RESEARCH ARTICLE

Conservation and divergence of YODA MAPKKK function in regulation of grass epidermal patterning

Emily Abrash1,‡, M. Ximena Anleu Gil1†, Juliana L. Matos1,* and Dominique C. Bergmann1,2,§

ABSTRACT

All multicellular organisms must properly pattern cell types to generate functional tissues and organs. The organized and predictable cell lineages of the Brachypodium leaf enabled us to characterize the role of the MAPK kinase kinase gene BdYODA1 in regulating asymmetric cell divisions. We find that YODA genes promote normal stomatal spacing patterns in both Arabidopsis and Brachypodium, despite species-specific differences in those patterns. Using lineage tracing and cell fate markers, we show that, unexpectedly, patterning defects in bdYODA1 mutants do not arise from faulty physical asymmetry in cell divisions but rather from improper enforcement of alternative cellular fates after division. These cross-species comparisons allow us to refine our understanding of MAPK activities during plant asymmetric cell divisions.

KEY WORDS: Asymmetric cell division, MAPK pathway, Stomata, Brachypodium, Comparative development

INTRODUCTION

The correct establishment of cell types during development is essential for the generation of cellular diversity and patterning of tissues, organs and organisms. Asymmetric cell division, the process that gives rise to daughter cells with different physical appearance and/or developmental fate, is a crucial mechanism that most eukaryotes employ to generate diverse but organized cell populations. Asymmetric cell divisions are necessary from the very first to the very last events in plant development, and have been studied extensively during embryogenesis and root, shoot and reproductive development in flowering plants (Van Norman, 2016; Marzec et al., 2015; Abrash and Bergmann, 2009) as well as in basal lineages (Harrison et al., 2009; De Smet and Beeckman, 2011), albeit in a more limited fashion. A number of regulatory players and mechanisms have been identified in specific cellular contexts; however, unifying players and modules used repeatedly among these different asymmetric cell division contexts are only just beginning to come to light (Abrash and Bergmann, 2009; De Smet and Beeckman, 2011).

The stomatal lineages of various flowering plants offer particularly rich and accessible model systems for the study of asymmetric division regulation. Stomata are epidermal valves consisting of paired guard cells (GCs) flanking a central pore, and they contract and relax to regulate gas exchange between the plant and its environment. In many plant species, asymmetric cell divisions produce one daughter cell that acts as a stomatal precursor and another that differentiates as an epidermal pavement cell (Fryns-Claessens and Van Cotthem, 1973; Dong and Bergmann, 2010; Lau and Bergmann, 2012). In broadleaf dicots like Arabidopsis, the asymmetric divisions are self-renewing, i.e. once a cell undergoes an asymmetric division, its progeny may also continue dividing asymmetrically, in a situation somewhat analogous to the transit-amplifying cells in animal lineages (Matos and Bergmann, 2014). In the grasses, by contrast, the stomatal lineage exhibits a less flexible pattern of divisions in which stomatal precursors undergo a single asymmetric division that yields a terminal precursor (the guard mother cell, or GMC) and a larger sister cell fated to become a pavement cell. Also, notably in grasses, the lateral neighbors of the GMC undergo unique additional asymmetric divisions, parallel to the long axis of the leaf, to form the subsidiary cells (SCs) that act to facilitate stomatal function (Hepworth et al., 2017). In Brachypodium distachyon, a forage grass related to wheat, asymmetric cell divisions accompany the creation of many epidermal cell types (Raissig et al., 2016). Here, both stomatal and hair cell lineage development are subject to tight temporal regulation and occur in a developmental progression from the base (younger tissue) to the tip (older tissue) of the leaf blade (Fig. 1A,B). The common deployment of asymmetric divisions in multiple epidermal lineages in grasses requires that stomatal fate be later superimposed in particular cell files to specify the smaller daughter cells as stomatal precursors (Raissig et al., 2016).

Asymmetric divisions during development are guided by fate, polarity, and signaling inputs. In Arabidopsis stomatal production, basic helix-loop-helix (bHLH) transcription factors, the polarity protein BASL and a signaling pathway comprising ligands, receptors and a mitogen-activated protein kinase (MAPK) cascade have been connected to these roles (Lau and Bergmann, 2012; Pillitteri et al., 2016). The MAPK pathway is headed by the MAPK kinase (MAPKKK) YODA (AtYDA) (Bergmann et al., 2004). Genetic and biochemical data indicate that AtYDA responds to positional information provided by peptide ligands of the EPIDERMAL PATTERNING FACTOR (EPF) family via transmembrane receptors TOO MANY MOUTHS (TMM) and members of the ERECTA (ER) family (ERF) (Nadeau and Sack, 2002; Kim et al., 2012; Hunt and Gray, 2009; Kondo et al., 2010; Sugano et al., 2010; Har et al., 2007; Shpak et al., 2005). AtYDA then relays this information through downstream kinases M KK4/5 and MAPK3/6 (Lampard et al., 2009; Wang et al., 2007) to phosphorylate and inhibit the bHLH transcription factor SPEECHLESS (AtSPCH), the primary regulator of entry into the stomatal lineage pathway (Lampard et al., 2008). Loss of AtYDA activity results in the production of excess stomata arranged in large
clusters, whereas overactivity suppresses development of stomata (Bergmann et al., 2004), phenotypes opposite of those ascribed to loss and gain of AtSPCH function (Lampard et al., 2009; Macalister et al., 2007).

