

Growth from two transient apical initials in the meristem of *Selaginella kraussiana*

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A major transition in land plant evolution was from growth in water to growth on land. This transition necessitated major morphological innovations that were accompanied by the development of three-dimensional apical growth. In extant land plants, shoot growth occurs from groups of cells at the apex known as meristems. In different land plant lineages, meristems function in different ways to produce distinct plant morphologies, yet our understanding of the developmental basis of meristem function is limited to the most recently diverged angiosperms. To redress this balance, we have examined meristem function in the lycophyte *Selaginella kraussiana*. Using a clonal analysis, we show that *S. kraussiana* shoots are derived from the activity of two short-lived apical initials that facilitate the formation of four axes of symmetry in the shoot. Leaves are initiated from just two epidermal cells, and the mediolateral leaf axis is the first to be established. This pattern of development differs from that seen in flowering plants. These differences are discussed in the context of the development and evolution of diverse land plant forms.

KEY WORDS: Meristem function, Leaf development, Lycophytes, Lineage analysis

INTRODUCTION

Plant architecture is determined by the activity of shoot apical meristems (SAMs). In all vascular plants, the SAM functions both to maintain itself and to produce lateral organs. However, the way in which the SAM contributes to final form differs dramatically in different plant lineages. In lycophytes, the earliest divergent vascular plant lineage, shoot branches are formed by bifurcation of the SAM (reviewed by Steeves and Sussex, 1989). By contrast, flowering plants branch through the outgrowth of axillary meristems that are themselves produced in association with leaves by the main SAM. Distinct leaf morphologies also distinguish these two plant groups in that lycophytes have microphyllous leaves typically with a single vascular trace, whereas flowering plants have megaphyllous leaves with complex venation patterns (Gifford and Foster, 1989; Kenrick and Crane, 1997).

In flowering plants, meristem structure and function is reasonably well understood. The SAM is both layered and zoned (reviewed by Steeves and Sussex, 1989). Cells in the outer L1 layer divide anticlinally to extend the epidermis of the plant, whereas cells in the subtending L2 and L3 layers divide randomly to contribute to inner tissues. Zones are superimposed upon layers, with cells of the central zone both self-renewing and contributing to the flanking peripheral zone, and cells of the peripheral zone contributing to determinate lateral organs. Throughout development, these zones and layers are maintained and meristem size is relatively invariant.

The balance between indeterminate shoot and determinate leaf growth in flowering plant SAMs is regulated by several interacting pathways. For example, KNOTTED1-like homeodomain (KNOX) proteins function to promote indeterminacy in the meristem, whereas ARP-type Myb transcription factors promote determinacy in leaf primordia by downregulating *KNOX* expression (Jackson et

al., 1994; Lincoln et al., 1994; Long et al., 1996; Nishimura et al., 1999; Sentoku et al., 1999). A failure to downregulate *KNOX* gene expression in the leaf can perturb both mediolateral (M-L) (Scanlon et al., 1996) and proximodistal (P-D) axis formation (Lincoln et al., 1994; Smith et al., 1992; Tsiantis et al., 1999), suggesting an interaction between meristem function and axis formation in the leaf. This link is further substantiated by the observation that class III HD-Zip genes, such as *PHABULOSA* (*PHB*), both promote meristem activity and confer adaxial leaf fate (McConnell et al., 2001). Aspects of both the KNOX-ARP and HD-ZIP pathways function in the lycophyte *Selaginella kraussiana* (Floyd and Bowman, 2006; Floyd et al., 2006; Harrison et al., 2005; Prigge and Clarke, 2006). However, the context in which these pathways operate is not yet clear because our current understanding of lycophyte SAMs is based solely on histological analyses.

Although anatomical descriptions provide some insight into how meristems function, interpretations of the data obtained are often conflicting owing to the limitations of analysing static views of a dynamic process. In the case of lycophyte meristems, competing hypotheses exist to explain how leaves are initiated on the flanks of the apex (Dengler, 1983; Hagemann, 1980; Wardlaw, 1957), how many and how apical initials contribute to shoot growth (Hagemann, 1980; Newman, 1965; Philipson, 1990; Popham, 1951; Von Guttenberg, 1966), and how meristem bifurcation occurs (Hagemann, 1980; Von Guttenberg, 1966). To resolve these issues, we have carried out a clonal analysis in *S. kraussiana*. This approach offers a distinct advantage over histological studies as it elucidates cell lineage relationships in a three-dimensional context and monitors the fate of individual cells. The choice of *S. kraussiana* as a model system reflects the evolutionary significance of lycophytes as the earliest diverging vascular plant lineage, recent genome initiatives and published 'evo devo' studies with this species (Floyd and Bowman, 2006; Floyd et al., 2006; Harrison et al., 2005). We show here that leaves are initiated from two adjacent epidermal cells on the flanks of the SAM, that shoots are primarily derived from the activity of two apical initials, and that these initials contribute directly to the two

