Independent recruitment of a conserved developmental mechanism during leaf evolution

C. Jill Harrison†, Susie B. Corley*, Elizabeth C. Moylan‡, Debbie L. Alexander†, Robert W. Scotland & Jane A. Langdale

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK

* These authors contributed equally to this work
† Present addresses: BioMed Central, Middlesex House, 32–42 Cleveland Street, London W1T 4LB, UK (E.C.M.); Carnegie Institution of Washington, Department of Plant Biology, 260 Panama Street, Stanford, California 94305, USA (J.L.A.)

Vascular plants evolved in the Middle to Late Silurian period, about 420 million years ago.¹ The fossil record indicates that these primitive plants had branched stems with sporangia but no leaves. Leaf-like lateral outgrowths subsequently evolved on at least two independent occasions²–⁴. In extant plants, these events are represented by microphyllous leaves in lycophytes (clubmosses, spikemosses and quillworts) and megaphyllous leaves in eudicots (ferns, gymnosperms and angiosperms). Our current understanding of how development is derepressed to operate during megaphyll formation. Because microphylls and megaphylls evolved independently, different mechanisms are required to account for each. Here we show that this is not so. Gene expression data from a microphyllous lycophyte, phylogenetic analyses, and a cross-species complementation experiment all show that a common developmental mechanism can underpin both microphyll and megaphyll formation. We propose that this mechanism might have originated originally in the context of primitive plant apices to facilitate bifurcation. Recruitment of this pathway to form leaves occurred independently and in parallel during different plant lineages.

Microphylls and megaphylls are determinate organs produced on the flanks of indeterminate shoot apical meristems (SAMS)². The formation of all vascular plant leaves therefore involves the addition of a determinate growth program to the indeterminate apical growth program. Microphylls develop simply with a single vascular trace, whereas megaphylls develop complex vascular and variable shape. The genetic basis of the developmental transition from indeterminate growth in the apex to determinate growth in the leaf has so far been studied only in eudicot species with megaphyllous leaves. Indeterminate apical growth is marked by class I knotted1-like homeobox (KNOX) gene expression³–⁴. Conversely, determinate leaf growth is marked by transcriptional repression⁵–⁷, of KNOX activity. In Arabidopsis, maize and Antirrhinum, MYB orthologues (ASYMMETRIC LEAVES1, ROUGH SHEATH2 and PHANTASTICA (ARP), respectively) maintain the KNOX-off state in leaves³–⁴, KNOX and ARP genes are expressed in mutually exclusive domains. In loss-of-function arp and gain-of-function KNOX mutants, ectopic foliar KNOX expression leads to indeterminate growth such that simple leaves become lobed³–⁴. Therefore, in these three species and also in tobacco⁸–¹⁰, KNOX–ARP interactions in the shoot apex regulate the balance between indeterminate and determinate growth.

KNOX–ARP interactions facilitate megaphyll formation in both monocots and eudicots, suggesting that aspects of leaf development are conserved between groups that diverged about 140 million years ago⁹. Involvement of the same mechanism in the formation of microphylls would imply the independent recruitment of identical processes in species that diverged more than 350 million years ago.

To investigate the meristem-to-leaf transition in a lycophyte, we have examined KNOX–ARP interactions in Selaginella kraussiana, a
Figure 1  KNOX–ARP relationships in *S. kraussiana*.  

**a**, Mature plant.  

**b**, Shoot showing position of large lower leaves (black arrow), small upper leaves (white arrow) and rhizophores (black arrowhead). Scale bar, 0.5 cm.  

**c**, Diagram of branching pattern seen in b; red lines depict major branches, black lines depict minor branches and circles depict rhizophores. Numbers indicate the number of leaf pairs on each branch.  

**d**, Scanning electron micrograph (SEM) of region outlined in b, showing shoot meristems (white arrows) and an emerging rhizophore (yellow arrow). Scale bar, 25 μm.  

