Handbook of Bioenergy Crop Plants

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Brachypodium

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23 Brachypodium

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23.1 THE NEED FOR A MODEL GRASS

In a 2005 feasibility study, the U.S. Departments of Energy and Agriculture predicted that within four decades, the United States could sustainably produce over 1 billion dry tons of plant biomass annually for the generation of energy and other products (DOE and USDA 2005). Crop and other residues from grasses, including 256 million tons of corn stover and 52 million tons of wheat straw, represent approximately 348 million tons of this total. An additional 368 million tons is expected to come from the cultivation of perennial grasses and trees as dedicated bioenergy crops (DOE and USDA 2005). Switchgrass (*Panicum virgatum*) and Miscanthus (*Miscanthus × giganteus*) have emerged as particularly attractive potential energy crops (DOE 2007; Dohleman and Long 2009). Given that the grasses being considered as energy crops are essentially undomesticated wild selections, there is considerable potential for improving them. In addition, with the exception of forest trees, many of the traits desirable in an energy crop (e.g., thicker stems, more cell walls) have not been selected for in traditional crops, in which, for the most part, breeding has focused on reproductive organs or digestible leaves, tubers, or stems. Unfortunately, breeding the grasses proposed as energy crops is complicated by their reproductive strategy (self-incompatibility or sterility) which prevents the development of inbred lines, selfing, etc. Basic research on the biology of grasses could be used to design rational approaches to breeding superior energy crops and to accelerate the domestication of these new crops. The most rapid way to gain this basic knowledge is through the use of an appropriate model system.

*Arabidopsis thaliana* serves as an extremely powerful generalized plant model; however, it is not suitable to study many aspects of grass biology because of the biological differences that have arisen between dicots and monocots in the 150 million years since they last shared a common ancestor. One example that is particularly relevant to the need for a model for bioenergy crops is the dramatic difference between grass and dicot primary 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; Vogel 2008). 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 argues strongly for the development of a truly tractable grass model system. Rice, with its sequenced genome and large research community, at first would seem to fill this need. Upon closer examination, the demanding growing conditions, large size, and long generation time of rice greatly increase the difficulty and expense of conducting high-throughput functional genomic experiments. Furthermore, the semiaquatic and tropical nature of rice limits the applicability of rice as a model for temperate grasses, especially in areas such as freezing tolerance and vernalization. *Brachypodium distachyon* (hereafter referred to as Brachypodium) is well suited to meet the need for an experimentally tractable model for the grasses. In this chapter, we provide a general introduction to Brachypodium, details about using Brachypodium as a model, a summary of genomic resources available for Brachypodium, and examples of how Brachypodium can be applied to the development of grasses as energy crops.

23.2 INTRODUCTION TO *B. DISTACHYON*

The utility of Brachypodium as a model system for the study of grasses was discussed in a 2001 paper that indicated that Brachypodium possesses 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 enable high-throughput functional genomic experiments. Large numbers of plants (1000 plants/m²) can easily be grown in growth chambers or greenhouses, allowing studies to be conducted under controlled environmental conditions. For comparison, the same space accommodates only 50 wheat plants, 36 rice plants, 13 sorghum plants, 6 maize plants, 6 switchgrass plants, or 2 Miscanthus plants (Rayburn et al. 2009) (Table 23.1 and Figure 23.1, a–c). As a group, the grasses are notorious for very
### Table 23.1
Comparison of Model and Crop Plants

<table>
<thead>
<tr>
<th>Common name</th>
<th>Brachypodium distachyon</th>
<th>Arabidopsis thaliana</th>
<th>Oryza sativa</th>
<th>Triticum aestivum</th>
<th>Zea mays</th>
<th>Panicum virgatum</th>
<th>Sorghum bicolor</th>
<th>Miscanthus × giganteus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height (cm)</td>
<td>15–20</td>
<td>15–20</td>
<td>100</td>
<td>50</td>
<td>155–215</td>
<td>200</td>
<td>170–320</td>
<td>400</td>
</tr>
<tr>
<td>Density (plants/m²)</td>
<td>1000</td>
<td>2000</td>
<td>36</td>
<td>50</td>
<td>6</td>
<td>6</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Growth requirements</td>
<td>Simple</td>
<td>Simple</td>
<td>Demanding</td>
<td>Simple</td>
<td>Simple</td>
<td>Simple</td>
<td>Simple</td>
<td>Simple</td>
</tr>
<tr>
<td>Generation time (weeks)</td>
<td>8–12</td>
<td>8–12</td>
<td>30</td>
<td>12</td>
<td>14–20</td>
<td>26</td>
<td>17</td>
<td>N/A</td>
</tr>
<tr>
<td>Genome size (Mbp)</td>
<td>272(^a)</td>
<td>119(^a)</td>
<td>382(^a)</td>
<td>16,000</td>
<td>2500</td>
<td>2400</td>
<td>758(^a)</td>
<td>6800</td>
</tr>
<tr>
<td>Ploidy</td>
<td>2x</td>
<td>2x</td>
<td>2x</td>
<td>6x</td>
<td>2x</td>
<td>4x–8x</td>
<td>2x</td>
<td>3x</td>
</tr>
<tr>
<td>N(_x) chromosome number</td>
<td>5</td>
<td>5</td>
<td>12</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>Cell wall type</td>
<td>Type II</td>
<td>Type I</td>
<td>Type II</td>
<td>Type II</td>
<td>Type II</td>
<td>Type II</td>
<td>Type II</td>
<td>Type II</td>
</tr>
</tbody>
</table>

\(^a\) Assembled genome sizes.

N/A, not applicable.
FIGURE 23.1 (See color insert) Adult plant comparisons and transformation. Flowering plants of maize (a), switchgrass (b), and Brachypodium (c) compared with the same 32-cm ruler, indicated with white arrows. The small size of Brachypodium is an advantage for a model system. (d–f) Comparison of B. distachyon and B. sylvaticum. (e) The annual species B. distachyon (left) next to its perennial relative B. sylvaticum (right). Bar is 15 cm. Inflorescences of B. distachyon (d) and B. sylvaticum (f). Note the exerted anthers of the outcrossing B. sylvaticum and the enclosed anthers of the inbreeding B. distachyon. (g) Embryogenic Brachypodium callus. The yellow structured regions are competent to regenerate plants and are used for transformation. Bar is 0.5 cm. (h) Plants regenerating from transgenic callus. After transformation and selection, a mixture of dying (brown) and healthy (yellow) callus can be seen. When placed in the light, healthy callus will turn green and regenerate plantlets. Plate is 10 cm in diameter. (i) Close-up of region in regeneration plate designated by the arrow in panel h.
large genomes. Fortunately, the now-sequenced 272-Mbp diploid Brachypodium genome is one of the smallest of any grass (International Brachypodium Initiative 2010). Brachypodium is self-fertile and does not typically outcross (Vogel et al. 2009). This feature is useful for breeding homozygous lines for many applications that require the maintenance of large numbers of independent genotypes (i.e., mapping experiments, mutant analysis, and studies of natural diversity). Furthermore, within the genus *Brachypodium*, there are species that may be useful to study polyploidy and perenniality.

