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The genetic basis for differences in leaf form between *Arabidopsis thaliana* and its wild relative *Cardamine hirsuta*

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A key question in biology is how differences in gene function or regulation produce new morphologies during evolution. Here we investigate the genetic basis for differences in leaf form between two closely related plant species, *Arabidopsis thaliana* and *Cardamine hirsuta*. We report that in *C. hirsuta*, class I KNOTTED1-like homeobox (KNOX) proteins are required in the leaf to delay cellular differentiation and produce a dissected leaf form, in contrast to *A. thaliana*, in which KNOX exclusion from leaves results in a simple leaf form. These differences in KNOX expression arise through changes in the activity of upstream gene regulatory sequences. The function of ASYMMETRIC LEAVES1/ROUGHSHEATH2/PHANTASTICA (ARP) proteins to repress *KNOX* expression is conserved between the two species, but in *C. hirsuta* the ARP-KNOX regulatory module controls new developmental processes in the leaf. Thus, evolutionary tinkering with KNOX regulation, constrained by ARP function, may have produced diverse leaf forms by modulating growth and differentiation patterns in developing leaf primordia.

Morphological innovations are often associated with altered expression of key developmental regulators[1]. However, it is unclear how such divergent expression arises, how it modifies growth to produce differences in form, or how the potentially pleiotropic effects of altered regulatory gene activity are constrained during evolution. Leaves of seed plants provide an attractive system to study the evolution of developmental mechanisms because they present considerable morphological variation. Leaf form can be described as simple (if the leaf blade is undivided) or dissected (if the blade is divided into distinct leaflets). Both simple and dissected leaves are initiated at the flanks of a pluripotent structure termed the shoot apical meristem (SAM). In simple-leafed species, such as *A. thaliana* and maize, ARP myb proteins act in the leaf to confine KNOX transcription factors to the meristem[2–6]. Conversely, many dissected-leafed species accumulate KNOX proteins in the leaf and ARP proteins in the meristem[7,8]. However, it is not known whether KNOX activity is required to produce a dissected leaf, or whether differences in ARP function or regulation are responsible for the divergent patterns of KNOX expression seen in different species.

To answer these questions, we analyzed dissected leaf development in *C. hirsuta*, a small crucifer related to the simple-leafed model species *A. thaliana* (**Fig. 1a–d**). Unlike many *A. thaliana* relatives, *C. hirsuta* has the distinct advantages of being a diploid, self-compatible plant that can be used for genetic analyses and transformed, thus allowing parallel genetic studies of leaf development to be conducted in species that diverged relatively recently[9]. To understand whether KNOX expression in the leaf is associated with dissected leaf form, we examined KNOX protein accumulation patterns in *C. hirsuta* and *A. thaliana* shoot apices. Class I KNOX proteins were expressed in the SAM but were excluded from the cells that comprise an initiating leaf primordium in both *A. thaliana* and *C. hirsuta* (**Fig. 1e**,**f**). However, in contrast to what we observed in *A. thaliana*, we observed nuclear expression of KNOX proteins in later leaf primordia of *C. hirsuta* (**Fig. 1e**,**f**), associated with leaflet initiation (**Fig. 1g**,**h**).

To investigate whether KNOX activity is required for leaflet initiation, we reduced expression of the *KNOX* gene *SHOOTMERISTEMLESS* (*STM*) in *C. hirsuta* by RNA interference (RNAi) (**Fig. 2**). *C. hirsuta STM* is expressed in the SAM of the embryo and mature plant and is repressed in the majority of cells that comprise initiating leaf primordia (**Fig. 2a**,**b**). However, in contrast to *STM* expression in *A. thaliana* (**Fig. 2c**), we also observed *C. hirsuta STM* expression throughout the outer cell layers at the base of initiating leaf primordia (**Fig. 2b**). In comparison to wild-type plants (**Fig. 2d**), strong RNAi lines produced shootless plants with fused cotyledons that often initiated leaves from ectopic positions (**Fig. 2e**). Thus, as in *A. thaliana*[10], *C. hirsuta STM* is required for SAM initiation and cotyledon separation in the embryo. Furthermore, in weak RNAi lines that developed a functional SAM, leaflet initiation was severely reduced (**Fig. 2f–h**, 0.8 ± 0.2 leaflets per leaf in *C. hirsuta STM* RNAi lines compared with 4.4 ± 0.2 in wild-type plants), demonstrating that *C. hirsuta STM* is required to initiate leaflets.

To investigate whether the control of leaflet initiation by *C. hirsuta* STM involves regulation of cell division, we assayed the effects of reducing *C. hirsuta* STM activity on expression of *HISTONE 4* (*H4*),

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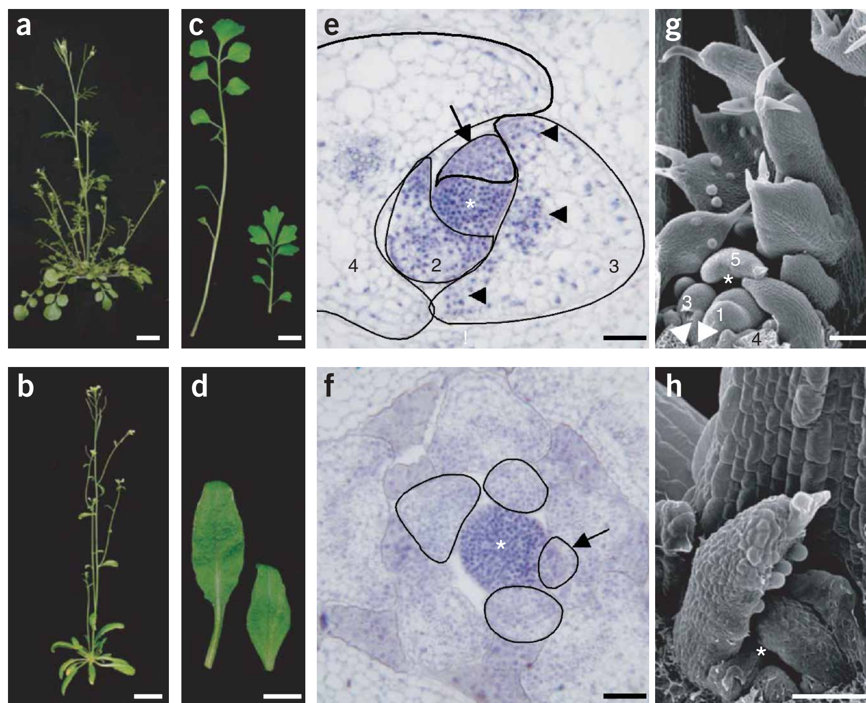