**e**, SEM of branching shoot. Arrow points to bifurcating meristem. Scale bar, 9 μm.  

**f**, SEM of shoot apex showing strip of large cells at the apex (flanked by white arrows). Scale bar, 8 μm.  

**g**, **h**, In situ hybridization of *SkKNOX1* (g) and *SkKNOX2* (h) in sequential sagittal sections of a Selaginella shoot apex. Black arrows point to the apical cells; blue arrows point to leaf primordia. In each case, the entire coding region 5′ to the ELK domain was used as a hybridization probe. Scale bar, 38 μm.  

**i**–**k**, Hybridization of *SkKNOX1* (i), *SkARP1* (j) and *SkKNOX2* (k) to sequential frontal sections of a bifurcating Selaginella apex. Black arrows point to the insertion point of the leaf vascular trace in the stem, and v marks the stem vasculature. The *SkARP1* full-length cDNA was used as a hybridization probe. Scale bar, 85 μm.
diploid lycophyte (Fig. 1a). Shoots have a dorsi-ventral organization, and leaf primordia arise in pairs, with one primordium giving rise to a large ventral (lower) leaf and the other to a small dorsal (upper) leaf. At maturity, leaves are ranked on the shoot with small leaves on the upper surface and large leaves on the lower side or in a lateral position (Fig. 1b). Shoots branch regularly after the formation of six or eight leaf pairs, and branch points are marked by the growth of a leaf pair and an aerial root-like structure known as a rhizophore on the upper surface (Fig. 1b–d). Branching involves bifurcation of the SAM (Fig. 1d, e) as opposed to outgrowth of axillary meristems as in angiosperms. A distinguishing feature of *S. kraussiana* meristems is the presence of a strip of large cells on the surface of the apex (Fig. 1f). How these cells relate to specific zones or layers of seed plant meristems is not yet clear.

*KNOX* and *ARP* gene copy number was assessed in *S. kraussiana* after gene isolation. Sequencing and hybridization analyses of genomic DNA revealed two class I *KNOX* genes (*SkKNOX1* and *SkKNOX2*), one class II *KNOX* gene (*SkKNOX3*) and a single *ARP* gene (*SkARP1*) (Supplementary Figs S1 and S2a, c). A single *ARP* gene was also isolated from a related species, *S. viticulosa* (Supplementary Fig. S2c). Phylogenetic analyses of 112 *KNOX* genes and 125 *MYB* genes demonstrated monophyly of the class I and class II *KNOX* clades (Supplementary Figs S2b and S3), and of the *ARP* clade (Supplementary Figs S2d and S4) respectively.

Expression patterns of *SkKNOX* and *SkARP* genes were assessed by *in situ* hybridization. *SkKNOX1* was expressed in cells subtending the large superficial apical cells (Fig. 1g, i), whereas *SkKNOX2* was expressed in internodal regions (Fig. 1h, k). These patterns strikingly resemble those seen in seed plant apices. Significantly, *SkKNOX* transcripts were not detected in leaf primordia. In contrast, *SkARP1* was expressed in leaf primordia and in the meristem (Fig. 1j). *SkARP1* expression in leaves is consistent with a mutually

**Figure 2** *KNOX–ARP* relationships in *O. regalis*. a, Mature plant. b, c, Immunolocalization of *KNOX* (b) and *ARP* (c) proteins in maize apices. White arrows indicate the meristem and black arrows leaf primordia. Scale bar, 40 μm. d, Immunoblot of proteins extracted from maize apex (A) and leaf (L) and from fern apex (A), young leaf (YL), mature leaf lamina (LL) and mature leaf rachis (LR) with anti-*KNOX* antibody. Proteins are detected in the maize apex but not the maize leaf. In *Osmunda*, proteins are detected in the apex and young leaf but not in mature leaf tissues. e, Control immunolocalization performed with no primary antibody. Scale bar, 170 μm. f, g, Immunolocalization of *KNOX* (f) and *ARP* (g) proteins in serial sagittal sections of *Osmunda* apices. h, i, the same sections as in f and g showing nuclei stained with 4,6-diamidino-2-phenylindole. Meristems (m) and plastochron 2 and 3 (P2 and P3) leaves are labelled. Scale bar, 180 μm.
exclusive KNOX–ARP relationship in microphylls. Mutually exclusive expression was also seen in parts of the stem, where ARP but not KNOX genes are expressed in the stem vasculature and in the leaf traces (Fig. 1i, j). In the meristem, however, expression domains of SkKNOX and SkARP1 overlap. This overlap might facilitate repression of SkKNOX at the midline of the apex during bifurcation.

