] are recommended for high-efficiency transformation (*see* **Note 1**); however, other *B. distachyon* accessions also can be transformed using this method with varying efficiencies [15, 16].

2.2 Binary Vector Constructs and Agrobacterium Strain

1. Many useful binary vectors are available [19]; however, vectors designed to achieve the goals of a particular project may be needed. In the binary vector, the selectable marker and the promoter driving selection greatly affect transformation efficiency. Hygromycin and paromomycin are suitable for production of transgenic *B. distachyon*. BASTA selection can be used, but transformation efficiency is substantially lower than when using the other two selective agents (*see* **Note 2**).
2. This method uses *Agrobacterium tumefaciens* strain AGL1 [21] containing a plasmid carrying the sequence desired for transformation. AGL1 is a hypervirulent strain with extra copies of some virulence genes. Other hypervirulent strains may be suitable, but we have not tested them. Carbenicillin should be added to growth media to maintain the plasmid containing the extra virulence genes (*see* **Note 3**).

2.3 Stock Solutions

1. Bleach (5.25 % NaOCl).
2. Triton X-100 stock solution (10 %, 100×).
3. $CuSO_4$ pentahydrate (0.6 mg/ml, 1,000×): prepared in water and stored at −20 °C.
4. 2,4-Dichloro-phenoxyacetic acid (2,4-D, 5 mg/ml, 2,000×): made by dissolving 50 mg 2,4-D in 10 ml 95 % ethanol. Solution is stored at −20 °C.
5. 3′,5′-Dimethoxy-4′-hydroxyacetophenone (acetosyringone, 200 mM, 1,000×): prepared by dissolving 0.392 g acetosyringone in 10 ml dimethyl sulfoxide (DMSO) and filter sterilized using a nylon syringe filter (DMSO will dissolve some non-nylon filters). Aliquot stock and freeze at −20 °C.
6. Synperonic PE/F68 (10 %, 100×; Sigma #81112, formerly Pluronic F-68): prepared in water and filter sterilized. Aliquot stock and store at −20 °C.
7. Kinetin (0.2 mg/ml, 1,000×): first prepare a 20 mg/ml stock in 100 % DMSO and then dilute to 0.2 mg/ml with 10 % DMSO. Store at −20 °C.
8. Antibiotic stock solutions for selection of the *Agrobacterium tumefaciens* strain containing the plasmid used in transformation (*see* **Note 3**). The antibiotic used will vary depending on the plasmid. Make 1,000× stock solutions such as 50 mg/ml spectinomycin, 100 mg/ml carbenicillin, or 50 mg/ml kanamycin. Antibiotic stocks should be filter sterilized and frozen at −20 °C in aliquots to avoid repeated freeze/thaw cycles.
9. Antibiotic/herbicide stock solutions for the selection of transgenic callus. The compound used will depend on the selectable marker used in the T-DNA region. Stock solutions (1,000×) are prepared in water, filter sterilized, and frozen at −20 °C in aliquots to avoid repeated freeze/thaw cycles. For hygromycin selection use 40 mg/ml hygromycin B; for BASTA selection use 60 mg/ml DL-phosphinothricin; and for paromomycin selection use 400 mg/ml paromomycin sulfate. Note that paromomycin precipitates when added to media containing the gelling agent phytogel (Sigma P-8169). When working with paromomycin selection, phyto agar should be used for solid media.
10. Timentin (ticarcillin, disodium salt/potassium clavulanate mixture 15:1; 150 mg/ml, 500×): prepared in water and filter sterilized. Aliquot stock and freeze at −20 °C.

2.4 Media

1. Callus initiation media (CIM): per L, add 4.43 g Linsmaier and Skoog (LS) basal medium (this may also be termed Murashige and Skoog minimal organics, MSMO), 30 g sucrose, and 1 ml 0.6 mg/ml $CuSO_4$. Adjust media to pH 5.8

with 0.1 N KOH. Add 0.5 ml of 5 mg/ml 2,4-D stock solution. Autoclave media on a liquid cycle for 45 min, cool, and store at 4 °C until needed. For solid media, add 2.5 g phytagel (Sigma P-8169) for hygromycin or BASTA selection or 5 g phyto agar (Research Products International Corp. A20300) for paromomycin selection to bottles before autoclaving. To prevent clumping, ensure bottles are dry before adding phytagel or agar. Autoclave media on a liquid cycle for 45 min. After autoclaving, cool media to 65 °C in a water bath. When media has cooled sufficiently, transfer it to a sterile hood. Just before pouring media into plates, add 2 ml Timentin stock solution and the appropriate antibiotic/herbicide stock solution (hygromycin, paromomycin, or BASTA) for selecting transgenic callus (*see* **Note 4**). Store plates at 4 °C until used.

