A WOX/Auxin Biosynthesis Module Controls Growth to Shape Leaf Form

Graphical Abstract

Highlights
- WOX1, 3, and 5 are required redundantly for lateral leaf growth and auxin biosynthesis
- YUC1 expression can partially bypass the requirement for WOX genes in leaf growth
- Time-lapse imaging allows quantitation of WOX effects on leaf growth
- WOXes shape a growth gradient that underlies the A. thaliana ellipsoid leaf shape

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In Brief
Zhang et al. discover that a genetic module comprising WOX homeobox proteins and the hormone auxin shapes Arabidopsis leaf form. They find that WOXes promote auxin biosynthetic gene expression. By using time-lapse imaging, they also show that this module generates a proximodistal gradient of growth that helps leaves grow laterally.

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A WOX/Auxin Biosynthesis Module Controls Growth to Shape Leaf Form

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SUMMARY

A key challenge in biology is to understand how the regional control of cell growth gives rise to final organ forms. Plant leaves must coordinate growth along both the proximodistal and mediolateral axes to produce their final shape. However, the cell-level mechanisms controlling this coordination remain largely unclear. Here, we show that, in A. thaliana, WOX5, one of the WUSCHEL-RELATED HOMEOBOX (WOX) family of homeobox genes, acts redundantly with WOX1 and WOX3 (PRESSED FLOWER [PRS]) to control leaf shape. Through genetics and hormone measurements, we find that these WOXs act in part through the regional control of YUCCA (YUC) auxin biosynthetic gene expression along the leaf margin. The requirement for WOX-mediated YUC expression in patterning of leaf shape cannot be bypassed by the epidermal expression of YUC, indicating that the precise domain of auxin biosynthesis is important for leaf form. Using time-lapse growth analysis, we demonstrate that WOX-mediated auxin biosynthesis organizes a proximodistal growth gradient that promotes lateral growth and consequently the characteristic ellipsoid A. thaliana leaf shape. We also provide evidence that WOX proteins shape the proximodistal gradient of differentiation by inhibiting differentiation proximally in the leaf blade and promoting it distally. This regulation allows sustained growth of the blade and enables a leaf to attain its final form. In conclusion, we show that the WOX/auxin regulatory module shapes leaf form by coordinating growth along the proximodistal and mediolateral leaf axes.

INTRODUCTION

How gene activity translates into distinct organ morphologies in complex eukaryotes remains poorly understood [1, 2]. Resolving this problem requires us to characterize the genetic modules that control different aspects of form and to understand how they influence the amount, direction, and duration of growth to shape final organ geometry [3]. Plant leaves are an attractive system in which to address these questions as they grow from almost indistinguishable leaf buds into shapes that vary tremendously among species. Leaves of eudicot plants typically emerge as simple leaves, with undivided margins, or complex with protrusions of different size and geometry. Yet even in simple leaf species, growth along the proximodistal (PD) and mediolateral (ML) axes results in a variety of shapes, such as elliptical in the model plant A. thaliana, lanceolate in willow, cordate in poplar, and linear in rosemary (Figure 1A). Recent work has uncovered broad principles as to how divergent leaf shapes emerge [1, 3–6]. However, there remain substantial gaps in our understanding of how genetically regulated growth produces key elements of leaf shape. For example, it remains unclear how regional differences in growth patterns emerge and contribute to the overall shape of the leaf blade, even for the simple ellipsoid form of A. thaliana leaves. Theoretical models derived from time-lapse imaging indicate that a basally emanating morphogen may shape A. thaliana leaf form, by influencing the amount and direction of cellular growth. However, it is unclear what specific genetic modules contribute to the gradient of lateral blade growth hypothesized in such models [3, 7].

In Arabidopsis, members of the WUSCHEL-RELATED HOMEOBOX (WOX) gene family, WOX1 and PRESSED FLOWER (PRF), and the homeodomain transcription factor WUSCHEL (WUS) are required to maintain the pluripotent state of leaf meristems and control leaf shape [3]. However, how these genes control the shape of leaves has been the subject of much debate. Recent studies have focused on how the location of auxin biosynthesis along the leaf margin controls leaf shape. Auxin is a key morphogen for leaf development, and recent work indicates that the regional control of auxin biosynthesis is important for leaf form [8].
Figure 1. WOX5 Acts Redundantly with WOX1 and WOX3 to Regulate Leaf Lateral Growth

(A) Left: the growth gradient of a leaf primordium (green color), relative to the mediolateral (ML) and proximodistal (PD) axes. Right: leaf primordia can give rise to varied leaf shapes in different plant species.

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(PRS/WOX3), regulate leaf blade outgrowth and marginal cell identity [8, 9, 10, 11]. The role of WOX genes in regulating leaf width appears to be broadly conserved among seed plants, as both monocot and eudicot loss-of-function WOX mutants display a narrow, strap-like leaf shape, indicative of aberrant lateral growth [8,9,11–14]. In Arabidopsis, WOX1/3/5 (PRS) have been proposed to promote lateral growth by influencing tissue identity [8]. Specifically, WOX action is thought to define a middle domain that contains the leaf margin in the growing leaf bud. This domain separates the abaxial and adaxial (dorsal and ventral) domains and regulates leaf blade outgrowth, downstream of adaxial/abaxial polarity [8]. However, how growth control contributes to this domain delimitation system is unknown. In addition, how WOX proteins regulate specific aspects of cellular growth in the leaf remains unclear. As such, we lack a clear understanding of how spatially distributed WOX action is translated into final leaf shape. During embryogenesis, WOX proteins promote SAM development by activating HD-ZIP III genes [15]. HD-ZIP IIs promote adaxial leaf identity and are one of the key components of the adaxial/abaxial delimitation system postulated to interact with WOXs to promote lateral growth [8]. This raises the question as to whether such WOX-mediated HD-ZIP III-activation is also deployed post-embryonically to support lateral leaf expansion. In addition, findings obtained in Medicago truncatula (medicago) indicate that WOX proteins might influence auxin homeostasis [13]. However, how WOX genes affect auxin homeostasis, and whether alterations in auxin homeostasis cause wox mutant phenotypes, is not known. Additionally, there is evidence that auxin signaling may act upstream of WOX genes [16, 17], raising the possibility that feedbacks between auxin signaling and biosynthesis contribute to WOX function.

Here, we investigate the WOX-mediated control of leaf shape in A. thaliana and show that WOX5 acts redundantly with WOX1 and WOX3 (PRS) to coordinate growth along the leaf PD and ML axes. We also show that WOX1/3/5 genes affect lateral growth and organize a proximally focused growth distribution in the leaf blade that generates the characteristic ellipsoid shape of A. thaliana leaves. Furthermore, we show that this action depends in part on the localized WOX-mediated activation of YUCCA auxin biosynthetic gene expression. In conclusion, we identify a regulatory module that coordinates growth along perpendicular axes by defining the spatial distribution of a broadly acting growth hormone.

RESULTS

WOX5 Acts Redundantly with WOX1 and WOX3 to Regulate Leaf Shape

In various angiosperms, including medicago, petunia, and tobacco, single wox1 or wox3/prs mutations cause an extremely narrow leaf phenotype [9, 13]. By contrast, wox13 double mutants in Arabidopsis show only a moderate reduction in leaf width compared to wild type (WT) [8] (Figures 1B, 1B’, 1D, and 1F–1H); this mild double-mutant phenotype suggests that considerable redundancy may exist among WOX genes in the Arabidopsis leaf. Consistent with this idea, we observed that loss of function of WOX5 significantly enhanced the narrow leaf defect of the wox13 double mutant (Figures 1B–1H, 1B’–1E’, and S1I). To investigate changes in leaf geometry in WOX single, double, and triple loss-of-function mutants, we used both univariate and multivariate shape analysis techniques (STAR Methods). This analysis showed that leaf size is reduced in wox13 leaves and that leaf blades became narrower, with a more triangular leaf shape (Figures 1B, 1D, and 1F–1H). Loss of WOX5 in wox13 mutants led to a further reduction in leaf size and enhanced the narrow leaf defect throughout the whole leaf blade but did not significantly affect leaf shape in WT or single-mutant backgrounds (Figures 1B–1H and S1A–S1D). To understand where WOX5 is active in the leaf, we characterized the expression of a transcriptional (pWOX5:GFP) [18] and a genomic fusion reporter (gWOX5-YFP) [19] during early leaf development (Figures 1I–1L). We found that pWOX5:GFP is expressed in the entire leaf blade except for a region encompassing the leaf margin (Figures 1I–1I3), with higher expression in the regions of presumed vascular development (Figure 1I3). pWOX5:GFP expression forms a gradient that decreases toward the leaf tip (Figures 1I and 1J), although this expression is variable in the epidermis at later development stages (~5–6 days after initiation [DAI]; Figure 1J). gWOX5-YFP expression patterns in developing leaves are similar to those of the pWOX5:GFP transcriptional reporter line (Figures 1K and 1L), but expression is much weaker (STAR Methods). The expression of pWOX5:GFP and gWOX5-YFP also largely overlaps with that of WOX1 and WOX3 (PRS), except in regions near the leaf margin where WOX1 and WOX3 (PRS) are expressed but WOX5 is not (Figures S1J–S1L) [8]. An overlap between WOX1 and WOX5 gene expression is also supported by in situ hybridization (ISH), in which we observed WOX5 transcript to be localized in the “middle leaf domain,” in a subdomain of that previously shown to be marked by WOX1 mRNA (Figure S1M) [8]. WOX5 mRNA expression is stronger in distinct foci that correspond to the presumed developing vasculature, consistent with WOX5 expression that also marks developing vascular strands (Figure 1K3). The somewhat broader expression seen with gWOX5 and particularly pWOX5:GFP relative to ISH may reflect differences in the capacity of the reporter genes to faithfully monitor WOX5 gene expression or simply reduced sensitivity of ISH relative to confocal microscopy. Overall, these observations indicate that WOX5 may act redundantly with WOX1 and WOX3 (PRS) in leaf development. This view is also
Figure 2. **WOX1, 3, and 5 Promote Auxin Biosynthesis**

