Keep on Growing: Building and Patterning Leaves in the Grasses

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Abstract

Monocot leaves have unique features that arise early in their development. Maturing leaves protectively enclose younger leaves and the meristem, the pool of founder cells from which a leaf emerges. Through the maturation process, proximal sheath and distal blade tissues differentiate and are separated by the ligule and auricle structures. Here we review current research focusing on the contribution of gene regulatory factors and phytohormones on the patterning and differentiation of monocot leaves primarily focusing on research in the grasses (*Poaceae*). The 10,000 members of the grasses include the true grain cereals (wheat, rice, maize, etc), biofuel crops such as sugarcane, pasture grasses, and bamboo. They are the most studied of the monocots due to their tremendous agricultural and agronomic importance.

Starting out: organogenesis and early leaf morphogenesis

Throughout the life of a land plant, above ground organs are derived from the shoot apical meristem, a pool of slowly dividing and undifferentiated cells (Fig1 a-c). The site of a new lateral organ is demarcated by a domain with a high concentration of the phytohormone auxin coupled with down regulation of KNOX (KNOTTED1-like homeobox) transcription factors [1,2]. Recent studies in maize and rice have begun to unravel a complex gene network regulated by KN1. Both maize KN1 and its rice homolog OSH1 were shown to bind the promoter region of thousands of genes while only modulating a subset of genes bound. Of the genes that are modulated and bound, the majority is attributed to hormone signaling or activity [3,4]. These results emphasize that precise spatiotemporal control of hormone levels, signaling and crosstalk is required for meristem maintenance and lateral organ initiation.

Characteristic differences in organ growth between monocots and eudicots are correlated with subtle differences in the patterning of these regulatory factors in the SAM and the leaf initial. In contrast to the leaf of the model eudicot *Arabidopsis*, the grasses exhibit parallel venation and a sheathing leaf base. Some of these characteristics can be attributed to differences in the arrangement of founder cells within the SAM; a monocot leaf emerges from a disc of insertion around the circumference of the meristem (Fig 1) while a eudicot leaf emerges from a point of insertion on the flank of the meristem [2,5].

Lateral organ initiation is correlated with creation of an auxin maximum [6]. The direction of auxin flow is visualized via the localization of the PIN1 family of auxin efflux transporters [7,8]. Convergence of auxin flow via active transport through polar localization of PIN1 creates an auxin maximum. In *Arabidopsis*, PIN1 functions as the primary auxin transporter during leaf initiation. In families outside of the Brassica, the gene family has expanded [9,10]. For example, in *Brachypodium*, *Pin1a*, *Pin1b* and *Sister of Pin1 (SoPIN1)* have unique accumulation patterns that appear to subdivide the effort of *Pin1* in *Arabidopsis* [9].

Patterning within a lateral organ begins as the provascular midvein is marked by the reorientation of PIN1 inward toward existing vascular strands in the stem. Organ outgrowth initiates along the axis created by the PIN1 marked provasculture and the underlying PIN1 depleted domain [5]. Computed tomography imaging of the developing maize leaf reveals that the pre-blade emerges from the disc of insertion with non-overlapping lateral margins and the pre-sheath domain with overlapping lateral margins [5]. A unique pattern of polar PIN1 localization pointing auxin flow toward the L1 cells from L2 cells is correlated with the overlapping pre-sheath margins; loss of this marginal gradient is hypothesized to result in the fused sheath phenotype associated with inhibition of auxin transport in leaves [5].

Crossroads: boundary formation along the proximal-distal axis

After initiation, an incipient grass leaf encircles the meristem and younger leaf primordia, while being encased by older leaves (Fig 1). Grass leaves develop basipetally, distal cells differentiate and mature while basal cells continue to divide and expand. Longitudinal

and transverse divisions occur contributing to proximal-distal as well as medial-lateral growth. The distinct tissues of grass leaves, blade, sheath and ligule, arise after the distal pre-blade has expanded considerably (Fig 2a). Cells in the preligule band take on distinct division and elongation patterns forming a line of small cells perpendicular to the proximal-distal axis dividing future blade and sheath. Within the preligule band, periclinal cell divisions produce the mature ligule fringe on the adaxial epidermis. Later in development, the adjacent auricle cells expand, tilting the leaf blade away from the stem (Fig 2b) [11]. Leaf angle is an important agronomic trait that is directly correlated with growth and differentiation of the ligule and auricle [12].