2. MG/L media: per L, add 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 5 g mannitol, 0.1 g $MgSO_4$, 0.25 g K_2HPO_4, and 1.2 g glutamic acid. Adjust pH to 7.2 with 1N NaOH. For plates add 15 g agar. Autoclave media for 45 min using a liquid cycle. After autoclaving, cool media to 65 °C in a water bath. When media has cooled sufficiently, transfer media to a sterile hood. Add appropriate antibiotics and pour into petri dishes (*see* **Note 4**).
3. Regeneration media (RM): per L, add 4.43 g Linsmaier and Skoog (LS) basal medium and 30 g maltose. Adjust to pH 5.8 with 0.1N KOH, and then add 1.0 ml of 0.2 mg/ml kinetin stock solution. Add 2.5 g phytagel for hygromycin or BASTA selection or 5 g phyto agar for paromomycin selection. To prevent clumping, ensure bottles are dry before adding phytagel or agar. Autoclave media on a liquid cycle for 45 min. After autoclaving, cool media to 65 °C in a water bath. When media has cooled sufficiently, transfer media to a sterile hood. Add 2 ml Timentin stock solution and appropriate antibiotic/herbicide for selection of transgenic callus, and pour media into petri dishes (*see* **Note 4**).
4. MS media: per L, add 4.42 g Murashige and Skoog (MS) basal medium with vitamins and 30 g sucrose. Adjust to pH 5.7 with 0.1 N KOH. Add 2.5 g phytagel or 5 g phyto agar to bottles. To prevent clumping, ensure bottles are dry before adding phytagel or agar. Autoclave media for 45 min using a liquid cycle. After autoclaving, cool media to 65 °C in a water bath. When media has cooled sufficiently, transfer media to a sterile hood. Add 1 ml Timentin stock solution and appropriate antibiotic/herbicide for selection of transgenic callus. Pour into sterile tissue culture boxes (*see* **Note 5**).

2.5 Other Solutions, Reagents, and Supplies

1. Fine forceps (points 0.1×0.06 mm).
2. Dissecting microscope.
3. Laminar flow hood.

4. Incubator set to maintain a constant 28 °C without lighting (*see* **Note 6**).
5. 0.2 μm nylon syringe filters.
6. Grade P4 7.0 cm circular filter paper, sterilized and then placed in 75 °C oven overnight.
7. Plant tissue culture incubator set to maintain a constant 28 °C with a 16 h light/8 h dark light cycle. Lighting to 65 μEm/m^2/s (*see* **Note 6**).
8. Soil for growing *B. distachyon*: mix 1 part sandy loam, 2 parts sand, 3 parts peat moss, and 3 parts medium grade (#3) vermiculite. The sandy loam can be moistened and autoclaved on a liquid cycle for 30 min, if desired. Before planting, mix into the soil the appropriate amount of a slow release fertilizer (Scotts Osmocote Plus 15-9-12 #903246) for the pot size used (0.25 tsp for a 2 in. pot or 1 tsp for a 6 in. pot) (*see* **Note 7**).
9. Growth chamber or greenhouse suitable for growing *B. distachyon*.

3 Methods

This procedure is based on the fact that very young, immature embryos will form a highly regenerable embryogenic callus when cultured on media containing the auxin 2,4-D. When cocultivated with *Agrobacterium*, this callus is very efficiently transformed. After cocultivation, the calluses are placed directly onto media containing antibiotics to kill the *Agrobacterium* and antibiotics or herbicide to kill untransformed callus. When sufficient transgenic callus has grown, the calluses are placed onto media containing a cytokinin—instead of auxin—to induce the formation of shoots. The transgenic shoots are then placed onto media lacking hormones and allowed to form roots before being transplanted into soil. All steps prior to moving plantlets to soil are performed under sterile, tissue culture conditions, and in the steps prior to regeneration, calluses are incubated in the dark.

