GENETIC TRANSFORMATION AND HYBRIDIZATION

High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3

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Abstract *Brachypodium distachyon* (*Brachypodium*) is a small grass with biological attributes (rapid generation time, small genome, diploid accessions, small stature and simple growth requirements) that make it suitable for use as a model system. In addition, a growing list of genomic resources have been developed or are currently under development including: cDNA libraries, BAC libraries, EST sequences, BAC end sequences, a physical map, genetic markers, a linkage map and, most importantly, the complete genome sequence. To maximize the utility of *Brachypodium* as a model grass it is necessary to develop an efficient *Agrobacterium*-mediated transformation system. In this report we describe the identification of a transformable inbred diploid line, Bd21-3, and the development of a transformation method with transformation efficiencies as high as 41% of co-cultivated calluses producing transgenic plants. Conducting the co-cultivation step under desiccating conditions produced the greatest improvement in transformation efficiency.

Keywords *Brachypodium* · Transformation · *Agrobacterium* · Biofuel · Model system

Abbreviations
CIM   Callus inducing media
EC   Embryogenic callus
EST   Expressed sequence tag
BAC   Bacterial artificial chromosome
2,4-D 2,4-Dichlorophenoxyacetic acid
LS   Linsmaier and Skoog basal medium
MS   Murashige and Skoog salts and vitamins

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Introduction

Despite the fact that humans derive the majority of their food from grasses either directly in the form of grains or indirectly from animals fed a diet of grains and forage, there is currently no truly tractable grass model system. While rice with its sequenced genome, genetic resources and large research community is useful for many experiments, it cannot be considered a truly tractable model system because its large size, long generation time and demanding growth requirements limit its utility, especially for high throughput genomic experiments. Thus, there is a need among grass researchers for a model grass with some attributes more similar to *Arabidopsis* than to rice. *Brachypodium distachyon* (*Brachypodium*) shares many characteristics with *Arabidopsis* including: small size, rapid generation time, small genome, diploid accessions and simple growth requirements. Thus, *Brachypodium* possesses physical and biological attributes necessary to serve as a model system (Draper et al. 2001). The promise of *Brachypodium* has led several groups to begin developing the resources necessary to fully realize the potential of *Brachypodium* as a model system. Resources currently available include: freely accessible inbred lines (Vogel et al. 2006b), cDNA libraries and EST sequences (Vogel et al. 2006a), BAC libraries and BAC end sequences (Hasterok et al. 2006; Huo et al. 2006, 2007). In addition, physical and linkage maps are nearing completion (M. Luo and D. Garvin, personal

communication). The promise of *Brachypodium* led the U.S. Department of Energy to call for the development of *Brachypodium* as a model system to help accelerate the development of superior grasses to serve as feedstocks for the emerging biofuel industry (DOE 2006). A concrete demonstration of DOE's desire to develop *Brachypodium* is evidenced by the initiation of the project to completely sequence the *Brachypodium* genome by the DOE Joint Genome Institute through their Community Sequencing Program. Genome sequencing is currently underway with a likely completion in early 2008. Thus, this small grass is on a fast track to becoming a powerful model that will be useful to researchers studying many aspects of grass biology.

High-efficiency transformation is an absolute requirement if *Brachypodium* is to reach its full potential as a model system. Fortunately, *Brachypodium* has proven receptive to in-vitro manipulations and transformation. Conditions for inducing embryogenic callus have been established (Bablak et al. 1995) and biolistic transformation has been demonstrated (Christiansen et al. 2005; Draper et al. 2001). However, biolistic transformation frequently results in complex insertions containing many copies of the inserted DNA often along with rearrangements of the native DNA (Kohli et al. 2003; Svitashev and Somers 2002). Thus, for many applications (i.e. sequencing DNA-flanking the insertion) particle bombardment is not suitable. *Agrobacterium*-mediated transformation typically results in much simpler insertions and has been used extensively to create insertional mutants of both *Arabidopsis* and rice (Feldmann 1991; Jeon et al. 2000). Previously, we reported a method to transform *Brachypodium* using *Agrobacterium* (Vogel et al. 2006b). However, the transformation efficiency for a diploid accession was too low for experiments that require efficient transformation such as insertional mutagenesis via T-DNA tagging. Here we describe a method that improves transformation efficiency ∼15-fold. This makes *Brachypodium* transformation efficiency comparable to rice transformation and makes T-DNA tagging feasible.