(A) GO term enrichment analysis of RNA sequence data shows that auxin-activated signaling pathways and biosynthetic processes are enriched among the genes downregulated in *wox135* mutants (GO terms enclosed in gray boxes).
consistent with previous findings that WOX1, WOX3 (PRS), and WOX5 can complement the narrow leaf defect of tobacco LAM1 (WOX1 ortholog) mutant [26]. Finally, we also observed that pWOX5:GFP expression expands to include this marginal region when it is expressed in wox13 or wox13wox5/+ backgrounds (Figures S1N–S1Q). This effect likely contributes to the functional redundancy between WOX1, 3, and 5.

Next, we investigated whether WOX genes act during leaf development via the same molecular pathways employed during embryonic development. To this end, we tested whether HD-ZIP III mediates WOX1/3/5 function in the leaf, as in embryos [15], by expressing either a microRNA-insensitive version of PHV (pWOX3:PHV) or a mimicry RNA against miR165/166 (pWOX3:MM165) in the wox135 mutant. In both cases, we found that increasing HD-ZIP III expression did not suppress the narrow leaf phenotype (Figures S1E–S1H). Although these findings do not preclude interactions between HD-ZIP III and WOX-dependent processes in developing leaf primordia [16], they suggest that, to shape leaf form, WOX genes act at least in part via different molecular pathways in the leaf and embryo.

WOX Genes Promote Auxin Synthesis in the Leaf

To investigate the molecular processes by which WOX1, 3, and 5 regulate leaf shape, we performed RNA sequencing (RNA-seq) analysis of 6-day-old WT and wox135 mutant seedlings. Compared to WT, 449 genes were upregulated (Table S1) and 778 genes were downregulated (Table S2) in wox135 mutants (STAR Methods). Gene Ontology (GO) term enrichment analysis, performed using Panther Gene Ontology (STAR Methods), showed that auxin signaling pathways and auxin biosynthesis genes are highly enriched among the downregulated genes (Figure 2A), although no obviously enriched pathway/process was found among the upregulated genes. We compared a list of 335 auxin-induced transcripts [21] and all the AUX/IAA genes [22] (most of which are upregulated by auxin) against our deregulated gene list. This analysis showed that most of the auxin-induced and AUX/IAA genes are downregulated in the wox135 mutant (Figures 2B and S2A). Consistent with our RNA-seq results, we found that the expression of the auxin activity reporter pDR5v2:NLS-tdTomato [23] was significantly reduced in mutant leaf primordia, coincident with the time that morphological defects first become apparent (Figures 2D–2J). Because auxin biosynthesis and signaling pathways are controlled via feedback loops [24], we reasoned that WOX genes might regulate growth by controlling auxin biosynthesis, which would then lead to changes in auxin responses. Consistent with this view, we measured auxin content of the wox135 shoots (2 weeks old) via liquid chromatography-mass spectrometry. The lower levels of the IAA conjugates IAAsp and IAAGlu as well as the IAA catabolite oxIAA in wox135 indicate that IAA biosynthesis is downregulated in this mutant (Figure 2O) [25]. Furthermore, the DR5 auxin reporter was upregulated by the WOX3 (PRS) promoter-driven expression of the auxin biosynthesis gene YUC1 (pWOX3:YUC1) in the wox135 mutant (Figures S2B–S2I). These results support the hypothesis that WOX genes regulate auxin biosynthesis and indicate that auxin signaling is at least partly functional in the wox135 mutant. To test whether yucca mutants display similar leaf development defects to those observed in multi-gene wox mutants, we analyzed leaf development in complex yuc mutants. We found that yuc146 triple and yuc1246 quadruple loss-of-function mutants develop narrower leaves, which partially mimic wox13 and wox135 mutant narrow leaf defects, albeit with more varied and asymmetric leaf shapes (Figures 2K–2P and 2K′–2M′). Taken together, these results suggest that auxin biosynthesis is impaired in wox135 mutants and that WOXs and YUCCAs control leaf development partly via similar pathways.

To investigate the spatial-temporal expression of auxin biosynthetic genes during leaf development, we analyzed the expression of transcriptional reporter lines of YUC1 (pYUC1:3xNLS-GFP; downregulated in our wox135 RNA-seq data) and YUC4 (pYUC4:3xNLS-GFP). Among the YUC family, YUC1 and YUC4 have previously been proposed to function in leaf shape [26]. Consistent with previous reports [26, 27], we found that, in WT plants, pYUC1:3xNLS-GFP is expressed in the marginal region of the leaf base (Figure 3A) and that pYUC4:3xNLS-GFP is strongly expressed at the tips of leaves and serrations from 2 to 4 DAI (Figure 3C). We also found that pYUC4:3xNLS-GFP is highly expressed in the marginal region of the leaf base (~2 DAI) and that this expression domain later expands distally along the margin (~3 DAI), eventually forming a gradient with the strongest expression at the leaf base (~4 DAI). By contrast, in wox135 mutants, the expression of these reporters is strongly reduced in the marginal regions of the leaf, although expression of YUC1 at the leaf petiole base and of YUC4 at the leaf tip is maintained (Figures 3B and 3D). Thus, it appears that WOX genes are specifically required for expression of YUC1 and YUC4 at the leaf margin. To test whether WOX genes are also sufficient to upregulate YUC1 and YUC4 expression, we mis-expressed WOX3 (PRS) in both WT and mutant plants under the RCO (REDUCED LEAF COMPLEXITY) [28] promoter (pRCO:WOX3-mCherry). The RCO gene regulates leaf complexity in the Brassicaceae, and its promoter is active at the base of the leaf blade in a domain
Figure 3. Auxin Partially Mediates WOX Function in Leaf Growth

(A–D) Expression pattern of pYUC1:3xNLS-GFP (pYUC1, green) and pYUC4:3xNLS-GFP (pYUC4, green) in WT (A and C) and wox135 (B and D) leaf primordia (at different DAI, as indicated). Dashed lines in (A)–(D) highlight regions where GFP expression levels differ between WT and wox135 primordia.

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broader than the basal expression of both YUC1 and YUC4 (compare Figure S2J to Figures 3A and 3C). RCO expression is maintained in wox135 mutant leaves, indicating that its expression is at least partially independent of WOX genes (Figure S2K). We found that pRCO:WOX3-mCherry ectopically induces pYUC1:3xNLS-GFP and pYUC4:3xNLS-GFP expression (Figures 3E–3L, S2L, and S2M–S2P, arrows), suggesting that WOX3 (PRS) is sufficient to induce the expression of YUC genes. Taken together, we conclude that WOX genes positively regulate the transcription of YUC genes at the leaf margin during early leaf development.

Auxin Partially Mediates WOX Function in Lateral Leaf Growth

To investigate how the WOX-dependent activation of YUC genes regulates leaf shape, we expressed YUC1 in the wox135 mutant using the WOX3 (PRS) promoter (pWOX3:YUC1). This promoter is active in the marginal regions of leaf primordium in both the WT and wox135 mutants (pWOX3:3xNLS-GFP reporter shown in Figures S3A–S3F). pWOX3:WOX3 rescues the leaf shape and area of wox135 (Figures S3G–S3I). pWOX3:YUC1 restores the narrow leaf shape of wox135 to a rounder WT-like leaf shape (Figures 3M–3Q and 3S) and partially restores leaf size (Figure 3R). Notably, introducing pWOX3:YUC1 into wox135 mutants does not rescue the marginal defects of wox135 mutants, because neither the adaxial marker AS2 (pAS2:3xNLS-GFP) nor the margin-specific gene LM1 (pLM1:3xNLS-GFP) [28, 29] were restored to their WT expression patterns by the expression of pWOX3:YUC1 (Figures S3G–S3R). These results suggest that the shape of the leaf primordium is more sensitive to perturbations of WOX-mediated auxin biosynthesis than the delimitation of adaxial/abaxial and marginal domains, as respectively indicated by AS2 and LM1 reporter gene expression, and that these two facets of WOX function are partly genetically separable. To address whether any auxin source in the leaf can mimic the local auxin synthesis that results from WOX-mediated YUC activation, we expressed YUC1 in the wox135 mutant using the epidermal layer-specific promoter AtML1, which is active in the meristem and leaf epidermis [30]. The pAtML1:YUC1 transgene was not sufficient to suppress the wox135 mutant narrow leaf shape (Figures S4A–S4G and S4A’–S4D’), indicating that development of the WT leaf blade does not simply depend on the accumulation of auxin in any location in the primordium. Thus, the local activation of YUC genes near the margin is important for leaf blade growth. The local requirement for YUC activity and auxin synthesis may also explain why the ubiquitous, exogenous application of auxin to medicago stf (wox1) plants did not rescue leaf shape [13].