3.1 Callus Initiation from Excised Embryos

1. Select immature seed heads when most of the seeds have started to fill out. The seeds should be filled with endosperm but still be soft and flexible when held in your fingers (Fig. 1a). Harvest the seed heads into a 50 ml Falcon tube with a small amount of water and cap to keep the seeds from drying out until they are processed. Seeds can be stored in this way for a few hours.
2. Remove individual seeds from the seed head. Remove the lemma by peeling it away using fingers to grab the long hair at the tip of the lemma and pull it back (Fig. 1b). The palea generally adheres too tightly to be removed without damage, but

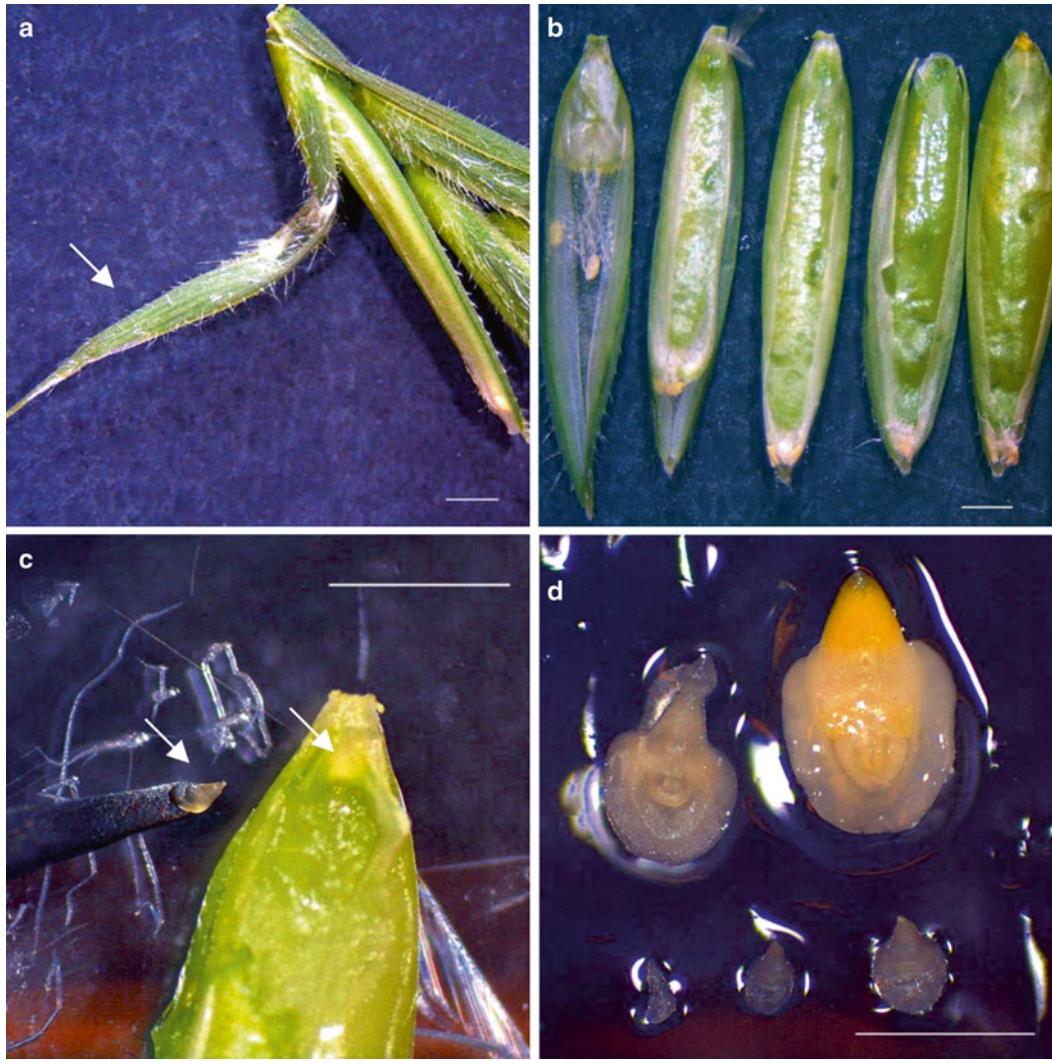

Fig. 1 *B. distachyon* seed and embryo sources for embryogenic callus. (**a**) The *arrow* designates the lemma as it is pulled away from an immature *B. distachyon* seed. After removing the lemma, the seeds are sterilized and the embryo is dissected out of the seed. (**b**) *B. distachyon* seeds are arranged so that the palea side is *down*, and the region containing the embryo is at the *top*, and the anthers are at the *bottom* of the panel. Note the seeds are upside down with respect to how they grow, but this is the most efficient orientation for dissecting out embryos. Seeds are arranged according to increasing maturity from *left* to *right*. The endosperm of the two *leftmost seeds* has not sufficiently filled out, indicating seeds containing embryos that are too small. The embryo in the *rightmost seed* is too mature to produce embryogenic callus, and *yellow color* is noticeable at the upper tip of the seed. The size of the embryos within the two remaining seeds is optimal for producing embryogenic callus. (**c**) The embryo on the tip of fine forceps (*left arrow*) that was just dissected out of the immature seed (*right arrow*) seen in the *center* of panel (**b**). (**d**) The *top* two embryos are *opaque* and *white to yellow in color*. These embryos are too mature to form embryogenic callus. The *bottom* three embryos are *colorless* and *translucent*. All three will form embryogenic callus, but the two on the *left* are optimal. Bars = 1 mm

sometimes it will come off as you peel the lemma from the seed. Place the peeled seeds in a Falcon tube with ~10 ml of water to prevent the seeds from drying out.