Materials and methods

Plant growth conditions

Plants were grown both in growth chambers and in a greenhouse. For growth chamber experiments the conditions were 20 h light:4 h dark photoperiod, 24°C during the day and 18°C at night with cool-white fluorescent lighting at a level of 150 $\mu E\ m^{-2}\ s^{-1}$. For greenhouse experiments, the greenhouse had no shading, was maintained at 24°C in the day and 18°C at night and had supplemental lighting to extend daylength to 16 h.

Seeds were sown in a soilless mix (sunshine mix #1, SunGro Horticulture, Vancouver, Canada) and fertilized once at planting time with a time release fertilizer containing micronutrients (Osmocote Plus 15-9-12, Scotts Co., Marysville, OH, USA). To encourage synchronous germination during growth-chamber experiments, seeds were stratified at 4°C for 1 week after sowing. To promote flowering of greenhouse-grown plants, seeds were sown in pots and placed at 4°C for 3 weeks after sowing for a combined stratification and vernalization.

Identification of transformable line Bd21-3

Forty seeds from accession PI 254867 were planted in individual pots and the resulting plants were allowed to self. Approximately 60 immature seeds from each subline were harvested, surface sterilized and placed on callus-inducing media as described (Vogel et al. 2006b). The remaining seeds were allowed to mature and were harvested to produce inbred lines. We attempted to transform three lines that produced embryogenic callus. To verify the diploid nature of Bd21-3, leaves were used for flow cytometric determination of *c*-values at the Benaroya Institute at Virginia Mason (Seattle, WA, USA). The method used was that of Arumuganathan and Earle (Arumuganathan and Earle 1991).

Callus initiation

Callus initiation was conducted essentially as described (Draper et al. 2001). Briefly, immature seeds were harvested when most of the seeds were just starting to fill. The lemma was removed using fingers (the palea adheres too tightly to be removed without damaging the seed) and the husked seeds were placed in a 15-ml polypropylene tube containing 3 ml of water to prevent desiccation. After the desired number of seeds were husked, they were surface sterilized by soaking in a solution of 10% household bleach (5.25% NaOCl) plus 0.1% triton X-100 for 4 min with occasional rocking. The seeds were then rinsed three times with sterile distilled water. Embryos were excised under a dissecting microscope in a laminar flow hood using fine forceps. Embryos <0.7 mm wide (Fig. 2a) produced the best callus and were placed on callus induction medium (CIM) [LS salts plus 3% sucrose, 11.25 μM 2,4-D, pH 5.8 and 0.2% phytagel (Sigma)] scutellar side down and incubated at 28°C in the dark. After 3–4 weeks the callus was subcultured onto fresh CIM taking care to select only yellowish embryogenic callus (EC) (Fig. 2). The embryogenic callus was carefully broken into pieces 2–3 mm in diameter taking care not to crush the callus and 25 pieces were placed on each 10-cm petri plate. After 2 weeks the callus was

subcultured again. After an additional week the callus was used for transformation.

Transformation

A super-virulent strain of *Agrobacterium*, AGLI, was used in this study (Lazo et al. 1991). Seven DNA constructs were used in this study: pOL001 (Vogel et al. 2006b) was a test construct containing GUS as a reporter gene inserted into the parent vector pWBVec8 (Wang et al. 1998); pGHyg, p3773 and p3612 were all derived from pGPro2 (Thilmony et al. 2006; GenBank accession EU147786); p#1, p#4, p#6 were derived from pCAMBIA1301 (CAMBIA, Canberra, Australia; GenBank accession AF234297). The details of the T-DNA portion of these vectors are presented in Fig. 1. To prepare the *Agrobacterium* inoculum, *Agrobacterium* was scraped off 2-day-old MG plates and resuspended in liquid CIM supplemented with 200 μM acetosyringone to an OD_{600} of 0.6. After the correct OD was attained, 10 μl of 10% Synperonic PE/F68 (Sigma) per 1 ml of inoculation media was added. One plate of *Agrobacterium* makes at least 20 ml of suspension.