WOX Genes Coordinate Leaf Growth by Locally Promoting Auxin Biosynthesis

We next sought to understand how WOX action shapes leaf form, which requires an understanding of how WOX genes affect organ-wide patterns of cellular growth. Although narrow-leaf wox mutant phenotypes are observed in many species [9, 13], such information is lacking, which prevents understanding how WOX proteins control leaf shape. To determine the cellular origin of the narrow leaf defect of wox135 and ascertain how the pWOX3:YUC1 transgene restores leaf shape when introduced into wox135 mutant leaves, we performed time-lapse imaging of the WT, wox135, and pWOX3:YUC1 wox135 lines. For each line, we imaged the first emerging true leaf from 2 DAI until 7 DAI (Figure S4H), when leaf shape divergence between genotypes becomes apparent. We constructed lineage maps from 2 DAI until 7 DAI (Figure S4I) and computed the key cell-level parameters that affect organ shape, including cell proliferation, cell growth, and cell area extension rates (Figures 4A–4I) using MorphoGraphX [31]. To connect cell-level parameters to tissue-level growth directions, we computed the growth of each clone with respect to an organ-wide coordinate system aligned with the PD and ML directions of the primordium at 2 DAI (Figure 4Q; STAR Methods). To identify the regional growth differences that underpin shape differences between our genotypes, we used growth alignment graphs, which allow cell growth at equivalent positions along the PD axis of leaf primordia to be compared (Figures 4J–4P and 4R; STAR Methods).

As WOX expression acquires a basally focused pattern of expression during leaf development (Figures 1J, 1K, S1N, and S1Q) [32], we reasoned that these genes may regulate the characteristic basipetal gradients of growth and proliferation (i.e., tip-to-base) observed in WT leaf blades [3, 4, 33]. To test this hypothesis, we examined the regional differences in growth and proliferation between WT and wox135. As expected, growth and cell proliferation had a basipetal gradient in WT leaves (Figures 4A, 4D, 4J, and 4K). However, in wox135 leaf blades, proximal growth and proliferation were reduced, resulting in almost uniform rates of growth and proliferation along the PD axis (Figures 4B, 4E, 4J, and 4K). Likewise, the contribution of proximal cells at 2 DAI to leaf area and cell number at 7 DAI was reduced (Figures 4L and 4M). Thus, WOX activity affects leaf shape by promoting a basipetal distribution of growth and proliferation that is higher in the proximal blade. Having found that WOX genes affect growth rates, we next sought to examine how WOX genes regulate growth directions by identifying how directional growth differs in wox135 mutants compared to WT (Figures 4N–4P and 4R). To obtain tissue-aligned growth directions, we decomposed cell growth into directions aligned with the PD and ML axes of the primordium.
Figure 4. A Specific Spatial Pattern of Auxin Biosynthesis Is Required for Lateral Leaf Growth

(A–I) Heatmaps of area extension (A–C; fold change in area), cell proliferation (D–F; fold change in cell number), and growth anisotropy (G–I) for the indicated genotypes over 5 days growth, visualized on 2 DAI and 7 DAI leaf primordia. In (G)–(I), white lines indicate the direction of growth anisotropy for cells with

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that, in the leaf blade of wox135 mutants, growth was anisotropic, similar to that of WT leaf blades (Figures 4G, 4H, and 4R), mirroring a reduction of growth in both tissue-aligned growth directions (with a slightly greater reduction in ML growth; Figures 4N–4P). By contrast, in the distal blade, anisotropy increased (Figures 4G, 4H, and 4R). Our quantifications indicate that, in the wox135 mutant, ML growth was substantially reduced throughout most of the leaf blade (Figure 4O), although PD growth was unchanged in the distal leaf blade (Figure 4N), coinciding with increased anisotropy in this location. Together, these results indicate that WOX genes influence directional growth differently in different regions of the leaf blade in WT leaves: at the leaf blade base, they promote both lateral and PD growth, although lateral growth is promoted broadly in the leaf blade.

Having characterized the growth differences between WT and wox135 mutants, we next sought to understand how pWOX3:YUC1 suppresses the wox135 mutant phenotype. Our analysis above (Figures 3Q and 3S) had shown that leaf shape is rescued in this background, producing a rounder leaf blade, but that leaf size is only slightly increased compared to wox135 plants (Figure 4R). This observation suggests that, although pWOX3:YUC1 only partially suppresses the growth phenotype of wox135 leaves, it nevertheless rescues components of growth that are critical for leaf shape. To identify these components, we analyzed the growth of pWOX3:YUC1 wox135 leaf blades and compared it to WT and wox135 plants. Growth alignments show that pWOX3:YUC1 wox135 leaf blades have a basipetal gradient of growth and proliferation, with this gradient being much less pronounced for proliferation (Figures 4C, 4F, 4J, and 4K). In this background, we observed an increase in the relative histogenic contribution of cells in the leaf base of 2 DAI to 7 DAI leaves compared to wox135 leaves (Figures 4L, 4M, S4L, and S4M), shifting the corresponding growth alignments toward those of WT leaves. Thus, expressing pWOX3:YUC1 in wox135 plants rescues the basipetal gradient of leaf blade growth (Figures 4J and S4J) but does not restore absolute growth (Figures 4A, 4B, and 4G) or proliferation rates to WT levels (Figures 4D–4F and 4S). In pWOX3:YUC1 wox135 plants, area extension and proliferation were also reduced in the distal blade compared to wox135 plants (Figures 4B, 4C, 4E, and 4F). Nonetheless, we observed increased area extension in the leaf blade base (Figures 4B, 4C, and 4J), which appeared to be the result of increased lateral growth (Figures 4N–4P and S4N–S4P; ML growth increased; PD growth decreased). This increase rescues the balance between PD and ML growth along the PD axis to WT levels (Figures 4P and S4P) and thus may explain the restoration of WT leaf shape in pWOX3:YUC1 wox135 leaves.

Interestingly, although expressing YUC1 in the pWOX3 domain reduces distal growth anisotropy toward WT levels, it did not change the growth anisotropy at the leaf blade base (Figure 4R). As such, our analysis of pWOX3:YUC1 wox135 compared to wox135 plants indicates that, although lateral growth is preferentially increased at the leaf blade base (Figure 4P), anisotropy in this region is largely unchanged. Together, these quantifications indicate a change in growth directions at the base of the leaf blade, which shifts from being closely aligned with the PD axis in wox135 to take on a more lateral orientation in pWOX3:YUC1 leaves (compare Figure 4H to Figure 4I). Together, our results indicate that WOX-induced auxin biosynthesis shapes the basipetal gradient of leaf growth. Furthermore, they show that auxin biosynthesis in the WOX domain promotes growth laterally at the leaf base (Figure 4O) while reducing growth at the leaf tip (Figures 4B and 4C). This differential growth appears sufficient to restore the broad leaf shape of WT leaves in narrow-leaved wox triple mutants. Notably, our results suggest that the relative balance of lateral versus PD growth may be more important to leaf shape than absolute growth rates. Nonetheless, the inability of WOX-induced, auxin biosynthesis to rescue leaf size or distal leaf growth indicates that these components of leaf shape depend on additional pathways downstream of WOX.
DISCUSSION

We have shown that WOX1, 3, and 5 act redundantly to coordinate cellular growth, proliferation, and differentiation in the developing leaf primordium of Arabidopsis thaliana (Figure 5). A single group of genes that control these processes might help to synchronize them during development to facilitate the emergence of leaves of the correct shape and size. We also provide evidence that auxin partially rescues basipetal growth in wox1, 3, and 5 mutants by mediating two aspects of WOX action that contribute to the development of an elliptical leaf shape. Specifically, we propose that WOX-dependent auxin synthesis: (1) promotes lateral growth at the leaf blade base (Figures 4J and 4P) and (2) inhibits growth of the distal leaf blade, thus generating a basipetal gradient of growth (Figure 5D). Furthermore, our results show that the uniform provision of auxin biosynthetic gene expression in the leaf epidermis does not rescue the narrow leaf defects of wox135. This suggests that the precise spatial deployment of the WOX-auxin module is of crucial importance for leaf growth and form and complements the transport-based regulation of auxin distribution [34, 35]. Given that pWOX3::YUC1 expression in wox135 did not restore growth and proliferation to WT levels (Figures 4J and 4K), WOX genes must also act through pathways that are independent of auxin biosynthesis. This conclusion is also consistent with the fact that the leaf phenotype of yuc triple and quadruple mutants (Figures 2N and 2P) does not fully phenocopy the effects of wox135 on leaf shape. Our observations indicate that pWOX3::YUC1 expression also affects distal leaf blade development, where growth and proliferation were decreased in comparison to both WT and wox135 mutants (Figures 4C, 4F, 4J, and 4K). Together, our findings show that WOX-dependent auxin production contributes to shaping cellular differentiation patterns in the leaf [3]. As there is evidence that WOX expression is in turn influenced by auxin signaling, our results raise the possibility that a feedback between auxin activity and WOX action are an additional part of this module [16, 17].

