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Agrobacterium-mediated transformation and inbred line development in the model grass *Brachypodium distachyon*

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Abstract

Brachypodium distachyon (*Brachypodium*) has been proposed as a model temperate grass because its physical, genetic, and genome attributes (small stature, simple growth requirements, small genome size, availability of diploid ecotypes, annual lifecycle and self fertility) are suitable for a model plant system. Two additional requirements that are necessary before *Brachypodium* can be widely accepted as a model system are an efficient transformation system and homogeneous inbred reference genotypes. Here we describe the development of inbred lines from 27 accessions of *Brachypodium*. Determination of c-values indicated that five of the source accessions were diploid. These diploid lines exhibit variation for a variety of morphological traits. Conditions were identified that allow generation times as fast as two months in the diploids. An *Agrobacterium*-mediated transformation protocol was developed and used to successfully transform 10 of the 19 lines tested with efficiencies ranging from 0.4% to 15%. The diploid accession Bd21 was readily transformed. Segregation of transgenes in the T_1 generation indicated that most of the lines contained an insertion at a single genetic locus. The new resources and methodologies reported here will advance the development and utilization of *Brachypodium* as a new model system for grass genomics.

Abbreviations: BA – benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; LS – Linsmaier and Skoog basal medium; MS – Murashige and Skoog salts and vitamins; CIM – callus inducing medium

Introduction

The value of the model system approach to modern plant biology has been powerfully illustrated by the dicot species *Arabidopsis thaliana*. Unfortunately, a dicot model is not suitable for answering questions about monocots where monocot and dicot biology diverge (e.g., cell wall composition). With its sequenced genome and large research community, rice can serve as a model monocot system for some questions. However, rice is not a good functional model species for temperate grasses because it is a semi-aquatic tropical grass that diverged from the most important forage grasses and temperate grains nearly 50 million years ago (Gaut, 2002) and thus is only distantly related. There are also logistical issues that limit the utility of rice as a model system. For instance, it is challenging to grow rice and have it flower under the conditions typically present in greenhouses in northern climates. Also, the large size of rice plants and the long generation

time of this species makes the completion of experiments expensive, particularly when large numbers of plants are involved.

Brachypodium distachyon has been proposed as a model temperate grass because it possesses all the characteristics required for a modern model organism: self fertility, small stature, simple growth requirements, short generation time, diploidy in some ecotypes, and a small genome size (Draper et al., 2001). The reported size of the haploid genome of diploid *Brachypodium* ecotypes varies from 0.15 to 0.36 pg (Shi et al., 1993; Draper et al., 2001; Bennett and Leitch, 2005). However, since the 0.15 pg is likely to be an underestimate (Bennett and Leitch, 2005), the haploid genome size is assumed to be approximately 0.36 pg. This puts the size of the *Brachypodium* genome between *Arabidopsis* (0.16 pg) and rice (0.495 pg) (Bennett and Leitch, 2005). These results suggest that *Brachypodium* possesses one of the smallest genomes of any grass and is suitable for studying both structural and functional genomics. An additional benefit of *Brachypodium* is that it belongs to a tribe in the grass family that is sister to the core pooids comprised of the four grass tribes that encompass most of the important cool-season cereal grains, forage grasses and turfgrasses (Kellogg, 2001). Thus, *Brachypodium* is far more closely related to these crops than is rice (Kellogg, 2001).

While the potential of *Brachypodium* to serve as a model system has been discussed (Draper et al., 2001), many steps remain before it can be fully utilized. Christiansen et al. (2005) reported genetic heterogeneity was present within collected accessions of *Brachypodium*. This is not surprising since no provisions were likely to have been taken to preserve individual plant identity when the species was initially collected; in fact the initial collections likely contained seeds from multiple plants in wild populations. Thus, one important step that needs to be taken is to develop homogeneous inbred lines from diverse diploid ecotypes that can serve as reference ecotypes, to permit integration of information generated by different laboratories.

An efficient transformation system is required to fully realize the power of a modern model plant system. Biolistic transformation of *Brachypodium* has been described (Draper et al., 2001; Christiansen et al., 2005). However, due to the nonspecific method of transgene integration, biolistic transformation typically results in complex transgene insertion patterns containing many copies of the transgene and substantial rearrangements of both the inserted DNA and chromosomal DNA (Svitashev and Somers, 2002). Complex insertions make subsequent genetic analysis, including cloning flanking plant DNA, difficult. In contrast to bioloistic transformation, the nature of T-DNA insertion during *Agrobacterium*-mediated transformation usually results in insertions at only one or at most a few loci. This has been demonstrated for both *Arabidopsis* and rice where studies of T-DNA populations have found an average of 1.5 and 1.4 T-DNA loci per transgenic line respectively (Feldmann, 1991; Jeon et al., 2000). *Agrobacterium tumefaciens* has been used to transform several grass species and has become routine for some (reviewed in Cheng et al., 2004).

Embryogenic callus is a desirable target tissue for transformation due to its high regeneration efficiency and its prolific growth that allows experiments to be easily scaled up. The ability to generate embryogenic callus varies greatly between species and among accessions of a single species. Embryogenic callus has been generated from immature *Brachypodium* embryos indicating that at least some *Brachypodium* genotypes can form embryogenic callus (Bablak et al., 1995; Draper et al., 2001; Christiansen et al., 2005). Here we report the development of a set of reference inbred diploid *Brachypodium* lines and identify growth requirements that greatly accelerate generation turnover for several of the lines. Further, we have characterized the ability to generate embryogenic callus from the inbred lines and describe an *Agrobacterium*-based method for efficient transformation of *Brachypodium*.