To begin co-cultivation, 100 callus pieces that had been sub-cultured 1 week earlier were placed in a 15-ml plastic tube and covered with the *Agrobacterium* suspension. The callus was incubated in the *Agrobacterium* suspension for 5 min with occasional gentle rocking. The callus was then poured into an empty petri plate and the *Agrobacterium* suspension was carefully aspirated off using a 1-ml pipettor taking care to remove as much liquid as possible. Plates for co-cultivation were prepared by placing a sterile 7-cm diameter p4 grade filter paper (Fisher, Waltham, MA, USA) in an empty 100 × 15 mm petri plate and adding 81 μl of sterile water to the center of the filter. The callus pieces were then gently distributed on the filter paper (Fig. 3a). One co-cultivation plate was used for two starting callus plates (50 initial callus pieces). The co-cultivation plates were then sealed with parafilm and incubated at 22°C in the dark for 3 days. The callus pieces were then transferred individually onto CIM supplemented with 150 mg/l Timentin (Phytotechnology, Shawnee Mission, KS, USA) to kill the *Agrobacterium* and incubated at 28°C in the dark for 1 week. Care was taken not to break any calluses at this stage to maintain their independence. The calluses were then transferred to CIM supplemented with 150 mg/l Timentin and 40 mg/l hygromycin and subcultured to the same media every 2 weeks for 6 weeks. At each subculture all the pieces from a single callus were kept together to maintain their independence.

To regenerate plants, healthy, rapidly growing callus was placed on regeneration media (LS salts plus 3% maltose, 0.93 μM kinetin, 40 mg/l hygromycin, pH 5.8 and 0.2%
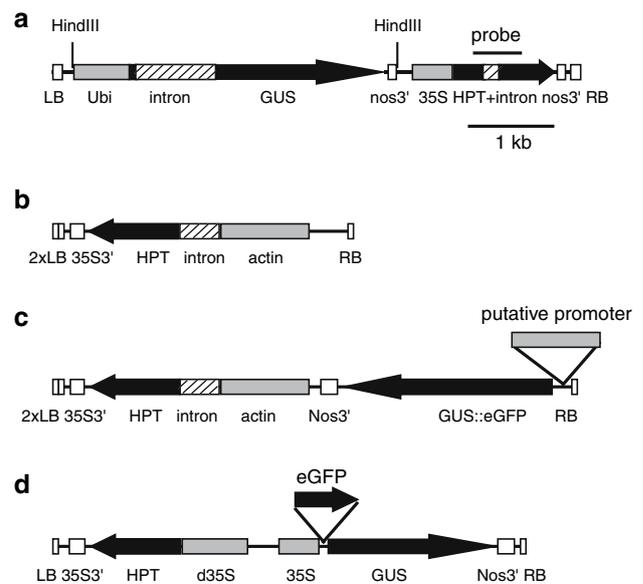
Fig. 1 Diagrams of the T-DNA region of the constructs used in this study. Open reading frames are shown as *black boxes*, promoters are *gray* and introns are *hatched*. **a** pOL001 with *Hind*III restriction sites shown. The location of the probe used for Southern analysis is indicated. **b** pGHyg differs from the parent vector pGPro2 in that the GUS::eGFP fusion has been removed. **c** Constructs p3773 and p3612 differ from the parent vector pGPro2 in that putative *Brachypodium* promoters have been inserted upstream of the GUS::eGFP fusion (**b** and **c**). Note that the *right* and *left* border sequences are synthetic and the *left* border is repeated. **d** Constructs p#1, p#4 and p#6 differ from the parent vector pCAMBIA1301 in that eGFP flanked by different recombinase recognition sites has been inserted upstream of the GUS open reading frame (**a–d**). Abbreviations: *LB* left border; *Ubi* maize ubiquitin promoter; *GUS* β-glucuronidase; *nos3'* terminator from nopaline synthase; *35S* CaMV 35S promoter; *HPT* hygromycin phosphotransferase; *RB* right border; *actin* rice actin promoter; *eGFP* enhanced green fluorescent protein; *d35S* double enhancer version of *35S* promoter

phytagel) and incubated under the following conditions: lighting to a level of 60 μE m^{-2} s^{-1}, a 16-h light:8-h dark photoperiod and a constant 28°C temperature. When plantlets were large enough to handle without damage (Fig. 3c), they were transferred to clear plastic sundae cups (Solo, Urbana, IL, USA) containing MS media (MS salts and vitamins, 3% sucrose, pH 5.7 and 0.2% phytagel) for continued growth and rooting. Even though these inexpensive cups are not certified sterile, we did not observe any contaminating microbial growth. When plantlets had established roots they were transplanted to soil and covered with a clear plastic dome. When new growth was evident, ∼4 days later, the dome was partially opened for 1 day and then removed.