In grasses, the stomatal lineage requires homologs of the bHLH transcription factors known from Arabidopsis, though sometimes employed in different ways (Raissig et al., 2016; Raissig et al., 2017; Liu et al., 2009). BdICE1, BdSPCH1 and BdSPCH2 are needed for stomatal lineage initiation, and the two SPCH homologs are expressed in epidermal cells before they undergo asymmetric cell divisions, but their expression becomes restricted to the smaller daughter cells after division (Raissig et al., 2016). EPF overexpression has been shown to arrest stomatal production in barley (Hughes et al., 2017), potentially acting upstream of these transcription factors, but whether EPFs enforce stereotyped asymmetric divisions and the ‘every other cell’ epidermal pattern in grasses is not yet known.

In a screen aimed at identifying mutations that affect stomatal patterning in Brachypodium, we have identified a mutation in BdYDA1 that displays clustered stomata similar to loss of AtYDA in Arabidopsis. The mutation in BdYDA1 also led to patterning and fate defects in other epidermal cell types and changes in overall plant morphology. These additional phenotypes were interesting in light of previous findings that AtYDA is not exclusively a stomatal lineage regulator; it was originally characterized for its role in asymmetric divisions of the zygote (Lukowitz et al., 2004) and was subsequently shown to act during development of the inflorescence (Meng et al., 2012) and anthers (Hord et al., 2008), in defense (Sopeña-Torres et al., 2018) and in specifying division plane orientation in the root (Směkalová et al., 2014). Based on work in Arabidopsis, YDA was surmised to establish physically asymmetric divisions and the ‘every other cell’ epidermal pattern in grasses is not yet known.

RESULTS

Mutations in BdYDA1 lead to severe disruptions in stomatal pattern

In wild-type Brachypodium distachyon Bd21-3 (WT), stomata are separated by at least one intervening non-stomatal cell (Fig. 1A,B). From an ethyl methanesulfonate (EMS)-mutagenized population of WT plants (described by Raissig et al., 2016), we identified a line segregating plants that, unlike WT, bore large groups of stomata in contact in a single row (Fig. 2A,B,H). The mutant did not exhibit ectopic stomatal rows, suggesting that the mutation affected processes occurring after specification of stomatal row identity. In addition to the stomatal patterning defects, mutants displayed whole plant growth defects, including compressed internodes and lateral organs, reduced overall stature, dark green coloration, and sterility (Fig. 2E). This combination of phenotypes was similar to that previously described for Arabidopsis plants bearing mutations in the MAPKKK-encoding gene AtYDA (Bergmann et al., 2004; Lukowitz et al., 2004). YDA orthologs can be identified in many plant species; in the grasses, YDA has been duplicated (Fig. S1). We sequenced the gene with the higher sequence similarity to AtYDA [BdYDA1 (Bradi5g18180)] and found a missense mutation (G2287A) predicted to confer a charge change (E460>K) in a conserved residue of the kinase domain (Fig. 21).

To determine whether the identified mutation was indeed causal for the phenotype, we generated BdYDA1pro:BdYDA1-YFP:Yt, a complementation construct consisting of ~5.1 kb of upstream sequence, the BdYDA1 genomic region (including introns), a YFP tag and ~1.5 kb of downstream sequence. The lateral organ and internode elongation defects of bdyda1-1 were rescued by this construct (Fig. 2E), as were the stomatal patterning defects (Fig. 2C, quantified in 2F,G). This was strong evidence that the mutation in BdYDA1 was responsible for the phenotype. Previous work on AtYDA and other MAPKKKs demonstrated that mutations in the kinase domain could lead to hypomorphic or null alleles (Sopeña-Torres et al., 2018; Lukowitz et al., 2004). We therefore also created an additional clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) mutation-based allele in the first exon of BdYDA1 to attempt to eliminate the protein altogether (Fig. S2A). bdyda1-2 is heteroallelic for frameshift mutations predicted to encode truncated proteins of

Fig. 1. Stomatal development in Brachypodium as model to study the progression of asymmetric divisions. (A) Simplified model of leaf blade epidermal development in Brachypodium. Specific cell files at predictable distances from veins (gray files) acquire stomatal lineage fate (stage 1) and undergo stomatal differentiation in a tip-to-base gradient. All cells in the epidermis then divide asymmetrically (ACD). In stomatal files, the smaller daughter cell of each division becomes a GMC (blue, stage 2). In all other files, these cells develop into hair cells (white circles in non-stomatal files). GMCs then recruit SCs (yellow, stage 3), divide once symmetrically to form two GCs (green, stage 4), and mature as four-celled complexes (stage 5). (B) Scanning electron micrograph of WT (Bd21-3) leaf epidermis. GCs and SCs are false-colored green and yellow, respectively. Co-existence of stomatal and hair fates in a single file is highlighted by white arrowheads. Scale bar: 50 μm.
41 and 95 amino acids in contrast to the normal BdYDA1 protein of 896 amino acids (Fig. S2C) and resulted in the same suite of stomatal and morphological phenotypes as \textit{bdyda1-1}; however, the magnitude of the clustering was increased (Fig. 2D, Fig. S2B). The difference between the phenotypes produced by the E460>K missense allele compared with the truncation allele suggests that \textit{bdyda1-1} is a hypomorphic allele. CRISPR/Cas9-induced mutations in the second \textit{YDA} homolog \textit{BdYDA2} (Bradi3g51380) did not result in any obvious stomatal or overall growth phenotypes (Fig. S3). To test whether there might be redundancy between \textit{BdYDA1} and \textit{BdYDA2}, we created early truncation \textit{bdyda2} CRISPR alleles in a \textit{bdyda1-1/+} background. In progeny homozygous for \textit{bdyda1-1} and \textit{bdyda2}, we did not see any novel leaf epidermal phenotypes, nor did we see enhancement of the \textit{bdyda1} phenotype (data not shown).

