Review

Leaf Development in Medicago truncatula

Liren Du, Samuel Adkins and Mingli Xu *

Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA; liren@email.sc.edu (L.D.); swadkins@email.sc.edu (S.A.)
* Correspondence: minglixu@sc.edu

Abstract: Forage yield is largely dependent on leaf development, during which the number of leaves, leaflets, leaf size, and shape are determined. In this mini-review, we briefly summarize recent studies of leaf development in Medicago truncatula, a model plant for legumes, with a focus on factors that could affect biomass of leaves. These include: floral development and related genes, lateral organ boundary genes, auxin biosynthesis, transportation and signaling genes, and WOX related genes.

Keywords: leaf development; morphogenesis; Medicago; leaflet number; leaf size; leaf shape

1. Introduction

Leaves are the primary organ for photosynthesis and provide energy to both plants and primary consumers. Although fruits and seeds produced by plants are more commonly thought of as food, many animals consume plant leaves as a source of energy and nutrients. The forage leaves that feed many agriculturally important herb animals are of particular interest. Forage yield is largely dependent on the number, size, and shape of forage leaves; therefore, plant leaf development has a great effect on proper forage yield results. Medicago truncatula (M. truncatula), an annual forage legume that is closely related to the world’s most economically important forage legume alfalfa (Medicago sativa), has been employed as a model plant for legumes because of its simpler genetics and easier growth habit [1]. In M. truncatula, normal leaf development procedures are divided into three stages. Leaves are first initiated as rod-like primordia from the flanks of the shoot apical meristems (SAM) before undergoing primary morphogenesis and secondary morphogenesis [2]. During primary morphogenesis—otherwise known as morphogenesis—the lamina is initiated and leaf marginal structures such as leaflets, lobes, and serrations are formed. During secondary morphogenesis—also referred to as differentiation—leaf tissues grow, differentiate, and mature, and the final leaf shape is formed [2]. Both M. truncatula and Medicago sativa undergo these three stages of leaf development and develop compound leaves in which more than one leaflet is attached to their petiole. As M. truncatula is a well-studied model plant for legumes, in this mini-review we will discuss factors that may affect the number of leaflets per petiole, and size and shape of leaves during the three leaf developmental stages of M. truncatula.

2. Regulation of the Number of Leaflets per Leaf

Simple and compound leaves are two basic forms of leaves in higher plants. Simple leaves have a single undivided blade per petiole, while compound leaves have multiple blade units, called leaflets, per petiole [2]. Compound leaves may have an evolutionary advantage, as they have more blades for photosynthesis and herbivory survival; therefore, mechanisms that determine simple and compound leaf variation are some of the major factors that determines number of leaflets per petiole, while other factors may also promote the presence of more or less leaflets per petiole.
2.1. KNOX Genes

In Arabidopsis thaliana, a plant with simple leaves, the class I KNOTTED1-LIKE HOME- 
OBOX (KNOXI) genes, including SHOOTMERISTEMLESS (STM), KNOTTED-LIKE FROM 
ARABIDOPSIS THALIANA 1 (KNAT1), KNAT2, and KNAT6, are expressed in the SAM 
and are excluded from the leaves, while MYB domain transcription factor ASYMTRIC 
LEAVES 1 (AS1), LOB domain transcription factor AS2, and BTB-POZ domain transcription 
cofactors BLADE-ON-PETIOLE 1 (BOP1) and 2 are expressed in the leaves and not in the 
SAM [3–7]. Loss-of-function as1 and as2 single mutants and bop1 bop2 double mutants 
cauised ectopic growth on their petioles, which resemble the compound leaf form [5–10]. 
Molecular and genetic analysis indicates that meristematic KNOXI genes STM, KNAT1, 
KNAT2, and KNAT6 are misexpressed in as1, as2, and bop1 bop2 leaves, and the inactivation 
of these KNOXI genes in these mutants resulted in reduced growth of their petioles. These 
results indicate that simple and compound leaf variation might be caused by different 
expressions of KNOXI genes in leaves [7,9–13]. This hypothesis was supported by studies 
in Cardamine hirsuta, where the expression of STM and KNAT1 is reactivated in its leaf 
primordia, which gives rise to compound leaves [14]. Further analysis showed that the 
ectopic expression of STM or KNAT1 in Cardamine resulted in more leaflets per petiole 
and reduced activities of STM resulted in less leaflets per petiole, which supports the 
hypothesis that controlling the expression of KNOXI genes in leaves could induce simple 
and compound leaf variation [14]. Surprisingly, studies carried out on legume models do 
not fully support this hypothesis. Both peas and soybeans produce compound leaves, but 
KNOXI genes behave differently. KNOXI genes are expressed in soybean leaf primordia, 
and an overexpression of such genes results in more leaflets per petiole in alfalfa [15]. 
However, these genes are not expressed in pea leaf primordia, nor in M. truncatula, a close 
relative to soybean that also has compound leaves [15–18] (Figure 1a). PHANTASTICA 
(PHAN) from Antirrhinum majus is an ortholog of AS1 from Arabidopsis and both promote 
abaxial–adaxial polarity in leaf development [19] (Figure 1a). KNOXI genes are reactivated 
in Arabidopsis as1 leaves [3,5]; however, KNOXI1, KNOXI2, and KNOXI6 are not reactivated 
in the M. truncatula phan mutant, though KNOXI7 is reactivated in M. truncatula phan petiole. 
The phan mutant did not show any changes in the complexity of leaves [17,18]. These 
udies suggest that the mechanisms for controlling the number of leaflets per petiole in 
M. truncatula differ from other compound leaf species.