Very few sheath-specific genes have been characterized in monocots and the distinct morphological differences between the sheathing monocot base and the narrow, stem-like petiole of eudicot models complicate comparative analysis of proximal tissues. In *Arabidopsis*, *Blade-on-petiole1/2* (*BOP1/2*) directly regulates the lateral organ boundary domain protein *Asymmetric leaves2* (*AS2*) that in turn aids the recruitment of the *Polycomb Repressive Complex 2* (*PRC2*) to silence *KNOX* promoter elements in the proximal leaf [13,14]. Loss of *BOP* family function results in distal blade tissue differentiating in the proximal leaf petiole [15,16]. Recent work characterizing the barley *Uniculme4* (*Cul4*) mutation reveals the affected gene encodes a *Blade-on-petiole* (*BOP*)-like gene. Loss of *Cul4* results in a liguleless phenotype and reduced tillering suggesting a conserved role for this protein in lateral organ initiation and proximal-distal patterning [17].

KN1 may also regulate the expression of key leaf specific transcription factors within the proximal base. The *KNOX* gene *Liguleless3* (*lg3*) is normally expressed in the maize sheath and is bound by KN1 [3,18]. The sheath is normal in *lg3* knockout lines and redundancy will likely complicate studies of individual *KNOX* proteins [18]. *Lg3* may interact with other leaf specific co-factors such as *bell2* and *bell4* that express at the proximal-distal boundary and leaf-SAM boundaries and are also bound and modulated by KN1 (Fig 2c-e) [3,19].

Although KN1 RNA is not expressed in the leaf, the protein has been detected in the proximal leaf base and it may affect proximal-distal patterning of leaves through regulation of downstream factors [20,21]. In maize, KN1 activates the GA catabolism gene, *ga2-ox*, which is high at the base of the leaf and in the unexpanded stem (below the SAM). The pattern of expression suggests that GA levels increase more distally in the leaf, and GA measurements are highest in cells at the transition from division to elongation [22]. In rice, the *KN1* homolog *OSH1* regulates catabolism of another phytohormone, brassinosteroid (BR) through direct transcriptional regulation of *OsCYP734A2*, *OsCYP734A4* and *OsCYP734A6*, three BR inactivating enzymes homologous to the *Arabidopsis* *BASI* cytochrome p450 [4]. BR response and metabolism has long been of agronomic interest in rice and is implicated in the regulation of several important traits such as leaf angle, biomass, grain size and yield [23]. Knockdown of *OSH1* or *OsCYP734A2*, *OsCYP734A4* and *OsCYP734A6* results in BR hypersensitive phenotypes in rice with an increased joint angle resulting from auricle overgrowth and decreased sheath length (Fig 2e). Overexpression of *OSH1* results in BR

insensitivity manifesting as upright leaves and decreased sheath length [4]. Recent papers in maize reveal consistent findings with those in rice. Mutants in BR synthesis show dwarf plants with upright leaves [24,25] as do RNAi lines that reduce expression of five BR receptors [26].

In rice, sensitivity to BR can be tested by incubating excised leaves in hormone containing solution and comparing leaf angle to controls. Incubation in BR leads to an increase in leaf angle for sensitive plants [4,27]. A new study has shed light on molecular mechanisms connecting BR response to control of cell proliferation. Evidence suggests that BR activates a *BES1*-like transcription factor that binds to the promoter of a boundary-specific cyclin, *CYCU4;1* inhibiting its expression (Fig 2d). This represses abaxial L2 sclerenchyma division coincident with increased cell expansion in the adaxial epidermis to create a larger angle. As another level of control, the *GSK3* kinase BIN2 can bind and phosphorylate *CYCU4;1* which is proposed to promote cell division of abaxial sclerenchyma cells. Proliferation of these cells promotes an upright leaf stature balancing adaxial cell expansion to sculpt final leaf angle [27].

