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Feature Review

Brachypodium: 20 years as a grass biology model system; the way forward?

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It has been 20 years since *Brachypodium distachyon* was suggested as a model grass species, but ongoing research now encompasses the entire genus. Extensive *Brachypodium* genome sequencing programmes have provided resources to explore the determinants and drivers of population diversity. This has been accompanied by cytomolecular studies to make *Brachypodium* a platform to investigate speciation, polyploidisation, perenniality, and various aspects of chromosome and interphase nucleus organisation. The value of *Brachypodium* as a functional genomic platform has been underscored by the identification of key genes for development, biotic and abiotic stress, and cell wall structure and function. While *Brachypodium* is relevant to the biofuel industry, its impact goes far beyond that as an intriguing model to study climate change and combinatorial stress.

Brachypodium: how it started

At the turn of the millennium, the plant science world was different. *Arabidopsis thaliana* (arabidopsis) was one of the major discovery workhorses based on the use of forward and reverse genetic approaches [1] and the genomes of arabidopsis and rice (*Oryza sativa*) were sequenced around that time [2,3]. However, the strategies used would not lend themselves to the sequencing of larger genome cereals that typically are rich in repetitive DNA. *Brachypodium distachyon* is phylogenetically related to the temperate cereals but has a small and compact nuclear genome with a low amount of repetitive DNA, which led to it being suggested as a genomic bridge to facilitate the assembly of larger genome cereals and as a functional genomics platform [4]. Driven by support from the US Department of Energy, the *B. distachyon* whole genome sequence was generated and it emerged as a bioenergy model, spurring significant interest in cell wall research [5]. The high collinearity of grass genomes [6] is in contrast to dicots, which do not show the same level of macrosynteny [7], and allowed *B. distachyon* to make a significant contribution to genome assembly in a range of species and was pivotal for the functional annotation of genes and proteins [8]. Thus, the *B. distachyon* **reference genome** (see Glossary) was an indispensable resource for mapping and cloning many important genes from the complex polyploid wheat genome (e.g., [9–13]). Moreover, the highly accurate *B. distachyon* genome played a vital role as a scaffold for the assembly of the barley [14] and wheat family (e.g., *Triticum urartu*-2x (AA) [15], *Aegilops tauschii*-2x (DD) [16], *Triticum durum*-4x (AABB) [17], *Triticum aestivum*-6x (AABBDD) [9,18] genomes. Since 2010, *B. distachyon* genomic resources have been used for comparative genomics, functional genomics, and comparative developmental analysis in, for example, other grasses (foxtail millet [19], sugar cane [20], *Panicum hallii* [21]).

Interest in *Brachypodium* has now expanded to the entire genus [8]. *B. distachyon* was initially considered to exist as three cytotypes of 2n = 10, 20, and 30 chromosomes, but these were later classified as three different species, respectively, *B. distachyon*, *Brachypodium stacei,* and *Brachypodium hybridum* [22]. The latter was shown to have arisen from allotetraploidisation events

Highlights

Brachypodium offers a unique combination of biological, ecological, phylogenetic, and cytological features. Many features of this model genus depend on its nondomesticated status.

These features are combined by well-developed resources such as diverse germplasm collections, sequenced genomes and pangenomes, and extensive mutant collections.

Brachypodium provides a solid foundation for diverse research on plant biodiversity, speciation, genome organisation at the cytomolecular level, cell wall organisation, response to abiotic and microbial interactions, including beneficial associations. These features are relevant to understanding ecological responses to environmental change.

Thus, despite improvements in genomic sequencing technologies and gene editing technologies in large-genome cereals, *Brachypodium* retains its value as a model nondomesticated grass genus.

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between *B. distachyon* and *B. stacei*. Now multi-omic resources are available for other *Brachypodium* species offering opportunities to investigate a range of processes, such as perenniality, that are not easily studied in other systems [8]. Therefore, 20 years after the publication of the first paper to systemically assemble a case for *B. distachyon* as a model grass [4], ongoing developments are underlying the continuing relevance of the genus to plant science. In this review, we highlight some significant avenues of ongoing *Brachypodium* research and concentrate on insights being gained into genotypic diversity, responses to environmental stress, and the structure and function of the cell wall. These subjects are directly relevant to research assessing the potential impact of climate change or responding to this challenge.

Brachypodium: a model to study biodiversity and speciation in grasses

The *Brachypodium* genus is quite small (~18 recognised species) and has emerged as one of the best test beds for investigating natural diversity, evolution, and speciation in plants [23] and, crucially, environmental adaptation (Box 1). The small genome sizes of the various *Brachypodium* taxa with low repetitive contents [22] have facilitated the assembly and annotation of reference genomes for nearly one-third of its species (https://phytozome-next.jgi.doe.gov/).

Further, **pangenomes** for the three annuals, *B. distachyon*, *B. stacei,* and their derived allotetraploid *B. hybridum*, as well as the perennial *Brachypodium sylvaticum*, have been generated, which

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Box 1. Exploiting genetic diversity of *Brachypodium* to study locally adapted traits