23.2.1 Phylogenetic and Syntenic Relationships of Brachypodium to Other Grasses

The phylogenetic relationship between Brachypodium and the other grasses has been evaluated a number of times with increasing amounts of data. Reports based on internal transcribed spacer (ITS) and 5.8S ribosomal DNA (rDNA) sequence, genomic restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers, and ITS sequence plus the chloroplast *ndfH* gene all placed Brachypodium between rice and a clade containing temperate grains such as wheat, barley, and *Secale* (Hsiao et al. 1994). Additional examinations of a much broader spectrum of grasses used ITS and the *ndfH* sequence, as well as morphological data 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 a “sister” to this economically important group of grasses. However, phylogenies based on single genes or small sets of genes can produce inconsistent phylogenetic trees (Rokas et al. 2003), and this phenomenon has been observed with rice (Kellogg 1998). Therefore, it was important to examine the phylogenetic relationships of Brachypodium using larger data sets. Analysis of a data set comprising 11 kb of sequence from 20 highly expressed genes verified the relationship between Brachypodium and the small grains (Vogel et al. 2006a). An even larger data set that is based on 335 bacterial artificial chromosome (BAC) end sequences provides further evidence to confirm the placement of Brachypodium within the grass family tree (Huo et al. 2007).

Several genomic regions of *B. distachyon* and *B. sylvaticum* have been compared to rice and wheat, and these have shown general colinearity. One comparison of a 371-kb genomic sequence from *B. sylvaticum* to the orthologous regions of rice and wheat showed perfect macrocolinearity between the three genomes. The order of the shared genes was the same in *B. sylvaticum* and wheat, whereas there was an approximately 220-kb inversion in rice, demonstrating variation in microcolinearity (Bossolini et al. 2007). Markers from a 140-kb region of rice also were compared with *B. sylvaticum* and wheat sequences in efforts to map the *Ph1* locus that controls the pairing of homologous chromosomes in wheat. In this case, the colinearity of *B. sylvaticum* and rice sequences permitted the localization of *Ph1* to a 2.5-Mb interstitial region of wheat chromosome 5B (Griffiths et al. 2006). Furthermore, only 17% of the genes from 55,221 paired *B. distachyon* BAC end sequences were not colinear with the orthologous regions in rice. A Brachypodium physical map covered 88% of the rice sequence and showed conservation of synteny across these genomes (Gu et al. 2009).

23.2.2 Related Brachypodium Species

The genus *Brachypodium* contains a relatively small number of grasses and is estimated to have diverged from *Triticeae* and *Poeae* 35–40 million years ago (Bossolini et al. 2007). The genus has been assigned to its own tribe, *Brachypodieae*, within the subfamily Pooideae (Catalán et al. 1995; Catalán and Olmstead 2000). Most of the 12–15 described *Brachypodium* species have been collected from Mediterranean and Eurasian locations, but representatives of this genus have been identified worldwide (Catalán et al. 1995; Catalán and Olmstead 2000). Species originating in the Mediterranean include *B. distachyon* (Figure 23.1 c–e), *B. retusum*, and *B. phoenicoides*. *B. sylvaticum* (Figure 23.1, e and f), *B. glaucovirens*, and *B. pinnatum* are from Eurasian locations, and the European representative
is B. rupestris. Seven additional taxa are reported from diverse origins: B. arbuscula (Canary Islands), B. boissieri (southern Spain), B. kawakami (Taiwan), B. mexicanum (Mexico to Bolivia), B. pringlei (Central and South America), B. bolusii (Africa), and B. flexum (Africa).

All members of the genus exhibit a set of common features that include lateral stem development from the coleoptile, small chromosomes, rDNA sequence, repetitive DNA families, and shared nuclear RFLPs. However, variation in morphology, life-cycle, and cytology is sufficient to clearly distinguish the species (Catalán and Olmstead 2000). B. distachyon is the only member to have an annual life-cycle, and it is also self-compatible, a trait that is shared with only two perennial species—B. mexicanum and B. sylvaticum (Khan and Stace 1999). With the exception of B. mexicanum, the perennial species are rhizomatous (Catalán and Olmstead 2000).

The phylogenetic relationships between eight Brachypodium species (B. arbuscula, B. distachyon, B. mexicanum, B. phoenicoides, B. pinnatum, B. retusum, B. rupestris, and B. sylvaticum) have been evaluated using multiple data sets: RFLP and RAPD markers, a chloroplast ndhF gene sequence, a nuclear rDNA sequence, and rDNA ITS sequence (Shi et al. 1993; Hsaio et al. 1994; Catalán et al. 1995; Catalán and Olmstead 2000). An EcoRI site was identified in the rDNA of most perennial species that could be used to distinguish them from B. distachyon and B. mexicanum, but this approach failed to identify sufficient variation to resolve the relationship between the perennial species. By using RAPD data along with ndhF and ITS sequences, B. distachyon was identified as the basal lineage of the group, followed by the divergence of B. mexicanum, B. arbuscula, B. retusum, B. rupestris, B. phoenicoides, B. pinnatum, and then B. sylvaticum (Catalán and Olmstead 2000).

Polyploidy is common among all taxa, and diploid, tetraploid, hexaploid, and octoploid species have been reported with base chromosome numbers ranging from 5 to 10 (Robertson 1981). In a recent report (Wolny and Hasterok 2009), cytogenetic analyses were performed on six species and two subspecies of Brachypodium. The researchers found that fluorescence in situ hybridization (FISH) could help identify the small chromosomes found in Brachypodium species that are otherwise difficult to distinguish. Evolutionary relationships between allopolyploid species were also investigated using genomic in situ hybridization (GISH) to assign chromosomes to putative ancestral genomes. Wolny and Hasterok (2009) concluded that B. pinnatum (2n=28) is an interspecific hybrid between B. distachyon and B. pinnatum (2n = 18) and suggested that B. distachyon is one of the putative ancestral species for both of the allopolyploids B. phoenicoides and B. retusum. Insight into the organization, phylogeny, and evolution of mechanisms that determine variation in chromosome number will become possible with the development of additional tools such as arm- and region-specific probes for cytogenetic analyses.

Perenniability and self-incompatibility are traits that are present in the wild grasses being developed into bioenergy crops (e.g., Miscanthus and switchgrass) and can also be found in a number of species within the genus Brachypodium (Khan and Stace 1999; DOE and USDA 2005). As a result of the close relationship between different Brachypodium species, researchers will be able to leverage the resources developed for B. distachyon to study these traits in other Brachypodium species.

### 23.3 BRACHYPodium AS AN EXPERIMENTAL SYSTEM

Brachypodium displays many traits that make it a tractable and powerful system for research targeted at improving grasses for use as food, feed, and fuel. A large collection of diverse accessions and described inbred lines, simple growth requirements, efficient transformation, and a compact genome make this small grass an attractive choice for an experimental system to understand basic questions in grass biology.

#### 23.3.1 GErMLASM AND NATURAL DIVERSITY

In contrast to the domesticated cereals which have been subjected to human selection for millennia, Brachypodium is a wild grass. Brachypodium germplasm, for which there are a number of collections,
Brachypodium thus provides an excellent resource for investigating natural diversity. The oldest collection consists of approximately 30 population samples dating back to the 1940s. These accessions are available from the U.S. Department of Agriculture (USDA) National Plant Germplasm System (NPGS) (www.ars-grin.gov/npgs/), and relevant passport data can be accessed at www.brachypodium.org. Twenty-seven of the NPGS accessions, 5 diploid and 22 polyploid, were used to generate inbred lines (designated with the prefix “Bd”) that are freely available to the research community (Vogel et al. 2006b). The diploid inbred lines include two lines [Bd21, the line used for genome sequencing, and Bd21-3, which was selected for efficient transformation (Vogel and Hill 2008)] that were derived from the same NPGS accession, PI 254867. These lines are genetically distinct and, thus, presumably were derived from different individuals collected at the same location (Vogel et al. 2009). Another collection of diploid and polyploid ecotypes (designated “ABR”) is maintained at the University of Wales, Aberystwyth. Some of the ABR ecotypes are unique, whereas others overlap with material in the NPGS collection. Synonymous ABR and NPGS designations are listed at www.brachypodium.org/stocks. A material transfer agreement governs the use of all ABR ecotypes.