The classic *liguleless1* (*lg1*) mutation knocks out an SBP type transcription factor that accumulates specifically at the proximal-distal boundary (Fig 2d). Mutant plants grow upright leaves devoid of ligule and auricle tissues with decreased tassel branch number and angle [28,29]. A transcriptomic analysis of laser-captured tissues from the sheath, boundary and blade of P7 wild-type and *lg1* leaves was performed to profile the factors important to the differentiation of maize leaf tissue types. In all cell layers, over 1700 genes were differentially expressed between the ligule and blade, 1000 between ligule and sheath and over 600 comparing blade to sheath [19]. The resulting database of factors is a valuable resource for characterizing known and unknown genes implicated in sheath and blade cell identity as well as boundary differentiation.

From this study, several genes known for their role in patterning organ boundaries were found to be up-regulated in the proximal-distal boundary [19]. For example, the maize homolog of *uniculme4* is expressed in the mature ligule as well as the developing proximal base of young leaves and in initiating axillary meristems (Fig 2e) [19]. *Cup-shaped cotyledon* (*CUC*) genes are factors required for boundary creation and organ separation in the Arabidopsis vegetative and floral meristem [30]. *ZmCUC2* is expressed at the proximal-distal leaf boundary as well as between the SAM and developing lateral organs (Fig 2f). These results suggest that conservation among regulatory gene cohorts at lateral organ boundaries across tissues types is highly likely.

An important domestication trait in rice is panicle branch angle; upright branches trap grains preventing pre-harvest grain drop better than widely spread branches. A small genomic region containing the *lg1* locus and 5' promoter elements from domesticated rice (*O Sativa* Nipponbare with a closed panicle) was recombined into undomesticated rice (*O rufipogon*, which otherwise would have open panicles) conferring a closed panicle trait [31]. Conversely, when this genomic region from an undomesticated line is transformed into a domesticated background, *lg1* expression and a spread panicle phenotype is restored [32]. Expression of *lg1* was equivalent in leaf tissues of both

domesticated and undomesticated varieties. Analysis of invariant residues within this genomic region of domesticated and undomesticated varieties implicated a single SNP associated with panicle-specific *lg1* expression in undomesticated lines [32]. Therefore, selective loss of *lg1* expression, only in the panicle, was a likely domestication strategy to affect changes in panicle branch architecture.

The *Wavy auricle in blade 1 (Wab1)* mutation affects a grass specific *tb1*-like TCP (*teosinte branched1 (tb1)/ Cycloidea/ Proliferation Cell Factor*) bHLH transcription factor. Dominant *Wab1* mutants show ectopic ligule outgrowth and narrow leaves caused by ectopic expression of *wab1* in the leaf blade [29,33,34]. This *wab1* expression in maize leaf blades is sufficient for LG1 protein accumulation [29]. Plants carrying loss of function mutations in *wab1* or in *lg1* show decreased tassel branch number and angle [29,35]. LG1 protein accumulates in the axil of wild-type tassel branches, but not in tassels of *wab1* loss of function mutants suggesting *wab1* is upstream of *lg1* in a pathway regulating tassel branch angle [29]. It is an intriguing possibility that WAB1 binds *lg1* regulatory elements to elicit tassel specific LG1 protein accumulation and open tassel branches.

Recent work supports the efficacy of experiments that compare and contrast the molecular mechanisms regulating boundary formation across tissue types. A genomics based approach was used to identify the gene networks regulating developmental transitions in several tissues during maize inflorescence development. As axillary meristems develop into flowers, the tassel initiates branches at its base while branching is repressed in the ear. An RNAseq survey of a mutant background where branching was de-repressed in the ear (a *ramosa1 (ra1)* mutant background) compared to wild type implicated a number of gene networks as promoters of branching. Of interest here, ChIPseq data was correlated with RNAseq data sets demonstrating that RA1 can bind an intron of *lg1* and that *lg1* expression is up-regulated in *ra1* ears. This signifies negative regulation of *lg1* by RA1 in wild-type ears as part of a pathway repressing branch outgrowth [36].

More work to do: Integrating the other axes of development

A number of genes that affect proximal-distal boundary patterning also affect medial-lateral leaf growth. *lg1* mutants are slightly narrower than normal sibs but *wab1* and *Liguleless narrow-R (Lgn-R)* leaves are very narrow [33,37,38]. *Lgn* encodes a transmembrane kinase that appears to be inactive in the dominant mutant. Heterozygous *Lgn-R* leaves are narrow and short and lack ligule and auricle except at the midrib. Tassel branches are few and upright and ears often are small or absent [38]. Expression of *lg1* and *liguleless2 (lg2)* is reduced in a *Lgn-R* background suggesting this kinase may function in the promotion of a signal required for proper ligule and auricle differentiation.

