

# Chapter 16

## *Brachypodium distachyon*, a New Model for the Triticeae

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**Abstract** *Brachypodium distachyon* (Brachypodium) is a small annual grass with biological, physical and genomic attributes (e.g. rapid cycling, small stature, inbreeding, small genome, diploid accessions) suitable for use as a modern model system. In pursuit of this goal, researchers have made rapid progress in developing genomic resources that will transform Brachypodium into a powerful model system including: facile *Agrobacterium*-mediated transformation methods, BAC libraries, physical maps, genetic maps, and germ-plasm resources. In addition, a preliminary 4x draft of the entire genome has been released, and completion of the final 8x assembly is anticipated in 2009. This chapter provides an overview of the advantages of Brachypodium as a model system and surveys the use and potential applications of this system to aid wheat, barley and *Lolium* research.

### 16.1 Model Systems in Biology

Biologists strive for a better understanding of the machinery that drives the living world and the great complexity of biological systems presents a challenge to their endeavors. In response, scientists have sought means to reduce the complexity of their particular systems of study in order to reveal the underlying basic design principles. Although reductionist methods are not appropriate for all experiments, in many areas of biology they are essential to achieve rapid progress. Research using relatively simple model organisms such as *E. coli*, yeast, fruit flies, and mice has led to innumerable discoveries that benefit the daily lives of billions of people. As scientists turn their attention toward more specialized areas of study, the number of model systems continually increases. For example, developmental biology has adopted *C. elegans* and zebrafish as

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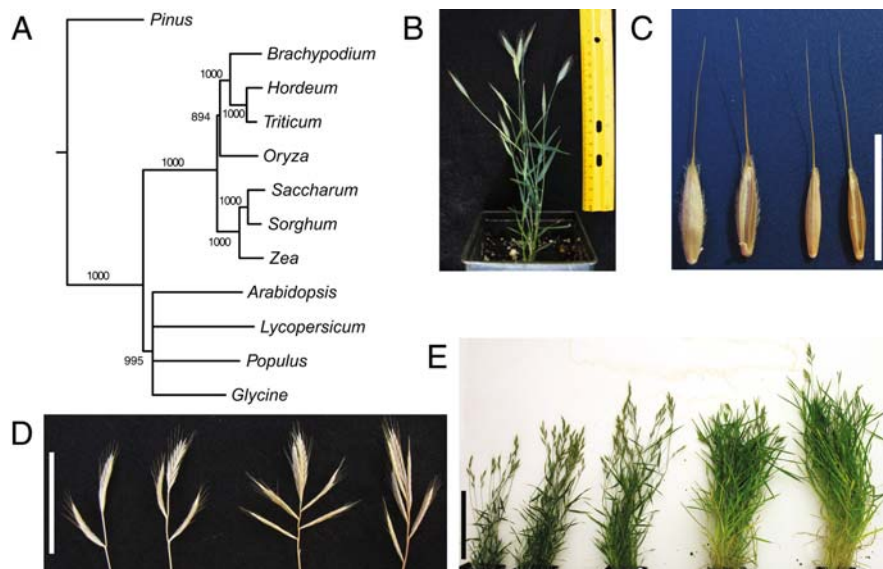
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model organisms. Laboratory manipulations of model organisms are facilitated by simple growth requirements, short generation time, the ability to inbreed, and small size. Additionally, characteristics of model systems that suit them for modern genomic methods include diversity of natural populations, facile transformation, diploidy, simple genetics, and a small genome size.

Plant biologists have widely adopted the small weedy species *Arabidopsis thaliana* as a generalized model plant and, due to its inherent biological attributes and the collegial nature of the research community that has sprung up around it, *Arabidopsis* has become an extremely powerful system. However, *Arabidopsis* is not suitable to study many aspects of grass biology due to the biological differences that have arisen between dicots and monocots in the 150 million years since they last shared a common ancestor. As an example, grass cell walls differ dramatically from dicot cell walls in terms of the major structural polysaccharides present, how those polysaccharides are linked together, and the abundance and importance of pectins, proteins and phenolic compounds (Carpita 1996). A partial list of additional areas in which *Arabidopsis* is not an appropriate model for the study of grasses includes: mycorrhizal associations, architecture of the grass plant, grain properties, intercalary meristems, and grass development. The tremendous importance of grasses as food, feed and, increasingly, as fuel, argue strongly for the development of a truly tractable grass model system. At a first glance, rice with its sequenced genome and large research community would seem to fill this bill. However, upon closer examination, the demanding growing conditions and large size of rice plants make it a poor choice for high-throughput genomic experiments in temperate regions. The fact that rice is a semi-aquatic tropical grass further limits its applicability as a model for temperate grasses, especially in areas like freezing tolerance and vernalization.

## 16.2 Introduction to *Brachypodium distachyon*

The utility of the small annual grass *Brachypodium distachyon* (hereafter referred to as *Brachypodium*) as a model system for the study of the Triticeae was first discussed in a 2001 paper that pointed out that *Brachypodium* displays all of the biological, physical and genomic attributes required for use as a model system (Draper et al. 2001). The small size and rapid generation time of *Brachypodium* enables high-throughput studies. Densities of 1,000 plants/m<sup>2</sup> can be easily achieved in growth chambers or greenhouses allowing growth of large numbers of plants under controlled environmental conditions (Fig. 16.1). For comparison, the same space accommodates only 50 wheat plants, 36 rice plants, or four switchgrass plants (Table 16.1). Furthermore, *Brachypodium* is self-fertile and does not typically outcross. This feature is useful for breeding and maintaining homozygous lines for many applications that require the maintenance of large numbers of independent genotypes (e.g. mapping



**Fig. 16.1** Relationships and phenotypes of *Brachypodium*. (A) Rooted phylogenetic tree based on the combined partial nucleotide sequences of 20 highly expressed genes (Vogel et al. 2006b). Branch length is proportional to sequence divergence. (B) A plant from line Bd21-3 flowering under 20 h light conditions. (C) Close-up of seeds from two accessions. Note that the seeds on the right are hairless and smaller than the seeds on the left. (D) Variation in inflorescence architecture in *four new inbred lines* from Turkey grown under the same conditions. Note the differences in spikelet number and angle. (E) Effect of vernalization on *inbred lines* from one location, Bismil, in Turkey. Plants were placed in the cold for 3 weeks and then moved into a growth chamber with 20 h daylength. The plants in the pot on the left flowered after 25 days in the growth chamber and the plants in the pot on the right flowered after 50 days. Note the range of flowering times even at one location. (D and E) Seeds of Turkish lines were kindly supplied by Metin Tuna (Namik Kemal University, Tekirdag, Turkey). The scale bar in (C) is 1 cm, in (D) is 5 cm and in (E) is 15 cm (See Color Insert)