Methods

Development of inbred lines of B. distachyon

Seeds of *B. distachyon* accessions deposited in the USDA National Plant Germplasm System (NPGS) were obtained for use in inbred line development. Seeds of each accession were germinated on filter paper and transferred to soilless mix in a greenhouse. After approximately one month, pots were transferred to a cold room (~5 °C) to vernalize for 5 weeks. Pots were then moved back

to a greenhouse to allow plants to flower. Seed of several individual plants for each accession was harvested and kept separate; these formed the basis of inbred line development. Subsequently, lines were advanced two or more additional generations in most cases by single seed descent, and then multiple plants from single inbred plants were selfed and seed bulked to produce inbred lines.

Determination of ploidy levels of B. distachyon *lines*

Young leaf tissue from several plants of each line was harvested and used for flow cytometric determination of c-values at the Benaroya Institute at Virginia Mason (Seattle, WA). The method used was that of Arumuganathan and Earle (Arumuganathan and Earle, 1991), with chicken red blood cells serving as a standard.

Growth conditions for transformation experiments

Plants were grown in a greenhouse with supplementary lighting to extend daylength to 16 h. The greenhouse had no shading and was maintained at ~24 °C in the day and ~18 °C at night. To promote synchronous germination, seeds were stratified at 4 °C for 1 week. To encourage flowering, diploid genotypes were vernalized by either sowing seeds in soil and keeping the pots at 4 °C for 6 weeks (combined stratification and vernalization) or by moving plants that had been grown in the greenhouse for 4 weeks after stratification to 4 °C under continuous fluorescent light for 4 weeks. The latter treatment results in larger plants that produce more seed.

Tissue culture conditions

Immature seeds were harvested 2–3 weeks after flowering when most of the seeds were large enough to handle without damaging them, but before they were fully filled. A range of seed maturities was sampled. The palea and lemma were manually removed and the naked seeds were placed in distilled water to prevent desiccation. Immature seeds were surface sterilized by soaking in a solution of 10% bleach (5.25% NaOCl) plus 0.1% triton X-100 for 4 min. Tubes containing the seeds were placed on a rocker to mix while soaking. After soaking in the bleach solution the seeds were rinsed three times with sterile distilled water. The surface-sterilized seeds (30 per 15 cm petri plate) were then randomly placed on the surface of callus induction medium (CIM) (LS salts (Linsmaier and Skoog, 1965) plus 3% sucrose, 11.25 μM 2,4-D and 0.2% phytagel (Sigma)) (Bablak et al., 1995). Each petri plate contained ~25 ml of CIM and was sealed with parafilm after plating the seeds.

Mature seed explants were prepared in a similar fashion except they were not placed in distilled water after removing the palea and lemma and they were surface sterilized in 15% bleach plus 0.1% triton X-100 for 30 min. Plates containing seeds were incubated at 28 °C in the dark. Embryogenic callus was removed from the seed and placed onto fresh CIM as soon as it was large enough to handle, about 4 weeks. The callus was then broken into small pieces and placed onto fresh CIM. The cultures were subcultured every 2 weeks by breaking them into small pieces (~2 mm diameter) and distributing onto CIM. At each transfer only compact embryogenic callus was transferred.

To regenerate plants, callus pieces were broken into pieces (~5 mm diameter) and placed on regeneration medium (LS salts plus 3% maltose, 0.93 μM kinetin and 0.2% phytagel (Sigma)) (Bablak et al., 1995). Regeneration plates were incubated at 28 °C with a 16 h light and 8 h dark photoperiod. Light intensity of 80 $\mu molm^{-2}s^{-1}$ was provided by fluorescent lights. Once the regenerated plants were large enough to handle (usually after 3 weeks) they were transferred to plantcon containers (MP Biomedicals, Aurora, OH) containing growth medium (MS salts and vitamins, 3% sucrose and 0.2% phytagel). After 2–3 weeks the regenerated plants were 3–6 cm tall and were transplanted to soil and placed on a mist bench in the greenhouse. New growth was obvious after 2 weeks at which time the plants were moved into a greenhouse without mist.

Transformation

Callus cultures were subcultured to fresh CIM one week prior to transformation taking care to select only embryogenic callus. A super-virulent strain of *Agrobacterium*, AGLI, was used in this study (Lazo et al., 1991). Three DNA constructs were used: pOL001 was a test construct containing

GUS as a reporter gene (Figure 2a), pT1 and pT2 were constructs designed for transposon tagging. All constructs contained a hygromycin phosphotransferase gene containing an intron in the 3′ region driven by a 35S promoter to confer hygromycin resistance to transformed plants. To prepare the *Agrobacterium* inoculum, *Agrobacterium* was scrapped off 2-day-old plates (MG medium (Garfinkel and Nester, 1980) supplemented with 50 mg l^{-1} spectinomycin and 100 mg l^{-1} carbenicillin) and resuspended in liquid LS medium supplemented with 200 μM acetosyringone to an OD_{600} of 0.6. The callus was immediately placed into the *Agrobacterium* suspension for 5 min. Callus pieces were then distributed onto solid CIM supplemented with 200 μM acetosyringone and incubated in the dark at 28 °C. After 3 days, calluses were transferred to CIM supplemented with 150 mg l^{-1} Timentin to kill the *Agrobacterium*. After 1 week the calluses were transferred to CIM supplemented with 150 mg l^{-1} Timentin and 40 mg l^{-1} hygromycin and subcultured onto the same medium every 2 weeks. At this stage the calluses often turned dark brown or black and healthy yellowish embryogenic callus would often emerge from dead looking calluses. Healthy callus was removed from the dying callus as soon as practical to ensure survival. Once healthy, hygromycin-resistant calluses were obtained, usually after 4–6 weeks, they were transferred onto regeneration medium supplemented with 40 mg l^{-1} hygromycin and handled as previously described. Throughout this process all calluses derived from each initial explant was kept together to ensure the independence of regenerated plants arising from different original explants.