Recently, 188 diploid inbred lines and a smaller number of polyploid inbred lines were generated from seeds collected at 53 sites across Turkey; these lines are being freely distributed to the research community (Filiz et al. 2009; Vogel et al. 2009). Inbred lines from seeds collected by M. Tuna were designated with a prefix corresponding to the first three letters of the collection location (e.g., “Tek” for the nearby town of Tekirdag) (Vogel et al. 2009). Lines from seeds collected by H. Budak were grouped based on phenotypic similarity and labeled with the prefix “BdTR” followed by the group number and a letter to designate the specific line (Filiz et al. 2009). To survey the genetic diversity of these newly generated lines, 43 simple sequence repeat (SSR) markers were used to genotype the lines, together with the six previously generated diploid inbred lines (Bd1-1, Bd2-3, Bd3-1, Bd18-1, Bd21, and Bd21-3) (Vogel et al. 2009). The SSR marker profiles were used to create an unrooted phylogenetic tree (Figure 23.2) (Vogel et al. 2009). Interestingly, lines that had been assigned to each BdTR group on the basis of phenotypic similarities clustered together in genetically related groups on the tree despite originating from many different locations. Conversely, lines originating from one location were often genetically distinct. Taken together, these data suggest that there is a significant amount of long-distance seed dispersal. The phylogenetic tree strongly supported a clade containing Bd1-1, the BdTR7 and BdTR8 groups, and the Tek accessions. These lines also shared similar phenotypes, including long vernalization requirement and small, nearly hairless seeds (Figure 23.2) (Vogel et al. 2009).

Diploid Brachypodium accessions have obvious phenotypic differences indicating that they can be exploited to study a number of traits relevant to biomass crop development. The diversity in the flowering times and vernalization requirements of Brachypodium lines is particularly striking. After 2–4 weeks of vernalization, the diploid inbred lines Bd2-3, Bd3-1, Bd21, and Bd21-3 flower relatively rapidly, within 2–3 weeks, under greenhouse conditions, whereas the Bd18-1 and Bd1-1 lines flower much later, even after longer vernalization (Vogel et al. 2006b, 2009). For some Bd lines (Bd2-3, Bd3-1, Bd21, and Bd21-3) extending the day length to 20 h eliminates the need for vernalization (Vogel et al. 2006b, 2009; Vogel and Hill 2008). Very long days do not trigger rapid flowering in any of the new Turkish inbred lines (Vogel et al. 2009). The Tek inbred lines, originating from northern Turkey, are especially late flowering and require 8–16 weeks of vernalization (Vogel et al. 2009). The molecular mechanisms underlying flowering-time regulation in Brachypodium are currently unknown. A report that expression of the floral repressor Terminal Flower 1 from perennial ryegrass, Lolium perenne, delays flowering in Brachypodium provides a starting point for future molecular-genetic studies (Olsen et al. 2006). Additionally, a recent analysis of inbred lines with diverse flowering times suggests that the putative Brachypodium VERNALIZATION2 and VERNALIZATION3 genes are involved in controlling flowering time (Schwartz et al. 2010). Other phenotypic differences between lines include the presence or absence of hairs and the number and angle of inflorescence branches (Opanowicz et al. 2008; Filiz et al. 2009; Vogel et al. 2009). Accessions also vary in the degree to which the seed disarticulates from the inflorescence, an agriculturally important trait known as
FIGURE 23.2 Neighbor-joining consensus tree for 187 Brachypodium inbred lines on the basis of 43 SSR markers. The unrooted tree was constructed from 100 shared-SSR-allele bootstrap trees. For major branches, bootstrap values >20 are shown. Note the considerable genetic diversity in this collection. (Adapted from Vogel, J. et al., *BMC Plant Biol*, 9, 88, 2009.)
Brachypodium also exhibits intraspecific diversity in chromosome number. Most diploid Brachypodium lines have a base chromosome number of 5 \((1n = 5)\) (Draper et al. 2001; Vogel et al. 2006b; Filiz et al. 2009). However, accessions with chromosome numbers of \(1n = 10\) and \(1n = 15\) have also been described (Draper et al. 2001; Hasterok et al. 2004). Studies utilizing FISH and GISH techniques indicate that the \(1n = 10\) and \(1n = 15\) cytotypes are not merely autopolyploids derived from the \(1n = 5\) cytotype (Hasterok et al. 2004). Chromosomes of the \(1n = 10\) accession ABR114 are smaller than those of the \(1n = 5\) accession ABR1, and the FISH-visualized pattern of rDNA loci in ABR114 is inconsistent with ABR114 being an autotetraploid arising from ABR1 (Hasterok et al. 2004). Thus, ABR114 seems to be a diploid with a base chromosome number of 10. Similar cytogenetic analyses have led to the idea that the \(1n = 15\) accession ABR113 is an allotetraploid that arose from the hybridization of ABR1- and ABR114-like parents, with base chromosome numbers of 5 and 10, respectively (Hasterok et al., 2004, 2006). Thus, the different Brachypodium cytotypes should probably be considered different species rather than a simple polyploid series. When examined carefully, the polyploids characterized to date can be easily distinguished from the \(1n = 5\) diploid accessions. For example, among the Turkish lines analyzed, one group of polyploids was distinguished by large seeds and thick, hairy stems and a second group of polyploids was characterized by a deep, longitudinal crease in the seed (Vogel et al. 2009). In both groups, anthers exerted more frequently than in the diploid lines. It should be noted that the \(1n = 5\) diploid is the cytotype being used for genome sequencing and resource development and that the \(1n = 10\) form is known from only one collection to date (Garvin et al. 2008).

23.3.2 GROWTH REQUIREMENTS

The simple requirements for growing Brachypodium make it easy to culture under laboratory conditions. Brachypodium can be grown in growth chambers or greenhouses used for *Arabidopsis*, wheat, barley, switchgrass, or other plants. 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 µEm⁻²s⁻¹. Our standard greenhouse conditions are no shading, 24°C in the day and 18°C at night, and supplemental lighting to extend day length to 16 h. Although Brachypodium grows well in a number of different soil types, we have observed that it is highly susceptible to *Pythium* root rot. Plants that are watered excessively or left in standing water often develop disease symptoms under our conditions. We also have observed disease symptoms that correlated with the use of one brand of commercial potting mix and recommend that Brachypodium growers test a few soil formulations before selecting one to grow large numbers of plants (Vogel and Bragg 2009).