Verification of transgenics and segregation of transgenes

To verify that plants putatively containing pOL001 were transgenic, T$_0$ plants were histochemically stained to detect
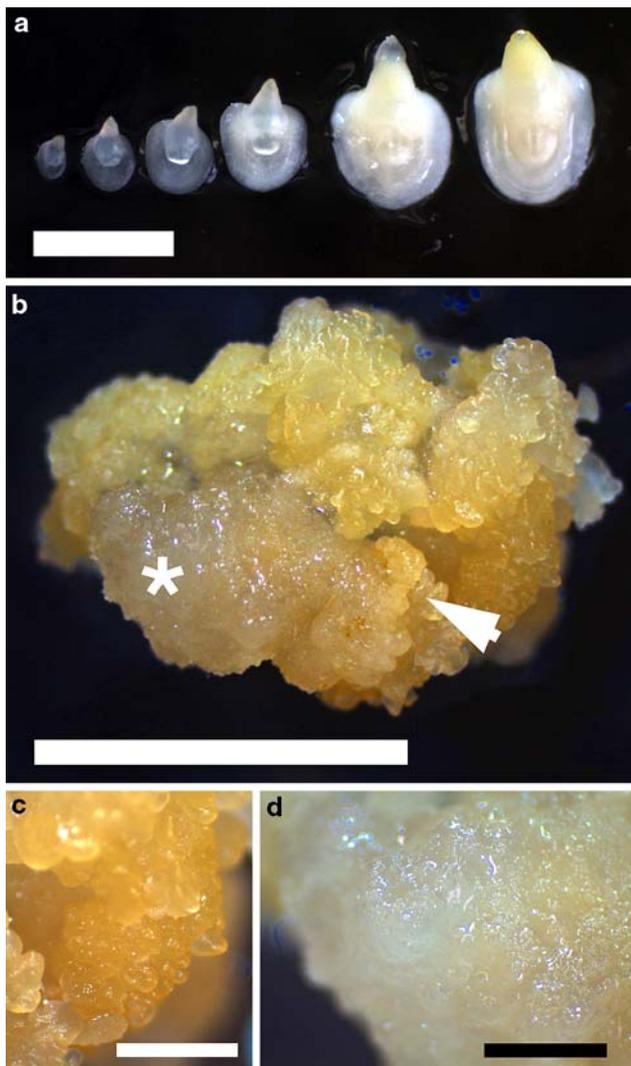
Fig. 2 Embryogenic callus initiation. **a** Immature embryos on CIM. The four embryos on the *left* are small enough to produce good embryogenic callus and the two large embryos on the *right* are too mature. *Scale* 1 mm. **b** Embryo after 3 weeks on CIM. The *yellowish* organized callus (*arrow*) is picked for subculture. Unorganized *white*, watery callus (*asterisk*) is discarded. *Scale* 5 mm. **c** Close-up of embryogenic callus. **d** Close-up of non-embryogenic callus *Scale* in **c** and **d** is 2 mm

GUS activity as described (Vogel et al. 2006b). To verify the transgenic status of plants containing the other constructs, T_0 plants were subjected to PCR as described (Vogel et al. 2006b). The Southern blot was performed using genomic DNA extracted from T_0 plants transformed with pOL001. Ten μg of DNA was digested with *Hind*III and run out on a 0.8% agarose gel. PCR using primers (FH51 GAATTCAGCGAGAGCCTGAC and FH52 ACATTGTTGGAGCCGAAATC), designed to amplify a 557-bp fragment of the *hptII* gene was used to prepare the probe (Fig. 1a). The probe was labeled with ^{32}P-dCTP using a DECAprime II labeling kit (Ambion, Austin, TX,

USA). Southern blot analysis was performed as described (Ausubel et al. 1996). To determine the segregation pattern of the transgenes in the T_1 generation, seeds from 13 lines containing pOL001 were planted and the presence of the transgene was inferred from GUS activity.