In conclusion, we propose that WOX expression promotes a fast-growing, proliferative state and inhibits the action of differentiation-promoting compounds (including WOX-dependent auxin) in the proximal leaf blade. More distally, where WOX genes are not expressed, auxin would be free to promote differentiation. WOXes may thus contribute to the PD differentiation gradient observed in time-lapse data [3, 4] by inhibiting differentiation proximally in the blade and promoting it distally. Given that WOXes also promote lateral growth, we propose that these interactions contribute to the elliptical leaf shape of Arabidopsis thaliana [1, 3, 4, 7]. This proposed role of the WOX/auxin module in shaping Arabidopsis thaliana leaf form can be conceptualized as a positional information system, with the following logic: an upstream regulator (WOX) promotes the synthesis of auxin, which serves as a long-range differentiation signal. This signal emanates from a basally localized growth zone within which the differentiation-inducing signal cannot act, allowing continued proliferation and growth. This basal dampening of differentiation promotes the continued activity of the growth zone despite the sustained production of the differentiation signal. As such, auxin may be one component of the distally acting morphogenetic signal that computational models predict is synthesized at the leaf base and regulates the basipetal progression of differentiation [7]. The expression of WOX proteins at the basal flanks of the developing leaf blade indicates that, in future work, it would be interesting to explore computational models that also incorporate morphogenetic signals originating from this domain.

Notably, our results with pWOX3::YUC1 in wox135 leaves also indicate that leaf shape can be restored to a shape very close to WT even if leaf size is only weakly rescued (Figures 1F and 1G). This observation suggests that the gradient of WOX-dependent leaf growth is at least as important for leaf shape as the absolute amount of growth. In this context, WOX/YUC-dependent lateral growth contributes to the increased width of the leaf blade compared to the petiole. The development of this aspect of leaf shape may involve an active change in growth directions owing to WOX/YUC-dependent changes in the polarity of cell growth [4, 37] or passive growth reorientation as a result of mechanical conflicts between the narrow petiole and the broader leaf blade [37, 38]. Distinguishing between these possibilities will require...
study of WOX and YUC genetic mosaics combined with tissue-level mechanically informed models of leaf growth. Use of genetic mosaics and tissue-specific gene editing [39] will also help clarify the cell-specific functions of different WOX genes, as well as their interactions with YUC genes and the degree of their non-cell-autonomous function in the leaf blade [40]. Given the role of auxin in vascular development [41] and the likely interplay between vasculature pattern and leaf form [6, 13, 42], further investigation of WOX function in the vasculature will be of particular interest [43]. An implication of our findings is that evolutionary modifications of this WOX-YUC module (for example, modifications that change the domain of WOX expression, the degree of local differentiation inhibition, the efficiency of WOX-mediated YUC activation, or the rate of distal auxin transport) have the potential to contribute to the natural variation of leaf forms.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.09.037.

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AUTHOR CONTRIBUTIONS

Conceptualization, Z.Z. and M.T.; Methodology, Z.Z. and M.T.; Investigation, Z.Z., R.A.M., D.K., M.K., X.G., P.H., and K.L.; Software, A.R., B.H., and S.S.; Formal Analysis, A.R.; Writing – Original Draft, Z.Z., A.R., and M.T.; Writing – Review & Editing, Z.Z., A.R., and M.T.; Visualization, Z.Z. and M.T.; Resources, M.T.; Data Curation, M.T.; Supervision, M.T.; Project Administration, Z.Z. and M.T.; Funding Acquisition, M.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Runions, A., and Tsiantis, M. (2017). The shape of things to come: from topology to predictive models for leaf diversity. Am. J. Bot. 104, 1437–1441.
2. Zuniga, A. (2015). Next generation limb development and evolution: old questions, new perspectives. Development 142, 3810–3820.
3. Kierzkowski, D., Runions, A., Vuolo, F., Strauss, S., Lymbouridou, R., Routier-Kierzkwoka, A.-L., Wilson-Sánchez, D., Jenke, H., Galinha, C., Mosca, G., et al. (2019). A growth-based framework for leaf shape development and diversity. Cell 177, 1405–1418.e17.
4. Kuchen, E.E., Fox, S., de Reuille, P.B., Kennaway, R., Bensmihen, A., Avondo, J., Calder, G.M., Southam, P., Robinson, S., Bangham, A., and Coen, E. (2012). Generation of leaf shape through early patterns of growth and tissue polarity. Science 325, 1092–1096.
5. Whitewoods, C.D., Gonçalves, B., Cheng, J., Cui, M., Kennaway, R., Lee, K., Bushell, C., Yu, M., Piao, C., and Coen, E. (2020). Evolution of carnivorous traps from planar leaves through simple shifts in gene expression. Science 367, 91–96.
6. Runions, A., Tsiantis, M., and Prusinkiewicz, P. (2017). A common developmental program can produce diverse leaf shapes. New Phytologist 216, 401–418.
7. Fox, S., Southam, P., Pantin, F., Kennaway, R., Robinson, S., Castorina, G., Sánchez-Corrales, Y.E., Sablowski, R., Chan, J., Grieniseisen, V., et al. (2018). Spatiotemporal coordination of cell division and growth during organ morphogenesis. PLoS Biol. 16, e2005952.
8. Nakata, M., Satsumo, N., Tsugeki, R., Rikirsch, E., Laux, T., and Okada, K. (2012). Roles of the middle domain-specific WUSCHEL-RELATED HOMEBOX genes in early development of leaves in Arabidopsis. Plant Cell 24, 519–535.
9. Vandenbussche, M., Horstman, A., Zethof, J., Koes, R., Rijpkema, A.S., and Gerats, T. (2009). Differential recruitment of WOX transcription factors for lateral development and organ fusion in Petunia and Arabidopsis. Plant Cell 21, 2269–2283.
10. Zhao, F., Du, F., Oliveri, H., Zhou, L., Ali, O., Chen, W., et al. (2020). Microtubule-mediated wall anisotropy contributes to leaf blade flattening. Curr. Biol. Published online September 10, 2020. https://doi.org/10.1016/j.cub.2020.07.076.
11. Nardmann, J., Ji, J., Wer, W., and Scanlon, M.J. (2004). The maize duplicate genes narrow sheath1 and narrow sheath2 encode a conserved homeobox gene function in a lateral domain of shoot apical meristems. Development 131, 2827–2839.
12. Scanlon, M.J., Schneeberger, R.G., and Freeling, M. (1996). The maize duplicate genes narrow sheath1 and narrow sheath2 encode a conserved homeobox gene function in a lateral domain of shoot apical meristems. Development 131, 2827–2839.
13. Tadege, M., Lin, H., Bedair, M., Berbel, A., Wen, J., Rojas, C.M., Niu, L., Tang, Y., Sumner, L., Ratet, P., et al. (2011). STENOFOLIA regulates blade outgrowth and leaf vascular patterning in Medicago truncata and Nicotiana sylvestris. Plant Cell 23, 2125–2142.
14. Cho, S.-H., Yoo, S.-C., Zhang, H., Pandey, D., Koh, H.-J., Hwang, J.-Y., Kim, G.-T., and Paek, N.-C. (2013). The rice narrow leaf2 and narrow leaf3 loci encode WUSCHEL-related homeobox 3A (OsWOX3A) and function in...
leaf, spikelet, tiller and lateral root development. New Phytol. 198, 1071–1084.

15. Zhang, Z., Tucker, E., Hermann, M., and Laux, T. (2017). A molecular framework for the embryonic shoot of stem meristem cells. Dev. Cell 40, 264–277.e4.

16. Caggiano, M.P., Yu, X., Bhatia, N., Larsson, A., Ram, H., Ohno, C.K., Sappl, P., Meyerowitz, E.M., Jönsson, H., and Heisler, M.G. (2017). Cell type boundaries organize plant development. eLife 6, e27421.

17. Guan, C., Wu, B., Yu, T., Wang, Q., Krogan, N.T., Liu, X., and Jiao, Y. (2017). Spatial auxin signaling controls leaf flattening in Arabidopsis. Curr. Biol. 27, 2940–2950.e4.

18. Sarkar, A.K., Luijten, M., Miyashima, S., Lenhardt, M., Hashimoto, T., Nakajima, K., Scheres, B., Heidstra, R., and Laux, T. (2007). Conserved factors regulate signaling in Arabidopsis thaliana shoot and root stem cell organizers. Nature 446, 811–814.

19. Pi, L., Aichinger, E., van der Graaff, E., Llavata-Peris, C.I., Weijers, D., Lin, H., Niu, L., McHale, N.A., Ohme-Takagi, M., Mysore, K.S., and Jacobs, T.B. (2019). CRISPR-TSKO: a technique for efficient mutagenesis in specific cell types, tissues, or organs in Arabidopsis. Plant Cell 31, 2866–2887.