To determine whether co-expression of KNOX and ARP genes is specific to S. kraussiana meristems or is more broadly representative of bifurcating shoots, we examined protein localization patterns in Osmunda regalis. O. regalis is a basal leptosporangiate fern with a clump-forming growth habit (Fig. 2a). Leaves arise spirally, and in the closely related species O. cinnamomea they are not determined until plastochron 8–10 (P8–10).21 As in Selaginella, the meristem has distinct apical cells on the surface, and bifurcates during branching.22 In control experiments, KNOX and ARP proteins were detected in meristems and leaves of maize, respectively (Fig. 2b, c). In contrast, both proteins were detected in meristems of Osmunda (Fig. 2e–i). Co-localization of KNOX and ARP proteins was also seen in Osmunda leaf primordia (Fig. 2f, g). KNOX protein accumulation in fern leaves has previously been reported in Anogramma chaeophylla, where no KNOX repression at P0 was seen.23 It was argued that this lack of repression indicated the recruitment of independent mechanisms of leaf formation in ferns and seed plants.23 We postulate instead that failure to repress KNOX expression at P0 reflects the delayed determinacy of fern leaves.21 Our immunoblot analysis supports this suggestion, because KNOX

Figure 3 KNOX–ARP relationships across land plants. Phylogenetic trees of class I KNOX (left) and ARP (right) genes showing KNOX expression patterns and KNOX–ARP interactions. Selaginella (red), monocot (green) and eudicot (black) sequences are placed. The trees are redrawn from more densely sampled trees shown in Supplementary Fig. S2. KNOX expression patterns fall into four classes, represented diagrammatically: expression in the central zone (blue circle), in the peripheral zone (blue oblongs), in the rib zone (blue oval) or absent from the meristem (empty). Asterisks indicate cases in which gene expression patterns have not been determined, and parentheses indicate genes that are also expressed in leaf tissue. Lines between the trees indicate the relationship between ARP and KNOX genes in particular species. When loss of ARP function leads to changes in KNOX gene expression, the line linking the two genes terminates in a vertical line. When KNOX gene expression is not altered by loss of ARP function the genes are linked by a straight line. KNOX expression patterns have been reported as follows: OSH1, OSH3, OSH6, OSH15, OSH43, OSH71 (ref. 9); rs1, kn1, knox3, knox8 (ref. 5); KNAT1 (ref. 6); STM; KNAT2 (ref. 7); AtSTM8; KNAT2 (ref. 27); Pskn1 (ref. 10); Pskn2 (J. Hofer, personal communication); invaginata, hirzina28; Tkn1 (ref. 11); Tkn2 (refs 29, 30). The effects of loss of ARP function have been reported as follows: Zmrs2 (ref. 14); AmPHAN1 (A. Hudson, personal communication); LePHAN; PsCRISPA (J. Hofer, personal communication).
proteins accumulate in *O. regalis* leaf primordia but not in mature leaves (Fig. 2d). We therefore propose that an overlap of KNOX and ARP expression in the meristem might facilitate shoot bifurcation whereas an overlap within leaf primordia confers meristematic expression in the meristem might facilitate shoot bifurcation.

