

# *Brachypodium distachyon*. A New Model System for Functional Genomics in Grasses<sup>1</sup>

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A new model for grass functional genomics is described based on *Brachypodium distachyon*, which in the evolution of the Pooideae diverged just prior to the clade of “core pooid” genera that contain the majority of important temperate cereals and forage grasses. Diploid ecotypes of *B. distachyon* ( $2n = 10$ ) have five easily distinguishable chromosomes that display high levels of chiasma formation at meiosis. The *B. distachyon* nuclear genome was indistinguishable in size from that of *Arabidopsis*, making it the simplest genome described in grasses to date. *B. distachyon* is a self-fertile, inbreeding annual with a life cycle of less than 4 months. These features, coupled with its small size (approximately 20 cm at maturity), lack of seed-head shatter, and undemanding growth requirements should make it amenable to high-throughput genetics and mutant screens. Immature embryos exhibited a high capacity for plant regeneration via somatic embryogenesis. Regenerated plants display very low levels of albinism and have normal fertility. A simple transformation system has been developed based on microprojectile bombardment of embryogenic callus and hygromycin selection. Selected *B. distachyon* ecotypes were resistant to all tested cereal-adapted *Blumeria graminis* species and cereal brown rusts (*Puccinia recondita*). In contrast, different ecotypes displayed resistance or disease symptoms following challenge with the rice blast pathogen (*Magnaporthe grisea*) and wheat/barley yellow stripe rusts (*Puccinia striiformis*). Despite its small stature, *B. distachyon* has large seeds that should prove useful for studies on grain filling. Such biological characteristics represent important traits for study in temperate cereals.

The past two decades have witnessed an explosion in the use of model eukaryotic organisms to aid studies on species of significant commercial or biological interest. Historically, specific eukaryotes (e.g. *Saccharomyces cerevisiae* and *Caenorhabditis elegans*) have attained the status of “models” because they reflect the individual characteristics of species of medical, industrial, or agricultural interest and are often small, easy to work with in large numbers, and cheap to maintain. More recently, the power of model species has been augmented by the development of whole genome sequencing programs. The new field of “functional genomics” provides further challenges for model organisms in the drive to understand the function of each gene in any genome. This requires a plethora of tools to allow an integrative examination of complex biological problems and relate phenotype to genotype. There are two distinct

categories of technology that need to be in place to exploit fully any proposed model organism in a functional genomics program (Table I). The technology associated with “physical genomics” is often standardized, generally organism independent, and can be developed theoretically for any species, given sufficient investment of time and resources. In contrast, the “biological genomics” capability associated with any particular organism is species dependent reflecting genome size, organization and degree of redundancy, breeding behavior, and in many cases, the development of gene transfer procedures and mutagenesis strategies (e.g. transposon tagging and gene trapping technology). Pragmatically, the utility of a model organism is dependent also on its possession of a range of biological features, for instance, small physical size, rapid life cycle, and undemanding growth requirements that make it amenable to high-throughput screening routines. Such characteristics allow easy adoption by many laboratories internationally, which accelerates the process of discovery and the development of bioinformatic tools (e.g. sequence databases) that are essential to the success of a functional genomic program.

All these features are exemplified in what is without doubt the most highly developed plant model system, the dicotyledon *Arabidopsis* (Meyerowitz and Somerville, 1994; Meinke et al., 1998). This species is a small crucifer that is an inbreeding annual with a rapid life cycle (Redei, 1970). The Arabi-

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**Table 1.** Model species requirements for functional genomics analysis

| Physical Genomics <sup>a</sup>                    | Biological Genomics <sup>b</sup>                                       |
|---------------------------------------------------|------------------------------------------------------------------------|
| Large insert libraries                            | Small, diploid genome                                                  |
| Genome sequencing                                 | Tractable genetics and mapping strategies                              |
| Expressed sequence tag databases                  | Mutagenesis techniques                                                 |
| Transcriptome, proteome, and metabolome data sets | Easily transformable and low-cost logistics for growth and maintenance |
| Bioinformatics tools                              | Exhibition of important biological traits                              |

<sup>a</sup> Technological infrastructure necessary to employ multidimensional analysis of a biological facet of the model species. <sup>b</sup> Biological attributes of a species that are required to be a valuable model species.

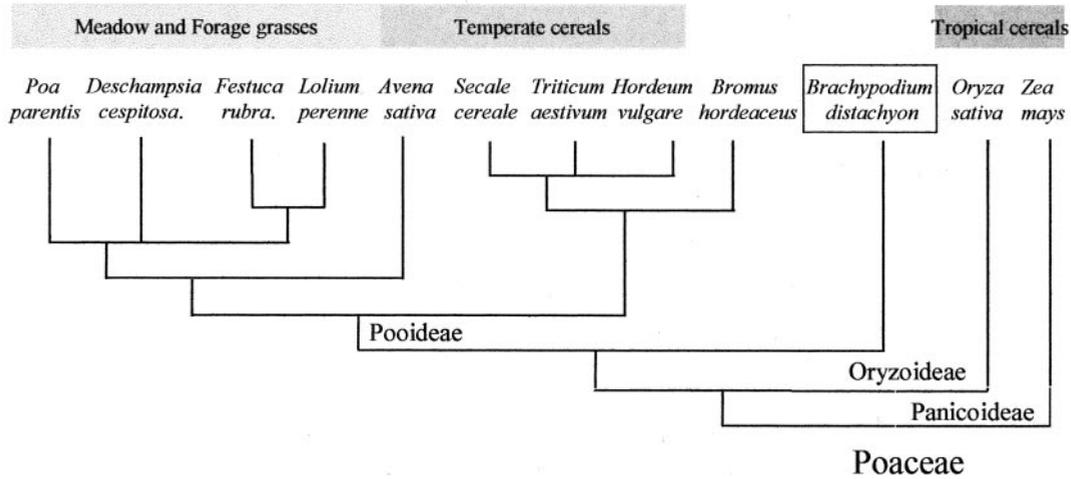
dopsis nuclear genome size has been estimated by flow cytometry to be 164 Mbp (Bennett et al., 2000), whereas a recent calculation following completion of the whole genome sequence (The Arabidopsis Genome Initiative, 2000) has indicated a figure as low as a 125 Mbp. However, phylogenetically, Arabidopsis is only distantly related to the Poaceae, which includes all of the world's major cereals crops and forage grasses (Keller and Feuillet, 2000). Hopes that Arabidopsis could serve as an "anchor" genome to help locate important chromosomal locations in cereal species have not been substantiated by recent studies (Bennetzen et al., 1998; Devos et al., 1999). Thus, it is clear that grass (Poaceae) model systems are a key requirement for the future identification of genes of agronomic interest from cereals and forage grasses.

With its international status as a staple food source and many years of intensive plant breeding, rice (*Oryza sativa*), with its compact genome (approximately 441 Mbp; Bennett et al., 2000) has been promoted as a model for cereal genomics (for review, see Havukkala, 1996; Goff, 1999). Considerable international effort has developed rice genetic maps, expressed sequence tag programs, and assembled a considerable germplasm collection (highlighted in McCouch, 1998), and the completed rice genomic sequence has just been announced (Dickson and Cyrnoski, 2001). Furthermore, the representation of all grass genome sectors by less than 30 linkage blocks (with centromeric sites often defining the break-points) within a consensus grass map "anchored" by reference to the rice genome offers the prospect of easing mapping in cereals with larger genomes (Gale and Devos, 1998). However, the value of rice as a model for the temperate cereals and forage grasses in the relatively distantly related Pooideae subfamily (see Fig. 1A) may be, on occasion, questionable. For instance, to effectively use the rice genomic map to isolate a corresponding genic region in the larger genomes of temperate cereals, "microsynteny" will

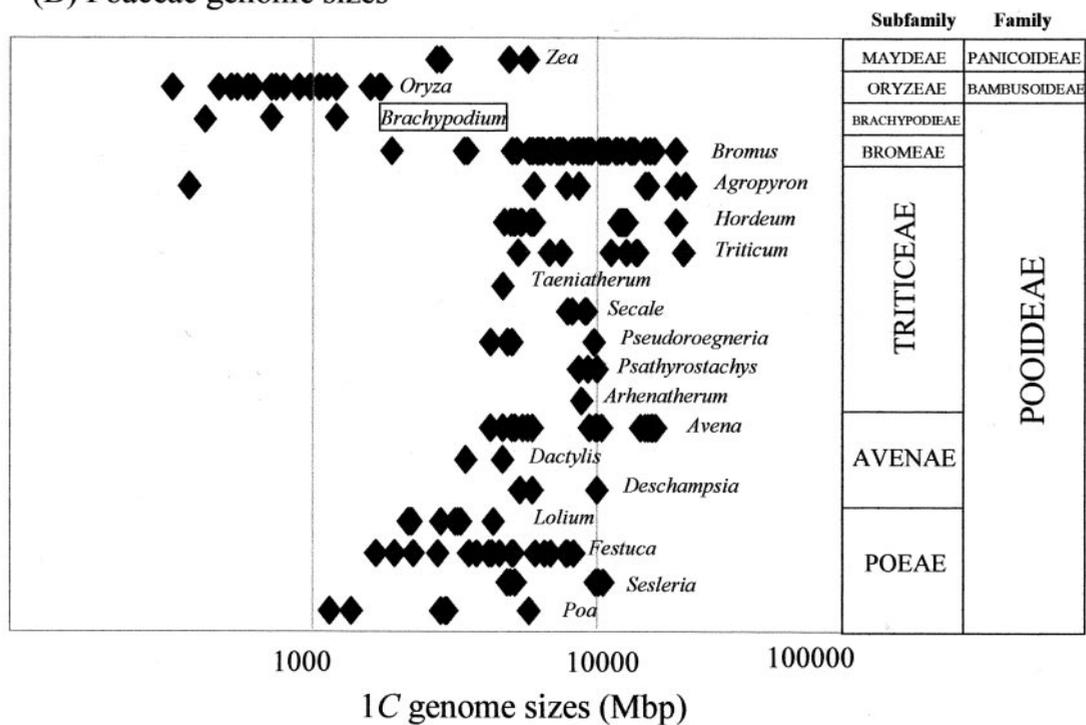
have to be conserved at the size of DNA inserts found in bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC) clones (approximately 100 kb and 0.5 Mb, respectively). This does not always appear to be the case. For example, although the *Sh2* locus appears to be colinear in many cereals (Chen et al., 1997), regions flanking *Adh1* locus from maize (*Zea mays*), sorghum (*Sorghum bicolor*), and rice were dissimilar (Bennetzen et al., 1998), and molecular analysis has detected multiple small rearrangements (Tikhonov et al., 1999). Also the "physical and biological genomics" infrastructure, though impressive, is incomplete. For instance, large scale, organized, and publicly available insertion mutagenesis resources (Izawa et al., 1997; Enoki et al., 1999) are not yet available, and a routine transgenic capability, even after decades of concerted effort, is restricted to a relatively few laboratories. Furthermore, the simple logistics of handling rice (as a large, outbreeding plant with a long life cycle and demanding growth requirements) will complicate the future development of rice as a model species for high-throughput functional genomics. Finally, and crucially, rice does not necessarily exhibit all the traits that are relevant to study in temperate crops, especially forage grasses. For example, resistance to specific types of pathogens, overwintering and freezing tolerance, vernalization, perenniality (including meristem dormancy mechanisms), wear and injury tolerance (amenity grasses), sward behavior, and post-harvest biochemistry of silage are all important areas of research relevant to temperate grasses, which are rarely studied in rice.

