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Regulation of Leaf Blade Development in *Medicago truncatula*

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Abstract

Leaves are the main photosynthetic organs for most flowering plants serving as solar panels capturing solar energy and converting it into chemical energy on which most heterotrophic organisms including humans depend. Leaves are confronted with two conflicting choices; while the broad and flat surface improves photosynthetic efficiency via facilitating capture of solar energy, it inadvertently promotes water loss via transpiration. Thus, plants strictly control the shape and size of their leaves depending on the environments in which they grow. In both simple and compound leaf species, leaf development starts with recruitment of leaf primordium founder cells from the peripheral region of the shoot apical meristem (SAM). The founder cells then undergo a series of well-organized cell proliferation and cell differentiation programs to allow the formation of a characteristically expanded lamina along with three polarized planes: proximal–distal, medial–lateral, and adaxial–abaxial. The legume model species *Medicago truncatula* has a compound leaf with three leaflets. Thus, the leaf founder initials, in addition to orchestrating growth in the three axes of the blade, are required for the initiation of lateral leaflets. In this review, we will summarize the major molecular factors that regulate leaf blade expansion in *M. truncatula* and highlight some recent findings in how leaflet initiation may be controlled.

9.1 Introduction

Leaves are the main photosynthetic organs of flowering plants assimilating solar energy and CO_2 into sugars on which most heterotrophic organisms depend for their livelihood. Leaves can be simple with a single blade or compound with more than one blade called leaflets. In both simple and compound leaf species, leaf development starts with recruitment of leaf primordium founder cells from the peripheral region of the shoot apical meristem (SAM) and a group of undifferentiated pluripotent stem cells at the shoot tips of plants. These small population of

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cells are strictly regulated by a complex and interconnected transcriptional network of genes and hormones. The Class 1 KNOTTED1-LIKE HOMEOBOX (KNOX1) family transcriptional regulators are expressed throughout the SAM and maintain the undifferentiated state in the SAM. Leaf primordium initiation requires localized accumulation of the phytohormone auxin as auxin maxima (Reinhardt et al. 2000, 2003; Braybrook and Kuhlemeier 2010) and repression of *KNOX1* gene expression by the ASSYMMETRIC LEAVES1 and 2 (AS1/AS2) complex (Long et al. 1996; Uchida et al. 2007; Guo et al. 2008; Jun et al. 2010) at the primordium initiation site. *KNOX1* genes modulate the cytokinin/gibberellin (GA) ratio in the SAM by activating cytokinin biosynthesis and repressing GA biosynthesis or activating GA catabolism (Jasinski et al. 2005; Bolduc and Hake 2009).

Once the leaf primordium initial cells are recruited from the SAM, the primordium organizes itself into defined cell layers through hierarchically programed cell division and cell expansion patterns forming a flattened blade along with three distinct growth axes: proximal–distal (length direction), medial–lateral (width direction) and adaxial–abaxial also called dorsal–ventral (thickness direction). Several elegant studies in Antirrhinum and Arabidopsis over the last 25 years have clearly demonstrated that polarity patterning along the adaxial–abaxial axis of the leaf blade is required for blade outgrowth (Waites and Hudson 1995; Waites et al. 1998; Byrne et al. 2000; Kerstetter et al. 2001; McConnellet al. 2001; Emery et al. 2003; Iwakawa et al. 2007; Husbands et al. 2009). The adaxial factors such as *PHABULOSA* (*PHB*) and *ASYMMETRIC LEAVES2* (*AS2*) are expressed on the adaxial/upper side of the leaf blade (McConnell et al. 2001; Iwakawa et al. 2002), whereas the *KANADI* (*KAN*) and *YABBY* (*YAB*) families of transcription factors are expressed on the abaxial side (Siegfried et al. 1999; Kerstetter et al. 2001). The adaxial and abaxial factors interact antagonistically to prevent expression in each other's domains, and this interaction is required for proper blade outgrowth (Husbands et al. 2009; Kidner and Timmermans 2010; Szakonyi et al. 2010). Polarity patterning is further fine-tuned by auxin response factors (ARFs), miRNAs, and tasi-ARFs, which also show polarized expression patterns (Efroni et al. 2010; Husbands et al. 2015; Merelo et al. 2016).

