Brachypodium distachyon as a Genetic Model System

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Abstract

Brachypodium distachyon has emerged as a powerful model system for studying the genetics of flowering plants. Originally chosen for its phylogenetic proximity to the large-genome cereal crops wheat and barley, it is proving to be useful for more than simply providing markers for comparative mapping. Studies in *B. distachyon* have provided new insight into the structure and physiology of plant cell walls, the development and chemical composition of endosperm, and the genetic basis for cold tolerance. Recent work on auxin transport has uncovered mechanisms that apply to all angiosperms other than *Arabidopsis*. In addition to the areas in which it is currently used, *B. distachyon* is uniquely suited for studies of floral development, vein patterning, the controls of the perennial versus annual habit, and genome organization.

> For every problem in a given discipline of science, there exists a species or other entity or phenomenon ideal for its solution. (Example: A kind of mollusk, the sea hare *Aplysia*, proved ideal for exploring the cellular basis of memory.) Conversely, for every species or other entity or phenomenon, there exist important problems for the solution of which it is ideally suited. (Example: bats were logical for the discovery of sonar.)
>
> - E.O. Wilson, *Letters to a Young Scientist*

INTRODUCTION

Why Another Model Organism?

The term model organism was rarely used before about 1970 and at the time was used to mean a model for a particular process [e.g., a slime mold as a model for photobiology (88), or a species of nematode as a model for aging (32)]. In other words, models were chosen to address particular questions. More recently, however, the term model organism has been used in a much more taxonomic or phylogenetic context, as a model for a clade rather than a model for a process or question. In addition, the term model now implies a system with a certain set of resources (e.g., a genome sequence and accompanying annotations, a collection of mutants, a set of wild accessions, transformations, and transcriptomes) and a set of biological characteristics (e.g., ease of growth in a laboratory, a short life cycle, and ease of crossing). In other words, a model is now one that permits discovery of the functions of particular genes, providing tools for connecting phenotype and genotype. As more and more species acquire the basic model tool kit, the idea that an organism is a model for a process or question is beginning to reappear.

As a representative of land plants, *Arabidopsis thaliana*, a eudicot in the Brassicaceae (Brassicales), is a proven system for answering questions about plant molecular biology, biochemistry, genetics, and physiology. *Arabidopsis* is useful for studying many aspects of plant biology that are shared among land plants (embryophytes), vascular plants (tracheophytes), seed plants (spermatophytes), and flowering plants (angiosperms), although in fact any flowering plant could be developed to fit this criterion. Other processes, however, are clade or species specific. For example, secondary chemistry and response to pathogens are familiar examples that vary enormously among families, genera, species, and even populations within species. For such aspects of plant biology, a generic model is less useful.

The cereal crops that feed most of the people of the world are morphologically, physiologically, and developmentally very different from *Arabidopsis*, and many aspects of their biology require a model plant that is more closely related. Although the crops themselves are excellent models for some purposes, many are inconvenient to work with because of either growth requirements (e.g., rice), large stature (e.g., maize), or aspects of their genomes. Some of the most challenging crops for genetic studies are members of the tribe Triticeae (wheat, barley, rye, and approximately 375 wild species), which have some of the largest genomes of the grasses. The haploid (1C) genome size of barley (*Hordeum vulgare*) is 5.1 Gb (50), approximately 60% larger than that of maize (50), and that of rye (*Secale cereale*) is approximately 8.1 Gb. Both barley and rye are diploids, so the difference in genome size is due almost entirely to the accumulation of transposons.

Brachypodium, and in particular the small annual species *Brachypodium distachyon*, is proving to be a valuable system for the study of specific processes, including dissecting the biology of the cell wall, the development of the endosperm, the controls of flowering, and the development of the inflorescence. *Brachypodium* was initially chosen as a model because of its phylogenetic position, which is more closely related to wheat and barley (both in the tribe Triticeae) than to rice or maize (10). In addition, it has the usual characteristics of a model system (rapid cycling, small stature,

small genome size, ease of growth). A useful table comparing the pros and cons of different model systems, including the crops themselves, can be found in Reference 10.

In much of the literature, the generic name *Brachypodium* is now widely used to mean *B. distachyon*. However, for clarity in the discussion here, the name *Brachypodium* is used to refer to the genus and *B. distachyon* to the model species.

Genetic and Genomic Resources

B. distachyon has many advantages as a model, and an extensive set of tools for use with this model is available (e.g., 6, 10, 20, 34, 77, 81, and references therein). These include a broad and diverse set of germplasm, microarrays, robust protocols for transformation, and a set of T-DNA insertion lines.

Several hundred wild accessions and inbreds are available from recent collecting efforts; approximately 250 lines are listed at *Brachypodium* Resources (54). Germplasm collection initially focused on native populations in Turkey, in the center of the native range of the species (26, 117), and these lines can be obtained from the U.S. Department of Agriculture's Germplasm Resources Information Network (GRIN). Some material is also available from other parts of the world. Extensive sets of simple sequence repeat markers have been developed for genotyping natural accessions and for use as markers in genetic maps (3, 31, 117).

A set of 171 wild-collected lines has been surveyed for plant height, spikelet disarticulation (shattering), and growth habit, and a subset of these has been assessed for cell wall characteristics and stem density (113). From these accessions, a core collection of 17 lines has been established and their genomes sequenced. To characterize the lines further, flowering time was synchronized by adjusting vernalization times. Variation remained in all characteristics, including height and stem density at maturity and seed size, traits that are important for biofuel and grain crops, respectively. Thus, natural diversity encompasses variation for dissecting numerous biological processes.