Vernalization has been shown to induce flowering in all diploid accessions studied to date, but, as noted above, the time required to induce flowering varies greatly between accessions (Vogel et al. 2006b, 2009; Vogel and Hill 2008). Providing the appropriate conditions to induce flowering is critical to preventing excessive vegetative growth. 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 (inbred lines Bd21 and Bd21-3 require 2–3 weeks of vernalization to reliably induce flowering unless grown under very long day lengths). After approximately 3 weeks in the cold, seeds begin to germinate. Thus, for vernalization times 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 more seed from individual plants is desired. Inbred lines Bd21 and Bd21-3 are particularly responsive to growth under very long day conditions (20 h light:4 h dark) 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 inbreeding nature of Brachypodium simplifies the maintenance of independent lines under laboratory conditions. The anthers of diploid accessions rarely exert, suggesting a low rate of outcrossing. This was confirmed by measuring pollen flow from transgenic to nontransgenic plants under growth chamber and greenhouse conditions. In a population of more than 2000 progeny, no outcrossing was observed (Vogel et al. 2009). The inbreeding nature of Brachypodium in the wild was confirmed by analyzing SSR profiles of 62 wild individuals. These individuals were overwhelmingly homozygous, despite the presence of multiple SSR alleles in the population, indicating that Brachypodium primarily self-pollinates in the wild (Vogel et al. 2009).

23.3.3 Transformation

The utility of a modern model plant system depends greatly on the development of efficient methods to introduce foreign DNA into its genome. The dicot model *Arabidopsis* benefits from an extremely facile transformation method in which flowers are simply dipped into a solution of *Agrobacterium tumefaciens* for several seconds (Clough and Bent 1998). As a result, the *Arabidopsis* research community has access to invaluable tools such as stable knockout lines for most genes in the genome (Pan et al. 2003). In contrast, grass transformation is a more challenging and labor-intensive endeavor. Routine transformation of grasses requires extensive tissue culture manipulations, and transformation of almost all grasses is very inefficient. Supporting its utility as a model system, Brachypodium has proven to be very responsive to in vitro culture, and current transformation efficiencies are on par with rice, the present gold standard for grass transformation. A major step toward achieving Brachypodium transformation was the development of a method for the induction of embryogenic callus (Figure 23.1g) from Brachypodium seeds and the regeneration of fertile plants (Figure 23.1, h and i) from this callus. In 1995, Bablak et al. established the optimal callus-inducing medium to contain LS salts, 3% sucrose, and 2.5 mg L⁻¹ 2,4-Dichlorophenoxyacetic acid (Bablak et al., 1995). Mature seeds from three diploid accessions (B200, B373, and B377) were incubated on callus-inducing media. All were found to produce embryogenic callus, along with several other types of callus, and regeneration was observed on several common media, indicating that Brachypodium had no unusual requirements for regeneration.

Particle bombardment and *A. tumefaciens*-mediated transformation are the two methods most commonly used for plant transformation, and both have been used to successfully transform Brachypodium. Each technique offers unique advantages and disadvantages.

23.3.3.1 Biolistic Transformation

The regeneration of plants from bombarded explants represents the primary determinant of successful biolistic transformation. The first published Brachypodium transformation involved particle bombardment of a polyploid Brachypodium accession (ABR100). In these experiments, the average efficiency was five transformants per gram of starting embryogenic callus (Draper et al. 2001). A subsequent, more detailed, account of biolistic transformation answered the question of whether a diploid accession could be transformed (Christiansen et al. 2005). In this study, the authors successfully transformed the diploid accession BDR018 with an average efficiency of 5.3% of bombarded calluses producing transgenic plants. The authors’ failed attempts to transform a second diploid accession (BDR001) demonstrate that, as for other plants, genotype plays a critical role in determining transformation efficiency. These initial Brachypodium studies compare favorably with early reports of biolistic rice transformation that showed an average efficiency of 3.75% (Christou et al. 1991). However, biolistic transformation commonly results in complex transgenic loci. Typically, these loci contain multiple copies of the inserted DNA, including truncated pieces of the target DNA interspersed with genomic DNA (Svitashev and Somers 2002; Kohli et al. 2003). These biolistic insertions often contain many repeats of inserted DNA and can span several megabases of host DNA (Svitashev and Somers 2002). The complexity of these insertions represents a major drawback of biolistic transformation because they can interfere with downstream applications 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).

### 23.3.3.2 Agrobacterium-Mediated Transformation

Compared with biolistic transformation, *Agrobacterium*-mediated transformation has been shown to yield much simpler and lower copy number insertion patterns in rice and *Arabidopsis* (for a direct comparison of methods see Dai et al. 2001 and Travella et al. 2005). Furthermore, transgenic plants contain an average of approximately 1.5 insertions per line, averting the challenges to downstream analyses encountered with the complex loci of the biolistic lines (Feldmann 1991; Jeon et al. 2000). Instead, the difficulties of establishing an efficient *Agrobacterium*-mediated transformation system reside in the host limitations of *Agrobacterium*. Fortunately, Brachypodium has proven amenable to *Agrobacterium*-mediated transformation. In the first report of *Agrobacterium*-mediated transformation, 16 polyploid accessions and 3 diploid accessions were evaluated for transformability (Vogel et al. 2006b). The highest transformation efficiency (14% of the callus pieces cocultivated 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%).

Subsequent studies have made considerable progress at improving the efficiency of the *Agrobacterium*-mediated transformation of Brachypodium. In 2007, three studies reported very high transformation efficiencies for three different Brachypodium lines. The first two papers used the inbred lines Bd21-3 (Vogel and Hill 2008) and Bd21 (Vain et al. 2008), which were separately derived from USDA accession PI 254867 as previously described in Section 23.3.1. The methods described in these two papers share a number of important similarities, including media types, *Agrobacterium* strains, and use of immature embryos as initial explants. Dissecting out immature embryos is the most labor-intensive step in these processes, and both methods take advantage of multiple subculture steps to amplify the callus before transformation so that each dissected embryo gives rise to many transgenic plants. The 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 appropriate callus type without the aid of a microscope, the use of very small embryos and copper sulfate to improve the quality of the Bd21 callus, and visual selection of green fluorescent protein (GFP) and the subculturing callus under a microscope to improve the efficiency of Bd21 selection. In these studies, transformation efficiency was calculated as the percentage of calluses cocultivated with *Agrobacterium* that produced fertile transgenic plants, and the average efficiencies achieved were 37% for Bd21-3 and 17% for Bd21. The third Brachypodium transformation paper reports an extremely high average transformation efficiency of 55% for accession BDR018 (Pácurar et al. 2007). The authors achieve this high efficiency by placing immature embryos on callus-inducing media for 17 days and then cocultivating those embryos with *Agrobacterium*. Efficiency is calculated from the percentage of dissected embryos that form fertile transgenic plants. The limitation of this method is that the embryogenic callus is not subcultured, and therefore no more than one independent transgenic line can arise from each dissected embryo. This increases the labor involved in generating transgenic plants when compared with the methods for Bd21-3 and Bd21 transformation.

The publication of three high-efficiency *Agrobacterium*-mediated transformation methods signals that Brachypodium transformation technology has matured, yet the lessons learned from these three papers inspire continued studies to optimize Brachypodium transformation. For example, adoption of the use of very small embryos (0.3–0.7 mm) and a callus initiation medium that includes 0.6 µg/mL copper sulfate has further increased the average transformation efficiency of Bd21-3 to more than 50% (J. Bragg unpublished; protocol available at http://brachypodium.pw.usda.gov/). Further improvements in Brachypodium transformation will undoubtedly emerge both from investigating the genetic diversity within the growing collections of Brachypodium accessions and from other technical advances. It is noteworthy that the rapid pace of Brachypodium transformation technology development compares very favorably with the development of high-efficiency transformation in
rice. In the initial reports, rice transformation efficiencies were less than 1%, and through the efforts of many groups over several years, efficiency was improved to the more than 40% commonly achieved today (Tyagi and Mohanty 2000).