2.2. Floral Development and Related Genes

Wild type M. truncatula has three leaflets per petiole, which was reduced to one by 
mutations in SINGLE LEAFLET1 (SGL1), suggesting that the roles of KNOXI genes in 
Cardamine may be replaced by SGL1 during evolution [20]. SGL1 encodes a LEAFY (LFY) 
ortholog from Arabidopsis, which is a master regulator for floral organogenesis. sgl1 also has 
defects in floral organogenesis, like lfy mutants in Arabidopsis [20]. SGL1 is detected at SAM 
and leaf primordia (Figure 1a,b). Close examination of leaf development in sgl1 mutants 
suggest that SGL1 promotes lateral lamina primordia—a hallmark of morphogenesis—and 
leads to the outgrowth of lateral leaflet on the petiole; conversely, the C2H2 zinc finger 
transcription factor PALMATE-LIKE PENTAFOLIATA1 (PALM1) functions to suppress 
lateral leaflet outgrowth in M. truncatula [21]. PALM1 is expressed in the lateral leaflet 
primordia to keep the expression of SGL1 at moderate levels and prevent extra leaflet 
outgrowth (Figure 1b). Mutation in PALM1 resulted in the outgrowth of two extra leaflets, 
creating a total of five leaflets instead of three [21,22]. Similar to PALM1, mutations in a 
BEL1-like homeodomain protein PINNATE-LIKE PENTAFOLIATA1 (PINNA1) also resulted 
in 5 leaflets per petiole. PINNA1 is expressed at the distal of the leaf primordia to restrict the 
expression of SGL1 at the base of leaf primordia (Figure 1b). pinna1 mutants have two extra 
leaflets below the terminal leaflet, which differs from the palm1 mutant which has two extra 
leaflets below the lateral leaflets. Mutation in PALM1 and PINNA1 simultaneously resulted 
in complicated leaves, suggesting that PALM1 and PINNA1 function synergistically in 
modulating the number of leaflets per petiole [22]. Another possible floral factor that
influences leaf development is a gene similar to AGAMOUS (AG) in *M. truncatula*. AG is a C-class function gene in *Arabidopsis* that controls the stamen and carpel identity in *Arabidopsis* flower development. AGAMOUS-LIKE FLOWER (AGLF) in *M. truncatula* controls both flower development and leaf development [23,24] (Figure 1b). In aglf-1 mutants, the petiole length is reduced, causing clustered leaflets in the mutant [24]. It is unknown if AGLF interacts with SGL1, PALM1, PINNA1, or other factors in regulating the complexity of *M. truncatula* leaves.

