The wild grass *Brachypodium distachyon* as a developmental model system

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The arrival of cheap and high-throughput sequencing paired with efficient gene editing technologies allows us to use non-traditional model systems and mechanistically approach biological phenomena beyond what was conceivable just a decade ago. Venturing into different model systems allows us to explore for example clade-specific environmental responses to changing climates or the genetics and development of clade-specific organs, tissues and cell types. We—both early career researchers working with the wild grass model *Brachypodium distachyon*—want to use this review to (1) highlight why we think *B. distachyon* is a fantastic grass developmental model system, (2) summarize the tools and resources that have enabled discoveries made in B. distachyon, (3) discuss a handful of developmental biology vignettes made possible by using *B. distachyon* as a model system. Finally, we want to conclude by (4) relating our personal stories with this emerging model system and (5) share what we think is important to consider before starting work with an emerging model system.

Brachypodium distachyon | development | grasses | stomata | flowering | emerging model system

An evolutionary perspective on *Brachypodium distachyon* as a model in the grass family (Poaceae)

For decades there have been just a few model plant organisms. For example, *Arabidopsis thaliana* (*A. thaliana*) for many years has been the plant of choice as a representative to model flowering plants (Koornneef and Meinke, 2010). Certainly, *A. thaliana* is useful for studying a number of different aspects of plant biology that are shared among flowering plants and five decades of *A. thaliana* research has provided great insights into how flowering plants develop and function (Provart et al., 2016). However, there is a great diversity of biology found within plants and *A. thaliana* is less useful for exploring specific phenomena unique to a given clade of plants or traits that have evolved independently multiple times (Kellogg, 2006). For example, *A. thaliana* does not have floral nectar spurs, which have evolved independently multiple times and are a defining characteristic of the beautiful columbine flower (Ballerini et al., 2020).

The grasses (Poaceae) are a large family of about 10,000 recognized species that have radiated to span the entire globe (Kellogg, 2001). Within this group of plants are cereal crops that provide the bulk of human caloric content world wide either directly through consuming grain or indirectly through feed for livestock (FAO Statistical Pocketbook, 2015). However, grasses are morphologically, physiologically and developmentally distinct from *A. thaliana* and many aspects of their biology require a model more closely related than *A. thaliana*, which last shared a common ancestor with the grasses around 140 million years ago (Figure 1; Kellogg, 2001). Maize has historically been utilized as a grass model and more recently so has rice; however, while they are useful in some respects, in many other regards they are not the ideal model organism due to their larger stature, more complex genomes and difficult growth requirements. Furthermore, despite being grasses, maize and rice are quite different from the pooid grass clade that contains the important crops wheat, oats, barley and rye (Figure 1). Pooid crops indeed are some of the more challenging crops for genetic studies, as many of the pooid cereals we eat are polyploid and have some of the largest genomes in the grass family (Kellogg, 2015). Additionally, the grasses originated as tropical forest understory and many of the extant species remain tropical including rice (Kellogg, 2001). However, Pooideae (temperate grasses) contain some 3850 species, which have radiated into higher latitudes and thus have unique characteristics not found in other grasses that facilitated their radiation into temperate regions such as cold tolerance (Figure 1; Grass Phylogeny Working Group II, 2012; Humphreys and Linder, 2013; Preston and Sandve, 2013).

The focus of this review will be on the emerging Pooid "model" grass *B. distachyon. B. distachyon* is placed phylogenetically within the Pooideae sub-family, sister to the agronomically important "crown pooids"— wheat, oat, barley and rye (Figure 1; Grass Phylogeny Working Group II, 2012). Furthermore, *B. distachyon* is a wild grass that has not undergone

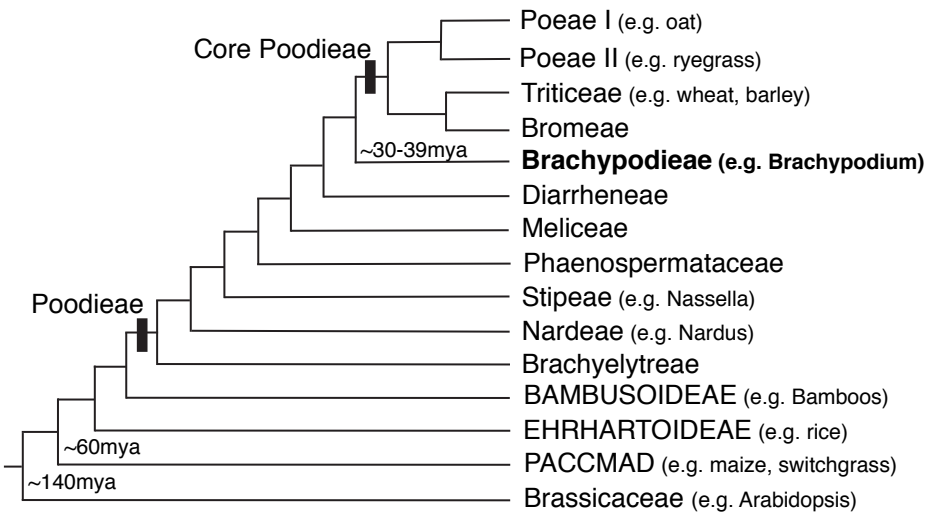

Figure 1. Pooideae phylogeny showing the eleven major tribes and delimitation of the core pooids based on (Schneider et al., 2009) and (Grass Phylogeny Working Group II, 2012). Outgroups are the closely related grass subfamilies, Bambusoideae and Ehrhartoideae, which together with Pooideae form the BEP clade. Sister to the BEP clade is the PACMAD clade that contains tropical cereals such as maize and switchgrass. Brachypodieae sister to the core pooid clade is in bold. Stem node age of *B. distachyon* based on studies from (Díaz-Pérez et al., 2018) and (Sancho et al., 2018). Mya= million years ago.

a number of genetic bottlenecks as a domesticated species and thus can provide additional allelic variation in genes that impact different developmental processes.

Genomics enabled developmental biology

B. distachyon has a number of attributes that make it an attractive developmental model system. While sounding simplistic, one of the key aspects for a successful model organism is that it is easy and cheap to grow. *B. distachyon* is a fraction of the size of its domesticated relatives barley and wheat (Figure 2) resulting in less required growth space enabling more replicates and experiments per growth area, which is notoriously sparse. Important aspects for plant models are that the species can self-fertilize to bulk and maintain stocks and that basic breeding protocols like cross-fertilization are established (see DOE-JGI resources website https://jgi.doe.gov/our-science/science-programs/plant-genomics/brachypodium/). Indeed, most of the *B. distachyon* accessions isolated to date are already highly inbred making it an attractive developmental genetics model system (Gordon et al., 2017). For functional genetic analyses it is essential to have efficient transformation protocols available. Grasses are notoriously hard to transform yet the main accessions used like Bd21-3 are readily transformable compared to other model grasses (Scholthof et al., 2018); https://jgi.doe.gov/our-science/science-programs/plant-genomics/brachypodium/). This allows for the generation of large collections of reporter lines and straightforward gene editing using CRISPR/Cas9. A recent high-efficiency transformation system for CRISPR/Cas9-mediated gene editing containing the wheat growth factors *TaGRF4* and *TaGIF1* (Debernardi et al., 2020) works efficiently in *B. distachyon* (DP Woods, personal communication). Examples of vectors and cloning systems that work well to generate reporter lines in *B. distachyon* include the pIPK and pANIC vector series based on Gateway cloning (Himmelbach et al., 2007; Mann et al., 2012) and the GreenGate system based on GoldenGate cloning (Lampropoulos et al., 2013).

Apart from horticultural and biotechnological protocols, the availability of genomic and genetic resources is fundamental to study developmental mechanisms. In 2010, the first fully assembled and annotated draft genome was published (Initiative, International Brachypodium, 2010). *B. distachyon* has a relatively small and simple diploid genome (272Mb) that is orders of magnitudes smaller in size compared with its close grass relatives such as hexaploid wheat (>15Gb) and diploid barley (>5Gb; Initiative, International Brachypodium, 2010; International Barley Genome Sequencing Consortium et al., 2012; International Wheat Genome Sequencing Consortium (IWGSC) et al., 2018). Since then pan-genome efforts have added *de-novo* assembled and fully annotated genomes of 54 additional accessions (Gordon et al., 2017); https://brachypan.jgi.doe.gov/). In addition, the diploid relative *B. stacei*, the perennial *B. sylvaticum* and the allotetraploid *B. hybridum* (hybrid of *B. stacei* and *B. distachyon;* Catalán et al., 2012) have been fully sequenced, assembled and annotated and are accessible as early release genomes in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and more perennial species will soon be sequenced (Community Science Program-Joint Genome Institute (CSP-JGI) Project ID 503006).