Against this background, it would seem appropriate to reexamine the Pooideae to identify a grass species that has the potential to be developed into a useful model representative of important temperate cereals and forage grasses. The Kew Gardens Angiosperm C- value Database (<http://www.kew.org/cval/homepage.html>) shows that the genus *Brachypodium* is distinct from other genera in the Pooideae in that all species examined to date have a narrow range of genome sizes with the smallest 1-C values being very similar to that of rice (Fig. 1B). Various molecular phylogenetic analyses have demonstrated that the genus *Brachypodium* diverged from the ancestral stock of Pooideae immediately prior to the radiation of the modern "core pooids" (Triticeae, Bromaeae, Poaeae, and Aveneae; see Fig. 1A), which includes the majority of important temperate cereals and forage grasses (Shi, 1991; Shi et al., 1993; Catalan et al., 1995, 1997; Catalan and Olmstead, 2000). Its phylogenetic position and small genome size prompted Moore and colleagues (Aragon-Alcaide et al., 1996) to use *Brachypodium sylvaticum* ( $2n = 18$ ) in a search for archetypal grass centromere sequences by screening for repetitive DNA conserved between wheat (*Triticum aestivum*), maize, and rice and *Brachypodium*. *Brachypodium* species have small chro-

(A) Phylogenetic relationships



(B) Poaceae genome sizes



**Figure 1.** A, Schematic phylogenetic relationship of *B. distachyon* to other Poaceae adapted from the data presented by Catalan et al. (1995, 1997; Catalan and Olmstead, 2000). B, Nuclear genome sizes (Mbp/1C) in different grass genera. Data derived from The Kew Gardens Angiosperm C- value database (<http://www.rbkew.org/cval/homepage.html>).

mosomes with a variable base number ( $x = 5, 7, 8,$  or  $9$ ), making them unusual in the Pooideae that tend to have large chromosomes and a base number of 7 (Shi et al., 1993). An analysis of ribosomal DNA structure revealed that *Brachypodium* had the smallest (150 bp) 5S rDNA spacer region found in the grasses (Shi, 1991; Catalan et al., 1995) and a very simple rDNA repeat unit with a low degree of methylation (Shi et al., 1993). Further characterization revealed that ge-

nomes of *Brachypodium* species contained typically less than 15% highly repeated DNA (Shi, 1991; Catalan et al., 1995). Therefore, we consider that the genus *Brachypodium* has several attributes in relation to its ancestry and genome characteristics that make it a useful focus for studies relating to the evolution of form and function in the Pooideae. The present paper describes the development of *B. distachyon*, the only true annual species within the genus *Brachypodium*,

as a potential model species for functional genomics and a representative of the temperate cereals and forage grasses.

## RESULTS

### Diploid Ecotypes of *B. distachyon* Have the Smallest Reported Genome Size in the Poaceae

The genome size of several species of *Brachypodium* was estimated in preliminary studies by scanning densitometry of Feulgen-stained nuclei prepared from root tip squashes (Table II). Representative accessions of each species had a 1C nuclear genome size of less than 0.5 pg, equivalent to  $\leq 410$  Mbp. Of particular note was the large discrepancy between the genome sizes of two ecotypes of *B. distachyon* ABR99 and ABR1 (0.3 pg/1C and 0.15 pg/1C, respectively).

Propidium iodide was used to stain nuclei isolated from our collection of 52 ecotypes of *B. distachyon*, and their genome size was determined by flow cytometry (Dolezel et al., 1989, 1998). To estimate genome size, the flow cytometer was calibrated against stained nuclei prepared from representative species with known genome sizes (Bennett et al., 2000). This analysis (Table II) revealed that the genome size in several *B. distachyon* accessions was indistinguishable from that of *Arabidopsis*, which is taken to be 164 Mbp/1C.

### Diploid *B. distachyon* Has a Simple, Distinctive Karyotype and Displays High Levels of Recombination

Somatic metaphase chromosomes were examined in seven ecotypes of *B. distachyon* (ABR1–ABR7), which had all been found to have a genome size of less than 175 Mbp. The analysis revealed that their karyotypes were morphologically the same (Fig. 2A),

which justified the construction of a consensus karyotype for this species. The chromosomes have been arranged in descending order of short-arm length and were designated 1 to 5, in accordance with normal conventions. Chromosome 1 is submetacentric, distinctly the largest of the complement, and unlikely to be confused with chromosome 2, which is more acrocentric and considerably smaller. The short-arms of chromosomes 2 and 3 are of similar length, but 3 is shorter and characteristically the only metacentric of the complement. Chromosome 4 shares similar features with 3, which could ordinarily be the source of potential mislabeling. However, FISH reveals that chromosome 4 only has a major 5S rDNA locus, which is proximally located in its long-arm (Fig. 2, B and C). Chromosome 5 is acrocentric and by far the smallest of the complement. In addition, it is the only chromosome to bear a major 45S rDNA locus, which occupies a distal site in the short arm (Fig. 2, B and C). In summary, each chromosome has diagnostic features enabling unequivocal identification of every chromosome of the complement (Fig. 2D).

Examination of 20 pollen mother cells at metaphase I of one ecotype (ABR1) revealed that 16 form five ring bivalents and four form four ring bivalents and one rod involving chromosome 5 in all cases. Two example cells are presented in Figure 2E, which also clearly shows that chiasmata are not strictly localized to any particular region of the chromosomes.

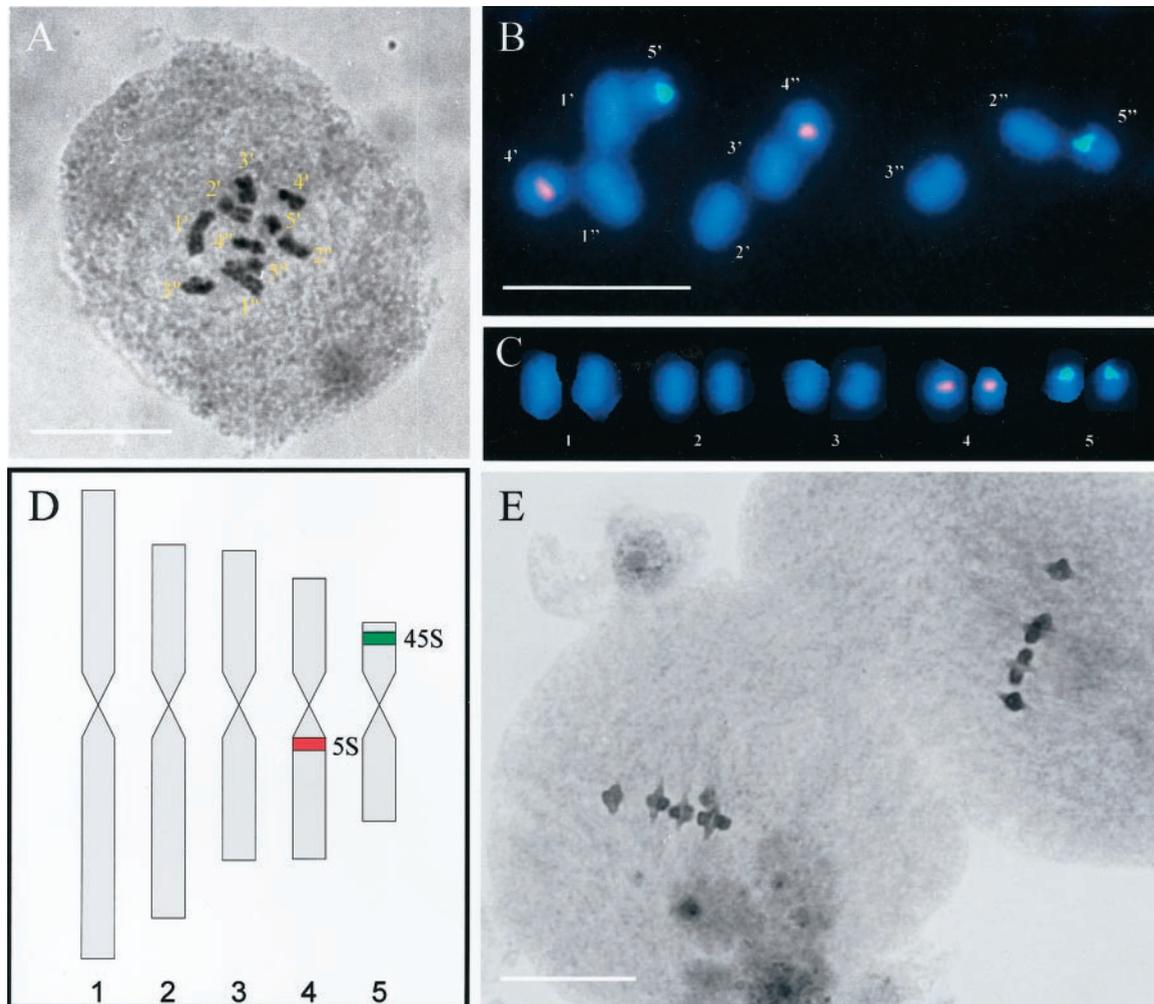
### Diploid *B. distachyon* Ecotypes Display Growth and Life Cycle Characteristics Suitable for High-Throughput Genetics