The large clusters of stomata in \textit{Arabidopsis yda} mutants arise from aberrant asymmetric divisions in the self-renewing precursor cells before they commit to becoming GMCs (Bergmann et al., 2004). Such self-renewing asymmetric divisions, however, are absent in grasses. An advantage of the more streamlined and rigid developmental trajectory in grasses is that we could generate a pseudo-timecourse by imaging a single \textit{Brachypodium} leaf from base to tip and observing the cells at different ontological stages in \textit{bdyda1-1} and WT (Fig. 3A-H). To our surprise, early epidermal asymmetric divisions appeared normal in the \textit{bdyda1-1} mutant (Fig. 3A). By the SC recruitment...
BdYDA1 acts primarily in the early fate decisions and not in stomatal GC differentiation.

To address how activity of BdYDA1 might enforce stomatal patterning in Brachypodium, we visualized the expression pattern of the complementing BdYDA1pro:BdYDA1-YFP:Yt reporter. BdYDA1-YFP signal was not specific to the stomatal lineage and appeared to be present at roughly comparable levels in all the different leaf epidermal cell types we monitored (Fig. 3I-L). Fluorescence was strongest in the cytoplasm and/or at the cell periphery and was not detectable in the nucleus. In some cells, BdYDA1-YFP fluorescence appeared to be concentrated at a single face of a cell, most frequently at the interface between a GMC and its neighbor cell that will give rise to an SC (Fig. 3I,J, arrowheads; Fig. S4). This broad expression pattern is similar to that of AtYDA, suggesting that, like in Arabidopsis, it is the presence of appropriate upstream activating signals and downstream targets that provides specificity to YDA-mediated signaling activity.

Cell fate marker expression suggests defects in fate reinforcement in bdyda1-1

To further dissect the defects during GMC specification and SC recruitment observed in bdyda1-1, we examined the expression and behavior of stomatal lineage fate markers. BdSCRM2pro:YFP-BdSCRM2 is a pan-stomatal lineage marker (Raissig et al., 2016). It can be visualized in nuclei from the time stomatal rows are specified; when these cells start dividing asymmetrically, BdSCRM2 is confined to the smaller daughter of these divisions (Fig. 4A) and remains restricted to stomatal complexes as they mature (Fig. 4B-D). In bdyda1-1, however, restriction of signal to the smaller daughter of an asymmetric division is lost (Fig. 4E,F). Specifically, we observed signal in larger cells between stomatal precursors (Fig. 4E, arrowheads). In some cases, these presumed pavement cell precursors underwent an ectopic asymmetric division generating a stomatal precursor positive for YFP-BdSCRM2 expression adjacent to the earlier-specified stomatal precursor (Fig. 4F, arrows).

To investigate later specification events and SC recruitment, we analyzed the expression pattern of BdMUTEpro:BdMUTE-YFP, which, in WT, starts in young GMCs and shows strong signal in mature GMCs and weak signal in adjacent subsidiary mother cell files (Fig. 4I). BdMUTE expression is maintained until after GMC division in both GCs and SCs and disappears during complex maturation (Fig. 4J,K). In bdyda1-1, young and mature GMCs showed marked patterning defects, with numerous BdMUTE-positive cells positioned adjacent to one another (Fig. 4L). Marker expression was also present in SCs even if they were recruited and formed abnormally. BdMUTE persisted in stomatal clusters through GMC division, but was extinguished rapidly as the GCs matured, as observed in WT (Fig. 4M,N).

Taken together, the reporter results agree with the morphological assessments and suggest that in bdyda1-1 mechanisms in place to control fate establishment and/or division potential early in the lineage are disrupted, but stomatal differentiation and morphogenesis are largely unaffected.

BdYDA1 regulates cell patterning in other cell lineages

In studying the effects of bdyda1-1 on the stomatal lineage, it became apparent that this was not the only epidermal cell lineage disrupted by the mutation. Brachypodium also produces regularly spaced hair cells in the leaf epidermis. These hairs arise via asymmetric divisions in a manner similar to stomata, and the two fates appear to be closely related and somewhat interchangeable.
[e.g. a cell file may contain a number of stomata, then a hair, then more stomata (Raissig et al., 2016); for an example, see Fig. 1B]. In *bdyda1-1* plants, the hair cell lineage displays defects very similar to the stomatal lineage, producing hair cells at higher density than in WT and often in longitudinal clusters (Fig. 5A,B, rescued in SC, and quantified in 5D,E). In addition, the epidermis of the leaf sheath contains crenellated pavement cells and round silica cells sometimes accompanied by a small lens-shaped cell and a small triangular cell (Fig. 5F). Patterning and proliferation defects were evident in all of these cell types in *bdyda1-1*, and were complemented by the BdYDA1 reporter (Fig. 5G,H). We quantified patterning and proliferation defects in the large, round silica cells because, among the sheath cell types, they were most unambiguously identified. Like stomata and hair cells, the silica cells were produced at a greater density than in WT and were found in numerous longitudinal clusters (Fig. 5LJ). Cross-sections of mature leaves revealed that the overall organization of the leaf was similar to WT, but bulliform, photosynthetic and vascular cells were present in slightly higher numbers (Fig. S5).