Figure 1 KNOX proteins accumulate in the dissected leaf of *C. hirsuta* but are excluded from the simple leaf of *A. thaliana*. (**a**,**b**) Mature plants of *C. hirsuta* (**a**), and *A. thaliana* (**b**). (**c**,**d**) Rosette (left) and cauline (right) leaves of *C. hirsuta* (**c**) and *A. thaliana* (**d**). *C. hirsuta* leaves are dissected into leaflets, each of which is borne on a petiolule attached to the rachis (**c**), whereas *A. thaliana* leaves are simple (**d**). (**e**,**f**) Immunolocalization of class I KNOX proteins in transverse sections of shoot apices shows nuclear expression of KNOX proteins in the SAM (*) but no expression in initiating leaf cells (arrow) of *C. hirsuta* (**e**) and *A. thaliana* (**f**). In *C. hirsuta* (**e**), nuclear expression of KNOX proteins is seen throughout plastochron (P) 2 (2), localized to the initiating leaflets and vasculature in P3 (arrowheads, 3) and limited to vascular-associated cells in P4 (4). No KNOX expression is seen in leaves of *A. thaliana* (**f**). (**g**,**h**) Scanning electron micrographs of the shoot apex of *C. hirsuta* (**g**) shows that the youngest leaf primordium (1) initiates on the flanks of the SAM (*) with a simple shape, and leaflets initiate at the leaf margins 1–2 plastochrons later (arrowheads, 3,4; the distal leaflet of P4 has been removed) in a basipetal manner. By contrast, leaf primordia in *A. thaliana* (**h**) initiate at the SAM (*) and continue to develop with a simple shape. Scale bars: 2 cm (**a**,**b**), 0.5 cm (**c**,**d**), 50 μm (**e**,**f**), 100 μm (**g**,**h**).

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which has previously been used to monitor cell cycle activity in *A. thaliana* lateral organs[11]. We observed that in *C. hirsuta STM* RNAi plants, fewer cells in developing leaf primordia express *C. hirsuta H4* (**Fig. 2j,k**, 23.5 ± 2.1 cells in *C. hirsuta STM* RNAi lines compared with 76.5 ± 3.2 cells in wild-type plants), and the epidermal cells are much larger in these leaves than in wild-type plants (**Fig. 2l,m**). This reduction in *C. hirsuta H4* expression and increased cell expansion suggests that *C. hirsuta* STM prevents the precocious exit of tissues from the cell cycle into differentiation pathways, thus promoting leaflet initiation. To investigate whether KNOX expression