With grasses covering more than 40% of the world land area, they naturally play a pivotal role in the function of a range of agroecosystems. Hence, characterising how this ubiquitous plant family adapt to challenging environmental conditions is of prime interest for fundamental and applied research. As it occurs naturally in the circum-Mediterranean region [23,31], which is a recognised hotspot for climatic change [142], *Brachypodium* is well placed to be the pre-eminent model to study how grasses may adapt to arid and oligotrophic habitats. After years of collaborative work, the genotypic tools are available to do this with a large diversity panel of genotypes spanning from Spain to Iraq (Figure I) [27,36–78], enabling better comprehension of population structure in *B. distachyon* and its drivers. As of today, five genetic clades whose divergence backdates to the upper Pleistocene have been described (Figure I) [37]. By exploiting niche modelling, genome-wide association studies, and genome-wide scans of selection analyses, recent works have defined how selection shaped genetic diversity at multiple temporal and spatial scales [31,143–145] and identified genes under selection and involved in climate adaptation [143–145]. This ever-growing diversity panel will undoubtedly allow further dissection of how gene–environment interactions shape fitness-related traits in *B. distachyon* and, therefore, more widely in grasslands. For instance, there is still an active debate on whether the difference in flowering times is an important driver of *B. distachyon* population structure and the relative contribution of stressors such as drought tolerance [37,38,78,146]. In this context, exploring diversity of flowering genes [147–151] in the diversity panel will allow testing of whether flowering time evolved through neutral processes or selection. Newer possible research avenues could be the relationship between stomata development [152,153] and their relationship to the environment, which could define stress resilience traits of agronomical interest.
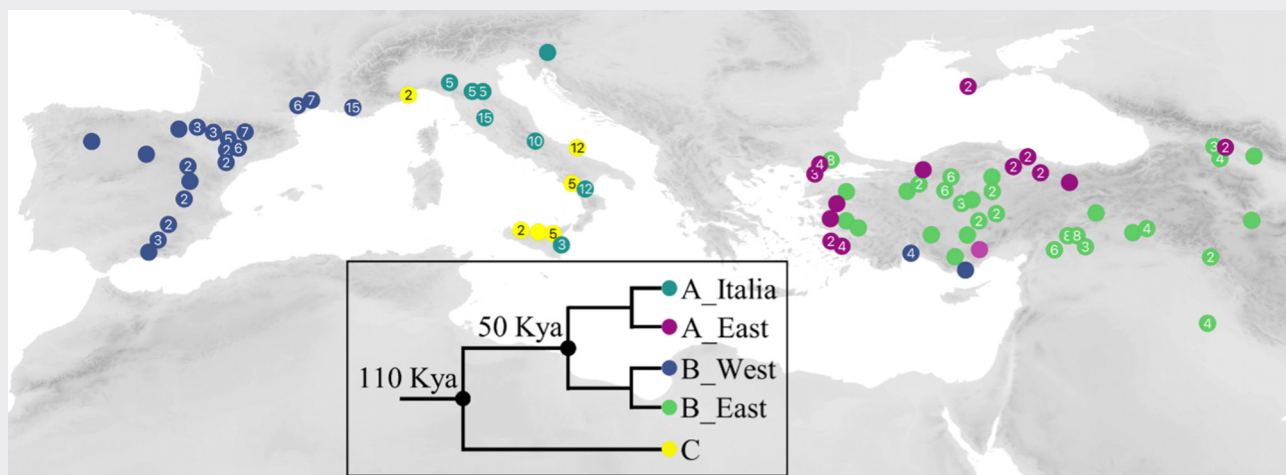
Figure I. *Brachypodium distachyon* as a model to study adaptation to local environments. Distribution and phylogeny of the sequenced genotypes (modified from [37]). Numbers indicate how many accessions were sampled per site (dots without numbers indicate that one accession was sampled from that site).

represents an enormously valuable resource in defining the origins and consequences of plant polyploidy and perenniality [24]. Important features such as **dysploidy**, **recurrent allopolyploidisation,** and extended **reticulation** [25] have made this genus an ideal model to identify the known and unknown diploid progenitor genomes of its polyploid species. Research into these could ultimately define generic features governing these processes. The basis for such studies are six polyploid species and cytotypes (*B. hybridum*-4x, *Brachypodium mexicanum*-4x, *Brachypodium boissieri*-6x, *Brachypodium retusum*-4x, *Brachypodium phoenicoides*-4x, *Brachypodium rupestre*-4x), which were shown to harbour three known and four **orphan ('ghost') progenitor subgenomes** (Figure 1A) [26]. Most important are reference genomes of accessions of the three annual *Brachypodium* species, *B. distachyon* Bd21, *B. stacei* ABR114, and *B. hybridum* ABR113 [5,27], which correspond to type specimens with distinctive morphological features and ecological traits [22,23]. Research has allowed their divergence, hybridisation, and polyploidisation events to be defined in other genera/lineages. This has included the analysis of artificially recreated autotetraploid progenitor species and allotetraploid *B. hybridum*-like lines [28]. Pangenomic and coalescence dated analyses detected multiple and bidirectional origins for the wild allotetraploid *B. hybridum*, with ancestral [*B. distachyon*-type (D) plastotypes] and recent [*B. stacei*-type (S) plastotypes] allotetraploids, originating 1.4 and 0.4 Ma, respectively (Figure 1B) [27]. These two different hybridisation and genome doubling events reflected a gradual polyploid genome evolution. Recent hybrids showed progenitor species subgenomes highly collinear with current *B. distachyon* and *B. stacei* genomes and old hybrids showing few rearrangements. This would not align with the 'polyploid genomic shock' hypothesis where allopolyploidisation has been suggested to lead to transposable element (TE) mobilisation and genome reshuffling in some plants [29]. Repetitive k-mer class analysis has indicated k-mers present only in the nuclear D and S subgenomes of the ancient hybrids and absent from both the remaining genomes of progenitor species and subgenomes of recent hybrids (Figure 1B) [27]. This suggested an exchange of k-mers between the two progenitor subgenomes in the old hybrids and postpolyploidisation evolution. This stated, the failure of these two types of stable allotetraploids to interbreed [27] may be indicative of a reproductive isolation barrier between these two potentially cryptic species. Thus, recurrent allopolyploidisations need not lead to the same speciation outcome but could end in separate polyploid species, as observed in other plant polyploids [30].

Several population-level studies have been conducted in *Brachypodium* that link ecology to phylogenetics. The mesic and drought-avoider *B. distachyon* differs from the aridic *B. stacei*. In contrast, the drought-escaper *B. hybridum* ecologically resembles its *B. stacei* progenitor species, although its climate niche overlaps with both parents whilst being statistically distinct [31,32]. This aligned with a niche competition hypothesis [33] that suggests the favouring of the newly formed allotetraploids in non-native habitats. Nonetheless, population analyses indicate that climate niche parameters and functional and phenotypic eco-physiological responses to environmental stress are more genotype-dependent within each species [32,34,35]. **Phylogenomics** of the pangenome has revealed up to five highly divergent Mediterranean lineages, which split from the common ancestor 0.16 Ma [27,36,37], showing some signs of geographic structure coupled with frequent long-distance dispersals. However, clear signatures of genome admixture and chloroplast capture between the highly diverging *B. distachyon* lineages [36,38] indicate that, despite its highly selfing nature, introgression is still acting within this species, preventing speciation [36]. Nonetheless, the extraordinary natural diversity of *B. distachyon* and its robust pangenome has served to launch a variety of studies into the sources of variation. These include potential interlineage isolation caused by different flowering times [36], the influence of the individual TEs in the genetic and epigenetic constitution of the host genomes [39] (Box 2), and drought stress [34]. Such studies illustrate ongoing intraspecific evolution of this *Brachypodium* species and how shifts in biodiversity could show the impact of environmental change.