The genome of accession Bd21 was sequenced in 2010 (51) using whole-genome shotgun sequencing. Cytogenetics, two physical maps, and sequenced bacterial artificial chromosomes (BACs) were used to verify the assembly. An updated sequence (v2.1) has been released, with an assembled size of ~272 Mb, consistent with sizes estimated by flow cytometry (85). The contig N50 of the assembly is now 6.4 Mb. The genome is predicted to include approximately 25,000 genes, a number comparable to that of other grass genomes (see 8, 54). Approximately 1,400 of the gene models (approximately 5.6%) appear to be grass specific, shared with rice and sorghum but not with eudicots.

Six lines of *B. distachyon*, including Bd21-3, the line most commonly used for transformation (116), were selected for resequencing on the basis of their phylogenetic and phenotypic divergence (35). As expected, the sequences uncovered many single nucleotide polymorphisms (SNPs), as well as hundreds of presence-absence variants. Transcriptomes of the six diverse lines permitted annotation of more than 2,000 genes that were not initially annotated in the reference sequence.

The *B. distachyon* genome encodes both conserved and novel microRNAs (miRNAs) (53, 118, 124). Recent sophisticated analyses of gene expression under 17 different environmental treatments identified a large set of miRNAs (53), many of which are differentially expressed. In addition, precise identification of cleaved targets of the miRNAs was possible using parallel analysis of RNA ends (PARE), a method that can identify targets in both annotated and unannotated regions of the genome (56).

The reference genome sequence was used to create an Affymetrix microarray covering introns and exons of all gene models, with an average coverage of 11 probes per region (10). This microarray has been used to investigate expression profiles of plants responding to stress (e.g., 87; see also below).

Current protocols for transformation of *B. distachyon* use *Agrobacterium* (114, 116), replacing older biolistic approaches (16, 20). Transformation efficiency is reported to be approximately 45% (54, 77).

B. distachyon is self-pollinated, with very little outcrossing in the wild or in the greenhouse. In general, plants are cleistogamous, and even if the flowers open, pollination has already occurred (117). Genotyping of wild-collected Turkish plants showed that most were homozygous at most loci, consistent with a high level of natural inbreeding (117). Thus wild-collected lines are already effectively inbred and insuring self-pollination is easy. Conversely, crossing requires some technical skill (protocols available at 54).

T-DNA insertion lines have been developed using a variety of vectors. Some of these have incorporated promoterless β-glucuronidase (GUS) sequences so they can serve as gene traps, whereas others have transcriptional enhancers so they can be used as activation tags. Over 22,000 lines, together including approximately 26,000 T-DNA insertion sites, are now available (9, 54, 107).

Relationships and Morphological Characteristics

Brachypodium is a member of the grass subfamily Pooideae (**Figure 1**), one of the 12 subfamilies in Poaceae, and a member of the large BEP (subfamilies Bambusoideae, Ehrhartoideae, and Pooideae) clade (58). The early-diverging subfamilies (Anomochlooideae, Pharoideae, and Puelioideae) include only a handful of species, with most other species of the family in the PACMAD (subfamilies Panicoideae, Aristidoideae, Chloridoideae, Micraroideae, Arundinoideae, and Danthonioideae) clade. The latter clade includes many important crops, such as maize, sorghum, sugarcane, and the various species of millet.

The subfamily Pooideae includes approximately 3,850 species in 177 genera, all characterized by cold tolerance and representing a radiation into temperate regions (21, 36, 46) (**Figure 1**, branch 1). Major clades within the subfamily share distinctive characteristics that distinguish them (and thus *Brachypodium*) from all other grasses, including rice and maize (58). Although the distribution of these traits is well documented, their functional significance is generally unknown. The styles are unfused in all members of the subfamily (**Figure 1**, branch 1) (36). Most species have a characteristic (pooid) embryo (90) in which the scutellum never separates from the coleorhiza; i.e., there is no scutellar cleft, and the embryonic leaf margins generally overlap. A small flap of tissue, the epiblast, forms on the side of the embryo away from the scutellum; the function of this structure is unknown. The epiblast is shared with other members of the BEP clade, whereas the lack of a scutellar cleft appears to be a uniquely derived character in Pooideae (36, 103).

The north temperate genus *Brachyelytrum* is sister to the rest of Pooideae, and Nardeae (*Nardus* plus *Lygeum*) is the next diverging clade (**Figure 1**). Meliceae, Stipeae, and Phaenospermateae (in the sense of Reference 58) diverged after Brachyelytreae and Nardeae but the order is uncertain (37, 49, 96, 97). Lodicules in all Pooideae except *Brachyelytrum* lack vascularization. Because there are no lodicules in *Lygeum* and *Nardus*, it is unclear whether vascularization was lost before (**Figure 1**, branch 2) or after (**Figure 1**, branch 3) the divergence of Nardeae. All Pooideae except for *Brachyelytrum*, *Lygeum*, and *Nardus* lack multicellular microhairs, so these must have been lost along branch 3 (**Figure 1**).

Parallel-sided subsidiary cells characterize the large clade made up of Brachypodieae, Bromeae, Triticeae, and Poeae (**Figure 1**, branch 4). *Brachypodium*, in its own tribe Brachypodieae, is clearly sister to the three tribes of the core Pooideae (**Figure 1**, branch 5), and this is supported by molecular and morphological data (36, 37, 103). After the divergence of *Brachypodium*, the common ancestor of the core Pooideae (**Figure 1**, branch 5) experienced major changes in genome size and organization (see below).