20. Chen, Y., Bai, Y., Li, J., Ji, Z., Cheng, Y., Fang, W., Yuan, F., and Lu, Z. (2010). Evidence for WOX10 regulation of leaf development in Arabidopsis. PLoS One 5, e11255.

21. Venera, C., Ravichandran, S.J., Sawchuk, M.G., Linh, N.M., and Scarpella, E. (2019). Coordination of tissue cell polarity by auxin transport and signaling. eLife 8, e51081.

22. Scarpella, E., Barkoulas, M., and Tsiantis, M. (2010). Control of leaf and vein development by auxin. Cold Spring Harb Perspect Biol. 2, a001511.

23. Ji, J., Strable, J., Shimizu, R., Koenig, D., Sinha, N., and Scanlon, M.J. (2010). WOX4 promotes procambial development. Plant Physiol. 152, 1346–1356.

24. Robert, H.S., Grones, P., Stepanova, A.N., Robles, L.M., Lokerse, A.S., Alonso, J.M., Weijers, D., and Firn, J. (2013). Local auxin sources orient the apical-basal axis in Arabidopsis embryos. Curr. Biol. 23, 2506–2512.

25. Yan, J., Gu, Y., Jia, X., Kang, W., Pan, S., Tang, X., Chen, X., and Tang, G. (2012). Effective small RNA destruction by the expression of a short tandem target mimic in Arabidopsis. Plant Cell 24, 415–427.

26. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.

27. Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. Genome Biol. 11, R106.

28. Mott, R., Yuan, W., Kaisaki, P., Gan, X., Cleak, J., Edwards, A., Baud, A., and Flint, J. (2014). The architecture of parent-of-origin effects in mice. Cell 156, 332–342.

29. Kuhl, F.P., and Giardina, C.R. (1982). Elliptic Fourier features of a closed contour. Comput. Graph. Image Process. 18, 236–258.

30. Kozlov, M.V., Wilesey, B.J., Koricheva, J., and Hautkoja, E. (1996). Fluctuating asymmetry of birch leaves increases under pollution impact. J. Appl. Ecol. 33, 1489–1495.

31. Chittwood, D.H., Headland, L.R., Ranjan, A., Martinez, C.C., Braybrook, S.A., Koenig, D.P., Kuhlemeier, C., Smith, R.S., and Sinha, N.R. (2012). Leaf asymmetry as a developmental constraint imposed by auxin-dependent phyllotactic patterning. Plant Cell 24, 2318–2327.

32. Chittwood, D.H., Mott, R., Thammapracharil, P., Woeger, A.C., Headland, L.R., and Sinha, N.R. (2012). Conflict between intrinsic leaf asymmetry and phyllotaxis in the resupinate leaves of Alstroemeria psittacina. Front. Plant Sci. 3, 182.

33. Hejnowicz, Z., and Romberger, J.A. (1984). Growth tensor of plant organs. J. Theor. Biol. 110, 93–114.

34. Dumais, J., and Kwiatkowska, D. (2002). Analysis of surface growth in shoot apices. Plant Physiol. 131, 229–241.

35. Dobrev, P.I., and Kaminek, M. (2002). Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. J. Chromatogr. A 950, 21–29.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, Peptides, and Recombinant Proteins** |
| Propidium iodide (PI) | Sigma-Aldrich | 87-51-4 |
| Phusion Taq | Thermo Fisher | F553S |
| Plant Preservative Mixture (PPM) | Plant Cell Technology | 250 |
| XmaI | New England Biolabs | R0180M |
| BamHI | New England Biolabs | R3136T |
| BglII | New England Biolabs | R0144M |
| PstI | New England Biolabs | R0140M |
| NotI | New England Biolabs | R3189L |
| 2,2'-Thiodiethanol | Sigma | 166782-100G |
| **Critical Commercial Assays** |
| CloneJET PCR Cloning Kit | Thermo Fisher Scientific | #K1231 |
| RNeasy Plant Mini Kit | Qiagen | #74904 |
| SuperScript VILO cDNA Synthesis Kit | Invitrogen | #11755-050 |
| **Deposited Data** |
| RNA seq | This study | PRJEB38272 |
| **Experimental Models: Organisms/Strains** |
| A. thaliana: Columbia-0 (Col-0) | N/A | N/A |
| A. thaliana: wox1-101 (wox1) | [8] | N/A |
| A. thaliana: wox3-2 (prs-2) | [8] | N/A |
| A. thaliana: wox5-1 (wox5) | [18] | N/A |
| A. thaliana: wox1-101 wox3-2 (wox13) | [8] | N/A |
| A. thaliana: wox1-101 wox5-1 (wox15) | This study | N/A |
| A. thaliana: wox3-2 wox5-1 (wox35) | This study | N/A |
| A. thaliana: wox1-101 wox3-2 wox5-1 (wox135) | This study | N/A |
| A. thaliana: pWOX5:GFP | Gift from Thomas Laux | N/A |
| A. thaliana: pWOX3:rPHV x wox135 | This study | N/A |
| A. thaliana: pWOX3:MIM165/166 x wox135 | This study | N/A |
| A. thaliana: pDR5v2:NLS-tdtomato x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pDR5v2:NLS-tdtomato x pWOX3:YUC1 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pDR5v2:NLS-tdtomato x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pDR5v2:NLS-tdtomato x pWOX3:YUC1 x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: yuc146 | [27] | N/A |
| A. thaliana: yuc1246 | [27] | N/A |
| A. thaliana: pRCO:3xNLS-GFP x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pRCO:3xNLS-GFP x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pyUC1:3xNLS-GFP x pUBQ10:acyl-YFP | This study | N/A |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| A. thaliana: pYUC1:3xNLS-GFP x pUBQ10:acyl-YFP x pRCO:WOX3-mcherry | This study | N/A |
| A. thaliana: pYUC1:3xNLS-GFP x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pYUC1:3xNLS-GFP x wox135 x pUBQ10:acyl-YFP x pRCO:WOX3-mcherry | This study | N/A |
| A. thaliana: pYUC4:3xNLS-GFP x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pYUC4:3xNLS-GFP x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pYUC4:3xNLS-GFP x wox135 x pUBQ10:acyl-YFP x pRCO:WOX3-mcherry | This study | N/A |
| A. thaliana: pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pWOX3:YUC1 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pWOX3:YUC1 x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pWOX3:3xNLS-GFP | This study | N/A |
| A. thaliana: pWOX3:3xNLS-GFP x wox135 | This study | N/A |
| A. thaliana: pLMI1:3xNLS-GFP x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pLMI1:3xNLS-GFP x pWOX3:YUC1 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pLMI1:3xNLS-GFP x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pLMI1:3xNLS-GFP x wox135 x pWOX3:YUC1 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pAS2:3xNLS-GFP x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pAS2:3xNLS-GFP x pWOX3:YUC1 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pAS2:3xNLS-GFP x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pAS2:3xNLS-GFP x wox135 x pWOX3:YUC1 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pAtML1:YUC1 x wox135 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pAtML1:YUC1 x pUBQ10:acyl-YFP | This study | N/A |
| A. thaliana: pWOX3:WOX3-GFP | This study | N/A |
| A. thaliana: pWOX3:WOX3-GFP x wox135 | This study | N/A |

**Oligonucleotides**

| Oligonucleotides | This study | Table S3 |
|------------------|------------|----------|

**Recombinant DNA**

| Recombinant DNA | This study | N/A |
|-----------------|------------|-----|
| pWOX3:rPHV      | This study | N/A |
| pWOX3:MIM165/166 | This study | N/A |
| pWOX3:WOX3-GFP  | This study | N/A |
| pDR5v2-NLS-tomato | This study | N/A |
| pWOX3:YUC1      | This study | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Mitos Tsiantis (tsiantis@mpipz.mpg.de).

Material Availability
Plasmids and seed generated in this study have been deposited in relevant collections of the Tsiantis lab in the Max Planck Institute for Plant Breeding Research and will be distributed upon request. Newly generated material is outlined in the Key Resources Table.

Data and Code Availability
The accession number for the RNA-seq reported in this paper is European Nucleotide Archive (ENA): PRJEB38272.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material and growth conditions
All transgenic plants and mutants were in Arabidopsis thaliana Col-0 background and listed in Key Resources Table. Plants were grown on soil in a growth chamber under long day conditions (16 h/8h light/dark, 110 \( \mu \text{E}\text{m}^{-2}\text{s}^{-1}) at 20 ± 2°C, with 65 ± 10% relative humidity. For time-lapse experiments, soil-grown plants were transferred 1 day after germination to half MS medium supplemented with 1% sucrose, 0.1% PPM and grown in long day conditions described above.