Brachypodium to study plant genome organisation at the cytomolecular level

One near unique feature of *Brachypodium* research is the key role that cytomolecular analyses have played and demonstrated the importance of such approaches in understanding speciation along with genome sequence assessments. Thus, *Brachypodium* karyotype structure and evolution has been extensively elucidated using **fluorescence *in situ* hybridisation (FISH)** with chromosome-specific low-repeat BAC clones as probes, known as **chromosome painting (CP)** or, sometimes, chromosome barcoding [27,40,41]. Together with phylogenomics, this approach identified some putative segmental allopolyploids and orphan genomes of some likely extinct ancestors in *Brachypodium* [41]. Although many kinds of DNA sequences make useful FISH probes [42], CP is particularly informative due to its high specificity (Figure 2A,B). The recent advent of single-copy oligonucleotides as probes (oligo-FISH) [43] made CP technically feasible in more plants, enabling comparative analyses not only of those with small genomes, like rice [44] but, to some

Reference genome: a fully assembled and annotated genome of an organism (including its nuclear and organellar genomes in eukaryotes) that serves as a reference for the species or taxon.
Reticulation: derivation of a lineage from the total or partial merging of two ancestral lineages.
Shell genes: genes that are shared by most or several of the genomes in a pangenome.
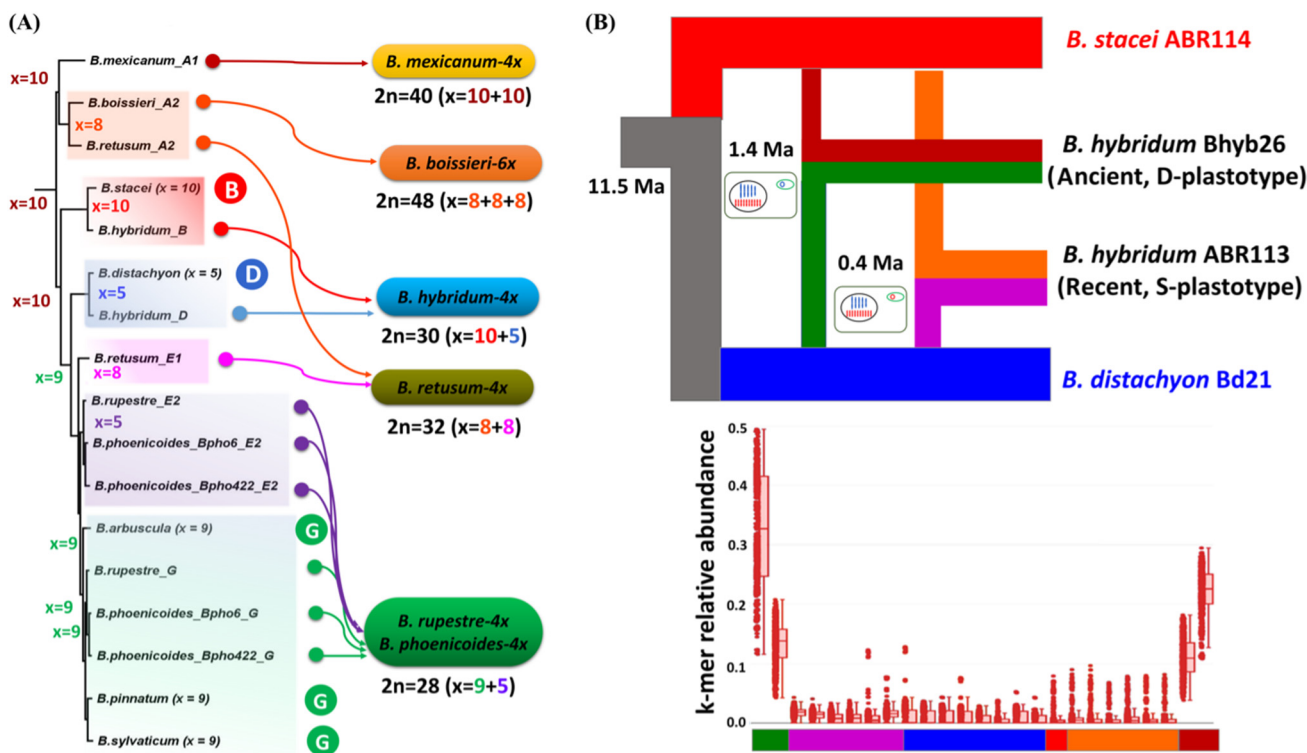
Figure 1. Inferred evolutionary speciation events of annual and perennial *Brachypodium* species. (A) Comprehensive evolutionary framework for the origins of *Brachypodium* diploids and polyploids based on the combined phylogenomic and comparative chromosome barcoding analyses [26]. Colours indicate the different types of (sub)genomes retrieved in the phylogenomic analysis using 322 gene-based phylogeny and subgenome detection algorithms; letters designate the karyotype profiles found in the diploids and polyploids. Allopolyploid species show different types of known (B, D, G) or orphan (A1, A2, E1, E2) diploid progenitor subgenomes [*Brachypodium hybridum*-4x (B, D), *Brachypodium retusum*-4x (A2, E1), *Brachypodium phoenicoides*-4x (E2, G), *Brachypodium rupestre*-4x (E2, G)], whereas putative autopolyploid (or segmental allopolyploid) species show the same type of orphan progenitor subgenome [*Brachypodium mexicanum*-4x (A1A1), *Brachypodium boissieri*-6x (A2A2A2)] (modified from [26]). (B) Evolutionary allopolyploidisation scenarios for the origins of the two types of *B. hybridum* lines; ancient *B. hybridum* (reference genome Bhyb26, showing maternal plastome D inherited from *B. distachyon*) and recent *B. hybridum* (reference genome ABR113 showing maternal plastome S inherited from *B. stacei*) were formed from distinct hybridisation and genome doubling events 1.4 and 0.4 Ma, respectively. Stylised plant cell diagrams with colour coding indicating the origins of the plastomes (circles) and nuclear genomes (haploid chromosome numbers) of ancient and recent *B. hybridum* lines (*B. distachyon*-type D genomes, blue; *B. stacei*-type S genomes, red). The datings were inferred from the coalescence-based and cross-bracing analyses of Gordon *et al.* 2020 (modified from [27]). Abundance of a repeat k-mer class that expanded in the D and S subgenomes of the ancient but not the recent hybrids, nor in the progenitor genomes, indicates a postpolyploidisation evolutionary novelty. Genomes and subgenomes are indicated by their respective colour codes in the bar below the x axis and correspond to those indicated in the above cladogram: from left to right, ancient *B. hybridum* D subgenome (green), recent *B. hybridum* D subgenome (purple); *B. distachyon* D genome (blue); *B. stacei* S genome (red); recent *B. hybridum* S subgenome (orange); ancient *B. hybridum* S subgenome (brown) (modified from [27]).