**Table 16.1** Comparison of models and crops

	Brachypodium	Arabidopsis	Rice	Wheat	Switchgrass
Height (cm)	15–20	15–20	100	50	200
Density (plants/m <sup>2</sup> )	1,000	2,000	36	50	6
Growth Requirements	simple	simple	demanding	simple	simple
Genome Size (Mbp)	300	165	430	16,000	2,400
Generation Time (weeks)	8–12	8–12	30	12	26
Reproduction	selfing	selfing	selfing	selfing	outcrossing
Cell Wall Type	Type 2	Type 1	Type 2	Type 2	Type 2

experiments, mutant analysis, and studies of natural diversity). As a group, the grasses are notorious for very large genomes. Fortunately, the ~300 Mbp diploid *Brachypodium* genome is one of the smallest of any grass. Since wheat is more closely related to *Brachypodium* than to rice, *Brachypodium* will serve as a more relevant model for wheat structural genomic studies. Like wheat, polyploid *Brachypodium* accessions exist and they may be useful as a model for polyploidy.

### 16.2.1 *Genome Size and Polyploidy*

A compact genome is one of the most important attributes of a modern model organism because it permits efficient positional cloning of genes, facilitates genome-wide insertional mutagenesis, and is a prerequisite for whole genome sequencing. Three publications reported the c-value of diploid *Brachypodium* to be approximately 0.36 pg, corresponding to a genome size of approximately 300–320 Mbp (Bennett and Leitch 2005; Vogel and Hill 2008; Vogel et al. 2006a). Although Draper et al. reported a c-value of only 0.15 pg, Bennett and Leitch (2005) obtained a c-value of 0.36 using the same accession, suggesting that the lower number may have been an artifact. An independent estimation of *Brachypodium* genome size was calculated in the range of 300 Mbp based on the frequency of recovering single-copy genes from two bacterial artificial chromosome (BAC) libraries (Huo et al. 2006), and the preliminary 4x genome assembly suggests a size no larger than 300 Mbp (unpublished). Thus, several lines of evidence indicate that *Brachypodium* has one of the smallest genomes of any grass.

Similar to wheat, polyploid *Brachypodium* accessions have been described, and 1n chromosome numbers of 5, 10, and 15 have been reported. Initially, these chromosome numbers appeared to fit a simple autopolyploid series: diploid, tetraploid, and hexaploid. However, careful analysis of c-values and fluorescence *in situ* hybridization (FISH) labeled karyotypes suggests otherwise. FISH analysis of 1n = 10 accessions revealed 10 small chromosomes more similar to the diploid *B. sylvaticum* than to the larger chromosomes found in 1n = 5 accessions (Hasterok et al. 2004). Thus, the 1n = 10 *Brachypodium* accessions appear to be diploid with a 1n = 10 base chromosome number similar to *B. sylvaticum* (Hasterok et al. 2004). The 1n = 15 accessions have c-values approximately twice as large as those of the diploid 1n = 5 accessions (Vogel et al. 2006a) and their karyotypes contain 5 large and 10 small chromosomes (Hasterok et al. 2004). The banding pattern of FISH labeling using a rDNA probe suggests that 1n = 15 accessions are actually allotetraploids with one parent containing a genome similar to diploid 1n = 5 accessions and the other parent containing a genome with a 1n = 10 chromosome number (Hasterok et al. 2004). Taken together, these data suggest that the *Brachypodium* 1n = 10 accessions are actually a separate species and the 1n = 15 accessions are derived

from an interspecific hybrid between one parent similar to the  $1n = 5$  diploid and one parent similar to the  $1n = 10$  diploid (Hasterok et al. 2006a,b). The  $1n = 15$  allotetraploid plants can be easily distinguished from diploid *Brachypodium* ( $1n = 5$ ) because the allotetraploids have larger seeds, grow to a larger size, have anthers that typically exert, and do not require vernalization for flowering. In addition, examination of SSR polymorphisms in an allotetraploid  $1n = 15$  accession revealed multiple bands which presumably correspond to the different genomes (unpublished).

### 16.2.2 Relationship to Other Grasses

The phylogenetic relationship between the genus *Brachypodium* and the other grasses has been evaluated a number of times with increasing amounts of data. *Brachypodium* has consistently been placed approximately halfway between rice and wheat (Fig. 16.1). Reports based on internal transcribed spacer (ITS) and 5.8 s rDNA sequence (Hsaio et al. 1994), genomic RFLP and RAPD markers (Catalán et al. 1995), and ITS sequence plus the chloroplast *ndfH* gene (Catalán and Olmstead 2000) all placed *Brachypodium* between rice and a clade containing temperate grains like wheat, barley and *Secale*. Additional examinations of a much broader spectrum of grasses used ITS and *ndfH* sequence as well as morphological and chloroplast restriction sites (Kellogg 2001) or the sequence of the *matK* chloroplast gene (Döring et al. 2007). These studies placed *Brachypodium* in the subfamily Pooideae just below the radiation of the small grains and forage and turf grasses making *Brachypodium* “sister” to the temperate grasses of greatest economic significance. However, phylogenies based on single genes or small sets of genes can produce inconsistent phylogenetic trees (Rokas et al. 2003), and this phenomenon as has been observed with rice (Kellogg 1998). Therefore, it was important to examine the phylogenetic relationship of *Brachypodium* using larger datasets. Analysis of a dataset comprising 11 kb of sequence from 20 highly expressed genes verified the relationship between *Brachypodium* and the small grains (Fig. 16.1; Vogel et al. 2006b). An even larger dataset based on 335 BAC end sequences provides further evidence to confirm the placement of *Brachypodium* within the grasses (Huo et al. 2007). Thus, the relationship of *Brachypodium* to cereal crops and other grasses has been firmly established.

### 16.3 *Brachypodium* as An Experimental System

*Brachypodium* is easy to grow in large numbers under controlled conditions, is easy to transform, is genetically tractable, and has a small genome. In addition, a large collection of diverse accessions and described inbred lines are currently

being created for *Brachypodium* and will allow researchers to exploit the power of natural diversity for understanding basic questions in biology.