3. Remove the water and surface-sterilize the seeds by soaking them in a solution of 10 % bleach plus 0.1 % Triton X-100 for 4 min. Gently mix the tube while the seeds are soaking to ensure all of the seeds are in contact with bleach solution.
4. Rinse the seeds 3 times with sterile water in the sterile hood. After the last rinse, add a small amount of sterile water and return the cap to the tube.
5. Dissect embryos out of immature seeds using fine forceps and a dissecting microscope in a laminar flow hood (Fig. 1c). Transfer seeds from the Falcon tube into the bottom half of a sterile petri dish, and place the lid of the sterile petri dish under the dissecting microscope to act as your dissection surface. Place seeds onto the lid palea side down. Anchor the seed by stabbing it with one set of forceps or your finger, and scrape away at the top layers to reveal the embryo inside. The embryo will be near the tip of the seed opposite from the anthers, just below the surface (Fig. 1b, c). Smaller embryos (<0.3 mm) work best to produce embryogenic callus (>90 % of small embryos will yield good quality embryogenic callus (Fig. 1d) (*see* **Note 8**)).
6. Transfer embryos to CIM plates, placing them on the media with the scutellar side down (the root primordium will be pointing up as in Fig. 1d). Seal plates with Parafilm and incubate at 28 °C in the dark for 3–4 weeks. The calluses produced from excised Bd21 and Bd21-3 embryos display a number of distinct morphologies. At first, the embryos start to form an amorphous soft, whitish callus. After 1–2 weeks on CIM, a yellow callus with organized structures begins to form. This is sometimes interspersed with amorphous white callus. After 3–4 weeks the yellow callus makes up greater than half of the callus volume. The yellow organized callus and the callus with yellow organized structures interspersed with amorphous callus both regenerate and are suitable for transformation (*see* **Note 1**).
7. The embryogenic callus can now be amplified by subculturing at least every 2 weeks. At the first subculture, it is important to select only the yellow organized callus for transfer (Fig. 2a). The callus is broken up into small pieces (approximately 2 mm) and transferred onto fresh CIM plates, leaving space between the pieces for the callus to grow (usually 25–30 pieces/plate) (*see* **Note 9**). The typical routine (Fig. 3) is to subculture embryogenic calluses generated from dissected embryos after 3–4 weeks on CIM. Subcultured calluses are left to grow for