Box 2. *Brachypodium* to study transposable elements and genome dynamics

Despite its small genome size, *Brachypodium distachyon* constitutes an ideal system to investigate genome and, more specifically, transposable element (TE) evolution. The near base perfect genome sequence of the reference Bd21 genotype has allowed the comprehensive annotation of the TEs [5,39]. As with many plant genomes, TEs in *B. distachyon* are represented mainly by LTR-retrotransposons [39], but their distribution along the chromosomes are much more pervasive than in Brassicaceae [154]. Their detailed characterisation revealed that many families display ongoing transpositional activity [39,155] and suggested that TE dynamics mediate **shell gene** genesis and regulation in this species [36]. However, methylation spreading around TEs is limited in *B. distachyon* [154,156], implying that the impact of TEs on nearby gene expression might involve additional regulatory mechanisms. Assessing TE insertion polymorphisms (TIPs) in genomes originating from Spain and Turkey further suggested that TE dynamics is shaped by a complex interplay between purifying selection and population demographic history in the wild [155]. Although relatively scarce in genic regions, TIPs also alter nearby gene expression and may contribute to accession-specific regulation patterns [154]. Therefore, with the current development of a large diversity panel spanning from Spain to Iraq (Box 1), *B. distachyon* is well-established as a model to understand plant genome evolution in changing environments.

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extent, also in previously intractable large-genome cereals, such as some Triticeae [45]. However, future research in *Brachypodium* is likely to focus on chromosomal localisation, genomic distribution, and the abundance of various satellites and TEs (Figure 2C1,C2). Repetitive DNA plays an essential role in organising any eukaryote genome and contributes to chromosome segregation, chromosome end protection, karyotype evolution, and reproductive isolation among species [46,47]. Recently, Li *et al.* [48] provided the first comprehensive insight into the cytomolecular characterisation of *Brachypodium* centromeres, the most repetitive chromosome regions [5]. Based on the presence or absence of *B. distachyon*-originated centromeric retrotransposons, species were classified into two distinct lineages. It will be interesting to test if the composition and organisation of various repeats within *Brachypodium* links to previous phylogenetic studies made using different approaches.

The spatial arrangement of chromosomes inside the interphase nucleus is another fascinating field due to its importance for gene regulation and genome stability [49], which could differ in responses to environmental change. Early studies in mammals revealed that individual decondensed chromosomes occupy **chromosome territories (CT)** (e.g., [50]). Their structure and distribution vary among vertebrates and often correlate with a given chromosome size and, in particular, its gene density [51,52]. Corresponding studies in plants are scarce, with the first demonstrating a predominantly random arrangement of CT in arabidopsis [53] but may depend on morphological features of a nucleus [54] that are conserved between related species [55]. Until only recently [56], *B. distachyon* was the only monocot for which the interphase arrangement of individual chromosomes had been studied. Using 3D preserved nuclei isolated from roots, four kinds of interactions between homologous CTs were observed, the frequency of which depended on morphometric parameters of chromosomes and the nuclear shape [57]. For example, the complete association of homologues was the most common for CTs of the large and metacentric chromosomes, chromosome Bd1 in particular. In contrast, in considerably shorter and more asymmetric chromosomes, such as Bd4, CTs were usually completely separated (Figure 2D,E). Whether CT arrangement varies among different organs or different representatives of the genus and could change with shifts in biodiversity with environmental change, remain open questions.

A novel spin-off from the studies on *B. distachyon* CTs concerns spontaneous or induced plant nuclear genome instability. Many physical and chemical agents cause micronuclei (MN) formation and assessing their frequency was commonly used to test genotoxicity [58]. It is probable that such stress could also be imposed as a result of climate change [59]. However, until our studies [60,61], there was no [62,63] or only a little [64] information about the MN composition. Our CP-based approach enabled better elucidation of mechanisms of their formation and, more indirectly, allowed us to infer on diverse susceptibility of specific genomic regions to genotoxic agents