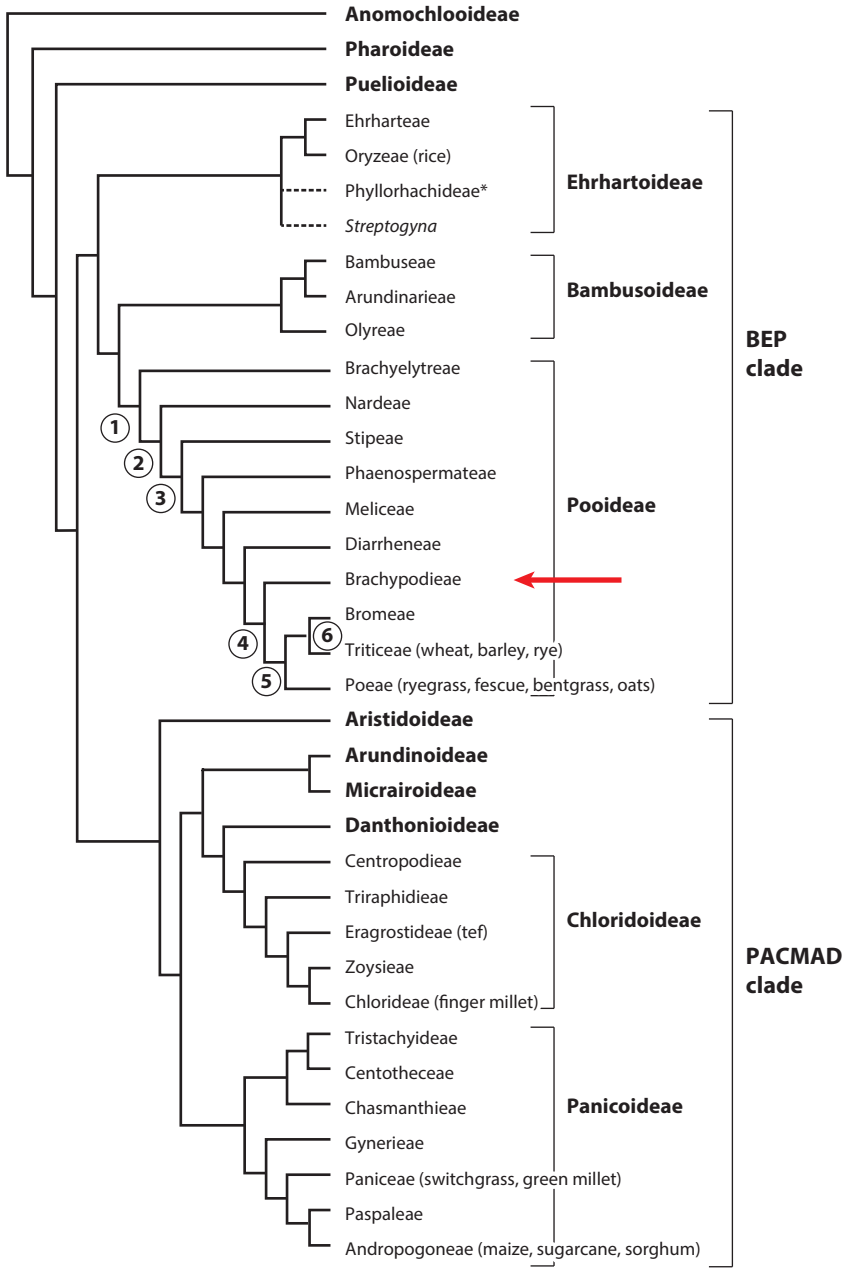

Figure 1

Phylogeny of the grasses. The phylogenetic relationships are based largely on those found in Reference 37. Modified with permission from Reference 58.

Brachypodium is often described as intermediate between wheat and rice, which is true in the sense that your sibling is intermediate between you and your first cousin. *Brachypodium* is clearly more closely related to wheat (*Triticum*), and thus any characters that originated in their common ancestor are more likely to be shared by *Brachypodium* and *Triticum* than by either one and rice (*Oryza*). However, for characters that changed along the branch leading to core pooids (**Figure 1**, branch 5), or to Triticeae (**Figure 1**, branch 6), species of *Brachypodium* are more likely to share the condition present in rice.

Brachypodium includes approximately 16 species in the temperate regions of Eurasia, Mexico, and Central and South America (17, 58). All species have unbranched inflorescences with the spikelets borne on short pedicels arising directly from the inflorescence axis (**Figure 2**). Most species are perennial and may be caespitose or rhizomatous. The annual species are clearly derived from perennial ancestors (15), suggesting that *Brachypodium* would be a good genus in which to explore the genetic basis of the perennial habit. *Brachypodium sylvaticum* is now being used as a point of comparison to *B. distachyon* with recently published transcriptomic data (29) and a BAC library (28).