Preliminary experiments established that all ecotypes performed well under standardized conditions (see “Materials and Methods”). The *B. dis-*

**Table II.** Estimation of genome size in *Brachypodium* species

| Species                           | Ecotype | 2n             | Microdensitometry |                           |                  | Flow Cytometry <sup>a</sup> |              |          |
|-----------------------------------|---------|----------------|-------------------|---------------------------|------------------|-----------------------------|--------------|----------|
|                                   |         |                | N                 | IC/pg                     | Mbp <sup>b</sup> | N                           | IC/pg        | Mbp      |
| <i>B. sylvaticum</i>              | B28     | 18             | 31                | 0.22 (0.026) <sup>c</sup> | 180 (21)         | 6                           | 0.27 (0.01)  | 221 (9)  |
| <i>B. pinnatum</i>                | B144    | 18             | 28                | 0.27 (0.019)              | 221 (16)         | – <sup>d</sup>              | –            | –        |
|                                   | B11     | 28             | 30                | 0.49 (0.067)              | 402 (55)         | 6                           | 0.57 (0.01)  | 468 (9)  |
| <i>B. phoenicoides</i>            | B39     | 28             | 40                | 0.45 (0.077)              | 328 (26)         | –                           | –            | –        |
| <i>B. arbuscula</i>               | B96     | 18             | 51                | 0.35 (0.039)              | 287 (32)         | –                           | –            | –        |
| <i>B. distachyon</i> <sup>e</sup> | ABR99   | ? <sup>f</sup> | 20                | 0.30 (0.024)              | 246 (19)         | –                           | –            | –        |
|                                   | ABR100  | 30             | –                 | –                         | –                | 6                           | 0.65 (0.01)  | 530 (12) |
|                                   | ABR1    | 10             | 43                | 0.15 (0.023)              | 123 (18)         | 6                           | 0.21 (0.001) | 172 (1)  |
|                                   | ABR2    | 10             | 20                | 0.15 (0.033)              | 123 (27)         | 6                           | 0.21 (0.004) | 172 (3)  |
|                                   | ABR3    | –              | –                 | –                         | –                | 6                           | 0.21 (0.002) | 165 (2)  |
|                                   | ABR5    | –              | –                 | –                         | –                | 6                           | 0.19 (0.004) | 158 (3)  |

<sup>a</sup> For the flow cytometry, genome sizes were calibrated from the standard genomes listed in “Materials and Methods” from which the following formula was derived: Genome size (Mbp) = Flow cytometer peak  $-7.5846/0.0265$  ( $R^2 = 0.9976$ ). Given this formula our results gave the genome size of *Arabidopsis* = 165 ( $\pm 9$  SE)  $n = 6$ . Results for genome sizes in Mbp are rounded up to the nearest whole number. <sup>b</sup> Scanning microdensitometry data (pg/IC) were converted to Mbp on the basis of 0.1 pg = 82 Mbp (Bennett et al., 2000). <sup>c</sup> Nos. in parentheses are the SE. <sup>d</sup> –, Not done. <sup>e</sup> *B. distachyon* is the only annual species. <sup>f</sup> ABR99 could not be revived from stocks and was replaced with ABR100, which had a chromosome complement of 30.



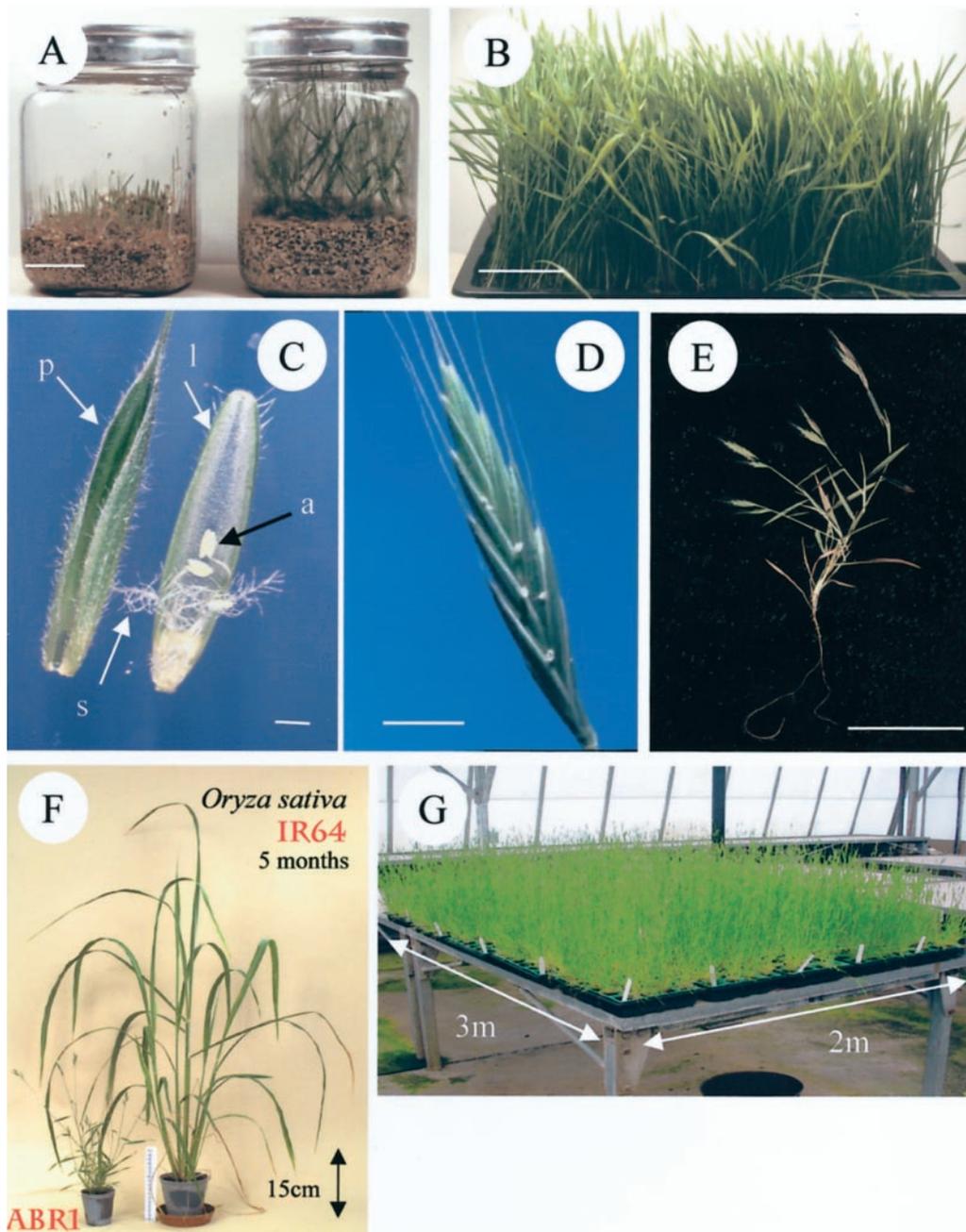
**Figure 2.** Somatic and meiotic chromosomes of *B. distachyon* ABR1 showing light micrograph of the somatic chromosome complement (A) in which the five pairs of chromosomes are readily identified and fluorescence in situ hybridization (FISH; B) of 5S rDNA (red) and 45S rDNA (green) to the five pairs of somatic chromosomes. C, Karyogram derived from the image shown in B. D, Idiogram showing the distinctive and diagnostic shapes and lengths of the haploid set of chromosomes. The sites of the two rDNA loci are indicated. E, Light micrograph of two pollen mother cells at metaphase I. Note the five ring bivalents in each. Bars = 10  $\mu\text{m}$ .

*tachyon* ecotypes were then characterized in terms of size at maturity, general growth habit, vernalization requirement, duration of life cycle, and amount of seed set. ABR1 was typical and exhibited undemanding maintenance requirements, growing successfully under sterile conditions in glass jars (Fig. 3A) on vermiculite supplemented with 0.5 $\times$  Hoagland solution (Draper et al., 1988) or at high-density (2,000 seedlings 18-  $\times$  30-cm tray<sup>-1</sup>) in compost (Fig. 3B). With only two exceptions (ABR13 and ABR15), all of the diploid ecotypes (including ABR1) benefited from a standard vernalization treatment (6 weeks at 5°C) to ensure synchronous induction of flowering and synchronized embryo development. Floral spikelet emergence occurs around 3 to 4 weeks after removal from the cold room (Fig. 3C). By 4 to 5 weeks following vernalization, visible anthesis occurs (Fig. 3D) with each spikelet eventually containing typi-

cally around 10 to 12 seeds. At maturity (4–5 mo), the diploid ecotypes ranged from 15 to 30 cm in height and very rarely shed any seed prematurely, thus aiding easy harvesting (Fig. 3E; Table IV). For all polyploids and two diploid ecotypes (ABR13 and ABR15), flowering is accelerated by approximately 6 weeks, as no vernalization stage is required (see Table IV). Furthermore, the smaller stature of *B. distachyon* compared with, for instance, rice (Fig. 3F), allows typical planting densities of at least 300 plants square meter<sup>-1</sup> in ordered arrays (Fig. 3G) for M2 mutant seed production or M3 mutant screening.