METHOD DETAILS

Plasmid construction
All constructs were generated with conventional restriction enzyme digestion and ligation (Key Resources Table). Newly amplified sequences were confirmed by sequencing. Primers used are listed in Table S3. WOX3 promoter (pWOX3) was amplified and ligated to the commercial plasmid pJet1.2 from CloneJET PCR Cloning Kit. pWOX3 was excised by XmaI and ligated to pBJ36 intermediate vector. For pWXO3:PHV, PHV fragment was excised from pWOX2:PHV by BamHI [15] and ligated to pBJ36 after the WOX3 promoter. For pWXO3:MIM165/166, MIM165/166 fragment was excised from MIM165/166 in pJet [45] by Bgill and ligated to pBJ36 after the WOX3 promoter. For pWXO3:WOX3-GFP, WOX3-GFP was synthesized and ligated in pUC57. WOX3-GFP was excised by Bgill and ligated to pBJ36 after the WOX3 promoter. For pWXO3:YUC1, YUC1 coding sequence was amplified and ligated to pJet1.2. YUC1 was excised by BamHI and ligated to pBJ36 after the WOX3 promoter. All these intermediate constructs were digested by NotI to obtain the targeted fragments and ligated to binary vector pMLBart. pMLBart gives Spectinomycin resistance in bacteria and Basta resistance in plants.
For pRCO:WOX3-mCherry, WOX3 coding sequence without stop codon was amplified and ligated together with mCherry in pJet1.2. WOX3-mCherry was excised by BamHI and BgIll and ligated to pBJ36 after pRCO [28]. pRCO:WOX3-mCherry was excised by NotI and ligated to pGreenII.

For pYUC4:3xNLS-GFP and pAS2:3xNLS-GFP, YUC4 promoter (pYUC4) and AS2 promoter (pAS2) were amplified and ligated to pJet1.2. pYUC4 and pAS2 were excised by Xmal and ligated to pBJ36 before 3xNLS-GFP. pYUC4:3xNLS-GFP and pAS2:3xNLS-GFP was excised by NotI and ligated to binary vector pMLBart.

For pWOX3:3xNLS-GFP, pWOX3 was excised from pWOX3 in pJet1.2 and ligated to 3xNLS-GFP in pGreenII. For pRCO:3xNLS-GFP, pRCO was excised from pBJ36 by PstI and BamHI and ligated to 3xNLS-GFP in pGreenII. For pLMI1:3xNLS-GFP, LMI1 promoter (pLMI1) was amplified and ligated to pBJ36. pLMI1 was excised by Sall and Xmal and ligated to 3xNLS-GFP in pGreenII. All these plasmids give Kanamycin resistance in bacteria and Methotrexate resistance in planta.

For DR5v2:NLs-tcottomato which was aimed to change plant resistance, DR5v2:tdtomato was excised from pGreenII by Sall and NotI (Kanamycin resistance in planta [23]) and ligated to pGreenII (Norflaxacin resistance in planta).

Plant transformations were performed using Agrobacterium tumefaciens strain GV3101 and the floral dipping method. All the transgenic plants were genotyped in the T1 generation for transgenes and/or mutant background. Plants carrying two transgenes or different mutant genotypes were produced by crossing and analyzed in the F1 generation.

For Figure S1G, 8 out of 8 independent transgenic lines displayed the phenotypes as shown in Figure S1G. For Figure S1H, 13 out of 13 independent transgenic lines displayed the phenotypes as shown in Figure S1H. For Figures 3Q–3S, 13 T2 families were analyzed for both pWOX3:YUC1 in wild-type and wox135. For Figures S3S and S3T, the first pair of leaves from 6 T2 transgenic families of pWOX3:WOX3 in wild-type and in wox135, respectively, were analyzed. For Figures S4E–S4G, the first pair of leaves from 11 and 17 T1 transgenic plants of pAtML1:YUC1 in wild-type and in wox135, respectively, were analyzed.

**RNA in situ hybridization**

RNA in situ hybridizations on 8 μm sections through fixed and paraaffin-embedded shoot apices of 2- to 3-wk-old short-day grown A. thaliana Col-0 plants were performed largely as previously described [28]. Digoxigenin-labeled antisense RNA probes to A. thaliana WOX5 (AT3G12610) were generated using a synthetic DNA template with the T7 RNA polymerase-binding sequence motif, 5’-CCCTATAGTGAGTCGTATTACGCAC-3’, added to its 3’ end (synthesized by BioCat GmbH, Heidelberg, Germany). The template represented positions 309-883 (5’-ACGAC .... ATTGA-3’) of NCBI Reference Sequence NM_111961.4. After hybridization and washing, the sections were covered with 70% TDE at pH = 9 and imaged with a Zeiss Axio Imager equipped with a digital color camera and DIC optics. To cover a broader hybridization pattern, three consecutive sections were registered and minimum projections were generated using the image processing package Fiji [16].

**RNA-seq**

6-day-old seedlings of wild-type and wox135 mutant on half MS medium were treated with Dexamethasone (10μM) for 4 hours. The cotyledons, root and half of the hypocotyl of seedlings were removed, and the remaining part including the leaves and ~2mm hypocotyl were collected. In total, 3 replicates for WT and wox135 (1 wox135 replicate failed in later RNA-seq analysis, therefore 2 replicates were left for transcriptome analysis) were prepared and total RNA was isolated using plant mini RNaseasy kit (QIAGEN) for sequencing.

Library preparation and sequencing have been performed at the Max Planck Genome Center Cologne, Germany (https://mpgc.mpipz.mpg.de/home/). 700 ng total RNA has initially been used for polyA enrichment with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). Subsequent library preparation has been performed with NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) according to the manufacturer’s instructions. Quality and quantity were assessed at all steps via capillary electrophoresis (TapeStation, Agilent Technologies) and fluorometry (Qubit, Thermo Fisher Scientific). Libraries were immobilized and processed onto a flow cell with cBot (Illumina) and subsequently sequenced on HiSeq2500 system (Illumina) with 2 × 100 bp paired end reads.

**RNA-seq analysis**

Paired-end reads were aligned to the reference genome TAIR10 for A. thaliana using TopHat2 (1) with parameters “--max-multithreads 10–coverage-search—microexon-search—mate-std-dev 40–library-type fr-secondstrand—max-intron-length 30000.” Raw read counts per gene were quantified with HTSeq v0.5.4p1 (http://www-huber.embli.de/users/anders/HTSeq/) using the “–t CDS -s reverse” option. Differential expression between samples from the same species was determined using DESeq [47]. We found the most sensitive parameter settings for the function estimateDispersions were method = “blind,” and sharingMode = “fit-only” [48]. The cutoff of deregulated genes is fold change > 1.5 and adjusted p < 0.05. GO term enrichment was analyzed via Panther Geneontology (http://pantherdb.org/webservices/go/overrep.jsp).

**Fluorescence imaging**

We used a SP8 upright confocal microscope equipped with a long working-distance water immersion objective (AP 20x/0.8 or AP 40x/0.8; Leica) to perform all imaging analyses. Excitation wavelengths for different fluorescence markers were as following: an argon laser with 486 nm for GFP, 514 nm for VENUS and YFP, and 561 nm for tdTomato. Images in a set of experiments which needed to be...
compared among each other were collected under the same settings. Chlorophyll autofluorescence was detected by excitation at 633 nm using a HeNe laser with 639-706 nm bandpass filter. For pWOX5:GFP and gWOX5-YFP (Figures 1I–1L), stronger laser power was used for the gWOX5-YFP reporter to detect the signal. The bandpass filter for pWOX5:GFP was 493-560 nm and 521-582 nm for gWOX5-YFP. Images were processed using LasX (Leica) analyzed using ImageJ or MorphGraphX software [31], and visualized by Photoshop (Adobe). For pDR5v2:NLS-tomato, leaf 1 to leaf 6 were taken for imaging. Signal intensity of pDR5v2:NLS-tomato was quantified via ImageJ using the cumulative signals from all the Z stacks of one leaf which were imaged under identical conditions.

Growth tracking experiment
After 1 day of germination, cotyledons of plants were carefully dissected out. Time-lapse imaging and analysis was performed according to the method described in [3]. Imaging was performed at 24h intervals, but at the last time point this interval was ~36h. Independent images were acquired at the indicated time-points.

Leaf shape analysis
To obtain leaf silhouettes and to quantify leaf shape, the first pair of leaves of 3-weeks-old plants were collected, flattened onto transparent adhesive paper, and scanned to obtain images. Leaf silhouettes were analyzed using Leaf Interrogator (LeafI), a GUI-based system providing an integrated pipeline for leaf shape analysis. LeafI is implemented in Python3.5, with a PyQt5 based GUI. LeafI was used to (1) extract leaf contours from images, (2) process contours to extract the leaf blade, (3) calculate simple shape measures (Figures 1G, 1H, 2O, 2P, 3R, 3S, S3T, S4F, and S4G), and (4) perform shape-space analysis and visualization (Figures 1F, 2N, 3Q, S3S, and S4E). Additional details are provided below.

1. Contour extraction: Image processing for the purpose of contour extraction was performed using OpenCV (V. 3:2.0.7). Binary thresholding was used to separate leaves from the image background, and a vector contour was extracted for each leaf (e.g., a sequence of 2D positions).

2. Contour processing: Contours were edited to delete the petiole and specify 2 common landmarks (the tip and base of the leaf-blade). The point of blade-petiole separation was identified manually by determining the point where the petiole begins to widen. Blade contours were resampled to obtain 60 points (semi-landmarks) on the contour intervals connecting the two-landmarks, yielding 122 points per contour (60 points on the left margin, 60 points on the right margin, and 2 landmarks). The 60 points were placed using an arc-length sampling of the contour (i.e., sample points were placed at equidistant points along each contour interval).