Finally, a growing repository of genetic resources and datasets facilitate functional genomic studies. There is a carefully curated diversity panel covering Spain, Italy and Turkey among which 54 accessions are fully sequenced, *de-novo* assembled and annotated (Gordon et al., 2017); https://brachypan.jgi.doe.gov/). A gene expression atlas has been built and integrated into the eFP browser (Sibout et al., 2017); http://bar.utoronto.ca/efp_brachypodium/cgi-bin/efpWeb.cgi). More than 23,000 T-DNA insertion lines are available (Bragg et al., 2012; Hsia et al., 2017) and searchable on Phytozome's J-Browse (https://phytozome.jgi.doe.gov/jbrowse/index.html). In addition, a heroic CSP-JGI (Project ID 1670) project whole-genome sequenced over 2000 mutant families mutagenized with sodium azide (NaN_3, hence the NaN population). Approximately 1.2 million variants were identified and accurately mapped to the reference genome Bd21-3 v1.1, which are annotated and searchable on Phytozome's J-Browse in Phytozome in the early release genome of Bd21-

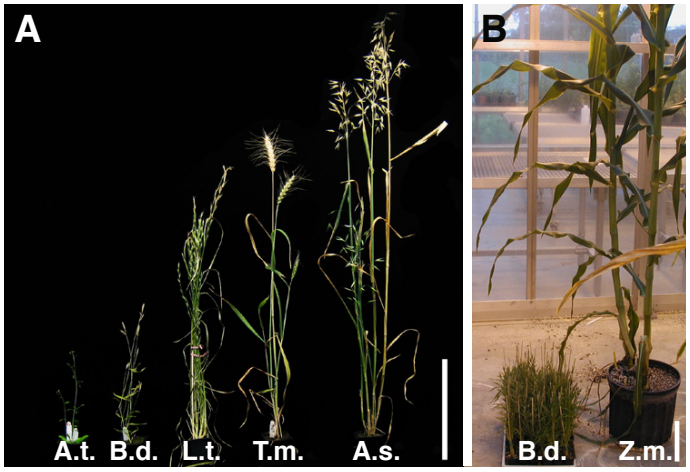

Figure 2. Growth habits of *B. distachyon* compared to *A. thaliana* and other grasses. **A.** A.t. *Arabidopsis thaliana*, B.d. *Brachypodium distachyon*, L.t. *Lolium temulentum*, T.m. *Triticum monococcum*, A.s. *Avena sativa*. **B.** A flat of one hundred *B. distachyon* plants next to a single *Zea mays* (Z.m.) plant. Scale bar, 36cm.

3 (https://phytozome.jgi.doe.gov/jbrowse/index.html). Finally, there are currently two additional CSP-JGI projects underway; one aims to generate extensive DNA Affinity Purification sequencing (DAP-seq) profiles of thousands of transcription factors (Project ID 504343) and another to use whole-genome sequence data to genotype 16 Recombinant Inbred Line (RIL) populations (Project ID 505702), which includes accessions that span the species range. For a detailed list of available resources see (Scholthof et al., 2018).

Brachypodium distachyon as a model for Pooid development

B. distachyon as a grass model has and will accelerate our understanding of the genetic and molecular basis of a number of Pooid grass developmental traits. Here, we will focus on the progress that has been made in *B. distachyon* to expand our understanding of the transition to flowering, organ initiation, development of stomatal pores, leaf epidermal patterning and root development (Figure 3). Particular focus will be on studies for which the use of *A. thaliana* or other grasses would not have been suitable to explore a given phenomena specific to Pooid grasses. Importantly, most of the work we will describe would not have been possible without the resources described above. There are a number of additional developmental processes being studied in *B. distachyon*; important contributions include for example, cell wall biology (see Coomey et al., 2020) and references therein), vascular development (Sakai et al., 2021; Smertenko et al., 2019), pleiotropic genes like the NOOT-BOP-COCH-LIKE genes (Magne et al., 2020), seed germination (Wolny et al., 2018), species-specific aspects of abscission zone formation (Yu et al., 2020), and even thigmomorphogenesis—the developmental response to touch (Coomey et al., 2021). We apologize for not being able to cover all the developmental work done with *B. distachyon* due to space limitations.

Transition to flowering

The transition from vegetative to reproductive development is a key decision for which the timing is often directly influenced by the environment (Amasino, 2010). This critical life history trait has been shaped over evolutionary time to maximize the ability to flower at a time that optimizes reproductive success. In crops, such as wheat, the timing of this transition has been modified multiple times as wheat expanded through multiple environments across the globe (Distelfeld et al., 2009; Dubcovsky and Dvorak, 2007). Furthermore, the timing of flowering is one of many traits that have been manipulated by humans for increased crop productivity (Greenup et al., 2009). Thus, the study of flowering-time is not only a fascinating topic from a basic research standpoint, but also has relevance for crop improvement particularly in a changing global climate and a growing human population.

In many plant species, flowering occurs at a particular time of year in response to the sensing of seasonal cues such as changes in day-length and temperature. Photoperiodism is the process by which the sensing of changes in day-length leads to flowering (Song et al., 2015). Plants such as *B. distachyon* and other pooid grasses that flower in the spring or early summer are often responding to lengthening photoperiods and are referred to as long-day (LD) plants (Ream et al., 2014). Many plants adapted to temperate climates have a winter annual or biennial life history strategy (Bouché et al., 2017). These plants become established in the fall, overwinter, and flower rapidly in the spring. Essential to this adaptive strategy is that flowering does not occur prior to winter, during which flowering would not lead to successful reproduction. Thus, plants have evolved ways to prevent fall flowering and sense the passing of winter to establish competence to flower (Chouard, 1960). The block to flowering can be alleviated by exposure to two hallmarks of winter, either prolonged exposure to cold temperatures and/or the exposure to a prolonged period of short days (Chouard, 1960; Heide, 1994; Woods et al., 2014a). The process by which flowering is promoted by cold exposure is known as vernalization and the process by which shorter photoperiods followed by growth in longer photoperiods promotes flowering is known as short-day (SD) vernalization (Figure 4; Woods et al., 2019, 2014a). A hallmark of cold-mediated vernalization is acquisition of *competence* to flower, not flowering *per se* (Chouard, 1960), and the SD vernalization phenomenon is similar: exposure to short days leads to competence, but plants must still be exposed to inductive long days to flower (Woods et al., 2019). These two processes exist in a diverse range of flowering plants, yet relatively little is known about the molecular nature of vernalization outside the eudicot model *Arabidopsis thaliana*, and only recently have molecular details been discovered about short-day vernalization in plants (*A. thaliana* does not have the short-day vernalization phenomenon; (Bouché et al., 2017; Woods et al., 2019). The characterization of vernalization in other species has revealed that different genes are involved in different plant groups, suggesting vernalization likely evolved

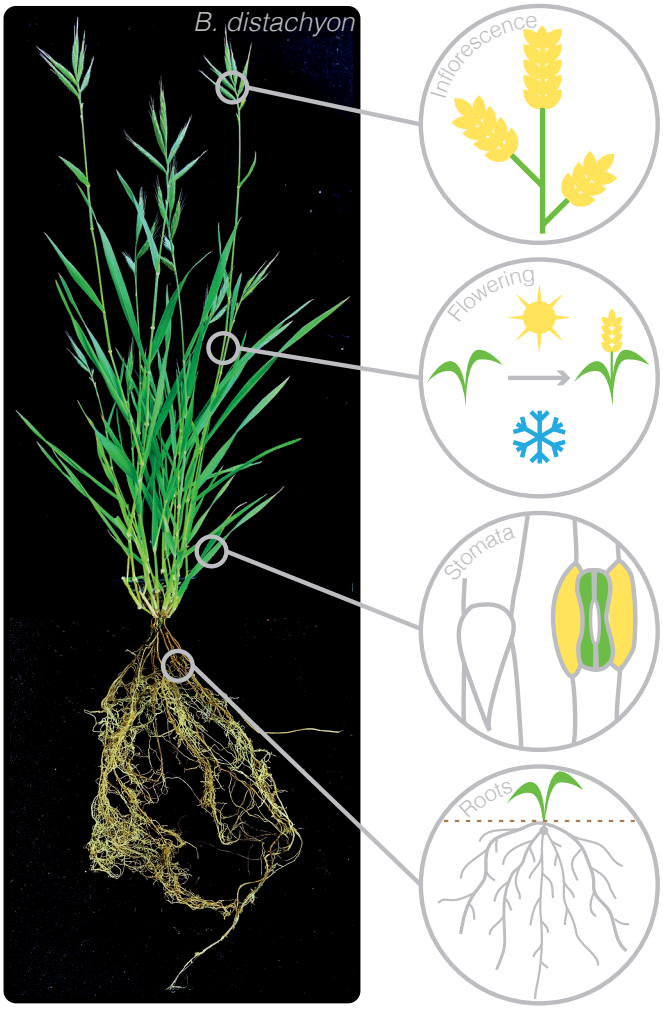

Figure 3. The wild grass *B. distachyon* as a developmental model system. On the left panel, the shoot and the root system of a 6 week old, flowering *B. distachyon* plant is shown. Highlighted are the four developmental systems this review primarily covers; flowering transition, inflorescence development, stomatal development, and root development.

independently multiple times throughout the diversification of flowering plants (Bouché et al., 2017; Ream et al., 2014; Zhong et al., 2017); thus, the rich information gained in *A. thaliana* cannot be directly applied to the grasses and studying vernalization in Pooid grasses provides an opportunity to explore the molecular basis of a classic example of convergent evolution. Furthermore, like vernalization, SD vernalization also likely evolved independently multiple times because a key gene required for SD vernalization is grass specific (Woods et al., 2019).