Molecular analysis and histochemical staining

T_0 transformants were verified by PCR using primers (FH51: GAATTCAGCGAGAGCCTG AC and FH52: ACATTGTTGGAGCCGAAA TC) designed to amplify a portion of the hygromycin resistance gene. DNA for PCR was extracted as described by (Edwards et al., 1991). Histochemical staining to detect expression of the GUS gene in leaves was conducted as described (Sundaresan et al., 1995) except that no chloramphenicol was used in the stain. Stained leaves were cleared in 95% ethanol at 65 °C to better visualize the stain.

Genomic DNA for Southern blot analysis was prepared as described by (Riede and Anderson, 1996). Two μg of DNA was digested with BamHI and run out on a 1% gel. PCR using primers (FH64 CTTGGCCGAAGCTTGCAT and FH65 TGGTTGGTGTCCGTTAGACTC) designed to amplify a 642 bp fragment of the maize ubiquitin promoter was used to prepare the probe. The probe was labeled with ^{32}P-dCTP using a Rediprime labeling kit (Amersham, Piscataway, NJ). Southern blot analysis was performed as described by (Ausubel et al., 1996).

Results and discussion

Line development and ploidy

Twenty-nine accessions listed as *B. distachyon* were obtained from the NPGS. One of these was eliminated from further line advancement because its flower morphology indicated that it was not *B. distachyon*. A second accession could not be induced to flower even after prolonged vernalization, and so it too was not advanced further. Thus, a total of twenty-seven accessions were used to develop inbred lines (Table 1). For most lines, a minimum of three generations of single seed descent was conducted, followed by selfing a set of plants grown from seeds of a single inbred plant, to obtain inbred lines. Further, for the majority of the accessions received, two independent inbred lines were developed (Table 1). The individual lines are expected to be highly homozygous across loci, while pairs of inbred sister lines derived from the same accession may retain some degree of molecular diversity that may be present in the original accession.

Ploidy determination was undertaken to identify diploid lines, which are of greatest interest for a model system. The c-values of inbred lines fell into two general groups; the majority of the lines had c-values between 0.65 and 0.91 (Table 1). However, lines derived from five of the accessions had c-values in a range between 0.39 and 0.49 (Table 1). Cross-referencing of the origins of the inbred lines to other available information (http://www.aber.ac.uk/plantpathol/germplasm.htm) and publications (Christiansen et al., 2005) helped to confirm that the lines with the lower c-values were diploid. Preliminary chromosome counts of one of

Table 1. Descriptive information on inbred *B. distachyon* lines developed from the USDA ARS Germplasm Resources collection

Inbred line (s) (Bd_)	Source PI accession	Geographic origin	C-values[a]	Putative ploidy
1–1	PI 170218	Turkey	0.47	Diploid
2–3	PI 185133	Iraq	0.49	Diploid
3–1, 3–2	PI 185134	Iraq	0.40, 0.39	Diploid
4–1, 4–2	PI 208216	South Africa	0.77, 0.79	Hexaploid
5–1, 5–2	PI 219961	Afghanistan	x[b], 0.8	Hexaploid
6–1, 6–2	PI 219965	Afghanistan	0.9, x	Hexaploid
7–1, 7–2	PI 219968	Afghanistan	0.87, 0.85	Hexaploid
8–1, 8–2	PI 219971	Afghanistan	x, 0.83	Hexaploid
9–1, 9–2	PI 220567	Afghanistan	0.80, 0.80	Hexaploid
10–1, 10–2	PI 226452	Iran	x, 0.8	Hexaploid
11–1, 11–2	PI 226629	Iran	0.82, 0.79	Hexaploid
12–1, 12–2	PI 227011	Iran	0.67, 0.72	Hexaploid
13–1, 13–2	PI 233228	Israel	0.87, 0.80	Hexaploid
14–1, 14–2	PI 239713	Iran	0.68, 0.75	Hexaploid
15–1, 15–2	PI 239714	Iran	0.79, 0.77	Hexaploid
16–1	PI 239715	Iran	0.85	Hexaploid
17–1, 17–2	PI 239716	Iran	x, 0.78	Hexaploid
18–1	PI 245730	Turkey	0.39[c]	Diploid
19–2	PI 250647	Pakistan	0.84	Hexaploid
20–1, 20–2	PI 253334	Morocco	0.76, 0.91	Hexaploid
21	PI 254867	Iraq	0.44	Diploid
22–1, 22–2	PI 254868	Iraq	0.80, 0.91	Hexaploid
23–1, 23–2	PI 287783	Spain	0.78, 0.77	Hexaploid
25–1, 25–2	PI 321403	Israel	0.76, 0.76	Hexaploid
26–1, 26–2	PI 372187	Uruguay	0.78, 0.71	Hexaploid
27–1, 27–2	PI 422452	Germany	0.72, 0.65	Hexaploid
28	PI 533015	Australia	0.79	Hexaploid

