Methods

DNA extraction, blotting, labelling and hybridization techniques were as described previously[5]. Genomic DNA sequences were determined by amplifying portions of the *hth* gene and directly sequencing the PCR product[30]. DNA samples for the PCR genotyping of embryos were obtained by dissecting individual embryos from siliques and surrounding maternal seed coat tissue, crushing the embryo with a plastic pestle in a Microfuge tube and resuspending the resulting homogenate in 20 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). A 1 μl portion of this sample was used in a standard PCR reaction.

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Independent recruitment of a conserved developmental mechanism during leaf evolution

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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 occasions[2–4]. In extant plants, these events are represented by microphyllous leaves in lycophytes (club-mosses, spikemosses and quillworts) and megaphyllous leaves in euphyllophytes (ferns, gymnosperms and angiosperms). Our current understanding of how leaves develop is restricted to processes that operate during megaphyll formation. Because microphylls and megaphylls evolved independently, different mechanisms might be required for leaf formation. 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 operated originally in the context of primitive plant apices to facilitate bifurcation. Recruitment of this pathway to form leaves occurred independently and in parallel in 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 programme to the indeterminate apical growth programme. Microphylls develop simply with a single vascular trace, whereas megaphylls develop complex vasculature 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 euphyllophyte species with megaphyllous leaves. Indeterminate apical growth is marked by class I *knotted1*-like homeobox (*KNOX*) gene expression[5–12]. Conversely, determinate leaf growth is marked by transcriptional[5–10], or possibly post-transcriptional[12], repression of KNOX activity. In *Arabidopsis*, maize and *Antirrhinum*, MYB orthologues (ASYMMETRIC LEAVES1, ROUGH SHEATH2 and PHANTASTICA (ARP), respectively) maintain the KNOX-off state in leaves[13–16]. *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 lobed[6,13–17]. Therefore, in these three species and also in tobacco[18], 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 ago[19]. 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