### 16.3.1 Growth Requirements and Flowering Triggers

One of the strengths of *Brachypodium* as a model system is its ease of culture under laboratory conditions. This is in contrast to rice whose demanding growth requirements, large size, and long generation time are a barrier to many researchers. Generally, *Brachypodium* can be grown in growth chambers or greenhouses used for *Arabidopsis*, wheat or barley. Our standard conditions for growth chambers are: 20 h light: 4 h dark photoperiod, 24°C during the day and 18°C at night with cool-white fluorescent lighting at a level of 150  $\mu\text{Em}^{-2}\text{s}^{-1}$ . Our standard greenhouse conditions are: no shading, 24°C in the day and 18°C at night, and supplemental lighting to extend daylength to 16 h. Providing the appropriate conditions to induce flowering is critical to prevent the plants from producing excessive vegetative growth. Vernalization has been shown to induce flowering in all diploid accessions studied to date. However, the time required to induce flowering varies greatly between accessions (Table 16.2). In general, accessions originating from colder northern regions (e.g. northern Turkey) require a longer period of vernalization to induce flowering (e.g. 8–12 weeks), and accessions originating from more southern regions, such as Iraq, require less vernalization (e.g. 2–3 weeks). For a combined stratification and vernalization treatment, we typically sow the seeds and then place them at 4°C for the desired number of weeks. After approximately 3 weeks in the cold, the seeds begin to germinate. Therefore, for vernalization times

**Table 16.2** Characteristics of select diploid inbred lines

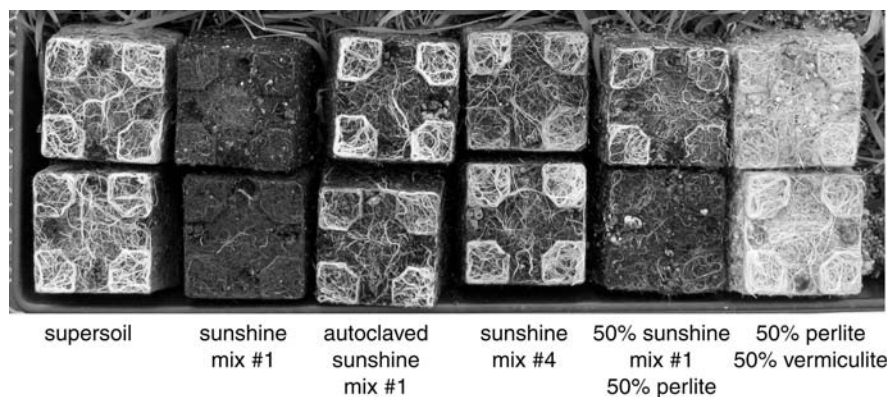
Inbred line	Geographic origin	Flower under 20 h day (weeks to seed) <sup>1</sup>	Vernalization requirement (weeks) <sup>2</sup>	Seed size
Bd1-1	Turkey	no	8	small
Bd2-3	Iraq	yes (12)	3	large
Bd3-1	Iraq	yes (10)	3	large
Bd18-1	Kaman Kirschir Province, Turkey (arid elev 3,000 ft)	no	8	large
Bd21 and Bd21-3	4 km from Salakudin on a road to Mosul, Iraq	yes (8)	3	large
na	northern Turkey	no	8–12	small
na	southern Turkey	no	4–6	large

<sup>1</sup>Plants were planted in soil, placed at 4°C for 1 week to synchronize germination and then moved to a growth chamber with 20 h days.

<sup>2</sup>Number of weeks of vernalization at 4°C needed to induce flowering under short day conditions.

greater than 4 weeks we place the pots under fluorescent lighting. Vernalizing seeds/seedlings induces the plants to flower quickly while still small. Alternatively, one can vernalize larger plants if a larger amount of seed from individual plants is desired. Growth under very long day conditions (20 h light 4 h dark) overcomes the need for vernalization in a few inbred lines (Bd2-1, Bd3-1, Bd21 and Bd21-3) (Vogel et al. 2006a; Vogel and Hill 2008). Among these, the Bd21 and Bd21-3 are the most responsive and go from seed to seed in as little as 8 weeks to yield nearly six generations per year. Under these conditions the plants flower and set seed when they are approximately 15 cm tall, a size that is compatible with high density planting.

The rate of out-crossing is an important consideration when generating and maintaining large numbers of independent lines. In this regard, *Brachypodium* is a superior model. The anthers of diploid accessions rarely exert suggesting a low rate of outcrossing. This was confirmed by measuring pollen flow from transgenic to non-transgenic plants under growth chamber conditions. In a population of more than 1,000 progeny, no outcrossing was observed (unpublished). While the inbreeding nature of *Brachypodium* is an advantage for maintaining homozygous lines, it must be overcome in order to make genetic crosses. A number of researchers have crossed *Brachypodium* lines, however, the process is currently inefficient. Since grasses often shed pollen at a specific time of day, identifying when *Brachypodium* pollen is shed may increase



**Fig. 16.2** *Brachypodium* as a model for root diseases. *Brachypodium* roots grown in different soils are shown. Plants were grown for 8 weeks and then removed from their pots and the roots photographed. Healthy white roots are evident in the plants grown in supersoil (a redwood sawdust-based mix), and 50% perlite/50% vermiculite. Plants grown in sunshine mix #1 (a peat-based mix) had severely diseased roots and *Pythium sp.* was isolated from these roots. The disease symptoms associated with sunshine mix #1 were largely eliminated by autoclaving the soil prior to planting. Incorporating perlite into the sunshine mix #1 to improve drainage also improved the symptoms. Plants grown in sunshine mix #4 (peat-based with more aggregate) were slightly diseased. Note these plants were not vernalized and thus grew much larger than plants grown for rapid seed set

crossing efficiency. This is because grass pollen is typically ephemeral and, since *Brachypodium* is normally inbreeding, *Brachypodium* pollen may be especially short lived.

Although *Brachypodium* grows well in a number of different soil types, plants that are watered excessively or left in standing water quickly develop disease symptoms. *Brachypodium* is highly susceptible to *Pythium* root rot and we have observed severe disease symptoms in some brands of commercial potting mixes (Fig. 16.2). Thus, *Brachypodium* growers may well be advised to test a few soil formulations before selecting one to grow large numbers of plants.

### ***16.3.2 Germplasm Resources and Natural Diversity***

A number of germplasm collections and several inbred lines are available to researchers. The USDA National Plant Germplasm System (NPGS) has ~30 accessions that are freely available ([www.ars-grin.gov/npgs/](http://www.ars-grin.gov/npgs/)). These collections were made many years ago and are population samples rather than inbred lines. Detailed passport data about these accessions can be found at ([www.brachypodium.org](http://www.brachypodium.org)). To increase the utility of this collection, inbred lines (designated by the prefix Bd) were created from five diploid and 23 polyploid NPGS accessions and have been widely distributed as a community resource (Table 16.2; Vogel et al. 2006a). A second collection assembled from many locations is maintained at the University of Aberystwyth ([www.aber.ac.uk/plantpathol/germplasm.htm](http://www.aber.ac.uk/plantpathol/germplasm.htm)), and the accessions are designated by the prefix ABR. This collection contains unique material as well as re-named material from NPGS and is available through a material transfer agreement, the conditions of which may not be agreeable to some institutions. A cross-referenced list of ABR lines derived from NPGS accessions is available at ([www.brachypodium.org/stocks](http://www.brachypodium.org/stocks)).