Results

Identification of transformable line Bd21-3

Our initial transformation experiments were conducted concurrent with the development of inbred lines (Vogel et al. 2006b). The seed used for our original transformation experiments was bulked from several individuals from accession PI 254867. Thus, this seed contained several genotypes that were present in the original collection. The passport data contained in the USDA-ARS Plant Inventory report for PI 254867 indicates that seeds were collected from a location 4 km from Salakudin on a road to Mosul, Iraq and that the plants were 12-cm high. Though not specifically stated, the seeds were almost certainly collected from several individuals at this location. The inbred line Bd21 was also developed from accession PI 254867 and is the line that is currently being sequenced. Unfortunately, Bd21 performed poorly in our tissue culture system and was difficult to transform. Only 15 of 500 whole immature Bd21 seeds plated on CIM produced embryogenic callus and that callus was of low quality that produced mainly albino shoots when placed on regeneration media. To create an inbred line that is readily transformable, we went back to our original seed stock and created 41 inbred lines. The ability of these lines to produce embryogenic callus was evaluated. The callus produced by these lines was variable. Eight lines produced highly regenerable embryogenic callus. We attempted to transform three of these lines and successfully transformed two (not shown). One line which we designated Bd21-3 was selected as the foundation for our inbred transformable line. To ensure genetic homogeneity we continued inbreeding this line and monitoring transformability.

The general appearance of Bd21-3 was very similar to Bd21 and differed markedly from all 19 polyploid accessions we have grown to date (Vogel et al. 2006b). In general, diploid accessions are smaller, have greater vernalization requirements, and their anthers rarely exert whereas polyploid accessions are larger, flower without vernalization and their anthers typically exert. Both Bd21-3 and Bd21 require either vernalization or long days to induce flowering as described in the methods section. When grown under 20-h days, Bd21-3 went from seed to seed in as little as 8 weeks. To confirm the diploid nature of Bd21-3, we determined the nuclear DNA content by
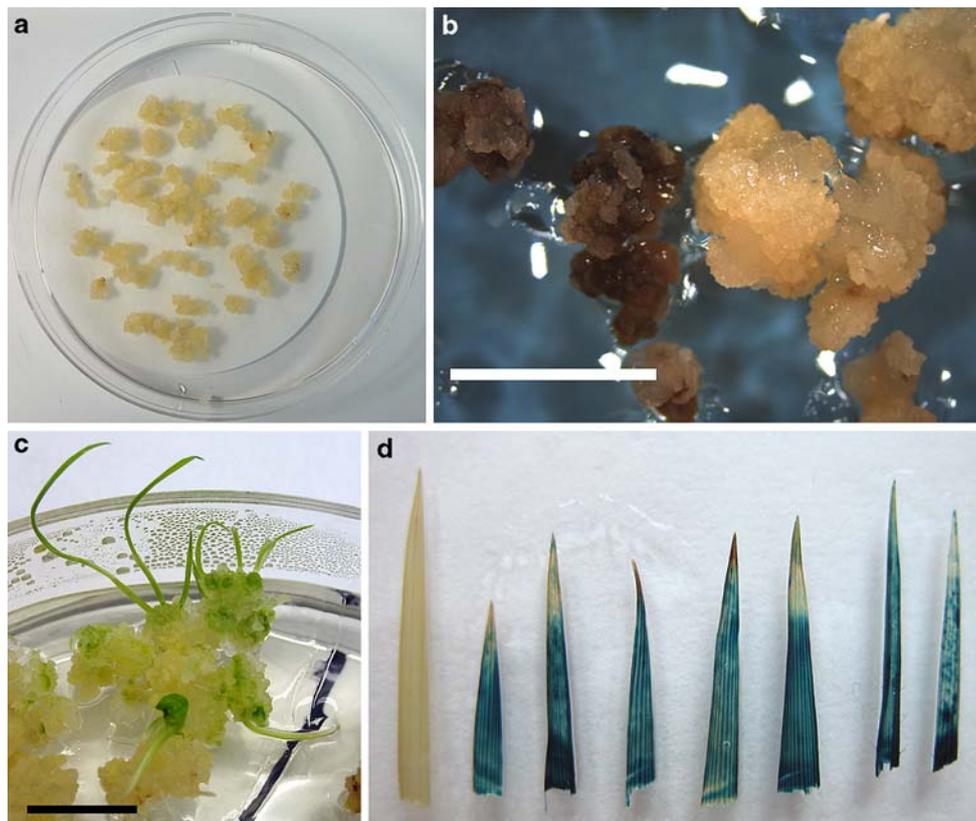
Fig. 3 Stages of transformation. **a** Co-cultivation on filter paper. Approximately 50 callus pieces are placed on each filter. The filter paper is 7 cm in diameter. **b** Selection of hygromycin resistant callus. *Scale* 5 mm. **c** After 2–4 weeks on regeneration media some of the calluses produce shoots. *Scale* 10 mm. **d** GUS stained leaves of seven independent transgenic lines. The non-transgenic control is on the *left*