[a] c-Values obtained on one of three different dates. The first value corresponds to the first inbred line listed in column 1, the second is for the second line listed where applicable. Values are rounded to the nearest hundredth.
[b] x: c-value not obtained for this line.
[c] Bd18 was used as a standard on all three dates that c-values were determined. The value in the table is the mean of the three individual values obtained (0.37, 0.35, and 0.46).

these lines (Bd3-2) indicated a somatic chromosome number of 10, supporting this contention. The mean c-value of the diploid lines across genotypes and dates was just over 0.4. This is higher than a recent report of 0.36 (Bennett and Leitch, 2005). However, the last run date produced values significantly higher than two previous dates. For instance, Bd18 gave c-values of 0.37 and 0.35 in two earlier runs, but a c-value of 0.46 in the third run. The c-values of three diploids (Bd1-1, Bd2-3, Bd21) came from the third run, and exhibited values similar to that of Bd18 on that date. Thus, the diploid c-values obtained only on the third run date are probably overestimates of the true c-values.

Interestingly, while the other lines had c-values that were approximately double that of the diploids, suggesting that they were tetraploid, cross-referencing the origin of these inbred lines with other literature suggests that in fact many of them are hexaploid. This is supported by our preliminary chromosome counts of one line (Bd12-2) in this group of putative hexaploid lines. This observation could reflect the process of genome diploidization (Wolfe, 2001), which in ploypoids can involve loss of genetic material. Alternatively, the hexaploid accessions could derive from hybridization of species with dramatically different c-values. Indeed, a recent publication (Hasterok et al., 2004) postulated that hexaploid ecotypes of

Brachypodium are allopolyploids, with one genome similar to diploid Brachypodium and the other genome from an as yet uncharacterized Brachypodium subtype or species with a base chromosome number of 10.

Acceleration of diploid line growth and development

The majority of the inbred line development was conducted in the greenhouse, and vernalization was used for many lines to induce flowering during inbred line development based upon recommendations in a previous publication (Draper et al., 2001). This included all of the diploid lines, which exhibited significant differences in vernalization requirement. In one experiment, each of the diploid accessions was grown in the greenhouse for one month and then vernalized for different lengths of time to more finely characterize differences in vernalization requirement. The relative vernalization requirements varied dramatically. For Bd3 and Bd21, 3 weeks of vernalization resulted in the initiation of flowering approximately 2 weeks after the treatment. In contrast, longer vernalizations (4 or 6 weeks) of Bd2 resulted in flowering in approximately 28 days. Both Bd18 and Bd1 required significantly longer vernalization; after 6 weeks of vernalization Bd18 flowered in approximately 5 weeks, while Bd1 began flowering in approximately 6 weeks.

The vernalization requirement is an impediment to rapid generation turnover in a model system, and thus efforts to identify conditions to accelerate flowering were initiated. Subsequently, research in growth chambers allowed us to more carefully control the growth conditions and evaluate the effect on plant development. We determined that long day lengths (≥20 h) obviated the vernalization requirement for Bd2-3, Bd3-1, and Bd21. Indeed, under these conditions, Bd21 begins to flower approximately three weeks after planting, Bd3-1 begins flowering in approximately 4 weeks, and Bd2-3 begins flowering in approximately 6 weeks (Figure 1a). Long daylengths were found to be ineffective in eliminating the vernalization requirement for Bd18 and Bd1-1 (Figure 1a). One benefit from this finding is that the need to vernalize three of the lines is obviated and flowering occurs more rapidly than that obtained with vernalization. Under long daylengths and limiting growth conditions (small soil volume, minimal fertilizer), mature seed for Bd21 can be obtained in approximately two months. This generation time is approximately twice as fast as what has been reported previously (Draper et al., 2001; Christiansen et al., 2005), and represents an important advance in the ability to manipulate this species as a model system. A second benefit from this discovery is that if desired, one can grow these same photoperiod-sensitive lines under shorter day lengths (e.g., 14 h) either to obtain a large amount of biomass, or to subsequently vernalize older plants to obtain many (>500) seeds from a single plant. The flexibility afforded by the ability to regulate flowering of several of the diploid lines extends the utility of *Brachypodium* to encompass research not only on temperate grain crops but also forage and turf grasses.

Initiation of embryogenic callus and regeneration of plants

The excellent regeneration efficiency of embryogenic callus make it a desirable target for *Agrobacterium*-mediated transformation. To simplify the process of generating embryogenic callus, we adapted a method based on excised immature embryo explants to use more easily obtained whole seed explants (Bablak et al., 1995). Preliminary experiments using Bd3, Bd21 and Bd14 indicated that callus grew faster at 28 °C than 22 °C (not shown). All subsequent experiments were conducted at 28 °C. For most genotypes, a watery callus began to appear from the embryo shortly after plating on CIM (Figure 1b). Embryogenic callus typically began to form 3–4 weeks after plating and was morphologically distinct from the watery callus to which it was attached (Figure 1c) (Bablak et al., 1995; Draper et al., 2001). Callus containing a high percentage of embryos was granular, light yellow and broke into pieces easily (Figure 1d and e). However, some genotypes only produced a few embryos embedded in non-embryogenic callus and care had to be taken to select only embryos during subculturing. It is possible to generate enough callus to do many transformations using callus derived from a single seed by subculturing the callus every 10–14 days.