Nevertheless, the phenotypes of some of the loss-of-function mutants of polarity genes in different plant species are variable and sometimes double and triple mutants are required to observe obvious leaf phenotypes. Recently, a new regulatory domain at the adaxial and abaxial juxtaposition was discovered (Tadege et al. 2011; Nakata et al. 2012), which is required for outgrowth of the leaf blade in the lateral direction. In this chapter, we will briefly revise the regulatory factors and their mechanism of action in the middle domain, as well as their connection to polarity factors with reference to *Medicago truncatula*. Also, since *M. truncatula* has trifoliate leaves, we will highlight some of the recent progress in the understanding of leaflet initiation, which has been obscured in the simple leaf Arabidopsis.

9.2 WOX Genes in Medio-Lateral Outgrowth of *M. Truncatula* Leaf Blade

WUSCHEL-related homeobox (WOX) family genes are plant-specific homeodomain transcription factors involved in a variety of plant developmental programs including shoot and root meristem maintenance, embryo development, and lateral organs growth (Mukherjee et al. 2009; van der Graaff et al. 2009). In Arabidopsis, the WOX family consists of 15 members, including the founding members of the WOX family WUSCHEL (WUS) and WOX1-WOX14 (Haecker et al. 2004). Arabidopsis WUS and WOX5 function in shoot and root meristem maintenance, respectively (Mayer et al. 1998; Sarkar et al. 2007).

WUS is expressed in the organizing center of SAM in Arabidopsis and the protein diffuses to neighboring stem cells in the central zone forming a gradient to function in pluripotent stem cells maintenance by activating *CLAVATA3* (*CLV3*) (Mayer et al. 1998; Yadav et al.

2010, 2011; Daum et al. 2014). CLV3, a peptide signal that activates CLV receptor kinase signaling, on the other hand, represses *WUS* to restrict its expression (Brand et al. 2000; Kayes and Clark 1998). This negative feedback loop regulation is required for meristem maintenance (Somssich et al. 2016). The *wus* mutant terminates the meristem and starts to regrow showing a ‘stop-and-go’ type of growth habit resulting in aberrant vegetative and inflorescence meristems (Laux et al. 1996; Kieffer et al. 2006; Wang et al. 2017). But the leaves of *wus* mutants display no discernible phenotype. However, we observed that when the *pressed flower* (*prs*) mutant is combined with the *wox1* and *wus* mutants, the *wox1 prs wus* triple mutant displayed much smaller and narrower leaf blade than the *wox1 prs* double mutant (Zhang and Tadege 2015), suggesting that Arabidopsis *WUS* may function in leaf development redundantly with other WOX genes.

In *M. truncatula*, the *WUS* homolog, *HEADLESS* (*HDL*) appears to have non-redundant function in leaf development. The *hdl* mutant displays a shorter and heart-shaped blade phenotype compared to wild type R108 (Meng et al. 2019; Wang et al. 2019), suggesting a role in proximo-distal growth. Unlike *WUS*, the *HDL* transcript is detected in leaf primordia in addition to the SAM (Meng et al. 2019), consistent with its direct involvement in leaf development. Interestingly, the *hdl* mutant also has other additional phenotypes compared to the *wus* mutant. The *hdl* mutant never make stems and as a result it never flowers (Meng et al. 2019; Tadege et al. 2015; Wang et al. 2019) but leaf primordia initiation is not affected as such, if any, more leaves are formed compared to WT. This finding suggests that the regions of the meristem may have different requirements for HDL function, in which the central meristem could completely fail while the peripheral meristem is fully functional. This also suggests that the aberrant leaf shape in the *hdl* mutant is not caused by failure in the recruitment of leaf founder initials from the SAM but rather by failure in specific functions of HDL during leaf proliferation and expansion after the leaf primordium is initiated. Nevertheless, the main function of HDL does seem to be in leaf development, as leaves in the *hdl* mutant are pretty normal despite modest alterations in shape. Other *WOX* genes may play a more determinant role in leaf blade development and the first such evidence came from sources other than Medicago.