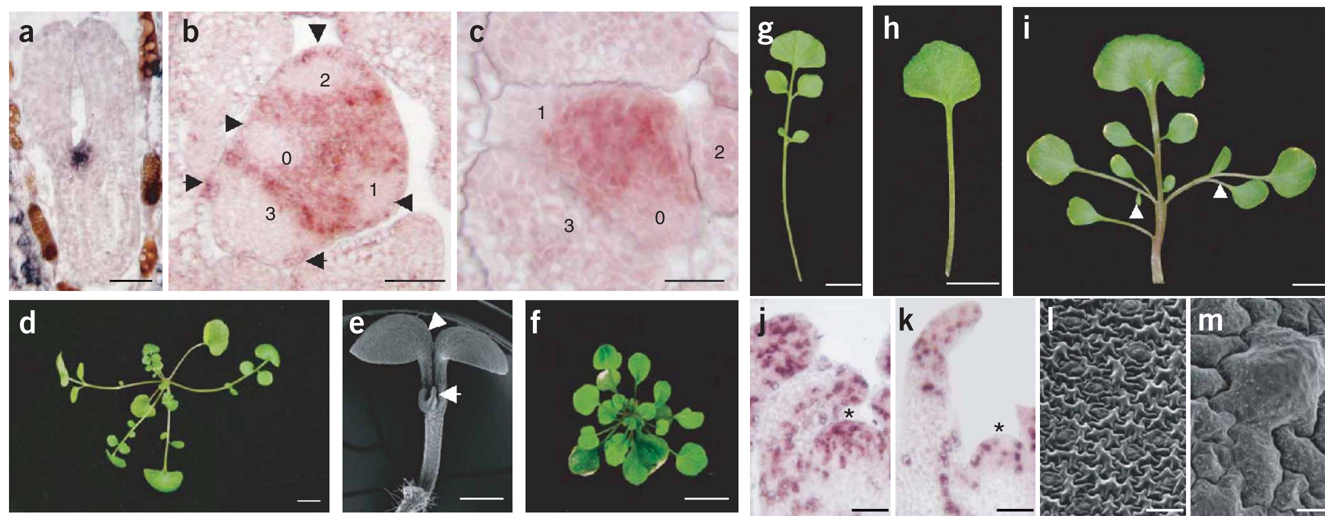

Figure 2 *C. hirsuta STM* expression in young leaf primordia is required for leaflet initiation. (**a**,**b**) *In situ* localization of *C. hirsuta STM* mRNA. *C. hirsuta STM* expression is restricted to the SAM of embryos (longitudinal section, (**a**) and vegetative shoot apices (transverse section, (**b**) but absent from most cells that comprise leaf primordia. However, expression is observed throughout the outer cell layers of P0 to P2 leaf primordia (arrowheads, 0, 1, 2) and in some cells of P3 leaf primordia (arrows, 3). (**c**) *In situ* localization of *STM* mRNA. Transverse section through an *A. thaliana* shoot apex shows expression is restricted to the SAM and absent from leaf primordia (0–3). (**d**–**f**) Vegetative *C. hirsuta* plants. (**d**) Wild-type. (**e**) Scanning electron micrograph (SEM) of a strong *C. hirsuta STM* RNAi line with fused cotyledons (arrowhead) and ectopic leaf initiation (arrow). (**f**) A weak *C. hirsuta STM* RNAi line with simple leaves. (**g**–**i**) *C. hirsuta* rosette leaves. (**g**) Wild-type with four lateral leaflets. (**h**) A weak *C. hirsuta STM* RNAi line lacking lateral leaflets. (**i**) *35S::KN1-GR* induced with 10^{-6} M dexamethasone with ectopic leaflets initiated upon leaflets (arrowheads). (**j**,**k**) *In situ* localization of *C. hirsuta H4* mRNA. Longitudinal sections through vegetative apices of wild-type (**j**) and *C. hirsuta STM* RNAi (**k**). * indicates meristem. (**l**,**m**) SEM of epidermal cells of the terminal leaflet of leaf three in wild-type (**l**) and *C. hirsuta STM* RNAi (**m**). Scale bars: 20 μm (**a**–**c**,**l**,**m**), 1 cm (**d**, **f**–**i**), 500 μm (**e**), 50 μm (**j**,**k**).