#### ***B. distachyon* Is Readily Responsive to Tissue Culture**

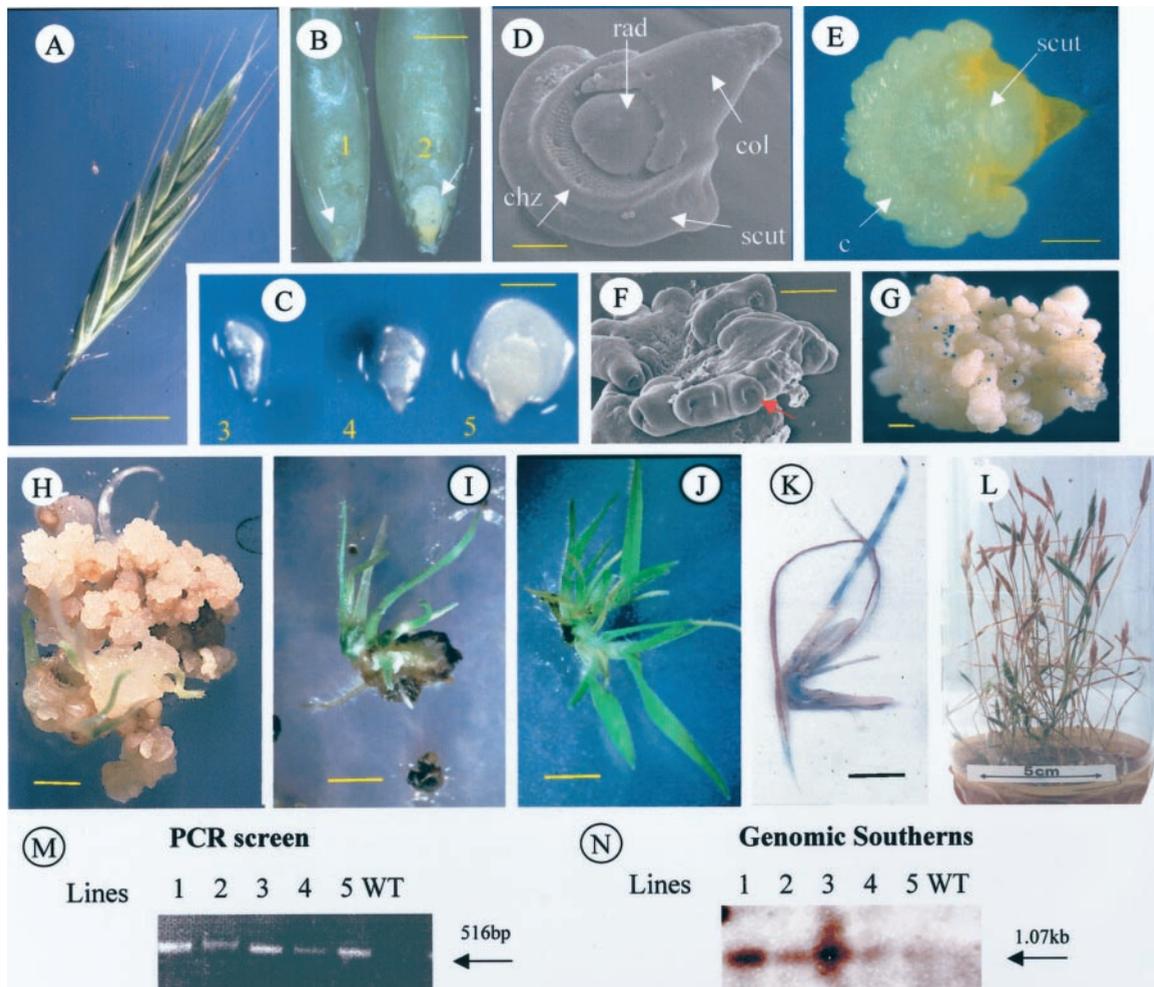
The diploid ecotype ABR1 was chosen for initial tissue culture studies as it was relatively compact and offered a high throughput of immature embryos



**Figure 3.** Growth habit and anatomy of the diploid *B. distachyon* ecotype ABR1 grown in glass jars (A) on vermiculite supplemented with 0.5× Hoagland solution (Draper et al., 1988) at 1 week (left jar) and 2 weeks (right jar), and at high density (B) in 30 × 18-cm trays grown on 1:1 mixes of Levington's:gravel. Bar = 5 cm. Flower morphology (C) prior to dehiscence and indicating hairy palea (p) and lemea (l), stigma (s), and anthers (a). Bar = 1 mm. Each plant has 6 to 10 flowering spikes that actually set seed and seed heads (D) have a “brome-like” appearance and typically carry 10 viable seeds. Bar = 4 mm. At maturity (E), ABR1 plants were 15 cm high with short nonrhizomatous roots. Bar = 5 cm. F, *B. distachyon* ABR1 (8 weeks post-sowing) is compared in size with rice cv IR64 (20 weeks post-sowing). G, 2,000 M1 progeny of  $\gamma$ -irradiated (at the Vienna Atomic Energy Institute) *B. distachyon* seeds grown under typical temperate greenhouse conditions.

for minimal growth space. A detailed analysis of embryo development in individual *B. distachyon* spikelets (Fig. 4A) revealed, as expected, a variation in maturity, with more mature embryos present in the center at 10 to 15 d post-visible anthesis (data not shown). Isolated immature embryos were divided

into one of five size classes (types 1–5; Fig. 4, B and C) and were placed on callus induction media (LS 2.5 and N6 2.5; Bablak et al., 1995), and typically after 10 to 15 d had formed a mass of callus on the surface of the scutellum (see Fig. 4, D and E). This included large areas of creamy-white, type II embryogenic



**Figure 4.** Tissue culture and transformation of *B. distachyon*. A, *B. distachyon* ABR1 seed head at 15 d postanthesis (bar = 4 mm) from which (B) isolated seeds have had their paleas and lemmas removed (embryos arrowed, bar = 0.5 mm) or (C) the embryos are isolated from endosperm to indicate embryo development (classes 1–5, bar = 0.5 mm). D, Scanning electron micrograph showing immature embryo structure. Indicated are the coleoptile (col), scutellum (scut), radical (rad), and coleorhiza (chz). Bar = 0.1 mm. E, Embryogenic callus (c) formation around the edge of the scutellum (scut) after 15 d of culture (bar = 0.1 mm), which (F) scanning electron micrograph revealed to contain many organized structures with a distinctive embryoid shape (arrowed; coleoptilar pore; bar = 20 µm). G, Propagated embryogenic callus of ABR100 stained for GUS activity 24 h following biolistic bombardment with pAct1GUSHm. Bar = 1 mm. H, Selection of ABR100 transgenic tissue on Hm- (40 µg mL<sup>-1</sup>) selective media. Bar = 3 mm. I, The second subculture of regenerating plants from bombarded embryogenic callus of ABR100 on selective media. Bar = 5 mm. J, Isolated Hm-resistant plantlet of ABR100 exhibiting tiller formation. Bar = 5 mm. K, Hm-resistant ABR100 shoot stained for GUS activity (bar = 1 cm). L, Rooted T<sub>0</sub> transgenic lines in soil producing viable seeds. M, The PCR amplification of *hgh* gene internal sequences (516-bp product) from five independent T<sub>1</sub> transgenic lines (1–5). N, Southern blot of *Bam*HI-digested genomic DNA from T<sub>1</sub> transgenic lines (1–5) and wild-type ABR100 probed with *hgh* sequences. Indicated is the 1.07-kb *Bam*HI internal *hgh* gene fragment.

tissue (dry, friable, and pale) easily identified by scanning electron microscopy (Fig. 4F). Immature embryos with the greatest potential for somatic embryogenesis were in the size range of 0.3 to 0.7 mm (see Table IIIA) and were generally off-white with a translucent scutellum margin and were suspended in a soft endosperm (see Fig. 4, B, class 2 and C, class 3). LS 2.5 medium proved superior for the induction of embryogenic callus, with around 45% of isolated embryos responding within this size range (Table IIIA). Type II callus could be separated from callus grow-

ing on the original explants and maintained on LS 2.5 for up to 9 to 12 mo after which it was normally discarded.

The regeneration potential of type II callus after subculture on LS 2.5 (approximately 6 weeks) was examined by inoculating onto a range of established regeneration media. Plantlets were produced on all media tested, with the highest regeneration potential realized on MS0 and N60 (Table IIIB). Although embryogenic callus could be obtained at a high frequency when immature embryos were placed on LS

**Table III.** Tissue culture and transformation in *B. distachyon*

| A. The responsiveness of immature ABR1 embryos to culture                          |                                                              |                                      |                                                                  |          |      |
|------------------------------------------------------------------------------------|--------------------------------------------------------------|--------------------------------------|------------------------------------------------------------------|----------|------|
| Embryo class <sup>a</sup>                                                          | 1                                                            | 2                                    | 3                                                                | 4        | 5    |
| Size (mm)                                                                          | <0.3                                                         | 0.3–0.4                              | 0.5–0.69                                                         | 0.7–0.89 | >0.9 |
| Medium                                                                             | Percentage of embryos producing type II embryogenic callus   |                                      |                                                                  |          |      |
| LS 2.5                                                                             | 4                                                            | 41                                   | 54                                                               | 16       | 5    |
| N6 2.5                                                                             | 16                                                           | 43                                   | 34                                                               | 12       | 2    |
| B. Plant regeneration from ABR1 embryogenic callus on different media <sup>b</sup> |                                                              |                                      |                                                                  |          |      |
| Medium                                                                             | Murashige and Skoog Salts (MSO)                              | 190-2                                | Rice Regeneration Medium (N6 0)                                  | MWW      | RM1  |
| No. of plants g callus <sup>-1</sup>                                               | 66                                                           | 25                                   | 71                                                               | 43       | 40   |
| C. Results of typical transformation experiments with ABR100 callus                |                                                              |                                      |                                                                  |          |      |
| Experiment                                                                         | No. of Independent Hm-Resistant calli g tissue <sup>-1</sup> | Percentage of Calli-Producing Plants | Transformation Frequency (no. of plants g tissue <sup>-1</sup> ) |          |      |
| 1                                                                                  | 8                                                            | 25                                   | 3                                                                |          |      |
| 2                                                                                  | 6                                                            | 50                                   | 7                                                                |          |      |
| 3                                                                                  | 7                                                            | 30                                   | 5                                                                |          |      |
| Average                                                                            | 7                                                            | 35                                   | 5                                                                |          |      |

<sup>a</sup> Embryo classes are illustrated in Fig. 4, B and C. 190-2, *Lolium* regeneration medium; MWW, winter wheat regeneration medium; RM1, barley regeneration medium (all are described in Bablak et al., 1995).