**DISCUSSION**

By characterizing a mutation that disrupted stomatal patterning in *Brachypodium distachyon*, we identified the *BdYDA1* gene, a *Brachypodium* ortholog of the *Arabidopsis* MAPKKK gene *AtYDA*. Like *atyda* (Bergmann et al., 2004; Lukowitz et al., 2004), the *bdyda1* mutant produces excess stomata arranged in clusters and displays a stunted growth phenotype characterized by compressed internodes and compact lateral organs. Strikingly, we could show that clusters in *bdyda1* arise from mis-specification of alternative fates in the epidermis, rather than being caused by alterations in the physical asymmetry of the divisions themselves. In addition, other non-stomatal epidermal cell types are also affected. Taken together, our results demonstrate that *BdYDA1* is a general regulator of cell fate establishment and enforcement, two processes that are crucial for the correct execution of asymmetric divisions during epidermal patterning of the grass leaf (Fig. 6A).

Asymmetric divisions are produced and oriented by a combination of extrinsic and intrinsic cues. *AtYDA* plays roles in the transduction of both sources of developmental information in *Arabidopsis* stomatal development. Positional (extrinsic) information conveyed by EPFL family ligands interacting with ERf/TMM receptors can activate the *AtYDA* MAPK cascade, leading to *AtSPCH* inhibition and repression of stomatal identity (reviewed by Pillitteri and Torii, 2012). More recently, a role in intracellular polarity via a physical association between *AtYDA* and *ERf/TMM* receptors can activate the *AtYDA* MAPK cascade, leading to differential signaling capacity; the cell with higher *AtYDA* has differential signaling capacity; the cell with higher *AtYDA* has higher *AtSPCH* levels and consequently loses stomatal identity (Zhang et al., 2015). The cortical *AtYDA/BASL* complex is preferentially inherited by the larger daughter cell of an asymmetric division, leading to differential signaling capacity; the cell with higher *AtYDA* has lower *AtSPCH* levels and consequently loses stomatal identity (Zhang et al., 2016).

Could BdYDA1 participate in similar signaling or intrinsic polarity modules? The observation of non-uniform BdYDA1-YFP distribution around cells might hint to it being localized by a differentially inherited polarity complex. However, two observations make this solution unlikely. First, the specific location of the BdYDA1-YFP enrichment, at the boundary between GC and prospective SC, is not consistent with it being preferentially segregated to the larger (non-stomatal) cell during normal development. Moreover, polar localization would require an as-yet-unknown polarity partner, as *BASL* homologs have not been detected in the grasses.
In terms of the cell-cell signaling response, Arabidopsis YDA and Brachypodium YDA1 proteins show appreciable sequence similarity (65% overall and 90% in kinase domain; Fig. S6) and components of the YDA MAPK pathway have clear orthologs in Brachypodium, including downstream kinases MKK4 (Bradi1g46880), MKK5 (Bradi3g53650), MPK3 (Bradi3g53650) and MPK6 (Bradi1g49100). Upstream signaling components include multiple EPFL family members, TMM (Bradi2g43940), and ERECTA, although the ERECTA family consists of only two members (Bradi1g46450 and Bradi1g49950). Although the presence of homologous signaling pathway genes makes participation in parallel signaling cascades possible for AtYDA and BdYDA1, there remains the issue of the distinct ontogeny of dicot and grass stomata. With no self-renewing divisions, mature complexes restricted to specific cell files, and consistent orientation of each complex along the leaf’s proximal-distal axis, the positional information required in grasses is very different from that in Arabidopsis in which distributed ‘point sources’ of signals and extensive neighbor-to-neighbor signaling are dominant patterning mechanisms (Torii, 2015) (Fig. 6B). As disorderly as epidermal identities become within bdyda1 cell files, they still obey tissue-wide fate arrangements, indicating that different factors control lateral (and proximal-distal) positional information in the leaf. Although none of the work with grass EPF homologs has yet demonstrated an effect on lateral patterning (Hughes et al., 2017; Yin et al., 2017), expanded expression of SHORTROOT, a factor normally involved in internal cell fates, leads to production of supernumerary stomatal rows in rice (Schuler et al., 2018), suggesting that lateral information may be provided through different pathways.