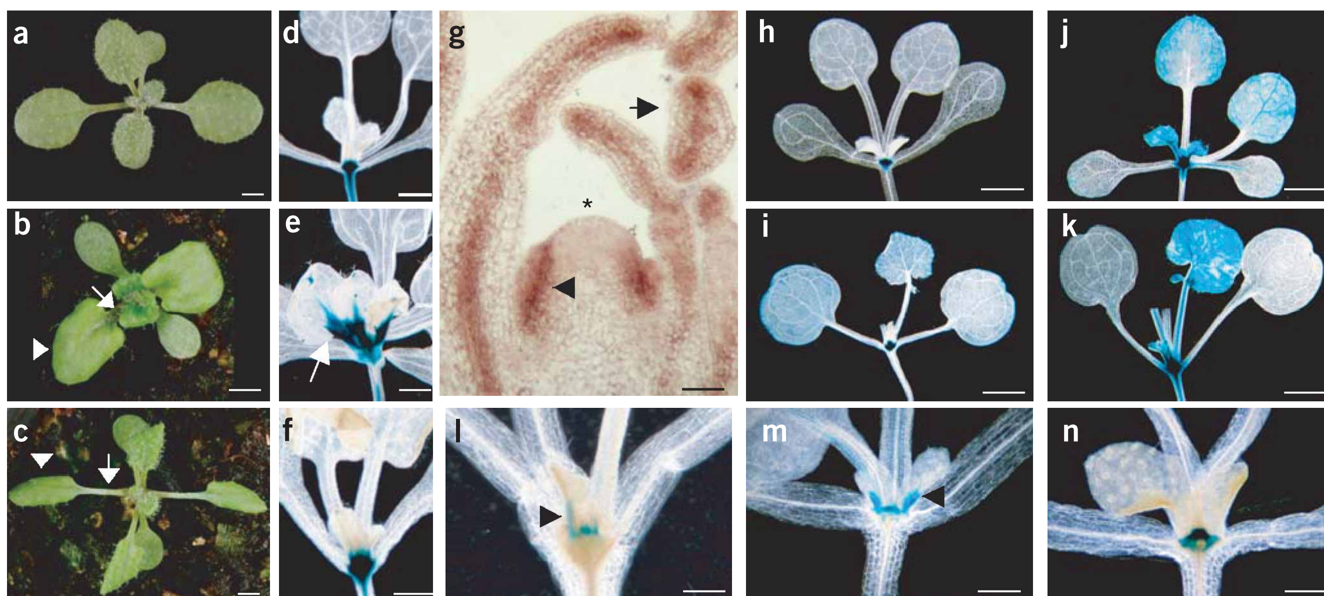

Figure 3 AS1 function is conserved between *A. thaliana* and *C. hirsuta*, whereas 5′ upstream regions of *KNOX* genes are sufficient to drive species-specific expression. (**a**–**c**) Rosettes of *A. thaliana* wild-type (Columbia) (**a**), *as1-1* showing an asymmetrical leaf lamina (arrowhead) and short, broad petioles (arrow) (**b**) and *as1-1;35S::ChAS1* transformants showing restoration of leaf shape (arrowhead) and petiole length (arrow) (**c**). (**d**–**f**) Seedlings of the same genotypes stained for *BP::GUS* expression. *BP::GUS* expression is observed only in the SAM of wild-type (**d**); ectopic expression is observed in *as1* leaves (arrow, **e**), and this ectopic expression is repressed in *as1-1;35S::cAS1* (**f**). (**g**) *In situ* localization of *C. hirsuta AS1* mRNA in the shoot apex of *C. hirsuta*, showing expression in initiating leaf primordia (arrowhead) on the flanks of the SAM (*) and in leaflets (arrow). (**h**–**n**) Seedlings stained for GUS expression. *BP::GUS* expression is restricted to the SAM in *A. thaliana* (**h**) and *C. hirsuta* (**i**). *ChBP::GUS* is expressed in both the SAM and leaves in *A. thaliana* (**j**) and *C. hirsuta* (**k**). Two leaves have been removed for clarity in **i** and **k**. *ChSTM::GUS* is expressed in both the SAM and the abaxial side of young leaves (arrowheads) in *C. hirsuta* (**l**) and *A. thaliana* (**m**), whereas *STM::GUS* expression is restricted to the SAM in *A. thaliana* (**n**). Scale bars: 0.5 cm (**a**–**c**, **h**–**k**), 1 mm (**d**–**f**), 50 μm (**g**), 0.5 mm (**l**–**n**).

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is sufficient for leaflet initiation, we expressed the maize KNOTTED1 (KN1) protein, which is able to rescue *A. thaliana stm* mutants[12], in *C. hirsuta* in a dosage-sensitive manner using a fusion with the rat glucocorticoid receptor (KN1-GR)[13]. A single induction of KN1 activity with 10^{-6} M dexamethasone resulted in reiteration of a second order of leaflets along the elongated petiolules of first-order leaflets (**Fig. 2i**). These results demonstrate that KNOX activity is not only necessary but also sufficient for leaflet initiation in *C. hirsuta*. Elevated KNOX expression in the dissected-leaf tomato plant can also increase leaflet number[14,15], suggesting that the requirement for KNOX activity in *C. hirsuta* leaf development may extend to other species where dissected leaf morphology has evolved independently[7].

We have shown that differences in KNOX expression contribute to the different leaf forms observed in *A. thaliana* and *C. hirsuta*, indicating that distinct mechanisms of *KNOX* gene regulation evolved in these two closely related species. In *A. thaliana*, AS1 represses *KNOX* gene expression in the leaf; therefore, loss of this regulation could be responsible for KNOX expression in *C. hirsuta* leaves. This scenario would be consistent with the coexpression of KNOX and ARP proteins observed in the shoot meristems of many dissected-leaf plants[8]. To investigate this possibility, we determined the extent of functional equivalence between *C. hirsuta* AS1 and *A. thaliana* AS1. Expression of *C. hirsuta AS1* under the control of the broadly expressed *CaMV 35S* promoter complemented the *A. thaliana as1* mutant phenotype (**Fig. 3a–c**) and repressed expression of the *KNOX* gene *BREVIPEDICELLUS* (*BP*) in *as1* leaves (**Fig. 3d–f**), indicating that the function of the two proteins is conserved. Moreover, *C. hirsuta AS1* mRNA was expressed in leaves and excluded from the SAM (**Fig. 3g**) in an equivalent pattern as *AS1* in *A. thaliana*[2]. Thus, it is unlikely that changes in either the function or expression of AS1 account for the differences in *KNOX* expression and leaf shape between *A. thaliana* and *C. hirsuta*.

We next investigated whether the differences in *KNOX* gene expression observed between the two species are attributable to differential activity of *KNOX* gene regulatory sequences. To test this idea, we analyzed 5′ upstream regions of the *KNOX* genes *STM* and *BP* (**Supplementary Fig. 1** online) and performed promoter swap experiments with these regions between *C. hirsuta* and *A. thaliana*. We reasoned that if the regulatory information necessary for species-specific expression is contained within the promoter regions, then each promoter should drive reporter gene expression regardless of the species into which it is transformed. If, however, species-specific activity of *trans* regulatory factors is required for correct *KNOX* gene expression, then each reporter should reflect the expression pattern of the species into which it is transformed. We found that each reporter reflected the endogenous gene expression pattern of its promoter in both the native and heterologous context (**Fig. 3**). That is, the *A. thaliana BP* promoter generated GUS expression in the SAM of both *A. thaliana* and *C. hirsuta* (**Fig. 3h,i**), and the *C. hirsuta BP* promoter generated GUS expression in both the SAM and leaves of both species (**Fig. 3j,k**). Swapping the *C. hirsuta STM* promoter region between *C. hirsuta* and *A. thaliana* gave similar results: the *C. hirsuta* promoter generated GUS expression in the SAM and the abaxial side of developing leaves in both species (**Fig. 3l,m**), whereas the *A. thaliana* promoter generated expression in the SAM only (**Fig. 3n**). These results indicate that differences in *KNOX* gene expression between *A. thaliana* and *C. hirsuta* are at least in part determined by differential activity of promoter sequences. KNOX activity in

