Homologies in Leaf Form Inferred from KNOXI Gene Expression During Development

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Homologies in Leaf Form Inferred from *KNOXI* Gene Expression During Development

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KNOTTEDI-like homeobox (KNOXI) genes regulate development of the leaf from the shoot apical meristem (SAM) and may regulate leaf form. We examined KNOXI expression in SAMs of various vascular plants and found that KNOXI expression correlated with complex leaf primordia. However, complex primordia may mature into simple leaves. Therefore, not all simple leaves develop similarly, and final leaf morphology may not be an adequate predictor of homology.

In simple-leaved species (maize, rice, *Arabidopsis*, tobacco, snapdragon) *KNOXI* genes are expressed in the SAM and unexpanded axis and are down-regulated after leaf initiation, suggesting fundamental differences between the indeterminate shoot and determinate leaves (*1*–*5*). Overexpression of KNOXI in simple-leaved plants results in distorted leaves and ectopic shoots (*6*–*8*). By contrast, in tomato, a complex-leaved plant, *KNOXI* genes are up-regulated in leaf primordia and down-regulated in the mature leaf (*9*–*11*). Overexpression of *KNOX1* in tomato results in increased leaf complexity (*9*–*11*). To determine how general these patterns of expression are, we studied KNOXI expression in developing simple- and complex-leaved shoots in different species of a genus, *Lepidium*, and within a single species, *Neobeckia aquatica* (both in Brassicaceae, eurosid II).

All species showed down-regulation of KNOXI protein at sites of initiation of leaf primordia (P_0), but differed in whether or not KNOX1 was expressed later in leaf development. The simple-leaved *Lepidium africanum* had high KNOXI protein expression in the SAM, but not later (Fig. 1, A and B), whereas in the complex-leaved *L. perfoliatum* and *L. hyssopifolium*, KNOX1 expression appeared later in leaf primordia (Fig. 1, C to H). *L. oleraceum* produces simple leaves (Fig. 1I) and unexpectedly shows KNOXI expression in its leaf primordia (Fig. 1K) [see supplementary material (*12*)]. Scanning electron microscopy revealed that the simple leaves of *L. oleraceum* had primordia that, in early development (primary morphogenesis), produced marginal outgrowths (Fig. 1J) typical of early complex leaf development (e.g., *L. hyssopifolium*: Fig. 1G) [see supplementary material (*12*)]. This early complex form of *L. oleraceum* was later subsumed by inner blade growth [by a process of secondary morphogenesis (*13*, *14*)], and the early marginal outgrowths were apparent only as coarse teeth in the simple mature leaf (Fig. 1I). That KNOXI expression correlates with early leaf development but not necessarily with final leaf form implies that other regulatory changes influence leaf blade expansion.

The species of *Lepidium* studied here share an ancestor with complex leaves, as inferred from parsimony reconstructions of mature leaf form (Fig. 1L) on a phylogenetic hypothesis for the genus (*15*). Within this lineage simple leaves arose in a group that included *L. africanum* and *L. oleraceum*, and later, there was a reversal back to complex leaves in *L. hyssopifolium* (Fig. 1L). We infer that the complex-leaved common ancestor had complex primordia with early KNOXI expression and that simple leaves evolved by either turning off KNOXI in the primordia (*L. africanum*) or modifying secondary morphogenesis (*L. oleraceum*) (*16*).

This correlation between KNOXI expression and primordium form was also observed within individuals of a single species, *N. aquatica* (Brassicaceae). This aquatic species has two kinds of leaves: simple or complex aerial leaves and submerged complex leaves. Emergent leaf form varies with light intensity: Simple leaves are produced under high light, and complex leaves under low light. KNOXI expression was absent in simple leaf primordia of emergent shoots under high light, but present in complex primordia made under low-light conditions (*17*). Thus, KNOXI expression can be modulated by light conditions, perhaps through hormonal changes that often accompany alterations in light quality and quantity (*18*).

Phylogenetic analyses of leaf evolution (Fig. 2) reveal that the ancestral angiosperm had simple leaves (*19*, *20*), and that complex leaves repeatedly arose from these simple-leaved ancestors (on average 29 "gains") and reverted (on average six "losses") to the ancestral simple form [see supplemental material (*12*)]. This indicates that neither all simple nor all complex leaves are homologous [similar owing to common ancestry (*21*)]. Complex leaves are generally assumed to be nonhomologous (*22*), but simple leaves are generally assumed to be homologous and, therefore, developmentally similar. Our observations in *Lepidium* suggest that the latter assumption may not always be correct.