Fig. 5. bdyda1-1 mutants exhibit disruption of cell fates in other asymmetrically dividing epidermal lineages. (A–C) DIC images of cleared WT (Bd21-3) (A), bdyda1-1 mutant (B), and bdyda1-1 rescued with BdYDA1pro:BdYDA1-YFP:Yt (C) leaf epidermis. Hair cells are false-colored magenta. WT and complemented bdyda1-1 images show sixth leaf from base (third from main tiller) at 27 dpg. bdyda1-1 images show epidermal peels of sixth leaf from base (third from main tiller) at 27 dpg. Scale bars: 40 μm. (D) Hair cell density of bdyda1-1 mutants compared with that of WT and rescued bdyda1-1 [sixth leaf from base (third from main tiller) at 27 dpg]. n=4 individuals for WT control and n=5 for rescued plants. For each sample, five different regions of the leaf were imaged and quantified. n=5 for bdyda1-1 mutants for which four different regions of the leaf were peeled, imaged, and quantified. ***P<0.001; n.s., not significant (based on Kruskal–Wallis test followed by Dunn’s multiple comparisons test). (E) Hair cell cluster profile as percentage of clustering of quantified hair cells in bdyda1-1 mutants, WT, and rescued bdyda1-1 (n=2286 hair cells for WT controls, n=3988 hair cells for rescue, n=2789 hair cells for bdyda1-1). Clusters of four or more hair cells were grouped in last category ‘4+-mer’. (F–H) DIC images of cleared WT (F), bdyda1-1 mutant (G), and rescued bdyda1-1 (H) sheath epidermis. Silica cells are false-colored orange. For all genotypes, images show the sheath of the sixth leaf from base (third from main tiller) at 27 dpg. Scale bars: 40 μm. (I) Silica cell density of bdyda1-1 compared with that of WT and rescued bdyda1-1 [sheath of the sixth leaf from base (third from main tiller) at 27 dpg]. n=4 individuals for WT control, n=5 for rescued plants, and n=5 for bdyda1-1 mutants. For all, four to six different regions of the sheath were imaged and quantified. ***P<0.001; n.s., not significant (based on Kruskal–Wallis test followed by Dunn’s multiple comparisons test). (J) Silica cell cluster profile as percentages of clustering of quantified silica cells in bdyda1-1 mutants, WT, and rescued bdyda1-1 (n=1328 silica cells for WT controls, n=2995 silica cells for rescue, n=1691 silica cells for bdyda1-1). Clusters of four or more silica cells were grouped in last category ‘4+-mer’.
When considering downstream targets of a BdYDA1-mediated MAPK cascade in cell fate reinforcement roles, the stomatal initiation module could be targeted to inhibit stomatal fate establishment in larger daughter cells of asymmetric divisions, much as it is in *Arabidopsis*, but the precise protein target in this complex may be different. In *Arabidopsis*, AtSPCH is the demonstrated target of MAPK regulation (Lampard et al., 2008), but in *Brachypodium*, BdICE1 may play a more dominant role as it possesses predicted high-fidelity MAPK target sites within the protein degradation-associated PEST domain, whereas BdSPCH1 and BdSPCH2 do not (Raissig et al., 2016). Furthermore, expression of ubiquitin promoter-driven YFP-BdICE1 accumulates only in the stomatal lineage cells of the leaf, indicating that this protein is subject to post-translational regulation, as one would expect from a target of a MAPK cascade (Raissig et al., 2016).

BdYDA1 also regulates fate re-enforcement in other non-stomatal epidermal cell lineages; however, the means by which it does so remain to be explored. It is likely that BdYDA1 has targets that could enforce fate asymmetry in all of these decisions, whereas the specific fate of the cells (stomata, hair or silica) would be determined by other information. Such is the case with *Arabidopsis* embryos and stomata in which signaling can work when swapped between these developmental contexts (Bayer et al., 2009), but downstream transcription factors provide unique cell identities (Jeong et al., 2011; Ueda et al., 2017). We noticed that in the early truncation allele *bdyda1-2* the degree of clustering is less in non-stomatal epidermal cells than in stomatal files (Fig. 2D, Fig. S2B) suggesting that BdYDA1-independent fate-determining mechanisms also exist in these lineages.

In *Arabidopsis* embryos, roots, and the stomatal lineage, loss or overactivity of AtYDA changes an asymmetric division into one for which daughters exhibit equalized cell fates and cell sizes. From these phenotypes, it is intuitive to imagine AtYDA’s role as one initiating asymmetry in the mother cell of these formative divisions (Fig. 6C). Furthermore, considering that YDA’s downstream effectors are the microtubule-regulating kinases AtMPK3 and AtMPK6, AtYDA was suggested to mediate cytoskeletal behaviors leading to the placement of division planes and creation of asymmetric divisions (Smékalová et al., 2014). In our present study, however, we showed that the physical asymmetry of divisions is not affected in the absence of *BdYDA1*, prompting us to re-evaluate whether AtYDA actually generates pre-divisional, physical asymmetry, or whether the failure to create different-sized cells in *atyda* mutants stems from a post-divisional failure in cell identity. High-resolution time-lapse imaging of developmental decisions in *Arabidopsis* would be needed to distinguish between YDA playing primarily a pre- or post-divisional role.
The work presented here emphasizes the value of comparative developmental studies, as the *bdyda1* mutations reveal roles for the YDA pathway in numerous cell fate decisions, some of which represent cell types not present in *Arabidopsis*. This work also provokes a conceptual shift in our emphasis on MAPK signaling as required for the creation of asymmetry to MAPK signaling required for the post-divisional enforcement of asymmetric fates. In a kingdom devoid of Notch-Delta lateral inhibition systems with scant evidence for any segregated fate determinants, and one characterized by exceedingly flexible development, it may be that plant cell fate decisions are rarely made in advance, but are subject to multiple rounds of confirmation through post-divisional communication and re-enforcement.