Embryogenic Bd2 callus grown on regular CIM contained a very low percentage of embryos. Since the diploid accessions are of particular interest for

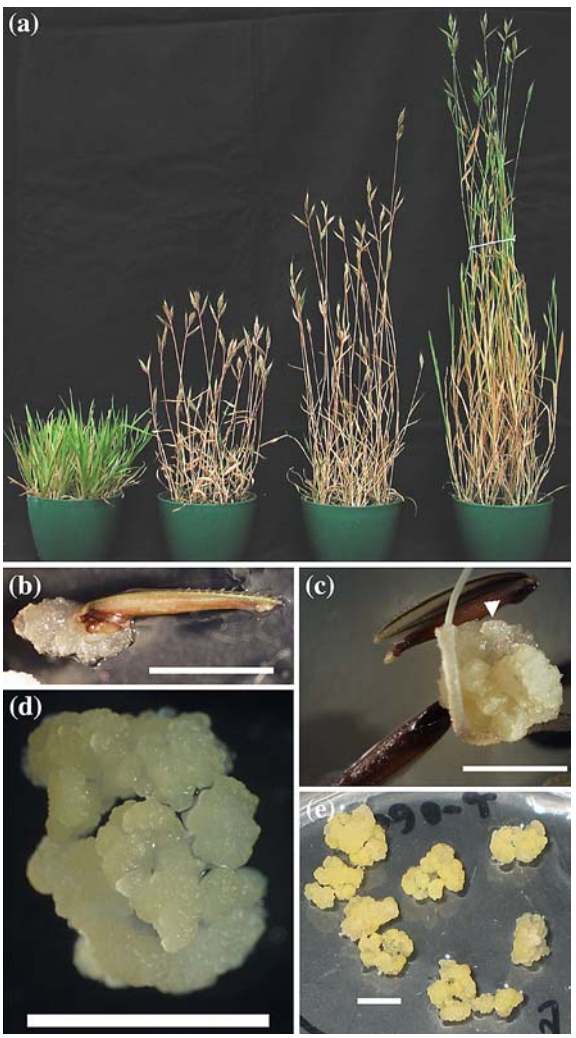

Figure 1. Response of *Brachypodium* to daylength and tissue culture. (*a*) Photograph of, from left to right, Bd1-1, Bd21, Bd3-1, and Bd2-3 after 8 weeks of growth under a 20 h light:4 h dark light cycle. Bd21 plants are 20 cm tall. The differences in maturity and height are but a few of the morphological traits that show variation between the inbred lines. (*b*) Watery, non-embryogenic callus emerging from an immature Bd21 seed. (*c*) Embryogenic callus emerging from an immature Bd21 seed. The white arrow is pointing at non-embryogenic callus growing adjacent to the embryogenic callus. (*d*) Close up view of Bd21 embryogenic callus. (*e*) Embryogenic Bd21 callus. Note the granular nature and yellow color (*b* and *c*). Immature seeds were placed onto CIM 4 weeks prior to being photographed. The seeds were green when plated and turned brown over time (*b*–*e*). The scale bar is 5 mm.

a model system, we optimized the concentration of the auxin 2,4-D and the cytokinin 6-benzylaminopurine (BA) to maximize callus initiation from immature seeds of Bd2, Bd3 and Bd21. No improvement over the 11.25 μM 2,4-D contained in the initial CIM used was noted for Bd3 or Bd21 (not shown). In contrast, immature Bd2 seeds cultured on medium containing 4.5 μM 2,4-D and 1 μM BA were 7.5 times more likely to form embryogenic callus than seeds incubated on medium containing only 11.25 μM 2,4-D (Table 2). Bd2 callus initiated on medium containing 4.5 μM 2,4-D and 1 μM BA had more embryos and less non-embryogenic callus than callus growing on other media. This allowed Bd2 callus to be subcultured and plants regenerated. For all subsequent experiments Bd2 callus was initiated on CIM containing 4.5 μM 2,4-D and 1 μM BA. For all other accessions callus was generated on CIM containing 11.25 μM 2,4-D.

We next compared the ability of 19 lines to form embryogenic callus from mature and immature seeds. The ability to form embryogenic callus was greatly affected by both the explant used and the genotype (Table 3). The percentage of seeds forming embryogenic callus ranged from a high of 33.6% to 0% with an average of 5.5% for mature seeds and 8.5% for immature seeds. In addition to a higher percentage of immature seeds producing embryogenic callus, the percentage of embryos in callus arising from immature seeds was higher than callus arising from mature seeds for most genotypes. However, Bd11-1 and Bd13-1 exclusively formed embryogenic callus using mature seeds indicating that the best explant depends on the genotype. It should be noted that the immature seeds used in this experiment spanned a range of developmental stages, only some of which may be competent to form embryogenic callus. Thus, it may be possible to improve the frequency of callus initiation by determining the best stage for each genotype. However, from a practical standpoint this is not necessary for most genotypes because the embryogenic callus originating from a single seed can be quickly bulked up to perform many transformations.