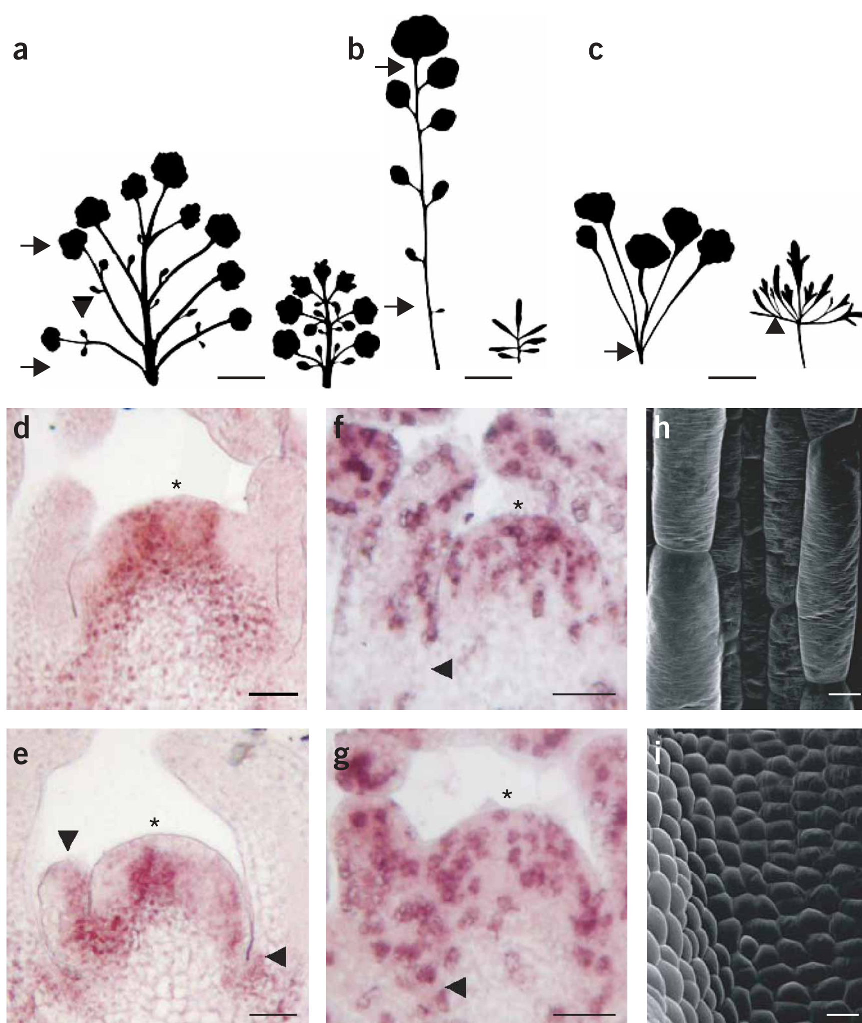

Figure 4 *C. hirsuta* AS1 delimits *C. hirsuta BP* expression and controls leaflet positioning by regulating growth. (**a**–**c**) Silhouettes of rosette (left) and cauline (right) leaves of *35S::KN1-GR* induced once with 10^{-6} M dexamethasone (**a**), wild type (**b**), and *chas1-1* (**c**). Arrows denote the length of the leaf rachis and arrowheads indicate extra leaflets. (**d**,**e**) *In situ* localization of *C. hirsuta BP* mRNA. Longitudinal sections of wild-type (**d**) and *chas1-1* (**e**) showing ectopic expression in the adaxial side and the base of developing leaves (arrowheads). (**f**,**g**) *In situ* localization of *C. hirsuta H4* mRNA. Longitudinal sections of wild-type (**f**) and *chas1-1* (**g**) showing *C. hirsuta H4*–expressing cells at the base of developing leaves in *chas1-1* but not wild-type (arrowheads). * indicates shoot meristem. (**h**,**i**) Scanning electron micrographs of epidermal cells on the adaxial surface of the leaf rachis at the position of leaflet insertion in wild-type (**h**) and *chas1-1* (**i**). Scale bars: 1 cm (**a**–**c**), 20 μm (**d**–**i**).

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developing leaf primordia is sufficient to elicit leaflet formation in the simple leaf of *A. thaliana* (**Supplementary Fig. 2** online), suggesting that *cis* regulatory changes may be sufficient to determine the differences in leaf form between *A. thaliana* and *C. hirsuta*. However, future work will determine if this is the case or if additional factors facilitate KNOX-dependent leaflet formation in *C. hirsuta*.

To understand how the pleiotropic effects (such as compressed proximodistal axis and supernumerary leaflets; **Fig. 4a**) of widespread *KNOX* expression in *C. hirsuta* leaves are constrained, we conducted a genetic screen to isolate recessive mutants that phenocopy the effects of KNOX overexpression. One such mutant initiated leaflets close together along an extremely compressed proximodistal axis and had additional orders of leaflets (**Fig. 4b**,**c**). This mutant phenotype was very similar to that observed in transgenic *C. hirsuta* lines when we reduced *C. hirsuta AS1* activity using RNAi (data not shown), suggesting that these phenotypic effects were a consequence of loss of *C. hirsuta* AS1 function. Molecular analysis confirmed that the mutant contained a premature stop codon at amino acid residue 170 in the *C. hirsuta* AS1 protein sequence that cosegregated with the mutant phenotype, and the allele was hence designated *chas1-1* (**Supplementary Fig. 3** online). Thus, *C. hirsuta* AS1 activity is required for development of the proximodistal axis of the leaf and for determining number and positioning of leaflets along this axis.

To investigate whether *C. hirsuta* AS1 controls dissected leaf form by defining the domain and level of *KNOX* expression, we analyzed KNOX protein and mRNA accumulation in *C. hirsuta AS1* RNAi lines and *chas1-1* mutants. We observed increased KNOX protein accumulation in *C. hirsuta AS1* RNAi leaves (**Supplementary Fig. 4** online) and observed ectopic expression of *C. hirsuta BP* but not *C. hirsuta STM* in *chas1-1* leaves (data not shown and **Fig. 4**). Notably, the pattern of ectopic *C. hirsuta BP* expression correlated well with the phenotypic perturbations observed in *chas1-1*. For example, in contrast to the wild-type expression of *C. hirsuta BP* (**Fig. 4d**), in *chas1-1*, we observed intense *C. hirsuta BP* expression in the adaxial domain and base of developing leaves (**Fig. 4e**), correlating with repression of growth and differentiation along the proximodistal axis (**Fig. 4b**,**c**) and adaxial rachis (**Fig. 4h**,**i**) of the leaf. The activity of *C. hirsuta* AS1 in controlling differentiation of the adaxial side of the leaf is shared by ARP proteins in other plant species[8,16,17]. Additionally, these results indicate that, at least in *C. hirsuta*, the roles of ARP proteins in axial patterning and *KNOX* repression are intimately intertwined.