The highly efficient transformation methods developed for Brachypodium lay a strong foundation for Brachypodium as a model system to study fundamental aspects of grass biology. In addition, efficient transformation means that Brachypodium is an excellent test bed for transgenic approaches in the grasses. By using Brachypodium, researchers can much more rapidly test constructs for expression, efficacy, etc., before moving into biomass or other grass crops.

### 23.3.4 Generation of Brachypodium Mutant Populations

Genome-saturating mutant populations provide a means to explore the relationship between a phenotype and a gene of interest, and both forward and reverse genetic strategies require large populations of mutagenized plants. Diverse approaches for the efficient generation of mutant populations include treatment with mutagenic chemicals, exposure to high-energy radiation, and random DNA insertions. Researchers have had success applying these protocols to Brachypodium, and it appears that there are no limitations in applying common mutagens used with other plants. The development of populations of mutants, similar to those available for *Arabidopsis* and rice, will provide valuable tools for the Brachypodium research community.

#### 23.3.4.1 Ethyl Methanesulfonate Mutagenesis

Ethyl methanesulfonate (EMS) is an efficient chemical mutagen that introduces single base changes and has been used widely to mutagenize plants. In *Arabidopsis*, an extensive review of mutations in 192 genes verified the random nature of EMS mutations and estimated the frequency at one mutation per 170 kb of genomic DNA (Greene et al. 2003). An advantage of the single base changes derived from EMS mutagenesis is that these simple changes 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 (Caldwell et al. 2004). The frequency of albino plants is often used to measure the success of EMS mutagenesis, and over a mutant population of 2000 M2 plants, we have observed 2% albinos. This rate is comparable to that typically observed in successful EMS treatments of *Arabidopsis* seeds (Kim et al. 2006). Our initial screens have focused on identifying mutations that alter cell wall and biomass phenotypes, and we have identified more than 25 mutants of interest to date, indicating that the EMS mutagenesis was a success. Visit [http://brachypodium.pw.usda.gov/](http://brachypodium.pw.usda.gov/) for our current EMS mutagenesis protocol.

#### 23.3.4.2 Fast Neutron Radiation

Fast neutron radiation (FNR) introduces short deletions into the genomic DNA and is therefore a complementary mutagen to EMS, which induces single base changes. The larger disruptions generated by FNR permit rapid cloning of the mutant loci using a genome tiling array. This is an advantage over EMS mutagenesis, in which the genes must be identified using map-based cloning. However, because the region deleted by FNR typically encompasses several genes, this method is not suitable for the identification of essential genes or genes located adjacent to essential genes. In early experiments, 1–2% of mutagenized plants were albinos, indicating that Brachypodium can be efficiently mutagenized by FNR (D. Laudencia-Chingcuanco and M. Byrne, personal communication).

#### 23.3.4.3 Insertional Mutagenesis

Insertional mutagenesis is a natural complement to chemical and radiation mutagenesis because, although the mutational load is low (only one or a few mutations per line), the ability to sequence DNA flanking the insertion site enables rapid identification of the affected genes, which is required for reverse genetic approaches. The insertion of transferred DNAs (T-DNAs) via *Agrobacterium*-mediated transformation and the movement of transposons are the two most common approaches
for transferring known DNA sequences into random sites in the host genome. In the resulting mutant lines, the T-DNA or transposon sequence serves as a tag that can be used to locate the DNA insertion site within the genome. Large, freely available collections of sequence-indexed, tagged lines have been an extremely valuable tool in Arabidopsis research, permitting both forward genetic screens for a particular phenotype within the mutant populations and reverse genetic studies in which researchers can identify disruptions of specific genes using a simple BLAST search. The value of a collection of sequence-indexed mutants to facilitate study of candidate genes selected from the enormous amount of sequence and bioinformatic data currently available is tremendous.

Each insertion event has the potential to cause a knockout phenotype; however, vectors can also be designed to achieve various research goals, including the identification of promoters and overexpression of nearby genes. In a gene trap construct, a promoter-less reporter gene (e.g., GUS or GFP) is placed at the end of the T-DNA sequence that is transferred to the plant genome (An et al. 2005). If the vector DNA integrates downstream of a promoter, reporter gene expression could be used to infer the expression pattern of the disrupted gene and provide clues about the role of the disrupted gene. Inclusion of splice acceptor sites adjacent to the reporter genes permits splicing should the vector DNA fall into an intron. Activation tagging constructs place transcriptional enhancers within the vector DNA to increase the transcription of genes close to the insertion site (Weigel et al. 2000; Fits et al. 2001; Nakazawa et al. 2003). Activation tagging is designed to overexpress nearby genes while still maintaining a wild-type expression pattern, and it is particularly well suited to studying genes with redundant functions in which knockouts in one family member fail to produce a phenotype. This strategy can also provide insight into complex processes such as cell wall biosynthesis because activation tagging can activate global control genes.

A large sequence-indexed Brachypodium mutant population would be a powerful research tool, and multiple groups have started assembling such a population. The choice of the most efficient method to produce these collections depends on the efficiency of transformation versus the efficiency of producing transposon-tagged mutants. Although the transposon approach has the potential to rapidly generate a large number of insertional mutants, this technique requires optimization before it will be a productive means of generating Brachypodium mutants. We have transformed Brachypodium with vectors containing the most commonly used transposons (Ac/Ds and En/Spm) and demonstrated that they were active in the Brachypodium genome. However, most transgenic plants died before setting seed, possibly because the transposons were too active or activated an endogenous transposon (J. Bragg, unpublished). In contrast, the efficiency of T-DNA tagging has increased with the optimization of transformation techniques, and one person can easily produce 100 lines per week. Using this approach, at least two substantial populations are in development. The BrachyTAG project at the John Innes Centre currently lists 4500 T-DNA lines for distribution to the public. Of these, 1005 have flanking sequence tags, and 61 have nearby genes identified. Additionally, we have developed over 8000 T-DNA lines that are available for distribution via the USDA Brachypodium Genome Resources site (see Table 23.2 for links to these resources).

### 23.3.5 Crossing Brachypodium

An efficient method of crossing Brachypodium is required for it to serve as a tractable model genetic system [e.g., to allow positional cloning and mapping of quantitative trait loci (QTLs)]. Brachypodium is primarily an inbreeding species, and flowers rarely open under greenhouse and growth chamber conditions (Figure 23.1e). In observations of rare open flowers, anthers have already dehisced on the stigma. This suggests that even open flowers primarily produce self-pollinations, a notion supported by the highly homozygous nature of wild Brachypodium accessions (Vogel et al. 2009). Two similar methods, one using a microscope and the other a jeweler’s loupe, have been developed for crossing Brachypodium. The protocols (available at [http://brachypodium.pw.usda.gov/](http://brachypodium.pw.usda.gov/) and [http://www.ars.usda.gov/pandp/docs.htm?docid=18531](http://www.ars.usda.gov/pandp/docs.htm?docid=18531)) contain detailed pictures of the flower stages appropriate for crossing and of each step in the procedure. These protocols are based
on the fact that immature anthers will dehisce shortly after removal from the plant. In brief, florets
with feathery stigmas and anthers that have not yet dehisced are suitable for crossing. One floret is
emasculated by peeling back the lemma and removing the anthers. The anthers that will serve as
pollen donors are dissected from florets and permitted to swell for 10–30 min before they release
fresh pollen for crossing. Pollen or entire anthers are then introduced to the emasculated floret, and
the lemma is closed to prevent desiccation and protect the developing seed. With practice, crosses
can be made in 5–10 min or less.