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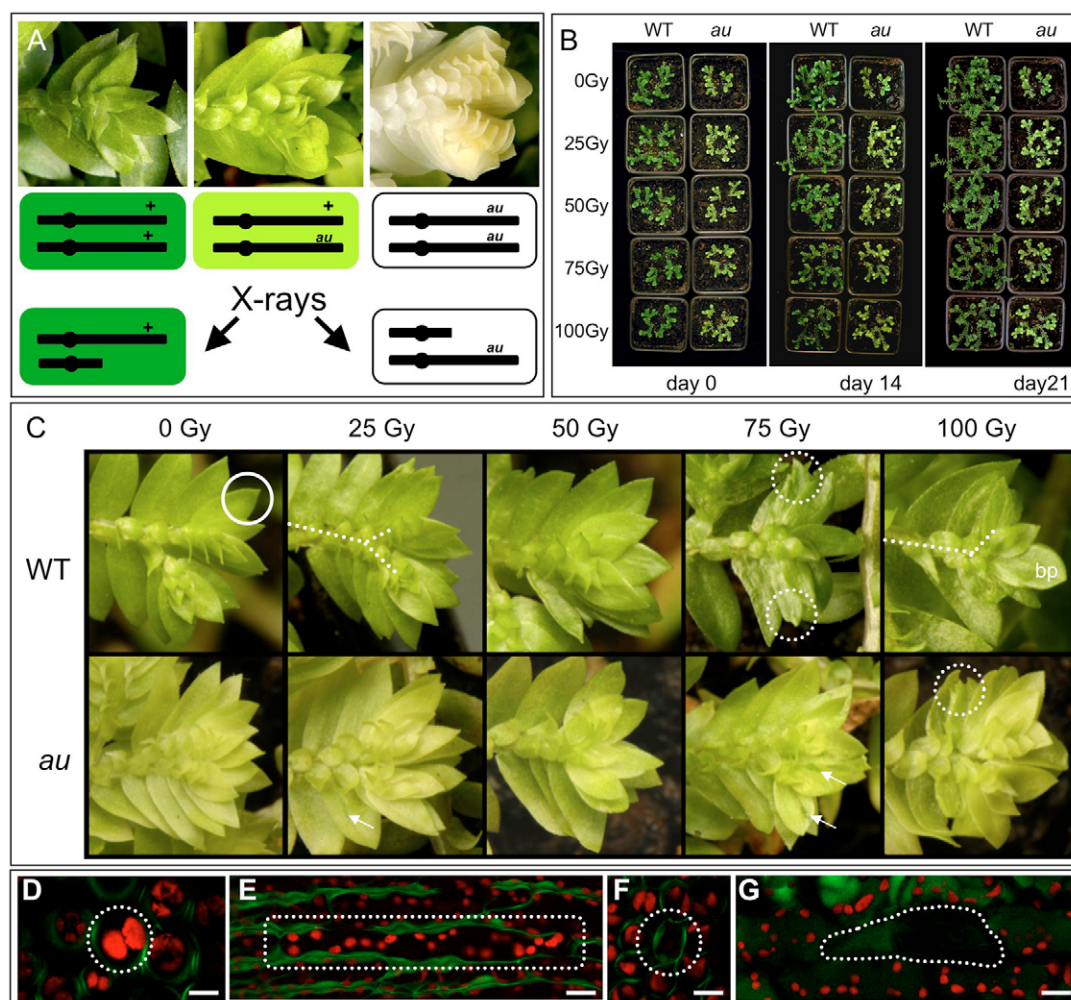


Fig. 1. X-ray irradiation of *S. kraussiana* var. *aurea* yields cell-autonomous sectors.

(A) Wild-type $+/+$ (dark green), heterozygous $au/+$ (pale green) and homozygous au/au (white) plants, and the predicted outcome of X-ray-induced aneuploidy. (B) Shoot morphology 14 and 21 days after irradiation with 0–100 Gy. (C) Growth perturbations in plants irradiated at 75 Gy and above. Note perturbations in leaf shape at high doses (dotted white circles), shoot termination at 100 Gy (dotted lines) and dark-green sectors (arrowed). (D–G) Single-cell $+/+$ (D,E) and $au/-$ (F,G) sectors in the epidermis (E,G) and mesophyll (D,F) layers. Scale bars: D,E,G, 10 μm ; F, 20 μm .

new meristems formed after bifurcation. This work provides a robust framework for future comparative studies of meristem and leaf development in land plants.