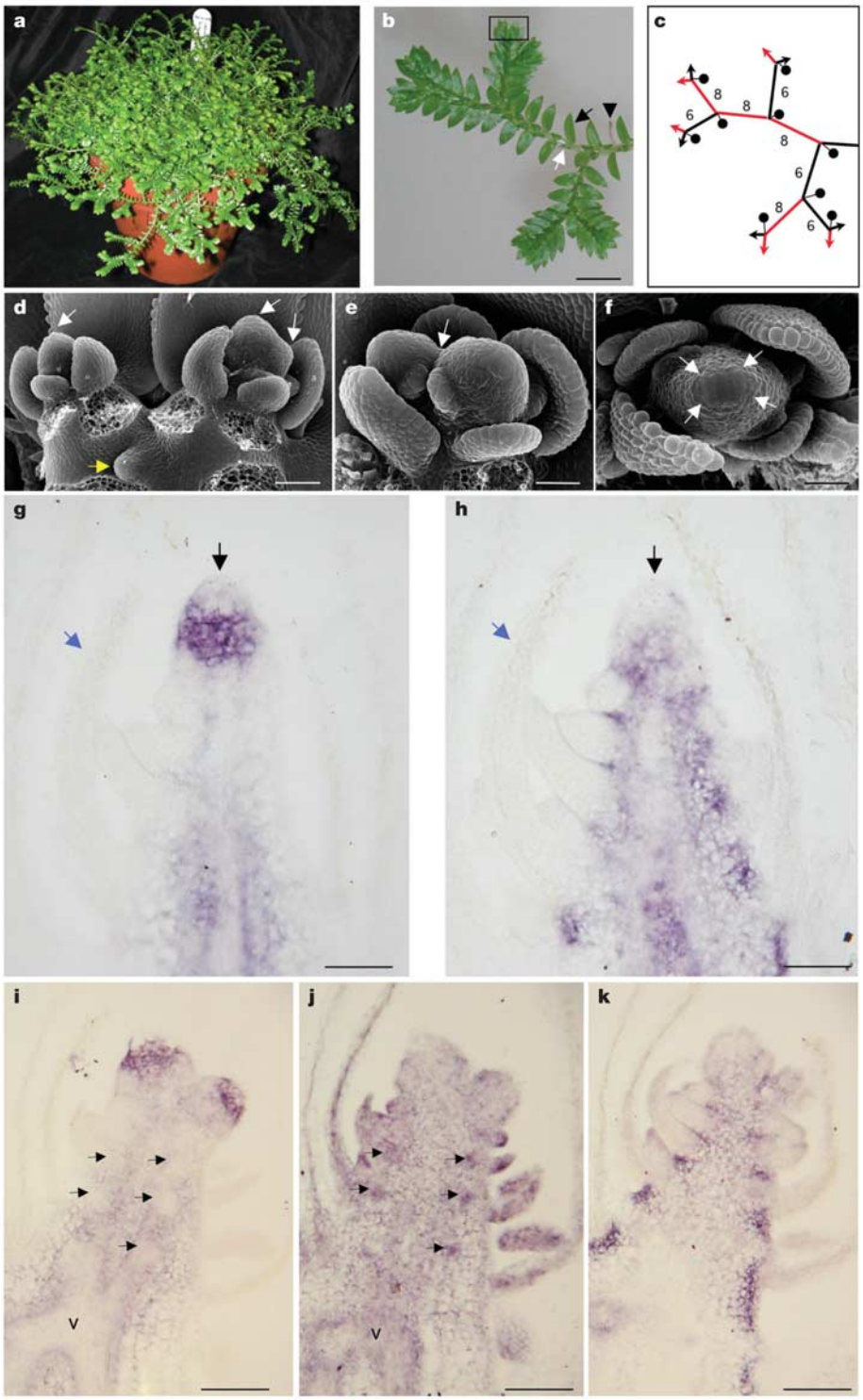

Figure 1 KNOX–ARP relationships in *S. kraussiana*. **a**, Mature plant. **b**, Shoot showing position of large lower leaves (black arrow), small upper leaves (white arrow) and rhizophores (black arrowhead). Scale bar, 0.5 cm. **c**, Diagram of branching pattern seen in **b**; red lines depict major 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[20]. 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

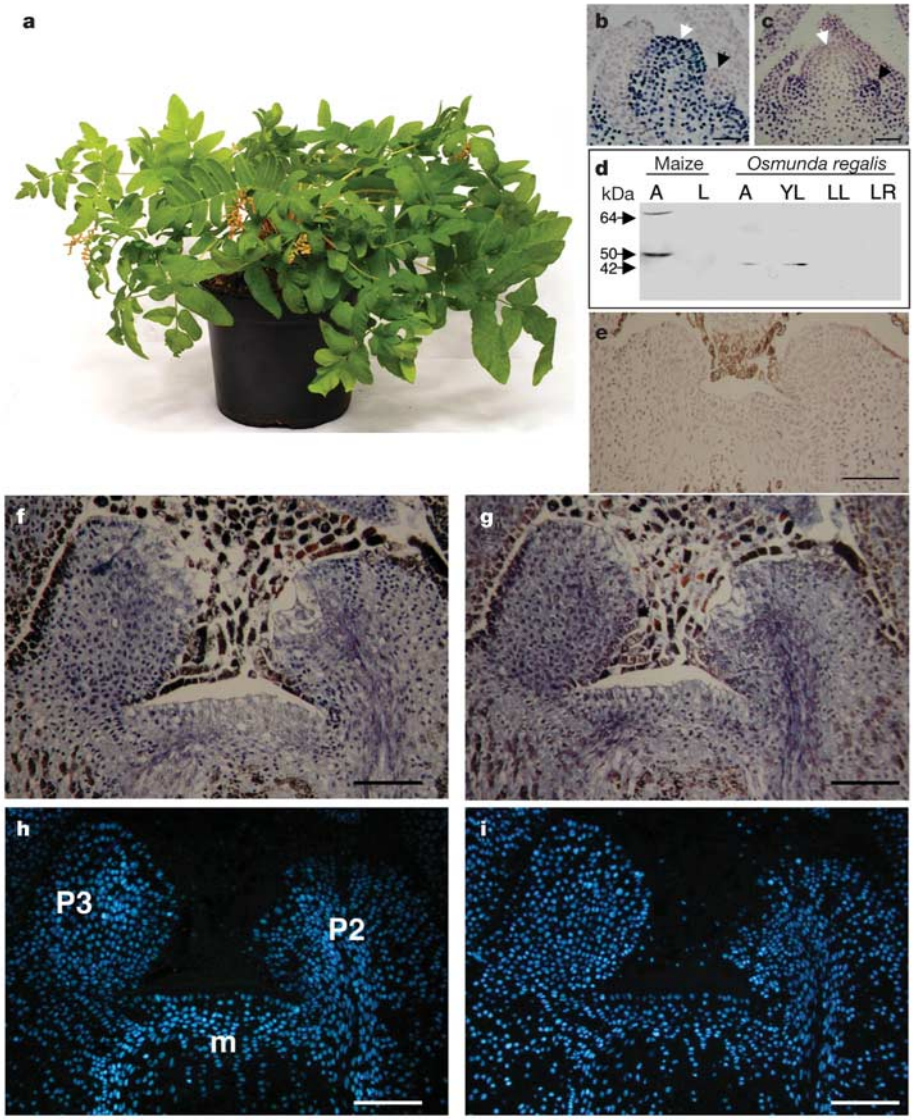

Figure 2 KNOX–ARP relationships in *O. regalis*. **a**, Mature plant. **b**, **c**, Immunolocalization of KNOX (**b**) and ARP (**c**) proteins in maize apices. White arrows indicate the meristem and black arrows leaf primordia. Scale bar, 40 μm. **d**, Immunoblot of proteins extracted from maize apex (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