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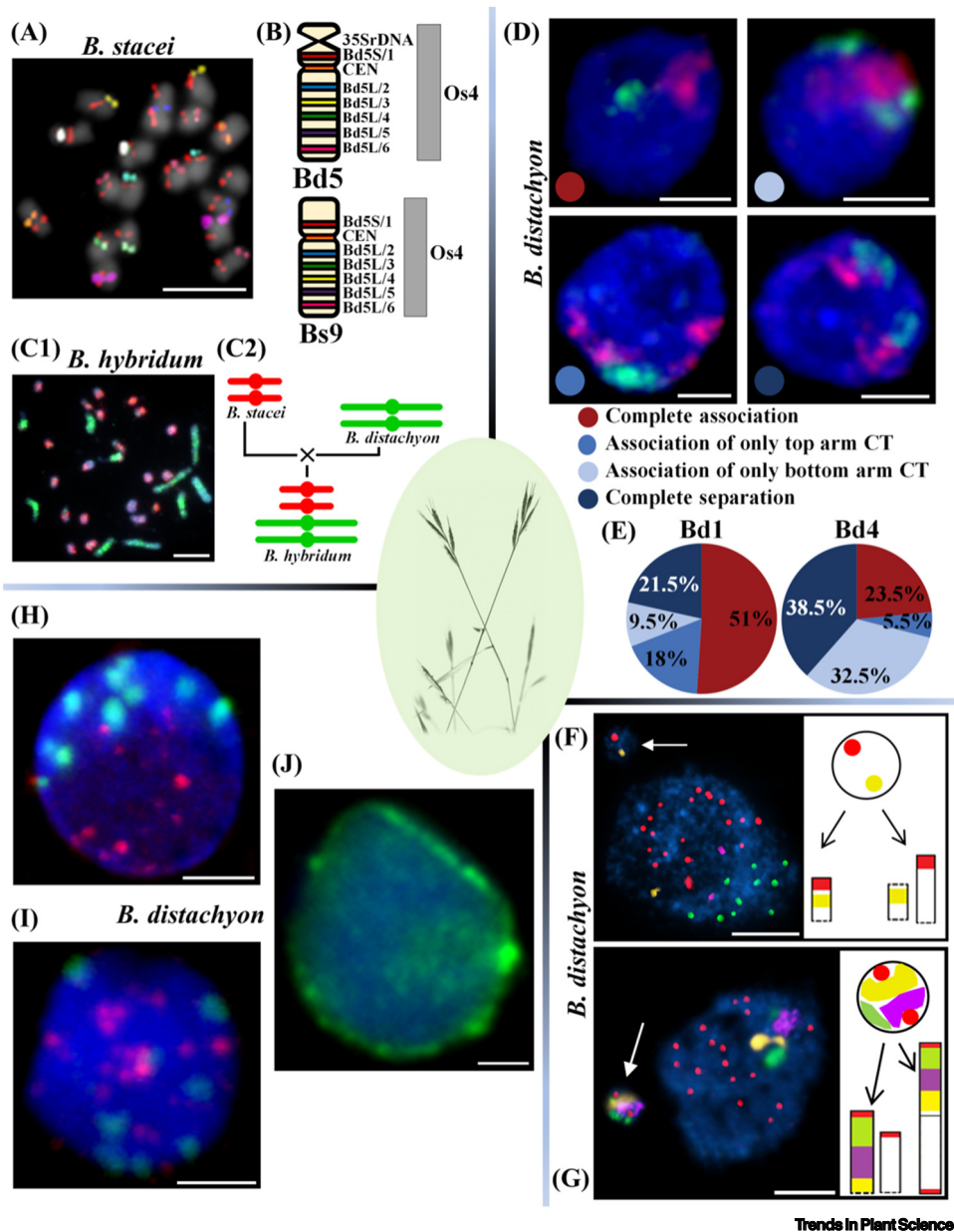
Figure 2. A simplified overview of main directions of recent cytomolecular research in *Brachypodium*. (A) Chromosome painting (CP) in *Brachypodium stacei* using six low-repeat BACs, the centromeric BAC, and two universal (5S and 25S) rDNA sequences as probes (adapted from [40]). (B) All BACs originate from *Brachypodium distachyon* genomic libraries, but they hybridise with chromosomes of other *Brachypodium* species, allowing dissection of the structure of individual chromosomes across the species, as exemplified by comparing chromosome Bd5 of *B. distachyon* and Bs9 of *B. stacei*. The chromosomes in extant *Brachypodium* species can be linked to so-called ancestral rice chromosome equivalents, Os4 in the exemplified case, enabling us to infer the evolution of entire karyotypes (modified from [27]). (C1) Dual-colour fluorescence *in situ* hybridisation discrimination of the *B. distachyon* (green) and *B. stacei* (red) subgenomes in *Brachypodium hybridum* using Bd genome-specific clone ABR1-63-E6 and Bs genome-specific clone 8P20, respectively. (C2) Schematic visualisation of *B. hybridum* subgenome discrimination shown in (C1) (both modified from [42]). (D) Four scenarios of interactions between homologous chromosome territories (CT) in root-tip interphase nuclei of *B. distachyon*, as demonstrated by CP using Bd4 short-arm- (green) and Bd4 long-arm-specific probes (modified from [57]). (E) Different frequencies of various interactions between homologues are shown for Bd1 and Bd4 chromosomes (modified from [57]).

(Figure legend continued at the bottom of the next page.)

(Figure 2F,G) [60,61]. In practical terms, it creates the opportunity for *B. distachyon* to become a standard in plant mutagenesis or in assessing environmental stress.

An intriguing aspect of 3D nuclear architecture is linked with the distribution of telomeric and centromeric domains. One well-known pattern is shown when all telomeres and centromeres are localised on opposite poles of the interphase nucleus. This so-called **Rabl configuration** is a natural consequence of the chromatid orientation at anaphase but is usually exhibited only in plants with large genomes and may act to reduce chromatin entanglement [65,66]. However, the studies in *Brachypodium* revealed that most of the root-tip nuclei in *B. distachyon* display the Rabl configuration (Figure 2H), while not in *B. stacei* and *B. hybridum* [67], despite small genomes in all species [22]. Furthermore, in *B. distachyon*, the proportion of the Rabl- to non-Rabl-arranged root-tip nuclei seems linked with nuclear shape and cell cycle phase. It cannot be ruled out that the Rabl configuration is also tissue/organ-specific since the leaf nuclei in *B. distachyon* do not display the polar arrangement of their telomeres and centromeres (Figure 2I) [67]. It is unknown why *B. hybridum* inherits the non-Rabl configuration found in the root-tip nuclei of *B. stacei* and not the Rabl configuration found in their counterparts in *B. distachyon*. Addressing this question could involve the 3D analysis of interphase nuclei using genome-specific probes, which efficiently cover the chromosomes at their entire length (Figure 2C1,C2) [42] together with telomeric and genome-specific centromeric probes [48]. The recent development of chromosome conformation capture techniques [49], Hi-C in particular [68], provides an attractive alternative to the cytomolecular approach in this respect. Alternatively, it may be that a fluorescence protein-tagged **CRISPR** system with deficient Cas9 could be used to track the telomeres *in vitro*, as demonstrated in *Nicotiana benthamiana* [69]. This would provide unprecedented insight into the dynamics of these particular chromosome domains by adopting both telomeric and (peri)centromeric sequences. However, the attempts to visualise telomeres in arabidopsis have not proven successful [70], so that live-cell CRISPR imaging could be problematic in small-genome plants. Considering underlying mechanisms, key nuclear envelope components (e.g., SUN proteins, Figure 2J) could be guiding the distribution of telomeric and centromeric regions during meiosis and at premeiotic interphase [71,72]. *Brachypodium* represents an excellent platform to assess the role of various nuclear envelope proteins through the availability of an efficient CRISPR-Cas9-based mutagenesis protocol [73] to dissect the key determinants of nuclear architecture, including the Rabl configuration.