Molecular understanding of flowering in temperate grasses from natural variation work in wheat and barley

To date, information about the vernalization response in grasses has largely been the result of studies of existing allelic variation in wheat and barley (Fjellheim et al., 2014). Wheat and barley varieties have been broadly classified into spring and winter varieties (Distelfeld et al., 2009). Spring varieties do not require vernalization in order to flower rapidly in inductive LD, whereas winter varieties have a vernalization requirement that must be met before LD can promote flowering (Distelfeld et al., 2009; Greenup et al., 2009).

The current molecular model of vernalization in grasses, which is based on studies in wheat and barley, consists of the genes, *VERNALIZATION1* (*VRN1*), *VRN2* and *VRN3*, forming a regulatory loop (Bouché et al., 2017; Distelfeld et al., 2009; Greenup et al., 2009). *VRN3* is orthologous to *FT1* which is a small mobile floral signal often referred to as florigen and from here on will be referred to as *FT1* (Yan et al., 2006). The regulatory loop is thought to exist in leaves where all three genes are expressed (Sasani et al., 2009; Yan et al., 2006, 2004). In the fall, prior to cold exposure, high levels of VRN2 repress *FT1* to prevent flowering (Figure 4; Yan et al., 2004). VRN2 is a CCT domain containing transcription factor and is part of the type VI *CO-like* family of genes (Griffiths et al., 2003; Yan et al., 2004). Prior to cold exposure, both *VRN1* and *FT1* are expressed at low levels (Greenup et al., 2009). However, during the cold of winter, *VRN1* is up-regulated proportionately to the length of cold experienced, and in turn, higher *VRN1* levels are thought to cause a decrease of *VRN2* expression (Dubcovsky et al., 2006; Sasani et al., 2009). *VRN1* is related to the *APETALA1/FRUITFULL* (*AP1/FUL*)-like class of MADS box transcription factors in *A. thaliana* (Yan et al., 2003) and arose from a duplication event at the base of the grass family (Preston and Kellogg, 2006). Upon repression of *VRN2* and in the presence of LD, *FT1* is activated by *Photoperiod-H1* (*PPD1*) a member of the pseudoresponse regulator gene family (Song et al., 2015; Turner et al., 2005; Yan et al., 2006). Once activated *FT1* up-regulates *VRN1* expression in leaves creating a positive feedback loop of *VRN2* repression and *FT1* activation (Figure 4; Distelfeld et al., 2009; Shimada et al., 2009; Yan et al., 2006). It is worth noting that while VRN1 can directly bind to the promoter region of *FT1* (Deng et al., 2015) this is not sufficient to cause *FT1* expression because during the cold *FT1* levels do not increase despite elevated levels of *VRN1* (Chen and Dubcovsky, 2012). As noted above a LD mediated photoperiod pathway via *PPD1* is required for *FT1* activation, however even if cold is provided under LD conditions *FT1* is still not activated perhaps because the photoperiod pathway does not work well in cold conditions.

Are the flowering pathways described in wheat and barley conserved in B. distachyon?

B. distachyon contains orthologs of all of the major flowering-time genes described so far in wheat and barley (Higgins et al., 2010; Woods et al., 2016). To a large extent these genes appear to be functionally conserved in *B. distachyon* (Higgins et al., 2010; Lv et al., 2014; Ream et al., 2014, 2012; Woods et al., 2016). For example, consistent with studies from wheat and barley, *BdFT1* and *BdVRN1* are promoters of flowering (Ream et al., 2014). Overexpression of *BdFT1* and *BdVRN1* results in rapid flowering (Ream et al., 2014) and knockdown of *BdFT1* and *BdVRN1* results in extremely delayed flower-

ing (Lv et al., 2014; Woods et al., 2016). *BdVRN1* expression is induced in leaves proportional to the amount of time in cold temperatures and expression remains high post cold treatment, which correlates with higher *BdFT1* levels and rapid flowering (Ream et al., 2014). Also, we observe high expression levels of *BdFT1* in lines overexpressing *BdVRN1* and high expression levels of *BdVRN1* in lines overexpressing *BdFT1* consistent with the positive feedback loop suggested from flowering studies in wheat and barley (Ream et al., 2014). Also, as in wheat and barley, *BdVRN2* acts as a floral inhibitor, but a key difference is that in *B.distachyon BdVRN2* is not repressed by cold or by *BdVRN1*; rather, *BdVRN2* is constitutively expressed (Woods et al., 2016). This suggests that *BdVRN2* came under control of *VRN1* after *B. distachyon* split from wheat and barley and that the regulation of *BdVRN2* by *BdVRN1* is a derived trait that evolved within Triticeae (McKeown et al., 2016; Woods et al., 2016). Furthermore, allelic variation at these genes probably contributes to natural variation in flowering responses in *B. distachyon* because they colocalize under quantitative trait loci controlling flowering-time variation (Bettgenhaeuser et al., 2017; Woods et al., 2017a).

B. distachyon as a model plant for flowering time gene discovery in temperate grasses

Although there has been great progress in understanding photoperiodic flowering and vernalization at a molecular level in grasses, much remains much to be learned including identifying additional components of the vernalization response and the extent to which vernalization pathways are conserved throughout grasses (Fjellheim and Preston, 2018). *B. distachyon* has many attributes that make it an attractive model to pursue flowering research (Ream et al., 2012). There are hundreds of inbred accessions that have been collected throughout the Middle-East and Europe that display a wide range of flowering behaviors that can be used to map genes underlying that flowering variation (Brkljacic et al., 2011; Gordon et al., 2017; Ream et al., 2014; Schwartz et al., 2010; Tyler et al., 2014). Another route to gene discovery is the use of *B. distachyon* to conduct forward genetic screens to identify novel genes which is not possible in polyploid crops such as wheat (Ream et al., 2012). We have streamlined mapping *B. distachyon* mutants and have identified the causative lesion of many EMS-induced flowering time mutants using a mapping by sequencing pipeline (Minevich et al., 2012; Woods et al., 2014b; Woods and Amasino, 2015; Woods et al., 2017; Woods et al., 2020). Interestingly, most of the genes we have mapped are in novel flowering-time genes —i.e, these genes have not been described before in other plants (Figure 4) or are in genes which, in other plants have a minor role impacting flowering time but play a major role in *B. distachyon* (e.g. Woods et al., 2017a, 2014b; Woods and Amasino unpubl.).

Sensing photoperiod

One of the first mutants isolated and mapped was an extremely delayed flowering mutant that is due to a lesion in the light receptor *PHYTOCHROME C* (*PHYC*; Woods et al., 2014b). We isolated three mutant alleles of *PHYC* all of which only flower sporadically after a year of growth in LD of 20h, which is a highly inductive photoperiod for flowering (wild type Bd21-3 flowers around 30 days after germination in 20h days; Ream et al., 2014). We found that *PHYC* is required for the transcriptional activation of all of the known genes within the photoperiod pathway including *FT1*, *CO* and *PPD1,* and ectopic expression of *FT1* in *phyC* results in rapid flowering (Woods et al., 2014b). Additionally expression of genes that comprise the circadian clock were also altered in the *phyC* mutant background (Woods et al., 2014b). Similar results were also obtained in studies of wheat and barley (Chen et al., 2014; Pankin et al., 2014). Thus, it appears that *phyC* is essential for photoperiodic flowering broadly across Pooideae. However, the essential role of PHYC in photoperiodic flowering is not universal as loss of *phyC* function in *A. thaliana* and rice has only small effects on flowering (Monte et al., 2003; Takano et al., 2005).