To investigate whether this reduction in growth along the *chas1-1* leaf rachis reflects a reduction in cell division or cell expansion, we analyzed *C. hirsuta H4* gene expression and cell size in developing leaves. Compared with wild-type (**Fig. 4f**), a greater proportion of cells in *chas1-1* leaf primordia express *C. hirsuta H4*, particularly at the leaf base (**Fig. 4g**). In addition, epidermal cells along the adaxial surface of the *chas1-1* leaf rachis fail to elongate, or differentiate a striated cell wall, as occurs in wild-type (**Fig. 4h**,**i**). Similar defects were observed in the leaves of *C. hirsuta* plants expressing *BP* under the control of the *35S* promoter (**Supplementary Fig. 5** online). These observations suggest that *C. hirsuta* AS1 regulates *C. hirsuta BP* expression within the *C. hirsuta* leaf, thereby defining the correct timing for leaf cells to exit the cell cycle and enter differentiation pathways. Thus, changes in *KNOX* promoter activity underpin differences in leaf shape between *A. thaliana* and *C. hirsuta* by allowing

KNOX proteins to become part of the regulatory toolkit that controls leaf growth and differentiation. However, potentially pleiotropic effects arising from KNOX activity in leaves are constrained by the repressive action of *C. hirsuta* AS1.

We found it striking that although the molecular function of *C. hirsuta* AS1 and *A. thaliana* AS1 to repress *KNOX* gene expression is conserved, the developmental significance of this repression is different for the two species. In *A. thaliana*, AS1 acts to safeguard leaf fate by maintaining the repression of *KNOX* expression in leaves. By contrast, in *C. hirsuta*, *KNOX* gene regulatory sequences drive expression in the leaf where KNOX activity is required for dissected leaf development. Within this different developmental context of the dissected leaf, *C. hirsuta* AS1 constrains the spatiotemporal domain of *KNOX* expression and hence leaflet number and arrangement. Our work identifies two processes that underpin the evolution of new morphologies in multicellular eukaryotes. First, changes in the expression domain of key developmental regulators offer the potential to alter morphology by changing tissue growth. Second, conserved molecular interactions of these regulators, within their new expression domains, can acquire new developmental significance and mold morphology to its final state. Differences between *C. hirsuta* and *A. thaliana* extend to many other aspects of their growth and development, including shoot branching and floral organ morphogenesis. Therefore, future research in these species will test how robustly these principles apply to the evolution of relevant developmental pathways.

METHODS

Plant growth conditions. Plants were grown in a greenhouse with supplemental lighting (days: 18 h, 20 °C; nights: 6 h, 16 °C).

Genetic stocks. Wild-type *C. hirsuta* seed was collected from wild populations in Oxford, UK; verified by internal transcribed spacer sequencing; and self-pollinated for seven generations before use (specimen voucher Hay 1 (OXF)). Wild-type *C. hirsuta* seed was X-ray–irradiated at 16 kR, sown and harvested in pools of five plants. Approximately 100 seed of 150 M2 pools, giving a total of 1,500 plants, were screened. Mutant characterization was performed after backcrossing to wild-type *C. hirsuta* twice.