2.5 or N6 2.5 media, albino shoots were recovered at a much higher frequency from tissues maintained on N6 2.5 (45% compared with 7% on LS2.5), and thus LS 2.5 was used for all future work on transformation.

### Transgenic Plants Can Be Recovered at a High Frequency from Embryogenic Callus Using Microprojectile Technology and Hygromycin (Hm) Selection

Tissue from the hexaploid accession ABR100 remained highly embryogenic when maintained on LS 2.5 medium and so was used to bulk up material for development of transformation technology. Embryogenic callus from ABR100 was bombarded with microprojectiles loaded with the plasmid pACT1-GUSHm (pAGH). A small sample of bombarded callus tissue with well-dispersed cauliflower mosaic virus 35S- $\beta$ -glucuronidase (GUS) activity is shown in Figure 4G. One week after bombardment, callus was subcultured to LS 2.5 medium containing 40 mg L<sup>-1</sup> Hm where it rapidly became brown and necrotic, but after 2 weeks, small nodes of actively growing cells were visible. Once these had developed to a diameter of 1 to 2 mm, these tissues were transferred twice at weekly intervals for further growth on selection medium containing 40 mg L<sup>-1</sup> Hm. In typical experiments, an average of seven Hm-resistant clones were recovered per gram of target tissue (Table IIIC).

Hm-resistant calli were removed from selection plates and were transferred onto regeneration medium (see "Materials and Methods") containing 30 mg L<sup>-1</sup> Hm and they produced plantlets within 7 to 10 d (Fig. 4H). Callus containing young shoots and

viable somatic embryos were transferred subsequently to germination medium (MS0) and were incubated in the light. An average of five Hm-resistant plants were recovered per gram of bombarded tissue (Table IIIC). The formation of roots was initially rather poor on the regeneration medium (Fig. 4I), but a well-developed root system could be produced following further subculture on the same hormone-free media once the plantlets had been separated from necrotic tissue (Fig. 4J). Histochemical staining with 5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronic acid of regenerated plantlets surviving on Hm revealed clear evidence of GUS activity (Fig. 4K). All rooted plants that were established successfully in soil produced fertile flowers and set seed (Fig. 4L). T<sub>1</sub> progeny of five independent transgenic lines were examined for the presence of the Hm gene by PCR and Southern blotting to confirm that stably transformed lines had been generated. PCR amplified a 516-bp fragment from all transgenic lines (Fig. 4M), which hybridized to a *hgh* probe following Southern blotting (data not shown). In addition, probing *Bam*HI-digested genomic DNA extracted from these transgenic lines with a *hgh* probe indicated hybridization to a 1.07-kb fragment that corresponded to the size predicted from the plasmid used for the initial bombardment (Fig. 4N).

### *B. distachyon* Displays Traits That Are Important for Temperate Cereal Research

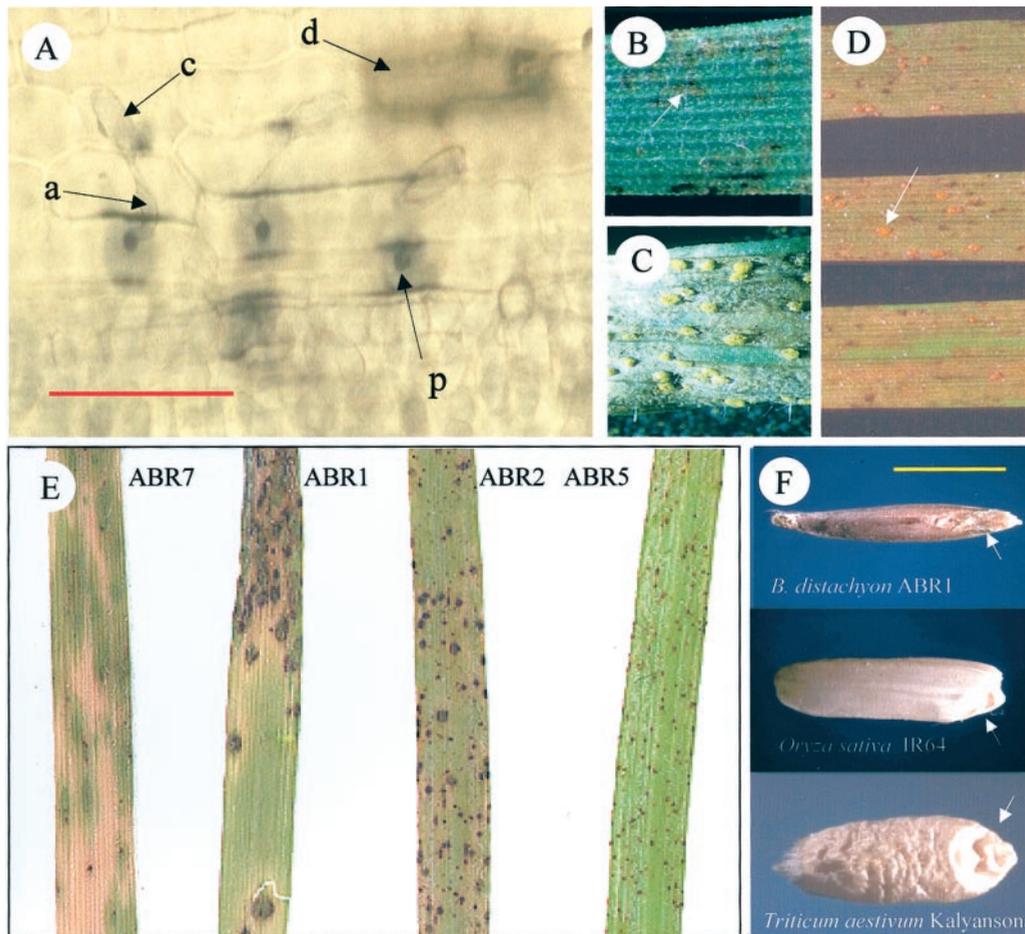
We have characterized the responses of *B. distachyon* ecotypes to challenge with a range of pathogens that are the cause of significant crop loss in

cereals. *Blumeria graminis* is the causal agent of powdery mildew on cereals and occurs in several *forma specialis* (f. sp.). Isolates of *B. graminis* f.s.p *hordei*, *avenae*, and *tritici* were used to challenge ecotypes (ABR1–ABR7 and ABR100) of *B. distachyon* and failed to elicit disease symptoms that were observable to the naked eye. However, light microscopic examination revealed single epidermal cell death and also papillae-based resistance that are typical of a hypersensitive response (Fig. 5A). Attempts to isolate a *Blumeria* sp. capable of establishing an infection on *B. distachyon* by exposing plants to the outdoor environment have, to date, been unsuccessful.

The responses of *B. distachyon* ecotypes (ABR1–ABR7, and ABR100, 101, 105, and 113) were examined following challenge with the rust pathogens *Puccinia recondita* f. sp. *hordei* and f. sp. *triticae* (barley and wheat brown rust, respectively) as well as *P.*

*striformis* f. sp. *hordei* and f. sp. *triticae* (barley and wheat yellow stripe rust, respectively). ABR100 displayed a “brown flecking” (BF) following challenge with wheat brown rust strains with all other tested ecotypes eliciting no macro- or microscopic responses. Ecotypes ABR1, ABR3, ABR100, and ABR105 also exhibited BF symptoms with barley brown rust strains. In contrast, all ecotypes exhibited some form of visible response to wheat and barley yellow stripe rusts ranging from BF (ABR1–ABR7, and 101 and 111; Fig. 5B) or the more extensive “brown tissue” response. It is interesting that ABR100 and ABR105 displayed uridinea (raised pustule) formation (Fig. 5C), indicating that the fungus has successfully colonized the host, though these formed within areas of extensive necrosis (Fig. 5D).

A range of symptoms were also elicited (Fig. 5E) following challenge of *B. distachyon* ecotypes (ABR1–



**Figure 5.** Targets for *B. distachyon* research. Responses to pathogen challenge and grain development. A, The responses of ABR1 to challenge with conidiophores of *Blumeria graminis* f. sp. *Triticae*. Bar = 0.1 mm. Arrowed are the condiophore (c), appressorial germtube (a) and papillae formation (p) and single cell-death (d) in the host cell. Challenging with *Puccinia striiformis* f. sp. *triticae* (wheat yellow stripe rust) elicits (B) localized necrotic flecking on ABR105 and (C) yellow uridinea (pustule) formation on ABR100 that develop from (D) extensive areas of necrotic tissue. (Uridinea forming within the necrotic tissue are arrowed.) E, Variable responses by *B. distachyon* ecotypes to challenge with *M. grisea* Guy-11. F, Comparison of mature seed size and morphology of *B. distachyon* ABR1 with rice and wheat cv Kalyansona). Embryos are arrowed on each seed. Bar = 2 mm.