Table 2. Effect of growth regulator concentration on callus initiation from Bd2

	2.25 μM 2,4-D	4.5 μM 2,4-D	11.25 μM 2,4-D
0 μM BA	1	0	2
1 μM BA	7.5	15	1

Percentage of immature seeds forming embryogenic callus. The number of seeds was between 66 and 100. The entire experiment was repeated once with similar results

Table 3. Callus initiation and regeneration of plants

Genotype	% mature seeds forming embryogenic callus (total seeds)	% callus pieces from mature seeds forming green shoots (total callus pieces)	% callus pieces from mature seeds forming albino shoots	% immature seeds forming embryogenic callus (total seeds)	% callus pieces from immature seeds forming green shoots (total callus pieces)	% callus pieces from immature seeds forming albino shoots
Bd2[a]	2.6 (191)	6 (100)	0	10.9 (320)	20 (30)	0
Bd3	0.7 (284)	20 (100)	0	4 (320)	40 (37)	0
Bd4-2	12 (225)	11 (100)	10	33.6 (300)	87.5 (16)	0
Bd5-2	0.9 (117)	29 (100)	0	11.7 (300)	9.4 (32)	0
Bd6-1	6.4 (249)	10 (100)	67	4.3 (300)	94 (16)	0
Bd8-2	16.4 (263)	0 (100)	100	15.6 (300)	11.1 (18)	61.1
Bd9-1	4.4 (272)	0 (100)	100	5.8 (104)	0 (18)	0
Bd9-2	7.4 (257)	10 (100)	50	3.7 (191)	44.4 (18)	0
Bd10-2	3.2 (249)	14 (100)	14	13.3 (300)	12.5 (16)	18.8
Bd11-1	4 (250)	10 (100)	70	0.0 (300)	NA	NA
Bd12-1	1.9 (260)	67 (100)	0	8.3 (300)	77.8 (18)	0
Bd12-2	1.7 (232)	0 (100)	100	5.3 (300)	0 (18)	0
Bd13-1	10.5 (247)	64 (100)	0	0.0 (300)	NA	NA
Bd14-1	5.1 (257)	48 (100)	20	4.3 (300)	25 (32)	0
Bd14-2	1.1 (263)	85 (100)	0	10.7 (300)	87.5 (16)	0
Bd16-1	1.2 (252)	55 (100)	0	7.7 (300)	15 (20)	85
Bd17-2	20.3 (237)	42 (100)	10	13.0 (300)	94.4 (18)	0
Bd19-2	2.4 (253)	77 (100)	0	1.7 (300)	0 (18)	0
Bd21	2.9 (242)	0 (100)	0	7.5 (320)	75 (32)	0

[a] The CIM for Bd2 contained 4.5 mM 2,4-D and 1 μM BA.

For Bd2, Bd3, Bd21, Bd14-1 and Bd17-2 the callus initiation experiment was repeated at least three more times with similar results. For all other genotypes the callus initiation experiment was repeated once with similar results. For Bd21, Bd14-1 and Bd17-2 the regeneration experiment was repeated at least 3 more times with similar results. For Bd2 and Bd3 the regeneration experiment was repeated once with similar results and once with the callus from immature seeds producing no shoots. For all other genotypes the regeneration experiment was repeated once with similar results.

The percentage of embryos within the embryogenic callus varied greatly between genotypes with Bd4-2, Bd6-1, Bd8-2, Bd9-1, Bd9-2, Bd10-2, Bd16-1, Bd17-2 and Bd21 callus composed almost entirely of embryos whereas Bd2, Bd3, Bd5-2, Bd12-2, Bd14-1 and Bd19-2 callus was composed of embryos distributed in a watery callus. Callus containing a low percentage of embryos was difficult to subculture because the percentage of embryos often decreased with time. Not surprisingly, lines that produced callus containing a low percentage of embryos regenerated and transformed poorly. However, some accessions that produced callus composed almost entirely of embryos regenerated poorly (e.g., Bd9-1). The source of the explant also affected the callus quality, especially for the diploids Bd2, Bd3 and Bd21 for which acceptable callus was only obtained from immature seeds.

An important test of callus quality is the ability to regenerate viable, fertile plants. To test this, we placed callus pieces onto regeneration medium to determine the percentage of callus pieces that produced at least one shoot (Table 3). Regeneration rates ranged from 94.4% to 0%. Both the genotype and initial explant source affected regeneration rates. Regeneration in this system appears to be primarily through adventitious shoots arising from the embryos because most regenerated plants initially consist of only leaves and a stem. Roots typically appear after the shoots are well formed, usually about 2 weeks after the shoots become recognizable. Overall, callus derived from immature seeds regenerated more readily, 37.3% of callus pieces formed green shoots, than callus derived from mature seeds, 28.8% of callus pieces formed green shoots. An extreme example was Bd21 callus, which exhibited a regeneration frequency of 75% when derived from immature seeds but failed to regenerate at all when callus was generated from mature seeds. This result correlates with the high percentage of embryos contained in the callus from immature seeds and the low percentage of embryos in the callus from mature seeds. Bd2 and Bd3 callus regenerated erratically. In some experiments a high percentage of callus pieces produced plantlets, in others a low percentage regenerated and in one exceptional experiment none of the calluses derived from immature seeds produced plants. This makes these genotypes recalcitrant and poor candidates for transformation unless further optimization reduces the variability in regeneration. Our results suggest that across all genotypes, there was no correlation between callus initiation frequency and regeneration. For example, Bd12-1, Bd14-2 and Bd16-1 had callus initiation frequencies $<2\%$ and high regeneration frequencies. Six plants for each genotype that regenerated were transplanted to soil and allowed to set seed in the greenhouse. A high percentage of regenerated plants ($>95\%$) grew when transferred to soil. All plants that grew in soil set seeds normally and produced seeds, which appeared normal. Regenerated plants, including the diploid Bd21, flowered without vernalization.