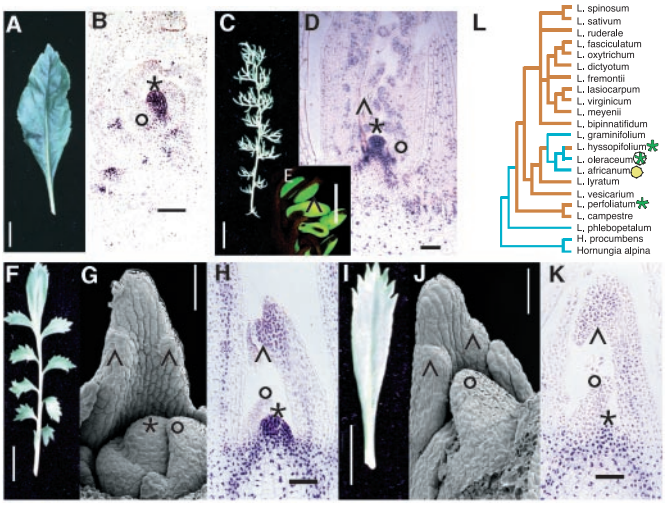

Fig. 1. Consistent correlation between leaf form and KNOX expression in Brassicaceae. (**A** and **B**) *L. africanum*; (**C** to **E**) L. *perfoliatum*; (**F** to **H**) *L. hyssopifolium*; (**I** to **K**) *L. oleraceum*. The final leaf form is shown in (A), (C), (F), and (I). Protein expression is present in (B), (D), (H), and (K) in the SAM (*); absent in P_0 and P1 (O); and present in developing leaflets of complex leaf (/\). The same symbols are used in Figs. 3 and 4. (E, inset) Whole-mount reverse transcription–polymerase chain reaction in situ hybridization shows expression of the *KNOX* gene *STM1* in developing leaflets. Scanning electron micrographs of *L. hyssopifolium* (G) and *L. oleraceum* (J) developing leaves. (**L**) Phylogenetic patterns of leaf evolution in *Lepidium* (Brassicaceae, eurosid I). Colors indicate ancestors reconstructed with simple (blue) or complex (red) leaves. Bars: (A, C, F, I) 1 cm, (G and J) 50 μm, (B, D, H, K) 100 μm. KNOX expression patterns: complex with no secondary simplification (asterisk), complex with secondary simplification (circled asterisk), or simple (filled yellow circle).

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Does the correlation between *KNOXI* expression and primordium form hold for a broad range of taxa? We surveyed simple and complex leaves across vascular plants and found that (i) modification of complex primordia through secondary morphogenesis is common in simple leaves across eudicots; (ii) the presence of KNOXI expression is associated with complex primordia and its absence with simple primordia; and (iii) KNOXI protein expression is down-regulated at the site of leaf initiation (P_0), except in ferns. Taxa were drawn from cycads, ferns, and various angiosperms that represent independent instances of origins of complex leaves or reversals to simple leaves.

KNOXI protein expression reflective of simple leaves was seen in the simple primordia of *Amborella trichopoda* (Fig. 3, A to C), a putative basal extant angiosperm (*23*–*25*), and in the simple primordia of grasses (*26*, *27*) [fig. S1 (*12*)]. Because primordia in basal angiosperms are simple (*28*), this expression pattern is inferred to represent the ancestral state in the angiosperms. This inference is reinforced by our observation of the "simple" pattern in the simple-leaved gymnosperm, *Welwitschia* [fig. S1 (*12*)]. This is the pattern in most simple-leaved species studied to date (*1*–*5*) and in this study (*L. africanum*, *Neobeckia*, *Amborella*) and contrasts with the pattern seen in complex leaves of tomato [fig. S1 (*12*)]. However, as in *L. oleraceum*, development of simple leaves from complex primordia through secondary morphogenesis was also observed in various eudicot lineages [euasterid II: Apiales, *Pimpinella* (Fig. 3, D to F); euasterid I: Gentianales, *Coffea*; rosids: Vitales, *Vitis* sp. (fig. S2) (*12*)]. This molecular developmental dissimilarity may