**MATERIALS AND METHODS**

**Plant material**

The *bdyda1-1* mutant was recovered from the M3 generation of an EMS mutagenesis of the Bd21-3 ecotype (seeds provided by Dr John Vogel, Joint Genome Institute, CA, USA; Raisig et al., 2016), and maintained through selection of heterozygous individuals that segregated the mutations in a typical 3:1 Mendelian recessive ratio. Bd21-3 was used as WT for all experiments described in this paper (Vogel and Hill, 2007). Plants were initially grown on half-strength MS agar plates in a 22°C chamber with a 16-h light/8-h dark cycle (110 μmol m⁻² s⁻¹), then subsequently transferred to soil and placed in a greenhouse with a 20-h light/4-h dark cycle (250-300 μmol m⁻² s⁻¹; day temperature: 28°C; night temperature: 18°C). Seeds were stratified for at least 2 days at 4°C before transferred to light.

**Generation of constructs**

All primer sequences are provided in Table S1. *BdASCRM2:YFP-BdASCRM2* was recovered by Raisig et al. (2016). To create *BdYDA1pro:BdYDA1-YFP:Yt* and *BdMUTEpro:BdMUTE-YFP*, sequences were amplified from BACs BD_BA00029F15 and BD_BA00042015/BD_AB0008512 (Arizona Genomics Institute; http://www.genome.arizona.edu), respectively. For *BdYDA1pro:BdYDA1-YFP:Yt*, 5.1 kb upstream sequence (primers *BdYDA1proF:1-1R* and *BdYDA1pro_AscI-1R*) and 3′ sequence (*BdYDA1term-1F* and *BdYDA1term-1R*) of the *BdYDA1* gene were cloned into pIPKb001 (Himmelbach et al., 2007). This then was recombined with a *BdYDA1-YFP* fusion in PENTR-D, composed of the *BdYDA1* genomic region (primers *BdYDA1proPac1-1F* and *BdYDA1cDNAscAscl-1R*) followed by AscI-flanked *Citrine* YFP.

For *BdMUTEpro:BdMUTE-YFP*, 1.1 kb upstream sequence of the *BdMUTE* gene (Bradi1g18400) (primers *BdMUTEproFWD* and *BdMUTEpro-REV*) was cloned into pIPKb001 (Raisig et al., 2016). Separately, the *BdMUTE* genomic sequence (primers *BdMUTE-DS-FWD* and *BdMUTE-DS-REV*) was cloned into pENTR-D with a poly-alanine linker (annealed primers Aaln linker-F and Aaln linker-R) and an AscI-flanked *Citrine* YFP inserted 3′ of the gene by AscI digest. Finally, the entry clone was recombined into the pIPKb001 vector.

CRISPR constructs were designed using the vectors pHUBi-cas9-9 and pOs-sgRNA (vectors and protocol described by Miao et al., 2013). The online server, CRISPR-P, was used to identify candidate spacer sequences (Lei et al., 2014). Spacers were generated by annealing oligo duplexes priMXA38F+39R for *BdYDA1* CRISPR sgRNA 6 (which generated *bdyda1-2*) and priMXA30F+31R for *BdYDA2* CRISPR sgRNA 11. Primers priMXA50 and 52 were used to genotype *bdyda1-2* and primers priMXA48 and 49 were used to genotype *bdyda2-1* and *bdyda2-2*. BdYDA1 CRISPRs were transformed into WT and BdYDA2 CRISPRs into both WT and *bdyda1-1/+* genotypes as described below.

**Generation of transgenic lines**

*Brachypodium* calli derived from Bd21-3 and *bdyda1-1/+* parental plants were transformed with AGL1 *Agrobacterium tumefaciens*, selected and regenerated according to standard protocols (https://jgi.doe.gov/our-science/science-programs/plant-genomics/brachypodium/). Plants and calli from *bdyda1-1/+* parents were genotyped using primers priMXA25, 26 and 27 in a single PCR reaction as described by Gaudet et al. (2009).

**Microscopy and phenotypic analysis**

For imaging on a Leica SP5 confocal microscope, leaves were carefully taken out from the surrounding sheath and stained with propidium iodide (PI; 1:100 dilution of 1 mg/ml stock) to visualize cell walls, then mounted in water. For differential interference contrast (DIC) imaging on a Leica DM6 B microscope of WT and rescue plant leaf (1.5-2 cm distal of the sixth leaf blade of 22 dpg leaves) and sheath (topmost centimeter of sheath tissue surrounding seventh leaf of 22 dpg plants), tissue was collected into 7:1 ethanol:acetic acid and incubated overnight to remove chlorophyll, then rinsed with water, and mounted in Hoyer’s medium to clear. The same was done for DIC imaging of *bdyda1-2*, except no sheath tissue was collected for it. For DIC imaging of *bdyda1-1*, epidermal peels were collected from the tissue of interest into Hoyer’s medium, mounted on slides, and examined. Cell numbers and extent of stomatal clusters were counted directly on a computer display attached to the microscope (0.29 mm² field of view for stomata and hair cell counts; 0.15 mm² field of view for silica cell counts). Only cells fully contained within the image frame were included in the respective analysis. In cases when a cluster expanded beyond the image frame, cells outside the image frame were included to correctly represent the number of cells part of the cluster. For DIC imaging of WT and *bdyda1-1* leaf cross-sections, fully emerged adult leaves were taken directly from a growing plant (~6 weeks old) then hand-sectioned and immediately mounted in Hoyer’s medium. For scanning electron microscope (SEM) images, WT leaves were taken directly from a growing plant, then introduced into an Environmental SEM (FEI Quanta 200) without any further treatments.