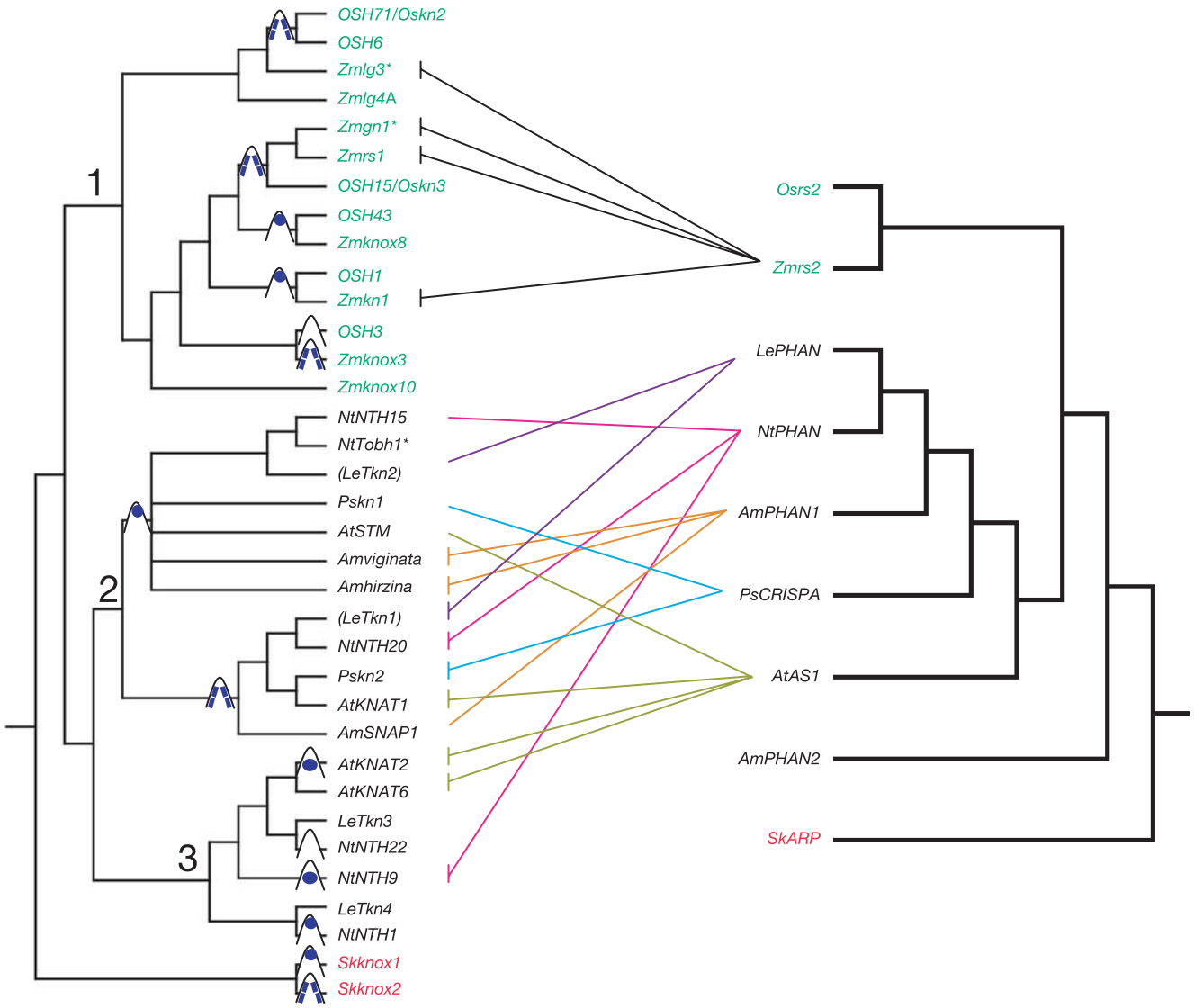

Figure 3 KNOX–ARP relationships across land plants. Phylogenetic trees of class I *KNOX* (left) and *ARP* (right) genes showing *KNOX* expression patterns and *KNOX–ARP* interactions. *Selaginella* (red), monocot (green) and eudicot (black) sequences are placed. The trees are redrawn from more densely 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*[8]; *KNAT2* (ref. 27); *Pskn1* (ref. 10); *Pskn2* (J. Hofer, personal communication); *NTH1*, *NTH9*, *NTH15*, *NTH20*, *NTH22* (ref. 7); *SNAP1* (A. Hudson, personal communication); *invaginata*, *hirzina*[28]; *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[15,16]); *AtAS1* (ref. 13); *NtPHAN* (N. McHale, personal communication[18]); *LePHAN*[25]; *PsCRISPA* (J. Hofer, personal communication).