ABR7) with the causal agent of rice blast, *Magnaporthe grisea* (strain Guy-11). On ABR7 and ABR1, an extensive and spreading necrosis was observed that was reminiscent of blast symptoms on rice. In contrast, highly localized necrotic lesions formed on ABR5 that did not change significantly in phenotype over time and were consistent with the exhibition of full resistance to *M. grisea* Guy-11.

Seed development in *B. distachyon* has yet to be extensively analyzed. However, in terms of overall size and external anatomy, mature seeds of the diploid ABR1 are very similar to those of the major grain crops rice and wheat, the only major structural difference being a smaller endosperm volume in *B. distachyon* (Fig. 5F).

## DISCUSSION

New “post-genomic” techniques are offering increasingly powerful ways to link plant phenotype to the transcriptome, proteome (Gura, 2000), or the metabolome (Fiehn et al., 2000), allowing the development of a “blueprint” of plant form and function (Chory et al., 2000). Optimal exploitation of these technologies is dependent on possession of suitable model species. It is now becoming clear that Arabidopsis cannot be considered as an ideal model for the grasses (Devos et al., 1999; Tikhonov et al., 1999; Keller and Feuillet, 2000), and there is significant international effort to develop rice as a model species more suitable for the Poaceae (Havukkala, 1996; Goff, 1999). However, against this background, the limitations of rice to provide such a model and the complementary opportunities offered by other species needs to be recognized.

## Analysis of the *B. distachyon* Genome Will Provide a Valuable Resource for Physical Genomics in Temperate Cereals and Forage Grasses

Differing analytical techniques pose different problems when attempting to estimate genome size. The commonly used flow cytometry technique is dependent on accurate calibration, and even apparently definitive DNA sequencing approaches do not easily include regions of highly repeated sequence and may therefore underestimate genome sizes. Nevertheless, we have taken The Arabidopsis Genome Initiative (2000) estimate (approximately 125 Mbp) as the more accurate and, given that we failed to distinguish any size difference between diploid *B. distachyon* ecotypes and Arabidopsis genomes by flow cytometry (Table IV), we propose that they should be considered to be approximately the same. This genome size is somewhat at odds with a previous report ( $4C = 3$  pg; Bennett and Leitch, 1995). However, our present data suggest the presence of a polyploid series within *B. distachyon* ( $2x = 10$ ,  $4x = 20$ , and  $6x = 30$ ) and, therefore, the discrepancy can be explained by the fact that these authors used a hexaploid accession for their determination (e.g. ABR100  $2n = 6x = 30$ ,  $4C$  approximately 2.6 pg, derived from Table II). The flow cytometry screen identified 12 diploids, eight tetraploids, and 32 hexaploids in our germplasm collection.

We recognize that the utility of *B. distachyon* will be augmented by the development of genetic and physical chromosome markers. Given the small chromosome size in this species, there is a surprisingly high chiasma frequency of at least  $9.8 \text{ cell}^{-1}$ . Furthermore, this chiasma frequency is likely to be an underesti-

**Table IV.** Comparison of “biological genomic” traits in established model plants and *B. distachyon*

|                                                                   | Arabidopsis          | <i>B. distachyon</i> (ABR1) | Rice                                |
|-------------------------------------------------------------------|----------------------|-----------------------------|-------------------------------------|
| Plant family                                                      | Cruciferae           | Pooideae                    | Oryzoideae                          |
| Chromosome no.                                                    | 10 (2n)              | 10 (2n)                     | 24 (2n)                             |
| Genome size (1C)                                                  | 164 Mbp <sup>a</sup> | 160 Mbp                     | 441 Mbp <sup>a</sup>                |
| Amount of repetitive DNA                                          | 16% <sup>b</sup>     | 12%–15% <sup>c</sup>        | ~20% <sup>d</sup>                   |
| Breeding strategy                                                 | Self fertile         | Self fertile                | Outbreeder                          |
| Life cycle (weeks) <sup>e</sup>                                   | 8–10 <sup>f</sup>    | 11–18 <sup>g</sup>          | 20–30 <sup>h</sup>                  |
| Height at maturity (m)                                            | ~0.2                 | ~0.2                        | 1–1.2                               |
| Planting density for M2 seed collection (plants m <sup>-2</sup> ) | ~300                 | ~300                        | 8–10                                |
| Seed yield plant <sup>-1</sup>                                    | >1000                | 80–200                      | >1,000                              |
| Seed yield m <sup>-2</sup>                                        | >300,000             | 24–60,000                   | >8,000                              |
| Growth requirements                                               | Simple               | Simple                      | Relatively specialized <sup>i</sup> |
| Transformation efficiency                                         | 0.5%–4% <sup>j</sup> | ~5                          | 3–8 <sup>k</sup>                    |

<sup>a</sup> Bennett et al., 2000. <sup>b</sup> Pruitt and Meyerowitz, 1986. <sup>c</sup> Catalan et al., 1995. <sup>d</sup> Deshpande and Ranjekar, 1980. <sup>e</sup> Life cycle defined as time from seed germination to harvesting of first seed. <sup>f</sup> Sommerville and Meyerowitz, 1994. <sup>g</sup> Depending on vernalization requirement. <sup>h</sup> Observed at the UW Aberystwyth. <sup>i</sup> To achieve fast growth and controllable flowering in temperate latitudes, many rice varieties require to be grown at high light intensity in a heated greenhouse with purpose-built irrigation/flooding systems. Specialized environmentally controlled growth cabinets are often required for the production of somatic embryos suitable for tissue culture. <sup>j</sup> Transformation frequency in Arabidopsis is presented as the proportion of transgenic to wild-type seeds recovered from florally “dipped” plants (Cough and Bent, 1998). <sup>k</sup> The comparative data concerning rice callus transformation is derived from Li et al. (1993) and Biswas et al. (1998) and is expressed as the number of transgenic plants produced 1 g embryogenic tissue<sup>-1</sup> bombarded with microprojectiles.

mate because of the technical problem of resolving more than one chiasma per chromosome arm at this meiotic stage. Thus, we expect that genetic maps of *B. distachyon* could be quickly generated given its high crossover frequency and given the development of high-throughput markers such as single nucleotide repeats (Ellis, 2000). In diploid *B. distachyon* ecotypes, the five pairs of chromosomes are sufficiently dissimilar to enable positive identification in routine squash preparations of somatic tissue. Meiotic prophase chromosomes are amenable to study in *B. distachyon*, and provide longer substrates for the hybridization in situ of fluorescent probes. Thus, it should be possible also to physically map DNA probes from large insert libraries (such as BACs) to meiotic prophase chromosomes and DNA fibers, as has been achieved in tomato (*Lycopersicon esculentum*; Zhong et al., 1999). If the chromosomes are probed simultaneously with landmarks such as rDNA and pericentromeric repeats originally isolated from *B. sylvaticum* (Abbo et al., 1995), it should be possible to construct rapidly physical linkage maps and assign contiguous megabase tracts of DNA to particular chromosome arms. Such an approach may aid future programs requiring the targeted sequencing of specific regions of the *B. distachyon* genome. As publicly available rice genomic sequences from the International Rice Genome Sequencing Project are only available for around 25% of the rice genome, with the more complete sequence only available through "research contracts" with Syngenta (Dickson and Cyranoski, 2001), then the rapid generation of sequence information from the *B. distachyon* genome could have great utility in the academic community.

The genus *Brachypodium* is considered a sister group to the "core pooid" clade, which contains all of the important temperate cereals, fodder grasses, and amenity grasses. We further propose that *B. distachyon*, by occupying this key phylogenetic position and by virtue of its exceedingly "compact genome" (allowing representation by a very small genomic library), could provide a significant resource for the analysis of the much bigger genomes possessed by important temperate grasses. For example, to aid long-distance chromosome walking and quantitative trait loci analysis in cereals with large genomes, mapping information from one grass genome can theoretically be used to locate a likely syntenic (or colinear) region represented in BAC/YAC clone contigs from a cereal with a smaller genome (Moore et al., 1993). In reality, there are as yet very few examples of this approach being successful (e.g. Chen et al., 1997), which is due largely to the breakdown of genome colinearity at the microsyntenic level (e.g. 50 Kb–1 Mb) where the species are relatively distantly related (Foote et al., 1997). However, considering the phylogenetic position of *Brachypodium* in the Pooideae, microsynteny is likely to be relatively more conserved within this subfamily and thus *B. distachyon*

could provide an archetypal map specifically for pooids to which other colinear genomes could be aligned. In practical terms, physical markers linked only loosely to important traits in the large genomes of species such as wheat and barley (*Hordeum vulgare*) might be located to relevant *B. distachyon* genomic library clones to provide rapid access to syntenic genomic regions.

#### Biological Characteristics Indicate That *B. distachyon* May Be Amenable for Large-Scale Mutagenesis Programs

Mutagenesis is commonly used to relate genotype to phenotype. Large-scale mutagenic techniques may employ chemical, physical, or genetic mutagens, and the latter especially has proved a powerful tool for gene tagging in Arabidopsis (for example, Tissier et al., 1999). Successful mutagenesis of a plant species is dependent on a small diploid genome, available mapping strategies, ease of crossing, transformability, and not inconsequential features such as a small physical stature, a rapid life cycle, and good seed yield, which eases mutant screening and collecting selected lines (Vizir et al., 1996). Table IV illustrates how Arabidopsis fulfills all these requirements, but *B. distachyon* also scores highly. As stated above, genetic maps now can be generated relatively quickly, and so the only remaining unfavorable feature is the smaller seed yield compared with Arabidopsis. However, compared with rice, *B. distachyon* has a more rapid life cycle, smaller stature, and an inbreeding reproductive strategy with anthers and stigmas enclosed tightly within the palea and lemma. The latter makes crossing different plants slightly more demanding technically. Floral spikelet emergence proved to be the best stage to remove the three anthers and apply pollen to the stigmas when conducting crossing experiments. However, this inbreeding reproductive behavior and the absence of seed head "shatter" in most *B. distachyon* ecotypes means that F<sub>1</sub> seed can be collected without the need for hand pollination or the time-consuming bagging of flowering plants. Thus, individual plants do not have to be isolated when grown in large populations to avoid outcrossing, which is a great advantage for mutagenesis studies. We have already generated  $\gamma$ -irradiated populations, and transposon-based mutagenic approaches are under development (J. Draper, L.A.J. Mur, G. Jenkins, and P. Bablak, unpublished data).