The interconversion rates of the active Pfr and inactive Pr forms of PHYC is important in floral regulation. Specifically, phytochromes detect the levels and ratio of red (R) and far-red (FR) light in the environment (e.g. Quail, 2002). Upon absorption of red light, phytochromes photoconvert to an active form that initiates a signal transduction cascade. Recently a *tour de force* of genomic and biochemical work in *B. distachyon* revealed that the dark reversion rate of the Pfr form of PHYC is likely to be important in the measuring of the night length, which has downstream consequences in modulating the accumulation of the ELF3 clock component, which is a direct transcriptional repressor of *PPD1* (Gao et al., 2019). This suggests that the hourglass model first described decades ago (Borthwick and Hendricks, 1960) to explain how plants sense the length of the day may in fact be how temperate grasses sense photoperiod, although more work is needed to determine to what extent this is true (Gao et al., 2019). This appears distinct from *A. thaliana* where evidence supports the external coincidence model of CO in photoperiod sensing (e.g. Andrés and Coupland, 2012). It is worth noting, however, that in wheat *CO* plays a minor role in the activation of *FT1* especially in the presence of *PPD1* (Shaw et al., 2020), thus, the CO-FT1 regulon critical to flowering in *A. thaliana* appears to play a lesser role in temperate grasses (Ballerini and Kramer, 2011). However, we cannot rule out if both models are occurring in temperate grasses, which likely depends on the genetic background and environment. In summary, PHYC appears to be the main light receptor required for photoperiodic flowering in temperate grasses and may have played a role in the evolution of LD flowering in temperate grasses.

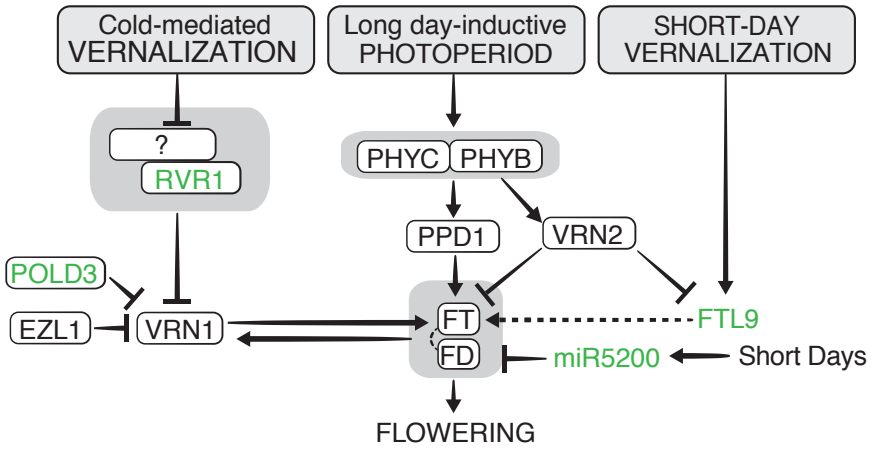

Figure 4. Current working model of *B. distachyon* flowering pathways. Genes in green were identified in *B. distachyon.* Cold mediated vernalization results in the induction of *VRN1* which promotes flowering by activating *FT1*. Before cold there are a number of genes involved in establishing a vernalization requirement such as *RVR1*, *EZL1* and *POLD3*. Also before cold, genes such as *VRN2* repress *FT-like* genes. Essential to long day flowering are the photoreceptors PHYC and PHYB. Long days increase *PPD1* expression that has downstream consequences on the activation of *FT1* and flowering. During short days miR5200 expression is elevated which keeps *FT1* from functioning under these environmental conditions. Also SD triggers the expression of *FTL9* whose expression in SD provides competency to flower allowing plants to flower once shifted into LD (SD vernalization) where *FT1* can become expressed. Dotted arrow indicates *FTL9* is involved in providing competency to flower by effecting *FT1* expression but this is likely indirect and requires *VRN1.*

Sensing the cold of winter

A key feature in the evolution of all vernalization systems is a system to perceive the length of cold exposure. In pooid grasses *VRN1* induction by prolonged cold is a key feature of vernalization (McKeown et al., 2016). We have isolated a number of mutants from forward genetic screens looking for mutants where *VRN1* is expressed and flowering occurs without cold exposure in conditions in which the wild type requires cold for flowering and *VRN1* induction (e.g. Lomax et al., 2018; Woods et al., 2020, 2017b; Woods and Amasino, 2015)). Consistent with the key role of *VRN1* expression in achieving competence to flower upon prolonged cold exposure, these mutants no longer have a vernalization requirement for rapid flowering. These mutants are, in a sense, constitutively vernalized, and the role of the functional alleles is to repress *VRN1* prior to cold exposure (Woods et al., 2017b). Indeed, the transcriptomes (via RNAseq) of several of our mutants overlap substantially with vernalized plants (Woods et al., 2020, 2017b; Woods unpubl.). We found that one of the mutations was in a gene with a Bromo-Adjacent Homology and a TFIIS domain; we refer to the gene as *REPRESSOR OF VERNALIZATION1* (*RVR1*; Woods et al., 2017b). *RVR1* homologs are present in all plants, but *RVR1*-like genes are not found outside of the plant kingdom. Interestingly, no mutant phenotypes resulting from a *RVR1* lesion have been described in any other plant system and null mutants in *A. thaliana* do not have any obvious phenotype (Woods et al., 2017b). In other organisms, proteins containing BAH domains are often repressors that "read" chromatin states, and as noted above the role of RVR1 is to repress *VRN1* (Yang and Xu, 2013). Indeed, we observe loss of the repressive chromatin marks H3K27me3 in the *rvr1* mutants around *VRN1* similar to what has been observed in the Polycomb Repressive Complex 2 (PRC2) mutant *ENHANCER OF ZESTE1/CURLY LEAF* (*ezl1*; Goodrich et al., 1997; Lomax et al., 2018), which is a histone methyltransferase and the catalytic subunit of PRC2 responsible for the deposition of H3K27me3 marks in plants (De Lucia et al., 2008). At present, it is unclear how RVR1 mediated repression of *VRN1* is overcome by vernalization because *RVR1* is constitutively expressed (Woods et al., 2017b). Perhaps, *RVR1* is abolished via a post transcriptional mechanism and/or *RVR1* could be part of a general repressive complex that has several subtypes with different target gene specificities, and loss of a flowering specific component of a *VRN1* repressing subtype after prolonged cold exposure could abolish *VRN1* repression (Figure 4).

Sensing the short days of winter

Another route to gene discovery is exploiting natural variation in flowering responses. With regards to the SD vernalization response, some *B. distachyon* accessions exhibit SD vernalization whereas others do not (Woods et al., 2019). From crosses between such accessions, we found that SD vernalization segregates as a single locus and the responsible gene is a paralog of *FT1* referred to as *FT-LIKE 9* (*FTL9*) that is expressed in SD (Woods et al., 2019). However, unlike *FT1*, *FTL9* is unable to directly cause flowering; rather, its expression in SD enables flowering once plants are shifted into LD

conditions (Woods et al., 2019). Accessions with a functional allele of *FTL9* have both the SD and cold vernalization responses, whereas accessions with loss-of-function alleles can only respond to cold vernalization (Woods et al., 2019). The adaptive value of an active allele of *FTL9* and the SD vernalization response is that in areas with a milder climate, SD is a more reliable indicator of winter than cold temperatures alone. In contrast, the adaptive value of loss of *FTL9* and the SD vernalization response might be to enable these *B. distachyon* accessions to grow in regions with more variable spring conditions. In these environmental conditions having a robust SD vernalization response may lead to premature flowering when there's still a chance of a hard freeze, which could damage the more sensitive floral organs. Consistent with this trend, accessions that lost the SD vernalization response also require longer periods of cold treatment in order to flower (Woods et al., 2019). Grasses have undergone an expansion of the *FT* gene family, and it is fascinating that a florigen-like gene acquired a role in creating *competence* to flower rather than directly inducing flowering as florigen (*FT1*) does. A recent study suggests that *FTL9* in some *B. distachyon* accessions might also be involved in actual SD flowering rather than SD vernalization, and thus, *FTL9* might have different functional roles depending on the *B. distachyon* accession (Qin et al., 2019). Taken together, the use of *B. distachyon* allowed the discovery of the first example of a gene required for SD vernalization— a phenomenon first described in the 1930s (Purvis and Gregory, 1937).