Fig. 2 Embryogenic callus and regenerated transgenic plantlets. (**a**) Embryogenic callus developing from an embryo 3 weeks after dissecting it from a seed. The *white arrow* points to a region of structured, *yellowish*, embryogenic callus. The *black arrow* points to a region of amorphous, *white*, watery callus that is not suitable for transformation. (**b**) Embryogenic callus spread onto filter paper after cocultivation with *Agrobacterium*. Plate diameter is 100 mm. (**c**) Embryogenic callus growing on selective media 3 weeks after cocultivation with *Agrobacterium*. The *top arrow* points to a region of callus that is not transgenic and is turning brown as the selective agent is killing off the cells. The *lower arrow* is pointing to a healthy, *yellow region* of transgenic embryogenic callus. (**d**) *Black arrows* point to transgenic plantlets (ranging in size from 0.5 to 1.5 cm tall) regenerating from transgenic callus 2 to 4 weeks after cocultivation. Note the presence of *black*, dying, non-transgenic callus. Bars = 1 mm (Color figure online)

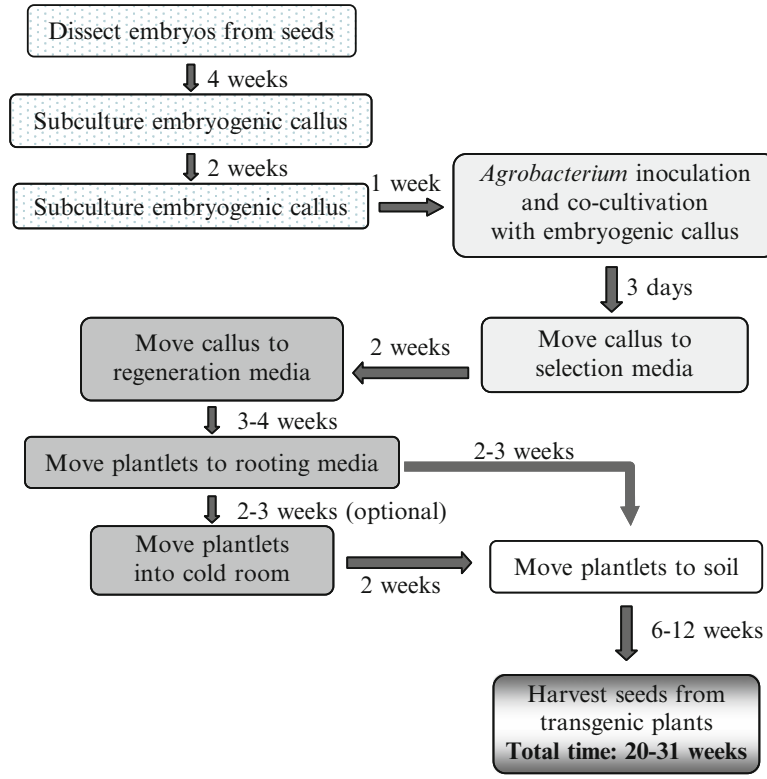

Fig. 3 Flow chart for *Agrobacterium*-mediated transformation of *B. distachyon*. The major steps in the transformation protocol are listed. Transgenic plants can be moved to soil 14–18 weeks after embryos are dissected from immature seeds, and seeds can be harvested from transgenic plants in 20–31 weeks

2 weeks and then subcultured a second time. The calluses from the second subculture are grown for 1 additional week before being used for transformation. The total time from embryo dissection to transformation is 6–7 weeks.

3.2 Transformation of Embryogenic Callus and Regeneration of Transgenic Plants

1. Two days prior to transformation, streak *Agrobacterium* from a frozen stock onto solid MG/L containing the appropriate antibiotics, and incubate the plate at 28–30 °C until needed (*see* **Note 3**). You do not want single colonies, and you do not need to add acetosyringone at this point.
2. At the time of transformation, transfer the callus pieces from the plates into sterile 50 ml Falcon tubes (try not to transfer media with the callus pieces), and cap the tubes to prevent callus from drying out (fill tubes no more than ¾ full). Prepare a suspension of *Agrobacterium* by scraping bacteria from the MG/L plate using a sterile loop or small spatula, and resuspend it by vortexing in liquid CIM to an $OD_{600} = 0.6$. After the OD is adjusted, add the acetosyringone stock solution (1,000×)

to the suspension to a final concentration of 200 μM. Likewise, add Synperonic PE/F68 stock solution (100×) to a final concentration of 0.1 % (*see* **Note 10**).