#### *B. distachyon*, A Potential High-Throughput Transformation System

A key technology for a model plant species is the availability of a facile transformation system. For many dicot species, high efficiency *Agrobacterium tumefaciens*-mediated transformation is well estab-

lished, but though the approach has been used to generate transgenic rice (Chan et al., 1992), wheat (Mooney et al., 1991), maize (Gould et al., 1991), and barley (Tingay et al., 1997), this technique is far from routine. For Poaceae, particle bombardment of embryogenic callus followed by selection of transgenic tissue (for review, see Potrykus, 1990; Hansen and Wright, 1999) has proven robust and has been used to transform maize (Klein et al., 1989; Fromm et al., 1990), wheat (Vasil et al., 1991), and rice (Cao et al., 1992). Nevertheless, the number of publications describing the use of transgenic cereals or forage grasses for basic studies in biology is still very limited in comparison with dicots. The success of the transformation techniques is dependent upon the optimal culture of highly embryogenic callus (Hansen and Wright, 1999). This is derived commonly from immature zygotic embryos, as this tissue is very responsive to in vitro culture and has a high plant regenerative capacity. Immature embryos from *B. distachyon* were not an exception. Large numbers of immature embryos can be isolated from *B. distachyon* plants grown under simple greenhouse conditions, without the need for specialist environmentally controlled growth chambers. For example, a single 18- × 30-cm tray containing 24 flowering plants can provide sufficient embryos to supply embryogenic cultures for a single researcher for 1 mo. The low frequency of gross morphological abnormalities (e.g. albinism and male sterility) suggested that significant somaclonal variation did not occur under the culture conditions used. Based on this behavior in tissue culture, we report a transformation system for a hexaploid ecotype of *B. distachyon* that is comparable with the best rice systems currently available in terms of ease of use and final transformation efficiency (Tables IIIC and IV; Li et al., 1993; Biswas et al., 1998). As testing a particular transgene in wheat can still take up to 5 years (Dunwell, 2000), given the rapid life cycle of *B. distachyon*, our development of a facile, low-cost transformation system will offer a rapid “test bed” for transgenic studies in grasses. We also demonstrate that embryogenic callus can be generated routinely from several diploid ecotypes, and current experiments aim to develop transposon tagging and gene trapping resources in diploid ecotypes of *B. distachyon* (J. Draper, L.A.J. Mur, G. Jenkins, and P. Bablak, unpublished data).

### ***B. distachyon* Displays Many Traits That Are Relevant for Cereal and Forage Grass Improvement**

In considering a potential model for temperate cereals, the value of any plant species obviously depends on whether the particular organism displays key traits representing those targeted currently in plant breeding and biotechnology programs. Crop loss through pathogen attack is significant and represents a key aspect for research. We also note that in

simple practical terms, a small plant, which is easy to maintain and which will support several classes of important cereal diseases may find utility in fungicide screening programs. All ecotypes of *B. distachyon* tested appeared to exhibit single cell death and papillae formation following challenge with powdery mildew pathogens adapted to cereals. These responses are symptomatic of resistance in other grass/cereal species (Carver et al., 1995) and may be indicative of lack of race structure. The molecular basis of such “non-host” HR is being investigated in model dicots (e.g. Lauge et al., 2000), and *B. distachyon* could serve in a similar role for the Poaceae, where race-specific resistance exhibits little longevity in the field. Different ecotypes of *B. distachyon* exhibited great variability in response to infection with individual rust species ranging from no visible symptoms or BF associated with a HR, to pustule formation (albeit associated with necrotic tissue). These data suggest that *B. distachyon* may prove to be a useful host for future studies on the molecular biology and genetics of these important plant-pathogen interactions. In contrast, we observed clear evidence of susceptibility and resistance exhibited by different ecotypes to challenge with *Magnaporthe grisea* Guy-11 and additionally, the phenotypes closely resembled those observed on rice cultivars (data not shown). *M. grisea* is undoubtedly one of the most virulent and economically devastating plant pathogens, causing losses of between 11% and 30% of the annual world crop and representing up to 157 million tons of rice (Baker et al., 1997). Thus, we are extensively characterizing the *B. distachyon*/*M. grisea* pathosystem to identify key resistance determinants and to understand defense-associated signaling (A.P.M. Routledge, L.A.J. Mur, and G. Shelley, unpublished data).

An even more important trait than pathogenic interactions for a Pooid model is the analysis of genetic and biochemical events underlying grain-filling and quality. The large size of the seeds in the genus *Brachypodium* has been highlighted previously (Catalan et al., 1997) and there have already been some basic studies on seed storage proteins (Khan and Stace, 1999). Thus, it is envisaged that resource allocation, grain filling, and endosperm development in particular will be a valuable focus for mutagenesis program in *B. distachyon*. Further features, including freezing tolerance, perenniality, repetitive injury (mowing and trampling) tolerance, meristem dormancy mechanisms, post-harvest biochemistry of silage and hay, mycorrhizae, and sward ecology—all of which are important to temperate forage grasses and many temperate cereals—are traits that are not exhibited by or are difficult to study in rice, but are possible targets for functional genomics in *Brachypodium*.

In summary, the present data demonstrate that *B. distachyon* has great potential to be adapted for high-throughput genetics. In many aspects of its biology such as genome size, chromosome number, height,

planting density, breeding system, and duration of life cycle, it is very similar to *Arabidopsis*. We propose that *B. distachyon* is best seen as a complementary model to rice offering, as it does, the opportunity to study grass traits that are not well exhibited by rice. In addition, the simpler logistics of handling large *B. distachyon* populations, as compared with rice, may well make *B. distachyon* an attractive prospect where investigative resources, such as access to controlled growth environments, are limited.

## MATERIALS AND METHODS

### Plant Growth Conditions

Full details of all *Brachypodium distachyon* ecotypes can be found at <http://www.aber.ac.uk/plantpathol/Brachyomics>. Seed samples of different *Brachypodium* ecotypes were obtained from Brachyomics (Botany Gardens, Aberystwyth, Ceredigion, UK) under a "Research Only" Materials Transfer Agreement. All *B. distachyon* ecotypes were grown under a 16-h light period at  $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . The plants were illuminated with 55W (Osram, Sylvania, Munich) high-frequency lighting tubes (4,580 lumen output) and were supplemented with  $2 \times 30\text{W}$  clear tube cooled lighting (Osram) and placed between 60 and 100 cm of the light bank. Light intensities always exceeded  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Plants were grown routinely on Levington's Universal Compost (Levington Horticulture, Suffolk, UK) supplemented with gravel (the longest axis was approximately 0.5 cm) to approximately 50% (v/v) to improve drainage. Plants were usually watered at 2-d intervals and were never allowed to stand in water.

### Nuclear Genome Size Estimations: Scanning Microdensitometry and Flow Cytometry

For microdensitometry, root tips were fixed in 4% (w/v) formaldehyde for 2 h, and were then washed with distilled water prior to acid-hydrolysis by treatment with 5 M HCl at  $20^{\circ}\text{C}$  for 45 min. Following washing with distilled water, Feulgen stain was added and the samples were kept in the dark for 45 min. The stain was washed off with  $\text{SO}_2$  water, and then root tip squashes were prepared. The density of well-stained prophase nuclei was scanned using an M86 scanning microdensitometer (Vickers Instruments, York, UK). The relative amount of DNA in each nucleus was calculated using the following formula: absorbance units of test species/absorption units of a standard species  $\times 4\text{C}$  value of standard species =  $4\text{C}$  value of test species. Mung bean (*Vigna radiata* var. Berken Mung Bean;  $2\text{C} = 1.05 \mu\text{g}$ ) was used as the standard species (seeds supplied by W. Atley Burpee Seed Company, Leicester, UK).

Nuclei were isolated for flow cytometry using the chopping technique developed by Galbraith et al. (1983) using LB01 buffer at pH 7.5 (Dolezel et al., 1989, 1998) supplemented with  $50 \mu\text{g mL}^{-1}$  propidium iodide and  $50 \mu\text{g mL}^{-1}$  RNase (Sigma, Poole, UK). The fluorescence of stained nuclei was analyzed using PA or III flow cytometers (Partec, Münster, Germany). Calibration standards

(Bennett et al., 2000) used to estimate the *B. distachyon* 1C genome size were *Lotus japonicus* (approximately 445 Mbp; Bennett and Smith, 1976), *Aesculus hippocastanum* (approximately 110 Mbp; Bennett et al., 1982), pea (*Pisum sativum*; approximately 4,150 Mbp; Bennett and Leitch, 1997), *Arabidopsis* (approximately 164 Mbp; Bennett et al., 2000), rice subsp. *indica* cv IR34 (approximately 441 Mbp; Bennett et al., 2000), and barley cv Sultan (approximately 4,116 Mbp; Bennett et al., 2000).