In addition to the progress that can be made using genetics, transcriptome studies have already begun to shed light on additional layers of flowering-time regulation such as the post-transcriptional regulation of *FT1* (Wu et al., 2013). An endogenous microRNA in *B. distachyon*, miR5200, has been shown to target *FT1* in SD and repress its activity (Wu et al., 2013). Consistent with miR5200's ability to repress *FT1*, miR5200 over-expressing plants have very low *FT1* levels even in LD conditions and delays flowering (Wu et al., 2013). Interestingly, miR5200 appears to be pooid specific as miR5200 is not detectable in other grasses or monocot species (McKeown et al., 2017). However, miR5200's expression pattern is conserved across pooid grasses with miR5200 more highly expressed in SD than in LD (Wu et al., 2013). That miR5200 is specific to the pooid lineage indicates that miR5200 represents a fairly recent evolutionary path to regulate *FT1* expression.

Flower development

The vast majority of grasses are characterized by having their flowers arranged in novel structures called spikelets that are absent from the closest grass relatives such as Joinvillea (Malcomber et al., 2006). Similarly, grass flowers with lodicules (homologous to petals), a palea and a lemma surrounding the stamens and pistil, with the overall spikelet being subtended by empty bracts called glumes are substantially different from the two alternating whorls of tepals surrounding the stamen and pistil typical of monocot flowers in the immediate grass relatives (Whipple and Schmidt, 2006). *B. distachyon* has an unbranched inflorescence with the spikelets arising directly on the inflorescence axis, called a spike (Kellogg et al., 2013). There are a number of different inflorescence architecture genes that have been identified in grasses (e.g. Kellogg, 2007; Whipple, 2017)); however, much less is known of the genes regulating unbranched inflorescence development.

Forward genetic screens have revealed mutants with more spikelets in *B. distachyon*. These mutants (called *more spikelets1*) had an increase in the number of axillary meristems and the causative gene for one of the more spikelets mutant phenotype is in an ethylene response factor *APETELA2* transcription factor orthologous to the *branched silkless* mutant in maize and *FRIZZY PANICLE* in rice (Derbyshire and Byrne, 2013). Therefore, it appears that the gene has a conserved role in determining spikelet meristem fate broadly in grasses. However, in *B. distachyon* it appears to have an additional role in the determinacy of the inflorescence meristem. This is potentially through the timing of the initiation of the terminal spikelet, which may be unique in grasses with a spike rather than a panicle for example (Derbyshire and Byrne, 2013). More recently it has been shown that in wheat and barley this gene affects the spike in a similar way and modifying the function of this gene could potentially be used to increase yield in these crops (Li et al., 2020; Poursarebani et al., 2015).

There is a great diversity in grass inflorescence architecture however, very few cross species functional comparisons between grasses have been done. Recently, a grass specific **T**eosinte branched1/**C**incinnata/**P**roliferating cell factor (TCP) transcription factor COMPOSITUM1 (COM1) was mapped in barley which plays a role inhibiting branch formation in the spikelet (Poursarebani et al., 2020). Interestingly, a role in inhibiting branches appears to be Tritaceae specific because in non-triticeae grasses such as *B. distachyon* and sorghum, COM1 actually promotes branch formation as well as cell differentiation in the palea (Poursarebani et al., 2020). The different functional roles of COM1 between grasses appear to be due to variation in coding sequence within *COM1*. Therefore, coding variation in genes such as *COM1* may contribute to the diverse inflorescence architectures found in the grass family.

Recently, auxin transport has been implicated in inflorescence development. In most plants, development requires the growth hormone auxin (e.g. Leyser, 2010; Mcsteen, 2009)). Auxin is actively transported to build sites with local auxin maxima, which are required for organ initiation (Mcsteen, 2010). Directional efflux of auxin is mediated by polarly localized PIN-FORMED (PIN) auxin efflux carriers (Gälweiler et al., 1998), which direct the flow of auxin to specific locations thereby orchestrating new organ formation. Thus, *pin*

loss of function mutants in *A. thaliana* fail to initiate organs resulting in a "pin" like inflorescence phenotype (Reinhardt et al., 2003, 2000). A phylogenetic analysis revealed that a duplication of *PIN1* in grasses produced the paralogs *PIN1a* and *PIN1b*, and, in addition, another *PIN-like* clade sister to *PIN1*, aptly named *Sister-of-PIN1* (*SoPIN1*; O'Connor et al., 2014). *SoPIN1* is present in all angiosperms except Brassicaceae, which contains *A. thaliana,* thus it was not functionally characterized before. To dissect the functional relevance of the PIN1 and SoPIN1 proteins, reporter lines and an auxin signalling output reporter (DR5) were generated in *B. distachyon*. It was found that SoPIN1 defines auxin convergence points in the inflorescence meristem whereas the PIN1a and PIN1b are implicated in leaf vein patterning (O'Connor et al., 2014). Consistent with the difference in the localization of the PIN1 and SoPIN1 proteins, double loss of function of *bdpin1a/b* have short internodes but no defects were observed in the grass spikelet whereas sopin1 loss of function mutants displayed the canonical "pin" phenotype and loss of organs (O'Connor et al., 2017). Likely, we would have missed the central role of *SoPIN1* in organ initiation if we relied on *A. thaliana* as the sole model for flowering plants. Some of the auxin-related reporter lines have recently been used to analyze auxin transport and signalling during grass embryogenesis in *B. distachyon* (Hao et al., 2021). In addition, stage-specific embryonic transcriptomes have been generated and identified common and diverged genetic programs during embryogenesis in *B. distachyon* and *A. thaliana* (Hao et al., 2021).

Development of stomatal pores and leaf epidermal patterning

The longitudinal grass leaf is morphologically and ontogenetically distinct from broad leaves typical for eudicots such as *A. thaliana* (Conklin et al., 2019). In grasses, leaf development follows a strict base-to-tip developmental gradient and patterning is highly stereotyped along the mediolateral leaf axis. Venation in grass leaves, for example, is parallel rather than reticulate (Conklin et al., 2019) and epidermal patterning is organized in semi-clonal longitudinal cell files that form different epidermal cell types like stomata, prickle hair cells, silica cells or pavement cells (Figure 3; Stebbins and Shah, 1960).

Stomata are tiny breathing pores on the leaf's surface that can open and close to enable carbon dioxide uptake for photosynthesis while simultaneously balancing water vapor loss. The graminoid stomatal complex of grasses shows a highly innovative, derived morphology consisting of two central, dumbbell-shaped guard cells (GCs) that are flanked by two lateral subsidiary cells (SCs; Figure 5A; Nunes et al., 2020). *A. thaliana*, on the other hand, features ancestral two-celled stomata, where two kidney-shaped GCs surround the central pore (Figure 5B; Nunes et al., 2020). Interestingly, the graminoid morphology is associated with faster stomatal opening and closing kinetics, which contribute to more water-use efficient photosynthesis (Franks and Farquhar, 2007; Lawson and Blatt, 2014; Lawson and Vialet-Chabrand, 2019; McAusland et al., 2016).

In *B. distachyon*, specific epidermal cell files adopt the capacity to form stomatal complexes (Figure 5C; McKown and Bergmann, 2020; Nunes et al., 2020; Raissig et al., 2016; Stebbins and Shah, 1960). Stomatal files always run parallel and next to leaf veins and are usually composed of two adjacent cell files. Non-stomatal cell files make other epidermal cell types like prickle hair cells or adaxial bulliform cells (Kellogg, 2015). At the base of the growing leaf stomatal rows transversely divide more rapidly than adjacent prickle hair rows (McKown and Bergmann, 2020; Nunes et al., 2020; Stebbins and Shah, 1960). Eventually a transverse asymmetric division in both the stomatal and the prickle hair cell rows in the *B. distachyon* epidermis generates a smaller daughter cell, which becomes a guard mother cell (GMC) or a prickle hair cell precursor, and a larger daughter cell that is destined to be a pavement cell (Figure 5C; McKown and Bergmann, 2020; Nunes et al., 2020). While the prickle hair cell precursor will grow out and form the typical shark tooth shape (Figure 3), the GMC induces its lateral neighbor cells to become subsidiary mother cells (SMCs), which polarize and asymmetrically divide longitudinally to contribute flanking SCs (Figure 5C; Gray et al., 2020; McKown and Bergmann, 2020; Nunes et al., 2020). Then the GMCs will longitudinally divide to form two equally sized GC precursors (Figure 5C; McKown and Bergmann, 2020; Nunes et al., 2020; Stebbins and Shah, 1960). Finally, pore formation separates the GCs, which then undergo dramatic morphogenesis to build the dumbbell-shaped GCs unique to graminoid stomata (Figure 5A, C; Galatis and Apostolakos, 2004; Spiegelhalder and Raissig, 2021).