Most of the material available in existing collections represents the  $1n = 15$  allotetraploid cytotype. The  $1n = 10$  cytotype is represented by only one accession suggesting that it may have a more restricted range, and the most useful diploid ( $1n = 5$  cytotype) is represented by a handful of collections of which only five are freely available. Given that few  $1n = 5$  cytotype diploids are available, a strong need exists for additional collections of diploid accessions. To begin to address this need, researchers have gathered >200 collections from many locations in Turkey (H. Budak and M. Tuna personal communication). Fortunately, much of this new Turkish material is diploid, and preliminary SSR marker analyses of these collections and existing inbred diploid lines (Bd1-1, Bd2-3, Bd3-1, Bd18-1, Bd21, Bd21-3) show a tremendous diversity both between and within wild *Brachypodium* populations (unpublished). Variability for several interesting morphological traits also has been observed among these diploid accessions including: vernalization requirements, flower initiation by



long days, size, seed pubescence, and inflorescence architecture (Fig. 16.1). The level of molecular and phenotypic diversity observed in *Brachypodium* collections indicate that *Brachypodium* can be used to identify genes responsible for natural variation in economically important traits. Furthermore, the level of polymorphism observed in SSR markers indicates that the generation of molecular markers will not be a limitation. Inbred lines have been generated from the new Turkish material, and these will be freely distributed as soon as sufficient seed is obtained.

### ***16.3.3 Chemical and Radiation Mutagenesis***

Forward and reverse genetic approaches both require large populations of mutagenized plants, and a number of protocols have been developed for using chemicals and radiation to efficiently generate large populations of mutants. Ethyl methanesulfonate (EMS) is an efficient mutagen that introduces single base changes and has been used widely to mutagenize plants. A thorough review of mutations in 192 *Arabidopsis* genes confirmed the random nature of EMS mutations and estimated the frequency at 1 mutation/170 kb of genomic DNA (Greene et al. 2003). Because EMS introduces single base changes, it can result in partial loss of function alleles that may be particularly useful when studying essential genes. We have mutagenized *Brachypodium* with EMS by adapting a method used to create a population of barley mutants that were used for TILLING (Caldwell et al. 2004). Early results indicate that EMS is an efficient mutagen for *Brachypodium* (unpublished).

Fast neutron radiation (FNR) is a complementary mutagen to EMS that introduces short deletions. Due to the larger disruptions generated by FNR, a gene disrupted by an FNR mutation may be rapidly cloned using a genome tiling array. However, since FNR typically deletes several genes, it does not lend itself to the identification of essential genes or genes located adjacent to essential genes. Early experiments indicate that *Brachypodium* can be efficiently mutagenized by FNR (D. Laudencia-Chinguanco and M. Byrne personal communication). Thus, there are no limitations in applying common mutagens to *Brachypodium*.

### ***16.3.4 Transformation and T-DNA Tagging***

Efficient transformation is a keystone of any modern model system and the extremely efficient floral dip method of *Arabidopsis* transformation is a key reason behind the tremendous success of this dicot model. Unfortunately, *Arabidopsis* is unusual in its ease of transformation and the methods used for grass transformation are much more laborious, involve extensive tissue culture and are often inefficient. Rice has set the benchmark for efficient grass

transformation and can be transformed by both *Agrobacterium* and biolistic methods at high efficiencies. A tremendous amount of work by many groups over several years increased rice transformation efficiencies from <1% in the first reports to the >40% efficiencies commonly achieved today (Tyagi and Mohanty 2000). For the other cereals (e.g. wheat and barley), however, transformation remains very inefficient. Fortunately, *Brachypodium* has proven to be very responsive to in vitro culture and current transformation efficiencies are on par with rice.

Embryogenic callus is a preferred target for transformation due to its highly regenerable nature. Thus, the development of a method for the induction of embryogenic callus from *Brachypodium* seeds and the regeneration of fertile plants from embryogenic callus was a major step toward developing *Brachypodium* transformation (Bablak et al. 1995). In this study, the optimal callus-inducing medium contained LS salts, 3% sucrose and  $2.5 \text{ mg l}^{-1}$  2,4-D. Three diploid accessions (B200, B373, B377) were found to produce embryogenic callus along with several other types of callus when mature seeds were incubated on callus-inducing media. Regeneration was observed on several common media indicating that *Brachypodium* had no unusual requirements for regeneration.

Both particle bombardment and *Agrobacterium tumefaciens* have been used to transform *Brachypodium* and each offers unique advantages and disadvantages. Particle bombardment is not dependent upon the biological limitations of *Agrobacterium*. The primary determinant of successful transformation by bombardment is the efficiency of the regeneration of plants from the bombarded explant. Thus, the transformation of embryogenic *Brachypodium* callus by particle bombardment was the next logical step in developing *Brachypodium* transformation. In the first published *Brachypodium* transformation, a polyploid accession (ABR100) was transformed via particle bombardment with an average efficiency of five transformations per g of starting embryogenic callus (Draper et al. 2001). This demonstrated that *Brachypodium* could be transformed at reasonable frequency, but raised the question of whether a diploid accession could be transformed. A more detailed account of biolistic transformation answered that question (Christiansen et al. 2005). In this paper the authors successfully transformed one diploid accession (BDR018) with an average efficiency of 5.3% of bombarded calli producing transgenic plants. They unsuccessfully attempted to transform a second diploid accession (BDR001) indicating that, similar to other plants, genotype has a substantial effect on transformation efficiency. The efficiency of this early transformation method compares favorably with the first reports of biolistic rice transformation that had an average efficiency of 3.75% (Christou et al. 1991). A serious disadvantage of biolistic transformation is the complexity of the resultant transgenic loci. Typically, these loci contain multiple copies of the inserted DNA including truncated pieces of the target DNA interspersed with genomic DNA (Kohli et al. 2003; Svitashv and Somers 2002). These complex biolistic insertions often contain many repeats of inserted DNA and can span several

megabases of host DNA (Svitashev and Somers 2002). Such complex insertions interfere with downstream application that require relatively simple insertions (e.g. cloning flanking DNA or promoter tagging) and may lead to silencing of transgenes in later generations. Attempts to minimize the complexity of biolistic loci by using linear DNA instead of circular plasmid DNA have produced mixed results (Fu et al. 2000; Loc et al. 2002).