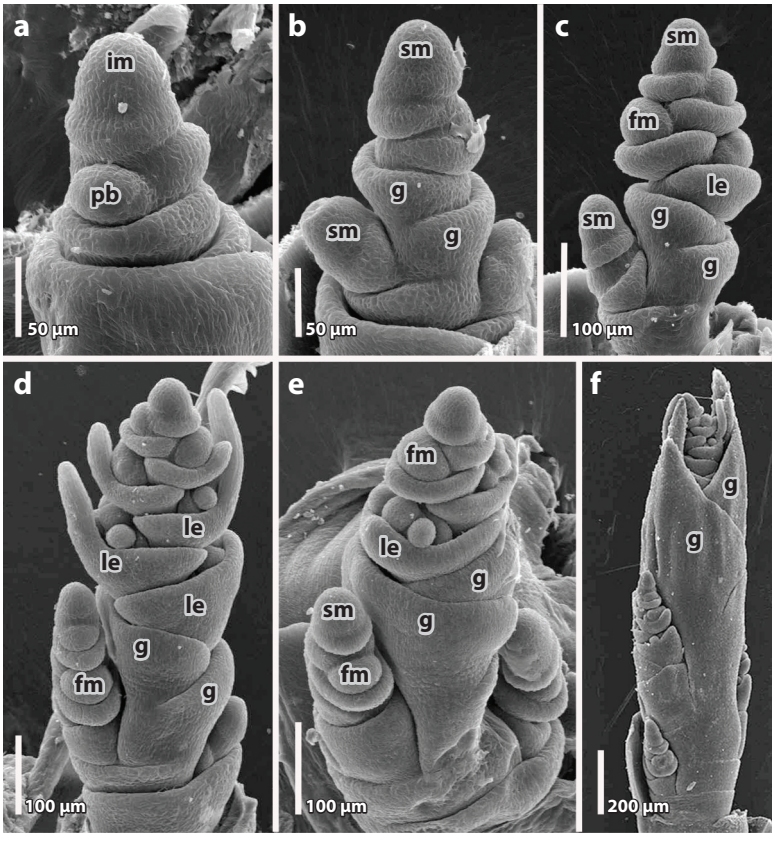

Figure 2

Early development of the inflorescence of *Brachypodium distachyon*. (*a*) Primary branch formation, showing distichous phyllotaxis. (*b*) Inflorescence meristem and uppermost branch meristem converted to spikelet meristems. (*c–f*) Successive stages of development, showing differentiation of the terminal spikelet well ahead of the lateral spikelets. Abbreviations: fm, floral meristem; g, glume; im, inflorescence meristem; le, lemma; pb, primary branch; sm, spikelet meristem. Reproduced with permission from Reference 60.

B. distachyon is native to the Mediterranean region but has become widespread and weedy in temperate regions worldwide, spread by human movement throughout the globe (15). It is one of three annual species in the genus, along with *Brachypodium stacei* and *Brachypodium hybridum* (15). [Note that some authors (e.g., 17) still place all three annual species into a single broadly defined *B. distachyon*, although this taxonomic resolution is not supported by recent data.]

B. distachyon has two or three anthers (15); reduction of anther number is common in the grasses and is generally associated with inbreeding and often cleistogamy (12). The genetic basis of anther number reduction is unknown, but *Brachypodium* would be an ideal system in which to dissect this character genetically.

Number of flowers per spikelet also varies in *B. distachyon*. Although most spikelets have seven flowers, the number can be as few as five or as many as nine (15). Because the number of flowers is fixed in all other model grasses (two in maize, sorghum, and *Setaria*, one in rice), *B. distachyon* is also a good system in which to dissect the controls and ecological trade-offs associated with this character.

Genome Size, Structure, and Collinearity

The ancestral chromosome number of Pooideae is probably 11 or 12 (36), and one or the other of these numbers occurs in most tribes and genera outside of *Brachypodium* and core Pooideae. However, the chromosome number must have been reduced in the common ancestor of *Brachypodium* plus the core Pooideae (**Figure 1**, branch 4). *Brachypodium* itself includes species with chromosome numbers of $2n = 10$, 14, 16, 18, 20, 28, and 30 (86), corresponding to base numbers of $x = 5$, 7, 8, 9, and 10 (15). *B. sylvaticum*, which is being studied as a perennial comparator to *B. distachyon*, has $2n = 18$ (91); reports of $2n = 16$ are considered unreliable (91). The sister lineage, the core Pooideae (**Figure 1**, branch 5), has a base chromosome number of $x = 7$ (36, 37, 61). Reduction to $x = 7$ correlates with a substantial increase in genome size generated by a massive expansion of transposable elements, leading to the largest genomes reported in the grasses (5, 59).

The genomes of rice and *B. distachyon* are broadly syntenic (51). *Brachypodium* chromosome 1 combines sequences from rice chromosomes 6, 7, and 3; *Brachypodium* 2 combines rice 1 and 5; *Brachypodium* 3 combines rice 2, 8, and 10; *Brachypodium* 4 combines rice 11, 9, and 12; and *Brachypodium* 5 is syntenic with rice 4. In several cases, the *B. distachyon* chromosome was clearly formed by insertion of one of the rice chromosomes into the centromeric region of another, a mechanism that has been documented in other grasses such as *Aegilops tauschii*, the presumed donor of the D genome of wheat (72).

Large blocks of collinear genes are also obvious in comparisons of *Brachypodium* to members of Triticeae (barley, wheat, and *A. tauschii*) (51). Comparisons between *B. distachyon* and *Agrostis stolonifera*, an allotetraploid member of tribe Poeae, also show good synteny but many disruptions of collinearity (2). However, the number of markers used to compare the *Agrostis* map with that of *B. distachyon* was relatively small, so local collinearity might still be seen in higher-resolution maps.

Genome rearrangements between *B. distachyon* and Triticeae are independent of rearrangements between *B. distachyon* and rice, consistent with the phylogeny. This suggests that the common ancestor of *Brachypodium* and the core Pooideae had a genome structure quite similar to that of rice. Subsequent reductions in chromosome number then must have proceeded independently in the two lineages.

The three annual species of *Brachypodium*, *B. distachyon*, *B. stacei*, and *B. hybridum*, were historically combined into a single polymorphic *B. distachyon* with three cytotypes of $2n = 10$, 20, and 30. Recent work (15) has shown that *B. distachyon* sensu stricto has $2n = 10$ and (fortunately) includes the inbred line Bd21, for which a genome sequence is available. *B. stacei*, in contrast, is

morphologically similar but can be distinguished by average spikelet and anther size; spikelets average 22 mm (range 15–41 mm) and anthers average 0.6–0.8 mm long in *B. stacei*, whereas in *B. distachyon* spikelets average 16 mm (range 12.5–22 mm) and anthers average 0.5–0.7 mm (15). More importantly, *B. stacei* has $2n = 20$ chromosomes and a genome size of approximately 0.282 pg/1C, roughly comparable to that of *B. distachyon* (15). *B. stacei* is thus not a polyploid derivative of *B. distachyon*. More likely, *B. stacei* has the ancestral chromosome number, and the chromosomes of *B. distachyon* represent fusions of the small *B. stacei*–like chromosomes. The $2n = 30$ cytotype is clearly an allopolyploid derivative of an ancestor similar to *B. stacei* and one similar to *B. distachyon*, and is now known as *B. hybridum*.