**Statistical analysis and plotting**

Statistical analysis was performed in R (R Core Team, 2017). The Shapiro–Wilk test (shapiro-test function) was used to check samples for normality; as many samples displayed non-normal distributions, the Kruskal–Wallis test (a nonparametric analog of ANOVA; kruskal.test function) was used, followed by Dunn’s Multiple Comparisons tests (dunn.test, dunnTest) to assess significance of pairwise comparisons.

**Acknowledgements**

We thank Akhila Bettadapur for technical assistance, Dr John Vogel (JGI) for creating *Brachypodium* resources, Dr Kathryn Barton (Carnegie, DPB) for use of her SEM, Emanuel Schorsch for consultation on statistics, and Dr Michael Raisig, Dr Annika Weimer, and other members of the Bergmann lab for discussions and comments on the manuscript.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


**Funding**

E.A. was supported by an National Science Foundation graduate research fellowship and a Stanford graduate fellowship. D.C.B. is an investigator of the Howard Hughes Medical Institute. Deposited in PMC for release after 6 months.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.165860.supplemental

**References**


Figure S1. Phylogenetic tree of YDA sequences from representative dicots, grasses, and basal plant lineages.

Bootstrap value (%) of 1,000 replicates is shown. Protein sequences were obtained from the Phytozome v11 database (https://phytozome.jgi.doe.gov/). On MEGA7 (Kumar et al., 2016), the sequences were first aligned by MUSCLE, then the tree was calculated using the maximum likelihood method (1,000 bootstrap replicates).
Figure S2. Additional characterization of a CRISPR/Cas9-induced allele of *BdYDA1*.

(A) Gene/Protein diagram of *BdYDA1*. The vertical magenta bar indicates the *bdyda1-1* EMS mutation. A green bar indicates the *bdyda1-2* CRISPR/Cas9 induced mutations. (two at same site). Models here and in Fig. S3A were generated in Gene Structure Display Server (Hu et al., 2015).

(B) Differential Interference Contrast (DIC) images of cleared *bdyda1-2*. T0 regenerants. Guard cells and subsidiary cells are false-colored green and yellow, respectively. Scale bar = 40 µm.

(C) Chromatogram of CRISPR-induced mutations in *bdyda1-2*. The WT sequence is indicated above the chromatogram, and the Sanger sequence is shown below the chromatogram.
Figure S3. Additional characterization of CRISPR/Cas9-induced alleles of *BdYDA2*.

(A) Gene/Protein diagram of *BdYDA2*. The vertical orange bar indicates the location of the *bdyda2-1* and *bdyda2-2* CRISPR/Cas9 induced mutations.

(B-C) Differential Interference Contrast (DIC) images of cleared *bdyda2-1* (B) and *bdyda2-2* (C). T0 regenerants. Guard cells and subsidiary cells are false-colored green and yellow, respectively. Scale bar = 40µm.

(C-D) Chromatogram of CRISPR-induced mutations *bdyda2-1* (B) and *bdyda2-2* (C). The WT sequence is indicated above the chromatogram, and the Sanger sequence is shown below the chromatogram.
Figure S4. A rescuing *BdYDA1*pro:*BdYDA1*-YFP:*Yt transgene co-localizes with PI.

Confocal images of *BdYDA1*pro:*BdYDA1*-YFP:*Yt in *bdyda1-1* (T1 plant; emerging 2nd leaf at 6 dpg) displaying (A) PI channel only and (B) merge of YFP and PI channel to show overlap of YFP signal with cell outlines in the leaf epidermis. YFP signal alone is shown in Fig. 3I-L. Scale bar = 10 µm. All images are oriented with the base of the leaf blade (younger cells) towards the bottom and the tip of the leaf (older cells) towards the top.
Figure S5. Cross sections of mature leaf anatomy in WT and bdyda1-1.