MATERIALS AND METHODS

Plant material, X-ray irradiation and sector measurements

Plants of *S. kraussiana* var. *kraussiana* (Kunze) A. Br. and *S. kraussiana* var. *aurea* (Webster and Tanno, 1980) were grown from cuttings in soil in a Sanyo MLR-350HT growth chamber with 70% humidity. After 2 months, plants were irradiated with single doses of 250 kV X-rays at a dose rate of 2.2 Gy per minute. The X-ray generator was operated at 14 mA, with 0.5 mm Cu and 1 mm Al added filtration (half value layer=1.32 mm Cu). An open-ended perspex applicator was used to restrict the area of the beam. Optimal dose determination was as described in the results. Plants were examined for sectors at weekly intervals after irradiation. Small leaf sectors typically became apparent about 1 month after irradiation, whereas sectors affecting the shoot became apparent after 2–3 months. Sected leaves and shoots were removed from the plant and examined using a Nikon SMZ800 dissecting microscope. Sector position and extent were determined using an eyepiece graticule and recorded both by photography and by hand on a line-drawn leaf or shoot template.

Chlorophyll fluorescence and confocal microscopy

Chlorophyll fluorescence in individual cell layers was measured by vacuum infiltrating leaves with a 10% calcofluor solution and then scanning through leaf layers using a LSM 510 META confocal laser scanning microscope with a Plan-neofluor 25 \times 0.8 NA DIC lens set for water immersion. Chlorophyll

was excited using the 543 line of a HeNe laser, and calcofluor was excited with a 405 nm blue diode laser. The primary dichroic was set to HFT 405/488/543 and fluorescent emission was separated using a NFT 490 secondary dichroic. A 435–485 nm band pass filter was used before channel 2 to detect calcofluor, and a long-pass 650 nm filter was used before channel 3 to detect chlorophyll. Pinholes were set to achieve a 1.5 μm optical section in each channel, with pixel dimensions of 0.29 \times 0.29 μm in x and y .

Calculation of apparent cell number (ACN)

Estimates of the ACN at the time of irradiation were obtained using the reciprocal of the proportion of the sectored part. Thus, where a sector occupied half of a plant part, one or two cells contributed to that part at the time of irradiation, depending on whether the cell(s) was in the G1 or G2 phase of the cell cycle [for further discussion see Poethig (Poethig, 1987)]. On this basis, if many plants are irradiated at different stages of development, individual sector types gain multiple representations in the data set and can be used to reconstruct patterns of cell division.

Histology and cell counts

Segments of fully expanded stem were fixed in 4% paraformaldehyde with 4% DMSO in PBS, and then dehydrated and embedded in wax or Technovit 7100 resin as described in the manufacturer's manual (Kulzer). Sections of 3 μm or 10 μm were cut and stained either with 0.1% aqueous Toluidine Blue for 30 seconds, rinsed in water and dried for storage and further examination, or with Safranin O and Fast Green as previously described (Berlyn and Miksche, 1976). Median longitudinal leaf sections were identified using three markers: the ligule, guard cell files and the leaf vein. Transverse section cell numbers were counted

at the widest point in the leaf. Epidermal and inner photosynthetic cell layer counts were obtained from stem tissue in longitudinal and transverse planes. The final numbers presented are averages of 25 counts. Sections were examined and photographed using a Leica DMRB microscope.

RESULTS

Cell-autonomous sectors can be induced in *S. kraussiana* without perturbing shoot development

The semi-dominant *aurea* (*au*) mutation confers pale green pigmentation on *S. kraussiana* chloroplasts (Webster and Tanno, 1980). To determine whether sectorial aneuploidy could be induced in heterozygous *au* mutants, plants were irradiated with X-rays. Irradiation led to the formation of white or dark-green sectors following presumed loss of the wild-type or mutant allele, respectively (Fig. 1A). Sectors were induced in all mutant plants irradiated at 25 Gy or more. No sectors were induced in non-irradiated controls, and few sectors were induced in irradiated wild-type plants (data not shown). To determine the dose at which the maximum number of sectors could be induced without deleteriously affecting plant growth, 8-week-old cuttings of both wild-type and mutant plants were irradiated with 0 to 100 Gy, at 25 Gy intervals. In both wild-type and mutant plants, overall growth was retarded following irradiation doses of 75 Gy or 100 Gy (Fig. 1B); phenotypic perturbations such as leaf splitting, change in leaf shape and termination of the meristem were also observed (Fig. 1C). The optimal dose for sector induction was therefore determined by irradiating new cuttings at 10 Gy intervals between 30 and 70 Gy. These experiments demonstrated that 40 Gy irradiation induced sectorial aneuploidy in *S. kraussiana au* mutant shoots at a sufficient frequency for clonal analysis without concomitant perturbation to growth patterns.