flow cytometry. The DNA-content of Bd21-3 was 0.38 pg/1C and of a Bd21 control plant was 0.35 pg/1C. These values are consistent with previously reported *c*-values of diploid *Brachypodium* lines (Bennett and Leitch 2005; Christiansen et al. 2005; Vogel et al. 2006b). We also used SSR markers to show that Bd21-3 differed from Bd21 (not shown). Thus, Bd21 and Bd21-3 are presumably derived from separate individuals originating from the same location.

Callus initiation

One factor that significantly impacts the overall efficiency of transformation in terms of person hours required per transgenic line produced is the ease of callus initiation. For *Brachypodium*, callus has been initiated using both whole seeds (Bablak et al. 1995; Vogel et al. 2006b) and isolated immature embryos (Christiansen et al. 2005; Draper et al. 2001). While whole immature seeds can be used as an explant to produce EC for Bd21-3, it is inefficient with only about 5% (8 of 147) of seeds producing callus. This is possibly due to the fact that it is difficult to select seeds

containing embryos of the right maturity and the fact that the embryo is not in direct contact with the CIM. By contrast, callus initiation from small (<0.3 mm) excised embryos and medium (0.3–0.7 mm) excised embryos (Fig. 2a), was highly efficient, 94% (50 of 53) of small embryos formed EC and 55% (21 of 38) of medium-sized embryos formed EC. Large embryos (>0.7 mm) rarely formed EC, 14% (3 of 21). Callus initiation from whole immature seeds was also slower taking 3 weeks longer than callus initiation from excised embryos. The greater percentage of explants that form EC and the more rapid callus initiation make initiating callus from excised embryos more efficient than whole immature seeds for Bd21-3 despite the added labor of dissecting out embryos. The initial callus formed on the embryos is subcultured two times. The resulting amplification means that for each embryo excised approximately 50 pieces of callus go into co-cultivation. While further subcultures would lead to even greater amplification, regenerability of the resulting callus decreases canceling out any benefit of extended subculturing.

The callus produced from excised Bd21-3 embryos displayed a number of distinct morphologies. First, the

embryos start to form an amorphous whitish soft callus. After 1–2 weeks on CIM yellowish callus with organized structures sometimes interspersed with amorphous white callus begins to form. After 3–4 weeks the yellowish callus makes up greater than half of the callus volume (Fig. 2b). The yellowish organized callus and the callus with yellowish organized structures interspersed with amorphous callus both regenerate and are suitable for transformation. At each subculture it is important to select only yellowish callus to transfer.

Improved transformation efficiency

In order to improve the efficiency of transformation we tested a number of different conditions. The most dramatic improvement resulted from conducting the co-cultivation under desiccating conditions similar to what has been used for *Agrobacterium*-mediated transformation of wheat (Cheng et al. 2003). Typically, the callus appeared somewhat dry after the co-cultivation but recovered rapidly and began to grow after being transferred to CIM containing Timentin. At this stage, callus co-cultivated under desiccating conditions was much healthier and grew much faster than callus co-cultivated on CIM possibly because there was much less bacterial growth under desiccating conditions.