3. Simple shape measures: The resampled contours were used to compute the area and narrowness index for each blade. The narrowness index (also called elongation) is calculated from the minimum area rectangle enclosing the contour by taking the ratio of the rectangle’s dimensions: 

$$ narrowness \ index = 1 - \frac{\text{width}}{\text{length}} \quad (\text{with length} \geq \text{width}) $$

Areas and narrowness indices were exported from LeafI as csv files, and plotted in R. Areas in LeafI are reported in pixels$^2$ and were converted to cm$^2$ based on image dimensions.

4. Shape space analysis and visualization: Shape spaces visualize symmetric variation in leaf blade shape (Figures 1F, 2N, 3Q, S3S, and S4E), and were obtained by performing Elliptical Fourier Descriptor (EFD) analysis on the resampled leaf blade contours [49]. Using symmetric variation eliminates asymmetric variation resulting from confounding factors including: environment and developmental noise (i.e., fluctuating asymmetry [50]), chirality of phyllotaxy [51], and variable phenotypes that obscured the overall shape of the blade (e.g., yuc126, yuc1246 mutants). Normalized EFDs were used for this analysis, as these provide a translation, rotation and scale invariant representation of the leaf contours. Rotation invariant coefficients were obtained by rotating contours to align the landmarks (leaf tip and leaf base) with the y axis before EFDs were computed [52]. Translation and scale-invariant EFDs were calculated as described in Kuhl and Giardina [49]. Principle Component Analysis was performed on the first 64 harmonics of the Fourier coefficients. To analyze symmetrical aspects of blade shape, Fourier coefficients capturing asymmetric variation about the main axis of the leaf (i.e., the line connecting the leaf tip and base) were set to zero prior to performing PCA [52]. The plots in Figures 1F, 2N, 3Q, S3S, and S4E were created using the Matplotlib library (V 2.0), and plotting the PCA values for the first two principal components. The first two components account for more than 94% of variance in all cases, indicating that they capture the majority of the symmetric shape variation of the leaf blade. Ellipses indicate half a standard deviation of the mean for each group, computed from the eigen-decomposition of the covariance matrix for that group.

Growth Alignment Graphs
To analyze the distribution of growth and proliferation in the blades of leaf primordium in A. thaliana wild-type, wox135 and pWOX3:-YUC1 wox135 plants between 2-7 DAI, we used growth alignment graphs [3]. Growth alignment graphs provide a method to compare equivalent regions along the Proximal-Distal axis (PD-axis) of leaf primordia in different backgrounds. Information on each clone was computed in MorphoGraphX [31], and exported as a csv file. This data was used to generate growth alignment graphs.
For each leaf primordium, segmented leaf surfaces at 2 and 7 DAI were used to quantify blade development based on lineage tracking information. MorphoGraphX was used to calculate clone area at 2 and 7 DAI, as well as growth parameters over the time interval of 2-7 DAI (area extension, proliferation and anisotropy; as described in [31]). To estimate the PD-position of each clone, the distance of the clone from the leaf base was computed at 2DAI (in microns) using Dijkstra’s algorithm. The organ aligned directions used for growth analysis (Figures 4N–4P and S4N–S4P) were estimated at 2DAI, when primordia are relatively radially symmetric. A line was used to approximate the PD-axis of the primordium. For each primordium, this line was placed to pass through the cell at the distal leaf tip as well as the center of the primordium base. For each cell in the abaxial leaf surface, the Medial-Lateral (ML) direction was determined by the vector orthogonal to the PD-axis which was tangent to the surface. For the Proximo-Distal (PD) direction, the vector tangent to the surface and orthogonal to the ML-direction was used. Organ aligned growth rates were determined using the growth tensor [53, 54] calculated for each cell. The growth tensor characterizes a cells 2D expansion over time, and was evaluated in the ML and PD directions to determine organ aligned growth rates. Finally, clones contributing to the leaf blade were marked by tracing the clones of leaf blade at 7DAI back to 2DAI. The list of clones comprising the primordium blade, as well as distances and other values computed in MorphoGraphX were exported as csv files.

Growth alignments for each primordium were created using a custom R-Script. The script loads the csv files exported from MorphoGraphX and eliminates invalid data-values. Normalized PD positions for each clone in a primordium are obtained using percentiles of the distance from leaf base at 2 DAII. Clones are divided into 7-bins based on their normalized PD-position, resulting in approximately the same number of clones in each bin. To obtain growth alignments for average proliferation, area extension, PD/ML growth and anisotropy we use bin-wise averaging (Figures 4J, 4K, 4N–4P, and 4R). By contrast, growth alignments for relative areal and cellular contribution (Figures 4L and 4M) allow the relative contribution of equivalently sized regions along the PD-axis to be evaluated. As in [3], the relative cellular contribution of cells in the ith bin at 2 DAII to the blade at 7 DAII is estimated using the formula:

$$CC_{i} = \frac{C_{init}}{C_{final}}$$

where \(C_{init}\) is the number of cells in the bin at 2 DAII, \(C_{final}\) is the number cells in the bin at 7 DAII and \(C_{clones}\) was the total number of clones present at 7 DAII. This estimate corrects for clones present at 2 DAII, which were not captured at all intermediate time-points. To compute relative areal contribution, clones were binned along the PD-axis based on their cumulative area at 2 DAII, binning the primordium into regions accounting for approximately the same total area. Using this binning, relative areal contribution is computed using a similar formula to that of cellular contribution, where \(C_{final}\) is replaced by the total area of the cells in a bin at 7 DAII. Both relative cellular and areal contributions are converted to percentages prior to further analysis and plotting.

Growth alignment graphs were plotted in R, using ggplot (n = 3 for each background, 7 PD-percentile positions). To estimate average alignments in each background (Figures 4J–4P and 4R), a cubic orthogonal polynomial was fit to the 21 (PD-percentile, value) pairs for each background. Cubic polynomials were used as a common model for all data, as they capture curvilinear trends in the data without overfitting. PCA analysis was performed in R using prcomp with centering and scaling (stats package) and plotted using ggbiplot (Figures S4J–S4P).

**Auxin metabolite quantification**

For endogenous auxin and its metabolites quantification, approximately 20 mg of aerial parts of 2-week-old seedlings were collected for each biological replicate. The plants were frozen immediately in liquid nitrogen after harvest and stored in –80°C upon further experimentation. Extraction was performed according to Dobrev and Kaminke [55]. Briefly, frozen samples with added internal standards (Olchemim, Czech Republic) were extracted and homogenized by bead mill (MixerMill, Retsch GmbH, Haan, Germany) and underwent subsequent solid-phase extraction using MCX cartridges (30 mg 1 cc, Waters, Milford MA, USA). The obtained eluates were then evaporated to dryness using a SpeedVac and dissolved in 40 μL of 10% methanol. Twenty microliters of each sample were then analyzed by a liquid chromatography–mass spectrometry (LC-MS/MS) system comprising of 1290 Infinity Binary LC System coupled to 6490 Triple Quad LC/MS System with Jet Stream and Dual Ion Funnel technologies, using Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA). The LC-MS/MS system parameters were optimized according to Novák et al. [25] and the concentrations were calculated using a standard isotope dilution method. All solvents used were of analytical or higher grade (SigmaAldrich-Merck GmbH, Steinheim, Germany).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed using R and boxplots in Figures 1G, 1H, 2O, 2P, 3R, 3S, S3T, S4F, and S4G were used to visualize data distribution. Error bars in Figure 2C represent standard deviation. All statistical tests and the numbers of how many samples have been analyzed (n) have been indicated in figure legends. The significance threshold used was \(p < 0.05\). Leaf contour shape-space plots in Figures 1F, 2N, 3Q, S3S, and S4E were created in Leaf (STAR Methods) based on Principle Component Analysis. PC1 and PC2 values for each contour (colored dots) were respectively plotted along the x- and y-axis as multiples of their respective standard deviations (% of explained variance is indicated). Crosses indicate genotype means; Ellipses indicate half the standard deviation.
Supplemental Information

A WOX/Auxin Biosynthesis Module Controls Growth to Shape Leaf Form

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Figure S1. *WOX* multiple mutant phenotypes and *WOX* gene expression, related to Figure 1.

(A-H) 5-week-old Arabidopsis plants of indicated genotypes. *WOX* single and double mutants (A-D) do not have obvious phenotypes compared to wild-type (Figure 1A). (E-H) *pWOX3:rPHV* (G) and *pWOX3:MIM165* (H) do not rescue the *wox135* narrow leaf phenotype (E-F). (I) Leaf 5
silhouettes of the indicated genotypes. *wox135* has a more pronounced narrow leaf defect than *wox13* mutants; n≥10 for each genotype. (J-L) Optical longitudinal sections of leaves from *pWOX5:GFP* (J-K) and *pWOX3:3xNLS-GFP* (L) reporter lines show that their expression overlaps in most of the primordium except for the marginal region. (L) is the same leaf shown in Figure S3B. (M) *In situ* hybridization of *WOX5* mRNA on transverse sections of developing leaves. Transcript is detected in the middle domain of primordia (see reference [S1] for definition of the middle domain) and the distinct foci are the presumed developing vasculature. (N-Q) The expression of *pWOX5:GFP* in wildtype (N-O) and *wox13* or *wox13wox5/+* mutants which are phenotypically indistinguishable (P-Q). Cross sections in (O) and (P) respectively correspond to the white dashed lines in (N) and (Q). Scale bars: 1cm in (A-H); 100µm in (J-Q)
Figure S2. WOX proteins promote auxin biosynthesis, related to Figure 2, Figure 3, Table S1 and S2.