For aneuploid sectors to inform accurately about cell division patterns, both wild-type and mutant gene products must act cell autonomously (Poethig, 1987). To determine the cell autonomy of dark-green and white sectors on heterozygous *au*+/+ shoots, we searched for single cell sectors. The position and extent of sectors within leaves was determined using chlorophyll autofluorescence as a marker of *au* activity. Chloroplasts fluoresce dark red in wild-type +/+ or aneuploid +/- cells, pale red in heterozygous *au*+/+ cells and do not fluoresce in homozygous *au/au* or aneuploid *au*-/- cells.

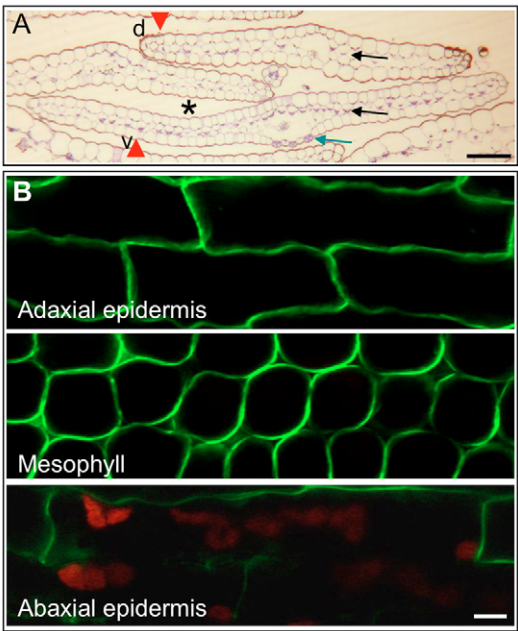


Fig. 2. *S. kraussiana* leaves comprise three chloroplast-containing cell layers. (A) Transverse section through leaves arranged around an apex showing the three main cell layers of the leaf, and the downwards orientation of chloroplasts in the mesophyll. The asterisk indicates the position of the meristem; red arrowheads indicate the abaxial surfaces of the dorsal (d) and ventral (v) leaves; black arrows indicate large chloroplasts in the mesophyll; the blue arrow indicates a guard cell. Scale bar: 40 μ m. (B) Scan through all three layers of the leaf showing a white *au*-/- sector in the upper two layers of the leaf. The abaxial epidermis is *au*+/+. Scale bar: 10 μ m.

Single-cell, dark-red (Fig. 1D,E) and non-fluorescent (Fig. 1F,G) sectors were identified in both the epidermal (Fig. 1E,G) and mesophyll (Fig. 1D,F) layers of the leaf, indicating that the *au* gene product acts cell autonomously. As such, *au*-/- and +/- sectors could reliably be used to elucidate cell lineage relationships in *S. kraussiana*.

Table 1. Within-leaf sector sizes

Sector width	Deduced M-L cell #	Cell layers traversed	Deduced Ad-Ab cell #	Range of sector lengths	% Full-length sectors	Deduced P-D cell #	Number of sectors scored
1/2	2	3	1	Full	100	1	7
1/4	4	3	1	Full	100	1	6
1/4	4	2	2	Full	100	1	1
1/6	6	3	1	1/3 to full	89	1-3	18
1/6	6	2	2	1/2 to full	80	1-2	5
1/6	6	1	3	1/4 to 1/2	0	2-4	4
1/8	8	3	1	1/4 to full	25	1-4	4
1/8	8	2	2	1/6 to full	21	1-6	14
1/8	8	1	3	1/8 to 1/2	0	2-8	10
1/12	12	3	1	1/4 to full	50	1-4	8
1/12	12	2	2	1/16 to full	9	1-16	11
1/12	12	1	3	1/16 to 1/2	0	2-16	16
1/16	16	3	1	1/6 to full	25	1-6	4
1/16	16	2	2	1/8 to 1/3	0	3-8	28
1/16	16	1	3	1/16 to 1/2	0	2-16	68

Sector number, width (proportion of circumference), depth (number of epidermal and mesophyll layers encompassed) and length are shown. Where sector lengths varied in any particular width or depth class, the proportion of those that were full length is indicated. From these measurements, the cell numbers (#) present at the time of irradiation were deduced for all three axes (M-L, Ad-Ab and P-D).