3. Once the suspension is prepared, immediately add *Agrobacterium* suspension to the tubes with callus pieces. Cap the tubes and invert gently to make sure that all of the calluses come into contact with the suspension. Incubate the calluses with the *Agrobacterium* suspension for 5 min (*see* **Note 10**).
4. During the incubation, prepare petri dishes (100 × 15 mm) for cocultivation. Place 1–2 pieces of sterile filter paper (circular, 7 cm) in each petri dish (*see* **steps 5** and **6**). A good rule of thumb is to prepare one plate for every 50 pieces of callus transformed.
5. After the 5 min incubation, remove as much of the *Agrobacterium* suspension from the callus as possible using a sterile pipet (*see* **Note 11**). Continue until no bubbles can be aspirated into the pipet. Once the *Agrobacterium* suspension is removed, invert the tube over a petri dish containing 2 pieces of filter paper and transfer the calluses to the plate by shaking or tapping the tube. Carefully remove any remaining liquid using a 1 ml micropipette.
6. Using sterile forceps, transfer the calluses to fresh petri dishes prepared with 1 piece of sterile filter paper (Fig. 2b). The calluses from one plate in **step 5** should be divided among multiple plates at this point. Generally, each plate is filled with approximately 50 pieces of callus; however, this will vary with the size of the callus pieces. Spread the calluses around to lightly cover the filter paper (Fig. 2b). The filter paper will be wet around the calluses, but should not be saturated. Leave the petri dishes open in a sterile hood to continue to dry the callus pieces if needed (up to 30 min, based on how wet the callus pieces are). Alternatively, the calluses can be transferred to a fresh petri dish prepared with 1 piece of sterile filter paper to achieve the desired dryness before sealing the plates with Parafilm (*see* **Note 12**).
7. Cocultivate the *Agrobacterium* and callus for 3 days in the dark at 22 °C. Place an empty plate on top of the stack of callus-containing plates to prevent accumulation of condensation in the uppermost plate (*see* **Note 13**).
8. Transfer callus pieces to CIM plates containing 300 mg/L Timentin (to kill *Agrobacterium*) and the appropriate selective agent to kill untransformed plant tissue. The callus pieces should recover quickly and grow rapidly, but you should see a mixture of healthy, yellow transformed tissue surrounded by the dying, brown tissue (hygromycin selection) or watery tissue

(paromomycin and BASTA selections) of the untransformed regions (Fig. 2c). Incubate plates at 28 °C in the dark for 2 weeks (*see* **Note 14**).

9. Transfer calluses to regeneration media containing 300 mg/L Timentin and the appropriate selective agent. At this stage, the callus tissue may look very unhealthy overall, but the small islands of healthy callus will produce transgenic plantlets even when surrounded by dead and dying non-transgenic callus. Incubate plates in the light (cool-white fluorescent lighting at a level of 65 μEm/m^2/s with a 16 h light/8 h dark cycle) at 28 °C. Callus will start to turn green and shoots should appear in 2–4 weeks (Fig. 2d).