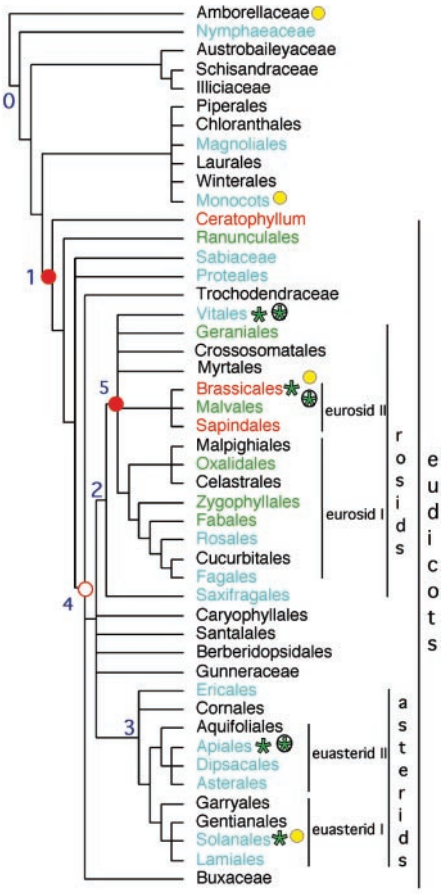

Fig. 2. Evolution of leaf form. Summary of phylogenetic patterns inferred from parsimony reconstructions of ancestral states with data on leaf form in 557 genera of angiosperms (*24*, *39*). The ancestral angiosperm (0), eudicot (1), rosid (2), and asterid (3) had simple leaves. Some groups at the tips (in red) are equally likely to have had ancestors with simple or complex leaves; within all other taxa in color there are multiple origins ("gains") and reversals ("losses") of complex leaves; taxa in black have only simple leaves. If reconstructions were done with terminals coded according to the state in the family (to include polymorphisms), then ancestors of taxa in green were reconstructed as having complex leaves. If polymorphic families were coded as having complex leaves, then the ancestral eudicot was equally likely to have had simple or complex leaves (1, closed red circle); if the ancestral eudicot is assumed to have had complex leaves, then a "loss" (4, open red circle) to simple leaves was followed by a new "gain" of complex leaves in the rosids (5, closed red circle). Under this scheme alone, it is possible that complex leaves of rosids (excluding Saxifragales) are homologous. KNOX expression patterns: as in Fig. 1.

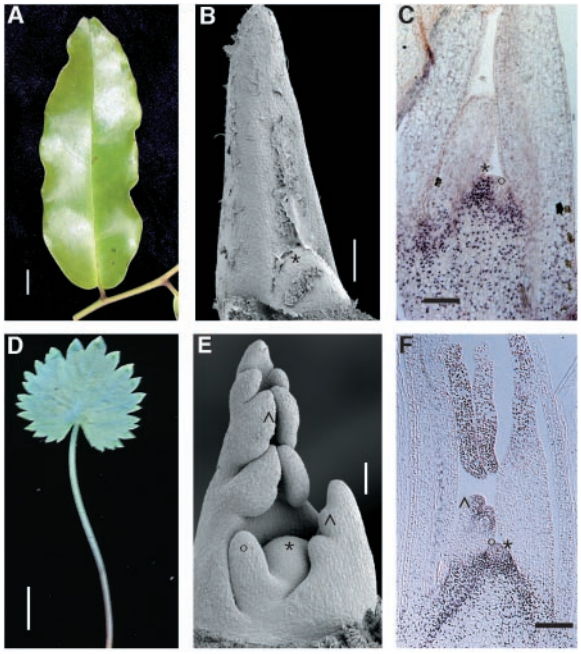

Fig. 3. Comparison of mature leaf form, leaf primordia, and KNOX1 immunolocalization pattern in angiosperms with simple leaves. "Simple" expression: (**A** to **C**) *A. trichopoda*. "Complex" expression: (**D** and **E**) *P. anisum* (Apiaceae). Scanning electron micrographs (B and E) show early development. KNOXI protein expression can be seen in shoot apices in (C) and (**F**). Symbols as in Fig. 1. Bars: (A and D) 1 cm, (B and E) 50 μm, (C and F) 100 μm.