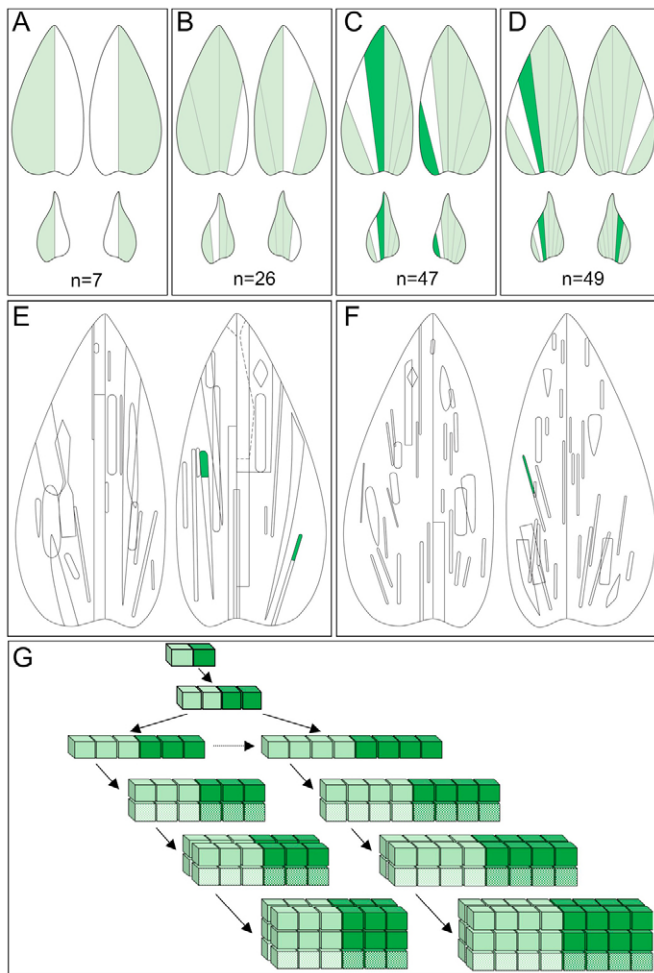


Fig. 3. Leaves are initiated from a pair of epidermal cells.

(A-D) Schematic of full-length leaf sectors that traversed all three Ad-Ab leaf layers and support a two-cell (A), four-cell (B), six-cell (C) and eight-cell (D) mediolaterally extended strip of cells that are recruited from the meristem to make a leaf. (E) Schematic of sectors occupying two of the three leaf layers (i.e. those that are formed when the Ad-Ab axis comprises two cell layers). Sectors are a maximum of a one-sixth leaf width indicating that the Ad-Ab axis is not established until at least six cells have been recruited in the M-L axis. Notably, some sectors traverse the full length of the leaf indicating that Ad-Ab axis formation precedes P-D axis formation. (F) Schematic of sectors occupying just one cell layer (i.e. those that are formed when the Ad-Ab axis comprises three cell layers). No sectors traverse the full length of the leaf showing that the P-D axis is established before divisions in the Ad-Ab axis are completed. (G) Summary of earliest cell division patterns in the leaf as inferred from 1362 sectors. At the six-cell stage, the next cell division can either be in the M-L axis to form an eight-cell strip, or in the Ad-Ab axis to produce 12 founder cells.

Leaves are initiated from a pair of cells on the flank of the *S. kraussiana* SAM

To assess cell lineage relationships within the leaf and to determine how leaves are initiated in the SAM, 1362 within-leaf sectors were examined. *S. kraussiana* leaves are arranged in ranks along a creeping dorsiventral axis, such that the small leaves are dorsal with the abaxial side facing upwards, and the larger leaves are ventral with the adaxial side facing upwards (Fig. 2A). Within each leaf

there are three cell layers: the adaxial epidermis, the mesophyll and the abaxial epidermis. Sectors were scored by measuring the proportion of leaf length encompassed, the proportion of leaf width encompassed, and by assessing chlorophyll fluorescence in each of the three leaf layers (Fig. 2B and Table 1).

The largest within-leaf sectors occupied half of the leaf width, traversed all three leaf layers and extended full leaf length (Fig. 3A). Only one sector was found to occupy a whole leaf, and this leaf was a dorsal branch-point leaf whose unusual initiation may be associated with angle meristem initiation. Therefore, leaves in *S. kraussiana* initiate from two adjacent cells that establish the M-L axis. One-quarter, one-sixth and one-eighth width sectors were also found to encompass all cell layers and to extend the full length of the leaf (Fig. 3B-D). Thus, the two starting cells divide circumferentially around the stem to form four cells. Either two of these cells then divide to form a strip of six cells, or all cells divide to form a strip of eight cells, thus extending the M-L axis prior to establishment of the adaxial-abaxial axis (Ad-Ab) or P-D axes (Fig. 3G).