After 1 week on CIM plus Timentin, all of the calluses were transferred to CIM containing hygromycin and Timentin. After 2 weeks under hygromycin selection most of the calluses began to die and were growing slowly. All of the calluses were transferred to fresh CIM containing Timentin and hygromycin. At this stage, it is important to transfer all of the calluses regardless of condition because healthy sectors will emerge from dying callus. At this and subsequent transfers, large callus pieces were broken up taking care to keep all pieces originating from one co-cultivated callus together to maintain independence. After another 2 weeks of growth under hygromycin selection a small fraction of the calluses were growing rapidly and were large enough to be placed on regeneration media (Fig. 3b). The rest of the calluses were subcultured taking care to only transfer pieces of callus that appeared to be growing. After a further 2 weeks of growth healthy callus pieces were placed onto regeneration media and unhealthy callus pieces were discarded. Using the same construct, pOL001, that we used in our previous transformation study we obtained an average transformation rate of 36.5% (Table 1). The transformation rate is the percentage of calluses originally co-cultivated that produce fertile transgenic plants. This is almost 15-fold higher than the 2.5% we obtained with standard co-cultivation conditions (Vogel et al. 2006b).

Currently, the greatest limitation to transformation efficiency is the failure of the majority of transgenic callus lines to regenerate plants. Previously we observed a decrease in fertility with the number of subcultures a callus culture underwent (Vogel et al. 2006b). To minimize this problem, we moved the callus as quickly as possible (as soon as we had a few healthy pieces of callus) onto regeneration media. Typically, this is after the second or third transfer onto CIM containing hygromycin. Since callus at this stage may contain non-transgenic sectors it is important that the regeneration media contain hygromycin. Using this approach, non-transgenic escapes were rarely encountered with most transformations having no escapes at all (Fig. 3d). However, even with this approach less than half of the transgenic callus lines produce green shoots.

Many other factors affect transformation efficiency, including the promoter driving the selectable marker. Most

Table 1 Transformation efficiency

Construct	Experiment[a]	Promoter[b]	Parent vector	Initial callus pieces	Fertile transformed plants	Transformation efficiency (%)[c]
pOL001	2	35S with intron	pWBVec8	100	41	41
pOL001	1	35S with intron	pWBVec8	100	32	32
PGHyg	3	Rice actin	pGPro2	100	14	14
p3612	2	Rice actin	pGPro2	100	17	17
p3773	2	Rice actin	pGPro2	66	11	17
p3822	3	Rice actin	pGPro2	100	10	10
p#1	4	d35S	pCAMBIA1301	na	6	na
p#6	4	d35S	pCAMBIA1301	na	4	na
p#4	4	d35S	pCAMBIA1301	na	3	na

[a] Transformations with the same number were performed on the same day with the same batch of EC

[b] Promoter driving the *hptII* gene to confer hygromycin resistance; d35S is the double enhancer version of the 35S promoter

[c] Calculated by dividing the number of fertile transformed plants by the number of initial callus pieces.

of our transformation development was conducted using vector pOL001 in which a 35S promoter drives the *hptII* gene that confers resistance to hygromycin. The *hptII* gene contains an intron to increase expression in monocots (Fig. 1a). To see if other promoters could be used for *Brachypodium* transformation, we tested constructs that contained two other promoters driving the *hptII* gene. Constructs pGHyg, p3773 and p3612 were all derived from the vector pGPro2, a newer version of pGPro1 (Thilmony et al. 2006). pGPro2 uses a rice actin promoter, including a 5′ intron, to drive *hptII* expression. The average efficiency of the transformations with pGHyg, p3773 and p3612 was 14.5%, substantially lower than that obtained with pOL001 (Table 1). This suggests that the rice actin promoter is less efficient for *Brachypodium* transformation.

Another question that arises is the necessity of the intron typically used for monocot transformation when using the 35S promoter. This is of particular interest because a huge number of constructs containing the 35S promoter without an intron have been made for dicot transformation. We successfully transformed *Brachypodium* with three constructs derived from pCAMBIA1301 indicating that an intron in the *hptII* gene is not an absolute necessity (Fig. 1a; Table 1).