Brachypodium to elucidate plant responses to environmental stress

As a nondomesticated plant genus, growing in climate change hotspots, *Brachypodium* lends itself to research into the mechanisms of responses to stress. This could be used for information on the development of new stress-tolerant major crops but also to report on the impact of environmental stress. Environmental change is likely to increase abiotic stresses such as drought, heat, and salinity in plants as well as changing pathogen dispersal patterns [74].

(F) The micronucleus (MN; marked by arrow) in *B. distachyon* root-tip cells shows the presence of one centromeric (red) and one 25S rDNA (yellow) signal. (G) The composition of MN in the same species was visualised using the centromeric probe (red) and pools of low-repeat BACs, specifically painting the distal (green), intercalary (purple), and proximal (yellow) segments of the top arm of chromosome Bd1. Diagrams on the right show putative ways of the MN formation (both adapted from [60]). (H) Spherical nucleus of *B. distachyon* root tip showing the Rabl configuration of centromeres (green) and telomeres (red). (I) Spherical nucleus of *B. distachyon* leaf without the Rabl configuration (both modified from [67]). (J) Immunolocalisation of the SUN1/2 nuclear envelope proteins (green) in the *B. distachyon* leaf nucleus. Chromatin stained with DAPI [blue, grey in (A)]. All bars, 5 µm. Photomicrograph (C1) is courtesy of Joanna Lusinska, (H–I) courtesy of Ewa Robaszkiewicz, and (J) courtesy of Dominika Idziak-Helmcke (Plant Cytogenetics and Molecular Biology Group, Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Poland). The photograph of the *B. distachyon* plant is courtesy of Anthony Pugh and Gwen Jenkins (Aberystwyth University, UK). Clone 8P20 is courtesy of Boulos Chalhoub (Institut national de la Recherche agronomique (INRA), Université d'Evry Val d'Essonne (UEVE), Evry, France, Institute of Crop Science, Zhejiang University, Hangzhou, China).

Although the literature describing drought responses in plants is extensive, *Brachypodium*-based research has made a contribution. At its most basic, an examination of *Brachypodium* inbred lines grown under range of soil % moisture contents indicated a nonlinear relationship, so simple pairwise comparisons between droughted and fully watered plants could be insufficient [75]. The variation in responses to drought in *Brachypodium* accessions has been assessed [34,76,77] and, in one case, the degree of drought tolerance related to rainfall in the original sampling sites [78]. The transcriptomic and metabolomic responses of *B. distachyon* to drought have been characterised and, amongst many changes, suggested the importance of the cell wall [76,79]. A proteomic approach described a network of drought-responsive proteins in leaves and roots and this highlighted changes in the bioenergetic and stress metabolism but also the cell wall [80]. *B. distachyon* is also an ideal test bed to identify and characterise the roles of genes linked to drought responses. Recent examples have included a large family of late embryogenesis-abundant (LEA) proteins and their responses to the important drought hormone abscisic acid (ABA) [81]. The impact of ABA on epigenetic changes under drought was revealed by overexpression of the histone deacetylases, BdHD1 (Bradi3g08060) [82], which interacts with the drought-responsive transcription factors, BdWRKY24, and BdMYB22 [83]. Drought stress generates many harmful metabolites and their detoxification by glutathione-*S*-transferases (GST) can be associated with tolerance. The small *B. distachyon* genome encodes a surprisingly large number of GST (a similar number to wheat) and it may be that BdGSTF8, BdGSTU35, and BdGSTU42 were influencing the relative tolerance to osmotic stress [84]. A link between physiology and drought tolerance was made in *B. distachyon* when the microtubule protein MAP20 was shown to be important in the function of pits in the cell walls of plant tracheary elements Here, researchers worked initially on the hybrid aspen (*Populus tremula* × *Populus tremuloides*), but when investigating tMAP20 in an annual species, the functional genomic properties of *B. distachyon* proved to be useful [85]. Other studies have adopted *Brachypodium* for similar reasons to characterise common responses to a wide range of abiotic stresses; for example, mechanical stress [86], heat shock proteins [87], the cystatin protease family [88], or phospholipase C genes [89]. With an increased appreciation of the need to undertake combinational stress studies, the value of relatively small *Brachypodium* plants as a valuable platform to assess such complex traits will surely increase.

The utility of *B. distachyon* in characterising responses to biotic stresses in cereals was one of the first suggested targets for research [4]. A range of important *Brachypodium*-based pathosystems have been developed and focused on *Magnaporthe oryzae,* the causal agent of rice blast disease [90], a range of rust species (*Puccinia graminis, Puccinia striiformis*, *Puccinia triticina*) [91,92], root rot caused by *Rhizoctonia* [93], head blight-forming *Fusarium* species [94], *Parastagonospora nodorum* causing *Septoria nodorum* blotch [95], or barley stripe mosaic virus [96]. Important development includes the definition of single, major gene-related host resistance [91,96], whilst resistance wheat-adapted *P. striiformis* f. sp. *tritici* was mapped to two loci, *Yrr1* and *Yrr2* [97]. Single gene-mediated resistance is notoriously ephemeral under field conditions. Therefore, it is of considerable importance that a recent study has dissected one example of multigenetic, non-host resistance [98]. Screening a population of recombinant inbred lines allows the differential responses to wheat stem rust (caused by *P. graminis* f. sp. *tritici*) to be rationalised into six quantitative trait loci. Grass genomic synteny could aid the translations of such findings into temperate cereals, as demonstrated when the synteny between wheat and *B. distachyon* not only allowed the fine mapping of resistance against powdery mildew (*Blumeria graminis*), but its introgression into bread wheat from a wild relative [99]. Equally, mutants of *B. distachyon* can be screened for increased susceptibility to non-host pathogens as with yAL6.2, which was mapped to *TIME FOR COFFEE* (*TIC*), a circadian rhythm gene, which has also been shown to influence pathogen resistance in arabidopsis [100,101].