### Karyotyping and FISH

After flow cytometry experiments, seeds of seven putative diploid accessions of *B. distachyon* (ABR1–ABR7) were germinated on filter paper moistened with tap water at  $22.5^{\circ}\text{C}$  in the dark. Whole seedlings with roots of about 1 cm long were incubated in a saturated solution of 1-bromonaphthalene at  $0^{\circ}\text{C}$  overnight, or in 2 mM aqueous 8-hydroxyquinoline for 1 to 2 h at room temperature. After washing and fixing for at least 2 h in a 3:1 mixture of methanol:acetic acid, root-tips destined for bright-field microscopy were hydrolyzed in 1 M HCl at  $60^{\circ}\text{C}$  for 10 min, stained in Feulgen solution, and mounted in 2% (w/v) orcein in 45% (w/v) propionic acid. Material for FISH was washed after fixation in 10 mM citrate buffer and was digested for 1.5 to 2 h at  $37^{\circ}\text{C}$  in a mixture comprising 1% (w/v) cellulase (Calbiochem, La Jolla, CA), 1% (w/v) cellulase (Onozuka RS, Yakult Honsha C. Ltd., Tokyo), and 20% (v/v) pectinase (Sigma, St. Louis). After washing, root-tips were detached and their meristems were dissected out into 45% (w/v) acetic acid and squashed onto microscope slides. Coverslips were removed by freezing and the preparations were post-fixed in 3:1 ethanol:acetic acid, dehydrated in absolute ethanol, and air dried. Immature inflorescences were fixed in Carnoy's solution. Anthers at first metaphase of meiosis were macerated in propionic orcein and squashed under coverslips. Somatic and meiotic chromosomes were photographed onto Imagemink HQ microfilm (Eastman-Kodak, Rochester, NY) with an MC100 camera attached to a Axioplan microscope (Zeiss, Welwyn Garden City, UK), and they were electronically scanned and processed in CorelDraw (Corel Corporation, Ottawa, Ontario, Canada).

The 5S rDNA probe was amplified and labeled with rhodamine-4-dUTP (Amersham Pharmacia, Uppsala) from the wheat clone pTa 794 (Gerlach and Dyer, 1980), using PCR with universal M13 sequencing primers under the following conditions:  $94^{\circ}\text{C}$  for 1 min, 35 cycles of  $94^{\circ}\text{C}$  for 40s,  $55^{\circ}\text{C}$  for 40s,  $72^{\circ}\text{C}$  for 1 min, and 1 cycle of  $72^{\circ}\text{C}$  for 5 min. The 45S rDNA probe was obtained by nick translation with digoxigenin-11-dUTP (Roche, Basel) of a 2.3-kb subclone of the 25S rDNA unit of *Arabidopsis* (Unfried and Gruendler, 1990).

FISH was adapted with some modifications from Schwarzacher and Heslop-Harrison (2000). In short, slides were pre-treated with RNase for 1 h at  $37^{\circ}\text{C}$ , post-fixed in 1% (w/v) aqueous formaldehyde in phosphate-buffered saline buffer for 10 min, and dehydrated in an ethanol series. Probe DNA was mixed to a concentration of 100 ng

slide<sup>-1</sup> with 50% (w/v) deionized formamide, 10% (w/v) Dextran sulfate, 2× SSC, and 1% (w/v) SDS. The probe DNA was allowed to hybridize with the chromosome preparations overnight at 37°C in an Omnislide in situ hybridization system (Thermo Hybaid, Ashford, Kent, UK). Slides were washed stringently in 20% (w/v) deionized formamide in 0.1% (w/v) SSC at 42°C, followed by detection of digoxigenin by fluorescein isothiocyanate-conjugated anti-digoxigenin antibodies. The chromosomes were counterstained with 4',6-diamino-phenylindole (2 μg mL<sup>-1</sup>), mounted in Vectashield (Vector Laboratories, Burlingame, CA), and photographed onto Provia 400 color reversal film (Fuji Photo Film, Tokyo) with an MC100 camera attached to a Axioplan epifluorescence microscope (Zeiss, Jena, Germany). Images were scanned electronically and processed using Micrografx Picture Publisher software.

### Tissue Culture and Transformation

All media were as described previously by Bablak et al. (1995) except, when required, maltose was substituted for Suc at a level of 30g L<sup>-1</sup>. Immature embryos or callus material was viewed by scanning electron microscopy as described previously (Bablak et al., 1995). Seedlings were germinated and grown as described above, and immature embryos were isolated from sterilized seeds (Bablak et al., 1995) under a dissecting microscope approximately 14 d after anthesis and their size was estimated (when required) using an eye-piece graticule. Approximately 50 embryos were cultured in the dark in a 9- × 1.5-cm petri dish containing 25 mL of LS 2.5 medium (Bablak et al., 1995). After 4 weeks, the developed calli were detached from the explants and were subcultured onto the same medium. In several experiments, LS 2.5 was substituted with N6 2.5 or SH 2.5 (Bablak et al., 1995). Type II callus (creamy-white, dry, and friable) was constantly selected at each subculture to bulk up tissue for regeneration and transformation studies. Media used for regeneration were MS0; 190-2 (*Lolium* regeneration medium), MWW (Winter wheat regeneration medium), and RM1 (Barley regeneration media; Bablak et al., 1995).

For microprojectile bombardment, 1.0-μm gold particles were coated with the plasmid and "fired" using the procedure described in the PDS1000/He biolistic gun instruction manual (Bio-Rad, Hercules, CA). The plasmid used was pAct1GUSHm (McElroy et al., 1991), which contained an Hm-resistance gene (*hgh*) driven by a cauliflower mosaic virus 35S promoter (35S) for transformant selection and a 35S-GUS gene for transient transformation assays. In preliminary transformation experiments, it was discovered that the substitution of Suc with maltose increased the regeneration potential of tissue bombarded with microprojectiles. The bombardment procedure was optimized by selecting conditions that achieved routinely >1,000 GUS-positive regions per target plate when examined by histochemical staining. Approximately 1.0 g fresh weight of embryogenic callus was centered in petri dishes containing LS 2.5 supplemented with 140 g L<sup>-1</sup> maltose and 2.5 g L<sup>-1</sup> of Phytigel and incubated for 2 h. Calli were bombarded

with a single microprojectile firing using a PDS1000 particle acceleration device (Bio-Rad) with a helium pressure of 1,300 psi, under a chamber pressure of 27 mm Hg at a distance of 13 cm below the microprojectile stopping plate. For transient assays, calli were stained for GUS activity (Warner et al., 1993) after 24 to 36 h, and only callus batches that exhibited 1,000 blue GUS spots or more were used for stable transformation experiments. For stable transformation, all target materials were bombarded once with pAct1GUSHm and were then transferred to LS 2.5 (with maltose as a carbon source) for 1 week in the dark at 25°C. Bombarded calli were then transferred to the same medium supplemented with 40 mg L<sup>-1</sup> Hm (Duchefa, Haarlem, The Netherlands). Two weeks later, growing tissue was picked out and transferred to fresh medium containing 40 mg L<sup>-1</sup> Hm, a process that was repeated twice at 7 d intervals. Resistant calli were transferred to regeneration medium (LS containing 0.2 mg L<sup>-1</sup> kinetin, 2.5 g L<sup>-1</sup> Phytigel, and 30 g L<sup>-1</sup> maltose) containing 30 mg L<sup>-1</sup> Hm. After 5 to 7 d, the callus sections containing germinating shoots and viable embryos were transferred onto MS0 medium (Bablak et al., 1995) supplemented with 30 mg L<sup>-1</sup> Hm and were placed in the light. When plantlets had reached a size of 3 to 4 cm in height, they were separated gently from any remaining necrotic callus and removed to one-half strength MS0 to enhance root development. The transformation frequency was expressed in terms of average number of transgenic plants derived per gram of tissue. After rooting, plants were transferred to soil as described previously (Bablak et al., 1995).

### Genomic Analysis of Transgenic *B. distachyon* Lines

Total genomic DNA was isolated from leaves using a modified cetyl-trimethyl-ammonium bromide protocol (Murray and Thompson, 1980) and was quantified after RNase treatment. Plants were screened using PCR amplification of the introduced *hgh* gene. PCR primers (5'-CCTGAAGTACCACCGGAC-3' and 3'-GCTCATCGAGAGCCTGC-5') were used to amplify a 516-bp fragment encoding the *hgh* gene, which was analyzed by electrophoresis in 0.8% (w/v) agarose/ethidium bromide gels. For Southern analysis, *Bam*HI-digested genomic DNA (10 μg lane<sup>-1</sup>) was separated on an agarose gel, blotted onto a membrane, and probed with a radioactive probe following a standard protocol (Sambrook et al., 1989). The radioactive probe was prepared by the random primer method (Feinberg and Volgstein, 1983), and consisted of a 1.07-kb *Bam*HI fragment containing the coding sequence of the *hgh* gene.

### Challenge with Fungal Pathogens

All fungal infections were performed on batches of 24 *B. distachyon* plants with each challenge repeated at least three times on seedlings aged from 3 to 4 weeks post-germination. Diseased cereal plants with confirmed infections of *Blumeria graminis* f. sp. *avenae*, *hordei*, or *triticeae* were used to infect *B. distachyon* ecotypes by simply shaking the conidiophores on the recipient plants. Challenging

with the rust fungal pathogens *Puccinia striiformis* f. sp. *hordei* (strains BWR 80-1 and PB 60-7), f. sp. *triticae* (strains WYR 95-6 and IPO 86053; yellow stripe rust), and *Puccinia recondita* f. sp. *hordei* (strains BBR 79-1 and PB60-2-2), f. sp. *triticae* (strains WYRP96-8 and WBRP90-25; brown leaf rust) involved powder spraying a talc/urideospore suspension on to 3- to 4-week-old *B. distachyon* seedlings. Plants were then bagged for 24 h in high humidity and were incubated at 10°C, after which the bag was removed and the temperature was increased to 24°C. Symptoms were first observed by 16 to 20 d following challenge. *M. grisea* strain Guy-11 was cultured on Potato Dextrose Agar (Sigma) and the spores were harvested into 0.2% (w/v) gelatin and diluted to 10<sup>5</sup> spore mL<sup>-1</sup>. The spore suspension was sprayed onto *B. distachyon* 3- to 4-week-old seedlings to run off and the challenged material was bagged for 24 h to maintain a high humidity. Symptoms were first observed 3 to 4 d after spraying.

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