To gain an overview of KNOX–ARP interactions in leaves, we mirrored ARP and class I KNOX phylogenies, and mapped KNOX expression patterns and the effect of loss of ARP function on to them (Fig. 3). Examining KNOX expression patterns in this context revealed parallel evolution of distinct patterns in the eudicot, monocot and lycophyte lineages. Eudicot KNOX expression patterns in one of the three main clades varied (clade 3), with some genes expressed in the central zone of the SAM and others in the rib zone. In a sister group (clade 2), two subclades were characterized by KNOX expression either in the central zone (CZ type) or the peripheral zone (PZ type). These two patterns predominate in monocots (clade 1), although their distribution varies between gene clades. Both patterns are also seen in *Selaginella* (Fig. 1). In the monocot maize (*Zea*), ARP function represses both CZ-type and PZ-type activities in the leaf. However, a different relationship emerges in eudicots. With the exception of *Antirrhinum*, loss of ARP function in eudicots leads to the ectopic expression of PZ-type but not CZ-type genes in the leaf. ARP function therefore constrains the activity of PZ-type but not CZ-type genes. Moreover, in *Arabidopsis* and tomato (*Lycopersicon*), PZ-type genes repress ARP expression, indicating a possible constraint in the opposite direction. The *Antirrhinum* exception might reflect functional divergence or switching of roles between the two ARP duplicates.

The functional significance of CZ-type versus PZ-type patterns has been explained in several flowering plants. In brief, CZ-type genes facilitate meristem formation and indeterminacy, whereas PZ-type genes facilitate the positioning of leaves and internode expansion. We propose that ARP-mediated repression of all KNOX genes in the leaf, as in maize, reflects the relatively simple and invariant leaf morphology found in the grasses. In contrast, the specialization of KNOX–ARP interactions in eudicots invokes a more complex repression mechanism, both between the meristem and the leaf and within leaf primordia. This complexity permits multiple outputs that are reflected in the array of eudicot leaf diversity. We predict that ARP genes regulate both CZ-type and PZ-type activities in lycophytes, in which leaf morphology and anatomy are simple and invariant.

Despite subtle differences in the nature of ARP–KNOX interactions, the monocot ARP gene *rs2* complements the eudicot *arp as1-1* (ref. 26). To determine the extent of functional equivalence between lycophyte and eudicot ARP genes, we introduced *SkARP1* into *as1-1* mutants under the control of a constitutive promoter. Compared with wild-type and *as1-1* controls (Fig. 4a, b), the phenotype of transformed plants varied from *as1*-like (Fig. 4c–e) to wild type (Fig. 4f–h). The degree of phenotypic rescue was inversely proportional to the number of transgenes (data not shown) and directly proportional to *SkARP1* transcript levels (Fig. 4i). These observations suggest that post-transcriptional silencing of the transgene occurred in lines that retained the *as1-1* mutant phenotype. The phenotype of non-silenced lines demonstrated that *SkARP1* is able to repress KNOX gene expression in the *Arabidopsis* leaf. In this context, *SkARP1* is therefore functionally equivalent to *AS1*.

The fossil record is insufficiently comprehensive for it to be stated unambiguously how many times leaves have evolved. However, it is generally accepted that determinate lateral outgrowths arose independently in ancestral lycophytes and euphyllophytes. Within the euphyllophytes, it is not clear how ‘leaf-like’ these lateral outgrowths were. Consequently, it has been suggested that leaves evolved independently in the sphenopsids, ferns, progymnosperms and seed plants (that is, four times within the euphyllophytes). However, a morphometric analysis of more than 600 fossils implied that a ‘common developmental process underpins leaf development in all four groups’. KNOX–ARP interactions fulfil this role in ferns and seed plants, with spatial and temporal changes in gene expression leading to variations in leaf morphology and growth patterns. Our data indicate that the same developmental mechanism might operate in at least one lycophyte and therefore that this mechanism was recruited at least twice independently during land plant evolution. The evolution of leaves was thus constrained by a fundamental developmental programme that might originally have operated in primitive plants to regulate shoot branching.

**Methods**

**Plant materials**

Plants were obtained from the Royal Botanic Gardens at Kew, the University of Oxford Botanic Gardens, local garden centres in Oxford, and the Nottingham Arabidopsis Stock Centre.