Both the recruitment of lateral SCs and the morphogenetic events foming dumbbell-shaped GCs do not exist in *A. thaliana*. A forward genetic screen in *B. distachyon* instead yielded several players required for grass stomatal development including factors that determine stomatal identity, recruit the SC lineage, differentiate GCs and pattern stomata (McKown and Bergmann, 2020; Nunes et al., 2020). Surprisingly, many of the genes identified also have a role during stomatal development in *A. thaliana*, however, their function, their genetic interactions, and their regulation have been changed to generate novel morphological features and accommodate for the diverse grass leaf ontogeny and form (McKown and Bergmann, 2020; Nunes et al., 2020).

Establishing and differentiating the guard cell and subsidiary cell lineages

One of the key mutants identified in a forward genetic screen using chemically mutagenized *B. distachyon* families was a mutant called *stomataless* (*stl*) that lacked stomatal complexes (Raissig et al., 2016). It resembled the *speechless (spch)* mutant in *A. thaliana,* which disrupts the bHLH transcription

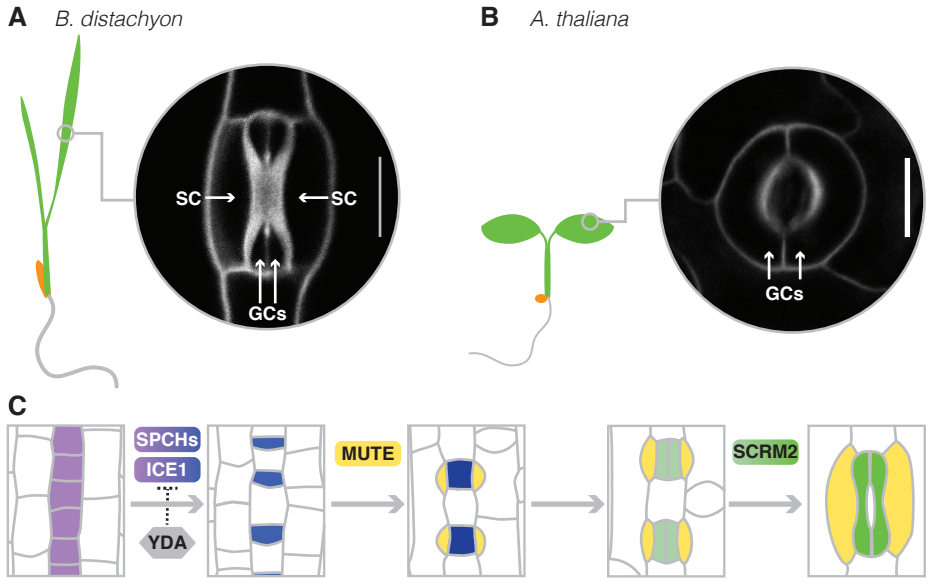

Figure 5. Stomatal development in *B. distachyon*. **A.** Graminoid stomata of *B. distachyon;* the central dumbbell-shaped guard cells (GCs) are flanked by lateral subsidiary cells (SCs); scale bar, 10*μ*m. **B.** Anomocytic stomata of *A. thaliana*; two kidney-shaped guard cells (GCs) surround the central pore; scale bar, 10*μ*m. **C.** Current working model of stomatal development in *B. distachyon*; the *Bd-SPCHs* and *BdICE1* together determine stomatal cell files (purple) and guide the smaller daughter cell towards GMC fate (blue). *BdMUTE* establishes the SC lineage and initiates polarization and asymmetric division that yields lateral SCs (yellow). After the symmetric division of the GMC, *BdSCRM2* orchestrates the differentiation and morphogenesis of the dumbbell-shaped, graminoid GCs (green).

factor *AtSPCH* that is required for entry into the stomatal lineage (MacAlister et al., 2007). Surprisingly the *B. distachyon stl* mutation affected the *INDUCER OF CBF EXPRESSION1 (ICE1)* ortholog (Raissig et al., 2016), which in *A. thaliana* acts as AtSPCH's heterodimerization partner and does not have a mutant phenotype due to its redundancy with *AtSCREAM2* (*AtSCRM2*; Kanaoka et al., 2008). Notably, a loss of function gene-edited *bdscrm2* allele revealed a role for *BdSCRM2* in differentiation and pore formation of the four-celled complex (Raissig et al., 2016). Thus unlike in *A. thaliana*, the paralogs *ICE1/SCRM1* and *SCRM2* are non-redundant in grasses with distinct roles in controlling stomatal identity establishment and stomatal differentiation, respectively (Figure 5C). In contrast, gene editing of the duplicated *SPCH* homologs *BdSPCH1* and *BdSPCH2* revealed these factors to be acting redundantly in establishing stomatal fate (Figure 5C; Raissig et al., 2016).

The same screen also identified the *subsidiary cell identity defective* (*sid*) mutant, which failed to recruit SCs and produced ancestral, two-celled stomata without SCs (Raissig et al., 2017). The *sid* mutation caused a 5bp deletion in the *B. distachyon MUTE* homolog (Raissig et al., 2017). *A. thaliana MUTE* commits stomatal meristemoids to become guard mother cells (GMCs) and *atmute* plants arrest stomatal development before the symmetric GMC division causing seedling lethality (Pillitteri et al., 2007). In *bdmute*, however, 70% of the stomatal complexes still formed functional GCs yet failed to recruit SC, suggesting that *BdMUTE* is primarily responsible to establish the SC lineage (Figure 5C; Raissig et al., 2017). Accordingly, overexpressing *BdMUTE* in *B. distachyon* leaves caused excessive asymmetric SC-like divisions yielding multiple subsidiary cells around stomatal pores (Raissig et al., 2017). In *A. thaliana*, however, overexpressing *AtMUTE* reprogrammed all epidermal cells to GMCs, which divided and formed two-celled stomata (Pillitteri et al., 2007). Much like AtMUTE, BdMUTE is initially expressed in GMCs, but unlike the *A. thaliana* homolog, acquired cell-to-cell mobility and moves laterally into neighboring, non-stomatal cells to establish SMC identity (Raissig et al., 2017). Finally, the availability of a SC-less genotype allowed for testing the functional relevance of SCs to the extremely fast stomatal kinetics observed for graminoid stomata. In fact, SC-less *bdmute* stomata were not only much slower in opening and closing, they also failed to open and close to the same degree as wild-type stomata (Raissig et al., 2017). Interestingly, this work was only possible using the wild grass *B. distachyon* since mutations in *MUTE* in the domesticated cereals maize and rice not only lack SCs but also fail to to make functional GC complexes leading to seedling lethality (Wang et al., 2019; Wu et al., 2019).

Patterning cell types in the leaf epidermis

Finally, a mutation that caused stomata to cluster within the stomatal cell file ("pearls on a string" phenotype) affected the mitogen activated protein (MAP) triple kinase *BdYODA1* (*BdYDA1*) (Abrash et al., 2018). Much like *yda* in *A. thaliana*, the mutant was also dwarfed and intensiley green (hence the gene name *YODA*; Lukowitz et al., 2004). Yet, while in *atyda*

only stomatal spacing was affected (Bergmann et al., 2004), in *bdyda1* not only spacing of stomata but also spacing of prickle hair cells and silica cells was affected (Abrash et al., 2018). This suggests a more general role for the grass MAPKKK *YDA1* homolog in patterning epidermal cell types. In *A. thaliana*, the YDA MAPK pathway directly targets and represses AtSPCH (Lampard et al., 2008) to inhibit stomatal lineage entry. Therefore, overexpressing *AtSPCH* without mutating the MAPK phosphorylation sites does not have an effect on stomatal density or number of entry divisions (Lampard et al., 2008). In *B. distachyon*, on the other hand, *BdSPCH2-YFP* could be efficiently overexpressed and was able to reprogram prickle hair cell precursor cells to generate ectopic stomatal complexes (Raissig et al., 2016). Indeed, peptide sequence analysis of SPCH homologs in *B. distachyon* and *A. thaliana* revealed differences in MAPK target sites and protein degradation domains (Raissig et al., 2016). Acquisition of high fidelity MAPK target sites in the protein degradation domain of BdICE1, on the other hand, together with the observation that BdICE1 was restricted to stomatal cell files even when overexpressed suggests that BdICE1 rather than the BdSPCH homologs might be targeted by the BdYDA1 MAPK pathway (Figure 5C; Raissig et al., 2016).