*Agrobacterium*-mediated transformation typically results in much simpler insertion patterns than biolistic transformation (for a direct comparison of methods see Dai et al. 2001; Travella et al. 2005). *Agrobacterium*-mediated transformation of both rice and *Arabidopsis* has been shown to produce low copy number transgenics with an average of  $\sim 1.5$  insertions per line (Feldmann 1991; Jeon et al. 2000). However, the host limitations of *Agrobacterium* add to the difficulty of establishing an efficient *Agrobacterium*-mediated transformation system. Fortunately, *Brachypodium* has proven amenable to *Agrobacterium*-mediated transformation and the first report of *Agrobacterium*-mediated transformation was published in 2006 (Vogel et al. 2006a). In this study, 16 polyploid accessions and three diploid accessions were evaluated for transformability. The highest transformation efficiency (14% of the callus pieces co-cultivated with *Agrobacterium* produced transgenic plants) was achieved with the polyploid line Bd17-2. A diploid accession, PI 254867, was transformed at a much lower efficiency, 2.5%.

At the end of 2007, three papers were published online that reported very high efficiency transformation of three different *Brachypodium* lines. Two papers used inbred lines, Bd21-3 (Vogel and Hill 2008) and Bd21 (Vain et al. 2008), that were derived from the same initial USDA accession, PI 254867. Bd21-3 was selected for transformability from accession PI 254867 which presumably represents multiple individuals collected at the same location. The methods described in these two papers share a number of important similarities: media types, *Agrobacterium* strains, use of immature embryos as initial explants and they both subculture the callus several times before transformation so that each dissected embryo gives rise to many transgenic plants. This is important because of the labor involved in dissecting out immature embryos. Differences between the methods lie in the following: the use of desiccating conditions to improve transformation of Bd21-3; the formation of a yellow embryogenic callus in Bd21-3 that allows selection of the proper callus type without the aid of a microscope; the use of very small embryos and copper sulfate to improve the quality of Bd21 callus; the use of visual selection of GFP and sub-culturing callus under a microscope to improve efficiency of Bd21 selection. Average transformation efficiencies achieved (expressed as percentage of calli co-cultivated with *Agrobacterium* that produced fertile transgenic plants) were 37% for Bd21-3 and 17% for Bd21. The third paper reports extremely high average transformation efficiency, 55%, of accession BDR018 (Păcurar et al. 2008). This remarkable achievement was obtained by placing immature embryos on callus inducing media for 17 days and then co-cultivating those embryos with *Agrobacterium*. The calculated efficiency is the percentage

of dissected embryos that form fertile transgenic plants. It is significant that the embryogenic callus is not subcultured and therefore no more than one transgenic plant can arise from each dissected embryo. This increases the labor involved in generating transgenic plants when compared to the methods for Bd21-3 and Bd21 transformation. Although continued improvements in transformation efficiency, including the identification of superior genotypes, will doubtless be made, the near simultaneous publication of three high efficiency *Agrobacterium*-mediated transformation methods signals the maturation of Brachypodium transformation technology.

### 16.3.5 Related Species

The relatively small genus *Brachypodium* is estimated to have diverged from sister tribes *Triticeae* and *Poeae* 35–40 mya, and this clade has been assigned to its own tribe, *Brachypodieae*, within the subfamily *Pooideae*. Although most of the 12–15 described *Brachypodium* species have been collected from Mediterranean, European, and Eurasian locations, representatives of this genus are distributed across the globe (Catalán et al. 1995; Catalán and Olmstead 2000). Species originating in the Mediterranean include *B. distachyon* as well as the *B. retusum* and *B. phoenicoides*. In addition, one species from southern Spain, *B. boissieri*, shows substantial similarity to *B. retusum*. A single European taxon is represented by *B. rupestre*, and three species, *B. sylvaticum*, the closely related species *B. glaucovirens*, and *B. pinnatum*, are from Eurasian locations. Reports describe six additional taxa of diverse origins: *B. arbuscula* (Canary Islands), *B. kawakamii* Hayata (Taiwan), *B. mexicanum* (Mexico to Bolivia), *B. pringlei* (Central and South America), *B. bolusii* (Africa), and *B. flexum* (Africa).

All members of the *Brachypodieae* exhibit a set of common features that include: lateral stem development from the coleoptile, small chromosomes, ribosomal DNA sequence, repetitive DNA families, and shared nuclear RFLPs. However, variation in morphology, life cycle, and cytology is sufficient to clearly distinguish between species (Catalán and Olmstead 2000). Within the group, an annual life cycle is unique to *B. distachyon*. This species is also self-compatible, a trait that is shared with only two perennial species, *B. mexicanum* and *B. sylvaticum* (Khan and Stace 1999). Most of the perennial species contain long-rhizomes, however *B. mexicanum* is distinguished by being non-rhizomatous (Catalán and Olmstead 2000). Polyploidy is common among all taxa, and diploid, tetraploid, hexaploid, and octaploid species have been reported with base chromosome numbers ranging between 5 and 10 (Robertson 1981; Hasterok et al. 2004).

The phylogeny of eight *Brachypodium* species (*B. arbuscula*, *B. distachyon*, *B. mexicanum*, *B. phoenicoides*, *B. pinnatum*, *B. retusum*, *B. rupestre*, and *B. sylvaticum*) has been evaluated using several data sets including RFLP and RAPD data, chloroplast *ndhF* gene sequence, nuclear rDNA sequence, and

rDNA internal transcribed spacer (ITS) sequence (Shi et al. 1993; Hsaio et al. 1994; Catalán et al. 1995; Catalán and Olmstead 2000). Shi et al. identified an EcoRI site present in the rDNA of most perennial species that could be used to distinguish them from *B. distachyon* and *B. mexicanum*, but the selected markers failed to identify sufficient variation to resolve the relationship between the perennial species. An analysis using *ndhF* and ITS sequences along with RAPD data identified *B. distachyon* as the basal lineage of the group followed by the divergence of *B. mexicanum*, *B. arbuscula*, *B. retusum*, *B. rupestre*, *B. phoenicoides*, *B. pinnatum*, and then *B. sylvaticum* (Catalán and Olmstead 2000). Because of the close relationship between species in the genus *Brachypodium* researchers will be able to leverage the resources developed for *B. distachyon* to study the perennial life cycle and self-incompatibility exhibited by most other *Brachypodium* species (Khan and Stace 1999). These traits are common in the wild grasses (e.g. *Miscanthus* and switchgrass) that are being developed into biomass crops.