![Figure 1](image-url)

**Figure 1.** (a) Gene networks controlling compound leaf development of *Medicago truncatula* at the initiation stage. KNOX I genes are expressed in SAM and down regulated in leaf primordia. PHAN and SGL1 are expressed at both SAM and early-stage leaf primordia during development. HDL is expressed in the organization center of the SAM. SLM1 and SGL1 are expressed at early-stage leaf primordia. FCL1, LLS1, and CUC genes are expressed at the boundary between SAM and leaf primordia. (b) Gene networks controlling the compound leaf development of *Medicago truncatula* at the morphogenesis stage. PALM1 is expressed at lateral leaflet primordia and PINNA1 is expressed at the distal region of terminal leaflet primordia. SGL1 is restricted to the basal region of the terminal leaflet primordia by PALM1 and PINNA1. LLS1 and CUC are expressed at the boundary between terminal leaflet primordia and lateral leaflet primordia. REV is expressed at the adaxial portion of leaf primordia to regulate auxin-related genes. AGO7 and PHAN repress ARF3, which represses PALM1 during leaf morphogenesis. STF is expressed at the boundary between the adaxial–abaxial domain to restrict the expression of AS2 and WOX9. (c) Genes control lamina size and shape at the differentiation stage. HDL promotes margin.
Cardamine was observed in the petiole of DON3 (domains and lacks the homeodomain, resulting in a distinct expression pattern KNOX2 CUC2 polar auxin transport efflux carrier primordia in several compound leaf species [26,27]. Ectopic expression of is expressed at the adaxial portion of leaf primordia to regulate auxin-related genes. AGO7 and PHAN repress ARF3, which represses PALM1 during leaf morphogenesis. STF is expressed at the boundary between the adaxial–abaxial domain to restrict the expression of AS2 and WOX9. (c) Genes control lamina size and shape at the differentiation stage. HDL promotes margin serration and lamina length/width ratio, while MIO1 promote cell proliferation to promote lamina size. TL: terminal leaflet. LL: lateral leaflets. ST: stipules. Ab: Abaxial. Ad: Adaxial.

2.3. Lateral Organ Boundary Genes

Deep leaf margin serration can cause a blade to divide, resulting in multiple leaflet-like structures at the distal of leaf [25]. The lateral organ boundary gene CUP-SHAPED COTYLEDON3 (CUC3) is expressed at organ boundaries in Arabidopsis and at the boundary of leaflet primordia in several compound leaf species [26,27]. Ectopic expression of CUC genes in Arabidopsis resulted in highly serrated leaf margins [26], and reducing expression levels of CUC in Cardamine resulted in smoother leaf margins and less leaflets [27]. These findings suggest that multiple leaflets could be caused from deep leaf margin serration. In M. truncatula, CUC2/NAM is expressed at the boundary between the cotyledons and SAM, and at the boundary between leaflets (Figure 1a,b); consequently, mutation in MUCUC2/NAM resulted in fused cotyledons and fused leaflets, mimicking simple leaves [28,29]. Fused leaflets are also observed in the class M KNOX gene mutant fused compound leaf 1 (fcl1) where FCL1 is expressed at incipient leaf primordia and at the boundary between SAM and leaf primordia in Medicago [16] (Figure 1a). FCL1 encodes a KNOX gene that contains the KNOXI and KNOX2 domains and lacks the homeodomain, resulting in a distinct expression pattern and function from KNOXI genes [16]. The sgl1 nam double mutant produced one leaflet, the same phenotype as the sgl1 single mutant, suggesting that sgl1 is epistatic to nam in regulating number of leaflets [29]. These results suggest that SGL1 and organ boundary genes have different roles in leaf development even though mutations in both resulted in simple leaves. SGL1 promotes lateral leaflet primordia formation while organ boundary genes promote leaflet separation during compound leaf development [16].