Transgenic construction. All primers are listed in **Supplementary Table 1** online. To construct the *C. hirsuta STM* RNAi vector, a 310-bp fragment was amplified from *C. hirsuta* shoot cDNA by PCR with the primers ChSTMrnai-F and ChSTMrnai-R. This fragment was cloned in both sense and antisense orientations in the PHANNIBAL vector[18] using the restriction enzyme pairs *Xba*I/*Cla*I and *Eco*RI/*Kpn*I. This RNAi cassette was transferred as a *Not*I fragment into the binary vector pMLBART[19], transformed into the *Agrobacterium tumifaciens* strain GV3101 and used to transform wild-type *C. hirsuta* plants by a modified floral dipping protocol. We analyzed 14 independent T1 lines. We constructed a *C. hirsuta AS1* (hereafter, *ChAS1*) RNAi vector in an identical manner using a 343-bp PCR fragment amplified from *C. hirsuta* shoot cDNA with the primers ChAS1rnai-F and ChAS1rnai-R, and we used it to transform wild-type *C. hirsuta* plants as above. We analyzed 16 independent T2 lines. The pMLBART vector alone was used to transform wild-type *C. hirsuta* plants as above, and all T1 lines were phenotypically wild-type. We used three independent T2 lines as wild-type comparisons for analyses of RNAi lines. Transcript levels were analyzed by RT-PCR (**Supplementary Fig. 6** online). A previously described *35S::KN1-GR* translational fusion[13] was used to transform wild-type *C. hirsuta* plants as described above. We analyzed 16 independent T2 lines. To construct the *35S::ChAS1* vector, we amplified a 1,117-bp fragment of the *C. hirsuta AS1* coding region by PCR using a proofreading *Taq* polymerase (Pyrobest, Takara) from a full-length cDNA clone using the primers ChAS1-F and ChAS1-R. The PCR product was cloned into the pCR Blunt vector (Invitrogen), sequenced to confirm fidelity and cloned as an *Eco*RI fragment behind the *CaMV* 35S promoter of the pART7 vector[19]. The *35S::ChAS1::ocs* cassette was transferred as a *Not*I fragment into the binary vector pMLBART and transformed into *as1-1* mutant plants by floral dipping. We analyzed 100 independent T1 lines for recovery of the *as1* mutant phenotype as described previously for *35S::AS1* (ref. 20). Five T2 lines with a single transgene copy were crossed to *as1-1;BP::GUS* plants[3], and GUS expression was analyzed in the F1. To make transcriptional fusions of *C. hirsuta BP* and *C. hirsuta STM* to the *uidA* (GUS) gene, a BAC library of *C. hirsuta* genomic clones was screened (a full description of library construction will be given elsewhere), and ∼6-kb *Eco*RI and *Xba*I restriction fragments of *C. hirsuta BP* and *C. hirsuta STM* DNA, respectively, were cloned into pBluescript (Stratagene). We amplified 4 kb of upstream sequence, including the 5′ UTR, by PCR using a proofreading *Taq* polymerase (Pyrobest, Takara) with the primers M13 reverse and ChBP-R with a *Pst*I restriction site introduced at the ATG, and cSTMpst-F and cSTMbam-R with a *Bam*HI restriction site introduced at the ATG. These sequences were transferred, as a *Pst*I fragment for *C. hirsuta BP* (hereafter '*ChBP*') and as a *Pst*I/*Bam*HI fragment for *C. hirsuta STM* (hereafter '*ChSTM*'), upstream of GUS in the pRITA vector[19]. Orientation and integrity of the sequence junctions were confirmed by sequencing. Transcriptional fusions were generated in a similar manner using ∼5 kb of upstream sequence, including the 5′ UTR of *BP* and *STM*. All four promoter-GUS cassettes were transferred as *Not*I fragments into the binary vector pMLBART, transformed into *A. tumefaciens*, as above, and used to transform both wild-type *A. thaliana* (Columbia ecotype) and *C. hirsuta*. GUS expression was analyzed in 97 independent T1 lines for *ChBP::GUS* and 96 lines for *BP::GUS* in *A. thaliana*, ten lines for *ChBP::GUS* and 12 lines for *BP::GUS* in *C. hirsuta*, 67 lines for *ChSTM::GUS* and eight lines for *STM::GUS* in *A. thaliana*, and eight lines for *ChSTM::GUS* in *C. hirsuta*. A previously described *35S::BP* construct[21] was transformed into *A. tumefaciens* and used to transform wild-type *C. hirsuta* plants as described above. We analyzed 18 independent T2 lines. *PHV*>>*BP*, *FIL*>>*BP* and *ANT*>>*BP* lines were generated by constructing *pVTOp::BP* (ref. 22) and transforming *PHV::LhG4*, *FIL::LhG4* (gift from Y. Eshed, Weizmann Institute of Science, Israel) and *ANT::LhG4* (ref. 23) plants with this construct. We analyzed 30 independent T2 lines for each construct.

5′ and 3′ RACE. *C. hirsuta AS1* full-length cDNA sequence was determined in wild-type lines and *chas1-1* mutants by 5′ and 3′ RACE. cDNA was generated using a SmartRace kit (BD Biosciences) according to manufacturer's protocols. We used 1 μg of total shoot RNA per reaction. PCR amplification was performed for 5′ RACE with the primer ChAS1-R1 and for 3′ RACE with the primer ChAS1-F1. *C. hirsuta AS1*-specific products were cloned, and two clones from each RACE reaction were sequenced for each genotype. In *chas1-1* mutants, a premature stop codon at amino acid 170 of ChAS1 introduces an *Acc*I site that is not present in wild-type plants. This sequence polymorphism was used to generate a cleaved amplified polymorphic sequence marker by amplifying a 600-bp product with primers ChAS1-F2 and ChAS1-R2, which yielded products of 425 bp and 175 bp after *Acc*I digestion of *chas1-1* but not after digestion of wild-type amplicons.

Leaflet and cell measurements. Average number of leaflets per leaf was determined for ten *C. hirsuta STM* RNAi plants and ten wild-type plants. Average number of *C. hirsuta H4*–expressing cells in adjacent longitudinal sections of the two youngest leaf primordia at the shoot apex was determined for *C. hirsuta STM* RNAi and wild-type plants (as described in ref. 11). Error shown in all cases is standard error.

Immunocytochemistry. Fixation and hybridization were carried out as previously described[24] on 8-μm paraffin sections using a previously described polyclonal antibody to KNOX[25] that detects class I KNOX proteins (encoded by a four-member gene family in *A. thaliana* and *C. hirsuta* (data not shown)).

Scanning electron microscopy. Fixation and dehydration were carried out as previously described[26]. Scanning electron microscopy was performed using a JSM-5510 microscope (Jeol).

***In situ* RNA localization.** Fixation and hybridization were carried out as previously described[10] on 8-μm paraffin sections using probes for *A. thaliana STM*[10], *C. hirsuta STM*, *C. hirsuta H4*, *C. hirsuta AS1* and *C. hirsuta BP*. To generate a probe to *C. hirsuta AS1*, a 373-bp fragment was amplified from *C. hirsuta* shoot cDNA using the primers AS1-F and AS1-R. To generate a probe to *C. hirsuta H4*, a 297-bp fragment was amplified from *C. hirsuta* shoot

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cDNA using the primers H4-F and H4-R. To generate a probe to *C. hirsuta STM*, a 986-bp fragment was amplified from a cDNA clone using the primers ChSTM-F and ChSTM-R. Three probes were generated to *C. hirsuta* BP by amplifying 306-bp, 387-bp and 351-bp fragments from a cDNA clone using the primer pairs ChBP457-F and ChBP762-R, ChBP700-F and Ch1086-R and ChBP5′-F and ChBP5′-R, respectively. All fragments were cloned into the pGEM T-Easy vector (Promega) and sequenced to determine orientation. Antisense and sense probes were transcribed and DIG labeled as previously described[10].