The recognition of three species within the formerly broadly construed *B. distachyon* offers some insight into the relative importance of autopolyploidy versus allopolyploidy. Before cytogenetic and DNA sequence data were available, the $2n = 20$ and $2n = 30$ cytotypes of *B. distachyon* (now *B. stacei* and *B. hybridum*) were assumed to be autopolyploids of the $2n = 10$ cytotype (91, 106). However, as shown by Hasterok et al. (47, 48), both *B. distachyon* and *B. stacei* are diploids with different numbers of chromosomes, and *B. hybridum* is clearly allopolyploid. This pattern is similar to that seen in panicoid grasses by Estep et al. (23), who identified many species formerly thought to be autopolyploid that are in fact allopolyploids detectable only with cytogenetic or genomic data. Thus, *B. distachyon* and its relatives could be a useful system in which to explore the genetic and genomic processes that make allopolyploidy morphologically cryptic in some taxa.

INSIGHTS FROM *BRACHYPODIUM* RESEARCH

Genome Dynamics and Positional Cloning

Synteny and broad collinearity of grass genomes have been shown repeatedly (e.g., 18, 30, 76), and data from *Brachypodium* have helped establish both the tempo and the mode of genome rearrangement. In general, collinearity of genomes is steadily lost through evolutionary time, and thus species that are more closely related will share more collinear regions (119). A comparison of *B. distachyon*, rice, and sorghum found 69% of genes to be collinear in all three species, whereas 82% were collinear between *Brachypodium* and rice, consistent with the phylogeny. Likewise, collinearity is more extensive between barley and *Brachypodium* than between barley and rice, particularly with regard to disease resistance (R) genes (19). Wicker et al.'s (119) study is particularly useful in that it defined collinearity precisely, considering two genes to be collinear if they are "found in a syntenic chromosomal region and four out of [their] eight closest neighboring genes also have [their] closest homologs in the same location and order in other species" (p. 1,230). The well-annotated *Brachypodium* genome helped show that the apparent movement of genes could be traced to duplication of genomic regions following double-stranded break repair. After such duplication, loss of the original gene at the donor site would lead to the appearance of movement.

B. distachyon has been useful for positional cloning in wheat and other Triticeae. Faricelli et al. (25) used positional cloning to locate the gene *earliness-per-se1* (*eps1*) in the genome of *Triticum monococcum*, a diploid wheat with an A genome; *eps1* had been mapped to a region approximately 5,600-kb long in *T. monococcum*. The corresponding region in rice is 58 kb, but the *Eps* region was absent, despite collinearity on either side. Corresponding regions in *A. tauschii*, the donor of the D genome of wheat, were localized to two noncontiguous BACs. The *Eps1* region could be localized to a single *Brachypodium* BAC, approximately 117 kb, and the candidates for *Eps1* narrowed to two possible genes.

Mapping in close relatives is particularly necessary to help identify genes conferring resistance to pathogens (R genes). The gene *Sr35* for resistance to stem rust *Puccinia graminis* f. sp. *tritici*,

a major pathogen of wheat (94, 125), is absent in *Triticum urartu*, the presumed donor of the A genome of wheat. However, the gene is in *T. monococcum*, which also has an A genome. Collinearity between the two *Triticum* species and *B. distachyon* provided markers to delimit the region with the resistance gene, and ultimately to clone the gene. Likewise, markers from *B. distachyon* were instrumental in cloning the gene *Lr34/Yr18*, which confers resistance to many rusts in wheat (63, 65, 104). *B. distachyon* was also used to clone the gene *Ppd-H1*, a photoperiod response gene, in barley (112).

Despite some success in using *B. distachyon* to aid in cloning resistance genes, most efforts to do so have failed. These gene families are known to evolve rapidly in both sequence and position in the genome (68, 73, 74), and presence/absence variation is common. For example, only 16 of 495 R-gene loci were found in syntenic locations in a comparison of rice, maize, sorghum, and *B. distachyon* (73), which means that the use of synteny for chromosome walking is not always successful. NBS-LRR (nucleotide binding site–leucine-rich repeat) and F-box genes are not found in syntenic locations when comparing *Brachypodium* with rice and sorghum (51). The locus *Ror1* (*required for mlo-specified resistance1*) in barley is one of a pair of loci that enhance the effect of the *mildew resistance locus o* (*Mlo*) in conferring resistance to powdery mildew (1). It maps less than 0.5 cM from the centromere of chromosome 1H. Numerous breaks in collinearity were found between barley, *B. distachyon*, rice, and sorghum. Given the long evolutionary time separating the last two from barley, this is not a great surprise, but the extensive rearrangements in *B. distachyon* were unexpected.

The higher chromosome numbers of the perennial *Brachypodium* species hint that their genomes may be less rearranged than those of *B. distachyon*, so they could provide useful tools for map-based cloning. For example, a BAC library constructed from the perennial species *B. sylvaticum* ($2n = 18$) was used successfully to identify the *Ph* (*pairing homoeologous*) locus in wheat (38). However, *B. sylvaticum* has not yet been widely used.

Cell Walls

B. distachyon is proving to be a useful system for discovering the controls of cell wall development. A major component of all cell walls is cellulose, whose synthesis depends primarily on the protein CESA (cellulose synthase A) (102). Like rice, *Brachypodium* has 10 *CesA* genes, one set of which produces cellulose in primary cell walls and the other set in secondary walls (42). Although disruption of such a critical protein might be expected to have a severe mutant phenotype, knockdown of specific *CesA* genes with artificial microRNAs created only modest defects in the walls of xylem and fiber cells in the stems and reduced the total amount of cellulose in cell walls.

In addition to cellulose, primary cell walls include hemicelluloses, pectins, and some proteins. In the grasses and other commelinid monocots, the hemicelluloses are predominantly arabinoxylans rather than xyloglucans as in eudicots, and there is relatively little pectin and protein (13). Secondary cell walls also contain lignin, the specific structure of which is distinct in grasses. Thus, a full understanding of cell wall biology, its role in normal development, and its utility for cellulosic biofuels requires a convenient grass model.