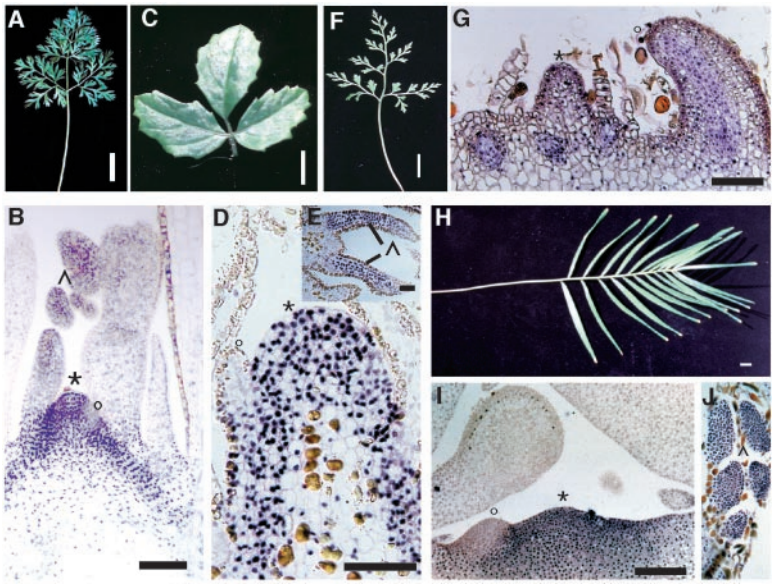

Fig. 4. Vascular plants with complex pattern and complex leaves. (**A** and **B**) *D. carota* (Apiaceae). (**C** to **E**) *C. congestum* (Vitaceae). (**F** and **G**) *Anogramma chaeophylla*. (**H** to **J**) *Zamia floridans*. Symbols as in Fig. 1. Bars: (A, C, F, H) 1 cm, (B, E, G, I), 100 μm, (D) 50 μm.

reflect the nonhomology of simple leaves in eudicots. KNOXI expression of the "complex" pattern was seen in the complex primordia of different eudicot lineages [euasterid II: Apiaceae–*Daucus carota* (Fig. 4, A and B); rosids: Vitaceae–*Cissus* (Fig. 4, C to E)]. Sampling suggests that the molecular hypothesis regarding an association of KNOXI activity with complexity of the leaf primordium is supported across most eudicots [fig. S3 (*12*)]. One exception is a group of legumes, including peas, which have complex leaves but no KNOXI expression in leaf primordia (*29*, *30*). We believe that, in this group, *KNOXI* genes ceased to be part of the genetic cascade leading to the complex leaf form and that a different gene, PEAFLO (*29*, *30*), became part of the cascade (*31*). The unusual KNOXI expression pattern in this group of legumes is striking, given our observation that the correlation of "complex" KNOXI protein expression with primordium complexity was present in ferns and gymnosperms (Fig. 4, F to J), representing stages early in the evolution of vascular plants.

Regardless of final leaf form, KNOXI expression is down-regulated at sites of leaf initiation (P_0) in flowering plants and gymnosperms [Figs. 1, 3, and 4; figs. S1 to S3 (*12*)]. This suggests a mechanism that denotes "determinacy" during initiation of the leaf. Unlike in seed plants, KNOXI is not down-regulated in the P_0 of ferns (Fig. 4G) (*32*). This result is consistent with current understanding that leaves of ferns and seed plants evolved independently (*33*) and may have different developmental characteristics (*34*).

Our results suggest that at least two different modes of development have evolved to generate simple leaves (e.g., *L. africanum* with simple pattern and *Pimpinella anisum* with complex pattern). By contrast, the same, complex, pattern of KNOXI expression characterizes independently evolved complex leaves (e.g., *Cissus congestum* and *Daucus carota*; *N. aquatica*, *L. perfoliatum*, and *L. hyssopifolium*). Complex leaves may thus be partially indeterminate. Several studies on vascular development in leaves suggest that leaf shape and venation patterns parallel each other. However, it is unclear whether one directs the other and, if so, which one. Analysis of venation patterns in developing leaves with secondary morphogenesis may provide some information on this aspect of leaf development. Our results are similar to those for Crustacea, a group of animals, in which Hox expression is correlated with the specialization of limbs into feeding appendages (*35*). As in that case, these results highlight the value of comparative studies in augmenting and/or refuting hypotheses that emerge from experimental studies, and in suggesting new hypotheses that may be tested experimentally.

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Amacrine-Signaled Loss of Intrinsic Axon Growth Ability by Retinal Ganglion Cells

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The central nervous system (CNS) loses the ability to regenerate early during development, but it is not known why. The retina has long served as a simple model system for study of CNS regeneration. Here we show that amacrine cells signal neonatal rat retinal ganglion cells (RGCs) to undergo a profound and apparently irreversible loss of intrinsic axon growth ability. Concurrently, retinal maturation triggers RGCs to greatly increase their dendritic growth ability. These results suggest that adult CNS neurons fail to regenerate not only because of CNS glial inhibition but also because of a loss of intrinsic axon growth ability.

Neurons in the CNS lose the ability to regenerate their axons early in development, but it is not known why. A currently prevailing view is that a strongly inhibitory glial environment causes regenerative failure in the adult CNS (*1*, *2*), as CNS glial cells, both astrocytes and oligodendrocytes, inhibit regenerating axons after injury (*3*–*6*). A crucial question is whether overcoming these inhibitory cues will be sufficient to promote rapid regeneration or whether adult CNS neurons have undergone a developmental loss of intrinsic regenerative ability (*7*–*11*). For example, CNS neurons in slices are less able to