Leaf silhouettes. Leaves were flattened onto clear adhesive, adhered to white paper and digitally scanned.

Chemical treatments. Dexamethasone (Sigma) was dissolved in water and applied at a concentration of 10^{-6} M with 0.02% silwet using a paintbrush.

Accession codes. GenBank: *C. hirsuta STM* mRNA, complete coding sequence (cds), DQ512732; *C. hirsuta AS1* mRNA, complete cds, DQ512733; *C. hirsuta BP* mRNA, complete cds, DQ630764; *C. hirsuta BP* gene, 5′ upstream region, DQ526379; *C. hirsuta STM* gene, 5′ upstream region, DQ526380.

Note: Supplementary information is available on the Nature Genetics website.

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The authors declare competing financial interests (see the *Nature Genetics* website for details).

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1. Carroll, S.B. Evolution at two levels: on genes and form. *PLoS Biol.* **3**, e245 (2005).
2. Byrne, M.E. *et al.* Asymmetric leaves1 mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature* **408**, 967–971 (2000).
3. Ori, N., Eshed, Y., Chuck, G., Bowman, J.L. & Hake, S. Mechanisms that control knox gene expression in the *Arabidopsis* shoot. *Development* **127**, 5523–5532 (2000).
4. Schneeberger, R., Tsiantis, M., Freeling, M. & Langdale, J.A. The *rough sheath2* gene negatively regulates homeobox gene expression during maize leaf development. *Development* **125**, 2857–2865 (1998).
5. Tsiantis, M., Schneeberger, R., Golz, J.F., Freeling, M. & Langdale, J.A. The maize rough sheath2 gene and leaf development programs in monocot and dicot plants. *Science* **284**, 154–156 (1999).
6. Timmermans, M.C., Hudson, A., Becraft, P.W. & Nelson, T. ROUGH SHEATH2: a Myb protein that represses knox homeobox genes in maize lateral organ primordia. *Science* **284**, 151–153 (1999).
7. Bharathan, G. *et al.* Homologies in leaf form inferred from KNOXI gene expression during development. *Science* **296**, 1858–1860 (2002).
8. Kim, M., McCormick, S., Timmermans, M. & Sinha, N. The expression domain of PHANTASTICA determines leaflet placement in compound leaves. *Nature* **424**, 438–443 (2003).
9. Koch, M., Haubold, B. & Mitchell-Olds, T. Molecular systematics of the Brassicaceae: evidence from coding plastidic matK and nuclear Chs sequences. *Am. J. Bot.* **88**, 534–544 (2001).
10. Long, J.A., Moan, E.I., Medford, J.I. & Barton, M.K. A member of the KNOTTED class of homeodomain proteins encoded by the SHOOTMERISTEMLESS gene of *Arabidopsis*. *Nature* **379**, 66–69 (1996).
11. Dinneny, J.R., Yadegari, R., Fischer, R.L., Yanofsky, M.F. & Weigel, D. The role of JAGGED in shaping lateral organs. *Development* **131**, 1101–1110 (2004).
12. Kim, J.Y., Yuan, Z. & Jackson, D. Developmental regulation and significance of KNOX protein trafficking in *Arabidopsis*. *Development* **130**, 4351–4362 (2003).
13. Hay, A., Jackson, D., Ori, N. & Hake, S. Analysis of the dompetence to respond to KNOTTED1 activity in *Arabidopsis* leaves using a steroid induction system. *Plant Physiol.* **131**, 1671–1680 (2003).
14. Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y. & Lifschitz, E. The making of a compound leaf: Genetic manipulation of leaf architecture in tomato. *Cell* **84**, 735–744 (1996).
15. Chen, J-J., Janssen, B-J., Williams, A. & Sinha, N. A gene fusion at a homeobox locus: Alterations in leaf shape and implications for morphological evolution. *Plant Cell* **9**, 1289–1304 (1997).
16. Waites, R., Selvadurai, H.R., Oliver, I.R. & Hudson, A. The PHANTASTICA gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in Antirrhinum. *Cell* **93**, 779–789 (1998).
17. Xu, L. *et al.* Novel as1 and as2 defects in leaf adaxial-abaxial polarity reveal the requirement for ASYMMETRIC LEAVES1 and 2 and ERECTA functions in specifying leaf adaxial identity. *Development* **130**, 4097–4107 (2003).
18. Wesley, S.V. *et al.* Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J.* **27**, 581–590 (2001).
19. Gleave, A.P. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**, 1203–1207 (1992).
20. Theodoris, G., Inada, N. & Freeling, M. Conservation and molecular dissection of ROUGH SHEATH2 and ASYMMETRIC LEAVES1 function in leaf development. *Proc. Natl. Acad. Sci. USA* **100**, 6837–6842 (2003).
21. Chuck, G., Lincoln, C. & Hake, S. Knat1 induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. *Plant Cell* **8**, 1277–1289 (1996).
22. Craft, J. *et al.* New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in *Arabidopsis*. *Plant J.* **41**, 899–918 (2005).
23. Lenhard, M., Jurgens, G. & Laux, T. The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in *Arabidopsis* shoot meristem regulation. *Development* **129**, 3195–3206 (2002).
24. Smith, L.G., Greene, B., Veit, B. & Hake, S. A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. *Development* **116**, 21–30 (1992).
25. Scanlon, M.J., Schneeberger, R.G. & Freeling, M. The maize mutant narrow sheath fails to establish leaf margin identity in a meristematic domain. *Development* **122**, 1683–1691 (1996).
26. Bowman, J.L., Smyth, D.R. & Meyerowitz, E.M. Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development* **112**, 1–20 (1991).