3.3 Growth of Transgenic Plants

1. When large enough to handle safely, transfer individual plantlets to tissue culture boxes containing MS sucrose with 150 mg/L Timentin (to kill *Agrobacterium*) for continued growth. At this point, continued selection through addition of the appropriate selective agent to kill untransformed plant tissue is useful to minimize escapes, but is not critical. Incubate boxes in the light (cool-white fluorescent lighting at a level of 65 μEm/m^2/s with a 16 h light/8 h dark cycle) at 28 °C.
2. When plantlets are 2–5 cm tall and have grown roots, carefully transplant to soil. Plants >2 cm can be placed directly into a growth chamber without protection and treated as seedlings. Plants that are transplanted when they are <2 cm should be covered with a clear plastic dome to prevent desiccation. When new growth is obvious, approximately 1 week, remove the dome and move to normal growth conditions. Note that even rootless plantlets have a high survival rate so these should not be discarded.
3. Under growth chamber conditions (20 h light/4 h dark, 24 °C during the day and 18 °C at night, cool-white fluorescent lighting at a level of 150 μE/m^2/s), vernalization is not required to induce flowering; however, plants transferred directly to greenhouse conditions (no shading, 24 °C in the day and 18 °C at night with supplemental lighting to extend day length to 16 h) require vernalization to promote rapid flowering. Vernalization under light at 4 °C (we use continuous cool-white fluorescent lighting, 4 μE/m^2/s) can be performed in tissue culture boxes or after the plants have been transferred to soil. Plants require 2–4 weeks of vernalization, depending on the season.
4. Growth chamber-grown T_0 plants begin to flower 2–3 weeks after transplanting to soil and have mature seed ready for harvest after 6–12 weeks (*see* **Note 15**). The typical yield is 50–150 T_1 seeds/T_0 plant.

4 Notes

1. We prefer line Bd21-3 because it forms a more strongly colored yellow callus with organized structures that is easier to see with the naked eye than that formed by line Bd21. For this reason, it is easier to select and subculture the correct callus using Bd21-3, and as a result, transformation is more efficient.
2. Hygromycin is by far the most efficient selective agent in this system. Paromomycin and BASTA selections typically require a greater number of subculturing steps to ensure the recovery of transgenic plants. Both the maize ubiquitin promoter and the cauliflower mosaic virus 35S promoter with a 3′ intron function well to drive the selectable marker, with the maize ubiquitin promoter having a measurable advantage over the 35S. A 35S promoter lacking the 3′ intron is about half as efficient as a 35S promoter with an intron. In our experience, the rice tubulin promoter did not provide good selection. Addition of a second left border sequence helps to minimize transfer of vector backbone sequences and therefore aids in the recovery of genomic sequence flanking the T-DNA insertion site.
3. *Agrobacterium* is typically grown with two antibiotics, one to maintain the helper plasmid in the strain and one for the binary plasmid. For derivatives of strain AGL1, use 100 mg/L carbenicillin (1,000× stock) for the helper plasmid and the appropriate antibiotics for the binary plasmid.
4. Adding antibiotics to media when it is too hot can result in inactivation of these compounds. In addition, antibiotics will degrade over time. We store our plates at 4 °C and use them within 2 weeks of pouring. If not pouring media on the same day as autoclaving, bottles should be swirled after autoclaving to evenly distribute the gelling agent and then left to solidify at room temperature. Media can be remelted by shaking the bottle to break up the gel, loosening the cap, and carefully microwaving in short intervals. Media should be swirled in between intervals to redistribute the gelling agent evenly.
5. Tissue culture boxes are required for growing plantlets to a size large enough for transplanting. We use sundae cups made for food service applications (Solo Corporation, Lake Forest, IL, Cat. # SOL-TS5 (cups) and SOL-DL-100 (dome lids)) in place of tissue culture boxes made specifically for plant tissue culture. These very inexpensive containers (current cost is a few cents vs. about $1 each for disposable plant tissue culture boxes) come in a range of sizes. Although they are not guaranteed sterile, we have not observed any contamination from these containers in many thousands of transgenics produced. If you are planning to do a lot of transformations, you might consider a local food service supplier for similar containers.