### 23.4 GENOMIC RESOURCES

An extensive infrastructure of genomic resources has been (and continues to be) assembled for
Brachypodium, including cDNA libraries, BAC libraries, a large expressed sequence tag (EST)

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Brachypodium collection, a high-resolution genetic linkage map, physical maps, SSR markers, bioinformatic resources, and most importantly the recently completed 8× genome sequence.

23.4.1 BIOINFORMATIC RESOURCES

The rapid development of Brachypodium genomic resources presents researchers with an approaching avalanche of genomic data, and it is necessary to develop the appropriate bioinformatic infrastructure to efficiently support and utilize these tools. The available web-based resources are summarized in Table 23.2 and are briefly described below. A Brachypodium-specific web portal, www.brachypodium.org, provides links to numerous sources of Brachypodium information and also houses a newsgroup that links the Brachypodium community. The 8× Brachypodium sequence is housed in three databases that 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. Three sites designed to support web-based comparative genomics studies—Phytozome, GrainGenes, and CoGe—also contain the Brachypodium 8× genome sequence. Other websites that contain project-specific information include the BrachyTAG and USDA-ARS Genomics and Gene Discovery Unit websites. Where appropriate, the sections below contain references to these resources, and readers are directed to these sites for the most detailed resources currently available.

23.4.2 ESTS AND MICROARRAYS

ESTs generated by randomly sequencing the ends of cDNA clones provide a quick and relatively inexpensive way to learn a great deal about an unknown genome (Adams et al. 1991). As a result, the first significant sequence resource for Brachypodium was the 20,440 ESTs deposited into GenBank in 2005 (Vogel et al. 2006). These ESTs were derived from five cDNA libraries and represent approximately 6000 genes. As part of the genome sequencing project, the U.S. Department of Energy-Joint Genome Institute (JGI) generated approximately 128,000 Sanger ESTs, approximately 2.3 million 454 sequences, and approximately 289 million Illumina ESTs (International Brachypodium Initiative 2010). These newly expanded EST collections were prepared from a diverse set of tissues and treatments (International Brachypodium Initiative 2010). EST sequences are useful for many applications, including microarrays, analyses of gene expression, and manual curation of gene models. The Brachypodium ESTs have already been used to refine the phylogeny of Brachypodium and to identify candidates for all of the genes involved in the biosynthesis of lignin monomers (Vogel et al. 2006a). Soon a Brachypodium whole-genome hybrid exon-scanning/expression/tiling microarray containing 6.5 million features/array will be available on an Affymetrix platform (T. Mockler, personal communication).

23.4.3 BAC LIBRARIES

Sequence data obtained from BAC libraries permit evaluation of genome content and complexity when a full genome sequence is not available and can aid in assembly of genome sequencing data. Furthermore, BAC libraries are useful for comparative genomic analyses that can be exploited to facilitate positional cloning of genes in related species. Eight BAC libraries have been constructed for diploid Brachypodium accessions. The first two libraries were constructed from accessions ABR1 and ABR5 and together contain a total of 9100 clones with an average insert size of 88 kb (Hasterok et al. 2006). These relatively small libraries represent approximately 2.7 haploid genome equivalents. Two BAC libraries made from inbred line Bd21, the line used for genome sequencing, contain a combined total of 110,592 clones with an average insert size of approximately 100 kb. These Bd21 libraries represent 38 haploid genome equivalents and provide greater than 99.99% likelihood a particular gene is included within the library (Huo et al. 2006). These Bd21 libraries
were used to generate BAC end sequences from 64,694 clones, and the resulting 38.2 Mbp of sequence covers approximately 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. In addition, the Arizona Genomics Institute (www.genome.arizona.edu) has constructed one library from the inbred line Bd3-1 and two libraries from Bd21 (M. Bevan, personal communication).

One BAC library exists for the perennial species *B. sylvaticum*. This library contains 30,228 clones with an average insert size of 102 kb (6.6 genome equivalents, on the basis of 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 among 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 greater size of the *B. sylvaticum* genome.

### 23.4.4 Maps and Markers

Mapping resources for Brachypodium are developing rapidly. A physical map has been constructed from two of the Bd21 BAC libraries mentioned above (Gu et al. 2009). This map contains over 67,000 BAC clones assembled into 671 contigs and can be accessed at http://phymap.ucdavis.edu:8080/brachypodium. In addition, a second physical map using two different Bd21 libraries has been constructed (M. Bevan, personal communication). A high-density linkage map based on 562 single nucleotide polymorphism (SNP) markers has also been constructed (N. Huo, unpublished). The markers fell into five linkage groups corresponding to the five chromosomes of the haploid Brachypodium genome. The resulting map was used to assemble the whole-genome shotgun sequence into chromosome-scale assemblies (International Brachypodium Initiative 2010).

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. 2006). A more extensive application of the technique will be highly instructive in verifying the whole genome assembly and for comparing the evolutionary relationships among genomes of various grasses (Wolny and Hasterok 2009).

Genetic markers are essential for many experiments, including positional cloning, mapping QTLs, association mapping, ECOTILLING, and analysis of genotypic diversity in populations. Polymerase chain reaction (PCR)-based markers are particularly useful because they are fast, easy to score, and can be used by any laboratory with routine molecular biology tools. A recent publication describes the development of 398 SSR markers for Brachypodium (Vogel et al. 2009). SSRs, also known as microsatellites, are genomic areas with simple, short repeat units. The number of repeats is highly polymorphic, making SSRs powerful markers. As previously discussed in Section 23.3.1, the utility of these SSRs was demonstrated by showing that genetic diversity in a large number of new Brachypodium accessions correlated with significant differences in easily scored phenotypes such as seed size, vernalization requirements, and the presence of hairs (Vogel et al. 2009). Another study used 12 SSR markers to examine introduced populations of polyploid Brachypodium and showed that there have been multiple introductions of Brachypodium into the state of California (Bakker et al. 2009).

### 23.4.5 Whole-Genome Sequencing

A completely sequenced genome underpins a host of tools, including efficient map-based cloning, sequence-indexed T-DNA populations, gene chips, and reverse genetic approaches such as TILLING.
and RNA interference (RNAi), and is therefore a requirement for a modern model system. Plans for the development of Brachypodium as a model to accelerate the domestication of grasses for use as biomass crops (e.g., switchgrass and Miscanthus) 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, JGI approved a proposal to sequence the Brachypodium genome with a whole-genome shotgun sequencing strategy through their Community Sequencing Program for 2007. In May 2009, JGI released the final 8x sequence and the version 1.0 annotation of the Brachypodium genome. The assembly incorporates all of the mapping and BAC sequence resources and has been shown to be of very high quality: Gaps in the assembly represent only approximately 0.4% of the genome, and Illumina EST data support more than 92% of the predicted protein-coding genes (International Brachypodium Initiative 2010). Through the Community Sequencing Program for 2009 (http://www.jgi.doe.gov/sequencing/cspseqplans2009.html), JGI has approved the resequencing of six additional Brachypodium lines using next-generation sequencing platforms. The information gained through this project will permit comparisons of the genetic diversity of phenotypically distinct Brachypodium lines and assist in identifying the genetic basis for phenotypes critical to the improvement of bioenergy and cereal crops as well as many other research interests.