(A) 11 out of 29 of AUX/IAA genes are downregulated in the wox135 based on RNA-seq data. Dashed lines indicate a 2-fold change in expression. (B-G) The expression of pDR5v2::NLS-tdtomato (red) is induced in both pWOX3:YUC1 WT (B-D) and pWOX3:YUC1 wox135 (E-G), compared to its expression in wild-type (Fig. 2D-F) and wox135 (Figure 2G-I), respectively. (H) Quantification of pDR5v2::NLS-tdtomato expression during early development of wild-type (blue dots, n=54) and pWOX3:YUC1 WT (red dots, n=54) leaves. Each dot represents one leaf. Y axis is average pixel per area in arbitrary unit. (I) Quantification of the expression pattern of pDR5v2::NLS-tdtomato during early leaf development in pWOX3:YUC1 wox135. Each dot represents one leaf, n=53. When the leaf primordium length is less than around 110µm (2DAI), DR5 is not ectopically expressed (brown dots), but it is ectopically expressed along the leaf margin when the leaf primordium length is greater than 110µm (from 2DAI on, green dots). (L) Quantification of YUC1 expression induced by pRCO:WOX3-mCherry during early leaf development (29≤n≤50, each dot represents one leaf). Dot color indicates genotype. “Yes” indicates that pYUC1:3xNLS-GFP is expressed in the leaf base where the RCO promoter is active (N-P), and “no” indicates that pYUC1:3xNLS-GFP expression was not observed at the leaf base where the RCO promoter is active (M-O). Note that in WT and wox135, pYUC1:3xNLS-GFP is never expressed in the RCO domain during leaf development. By contrast, when WOX3 is ectopically expressed from the RCO promoter, either in WT or wox135, pYUC1:3xNLS-GFP is induced in the RCO domain when the leaf primordium length is greater than 90µm (from 1-2 DAI on). (J-K) The expression pattern of pRCO:3xNLS-GFP (green) in wild-type and wox135 plants during early leaf development. (M-P) pRCO:WOX3-mCherry (pRCO:WOX3) induced the
expression of \textit{pYUC1:3xNLS-GFP (pYUC1)} in 3 DAI leaves of both WT (M and N) and \textit{wox135} (O and P). Arrows highlight cells where \textit{pYUC1:3xNLS-GFP} is ectopically induced. Cell membranes in (B-G (red), and J-P (green)) are outlined by a plasma membrane marker (\textit{pUBQ10:acyl-YFP}). Scale bars: 50\mu m in (B and E) and in (1 DAI, J-K); 100\mu m in (C-D, F-G, M-P) and in the remaining panels of (J-K).
Figure S3. *pWOX3:YUC1* does not rescue the cell identity defects of *wox135* mutant, related to Figure 3.

(A-F) The expression pattern of *pWOX3:3xNLS-GFP* (*pWOX3*, green) during early leaf development in wild-type (A-C) and *wox135* (D-F) plants. PI staining is used to visualize cell walls (red). (G-J) The expression pattern of *pLMI1:3xNLS-GFP* (*pLMI1*, green) in the leaves of indicated genotypes. *pWOX3:YUC1* did not rescue the expression in *wox135*. (K-R) The expression pattern of *pAS2:3xNLS-GFP* (*pAS2*, green) in 1 and 3 DAI leaves of the indicated genotypes. At 1 DAI, the expression patterns are similar across all genotypes. *pWOX3:YUC1* does not restore the pattern of *AS2* expression in 3 DAI leaves of *wox135*. Stars in (K-R) indicate the marginal cells between adaxial and abaxial cells. Cells in (G-R) are visualized using a plasma membrane marker (red, *pUBQ10:acyl-YFP*). (S) Leaf shape-space plot for leaf silhouettes of the indicated genotypes based on principle component analysis (see Figure 1F and STAR Methods), 25≤n≤34. (T) Quantification of the leaf blade area using the same data as in (S). The letters a-c in (T) show statistically significant groups (ANOVA followed by Tukey’s test). (U-X) The first leaves from WT (U), *pWOX3:WOX3;WT* (V), *wox135* (W), and *pWOX3:WOX3;wox135* (X). Scale bars: 50µm in (A, D, K-R); 100µm (B-C, E-F, G-J); 1cm (U-X).
Figure S4. *YUCCA1* expression in the *WOX3* domain can partially restore lateral growth in *wox135* mutants, related to Figure 3 and Figure 4.

(A-D’) 4-week-old plants of the indicated genotypes (A-D) and their corresponding leaf silhouettes (A’-D’). Scale bars: 1cm. (E) Leaf shape-space plot for leaf silhouettes of the indicated genotypes based on PCA (see Fig. 1F and STAR Methods); 22≤n≤37. (F-G) Quantification of the leaf blade area (F) and narrowness index (G, see Figure 1H) using the same data as (E). *pAtML1:* *YUC1* does not restore *wox135* leaf size or width. The letters a-c in (F-G) show statistically significant groups (ANOVA followed by Tukey’s test). (H) Time-lapse images of the wild-type leaf over 6 days with ~24h intervals for the first 4 days, ~36h for the last interval. (I) Lineage tracing over 5 days using MorphoGraphX. Sectors with the same color over days originate from the same cell, as at 2 DAI. (J-P) Growth alignments of growth and proliferation for WT (green), *wox135* (blue) and *pWOX3:* *YUC1;wox135* (orange) between 2 and 7 DAI were computed (n=3 independent time-lapse experiments for each background). Growth alignments of growth and proliferation plotted as a PCA plot for mean cellular area extension (J), proliferation (K), relative areal (L), and cellular contribution (M), as well as for PD growth (N), ML growth (O) and the ratio of PD/ML growth (P). PCA plots in (J-P) show the first two principal components, ellipses indicate one standard deviation of the mean of the Gaussian fitted to each distribution.
| Oligonucleotides | Name          | Sequence                                      |
|-----------------|---------------|-----------------------------------------------|
| wox1-101F:      |               | GGATCCATGTGGACGATGGGTACACG                    |
| wox1-101R:      |               | GATATATAGCTCTGGTTGCG                         |
| wox1-101M:      |               | CGTCAATTTTTTACACCCAC                        |
| wox3-2F:        |               | CTAAGTGTTTTGAGATGACATCATCAC                  |
| wox3-2R:        |               | ACATGGGAGAAGGAGAGACG                         |
| wox3-2M:        |               | AGCTGTTTGCCTTACTGAGGTG                       |
| wox5-1F:        |               | AATCTTATATATATATGAGATATATACACAGGGCC         |
| wox5-1R:        |               | AATCTGATGCTTGGAGAATTTCAGGAGCAG              |
| wox5-1M:        |               | TGGTTACACGTAAGTGAGCCATCG                     |
| pYUC4-F:        |               | CCCGGGTTTTTTGGTTCTATATACAGAAGGTGAC          |
| pYUC4-R:        |               | CCCGGGTTTTGACTATAAAAAAGCAGAGAG             |
| pAS2-F:         |               | CCCGGGTATAAGTGAGGAGAGAGGAGGATT             |
| pAS2-R:         |               | CCCGGGTTTATTAGACTTTGAGAACATGGGAG            |
| pWOX3-F:        |               | CCCGGGCTACAAACCAACATGCACTTTTCG              |
| pWOX3-R:        |               | CCCGGGTCTCCGTTGACACAAAATTAGATGC            |
| WOX3CDS-F:      |               | GGATCCATGAGTCTCTGTTGAGCAGCC                |
| WOX3CDS-R:      |               | GGATCCCTAAGGGTTTGCTCTTGGTGGTGGG             |
| pAtML1-Xmal-F:  |               | CCGGGGCTTATCAAAAGAAGGAACACAGAC             |
| pAtML1-Xmal-R:  |               | CCGGGAACCGGTGATGGACCTGAGGAGTTTC            |
| YUC1CDS-F:      |               | GGATCCATGGAGGTCTCTCACAACAAAACCTGACC        |
| YUC1CDS-R:      |               | GGATCCCTAGGATAGGTAAGAGCAAAAAACG            |
| pAtLMII:        |               | GTCGACCGCGTGACTGAGGAGATGAGT                |
| pAtLMII:        |               | CCCGGGTCCTCTTCTTCTTCTGAGTTTC              |

Table S3. Oligonucleotides used in this study are listed, related to Star Methods.
Supplemental References

S1. Nakata, M., Matsumoto, N., Tsugeki, R., Rikirsch, E., Laux, T., and Okada, K. (2012). Roles of the Middle Domain–Specific WUSCHEL-RELATED HOMEOBOX Genes in Early Development of Leaves in Arabidopsis. The Plant Cell 24, 519.