Interestingly, the genomic environment of the *Lgn-R* mutation affects expressivity of the phenotype; the phenotype described is in the B73 inbred background while crosses to another inbred, Mo17, give nearly complete rescue. In this background, leaves are wider, plants make ears and the ligule and auricle are restored. Using recombinant inbred lines, a major QTL was discovered that is responsible for the rescue [39]. The rescue is even

more dramatic when plants are grown at higher temperatures. Using *Lgn-R* as a sensitized line has provided an inroad into a genotype by environment effect.

The *Lgn-R* mutation affects placement of the proximal-distal boundary but the phenotype only manifests in medial domains. Conversely, the well-characterized *lg2-R* mutation also affects the proximal-distal boundary but the phenotype only manifests in the lateral domains. The LG2 protein has been proposed to function non-cell autonomously and to coordinate (or to be) a signal moving laterally away from the midrib to delineate the proximal-distal boundary [40,41]. Interestingly, loss of *lg1* or overexpression of *lg1* (as seen in the *Wab1-R* mutant background) results in narrower leaves without affecting blade length [29,38]. The correlation of narrow leaf phenotypes manifesting in mutant backgrounds where proximal-distal patterning is disrupted suggests that the growth and patterning of these axes is intricately linked.

In the last few years, the rice *narrow leaf1* (*nal1*), *nal2* and *nal3* mutants have been characterized and cloned [42,43]. Loss of *nal1*, a trypsin-like protease, results in an overall reduction in leaf width and length correlating with significant reductions in several cell cycle regulatory genes [43]. Double mutants of the WUSCHEL-related homeobox genes *nal2/nal3*, homologs to the maize *narrowsheath1* (*ns1*) and *ns2* pair [44], have very narrow leaves and lack marginal saw-tooth hairs without affecting leaf length [42]. Although all three are called “narrow leaf” and have pleiotropic phenotypes, *nal1* generally affects growth through regulation of cell division throughout the plant while *nal2/nal3* and *ns1/ns2* play a specific role in patterning the lateral domain of a leaf. Indeed, *ns1* and *ns2* are expressed specifically in the lateral domains of leaves [44].

In addition to the proximal-distal and medial-lateral axes, the adaxial-abaxial axis exists at organ inception. Most research studying the regulation of adaxial and abaxial cell fate has been conducted in eudicot species with a few reports in rice and maize and other models of interest; progress of note has been thoroughly reviewed elsewhere (Box 1).

Conclusions and Directions

Several research topics have pushed the study of leaf patterning and differentiation in recent years (Box 1). Continued investment in grasses as new model systems provides inroads towards robust comparative studies [45]. Cutting edge sequencing and computational power has become more affordable and widespread providing large data sets and tools for the research community (Box 2). We have focused on several papers that describe the gene regulatory circuits connecting phytohormone signaling and perception to leaf patterning and development. We also draw attention to the recent surge of interest in proximal-distal patterning of grass leaves and the broad roles that many of these factors play at other organ boundaries throughout the plant.

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Box 1: reviews of interest:leaf initiation, growth and development:

Bar M, Ori N.: Compound leaf development in model plant species. *Curr Opin Plant Biol* 2015, 23: 61-9.

Dkhar J, Pareek A.: What determines a leaf's shape? *Evodevo* 2014, 5(47): 1-19.

Fukushima K, Hasebe M.: Adaxial-abaxial polarity: The developmental basis of leaf shape diversity. *Genesis* 2014, 52(1):1-18.

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Asymmetric cell divisions and stomatal patterning:

Arnaud D, Hwang I. A sophisticated network of signaling pathways regulates stomatal defenses to bacterial pathogens. *Mol Plant* 2015, 8(4): 566-81.

Serna, L: The role of brassinosteroids and abscisic acid in stomatal development *Plant Science* 2014, 225: 95-101.

McLachlan DH, Kopischke M, Robatzek S.: Gate control: guard cell regulation by microbial stress. *New Phytol* 2014, 203(41): 49-63.