In the maize *narrow sheath1* (*ns1*) and (*ns2*) double mutant, the lateral domain of the leaf blade is ablated leading to severely narrow blades (Scanlon et al. 1996; Scanlon and Freeling 1997). Both NS1 and NS2 were found to be homologs of Arabidopsis WOX3/PRS (Nardmann et al. 2004), demonstrating a key role for WOX genes in regulating medio-lateral outgrowth of the leaf blade. In the *ns1 ns2* double mutant, *KNOX* genes are not down regulated in the founder cell domain and as a result *NS1* and *NS2* are supposed to function in direct recruitment of marginal founder cells from the SAM (Scanlon 2000). Similarly, in rice, the homologous *nal2 anl3* double mutant causes narrow leaf and other pleotropic phenotypes (Ishiwata et al. 2013; Cho et al. 2013), confirming the function of *NS/WOX3* in leaf development at least in monocots.

In *M. truncatula*, medio-lateral outgrowth of the leaf blade is regulated by another *WOX* gene named *STENOFOLIA (STF).* Loss of function of *STF* and its homolog *LAM1* in *Nicotiana sylvestris* results in severely narrow leaf blades and flower petals, and aberrant blade vasculature due mainly to defects in cell proliferation (McHale 1992; Tadege et al. 2011; Lin et al. 2013a). STF is a homolog of Arabidopsis WOX1, which redundantly with WOX3/PRS controls lateral expansion of the leaf blade but the *wox1* mutant alone has no visible phenotype (Vandenbussche et al. 2009; Nakata et al. 2012). Histological analysis by in situ hybridization and GUS staining demonstrated that *STF* is specifically expressed at the adaxial–abaxial junction of leaf primordia and expanding leaves but excluded from the SAM (Tadege et al. 2011). The Medicago *stf* and *N. sylvestris lam1* mutants are drastically affected in blade width (medio-lateral growth), the *lam1* mutant showing the more severe phenotype with nearly naked midrib and vestigial blade strips, but proximo-distal growth (leaf length) is unaffected (Tadege et al. 2011; Tadege 2014). The phenotypes are primarily caused by reduced cell proliferation. This demonstrates that a factor that is neither adaxial nor abaxial in expression pattern critically regulates medio-lateral growth by activating cell proliferation. Phylogenetic analysis revealed that STF/WOX1 homologs are specific to eudicots and the ancestral *Amborella trichopoda* but absent from monocots (Vandenbussche et al. 2009; Tadege et al. 2011; Zhang et al. 2014). Despite the *WOX1* and *WOX3/PRS* redundancy in Arabidopsis leaf blade development, most STF/WOX1 homologs, in addition to *lam1*, display phenotypes as single mutants including *maw* in petunia (Vandenbussche et al. 2009), *lath* in pea and *naol* in Lotus (Zhuang et al. 2012), *mf* in cucumber (Wang et al. 2020), and *Sllam1* in tomato (Wang et al. 2021). Moreover, the WOX3 homolog in Medicago has a flower but no leaf blade phenotype in the *lfl* mutant (Niu et al. 2015). These observations suggest that *STF* and its homologs are key regulators of lateral blade expansion in eudicots, but this function is performed by *WOX3* and its homologs in monocots.

9.3 Mechanism of STF Function

9.3.1 STF Acts as a Transcriptional Repressor

The STF protein has two highly conserved motifs of about 10 aa each in length near the 3’ end; WUS box and STF box (Tadege et al. 2011). These motifs additively confer repressive activity to STF, and mutational analysis revealed that this activity is required for STF function in promoting blade outgrowth (Zhang et al. 2014). Thus, STF is a transcriptional repressor required for lateral organ (including leaves and petals) expansion and vascular patterning (Lin et al. 2013a, b; Zhang and Tadege 2015). To perform its repressive function, STF physically interacts with the MtTPL family of transcriptional corepressors with its WUS box and STF box (Zhang et al. 2014). The STF-MtTPL interaction is required for STF-mediated cell proliferation at the adaxial–abaxial junction and medio-lateral growth of the leaf blade, suggesting that STF probably works in a protein complex to carry out its central function of transcriptional repression to allow cell proliferation in the specified region that is not yet differentiated into adaxial or abaxial tissue. STF is also demonstrated to physically and genetically interact with transcriptional co-activators MtAN3 and MtLUG in the regulation of leaf and inflorescence development (Zhang et al. 2019), although the mechanism of this interaction requires further investigation.