Lignin. Lignin is a complex polymer produced by the phenylpropanoid pathway, which produces monolignols and coniferyl, coumaryl, and sinapyl alcohols. These alcohols in turn create the G (guaiacyl), H (*p*-hydroxyphenyl), and S (syringyl) subunits, respectively, of lignin. Grasses have a smaller fraction of H subunits than eudicots do (7). Late steps in the biosynthesis of lignin monomers are catalyzed by cinnamyl alcohol dehydrogenase (CAD) and caffeic acid O-methyltransferase (COMT). Genes encoding these enzymes have been identified in *B. distachyon* and are providing tools for further dissection of the pathways leading to lignin biosynthesis (109).

In the grasses and other commelinid monocots (including bananas, gingers, other Zingiberales, Commelinales, and Poales), cell walls include both *p*-coumaric acid and ferulic acid (44, 45). Before incorporation into the lignin polymer, the monolignols, particularly the syringyl units, are often acylated with *p*-coumarate. A grass-specific enzyme, first identified in rice, functions as a *p*-coumaryl:monolignol transferase (PMT1), and is a member of the benzylalcohol acetyl-, anthocyanin-*O*-hydroxy-cinnamoyl-, anthranilate-*N*-hydroxy-cinnamoyl/benzoyl-, deacetylvindoline acetyltransferase (BAHD) superfamily of acyl-CoA-dependent transferases (121). Although its function was identified initially by an in vitro assay using the rice enzyme, in planta characterization was done in *B. distachyon* using a reverse genetics approach (84). This work confirmed the function of OsPMT-BdPMT as responsible for binding *p*-coumarate to monolignols. On the basis of the phylogenetic relationships of BAHD genes, Molinari et al. (75) hypothesized these and closely related genes are all responsible for binding *p*-coumarate.

trans-Ferulic acid (ferulate) is ester-linked to glucuronoarabinoxylan (GAX), a hemicellulose that is itself unique to the commelinids. The ferulate can then link GAX to other GAX molecules or to lignin. Good candidates for enzymes creating the ferulate-GAX linkage are found in a subclade of the BAHD proteins different from that of BdPMT1. Although acyl-CoA transferase activity has not been proven for proteins in this subclade, coexpression studies are consistent with the involvement of these genes (75).

Polysaccharides. In the graminid clade of monocots, including the grasses and their closely related families (Anarthriaceae, Restionaceae, Centrolepidaceae, Flagellariaceae, and Ecdeiocoleaceae), cell wall polysaccharides include those with both β-1-3 and β-1-4 linkages, giving rise to the name mixed-linkage glycans (101). In cell walls in the endosperm, the mixed-linkage glycans are mixed with arabinoxylans, the relative proportions of which vary among pooid grasses and even among varieties of wheat (24). For example, wheat endosperm cell walls are predominantly arabinoxylans, whereas those of barley and oats are enriched in mixed-linkage β-glucans. In *B. distachyon* endosperm, approximately 60% of polysaccharides are found in the cell wall, a proportion much higher than in wheat, oats, or barley (10–20%) (39), and cellular starch content is correspondingly low. Guillon et al. (40) hypothesize that (1-3)(1-4)-β-glucan serves a storage function in *Brachypodium*.

In the endosperm, *B. distachyon* cell walls are unusually thick compared with those of wheat and barley, suggesting the possibility of a direct trade-off between β-glucan content and starch composition (110). Trafford et al. (110) investigated 32 species of Pooideae in the tribes Meliceae, Brachypodieae (i.e., *Brachypodium* species), Bromeae (i.e., *Bromus* species), Triticeae, and Poeae. All species of *Brachypodium* investigated had <20% starch in their endosperm but >20% β-glucan. In contrast, in Meliceae, Triticeae, and Poeae the numbers were >30% and <5%. *Bromus* was intermediate in value. The inverse correlation could be observed within individual grains as well, with higher starch in the proximal region near the embryo and high β-glucan in the distal region; this pattern was not seen in wheat and barley.

Grain Development

More than 50% of the world's calories are provided by the endosperm of cereal grasses. *Arabidopsis* is limited for studying endosperm development because the endosperm is a transient tissue with nutrients rapidly transferred to the developing embryo. In contrast, most members of the commelinid clade have a well-developed endosperm that persists in the seed (105). Thus, deep understanding of endosperm requires not only a monocot but a commelinid monocot.

Grain structure and endosperm formation. Because of *Brachypodium*'s close relationship to Triticeae, it has been important to characterize endosperm formation to determine similarities and differences. Provisioning of seeds is important for fitness in the wild as well as for yield in agricultural settings, so both natural and human selection are likely to have affected endosperm structure and content. Indeed, increasingly detailed comparisons are revealing intriguing differences among species.

As with other cereals that have been studied (11, 93), endosperm nuclei in *B. distachyon* begin to divide soon after fertilization, initially forming a syncytium lacking cell walls and surrounding a large central vacuole (80). Beginning with the outside layers of endosperm, the nuclei become surrounded by cell walls, a process that continues until the entire central cavity is fully cellularized, about seven days after fertilization in *B. distachyon* (40). The process of cellularization in *B. distachyon* and wheat differs somewhat, and may be worth exploring in other pooid grasses (80). At maturity, the *B. distachyon* grain is flattened in cross section, similar to many wild grasses but unlike domesticated wheat (40).

The aleurone corresponds to the outer layer of endosperm cells in the grasses as well as other angiosperms. In *B. distachyon*, the aleurone is one to three layers thick, as in rice, whereas there is only one cell layer in wheat, rye, oats, sorghum, and maize, and three in barley (22, 39, 80, 93). In wheat, the aleurone remains attached to the inside of the pericarp when the grain is crushed during roller milling (24), whereas it is attached to the endosperm in *B. distachyon* (80). Although the attachment of the aleurone is significant for milling properties of wheat grains, its significance in a natural setting is unclear. In general, the aleurone in *B. distachyon* is less clearly differentiated from the rest of the endosperm than it is in wheat (80).