**Gene isolation**

An *SKNOX* fragment and *SkARP1* and *SwARP1* sequences were isolated by genomic polymerase chain reaction (PCR) with degenerate primers. Full-length sequences of *SKNOXI*, *SKNOX2* and *SKNOX3* were obtained by screening a *Selaginella* shoot complementary DNA library with the PCR-amplified fragment.

**Phylogenetic analyses**

To maximize sampling in KNOX and MYB phylogenies, conserved homeodomain and MYB sequences from *Arabidopsis* KNOX and ARP genes were used in *BlastX* searches of the following genome databases: http://blast.genome.jp/, www.ncbi.nlm.nih.gov/, www.plantgdb.org/ and www.moss.leeds.ac.uk/. Only EST or cDNA sequences were retrieved to avoid alignment problems with introns.

**Transgenic Arabidopsis**

The full *SkARP1* coding sequence was amplified by PCR. The PCR product was inserted into the pART27 binary vector by using the pART7 shuttle vector. Thus, *SkARP1* was flanked by the cauliflower mosaic virus 35S promoter and a transcriptional terminator. Constructs were introduced into *Agrobacterium* strain GV3101 by electroporation and then into *Arabidopsis* as1-1 mutant plants by floral dipping.

Received 7 December 2004; accepted 28 January 2005; doi:10.1038/nature03410.


Regulation of bone resorption by the sympathetic nervous system and CART

Florent Elefteriou1,2,3, Jong Deok Ahn1,2, Shu Takeda1,2,4,5, Michael Starbuck1,2, Xiangyi Yang1,2, Xiuyun Liu1,2, Hisataka Kondo1,6, William G. Richards1, Tony W. Bannon1, Masaki Noda6, Karine Clement6, Christian Vaisse7 & Gerard Karsenty1,3

1Department of Molecular and Human Genetics, 2Bone Disease Program of Texas, 3Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas 77030, USA
4Department of Orthopedics, 5Center of Excellence Program for Frontier Research on Molecular Destruction and Reconstruction of Tooth and Bone, 6Department of Molecular Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 101-0062, Japan
7 Amesgen Inc., Neuroscience, Thousand Oaks, California 91320, USA

Bone remodelling, the mechanism by which vertebrates regulate bone mass, comprises two phases, namely resorption by osteoclasts and formation by osteoblasts; osteoclasts are multi-functional cells also controlling osteoclast differentiation. Sympathetic signalling via β2-adrenergic receptors (Adrb2) present on osteoblasts controls bone formation downstream of leptin. Here we show, by analysing Adrb2-deficient mice, that the sympathetic nervous system favours bone resorption by increasing expression in osteoblast progenitor cells of the osteoclast differentiation factor Rankl. This sympathetic function requires phosphorylation (by protein kinase A) of ATF4, a cell-specific CREB-related transcription factor essential for osteoblast differentiation and function7. That bone resorption cannot increase in gonadectomized Adrb2-deficient mice highlights the biological importance of this regulation, but also contrasts sharply with the increase in bone resorption characterizing another hypogonadal mouse with low sympathetic tone, the ob/ob mouse8. This discrepancy is explained, in part, by the fact that CART (‘cocaine amphetamine regulated transcript’), a neuropeptide whose expression is controlled by leptin and nearly abolished in ob/ob mice9, inhibits bone resorption by modulating Rankl expression. Our study uncovers that leptin-regulated neural pathways control both aspects of bone remodelling, and demonstrates that integrity of sympathetic signalling is necessary for the increase in bone resorption caused by gonadal failure.

Leptin antiostogenic function is mediated by the sympathetic nervous system (SNS) acting through Adrb2, the only adrenergic receptor expressed in osteoblasts10. We hypothesized that Adrb2-deficient mice would demonstrate increased bone mass, comprised of increased bone formation downstream of leptin. These results indicate that bone resorption (BR) is controlled by sympathetic function, and the sympathetic neuron soma is a key regulator of leptin downstream signalling, providing an important role for sympathetic signalling in bone resorption.

...