B. distachyon can also be used to dissect features of the host and pathogen interaction. An early example of this was the detoxification of the *Fusarium* head blight toxin, deoxynivalenol, by host uridine diphosphate glycosyltransferases [102]. More recently, fungal small RNAs encoded by *Fusarium graminearum* were shown to suppress host defence gene expression [103] and a similar approach could also be a feature of the *Magnaporthe grisea infections* [104]. *B. distachyon* also lent itself to the development of an infection system with *Rhizoctonia solani*, based on which 61 secreted protein effectors were identified that could manipulate the host [105] via WRKY transcription factors BdWRKY38 and BdWRKY44 [106,107].

A recent comprehensive review of *B. distachyon*–viral interactions has highlighted the value of this model in elucidating complex virus–virus host interactions [108]. Viral interactions can be synergistic, interfering, cooperative, or attenuating and some have been described in panicum mosaic virus (PMV) infections of *B. distachyon*. Importantly, *B. distachyon* was used to show that the PMV satellite virus is modified at its 3′ end by the host to reduce disease symptoms [109].

The importance of beneficial microbial interactions is well appreciated in, for example, mobilising nutrients to the plant as well as influencing plant physiology. Using controlled mesocosms and stable isotopes showed surprisingly large amounts of fungal and plant N had moved below ground as gaseous ammonia, with 6–9% of total plant N having moved by this mechanism [110]. Importantly, these interactions will also be influenced by climate change [111] and require characterisation in a nondomesticated model that retains the richness of rhizospheric associations [112], hence the value of *Brachypodium*. Several studies demonstrated that *B. distachyon* growth could be enhanced by previously described beneficial bacteria [113–115]. Two studies focused on transcriptomic or metabolomic responses provided data to hypothesise mechanisms involved in two beneficial plant–bacterial interactions [116,117]. The beneficial effects of mycorrhizal associations with *B. distachyon* have been used to show differential responses, depending on plant or fungal genotype [118,119]. *B. distachyon* mutants have been used to explore the role of auxin in mycorrhizal associations [120] and to show the importance of the GRAS transcription factor, RAM1, previously shown to be essential for arbuscule formation in three dicots and a grass [121]. Given the importance of reducing synthetic fertiliser use with crops such as cereals, such *Brachypodium*-based studies will increase. This is especially the case with the development of approaches to describe the **microbiome** (Box 3), both in its characteristics and function, in soil and rhizospheric interactions.

Brachypodium to study the grass cell wall

The cell wall plays a central role in plant structure and function and its complexity has been extensively assessed in *B. distachyon.* The primary and secondary cell walls in grasses are distinct in several ways from eudicots (Figure 3). The cellulose fraction is equivalent, with (1,4)-beta-D-glucan polymers assembled in parallel at the plasma membrane to form microfibrils, but not so with the hemicellulose fractions. In eudicots, hemicellulose tends to be comprised of (1,4)-beta-D-glucans that are considerably shorter than cellulose and amended with xylose decorations. Grasses are most abundant in heteroxylans with a (1,4)-beta-D-xylose backbone decorated with xylose, arabinose, glucuronic acid, and phenolic side chains of hydroxycinnamates [122]. *B. distachyon* has helped to characterise enzymes that elongate the xylan chain, including BdGT43A [123] and XYLOSYL ARABINOSYL SUBSTITUTION OF XYLAN such as BdGT61 [124].

A qualitative distinction in grasses from eudicots is the presence of mixed linkage glucan (MLG), (1,3;1,4)-beta-D-glucan. It is synthesised by three classes of the cellulose synthase (Ces) superfamily, CslF, CslJ, and CslH [125]. Biochemical and cytological techniques have localised MLG synthase activity and the most active isoform CslF6 to the maize Golgi membrane alone [126] and to the plasma membrane in wheat and barley [127]. In *B. distachyon*, yellow fluorescent

> **Box 3. *Brachypodium* to study plant–environment–microbiome interactions**
>
> The importance of the microbiome for plant and animal health and productivity has become increasingly apparent over the past few years and *Brachypodium* is making a significant contribution to such studies. *B. distachyon* root microbiome and root exudate profiles are similar to wheat [157] and the location along the root greatly affected microbiome composition [158]. While these microbiome studies provide correlations between microbiome composition and plant phenotypes, they cannot definitively assign function to either microbes or genes. To address this, researchers have developed controlled systems that maintain sterility and allow for nondestructive imaging, which, when synthetic microbial communities are used to test the effects of relatively small numbers of microbes, begin to uncover mechanisms. One such system developed using *B. distachyon* is called EcoFAB and consists of a small chamber attached to a microscope slide, such that roots grow into a chamber containing liquid or solid growth media, where they can be visualised from below. Ports on either end allow media to be added and samples removed while maintaining sterility [159]. While this system can be used for any small plant, it has been optimised using *B. distachyon* and is large enough for *Brachypodium* plants to nearly complete their lifecycle under controlled conditions. The reproducibility of plant growth and root exudation in EcoFABs in response to nutrient deficiency and soils extracts was demonstrated by a ring trial among four laboratories [160] and the EcoFAB has been adapted for high-resolution microscopy [161]. To increase throughput and reproducibility, a robotic system has been developed that can move individual EcoFABs between a growth chamber, a liquid handler to change media and remove samples, a microscope that allows for imaging of entire root system as well as individual bacteria, and a hyperspectral camera to measure above-ground growth and composition (https://ecofab-teams.lbl.gov/the-ecobot/). When coupled with novel systems like the EcoFAB, the small size and experimental tractability of *B. distachyon* set the stage for rapid discovery.

protein-tagged BdCSLF6 localised to the Golgi membrane [128,129]. The relative ease of stable genetic transformation in *B. distachyon* compared with other grasses was key in establishing this study and identifying the first protein to directly regulate the transcription of a *CSLF* gene [130]. *BdTHX1* binds the GT-element in the first intron of *BdCSLF6* and the promoter region of *BdXTH8*, a grass-specific endotransglucosylase/hydrolase. While characterisation was facilitated by generating *BdTHX1* overexpression lines, 50 plants regenerated from calli died after 1–2 weeks, which was an outcome attributed to the transgene, another example of easy transformation, facilitating discovery. An example of a grass cell wall regulator not present in arabidopsis, but characterised in *B. distachyon*, is *SECONDARY WALL ASSOCIATED MYB1* (*SWAM1*) [131], which binds the AC-element to activate secondary wall-associated gene expression. Another regulatory factor identified in *B. distachyon* is the diurnal regulation of secondary cell wall-associated transcripts, which are controlled by daily rhythms of temperature change [132].