9.3.2 Relationship Between STF and Polarity Factors

Because leaf polarity patterning is a prerequisite for blade lateral outgrowth, we wondered if and how STF affects polarity patterning. In the *stf* and *lam1* mutants, differentiation of adaxial and abaxial cells is weakened but not abolished (Tadege et al. 2011). Neither adaxialization nor abaxilization was observed in these mutants, suggesting that STF-mediated blade outgrowth may be orchestrated downstream of polarity specification. Genetic studies in Arabidopsis suggest that the middle domain-specific WOX1 and PRS may repress both adaxial and abaxial factors to establish boundary and prevent the mixing of these two cell types (Nakata et al. 2012; Nakata and Okada 2012, 2013). In Medicago, this proposal holds true at least for the STF and *MtAS2* interaction. MtAS2 is a LOB domain adaxial polarity factor required for leaf adaxial identity in Medicago. In vitro and in vivo studies uncovered that STF directly binds to the *MtAS2* promoter and prevents its expression at the adaxial–abaxial boundary in the leaf margin (Zhang et al. 2014). In the *stf* mutant leaf primordium, the expression of *MtAS2* expands to the adaxial–abaxial junction at the leaf margin, consistent with the Arabidopsis model. Interestingly, repression of the *MtAS2* promoter by STF in transgenic Arabidopsis cannot occur in the absence of TPL, and it also requires histone deacetylation at the target promoter site (Zhang et al. 2014). This indicates that the STF-mediated repression of the adaxial polarity factor *MtAS2* at the adaxial–abaxial junction of the leaf margin involves a transcriptional repressor complex that includes corepressors and chromatin modifiers.

In this connection, *MtWOX9-1* is identified to be an abaxial factor required for proper blade outgrowth in Medicago, and STF represses *MtWOX9-1* expression by directly binding to its promoter at multiple sites (Wolabu et al. 2021). It is, therefore, likely that STF establishes and maintains a cell proliferation zone at the adaxial–abaxial junction in the middle mesophyll and leaf margin by keeping adaxial and abaxial polarity factors away from this region. Preventing polarity factors from expressing in the margin may help to keep cells in the undifferentiated state so that they can continue dividing and supply cells for differentiation into both adaxial and abaxial tissues. In this way, the STF transcriptional repressor complex may maintain a cell proliferation zone at the adaxial–abaxial boundary and perhaps also contribute to cell proliferation per se.

Overexpression of *STF* in transgenic switchgrass, rice, wheat, and *Brachypodium* leads to increased biomass yield attributed to increased leaf lateral expansion caused by enhanced cell proliferation (Wang et al. 2017; Liu et al. 2018). At least in transgenic rice, STF directly binds to the promoters of some cytokinin oxidase/dehydrogenase enzymes (*CKXs*) that degrade cytokinin and represses their activity leading to increased active cytokinin contents (Wang et al. 2017), which promotes cell proliferation. This suggests that the STF repressor complex at the adaxial–abaxial junction maintains a cell proliferation zone and promotes cell proliferation at the same time. In Arabidopsis, *WOX1* and *PRS* are known to be activated by auxin and the auxin response factor MONOPTEROS (MP) and dominant negative expression of *MP* leads to narrow leaves (Guan et al. 2017). Although this has not been shown directly in Medicago, the involvement of auxin in the STF pathway was predicted from microarray experiments and *lam1* mutant treatment with auxin and cytokinin (Tadege et al. 2011; Tadege and Mysore 2011). Thus, phytohormones, especially the auxin–cytokinin crosstalk may be important for the STF-mediated regulation of medio-lateral growth through activation of cell proliferation.