In many core Pooideae, particularly Triticeae, the vascular strand on the adaxial side of the ovary projects into the nucellus and is surrounded by a set of cells known as the nucellar projection. Pooideae are described as having a linear hilum, although the term hilum is definitely a misnomer in this context. The linear hilum is shared by all pooid grasses except for the tribe Poeae, in which the hilum is punctate (36). In wheat, the projection of the vascular strand creates a structure known as the crease, which affects milling properties of grain by making the separation of the endosperm from the rest of the caryopsis difficult (24). Although in *B. distachyon* the vascular strand also extends the length of the grain, the endosperm does not develop to form a deep projection (39, 43), so *B. distachyon* is of limited use in the study of this important aspect of grain structure. The aleurone surrounding the nucellar projection is modified cytologically in wheat and barley (24), whereas in *B. distachyon* the aleurone in this position forms a continuous layer of living cells even at grain maturity and is not modified (80).

The nucellar epidermis in wheat breaks down while the aleurone differentiates (82), whereas in *B. distachyon*, as in rice, the nucellar epidermis is persistent (22). This pattern suggests that the condition in *B. distachyon* is a retained ancestral state. The nucellar cells in *B. distachyon* are connected by extensive plasmodesmata (80). In studied cereals, including *B. distachyon*, the cells of the starchy endosperm undergo endoreduplication soon after cellularization (62, 78, 92, 93). However, in *Brachypodium* endosperm, the cells do not achieve the very high ploidy levels seen in other species (110).

Composition of the endosperm. The composition of cereal endosperm was first studied by Beccari (4), who showed that it could be divided into a water-soluble fraction (starch) and a water-insoluble fraction, which he called glutinin. Starch is detectable in the *Brachypodium* endosperm approximately 13 days after fertilization (40) and continues to accumulate during grain development. The starch granules are ellipsoid and uniform in size (40, 80), in contrast to

Triticeae, in which granule size is bimodal (108). Also unlike that of Triticeae, starch constitutes only approximately 10% of the *Brachypodium* grain. The lipid content of the *Brachypodium* grain is also low compared with that of most cultivated cereals, at approximately 1.6% of the weight of the mature grain (40). Early in development of the *B. distachyon* grain, starch accumulates in the pericarp (40, 110) but later declines, presumably being mobilized for metabolic functions. Both the percentage of lipid and its fatty acid components resemble those of *Avena sativa* (oats). The percentage of lipid in wild grasses in general is unknown, so it is unclear whether this simply reflects domestication processes or natural interspecific variation.

Endosperm proteins are traditionally classified according to their solubility, with the globulins being soluble in water, albumins in salt solutions, and prolamins in alcohol (99). The major proteins in the *B. distachyon* endosperm are 12S- and 7S-type globulins (67). The 7S proteins are similar to ones in maize that are localized to the embryo and aleurone, so the location in *B. distachyon* is assumed to be the same, although this has not been tested (66). The 7S globulins are encoded by two genes (40). Within individual endosperm cells, small protein bodies surrounded by starch granules sit near the periphery of the cell; these protein bodies generally contain the globulins (40). Albumins were not detected in *B. distachyon* endosperm (66). Glutelins (salt-insoluble globulins) are found in large vacuoles in endosperm cells (40) and are encoded by four paralogous genes. Prolamins are only a minor component of the *B. distachyon* endosperm. This arrangement is similar in rice, in which prolamins are also a small component of the protein repertoire of the endosperm, but markedly different in Triticeae, in which prolamins predominate (40). Thus, *B. distachyon* may be a poor model for the study of prolamin accumulation but could be valuable for exploring regulation of differential protein accumulation in the grain.

Cold Tolerance, Vernalization, and Control of Flowering

Members of Pooideae represent a radiation into cool temperate regions, a habitat quite different from that of other grasses, which originated in warm, moist, shady environments (21, 36). For this radiation to occur, plants had to acquire tolerance for cool or cold temperatures. In *B. distachyon*, several hundred genes are upregulated in response to cold stress, whereas relatively few are downregulated (87); many of these differentially regulated genes also respond to drought and salt. The average rate of nonsynonymous substitution in genes induced by low temperatures is higher in *B. distachyon* and four core Pooideae than it is in four warm-season grasses, indicating possible positive selection and adaptation (115).

Among the well-characterized cold-responsive genes are the ice-recrystallization inhibition proteins (IRIPs), fructosyl transferase proteins (FSTs), and C-repeat binding factors (CBFs). IRIPs reduce formation of ice crystals in the apoplast (111, 123). The gene family encoding these proteins in *B. distachyon* has expanded via duplication (69), independent of a similar expansion in ryegrass, wheat, and barley (core Pooideae) (64, 95). Thus, the IRIPs in *B. distachyon* are nonorthologous to those in the core Pooideae. This is perhaps not surprising in that the reference accession of *B. distachyon*, Bd21, generally overwinters as a seed rather than as a plant. The evolution of IRIPs in perennial, frost-tolerant species of *Brachypodium* would be of interest, as is the evolution of the corresponding genes in the genera of Pooideae that diverged before *Brachypodium* (e.g., *Brachyelytrum*, *Nardus*, *Diarrhena*, many Stipeae and Meliceae), most of which are perennials and all of which occur in regions with cold winters.

As with IRIPs, FSTs are associated with responses to cold in members of core Pooideae. These proteins create polymers of fructose molecules from sucrose (70), and are upregulated in response to cold treatments in oats (71), bluegrass (89), and wheat (57). However, orthologous proteins are

not found in *B. distachyon* (69), indicating that *B. distachyon*—at least the reference accession—is not a useful model for this aspect of cold response.