Full-length leaf sectors that are one-sixth or one-eighth of the leaf width and extend through two of the three leaf layers suggest the subsequent establishment of the Ad-Ab axis by a symmetrical and downwards division (Fig. 3E,G). The resultant leaf founder-cell population for each type of leaf is therefore a group of twelve or sixteen epidermal cells. As sectors that mark only one Ad-Ab leaf layer extend from one-half to one-sixteenth leaf length (Fig. 3F,G), 1-15 cell divisions must occur in the P-D direction prior to formation of the internal mesophyll layer. Subsequent rounds of cell division give rise to dorsal leaves of 31 ± 5.6 (M-L) \times 3 (Ad-Ab) \times 45 ± 12.2 (P-D) cells and ventral leaves of 62 ± 7 (M-L) \times 3 (Ad-Ab) \times 133 ± 10.7 (P-D) cells at maturity.

S. kraussiana shoots have four axes of symmetry

To ascertain the anatomical context in which to assess lineage relationships within the *S. kraussiana* SAM, general growth patterns were first documented. Following germination, and a short period of juvenile growth where leaves are initiated in spiral phyllotaxy, *S. kraussiana* SAMs branch to form two new growth axes – a thicker major stem and a more slender minor stem (Fig. 4A). Each stem is flattened dorsiventrally and is ellipsoid in transverse section, such that stems have both left-right and dorsiventral planes of symmetry. On each stem, diagonal and intersecting axes mark the position of leaf initiation, and six or eight opposite and decussate leaf pairs are produced before each SAM bifurcates again. New axes form on alternate sides of the shoot at successive branch points giving the shoot a characteristic zig-zag growth pattern. Stem growth, leaf formation and branching therefore require the establishment and maintenance of a dorsiventral, two-diagonal and a left-right axis of symmetry within the shoot (Fig. 4B).

Shoots that are not bifurcating derive from the four distal-most cells in the SAM

To understand how cell division patterns might establish symmetry in the *S. kraussiana* SAM, transverse sections of shoot apices were examined by light microscopy. Sections cut across the top of the apex showed four large, lightly staining cells (Fig. 4C,G). The inner two divide in parallel to the left-right axis to establish dorsiventrality in subtending cell layers, whereas the two lateral cells divide radially to establish the diagonal axes of symmetry (Fig. 4D,E,H,I). Leaves arise around five cell layers below the tip, at the boundary between daughter cells of an inner and an outer distal-most cell of the four apical cells (Fig. 4F,J). Left-right, dorsiventral and diagonal growth

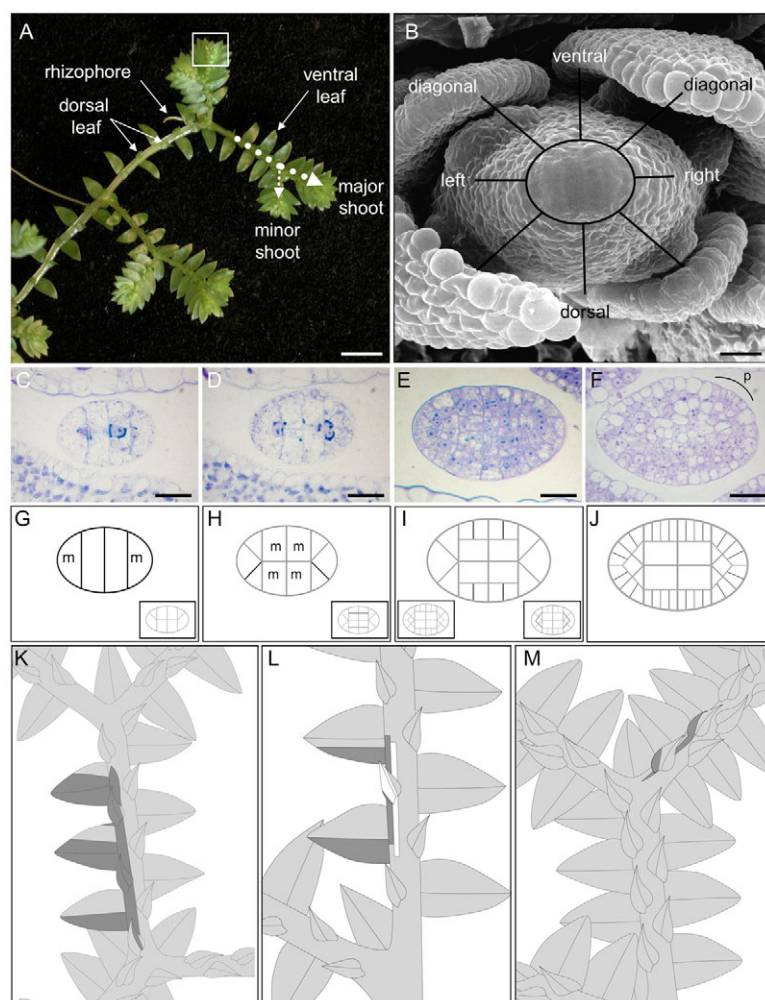


Fig. 4. Shoot growth occurs from two apical initials.