9.4 Leaf Blade Length Growth Regulators in *M. Truncatula*

The fact that the *stf* mutant and even the more severe *lam1* mutant are not affected in leaf length suggests that proximo-distal and medio-lateral growth are likely controlled by separate genetic factors. However, mutants specifically affected only in leaf length are not described as such in Medicago. Mutant leaves with proximo-distal defect are generally small indicating that medio-lateral growth is also restricted. This might be because of the nature of leaf growth. When the primordium first emerges from the meristem, it appears as a peg-like structure and assumes a rod shape (Ge et al. 2014). As cell division and differentiation continues, the blade starts to flatten because of lateral expansion. It is also clear that *STF* is never expressed in any region of the meristem, central or peripheral, but the first detectable expression is in visible primordium, about stage P1. STF is, thus, unlikely to be involved in the recruitment of primordial founder cells from the meristem and in the initiation of the primordium. These two observations, the rod shape of the primordium at the beginning and appearance of *STF* after primordium initiation, suggest that proximo-distal growth is primary and medio-lateral growth is secondary. This assumption explains why proximo-distal growth defect has impact on medio-lateral growth but not necessarily vice versa.

Irrespective of the reason, there are Medicago mutants with small leaves that may be either genuinely affected in both length and width growth or caused by consequences of defects in proximo-distal growth alone. The *hdl* mutant may be an exception in this regard because the defect appears at the distal tip of the blade where it fails to elongate without any impact on leaf width (Meng et al. 2019; Wang et al. 2019) altering the blade shape. The *agamous-like flower* (*aglf*) mutant though described for its primary defects in floral organ development also appears to have defect specifically in leaf proximo-distal growth that results in short blade, rachis, and petiole (Zhang et al. 2019).

In Arabidopsis, two BTB/POZ domain-ankyrin repeat proteins BLADE-ON-PETIOLE1 (BOP1) and BOP2 are reported to regulate proximo-distal patterning (Ha et al. 2003; Hepworth et al. 2005). The Medicago *BOP1* homolog, *NOOT*, loss-of-function phenotype is primarily in the nodule, stipules and flower petals without clear indication of proximo-distal defect in the petiole or blade (Couzigou et al. 2012). Homologs of the *ROTUNDIFOLIA3* (*ROT3*) and *ROT4* genes, another group involved in proximo-distal polarity patterning in Arabidopsis, have not been characterized in Medicago. However, *ROT3* encodes a member of the cytochrome P450 family involved in brassinosteroid (BR) synthesis (Kim et al. 1998, 2005), and brassinosteroid signaling has been shown to regulate overall plant growth including leaf expansion in Medicago. The brassinosteroid receptor (*mtbri1*) mutants of Medicago are extremely dwarf in all aspects of plant architecture with nearly rounded leaf blades (Cheng et al. 2017), suggesting that the primary defect in the leaf blade may be in cell proliferation and/or expansion in the proximo-distal axis.

Dwarf and Increased Branching 1 (*DIB1*) a GA3ox1 enzyme (Zhang et al. 2020) and *mini plant 1* (*mnp1*) a CPS enzyme (Guo et al. 2020) in the gibberellin biosynthetic pathway are extremely dwarf plants with defects in cell length expansion, and show slightly more oval-shaped leaves than WT. The auxin efflux carrier mutant *smooth leaf margin1* (*slm1*) also called *mtpin10* has slightly shorter leaf blades but also with defects in leaflet number and leaf blade margin serration (Peng and Chen 2011; Zhou et al. 2011). Another Medicago gene, *SMALL LEAF AND BUSHY1* (*SLB1*)/ *MINI ORGAN1* (*MIO1*), is recently described to affect organ size (Yin et al. 2020; Zhou et al. 2021). *slb1/mio1* mutant leaves are small but relatively shorter, suggesting a primary defect in proximo-distal growth. *SLB1/MIO1* encodes an F-box protein assumed to be part of the SKP1/Cullin/F-box (SCF) E3 ubiquitin ligase complex and promotes leaf size by activating cell proliferation through targeting the cell division repressor *BIG SEEDS1* (*BS1*) for degradation (Yin et al. 2020; Zhou et al. 2021). *BS1* encodes a TIFY family transcriptional regulator that controls organ size by negatively regulating primary cell division (Ge et al. 2016).These observations suggest that the phytohormones, auxin, GA, brassinosteriodes and their crosstalk, and the ubiquitin ligase proteasome pathway are involved in modulating proximo-distal growth in Medicago leaves, but due to their effects on growth in the other axes as well, and consequences of proximo-distal growth defects on medio-lateral expansion, these defects generally result in small leaves affected in both length and width directions.