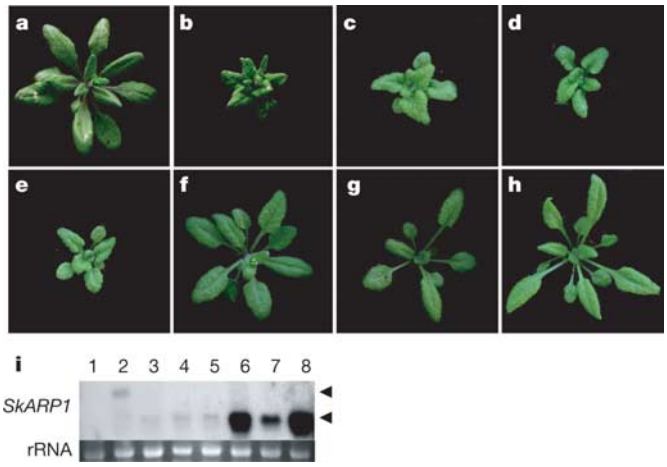

Figure 4 Complementation of *Arabidopsis as1-1* mutants by *SkARP1*. **a**–**h**, Vegetative phenotype of wild-type (**a**), *as1-1* (**b**) and 35S::*SkARP1*;*as1-1* (**c**–**h**) plants. **i**, Northern blot analysis of *SkARP1* transcript levels in *as1-1* (lane 1), *S. kraussiana* (lane 2) and 35S::*SkARP1*; *as1-1* (lanes 3–8) plants. Lanes 3–8 correspond to the lines shown in **c**–**h**, respectively. RNA was hybridized to the entire coding region of *SkARP1*. Arrows point to the endogenous *SkARP1* transcript in *S. kraussiana* and to the transgene transcript in *Arabidopsis*. Ethidium bromide fluorescence of ribosomal RNA is shown as a loading control.

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 properties on the leaf and enables the generation of complex leaf morphologies[24].

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 in either 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*), CZ-type genes repress *ARP* expression, indicating a possible constraint in the opposite direction[13,25]. 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[20]. 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* mutant *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[4]. 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[3]. 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 *SkKNOX* fragment and *SkARP1* and *SvARP1* sequences were isolated by genomic polymerase chain reaction (PCR) with degenerate primers. Full-length sequences of *SkKNOX1*, *SkKNOX2* and *SkKNOX3* 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 tBLASTX 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.

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Leptin regulation of bone resorption by the sympathetic nervous system and CART

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Bone remodelling, the mechanism by which vertebrates regulate bone mass, comprises two phases, namely resorption by osteoclasts and formation by osteoblasts; osteoblasts are multifunctional cells also controlling osteoclast differentiation. Sympathetic signalling via β2-adrenergic receptors (Adrb2) present on osteoblasts controls bone formation downstream of leptin[1]. 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 function[2]. 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 hypogonadic mouse with low sympathetic tone, the *ob*/*ob* mouse[3]. 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* mice[4], inhibits bone resorption by modulating *Rankl* expression. Our study establishes 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 antiosteogenic function is mediated by the sympathetic nervous system (SNS) acting through Adrb2, the only adrenergic receptor expressed in osteoblasts[1] (Supplementary Fig. 1). If both arms of bone remodelling are regulated by similar mechanisms, these results imply that bone resorption (BR) is controlled by neural means. To test this hypothesis, we used mutant mice in which pathways acting downstream of leptin signalling were disrupted.

Adrb2$^{-/-}$ mice have normal body weight and fat pad weight[5], and none of the endocrine abnormalities observed in mice lacking leptin (*ob*/*ob*) or noradrenaline (*Dopamine-β-hydroxylase (Dbh)*$^{-/-}$ mice, Fig. 1a)[6,7]. Analyses of vertebrae and long bones revealed two unanticipated features in 6-month-old male and female *Adrb2*$^{-/-}$ mice. First, *Adrb2*$^{-/-}$ mice had a more severe high bone mass phenotype (HBM) than *ob*/*ob* or wild-type (WT) mice receiving β-blockers[1,3] (Fig. 1b, c). Illustrating the importance of sympathetic signalling in bone remodelling, this HBM also affected *Adrb2*$^{+/-}$