## 16.4 Genomic Resources

In order for a model system to be widely adopted, a comprehensive infrastructure of genomic resources and methods must be developed. Numerous resources have been or are currently being assembled for *Brachypodium* including: cDNA libraries, BAC libraries, a large EST collection, BAC end sequences, a high-resolution genetic linkage map, a physical map, bioinformatic resources, and most importantly, the complete genome sequence.

### 16.4.1 ESTs

Randomly sequencing the ends of cDNA clones to generate expressed sequence tags (ESTs) is a quick and relatively inexpensive way to learn a great deal about an unknown genome (Adams et al. 1991). Thus, it is no surprise that the first significant sequence resource for *Brachypodium* was the 20,440 ESTs deposited into Genbank in 2005 (Vogel et al. 2006b). These ESTs were derived from five cDNA libraries and represent approximately 6,000 genes. As of December 14, 2007, there were 10 grasses (including *Brachypodium*) with >20,000 ESTs in Genbank. Three of those grasses (rice, wheat, maize) had >1 million ESTs. These sequences are useful for many applications including microarrays, analysis of gene expression, and annotation of genomic sequence. The *Brachypodium* ESTs have been used to refine the phylogeny of *Brachypodium* and to identify candidates for all the genes involved in the biosynthesis of lignin monomers.

Although the initial set of *Brachypodium* ESTs are very useful, many more are required for *Brachypodium* to reach its full potential as a model system. As

part of the genome sequencing project, the U.S. Department of Energy Joint Genome Institute (JGI) is currently sequencing >180,000 additional EST sequences. To maximize the utility of this newly expanded EST collection, cDNA libraries prepared from a diverse set of tissues and treatments are being used (Todd Mockler personal communication).

### ***16.4.2 BAC Library Resources***

Bacterial artificial chromosome (BAC) libraries are useful tools for genomic analyses. Sequence data obtained from BAC libraries permit evaluation of genome content and complexity when full genome sequence is not available and can aid in assembly of genome sequencing data. Furthermore, BAC libraries are useful for comparative genomic analyses of synteny of genes in different species, and this information can be exploited to facilitate positional cloning of genes in related species. Therefore, the development of BAC library resources for *Brachypodium* has the potential to be very useful in studies of temperate cereal crops due to the close relationship between these species.

To date, six BAC libraries have been constructed for diploid *Brachypodium* accessions. The first two libraries contain a total of 9,100 clones with an average insert size of 88 kb and were derived from the genomes of accessions ABR1 (5,968 clones) and ABR5 (3,132) (Hasterok et al. 2006b). These relatively small libraries represent approximately 2 haploid genome equivalents. Two BAC libraries were constructed from inbred line Bd21, the same line that is being sequenced (Huo et al. 2006). One library was generated from HindIII digested genomic DNA and contains 36,864 clones with an average insert size of 100 kb. The other library contains 73,728 clones with an average insert size of 105 kb and was derived from BamHI digested genomic DNA. In combination, these Bd21 BAC libraries represent 29 haploid genome equivalents and provide greater than 99.99% likelihood a particular gene is included within the library. The Bd21 libraries were also used to generate BAC end sequences (BES) from 64,694 clones (average size 583 bp), and the resulting 38.2 Mbp of sequence covers ~11% of the *Brachypodium* genome (Huo et al. 2007). This sequence was used to anchor the BAC clones to the rice genome and indicated that the *Brachypodium* genome contains 45.9% GC content, approximately 18% repetitive DNA (11% with homology to known repetitive sequence and 7.3% unique to *Brachypodium*), and 21.2% coding sequence. Comparison of the BES data to *Brachypodium* and cereal crop EST databases revealed that 40% of the sequence matched ESTs with a greater number of hits within the wheat database than in the maize database. The Arizona Genomics Institute ([www.genome.arizona.edu/](http://www.genome.arizona.edu/)) has constructed a library from the inbred line Bd3-1 that represents 10 genome equivalents within 36,864 clones with an average insert size of 130 kb. Two additional libraries with an average insert size of 130 kb

have been prepared from Bd21 by the Arizona Genomics Institute (M. Bevan personal communication).

An additional library exists for the perennial species *B. sylvaticum* ([www.jicgenomelab.co.uk](http://www.jicgenomelab.co.uk)). This library contains 30,228 clones with an average insert size of 102 kb (6.6 genome equivalents, based on a genome size of 470 Mbp) (Foote et al. 2004). From this library, repetitive DNA content was estimated to be approximately 50% and analyses demonstrated that synteny was maintained between rice, wheat, and *B. sylvaticum* BAC contigs over several regions of chromosome 9. The percentage of repetitive DNA in *B. sylvaticum* is much higher than in *B. distachyon* and largely explains the larger size of the *B. sylvaticum* genome.

### 16.4.3 *Physical and Genetic Maps*

There are currently no published physical or genetic maps for *Brachypodium*. However, much progress in this direction has been made and it is anticipated that both a physical and a genetic map will be published shortly. A physical map has been constructed from two of the Bd21 BAC libraries mentioned above (Huo et al. 2006, 2007). This map contains over 50,000 BAC clones assembled into ~600 contigs (M. Luo personal communication). It is anticipated that the map will be published in early 2009 and become available at: ([phymap.ucdavis.edu:8080/brachypodium/](http://phymap.ucdavis.edu:8080/brachypodium/)). In addition, a second physical map using two different Bd21 libraries has recently been constructed (M. Bevan personal communication). Rapid progress toward a genetic map is also being made. A large community collaboration is using ~200 markers to create the first genetic linkage map, and it is anticipated that this effort will be complete shortly (D. Garvin personal communication). As a measure of the rapid progress in *Brachypodium* research, a National Science Foundation funded project to create a high-density SNP based map is progressing rapidly even before the first generation linkage map is finished. The goal of this project is to map ~1,000 SNP markers. As of January 2008, 1,900 SNPs at 625 loci have been identified and the map is anticipated to be completed by mid 2008 (unpublished). These mapping resources will greatly aid in the final assembly and verification of the complete genome sequence and also will aid positional cloning experiments.

Linking individual BACs contained in physical contigs and ultimately genomic sequences to specific chromosomes can be accomplished through a technique called “BAC landing.” In this technique, entire BACs are fluorescently labeled and used for FISH. In this fashion, BACs were assigned to specific chromosomes, and 32 of 39 BACs hybridized to a single locus underscoring the compact nature of the *Brachypodium* genome (Hasterok et al. 2006b). A more extensive application of the technique will be highly instructive in verifying the whole genome assembly.