23.5 Brachypodium as a Model for Bioenergy Grasses

In the development of biofuel feedstocks, two key considerations are biomass quantity and biomass quality (Carpita and McCann 2008). Biomass quantity refers to the amount of feedstock produced per unit of land. Biomass quality reflects the efficiency with which the feedstock can be converted to the desired biofuel. Biomass quality encompasses feedstock recalcitrance and the feedstock’s suitability for the conversion process used. Thus, plant materials that exhibit less resistance to degradation or contain larger relative amounts of easily fermentable sugars are of higher “quality.” Brachypodium’s typical grass characteristics, together with the many resources available for its study, make Brachypodium a suitable system for investigating the factors that contribute to the quantity and quality of feedstocks.

23.5.1 Factors Influencing Biomass Quantity: Plant Architecture

Genetically influenced traits such as growth habit, stem density, and plant height affect the quantity of biomass that can be harvested. The small size and ease of growth of Brachypodium will facilitate the application of forward and reverse genetic approaches to study these traits. In addition, natural variation in these traits has been demonstrated in recently cataloged collections of Brachypodium accessions, providing researchers with another tool to investigate phenotypes of interest for increasing biomass (Vogel et al. 2009). For example, some accessions have an erect growth habit, whereas others exhibit extensive spreading under the same environmental conditions (Figure 23.3). This trait is relevant to biomass quantity; more erect plants can be planted at higher densities and may be less subject to crop losses due to lodging.

Plant height is another major contributor to biomass yield. Assuming there are no accompanying deleterious characteristics, taller plants will have more usable biomass. For example, a comprehensive study of biomass traits in sorghum recombinant inbreds lines found a positive and highly significant correlation between plant height and biomass yield (Murray et al. 2008). Pathways associated with the growth-promoting hormones gibberellins and brassinosteroids are potential targets for increasing plant height (Fernandez et al. 2009). The idea of substantially improving yield by altering phytohormone pathways has a historical precedent: Semi-dwarf rice and wheat varieties were key components of the “Green Revolution” of the 1960s and 1970s, during which the grain yields of cereal crops in developing countries increased dramatically (Evans 1993; Conway 1997; Hedden 2003). These dwarf varieties contained a loss-of-function mutation in a gibberellin (GA) biosynthetic gene in rice (Sasaki et al. 2002; Spielmeyer et al. 2002) and a gain-of-function mutation.
in a GA response repressor in wheat (Peng et al. 1999). The resulting disruptions in GA synthesis and signaling enabled the shorter varieties to resist lodging, or falling over, and to channel applied nitrogen fertilizer to grain production rather than stem growth (Evans 1993; Conway 1997; Sasaki et al. 2002; Hedden 2003). The converse goal of increasing vegetative biomass might be achieved through the opposite approach, i.e., upregulating GA biosynthesis and/or de-repressing GA growth responses (Sasaki et al. 2002; Fernandez et al. 2009).

The dwarfing alleles in hexaploid, Green Revolution wheat correspond to the \textit{Rht-B1}/\textit{Rht-D1} genes, which encode DELLA-motif containing proteins (Peng et al. 1999). A loss-of-function mutation in the orthologous rice gene \textit{SLR1} results in increased plant height (Ikeda et al. 2001). BLAST searches of the Brachypodium genome revealed that Brachypodium, like rice, contains a single DELLA-encoding gene and that the Brachypodium DELLA protein exhibits 86% amino-acid identity to \textit{SLR1}; this finding suggests that the mechanisms governing GA-regulated growth responses are likely to be similar in Brachypodium and the other grasses. Components of the GA pathway and additional candidate genes of interest can be easily identified, cloned, and functionally characterized in Brachypodium thanks to a sequenced genome and an efficient transformation protocol. Brachypodium’s short life-cycle and simple growth requirements will further facilitate rapid hypothesis testing for genetic manipulations aimed at improving biofuels feedstocks.

\subsection{23.5.2 Factors Influencing Biomass Quantity: Interactions with the Environment}

In addition to morphological characteristics, a plant’s interactions with the environment also affect biomass yield. Thus, adaptability to a range of soil conditions, efficient water and nutrient use, and resistance to abiotic and biotic stresses are all desirable traits in a bioenergy crop (DOE 2007). For investigating these traits, Brachypodium also provides a wealth of resources. The geographical range of Brachypodium encompasses a diversity of habitats (Garvin et al. 2008; Opanowicz et al. 2008; Vogel et al. 2009), providing the opportunity to investigate adaptations to different environments with a genetically tractable organism. The first proposal of Brachypodium as a model grass included the observation that different Brachypodium ecotypes varied in their responses to the agriculturally important fungal pathogens \textit{Puccinia striformis} f. sp. \textit{tritici} and \textit{Magnaporthe grisea}, the causative agents of wheat yellow stripe rust and rice blast, respectively (Draper et al. 2001). Infections with \textit{Fusarium graminearum} (head blight) (D. Garvin, personal communication)
Brachypodium and *Pythium* species (root rot) (Vogel and Bragg 2009) have also been noted. Characterizations of viral infections in Brachypodium are extremely limited—with a report of an unidentified virus on *Brachypodium sylvaticum* (Edwards et al. 1985) and preliminary studies using barley stripe mosaic hordeivirus (A. Jackson, personal communication).

To examine *Brachypodium*-*M. grisea* interactions in detail, Routledge et al. (2004) challenged 21 Brachypodium ecotypes with four strains of *M. grisea*. The Brachypodium ecotypes exhibited responses ranging from resistance to susceptibility. Significantly, plant cell death was associated with resistance, as seen in rice, and the development of disease in a susceptible ecotype paralleled disease progression in rice (Routledge et al. 2004). Analysis of the progeny obtained by crossing the resistant Brachypodium ecotype ABR5 with the susceptible ecotype ABR1 indicated that a single, dominant locus—likely an *R* gene—conferred resistance (Routledge et al. 2004). After developing a protocol for infecting Brachypodium, rice, and barley with *M. grisea* (Parker et al. 2008), researchers compared metabolite patterns during the early stages of infection and found evidence that *M. grisea* suppresses the production of reactive oxygen species and defensive lignin compounds in all three of the host grasses (Parker et al. 2009). Collectively, this work supports the development of the *Brachypodium*-*M. grisea* pathosystem as a tool to study the cellular events, gene-for-gene interactions, and other processes underlying plant defense and infection.

A number of observations suggest that Brachypodium can be used to study other aspects of responses to biotic and abiotic stresses. The lack of macroscopic disease symptoms in Brachypodium exposed to the causative agent of powdery mildew on wheat, *Blumeria graminis*, has lead to the proposal that Brachypodium would be a good model for studying non-host resistance to this fungal pathogen (Draper et al. 2001). A Brachypodium proteinase inhibitor gene (*Bdpin1*) that is induced by wounding, methyl jasmonate, and *M. grisea* has been identified (Mur et al. 2004). Because *Bdpin1* belongs to a class of molecules that serve as markers of the wounding response and protect against insect damage, this research suggests that Brachypodium could be suitable for studies not only of plant-microbe interactions, but also of wounding and insect herbivory (Mur et al. 2004).