6. Plant tissue culture incubators are optimal because they circulate air below the plates and minimize condensation on the plate lids. However, other growth chambers, such as still air incubators usually used for bacteria, can be used provided that plates are placed on wire racks to minimize temperature differentials in the plates and reduce condensation. In this case, an unused plate containing media can be placed on top of each stack of plates to minimize condensation on the top plate lid. While these incubators are not ideal due to condensation formation, they can be used to obtain small numbers of transgenics.
7. *B. distachyon* grows well in sandy, properly draining soil, but the soil should not be permitted to dry out completely. It is a good idea to check for healthy, white roots to determine whether your soil mixture provides the balance of moisture and aeration that is needed for healthy *B. distachyon*. We have observed phytotoxicity with some commercial soil-less potting mixes. The symptoms appear very similar to a fungal disease. If your *B. distachyon* plants are not thriving, it would be a good idea to try a couple of different soils.
8. If you use your finger to anchor the seed, keep it away from the embryo end of the seed to avoid contamination of the dissected embryo. Practice is required to find the small embryos because, in addition to being small, they are translucent and are hard to see until you become familiar with them. Embryogenic callus can also be obtained from medium-sized embryos (0.3–0.7 mm) >50 % of the time. In addition to being larger, these embryos have started to become opaque; however, they have not yet turned white. Large, older embryos (>0.7 mm) can be easily identified by their white or yellow color. These embryos work very poorly and should not be used (Fig. 1d). To gain experience dissecting embryos, it is useful to start with more mature seeds containing larger embryos and gradually move to less mature seeds to get a feel for where the embryo lies in the seed.
9. The best way to become familiar with identifying embryogenic callus is to test its ability to regenerate by transferring some untransformed callus onto regeneration media with no antibiotics or herbicides. A microscope is helpful to verify that you are choosing the correct material. Once you are familiar with identifying embryogenic callus, subculturing can be performed without the aid of a microscope. The size of the pieces is not crucial and will depend on how friable the callus is. Yellow callus is again selectively transferred at the second subculture. It is not recommended to subculture more than a few times because with extended time in culture, the ability of *B. distachyon* calluses to regenerate declines, and the incidence of sterility and albinism in regenerants increases.

10. An easy way to prepare the *Agrobacterium* suspension is to add an excess of bacteria to the media, vortex the suspension until all of the clumps of bacteria have dispersed, check the OD_{600}, and dilute the suspension as necessary. An $OD_{600} = 0.6$ is optimal for transformation; however, this value is not critical. We typically use suspensions with OD_{600} ranging from 0.55 to 0.65 without a noticeable change in transformation efficiency. The volume of suspension prepared will depend on the volume of callus being transformed. Enough suspension should be prepared to cover all of the calluses. One plate of *Agrobacterium* should be enough make at least 20–50 ml of suspension, depending on the amount of bacterial growth. As a guide, 5 ml of suspension is typically sufficient for 50 pieces of callus (25 ml of suspension is typically sufficient for a 50 ml Falcon tube filled ¾ full). In this step, the time of callus incubation with *Agrobacterium* is not critical and can be extended to allow time to transfer additional pieces of callus.
11. The size of the opening of a 50 ml pipet is optimal for removing the *Agrobacterium* suspension. The opening is large enough that small calluses do not clog it, but small enough that large pieces are not taken up into the pipet. A 10 ml pipet may be used with care to avoid clogging by small callus pieces.
12. Desiccation during cocultivation is critical to achieve high-efficiency transformation. It is important to remove as much of the *Agrobacterium* suspension as possible before transferring/splitting the callus between petri dishes. Transferring too much callus to a single plate will result in excess moisture that prevents the desiccation required for efficient transformation and should be avoided. To evaluate the desired moisture level in the plate, look at the filter paper. It can be slightly wet in the area immediately around the callus pieces, but should not be saturated.
13. To achieve a steady 22 °C, we place a small still air incubator (the type typically used for bacterial cultivation) in a cold room.
14. You may wish to subculture your healthy, transformed callus to increase the time of selection and bulk up healthy callus before transferring to regeneration media. In this case, incubate plates at 28 °C in the dark for only 1 week after cocultivation. Subculture callus pieces onto fresh CIM containing 300 mg/L Timentin (to kill *Agrobacterium*) and the appropriate selective agent to kill untransformed plant tissue. At this point, sectors of healthy, growing callus representing independent transformation events should be kept distinct from one another and can be delimited on CIM plates by drawing lines in the media using forceps. Incubate in the dark at 28 °C and continue to subculture every 2 weeks until you have a sufficient number of

healthy pieces for regeneration. However, it is best to transfer calluses to regeneration media as soon as possible (between 3 and 5 weeks after cocultivation). With hygromycin selection, it is not necessary to obtain a callus with only healthy transgenic tissue, because even small pieces of healthy callus surrounded by dead and dying callus will produce plantlets efficiently (*see* **Note 2**).

15. Updates to this method will be posted to http://*Brachypodium*.pw.usda.gov/.

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