DIC images of cleared hand-sectioned WT (Bd21-3) and bdyda1-1 leaves to show that inner tissues are also affected in the mutant. Compared to WT, bdyda1-1 leaves have a thicker mesophyll due to the presence of additional cells and display higher numbers of ground tissue cells around the vasculature.
Figure S6. Protein sequence alignment of AtYDA, BdYDA1, and BdYDA2.
Detailed protein alignment of the YDA orthologues in *Arabidopsis* and *Brachypodium*. Kinase domains are shown in blue; site of EMS-generated mutation in *bdyda1-1* is bolded. Alignments were done using Clustal Omega at EMBL-EBI (Sievers et al., 2011).
Table S1. Primers used in construction of transgenes and genotyping of alleles (all shown 5’ → 3’).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>AscI_FP_noATG- 1F</td>
<td>GGCGCCGCGGTGAGCAAGGGCGAGGAG</td>
<td>Citrine YFP cloning</td>
</tr>
<tr>
<td>AscI_FP_stop-1R</td>
<td>GGC CGCCTTACGCTCTCGTCCATGC</td>
<td>Citrine YFP cloning, BdYDA1 cloning</td>
</tr>
<tr>
<td>BdYDA1term-1F</td>
<td>GATTAATTTAATAATTTTTAAGCCAAGTGG</td>
<td>BdYDA1 terminator cloning</td>
</tr>
<tr>
<td>BdYDA1term-1R</td>
<td>CTGAGTCGTACATATGGCTGTTCC</td>
<td>BdYDA1 terminator cloning</td>
</tr>
<tr>
<td>BdYDA1pro5.1kb -1F</td>
<td>CACCGCGCGCCGCTGTTGGTTTGTGATCC</td>
<td>BdYDA1 promoter cloning</td>
</tr>
<tr>
<td>BdYDA1pro_AscI -1R</td>
<td>GGC CGGCATCGAAATGGAGAATTTGTTGTTG</td>
<td>BdYDA1 promoter cloning</td>
</tr>
<tr>
<td>BdYDA1proPacI- 1F</td>
<td>GATTAATTTAATAATGCATGGGATTTTTTCCAAGC</td>
<td>BdYDA1 genomic cloning</td>
</tr>
<tr>
<td>BdYDA1cDNAns cAscI-1R</td>
<td>CTGGCGCGCCGTTATCATGGCTGACTGGAAGG</td>
<td>BdYDA1 genomic cloning</td>
</tr>
<tr>
<td>BdYDA1-1F</td>
<td>CACCATGTGCTGAGTATTTTAGACGG</td>
<td>BdYDA1-YFP cloning</td>
</tr>
<tr>
<td>Ala_linker-F</td>
<td>CGCGCAGCAGCAGCAGCGCTGCCGCTGCCAGCGAGGG</td>
<td>Poly-alanine linker</td>
</tr>
<tr>
<td>Ala_linker-R</td>
<td>CGCGCCCTGCTGCCAGCGCGCCAGCGCTGCTGCTG</td>
<td>Poly-alanine linker</td>
</tr>
<tr>
<td>BdMUTEpro- FWD</td>
<td>TTACTAGTCAGCTAGCAGCAGCAGCTATT</td>
<td>BdMUTE promoter cloning</td>
</tr>
<tr>
<td>BdMUTEpro- REV</td>
<td>TAAGCTTGATCGTGTCGTTCTTC</td>
<td>BdMUTE promoter cloning</td>
</tr>
<tr>
<td>BdMUTE-CDS- FWD</td>
<td>CACCATGTCGACATCGC</td>
<td>BdMUTE genomic cloning</td>
</tr>
<tr>
<td>BdMUTE-CDS- REV</td>
<td>ATTGATCATGATGTCGCC</td>
<td>BdMUTE genomic cloning</td>
</tr>
<tr>
<td>priMXA38</td>
<td>GGCAGGAACCACAAAGTCAAACG</td>
<td>bdyda1-2 CRISPR (sgRNA_6) generation, F</td>
</tr>
<tr>
<td>priMXA39</td>
<td>AAACGGTTTTGACTTGTGTTCC</td>
<td>bdyda1-2 CRISPR (sgRNA_6) generation, R</td>
</tr>
<tr>
<td>priMXA30</td>
<td>GGCACTAGGATTGGTGGAACACTC</td>
<td>bdyda2-1, bdyda2-2 CRISPR (sgRNA_11) generation, F</td>
</tr>
<tr>
<td>primXA31</td>
<td>AAACGAGTGTTCACCAATTCCTAG</td>
<td>bdyda2-1, bdyda2-2 CRISPR (sgRNA_11) generation, R</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>primXA50</td>
<td>TGGCTGACATTGTGGTGAGT</td>
<td>bdyda1-2 genotyping, F</td>
</tr>
<tr>
<td>primXA52</td>
<td>GGACTTCTAGATGGGCTTGACA</td>
<td>bdyda1-2 genotyping, R</td>
</tr>
<tr>
<td>primXA48</td>
<td>AAGCCCTTCTCAAAAGCCCAA</td>
<td>bdyda2-1 and bdyda2-2 genotyping, R</td>
</tr>
<tr>
<td>primXA49</td>
<td>AGGCGGGACTCTATTCCAAA</td>
<td>bdyda2-1 and bdyda2-2 genotyping, R</td>
</tr>
<tr>
<td>primXA25</td>
<td>CAAAGCAGTTGGGCGAGG</td>
<td>bdyda1-1 genotyping, F</td>
</tr>
<tr>
<td>primXA26</td>
<td>AGCATAGATAATGGCAAGCGCTCAAGATGATGTTTC</td>
<td>bdyda1-1 genotyping, WT R</td>
</tr>
<tr>
<td>primXA27</td>
<td>TTCAGAAGCGGCTCAAGATGATGTTTT</td>
<td>bdyda1-1 genotyping, mut R</td>
</tr>
</tbody>
</table>