### **16.4.4 Whole Genome Sequencing**

A completely sequenced genome is a requirement for a modern model system and underpins a host of tools including efficient map-based cloning, sequence indexed T-DNA populations, gene chips and reverse genetic approaches including TILLING and RNAi. Plans for the development of *Brachypodium* as a model to accelerate the domestication of grasses (e.g. switchgrass and *Miscanthus*) for use as biomass crops were spelled out in a U.S. Department of Energy report on the research needed to establish a domestic biofuel industry (DOE 2006). As a result, the JGI ([www.jgi.doe.gov/](http://www.jgi.doe.gov/)) approved a proposal to sequence the *Brachypodium* genome through their Community Sequencing Program for 2007. The JGI is using a whole genome shotgun sequencing approach based upon Sanger sequencing technology with a final target of 8x genome coverage. The final assembly will incorporate all of the mapping and BAC sequence resources and promises to be of very high quality. A preliminary 4x draft sequence has been released through ([www.brachypodium.org](http://www.brachypodium.org)) and ([www.modelcrop.org](http://www.modelcrop.org)). It is anticipated that the final 8x genome assembly will be completed in 2008.

### **16.4.5 Bioinformatic Resources**

To fully utilize the approaching avalanche of genomic data, it will be necessary to develop the appropriate bioinformatic infrastructure. A *Brachypodium*-specific web portal ([www.brachypodium.org](http://www.brachypodium.org)) that provides links to numerous sources of *Brachypodium* information has been established. This website also houses a newsgroup that links the *Brachypodium* community. As mentioned above, the 4x *Brachypodium* sequence is housed on two databases ([www.brachybase.org](http://www.brachybase.org) and [www.modelcrop.org](http://www.modelcrop.org)). In addition to the 4x sequence, these databases contain or will contain tracks that place genetic markers, BAC clones, ESTs, T-DNA insertion sites, sequences from other species, and other applicable data in the context of the *Brachypodium* genomic sequence. Readers are directed to these sites for the most detailed resources available. Other websites that contain project specific information include the “Brachyomics” website ([www.aber.ac.uk/plantpathol/brachyomics.htm](http://www.aber.ac.uk/plantpathol/brachyomics.htm)) and a website describing projects underway at the USDA-ARS Genomics and Gene Discovery Unit ([brachypodium.pw.usda.gov/](http://brachypodium.pw.usda.gov/)).

## **16.5 Applications of *Brachypodium* as a Model for Grass Research**

The close relationship of *Brachypodium* to wheat holds the promise that the simple genome of *Brachypodium* can be used as a roadmap to navigate the complex wheat genome. The application of *Brachypodium* as such a structural model is dependent upon the conservation of colinearity between wheat and



Brachypodium. Thus, since wheat is more closely related to Brachypodium than to rice, it is anticipated that Brachypodium will serve as a better structural model than rice. However, colinearity will vary from locus to locus, and the utility of Brachypodium will vary depending upon the region examined. Thus, including the rice genome in a three-way comparison with Brachypodium and wheat may be more informative in some situations.

In addition to serving as a structural model for wheat, Brachypodium can serve as a functional model for grasses in general. In this capacity, it is not necessary to have an extremely close evolutionary relationship. It is only necessary to share the traits/properties and genes under study. For example, Brachypodium possess the type 2 cell wall (Carpita 1996) typical of all grasses and thus would be a suitable model to study this facet of grass biology. By contrast, Arabidopsis contains a type 1 cell wall typical of the dicots and therefore would be a poor choice to study the unique aspects of the grass cell wall.

### ***16.5.1 Brachypodium as Structural Model for Wheat and Barley Genomics***

The polyploid nature, large size and highly repetitive nature of the wheat genome present extreme challenges to researchers studying specific genes or genomic regions. A simpler, yet closely related, genome could serve as a roadmap to accelerate research on the complex wheat genome by providing a frame of reference and sequences from which markers in intervals of interest can be developed. Both *B. sylvaticum* and *B. distachyon* have already been used for this purpose. With the availability of the complete Brachypodium genome sequence, Brachypodium will be increasingly employed in this capacity. The use of a simple genome as a model for more complex genomes is dependent on a high degree of colinearity. As the phylogenetic distance increases between two species, assessment of colinearity becomes increasingly difficult, and within 1 Mb of sequence, the comparison of gene order between rice and wheat becomes less reliable (Foote et al. 2004). In this regard, Brachypodium has an advantage over rice because rice and wheat diverged approximately 50 million years ago (Gaut 2002) whereas wheat and Brachypodium diverged about 35 million years ago (Bossolini et al. 2007). As predicted based on evolutionary distance, a comparison of a 371 kb genomic sequence from *B. sylvaticum* to the orthologous rice sequence and to the segregation of wheat genes in this interval revealed that the gene content and gene order of the wheat region was closer to *B. sylvaticum* than to rice (Bossolini et al. 2007). Specifically, of the 15 wheat genes found in this interval, 10 had orthologs in the *B. sylvaticum* sequence and nine had orthologs in the rice interval. The order of the shared genes was the same in *B. sylvaticum* and wheat whereas there was a large inversion in rice. This suggests that Brachypodium will be a better model of the wheat genome than is rice.

Three examples of using *B. sylvaticum* sequence to map or clone wheat or barley genes have been published. *B. sylvaticum* BACs and rice genomic sequence were used in combination to clone the *Ph1* locus from wheat (Griffiths et al. 2006). In this study the authors used markers in a region of rice to define the breakpoints of wheat deletion lines that defined a region containing the *Ph1* locus. However, they were unable to map half of the rice markers in the wheat genome due to lack of sequence conservation. To map the remaining markers, they used the rice sequences to obtain the orthologous sequences from a *B. sylvaticum* library. They were then able to map all of these *B. sylvaticum* markers in wheat to narrow the interval containing the *Ph1* locus. That *B. sylvaticum* sequences could be mapped directly onto the wheat genome where the orthologous rice sequences failed underscores the close relationship of *B. sylvaticum* to wheat. The *B. sylvaticum* genome was also used to narrow the interval containing the *Lr34/Yr18* disease resistance locus in wheat (Spielmeyer et al. 2007). In this case, one of the *B. sylvaticum* BACs sequenced by Bosollini et al. 2007 (discussed above) was found to overlap the *Lr34/Yr18* region in *B. sylvaticum* and the authors were able to use this sequence to narrow the interval containing *Lr34/Yr18*. *B. sylvaticum* BACs were also used in the cloning of the *Ppd-H1* gene from barley though in this case the *B. sylvaticum* sequence primarily confirmed results obtained with rice sequence (Turner et al. 2005). From these examples, it is apparent that Brachypodium will be very useful as a roadmap for wheat. The extent of this utility will become apparent when a larger comparison of the synteny between wheat and Brachypodium is made.