Another prevailing component of plant secondary cell wall is lignin. Among the enzymes in the lignin biosynthesis pathway, more than half have been characterised in *B. distachyon* [phenylalanine ammonia lyase (PAL), phenylalanine tyrosine ammonia-lyase (PTAL), C3H, 4CL, COMT, F5H, CAD, and PMT], and these included the first *bona fide* PTAL [133]. Grass lignins tend to have a greater proportion of S- to G-unit monolignols than eudicots and analysis of *BdPTAL1* downregulation by RNAi was found to be mainly involved in the biosynthesis of S-unit lignins. Another PAL output that is a defining aspect of grass walls is the presence of *p*-coumaric acid and ferulic acid. These hydroxycinnamic acids provide linkages between arabinoxylans and lignins and hemicelluloses via acylation by the plant-specific BAHD family of acyl-CoA-dependent acyltransferases [134]. The analysis of overexpression and RNAi transgenic plants suggests that BdBAHD01/BdAT9 and BdBAHD05/BdAT1 are involved in the feruloylation of arabinoxylan [135,136]. *p*-Coumaroylation by BAHDs was first demonstrated by genetic gain-of-function in rice for *OsBAHD10/OsAT10* [137] and by genetic loss-of-function in *B. distachyon* for *BdBAHD09/BdAT3/BdPMT1* [138]. These studies serve as another example where *B. distachyon* facilitated the dissection of salient traits in grasses.

Looking forward, these excellent mechanistic studies will inform applied science focusing on aspects such as cell wall digestibility. Such studies have been initiated in *B. distachyon* with, for example, manipulations of enzymes in the UDP–sugar interconversion pathway, which alter
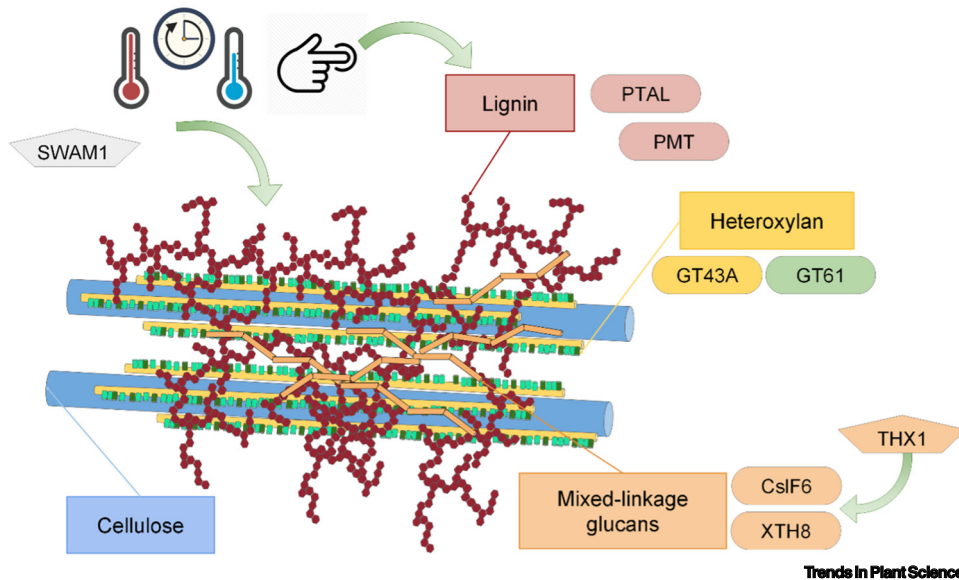
Figure 3. Schematic of grass secondary cell wall polymers highlighting enzymatic and transcriptional components uniquely defined using *Brachypodium distachyon*. Transcription factors are pentagons and cell wall biosynthesis genes are overalls. Hand denotes mechanical stress and thermometers denote the daily thermocycles that regulate wall thickening.

Outstanding questions

Can mechanisms of environmental stress tolerance defined in *Brachypodium* be translated to major crops?

How do the environment, stress, and nonstress conditions, influence cell elongation and wall thickening in the grasses?

What is the functional relevance of mixed linkage glucan in grass cell walls?

Could *Brachypodium* serve as a model genus to identify orphan subgenomes in polyploid plant groups with unknown ancestral diploid progenitor species?

Could the annual *Brachypodium* polyploid complex be used as a functional system for other plant polyploids?

Could the *Brachypodium* microbiomes indicate mechanisms that improve nutrient uptake in cereals through improved interaction of the rhizosphere?

Does the chromosome territory arrangement vary among different organs or different species of *Brachypodium*, or even alter in response to the environment?

Could changes in the diversity of *Brachypodium* populations be an indicator of environmental change, so that they could be considered 'sentinel' species?

cell wall sugars [139]. Targeting a UDP-arabinopyranose mutase through an RNAi approach resulted in a twofold greater release of total carbohydrate with xylanase. Similarly, through knockdown and mutation of *PAL*, *LACCASE5* and *8*, *PMT*, and *COMT6*, cell walls were more digestible [140]. Furthermore, the genetic resources available for *Brachypodium* have been exploited to characterise natural genetic variation within accessions, which varied in the composition of most cell wall components, the determinants of which could be identified with availability of recombinant inbred lines to help in the derivation of more digestible biofuel crops [123,140,141].

Concluding remarks and future perspectives

This overview has highlighted aspects of *Brachypodium* research that show its continuing relevance. The features of *B. distachyon* that led it to be first suggested as a grass model have now been extensively studied. Important developments not envisaged in the 2001 paper [4] were the extensive characterisation of wild *B. distachyon* populations and expansion of the model to represent entire the genus. Crucially, the opportunity to do such studies arose directly from *Brachypodium* being a nondomesticated genus. Going forward, *Brachypodium* populations will undoubtedly continue to provide the resources of indicating evolutionary impacts on genomes and physiology and, perhaps most importantly, some of the drivers of environmental adaptation. *Brachypodium* will also continue to be a 'characterisation platform' for, for example, genes linked to responses to stress, the cell wall development, and influencing the microbiome. Set against a background of climate change, such studies continue to be vital. With improvements in genomic sequencing and gene editing technologies, crops are taking on some of the properties of model species; however, *Brachypodium* has now emerged as the prime model because it is a nondomesticated genus (see Outstanding questions).

Declaration of interests

The authors declare no conflict of interest.

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