2.4. Auxin

The phytohormone auxin has many roles in plant development, including leaf, flower, and root development [30,31]. Simple leaf species Arabidopsis has a smooth petiole during morphogenesis, and there is no auxin maximum in the petiole; conversely, auxin maximum was observed in the petiole of Cardamine during leaf morphogenesis and mutations in the polar auxin transport efflux carrier PINFORMED1 (PIN1) resulted in less leaflets, suggesting that the formation of multiple leaflets during morphogenesis requires auxin accumulation in the petiole [32]. Several lines of evidence suggest that auxin is also required for multiple leaflet outgrowth in M. truncatula. SMOOTH LEAF MARGIN1 (SLM1) is an ortholog of the auxin efflux carrier PIN1 from Arabidopsis that is expressed at the leaf primordia and leaf margins of M. truncatula (Figure 1a,c). Mutation in SLM1 resulted in smooth leaf margins and more than 3 leaflets per petiole, and the slm1 sgl1 double mutant has more leaflets than sgl1 and less leaflets than slm1, suggesting antagonistic interactions between SLM1 and SGL1 [33]. LATERAL LEAFLET SUPPRESSION1 (LLS1) in Medicago is an ortholog to auxin biosynthetic enzyme YUCCA1 from Arabidopsis. LLS1 is expressed in early-stage leaf primordia and at the basal regions between the terminal leaflet and lateral leaflet primordia (Figure 1b). Mutations in LLS1 resulted in suppression of the lateral leaflet outgrowth, like SGL1 [34]. lls1 could not change the phenotypes in sgl1 but reduced the number of leaflets in palmi1, suggesting that LLS1 functions synergically to SGL1 and antagonistically to PALM1 [34]. AUXIN RESPONSE FACTORS (ARF) function downstream in auxin signaling and mediate auxin effects [31]. Yeast one-hybrid screening revealed that
MtARF3 physically binds to PALM1 promoter, suggesting that auxin is associated with PALM1. AGO7 is expressed at the adaxial portion of leaf primordia, and PHAN is expressed in both the adaxial and abaxial portion of leaf primordia [35]. Together, PHAN and AGO7 restrict ARF3 at the abaxial portion of the leaf primordia. Simultaneous mutations in AGO7 and PHAN, two repressors for ARF3 (Figure 1b), resulted in downregulation of PALM1 and 5 leaflets per petiole, resembling the palmi1 mutant [35]. Auxin homeostasis, number of leaflets per petiole, and leaflet shape are also regulated by HD-ZIPIII gene REVOLUTA (MtREV), which is specifically expressed in the adaxial portion of leaf [36] (Figure 1b). These studies suggest that leaf polarity regulators are key regulators for auxin signalling and response, and likely regulate leaflet number through auxin. Finally, HEADLESS (HDL), encoding a WUSCHEL (WUS) ortholog from Arabidopsis, has roles in forming shoot apical meristems, leaf margin serration, and controlling the number of leaflets per petiole, likely through its regulation on auxin efflux carrier SLM1/PINT [37,38].

3. Regulation of the Size and Shape of the Leaf

The size and shape of leaves also determines the biomass of leaves and therefore affects forage yields. In Arabidopsis, WUS controls meristem activity in the shoot apical meristems and flowers, while WLUS-related homeobox (WOX) genes have been found to regulate cell proliferation in leaves in both monocots and dicots. WOX1 and WOX3/PRESSED FLOWER (PRS) are expressed in Arabidopsis leaf primordia at the adaxial and abaxial boundary while the wox1 prs double mutant has narrower lamina than the wild type, indicating the roles of WOX genes in promoting cell proliferation along the mediolateral axis in leaves [39]. As with WOX1 and WOX3, mutations in the maize WOX3 ortholog NARROW SHEATH1 (NS1) and NS2, also resulted in exceptionally narrow leaves [40]. Likewise, the WOX1 ortholog in M. truncatula STENOFOLIA (STF) is expressed at the boundary between the adaxial–abaxial domain and has important roles in lamina expansion by promoting cell proliferation in leaves [41] (Figure 1b). Mutations in STF resulted in narrow and long leaves (in Nicotiana sylvestris) or leaflets (in M. truncatula), while overexpression of STF resulted in wider lamina and greater biomass in Brachypodium and switchgrass [42]. STF also interacts with TOPOLESS (TPL) to promote blade expansion through the MtAS2 dependent pathway and cytokinin pathways [42,43]. Additionally, ANGUSTIFOLIA3 (AN3) is a transcription coactivator and LEUNIG (LUG) is a transcription corepressor with both having roles in leaf development [44,45]. Studies showed that STF interacts with both AN3 and LUG physically and LUG may function as transcription coactivator to regulate lamina expansion similar to AN3 [46]. In leaf blade expansion, STF also directly represses WOX9, another member of the WOX gene family that is expressed at the leaf’s abaxial domain [47] (Figure 1b). HDL is an Arabidopsis WLUS ortholog and is expressed at leaf margin serrations. Mutation in HDL resulted in shorter blades and higher blade width/length ratio [38] (Figure 1c). This suggests that HDL is involved in promoting lamina shape along the proximal–distal axis, but the mechanism remains unknown.