During regeneration we noted that some lines produced albino shoots. The severity of this problem depended both on genotype and initial explant. Only three of 17 lines formed albinos when the callus was derived from immature seeds. By contrast, 10 of 19 lines formed albinos when the callus was derived from mature seeds. In the most extreme cases, callus derived from mature seeds of Bd8-2, Bd9-1 and Bd12-2 only produced albino shoots. Fortunately, the callus derived from immature seeds of 9 lines regenerated only green shoots indicating that these genotypes are good candidates for transformation.

Transformation

We sought to transform the 14 accessions that formed sufficient quantities of embryogenic callus derived from immature seeds (Table 4). In addition, callus derived from mature seeds of one line, Bd12-1 was transformed. Ten lines were successfully transformed with transformation efficiencies ranging from 0.4% to 15% of initial callus pieces yielding fertile transgenic plants. The hexaploid accession Bd17-2 exhibited the highest transformation competence, with an average transformation efficiency of 13.6%. The diploid accession Bd21 had the third highest average transformation efficiency, 3.2% (excluding the experiment where the callus was sub-cultured for 9 months, see below). Less than 5% of all putative T_0 plants failed to produce the expected band when subjected to PCR using primers designed to amplify a portion of the hygromycin gene. Thus, the hygromycin selection was operating efficiently.

Table 4. Transformation efficiency

Genotype	Experiment[a]	Fertile transformed plants	Transformation efficiency[b]	Transformed infertile plants
Bd2	1	0	0.0	0
Bd3	1	0	0.0	0
Bd4-2	1	0	0.0	0
Bd4-2	2	1	0.4	0
Bd5-2	1	0	0.0	0
Bd5-2	2	0	0.0	0
Bd6-1	1	8	3.3	9
Bd6-1	2	1	0.4	0
Bd8-2	1	0	0.0	0
Bd8-2	2	2	0.8	0
Bd9-2	1	0	0.0	0
Bd9-2	2	0	0.0	0
Bd10-2	1	10	4.2	5
Bd10-2	2	0	0.0	0[d]
Bd12-1	1	0	0.0	0
Bd12-1	2	10	4.2	0
Bd12-1[c]	4	31	13	1
Bd14-1	1	0	0.0	0
Bd14-1	2	1	0.4	0
Bd14-2	1	5	2.1	0
Bd14-2	2	1	0.4	0
Bd16-1	1	7	2.9	0
Bd16-1	2	0	0.0	0[d]
Bd17-2	1	36	15	2
Bd17-2	2	29	12.1	1
Bd21	1	1[e]	0.4[e]	30[e]
Bd21	2	6	2.5	1
Bd21[f]	3	10	4.2	0
Bd21[g]	3	7	2.9	2

[a] Construct pOL001 was used for experiments 1 and 2.
[b] Calculated by dividing the number of fertile transformed plants by the 240 initial callus pieces.
[c] Callus was derived from mature seeds. For all other lines the callus was derived from immature seeds.
[d] Numerous albino shoots regenerated.
[e] In this experiment the callus was sub-cultured for 9-months period prior to transformation. For all other transformations the callus was subcultured for less than 2 months.
[f] Construct pT1 was used for this transformation.
[g] Construct pT2 was used for this transformation.

Sterility in the T_0 generation was observed in some genotypes and was more pronounced when the callus had been sub-cultured for extended periods. For example, the first transformation experiment with Bd21 used callus that was sub-cultured for 9 months prior to transformation and resulted in 1 fertile plant and 30 infertile plants while the third Bd21 transformation experiment using callus that had been sub-cultured for 1 month produced 19 fertile plants and two infertile plants. Note that sterility was not observed when plants were regenerated from untransformed callus, described above, suggesting that sterility is either caused by the transformation process itself or the additional sub-culturing required during the transformation process.

The results from repeated experiments agreed very well and the lines with the highest transformation rates remained the same. Two exceptions were Bd10-2 and Bd16-1 that each had a high transformation efficiency in the first experiment and zero transformation in the second experiment. However, there were numerous albino plants produced in the second experiment indicating that

regeneration of viable plants rather than transformation itself may have been the problem. Another exception was Bd12-1 that had 0, 4.4 and 13.3 % transformation rates. The transformation efficiency correlated with the percentage of embryos in the callus that was low in the first experiment and higher in the second and third experiments. Variability in callus quality may have had a greater impact on Bd12-1 transformation efficiency because the low callus initiation frequency limited the choice of callus line. Overall the best performing line was Bd17-2 because it had the highest transformation efficiency, initiates callus at a high percentage from immature or mature seeds and grows well in culture. The diploid Bd21 also performed well overall and is our choice of genotype when it is desirable to use a diploid. Since only three diploid accessions were evaluated for transformation, it is likely that accessions with higher transformation rates can be identified.

Characterization of transformants

To determine if the transformants were stably transformed we examined the T_1 generation for segregation of the transgene using histochemical staining to determine expression of the GUS gene contained on the T-DNA. Progeny from 5 lines originating from 4 different accessions were examined (Table 5). Segregation of GUS expression in four transgenic lines fit a 3:1 ratio indicating that the insertion segregates as a single genetic locus. Segregation in one line did not fit a 3:1 ratio suggesting insertions at more than one locus. This data is consistent with that found in other systems where *Agrobacterium*-mediated transformation results in transgene integration at an average of 1.5 loci per line for *Arabidopsis* and 1.4 loci for rice (Feldmann 1991; Jeon et al., 2000).