Recently, quantitative polarization measurements of a BdYDA1 reporter revealed polarized localization at the base of the larger daughter cell, where stomatal fate needs to be repressed (Gong et al., 2021). This suggests that much like in *A. thaliana* a polarity scaffold in the larger, non-stomatal daughter cell might concentrate MAPK signalling components to efficiently downregulate key stomatal identity genes (Gong et al., 2021; Zhang et al., 2015).

In conclusion, a forward genetic screen in *B. distachyon* facilitated by its small size and small genome for efficient mapping by next-generation sequencing have significantly contributed to our understanding of how graminoid stomata form. Importantly, studies on *MUTE*'s function in SC development and SC function would not have been possible in domesticated grass models since *mute* mutants in both rice and maize are seedling-lethal (Wang et al., 2019; Wu et al., 2019). Taken together, the relatively efficient *B. distachyon* transformation protocols, which facilitated the generation of dozens of stomatal reporter and overexpression lines, coupled with stomatal developmental genetics will continue to reveal mechanistic insights into the development of graminoid stomata in the grasses.

Development of monocotyledonous root systems and root hair patterning

Monocotyledonous root systems are different from dicotyledonous root systems; in eudicots, primary tap roots and their lateral branches compile the root system. In monocots on the other hand, root systems are more complex and are dominated by adventitious, post-embryonic roots (Figure 3). *B. distachyon* forms a seminal primary root and at least two adventitious roots from the coleoptile node below ground , which all persist throughout the whole life cycle (Hardtke and Pacheco-Villalobos, 2016). Depending on environmental conditions, the genetic background of specific accessions and tiller proximity to the soil, the above-ground leaf node can form adventitious roots, too (Hardtke and Pacheco-Villalobos, 2016). In fact, wind-stimulated *B. distachyon* shoots formed leaf node roots at tiller positions in proximity to soil and this process could be significantly inhibited when auxin transport was inhibited in wind-stimulated plants (Nam et al., 2020). Both seminal and adventitious roots can form lateral roots and root hairs (Hardtke and Pacheco-Villalobos, 2016). Therefore, *B. distachyon* is an ideal model to study monocotyledonous root system formation in grasses as it possesses all root types of domesticated cereals but tends to be less complex and smaller allowing efficient imaging and root growth analysis. Furthermore, *B. distachyon* is able to functionally associate with arbuscular mycorrhiza, which requires substantial transcriptional reprogramming in root cortex cells and the development of symbiotic interfaces between plant and fungi (Buendia et al., 2019b; Hong et al., 2012; Müller et al., 2020; Torabi et al., 2021). It was shown, for example, that microbial symbiotic signals like Lipo-chitooligosaccharides stimulate lateral root formation in *B. distachyon* much like in legumes (Buendia et al., 2019a).

Auxin and root growth regulation

Due to distinct redundancy levels, whole genome duplications and diversification of life histories, mutant phenotypes of homologous genes can be substantially different between *A. thaliana* and grasses. A mutation in the auxin biosynthesis gene *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1-RELATED 2-LIKE* (*BdTAR2L*) and the ethylene signaling gene *ETHYLENE INSENSITIVE-2-LIKE-1* (*BdEIN2L1*) display a longer root phenotype (Pacheco-Villalobos et al., 2013). In *A. thaliana* the respective mutants show either no phenotype due to redundancy or shorter roots, respectively (Stepanova et al., 2008; Tao et al., 2008). The longer root phenotype results from enhanced cell elongation and cell anisotropy in the root, and, counterintuitively, is accompanied by higher rather than lower auxin levels (Pacheco-Villalobos et al., 2013). This is caused by an alternative wiring of ethylene-auxin crosstalk and by the common dependency of ethylene and auxin biosynthesis on the product of Tryptophan aminotransferases, IPA. In *A. thaliana*, ethylene positively regulates *TAA1* and the *YUCCA* genes and, consequently, auxin production, whereas in *B. distachyon*, ethylene signalling represses the *YUCCA* genes (Pacheco-Villalobos et al., 2013). Therefore, the *bdtar2l* mutant leads to lower IPA, and thus, lower ethylene, which hyperactivates the YUCCAs and leads to higher auxin levels (Pacheco-Villalobos et al., 2013). Similarly, *bdein2l1* mutants fail to repress YUCCAs in an ethylene-dependent manner resulting in higher auxin levels (Pacheco-Villalobos et al., 2013). Together, both mutations lead

to increased auxin levels, exaggerated cell elongation and, thus, longer roots (Pacheco-Villalobos et al., 2013). The isolation of these mutants also facilitated testing the effect of high cellular auxin levels on transcriptional changes and whether auxin indeed stimulates cell wall acidification to promote cell elongation via expansin-mediated cell wall loosening. Transcriptionally, high cellular auxin upregulated cell wall remodelling factors including expansins and it was shown that the arabinogalactan content was reduced in the mutants (Pacheco-Villalobos et al., 2016). However, proton extrusion was not increased due to high auxin levels and, much like in *A. thaliana* , root elongation is very well buffered against external pH changes (Pacheco-Villalobos et al., 2016).

Similar to the auxin biosynthesis mutant *bdtar2l,* mutants in the auxin importer *BdAUX1* also caused roots to elongate more. In addition, *bdaux1* showed shoot dwarfism, flower developmental defects and reduced fertility (van der Schuren et al., 2018). While in *A. thaliana*, *AtAUX1* and its three *LIKE-AUX1* (*LAX*) homologs are partially redundant, *BdAUX1* seems to play a more central role (van der Schuren et al., 2018). Importantly, *bdaux1* mutant phenotypes are also more severe than corresponding *AUX1* single mutants in other grasses like *osaux1* in rice, *zmaux1* in maize or *svaux1* in *Setaria viridis*, where much milder shoot and inflorescence defects were observed (Huang et al., 2017; Zhao et al., 2015). This might be due to the presence of five *AUX1* homologs in rice, maize and *S. viridis* rather than just the three in *B. distachyon* (van der Schuren et al., 2018).

Root hair patterning

Much like the leaf epidermal cell types, root hairs alternate with non-hair cells within a longitudinal epidermal cell file (Dolan, 2017). In *A. thaliana*, only specific root epidermal cell files can form root hairs and the root hair forming files are flanked by at least two hairless cell files (Dolan, 2017). In grasses, however, all root epidermal cell files make root hairs (Dolan, 2017).

In *B. distachyon* a terminal, physically asymmetric division generates a smaller daughter cell that will develop into a root hair and a larger daughter cell making a non-hair cell (Kim and Dolan, 2011). In contrast, the domesticated cereals rice and barely execute a physically symmetric terminal division and differential elongation creates the size dimorphism between root hair and non-hair cells (Kim and Dolan, 2011; Marzec et al., 2013). In *A. thaliana*, *ROOT HAIRLESS SIX LIKE* (RSL) class I basic helix-loop-helix transcription factors generate root hair formation competency in specific cell files and activate RSL class II genes, which are necessary and sufficient for root hair outgrowth and elongation (Dolan, 2017). In *B. distachyon*, the three RSL class I genes are preferentially expressed in the smaller daughter cells and future hair cells and double knock-down lines showed shorter root hairs (Kim and Dolan, 2016). Unlike in *A. thaliana*, overexpression of RSL class I genes was sufficient to induce root hair outgrowth suggesting that root hair morphogenesis might not require RSL class II genes in *B. distachyon* (Kim and Dolan, 2016). Overexpression lines showed three-fold root hair elongation and could increase phosphorus uptake, yet showed decreased overall biomass (Zhang et al., 2018).

Taken together, *B. distachyon* is well suited to answer fundamental questions specific to root development in grasses. Again, the small stature and the reduced gene redundancy space compared to other, domesticated grasses and even *A. thaliana* paired with a simplified yet complete monocotyledonous root system will allow for the identification of additional developmental mechanisms shaping root systems in grasses.

Joys and pains of working with a new model system - a personal perspective

B. distachyon *& Daniel*

Like many biologists, Daniel is interested in the diversity of life. He began investigating this diversity in Simon Malcomber's plant evolutionary developmental laboratory. Here, he explored the evolution of genes involved in the regulation of auxin transport and how this affects axillary meristem formation (Woods et al., 2011). It was here that he became interested in the evolution of the novel grass spikelet. At the time the evolution of grass spikelet formation was mainly investigated by doing comparative expression work in candidate genes that affect spikelet formation across a variety of grasses and near grass relatives. Particular focus was paid to genes that were recently duplicated in the grasses, which could potentially lead to neo/subfunctionalization of the gene. Yet, while documenting changes in expression of a given candidate gene in a specific tissue is a proxy for a change in gene function, functional work was not possible outside of maize and rice. This prompted the move during his PhD studies in joining Richard Amasino's lab and the Brachypodium community. He was particularly excited at the prospect of using genetics to accelerate gene discovery and to not rely on a candidate gene approach identified in other model organisms. The lab traditionally had worked in *A. thaliana* but a small group in the laboratory began working in *B. distachyon*. At that time there was little to no molecular understanding of flowering in *B. distachyon*, but it was clear this provided the opportunity to discover new genes previously not identified in other plants. When very little is known about a given phenomenon genetics offers a powerful tool to identify the important genes controlling a given trait. In particular doing forward genetics screens to identify mutants with altered flowering times and exploiting natural variation in flowering behaviours across *B. distachyon* accessions was an exciting avenue for gene discovery.