Mycorrhizal fungi can aid plants in the uptake of nutrients, especially phosphate, thereby reducing the cost of agricultural inputs such as fertilizers and facilitating plant growth on marginal land (Morgan et al. 2005). These interactions will be especially important in the context of biomass crops because it is projected that these crops will be grown on marginal lands with minimal fertilizer inputs. Brachypodium, unlike *Arabidopsis*, forms mycorrhizal associations (M. Harrison, personal communication) and thus enables studies of these symbiotic relationships in a model system.

### 23.5.3 Factors Influencing Biomass Quality: Cell Wall Structure and Composition

Plant-derived biofuel feedstocks primarily consist of plant cell walls. Key cell wall components relevant to fuel production include the carbohydrate polymers cellulose and hemicellulose and the phenylpropanoid polymer lignin. These polymers are cross-linked together to form a composite material that is the cell wall. The production of liquid fuels or specialty chemicals from biomass first requires the deconstruction of the glucan portion of this polymer-composite matrix into its constituent parts, namely monosaccharides. The inherent recalcitrance of the plant cell wall to degradation adds considerably to the expense of this step (DOE 2006). Thus, comprehensive knowledge of the genes that determine grass cell wall structure and composition could be used to design superior feedstocks and accelerate the domestication of the grasses proposed as energy crops (e.g., switchgrass and Miscanthus). The most rapid way to acquire this knowledge is through the use of a model system such as Brachypodium.

Because the compositions of dicot and grass cell walls differ substantially, *Arabidopsis* cannot be used to study all aspects of the grass cell wall (Carpita 1996; Vogel 2008). Commelinoid
monocots, including the grasses, have type II primary cell walls. Dicots, such as *Arabidopsis*, and noncommelinoid monocots, including orchids and lilies, have type I primary cell walls. Cell wall composition varies from species to species and even among cell types within a single plant (Knox 2008; Popper 2008). Although the primary and secondary walls of both grasses and dicots contain cellulose, the major hemicellulose is glucuronoarabinoxylan (GAX) in grasses and xyloglucan in dicots. Grass cell walls also contain ferulic acid and p-coumaric acid, hydroxycinnamate compounds that are only minor components of most dicot walls. Ferulate-mediated cross-linking of GAX and lignin decreases the digestibility of monocot walls (Grabber 2005) with direct implications for biofuel production. In addition to the differences in hemicellulose and hydroxycinnamates mentioned above, grass primary walls, unlike their dicot counterparts, incorporate mixed linkage (β1,3- and β1,4-linked) glucans and contain few structural proteins and little pectin compared with the large amounts of pectin—up to 35% dry weight—found in type I walls (Vogel 2008). Supporting the use of *Brachypodium* as a model for grass cell walls is the finding that the monosaccharide profiles of *Brachypodium*, wheat, barley, and Miscanthus cell walls are similar to each other but different from the profile of *Arabidopsis* (Gomez et al. 2008).

Cell wall composition is critical in the context of the biofuel production process. The conversion of lignocellulosic biomass into liquid fuels generally proceeds via four steps: (1) mechanical and/or thermochemical pretreatment of feedstocks to make the cell wall components more accessible, (2) enzymatic hydrolysis of the pretreated biomass to release sugars from the carbohydrate polymers of the wall, (3) microbial fermentation of the released sugars to produce liquid fuels, and (4) recovery of the biofuels from the fermentation medium (for example, by distillation) (Himmel et al. 2007; Wyman 2007; Kumar et al. 2008). The first two steps—pretreatment and hydrolysis—are necessary to overcome the recalcitrance of plant materials (i.e., their resistance to degradation). Recalcitrance is arguably the largest obstacle to the economical, efficient, and environmentally friendly production of biofuels (Himmel et al. 2007; Wyman 2007). There are various pretreatment options, including treatment with dilute acid, washing with large volumes of hot water, and ammonia fiber expansion (Wyman et al. 2005). Although each pretreatment method has distinct advantages and disadvantages, pretreatments in general require substantial inputs of energy, chemicals, or other resources (Wyman et al. 2005). Also, the production and use of hydrolytic enzymes can be both expensive and limiting (Wyman 2007; Kumar et al. 2008). Additional considerations include the possibility that pretreatments can release or produce inhibitors of fermentation (Himmel et al. 2007) and that the most commonly used fermentative microbes—primarily the budding yeast *Saccharomyces cerevisiae*, but also species such as the anaerobic bacterium *Clostridium thermocellum*—use only six-carbon sugars such as the glucose monomers of cellulose, not five-carbon sugars such as the xylose found in hemicelluloses (Demain et al. 2005; van Maris et al. 2006). From a feedstock development perspective, biomass recalcitrance might be decreased in a number of ways, e.g., by altering the structure or cell-cell-adhesion properties of plant tissues to allow greater access by chemicals and hydrolytic enzymes or by reducing the degree of cross-linking of the cell wall components, the crystallinity of cellulose, or the amount of lignin. Increasing the amount of cellulose or the percentage of hexoses in other cell wall polymers could also improve production efficiency by providing more substrates for microbial fermentation.

The resources available for *Brachypodium* will be useful for addressing these issues. Because cell walls support and protect plants, there is always the concern that modifying the wall will negatively affect plant fitness. In this respect, the natural variation documented for *Brachypodium* could prove especially valuable. If an accession with improved digestibility and/or fermentability can be found, its cell wall characteristics could reveal new avenues for altering wall composition and structure without severely decreasing viability. In a complementary approach, the effects of targeted modifications or randomly induced mutations can be analyzed more rapidly in *Brachypodium* than in grasses with longer generation times. However, to identify naturally occurring or mutant plants with desirable cell wall traits, new screening procedures must be implemented. As a first step, Gomez et al. (2008) showed that treatments with hot, dilute sulfuric acid resulted in limited
hydrolysis of Brachypodium cell wall material. This work thus establishes a pretreatment method that can be used to screen for variations—even subtle ones—in the recalcitrance of Brachypodium biomass (Gomez et al. 2008). Further studies applying the tools of cell wall analysis and the techniques of biofuel production to Brachypodium will help to meet the challenge of improving bioenergy feedstocks.

23.6 FUTURE PERSPECTIVES

Brachypodium is rapidly gaining in utility and acceptance as a model grass species. A critical mass of resources and researchers using Brachypodium has been achieved, and this trend will likely accelerate with the completion of the genome sequence. In addition to Brachypodium’s relevance to bioenergy, over 30 research papers in the previous 3 years alone have used Brachypodium to investigate topics as diverse as microRNAs (Unver and Budak 2009; Wei et al. 2009), comparative genomics (Bortiri et al. 2008; Huo et al. 2009; Kumar et al. 2009), characteristics of introduced populations (Bakker et al. 2009), and seed storage proteins (Laudencia-Chingcuanco and Vensel 2008). The interest in Brachypodium is also reflected in the large number of people—approximately 75 individuals from more than 20 laboratories—who have recently contributed to the Brachypodium genome annotation effort (International Brachypodium Initiative 2010). In summary, Brachypodium combines the desirable attributes of a model organism with many of the traits of interest for the development and improvement of biofuel feedstocks. Although not itself a bioenergy crop, Brachypodium is an accessible, informative representative of the grasses, a group of plants that are tremendously important to the future of bioenergy.

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Brachypodium