We performed Southern blot analysis on four independent lines to demonstrate that the transgene was inserted into genomic DNA (Figure 2b). Genomic DNA was digested with BamHI and probed with a sequence between the left border and the internal BamHI site. Bands detected by this probe would be expected to contain 2.5 kb of DNA from the T-DNA and an unknown length of flanking DNA. This allowed us to determine the number of T-DNA insertions containing the left border region. Two lines contained single bands larger than 2.5 kb suggesting single T-DNA insertion events. One line contained two bands, one larger and one smaller than 2.5 kb. The simplest interpretation of this is that there was one complete T-DNA and one partial T-DNA inserted in this line. This is not surprising as T-DNA insertions in other species commonly contain complete and partial T-DNAs, often arrayed in tandem. The last line contained three bands suggesting there are at least 3 T-DNAs inserted in this line. Overall, the insertion patterns observed on the Southern blot are consistent with the relatively simple T-DNA insertions typical of *Agrobacterium*-mediated transformation.

The tools described in this paper represent significant steps forward in establishing *Brachypodium* as a model plant system. In particular, the development of inbred lines that exhibit significant phenotypic variation in a variety of traits can serve as the foundation for studies focused on natural variation and induced mutations. The identification of growth conditions that permit rapid generation cycling of most of the diploids will permit *Brachypodium* research to be conducted at a much faster pace greatly reducing costs. This may allow researchers to undertake high-risk experiments that would be too costly to pursue in a monocot crop like wheat, barley or even rice.

Table 5. Segregation of GUS expression the T_1 generation

Parental line	Transformant	GUS positive plants	GUS negative plants	χ^2 value for 3:1 ratio	*p*-value
Bd6-1	Bd	14	3	0.49	0.48[a]
Bd10-2	Ea	26	1	6.5	0.01[b]
Bd14-1	Aa	21	6	0.11	0.74[a]
Bd17-2	Bc	36	11	0.063	0.8[a]
Bd17-2	Be	29	5	1.92	0.17[a]

[a] Consistent with a 3:1 segregation ratio.
[b] Not consistent with a 3:1 segregation ratio.

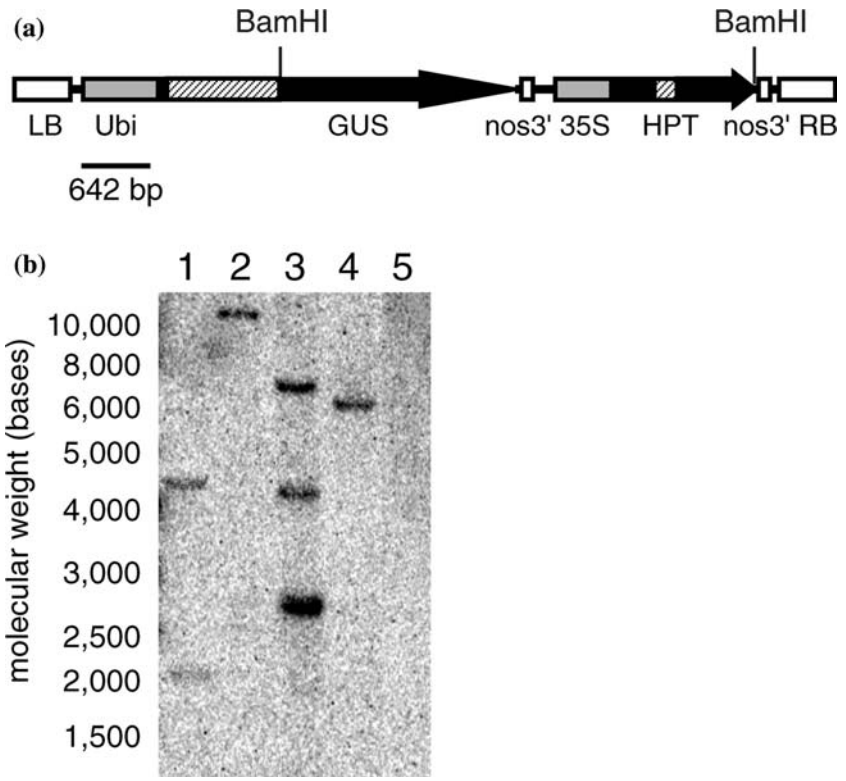

Figure 2. Molecular analysis of transgenic lines. (*a*) Diagram of pOL001 with BamH1 restriction sites shown. Coding sequences are shown as black boxes, promoters are gray and introns are hatched. (*b*) Southern blot of four transgenic lines. Lanes 1–4 are independent transgenic lines arising from Bd14, Bd21 and Bd17-2 (2 lanes). Lane 5 is untransformed Bd21. Genomic DNA was digested with BamH1 and probed with the 642 bp fragment shown in (*a*).

Finally, the development of a high-efficiency *Agrobacterium*-mediated transformation system that works on both diploid and polyploid accessions will allow the application of biotechnological tools to *Brachypodium*. This is particularly useful for applications where the relatively simple, predictable transgene insertions produced by *Agrobacterium* are desirable (e.g., insertional mutagenesis and stable expression of transgenes).

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