There are a lot of unknowns at the beginning of any research endeavor; however with a new model system this is amplified. For example, it was unclear which might be the

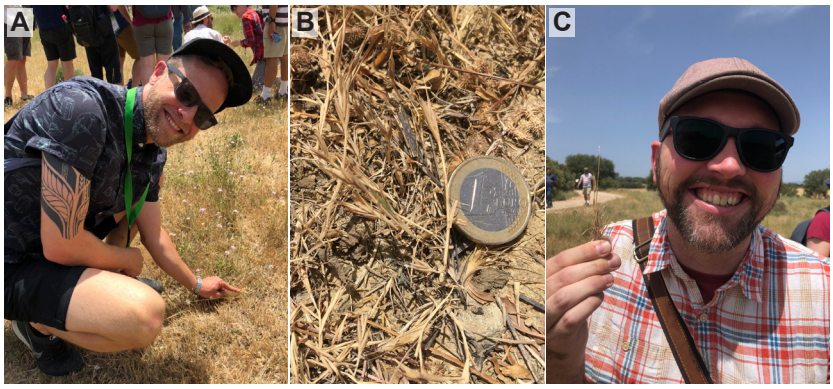

Figure 6. Two plant developmental geneticists in the field looking for their favorite plant model during the 4th International Brachypodium Conference 2019 in Huesca, Spain. The authors Michael (**A**) and Daniel (**C**) were surprised to see how small the growth habitus of *B. distachyon* is, when it grows in its natural habitat in Northern Spain (**B**). Middle photo by David Lowry.

best mapping partner to generate mapping populations or if EMS polymorphisms would need to be used for mapping in backcrossed populations. Fortunately, they identified a mapping partner in an accession that has a similar flowering behavior as the mutant background but also enough sequence variation that the density of markers would not limit mapping resolution. This required the community resources of a collection of diverse inbred germplasm and also whole genome sequence information across those accessions. Despite the many unknowns, too many to list here, the excitement of identifying new genes involved in flowering pushed the research forward.

B. distachyon *& Michael*

During his PhD studies Michael investigated how the maternal and paternal genomes differentially affect embryo formation and seed development in *A. thaliana* with a particular focus on genomic imprinting and zygotic genome activation. After having spent half of his PhD sitting in front of a custom-made embryo isolation microscope (Raissig et al., 2013) to harvest minuscule embryos deeply embedded within developing seed tissue, he decided to change to a developmental system that is more readily accessible. He thus joined Dominique Bergmann's lab to study how stomata—tiny breathing pores on the plant's surface—develop and mediate gas exchange between the plant and the atmosphere. While the Bergmann lab was and is mostly focused on stomatal development in *A. thaliana*, a small subgroup ("Team Brachy") led by the fantastic PhD student Emily Abrash was establishing *B. distachyon* as a model system to study grass stomatal development. Michael was immediately hooked by the unique morphology and cellular composition of grass stomata, their linear, cell-file specific patterning, and the strict developmental gradient that grass leaves present, so that he was willing to take the risk to work with a novel model system. He was extremely fortunate that the *B. distachyon* biotechnological (i.e. transformation), molecular and horticultural protocols were already up and running in the lab and very lucky that a handful of mutant phenotypes had already been isolated in a forward genetic screen. So upon arrival Michael could immediately start "doing the biology" and applying his developmental genetics background to map mutants and characterize gene functions while learning new techniques in cell biology, imaging and gas exchange physiology.

Together, it was rather accidental that Michael started working with *B. distachyon* and his reasoning to do so was mostly motivated by the compelling fascination of studying a derived stomatal morphology with improved functionality rather than an active choice of an alternate model system. In other words, the biological question, the phenomenon of graminoid stomatal morphology motivated Michael to embark on a journey with a novel model system. Clearly, the prospect of entering a research field that was wide open and joining the *B. distachyon* community, which is known to be open and supportive and which freely shared reagents, resources and datasets way ahead of publication were additional motivations.

Trials & tribulations

Working with a novel model system is risky and—like experimental research in general—requires an absurd amount of luck, timing and, well, luck again to succeed. And it strongly depends on the people around you, your mentors, your collaborators and your colleagues. We were both extremely fortunate to have had incredible mentors that created a supportive and vibrant research environment and fantastic collaborators within the Brachypodium community. We both want to stress how important it is to be part of an open, sharing and caring research community that puts the progress of the field before personal interests. We are very fortunate and grateful that it has always been at the core of the International Brachypodium Initiative to grant free access to genomic and genetic resources, where datasets and protocols are shared ahead of publication. In addition, the availability of molecular, genetic, genomic and biotechnological ressou-

rces facilitate deciphering developmental mechanisms in any species, so ideally you do not have to start from scratch.

With emerging model systems it can be difficult to fit in with established communities; we keep claiming that *B. distachyon* is the "Arabidopsis of grasses", yet we are lagging at least one if not two decades behind the *A. thaliana* research community in terms of technologies, reagents, datasets and the sheer size of the community. On the other hand, we are not working on a domesticated grass relevant to agriculture, which can hinder us from tapping into some of the funding opportunities that support cereal research and can evoke comments regarding why we did not work on a "real grass" or with a crop "that matters". We hope this review provides some reasons for the utility of using an emerging model over either an established model system or domesticated crops with complex genomes and growth requirements.

Novel model systems can be frustrating at times; frustrating because required reagents and technologies have not been developed yet (and frankly, usually you have to develop these things yourself, because nobody else is going to do it). And of course it can be frustrating because some things are just harder in your favorite model system than in the established ones. Crossing *B. distachyon*, for example, is a complete pain in the back compared to crossing *A. thaliana*. In *A. thaliana*, dehisced pollen is viable for several days and one cross-pollinated flower produces approximately 50 seeds in a silique. In *B. distachyon*, however, pollen is only viable for about 30 minutes after anther dehiscence and one cross-pollinated floret produces only one seed! Michael's crossing success rate is around 20% so he gets one seed per five crosses. Furthermore, while Michael was able to cross 50 Arabidopsis flowers in an hour (so 2.5k seeds!) it is approximately 10 florets for *B. distachyon* (so 2 seeds at a success rate of 20%), which means crossing *B. distachyon* is a thousand times less efficient than crossing *A. thaliana*. And we do not even want to get started comparing the benefits of plant transformation via floral dipping with the tediousness of plant transformation via embryonic tissue cultures.

That being said, it can also be extremely rewarding to work with an emerging model system. First of all, we both are convinced that this is the right time to start thinking and researching questions that go beyond the classical model systems. Recent biotechnological progress like the identification of precise gene editing tools such as CRISPR/Cas9 and affordable, accurate and efficient next-generation sequencing technologies means we can read the stored and expressed genetic information of any given species, and, if it is transformable, can mutate any given gene. This allows for a purely phenomenon-driven research approach, where unique biological aspects can be investigated that cannot be studied in established model systems because they simply do not have this cell type (i.e. stomatal SCs) or environmental response (i.e. short-day vernalization flowering). Studying unique biological aspects also means that there are so-called "low-hanging fruits"—significant discoveries that can define novel new research directions. Finally, it is extremely rewarding to be embedded within, be supported by, contribute to and help shape a pioneering research community. Both authors irrespective of their junior positions serve on the International Brachypodium Research Steering committee since 2020.

In summary, we are both strong advocates of curiosity-driven, phenomenon-oriented and somewhat untargeted fundamental basic research, because we think it is both fun and has the potential for real breakthrough discoveries. The research community studying bacterial immunity would never have anticipated that their findings would create a multi-billion dollar patent war over who owns the CRISPR/Cas9 gene editing technology (Ledford, 2017). Also in 1928, Andrew Fleming did not intend to alter modern medicine by sorting through old plates containing Staphylococcus colonies and by chance finding one plate with the antibiotic-secreting fungus *Penicillium notatum* (Aldridge, 1999). Therefore, if you are fascinated by an unusual biological structure, an unexpected environmental response, or a unique biotic interaction, you should try to find an appropriate model species with decent growth requirements and let the adventure begin.

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