(A) Dorsal view of the flattened *S. kraussiana* shoot showing major and minor branch structures. White square indicates meristem position. (B) Scanning electron micrograph of a meristem, and schematic showing the four axes that are established during shoot growth. The oval indicates four cells at the tip. (C–F) Transverse sections through the shoot apex at 3 μm intervals showing: two central initials flanked by two lateral merophytes on the surface (C); the division of the two central initials to produce dorsal and ventral merophytes, and radial division of the two lateral merophytes (D); further radial divisions in the epidermal cell layer (E); and finally, at five cell layers below the surface, approximately 30 large epidermal cells, and a ventral region of five to six small epidermal cells in the predicted position of leaf initiation (p) (F). (G–J) Schematics of sections C–F showing epidermal cell division patterns deduced from both histology and sector analysis. Black lines indicate the most recent division, and insets indicate stages that are not apparent in the sections shown but that must have occurred between the layer shown and the next layer down. m, merophyte. (K–M) Schematic representation of shoot sectors occupying one-half (K), one-quarter (L) and one-tenth (M) of the shoot circumference. The sector in L is a twin spot, most likely induced by somatic recombination rather than by independent aneuploidy events in two neighbouring cells. Scale bars: A, 5 mm; B, 22 μm ; C–F, 18 μm .

axes are therefore established within the top five cell layers of the meristem, and the two cells that initiate each leaf are descendants of two different surface cells.

To assess the functional significance of the deduced cell division patterns, lineage relationships in the SAM were determined by examining sectors that contributed to all or part of the shoot. No cylindrical sectors or sectors that occupied the medial third of the shoot were detected, suggesting the absence of a single apical initial. Half-shoot sectors typically affected the dorsal and ventral leaf rank on the left or right side of the stem, as opposed to either the dorsal or ventral leaf ranks (Fig. 4K). One-quarter-shoot sectors similarly affected the left or right side of the stem. However, these sectors were only observed in the left or right lateral quarters and were never observed in the dorsal and ventral medial quarters (Fig. 4L). This observation suggests that left-right shoot symmetry is the first to be established in the SAM and demonstrates that the inner two distal-most cells divide both to self-renew and to give rise to the two lateral cells. This capacity for self-renewal identifies the inner two distal-most cells as apical initials, and thus the two lateral cells are daughters of initials, i.e. merophytes (Lyndon, 1990). Sectors on the dorsal or ventral side of the stem occupied either medial or lateral parts of the shoot, with a maximum girth of about one-tenth (Fig. 4M). Consistent with deduced cell division patterns (Fig. 4H), these sectors must have arisen in one of the eight dorsal or ventral descendants of the four distal-most cells. In combination, these data suggest that a strip of four cells on the surface of the SAM gives rise to most of the shoot. The

two inner initials divide both in the left-right axis to produce the two lateral merophytes (Fig. 4G), and alternately in the dorsal and ventral planes to yield a further two pairs of merophytes in the subtending cell layer (Fig. 4H). The lateral merophytes contribute to growth at each side of the shoot, whereas the subtending merophytes give rise to the dorsal and ventral sides of the shoot.

Apical initials are not permanent

Of the 38 shoot sectors identified, all of those that occupied less than the whole shoot width occupied half or less of a branch length. This observation suggests that apical initials persist for relatively short periods of time, contributing to 1–4 internodes before being replaced. To determine the mechanism of initial replacement, we identified and analysed sectors that occupied different proportions of the shoot width at different points along the length of a branch. An informative example is shown in Fig. 5A, where the sector transiently occupies half of the branch width before occupying the whole width. This sector is consistent with the idea that one of the apical initials ceases to function as an initial after the production of three nodes. As a consequence, either the remaining initial divides both to replace itself and to produce a daughter that displaces the non-functional initial (Fig. 5B,C), or shoot growth now proceeds solely from the remaining initial (Fig. 5D,E). Either way, because sector displacement has been seen from both left to right and right to left, the two initials originally have equivalent developmental potential. However, because the change from half-shoot to whole-shoot