Although WOX and related genes were found to control leaflet shape, an F-box protein MINI ORGAN1 (MIO1)/SMALL LEAF AND BUSHY1 (SLB1) was discovered to regulate leaflet size in Medicago (Figure 1c). The morphology of leaflets and flowers was not changed in loss-of-function mio1, but the size of leaflets and flowers were reduced, which resulted in smaller leaflets and flowers; conversely, overexpression of MIO1 resulted in larger leaflets [48]. Mutations in STERILE APETALA (SAP)/SUPPRESSOR OF DA1 (SOD3), the Arabidopsis ortholog of MIO1/SLB1, also resulted in smaller leaves, and ectopic expression of MIO1 in Arabidopsis (35S::MIO1) could rescue the small leaves in sod3, indicating conserved roles of MIO1/SOD3 in plants [48]; however, MIO1 is expressed in leaf primordia and localized in nucleus to regulate cell division, not cell expansion, to regulate leaflet size [48]. The targets of MIO1 have yet to be determined, as is the mechanism of regulation.
4. Challenges and Perspectives

Forage yield is determined largely by leaf development in plants. In this mini-review, we summarized leaf development factors that could potentially affect overall leaf yield in the forage model plant *M. truncatula*. Number of leaflets per petiole is a critical influencer on the overall production of leaves, and several factors are involved in regulating the number of leaflets per petiole. These include floral development orthologs *SGL1* and *AGLF*, and factors that interact with them (such as *PALM1*, *PINNA1*); Auxin and auxin signaling related genes; organ boundary gene *CUC2/NAM*, and *FCL1*. The shape and size of leaflets may also affect leaf yield. The WOX gene family seems to have essential roles in promoting blade expansion and STF (WOX1) represses the leaf adaxial–abaxial polarity genes *AS2* and *WOX9* to promote cell division along the medial–lateral axis. MIO1 promotes leaflet size by promoting cell division activities in leaf primordia; however, the mechanisms of how MIO1 acts remain to be determined. In *Arabidopsis*, a series of genes have been found to regulate final leaf size by regulating cell proliferation, including GROWTHREGULATING FACTOR1 (GRF1), GRF2, and GRF5, ANGUSTIFOLIA3 (AN3)/GRF-INTERACTING FACTOR, KLUH, AVP1, JAW, BR11, and GA [49–52]. In maize, leaf size is regulated by ARGONAUTE, MADS box, SQUAMOSA PROMOTER-BINDING PROTEIN (SBP), GRF, bZIP and TCP transcription factors, and epigenetic factors [53]. It will be of particular interest to know if these life size regulators in *Arabidopsis* and maize function in *Medicago*. A third factor possibly affecting leaf yield is the rate of leaf initiation, or the plastochron length. Faster leaf initiation or smaller plastochron length could result in more leaves produced and higher forage yield during vegetative development. The microRNA *miR156* and its targets SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes are involved in regulating plastochron length and leaf shape in *Arabidopsis* [54,55]. Several lines of evidence suggest that the miR156-SPL module could be a promising tool for improving forage yield. The leaf complexity in *Cardamine* is age-dependent and is correlated to expression levels of the floral repressor FLOWERING LOCUS C (FLC) [56]; it is also likely that effects of FLC are mediated through SPL genes [56]. It is reported in *Medicago sativa* (alfalfa) that plants overexpressing *miR156* show a bushy phenotype and enhanced biomass yield [57]; however, it is unknown if these phenotypes are related to plastochron length. Mutations in *MtSPL8* and *MtSPL13* caused more branching and increased biomass in *M. truncatula* [58,59], and mutations in *MtSPL4* caused more leaflets per petiole. Future research may determine if the miR156-SPL module also regulates plastochron length in addition to regulating branching and number of leaflets per petiole in *Medicago*, adding an additional layer to increase forage yield. In *Cardamine*, a ribosome-associated protein SIMPLE LEAF 3 (SIL3) was reported to affect both leaflet number and plastonchron length through regulating auxin accumulation and KNOXI genes [60]. Ribosome encoding proteins PIGGYBACK1 (PGY1), PGY2, and PGY3 are involved in establishing leaf adaxial–abaxial polarity in *Arabidopsis* [61]. It will be interesting to understand how the ribosome related proteins function in *Medicago*, particularly if they regulate leaflet number or leaf size that could affect forage yield.

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