Kajala K, Ramakrishna P, Fisher A, Bergmann DC, De Smet I, Sozzani R, Weijers D, Brady SM:

Omics and modelling approaches for understanding regulation of asymmetric cell divisions in arabidopsis and other angiosperm plants. *Ann Bot* 2014, 113(7):1083-1105.

Facette MR, Smith LG. Division polarity in developing stomata. *Curr Opin Plant Biol* 2012, 15(6): 585-92.

Box 2: large data tools:

A: Quantative trait loci mapping of maize leaf characteristics in recombinant inbred lines.

Guo et al.: Genetic analysis and major quantitative trait locus mapping of leaf widths at different positions in multiple populations. *PLOS one* 2015,10(3).

Li et al.: Genetic control of the leaf angle and leaf orientation value as revealed by ultra-high density maps in three connected maize populations. *PLOS one* 2015,10(3).

B: Studies using a Genome-wide association (GWAS) mapping approach in the maize nested association mapping population of recombinant inbred lines.

Tian et al.: Genome-wide association study of leaf architecture in the maize nested association mapping population. *Nat Genetics* 2011, 43 (2): 159-162.

Brown et al.: Distinct genetic architectures for male and female inflorescence traits of maize. *PLOS Genetics* 2011, 7(11).

C: Models for C4/ C3 research

Wang L. et al.: Comparative analyses of C4 and C3 photosynthesis in developing leaves of maize and rice. *Nat Biotech* 2014, 32(11): 1158-1165.

Wang P. et al.: Genome-wide transcript analysis of early maize leaf development reveals gene cohorts associated with the differentiation of C4 Kranz anatomy. *The Plant Journal* 2013, 75: 656-670.

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D: Proteomics and phosphoproteomics database for maize leaf development

Facette M et al.: Parallel proteomic and phosphoproteomic analyses of successive stages of maize leaf development. *The Plant Cell* 2013, 25: 2798-2812.

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Figure legends

Figure 1. The Shoot Apical Meristem generates leaves. **(a)** A diagrammatic representation from a lateral view of a maize shoot dissected to reveal the meristem (M), disc of insertion for the emerging leaf (P0) and the first and second leaf primordia (P1, P2). The older leaves sheath the younger leaves protecting them and the meristem. **(b)** A simplified diagram of a longitudinal section through the representation in Figure 1A. **(c)** A simplified diagram of a transverse section through the meristem and surrounding leaves. The midvein forms in P1 and secondary veins appear in older leaves initiating towards the lateral margin in parallel lines and maturing basipetally.

Figure 2. The maize leaf and expression domains of important regulators. **(a)** Photograph of a wild-type maize leaf of the B73 inbred highlighting different tissue types. The proximal-distal boundary separates the blade (B) and sheath (S). **a'** At the boundary, the ligule (L) and auricles (A) differentiate, **(b)** A diagram of WT leaf angle compared to an upright leaf (left) and an outward leaf (right). Upright leaves are seen in mutant phenotypes of *lg1*, *lg2*, *Lgn*, *cul4* and *Bril* RNAi. An outward leaf with leaf angles approaching 90° are found in *Wab1-R* and RNAi of *cycu4;1*. **(c)** A diagram of a longitudinal section through a wild-type maize vegetative shoot apical meristem (SAM) with the upper portion of blades not shown. The 4th, 6th and 8th youngest leaves are diagrammed and labeled as the medial side (m) or the lateral side (l). At P4, the ligule has yet to be defined. By P6, the preligule band has formed and by P8, the sheath has greatly expanded and mature ligule has emerged from the adaxial epidermis. **(d-f)** Expression atlas for many of the factors described in this review are highlighted in blue. Expression in a domain does not strictly correlate with a mutant phenotype **(d)** A diagram of proximal-distal boundary specific expression patterns described for *lg1*, *bell2*, and *cycu4;1* [19,27,29]. **(e)** A diagram of factors with both proximal-distal boundary and sheath specific expression patterns described for *cul4*, *bop*, *lg3*, *bell4* and *cyp734A6* [4,17-19]. **(f)** A diagram of *cuc2* expression seen at both the proximal-distal and leaf/stem boundary [19].

Figure 1

Figure 2