### ***16.5.2 Brachypodium as a Functional Model***

A systematic approach to analyses of gene function is the goal that drives the development of functional model systems. The close relationship to temperate cereals and forage grasses paired with available and emerging genomic tools make Brachypodium an attractive model for these purposes. Examples of the merit of Brachypodium as a functional model are beginning to emerge. A recent study of the floral repressor *Terminal Flower 1* demonstrated that the orthologous genes from Arabidopsis (*TFL1*) and *Lolium perenne* (*LpTFL1*) both function to delay flowering in Brachypodium (Olsen et al. 2006). The grass gene, *LpTFL1*, mediated on average a 14 day longer delay in flowering than the gene from the dicot Arabidopsis suggesting that Brachypodium is a more appropriate system in which to pose questions regarding regulatory pathways of monocots. Furthermore, the inbreeding life cycle and short generation time of Brachypodium significantly accelerated the analysis of transgenic plants. Within one year of transformation, T<sub>1</sub> transgenic plants were ready for analysis, a feat that could not be accomplished with *Lolium perenne* due to low transformation efficiency, the requirement for extensive vernalization, and

self-incompatibility. Another application of *Brachypodium* to study development was the isolation of *Brachypodium* orthologs of *WUSCHEL* homeobox (WOX) gene family members (Nardmann et al. 2007). The authors had previously identified the members of the WOX gene family from rice and maize and thus with the *Brachypodium* sequences they were able to construct a phylogenetic tree containing members from the three major radiations of the family Poales. This comparison revealed gene duplications common to all grasses that were not present in dicots.

Initial surveys of *Brachypodium* responses to pathogen challenge indicate that this species is also well suited to the investigation of host-pathogen interactions in grasses. In the most comprehensive study to date, twenty-one diploid *Brachypodium* accessions were screened for susceptibility to three strains of the rice blast pathogen *Magnaporthe grisea* (Routledge et al. 2004). These experiments revealed responses ranging from a highly localized hypersensitive response to full susceptibility with cytology similar to that observed in rice. One accession, ABR5, presented full resistance to the *M. grisea* strain Guy-11. Based on segregation of resistance in a cross with the susceptible accession ABR1, the resistance appears to be mediated by a single locus. Hallmarks typically used to measure disease resistance in *Arabidopsis*, such as PR protein expression, callose deposition, and the appearance of autofluorescence and granular cytoplasm in infected regions, were also evident in the *Brachypodium* response to *M. grisea*. Virulence tests for several rust pathogens (*Puccinia striiformis hordei*, *Puccinia striiformis triticae*, *Puccinia recondita hordei*, *Puccinia recondita triticae*, *Puccinia coronata*) revealed substantial variation in resistance response between different *Brachypodium* accessions (Draper et al. 2001), and susceptibility to head blight caused by *F. graminearum* (Garvin 2007) and root rot resulting from infection by *Pythium* species also have been observed (Fig. 16.2).

In contrast to interactions between *Brachypodium* and *M. grisea*, the powdery mildew pathogen *Blumeria graminis* failed to elicit any observable disease symptoms on *Brachypodium* accessions (Draper et al. 2001). This suggests a non-host rather than race-specific resistance to this pathogen. Robust, non-host resistance it is thought to be mediated by multiple genes that together control a wide range of functions including the production of toxic agents or the absence of metabolites or signaling molecules required by the pathogen. Therefore, inactivation of multiple components would be required to render a plant susceptible to infection. Study of non-host resistance to *B. graminis* is being pursued in *Arabidopsis* as an alternative to the introduction of short-lived, race-specific disease resistance genes to crop species (Collins et al. 2003). These results suggest that *Brachypodium* can serve as a model to initiate similar studies in grasses.

Despite the agronomic importance of cereal viruses, information regarding interactions of these pathogens with *Brachypodium* is conspicuously lacking. One undefined, spherical virus has been described in *B. sylvaticum* (Edwards et al. 1985), and initial studies in *Brachypodium* have identified accessions that

are either susceptible or resistant to challenges with *Barley stripe mosaic hordeivirus* (A. Jackson, personal communication).

In addition to serving as a model for plant-pathogen interactions, *Brachypodium* can serve as a model for the interaction between plants and mycorrhizal fungi. Since mycorrhizal interactions can greatly improve plant productivity and decrease costs associated with the application of fertilizer, this is a very important area for plants in general and for the use of grasses as biomass crops in particular. Despite its importance, this area has been understudied in part because of the difficulties of working with roots and in part because *Arabidopsis* does not form mycorrhizal associations. Since *Brachypodium* forms robust mycorrhizal interactions (M. Harrison personal communication) it will no doubt serve as a powerful model these symbiotic relationships.

*Brachypodium* is also being used as a model for responses to wounding and insect attack. A proteinase inhibitor, *Bdpin1*, was identified from a cDNA library prepared from wounded *Brachypodium* leaves (Mur et al. 2004). Local and systemic expression of *Bdpin1* in response to wounding demonstrated the presence of long-distance signaling in grasses. *Bdpin1* was also induced in response to methyl jasmonate and *M. grisea*, but not in response to the salicylic acid analog benzothiadiazole. Taken together, these results indicate that *Brachypodium* can serve as a model for grass development and the interactions of grasses with pathogens, insects and symbionts.

## 16.6 Future Prospects and Directions

The future of *Brachypodium* as a model system is very bright. The development of a base suite of tools (e.g. complete genome sequence, inbred lines, BAC and cDNA libraries, facile transformation methods, physical and genetic maps) that allow researchers to rapidly utilize *Brachypodium* to study their questions of interest has been extraordinarily rapid. This is due to a favorable alignment of several factors including: an increased interest in grasses as feedstocks for bioenergy; the need for a tractable grass model as a surrogate for the large, difficult to handle grasses proposed as biomass crops; the recognition that the *Brachypodium* genome could be used as a comparative tool to probe the extremely large and highly repetitive wheat genome; the availability of low cost high-throughput sequencing capacity through the Community Sequencing Program; and the willingness of a handful of researchers to invest in developing freely-available community resources. The momentum of these initial developments has carried over into the development of next generation resources that are either currently in early stages of development (e.g. insertional mutants, TILLING resources, and elucidation of small RNAs and microRNAs) or being planned and proposed to funding agencies (e.g. microarrays, resequencing of additional lines). This rapid progress is likely to continue for the foreseeable future as the number of researchers using *Brachypodium* continues to grow exponentially.

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