CBFs are transcription factors with an AP2/EREBP domain that binds to so-called C-repeats (CCGAC) in the promoters of genes involved in response to low temperatures and drought stress (e.g., 41, 52, and references therein). The gene family has expanded greatly in the grasses (e.g., 100) compared with that in *Arabidopsis* (33, 41). The history of these genes is complex, with some clades apparently expanded in *B. distachyon* independent of those in the core Pooideae (69).

Associated with the shift of the pooid lineage to cooler climates is a requirement for vernalization, which has been found in some *B. distachyon* accessions and many core Pooideae (27, 98). Bd21, the source of the reference genome, requires no vernalization, whereas many other accessions do require a period of cold. Fjellheim et al. (27) suggest that investigation of perennials may be critical for understanding the evolution of the controls of flowering time in the grasses.

Expansion into temperate zones is associated with changes in day length requirements. *B. distachyon* flowers rapidly during long days, whereas flowering is considerably delayed during short days (98). However, accessions of *B. distachyon* vary in their response to day length. *B. distachyon* contains two loci, FTL1 and FTL2, annotated as orthologs of Flowering Locus T, or florigen. Both are cleaved by miR5200 (122), a microRNA that has been identified only in *B. distachyon*, wheat, and barley.

Development

In all pooid grasses except *Brachyelytrum*, the primary branches in the inflorescence initiate in a distichous phyllotaxis (60) (**Figure 2**). This is in contrast to most other grasses, in which the transition to flowering is accompanied by a change in phyllotaxis of the shoot apical meristem from distichous (the phyllotaxis of the leaves) to polystichous. The developmental retention of distichous phyllotaxis thus originated after the divergence of *Brachyelytrum* (**Figure 1**, branch 2). The genetic basis of this change is unknown, but *B. distachyon* would be a good system in which to determine it. The inflorescence of all species of *Brachypodium* is unbranched, with the multi-flowered spikelets on short pedicels attached to the main inflorescence axis (58). The axis terminates in a spikelet and spikelet maturation is basipetal (from the top downward) (60) (**Figure 2**).

A novel developmental gene that characterizes all angiosperms except Brassicaceae was recently identified and characterized in *B. distachyon*, illuminating details of auxin-related patterning in the inflorescence (79). PIN-FORMED1 (PIN1) is an auxin efflux carrier that controls formation of auxin gradients and thus many aspects of plant development (83, 120). A phylogenetic analysis showed that PIN1-like genes in the grasses were duplicated in the well-documented whole-genome duplication at the origin of the family to produce the paralogs PIN1a and PIN1b. Unexpectedly, however, O'Connor et al. (79) discovered another locus, *Sister of PIN1* (*SoPIN1*), which is shared by all angiosperms except Brassicaceae and which proved central to understanding auxin distribution. Marker lines were created with fluorescent-labeled proteins that could be followed during development of the *B. distachyon* inflorescence. SoPIN1 is deployed to define auxin convergence points and thus appears to be responsible for organ initiation, whereas the grass duplicates of PIN1 (PIN1a and PIN1b) are implicated in vein patterning. A computational model of the action of PIN1a, PIN1b, and SoPIN1 resolves a paradox in the various functions of the PIN proteins and promises to illuminate development in most other angiosperm systems. Thus, the role of SoPIN1 is likely to be central to angiosperm development, but its function is characterized only in *B. distachyon*.

CONCLUSIONS

In summary, *B. distachyon* is proving to be a tractable and highly useful model for a variety of biological processes, some of which are unique to the grasses, to the subfamily Pooideae, or to clades within the subfamily. We have outlined the available tools to aid positional cloning in the large genome cereals in Triticeae. But perhaps even more exciting in the long run will be functional studies on the following:

- protein and starch production in the endosperm and the controls of the different components
- cold tolerance
- vernalization requirements and controls of flowering
- floral development and number of flowers per spikelet
- vein patterning
- cell wall components and their biosynthesis
- controls of the perennial or annual habit
- genome organization

Information cannot be simply extrapolated from eudicots for any of these characters. In many cases, the genes themselves are unique to grasses or specific clades within the family. Although some evolutionary biologists have argued that all taxa share the same genetic tool kit and differences are largely regulatory (e.g., 14), the many studies cited above show that grasses are not simply differentially regulated *Arabidopsis*. Novel genes with unique functions are involved in controlling unique and important biological processes. In addition, the pooid grasses share distinctive characteristics, including lack of bicellular microhairs, distinctive embryo morphology, loss of vascularization in the lodicules, parallel-sided subsidiary cells, and particular chromosomal arrangements, whose regulation and function are completely unknown. At the moment, these characteristics are simply observations without functional context, but it seems likely that they have some significance yet to be discovered. In other words, to paraphrase the quote from E.O. Wilson that begins this review, there exists a set of phenomena for the study of which *B. distachyon* is ideally suited.

SUMMARY POINTS

1. *B. distachyon* has an impressive set of genetic tools.
2. The genome of *B. distachyon* is broadly syntenic with that of rice but is generally more similar to that of the Triticeae and cool-season grasses (tribes Bromeae and Poeae).
3. *Brachypodium* originated before the major genomic revolution that characterizes the core Pooideae.
4. *B. distachyon* can help positional cloning in core Pooideae, especially Triticeae.
5. *B. distachyon* is a model for understanding the biology of the cell walls of grasses, which are distinct from those of eudicots.
6. *B. distachyon* provides a model for regulation of endosperm development and chemical composition.

7. *B. distachyon* can be used for the dissection of the genetic controls of frost tolerance, although it is distinct from the core Pooideae in some aspects and may not be a substitute for looking at a truly frost-tolerant species, including perennial species of *Brachypodium*.
8. *B. distachyon* is a model for angiosperm developmental morphology.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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