Analysis of T-DNA insertions

One of the major advantages of *Agrobacterium*-mediated transformation is the relative simplicity of the T-DNA loci. Southern blot analysis was used to estimate the number of T-DNA insertions in 13 independent lines (Fig. 4). The DNA for the Southern blot was digested with *Hind*III and probed with a DNA fragment contained in the area between a *Hind*III site and the right border (Fig. 1a). Thus, the number of fragments on the Southern represents the number of T-DNA insertions containing this fragment. Three of the 13 lines examined contained a single copy of the T-DNA and 10 of the lines contained 3 or fewer insertions (Fig. 4). To estimate the number of genetic loci we determined the segregation of GUS activity in the T_1 generation of 13 independent lines containing pOL001. This was not the same set of 13 lines used for the Southern blot. For 10 of the 13 lines examined the segregation pattern fits a 3:1 Mendelian ratio (Table 2). Since *Agrobacterium*-mediated transformation commonly results in multiple insertions at the same genetic locus, it is not surprising that the average number of T-DNA inserts observed by Southern analysis is higher that the number of genetic loci. Similar to *Arabidopsis*, rice and our initial results with *Brachypodium*, the current transformation method results in relatively simple insertion patterns (Feldmann 1991; Jeon et al. 2000; Vogel et al. 2006b).
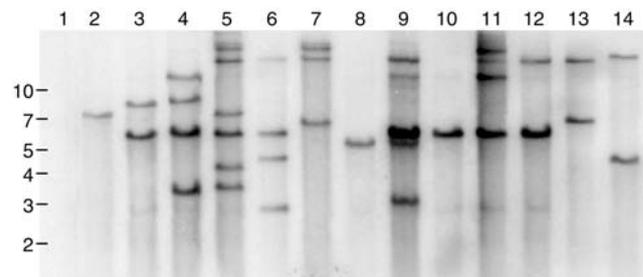
Fig. 4 Southern blot of 13 transgenic lines. *Lane 1* is untransformed Bd21-3. *Lanes 2–14* are independent transgenic lines transformed with pOL001. The blot was probed with a 557-bp fragment amplified from the *hptII* gene indicated in Fig. 1a. The *scale* is in kb

Discussion

A simple, efficient transformation system is required to fully utilize *Brachypodium* as a model system. While transformation of diploid *Brachypodium* accessions via *Agrobacterium*-mediated and biolistic methods has been previously demonstrated, the efficiency was not high enough for many applications. We improved on our previous transformation method by using excised immature embryos to initiate EC cultures and, most importantly, by altering the co-cultivation conditions. These improvements increased our transformation efficiency ∼15-fold. This brings *Brachypodium* transformation into the same range as the highly efficient rice transformation system (reviewed in Tyagi and Mohanty 2000). Furthermore, the time from callus initiation to transgenic plants in soil can be as short as 16 weeks and the time from non-transgenic seed to transgenic seed is as short as 30 weeks.

Table 2 Segregation of GUS expression in the T_1 generation of plants transformed with pOL001

Transgenic line	GUS positive plants	GUS negative plants	χ^2 value for 3:1 ratio	P value	Fits 3:1 ratio ($P = 0.05$ cutoff)
7C	15	5	0.00	1.00	Y
15B	18	8	0.46	0.50	Y
21B	20	1	4.59	0.03	N
46D	19	5	0.22	0.64	Y
51C	17	3	1.07	0.30	Y
56A	17	2	2.12	0.15	Y
61C	19	5	0.22	0.64	Y
62A	14	6	0.27	0.61	Y
66B	20	8	0.19	0.66	Y
92C	18	0	6.00	0.01	N
132A	13	5	0.07	0.79	Y
134A	16	11	3.57	0.06	Y
148B	15	0	5.00	0.03	N

While transformation efficiency in terms of the percentage of calluses that produce transgenic plants is important, what really matters is the amount of work in terms of time and the number of transfers that are required to generate each transgenic line. One of the most demanding steps in the transformation process is the establishment of the EC cultures. We observed that a very high percentage of small immature Bd21-3 embryos formed EC. Since embryo dissection is labor intensive, it is important to note that by sub-culturing the EC two times we amplified the number of callus pieces arising from each embryo by a factor of ~ 50. Thus, at a transformation efficiency of 40% ~ 20 independent transgenic lines are generated from each dissected embryo.

In addition to the plant genotype and the transformation method, properties of the T-DNA vector also affect transformation efficiency. To begin examining this facet of transformation we performed transformations with constructs that differed in the promoter driving the selectable marker. We observed that a construct in which the 35S promoter drives an *hptII* gene containing an intron was more efficient than constructs using the rice actin promoter with a 5′ intron. We also demonstrated that a 35S promoter without an intron could be used to successfully transform *Brachypodium*. Thus, many constructs designed for dicot transformation using 35S promoters can be used without modification to transform *Brachypodium*.

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