9.5 Control of Compound Leaf Development in *M. Truncatula*

M. truncatula has a characteristic trifoliolate (trifoliate) compound leaf with three leaflets; a single terminal leaflet (TL) distal to the petiole and two lateral leaflets (LL) below it separated from the TL by a short petiole-like structure called rachis, with the exception of the first leaf, which is always simple. In the *single leaflet1* (*sgl1*) mutant, all adult leaves are converted into simple leaves with single terminal leaflets (Wang et al. 2008). SGL1 is the homolog of Arabidopsis LFY, snapdragon FLO and pea UNI. *SGL1* is expressed in the SAM and leaf primordia (Wang et al. 2008), likely providing morphogenetic competence to primordial cells to initiate lateral leaflets. Another transcriptional regulator, FUSED COMPOUND LEAF1(FCL1), is required for boundary separation between leaflets as the leaflets appear fused in the *fcl1* mutant (Peng et al. 2011). *FCL1* encodes a class M KNOX protein that lacks a homeodomain (Peng et al. 2011) and presumably promotes cell proliferation at the boundary to enable competence and leaflet separation. *PALMATE-LIKE*

PENTAFOLIATA1 (*PALM1*) encoding a C2H2 zinc finger transcription factor is required to maintain the trifoliate identity by negatively regulating the expression of *SGL1* (Chen et al. 2010). In *palm1* mutants, two additional leaflets are initiated converting the three leaflet into five, in which the *SGL1* transcript is highly upregulated. Thus, PALM1 provides determinacy to leaflet initiation by spatial and temporal control of *SGL1* expression (Chen et al. 2010).

Another determinacy factor, *PINNATE-LIKE PENTAFOLIATA1* (*PINNA1*), has been described recently to regulate morphogenetic activity and leaflet number by directly repressing *SGL1* during *M. truncatula* leaf morphogenesis (He et al. 2020). *PINNA1* encodes a BEL-like homeodomain protein and functions alone in the TL region but synergizes with *PALM1* at the LL region to define the spatiotemporal expression pattern of *SGL1* (He et al. 2020). In the *pinna1* single mutant, the two new leaflets appear at the TL region making five leaflets, indicating that PALM1 function is sufficient to suppress extra leaflet initiation at the LL region, but in the *pinna1 palm1* double mutant, supernumerary leaflets are formed at the LL region, increasing the total number of leaflets to 12 (He et al. 2020). This indicates that the action of PINNA1 and PALM1 at the LL region is additive in suppressing lateral leaflet development, and this has been attributed to combined control of the indeterminacy factor SGL1 activity (He et al. 2020).

These observations together suggest that lateral leaflet initiation is a default pathway in leaf primordia orchestrated by *SGL1*, but *PINNA1* and *PALM1* control the spatial and temporal distribution of *SGL1* individually and in combination to achieve the characteristic trifoliate identity with one terminal and two lateral leaflets. Although *SGL1* is expressed in the SAM, it is not required for the formation of the first unifoliate leaf and the terminal leaflet since these structures appear unaffected in the *sgl1* mutant. It would be interesting to know the identity of the genetic factor that recruits leaf founder cells from the peripheral SAM in the first place and imparts leaf primordial identity in *M. truncatula* and other plants.

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