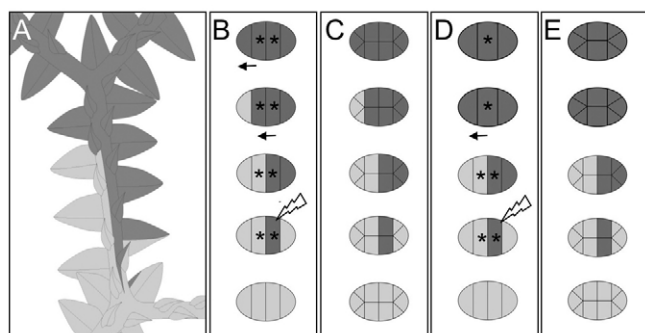


Fig. 5. Apical initials are not permanent. (A) Schematic of a shoot sector that first occupies segments of the front and back of the shoot, then occupies half of the shoot, and finally takes over the whole shoot. (B-E) Hypothetical patterns of inheritance of the mutant sector if two apical initials are functional throughout shoot growth (B,C), or if the number of initials decreases from two to one (D,E). Initials are indicated with an asterisk and the direction of cell division with arrows; the jagged arrow indicates an X-ray-induced aneuploidy event in one initial; light grey depicts *au/+* tissue and dark grey depicts *+/-* tissue. Cell patterns in the surface (B,D) and subtending (C,E) layers of the apex are depicted.

occupation can be so abrupt (Fig. 5A), when an initial ceases to function it can be rapidly depleted. In summary, these sectors suggest that two developmentally equivalent apical initials can contribute to all shoot tissues. Over time, however, one of the initials may cease to function; growth then proceeds from either one or two direct descendants of the remaining initial.

Apical initial number varies during shoot development

Having established the cell division patterns in the SAM during growth of an individual minor or major stem, we then assessed what happens in the SAM when two new stems are formed. To determine the cellular context within which bifurcation occurs, longitudinal sections of bifurcating apices were examined. Frontal longitudinal sections of recently bifurcated shoots show four or five lightly stained large cells in each SAM (Fig. 6A,E,F). After the SAM has initiated three leaf pairs, division and lateral expansion yields an increase in the number of these cells to around six, and a broadening of the apex (Fig. 6B). After the initiation of four leaf pairs on the major axis or three leaf pairs on the minor axis, further asymmetric divisions increase the cell number to seven or eight (Fig. 6C). When the apex shows visible signs of bifurcation, there are around 11–13 large cells (Fig. 6D). Of these, the four centrally located cells appear to become determinate whereas the two lateral groups of three or four appear to constitute the large distal cells of the new major and minor SAMs (Fig. 6E,F).

To assess the lineage relationship between apical initials in the parent SAM and those in the two new SAMs, sectors were identified that spanned a branch point. Four sectors permitted a deduction of lineage relationships during branching (Fig. 6G–N). First, where sectors occupied lateral portions of the new shoot, the maximum circumferential proportion occupied was one-quarter for the major shoot (Fig. 6G) or one-third for the minor shoot (Fig. 6G,H). These proportions suggest a contribution of four or three cells to the major and minor shoots, respectively. These proportions are also consistent with the observed increase in cell number of three or four to six or seven prior to branching (Fig. 6B–D), and with the deduced patterns of within-branch growth (Fig. 5). Second, the frequent association of shoot sectors with the ventral branch leaf, and the fact that branch

leaf sectors usually occupied half a leaf (Fig. 6G,I,J), suggest that branch leaves are derived from a pair of centrally located cells in an even-numbered strip of four or six cells. Third, sectors on the inner side of new branches do not go on to occupy the whole shoot (Fig. 6G), suggesting that they must be derived from newly specified lateral merophytes. Fourth, sectors on the outside of the original stem can extend across to the new stem (Fig. 6H), suggesting that lateral merophytes in the parent SAM can persist across the branch point. In summary, these observations suggest that stem growth occurs from either one or two initials that are flanked by a pair of lateral merophytes, that initial number transiently increases to four or five during bifurcation of the SAM, and that initials in the new branch SAMs are direct descendants of those in the parent SAM.

DISCUSSION

S. kraussiana shoot development in the context of land plant evolution

Previous studies have distinguished land plant meristems on the basis of apical cell numbers, cleavage patterns from apical cells, and arrangements of cells within the apex (Newman, 1965; Philipson, 1990; Popham, 1951). The simplest type is the monoplex meristem that is capped by a single tetrahedral apical initial that cleaves spirally to contribute to stem growth (Newman, 1965), whereas the most complex are arranged into distinct zones and layers with specialised function. Monoplex meristems are reportedly found in mosses and ferns, and complex meristems are found in seed plants. Lycopphyte meristems are intermediate between these types but are typically placed at the ‘simple’ end of the scale. Historically, the concept of monoplex apices has been linked to the notion of a slowly dividing persistent apical cell (Newman, 1965). By contrast, complex meristems of seed plants have a small number of impermanent apical initials in each layer (Bossinger et al., 1992; Dulieu, 1969; Furner and Pumfrey, 1992; Irish and Sussex, 1992; Jegla and Sussex, 1989; Korn, 2001; McDaniel and Poethig, 1988). Our data clearly show that apical initials in the *S. kraussiana* apex are impermanent and thus it is reasonable to propose that mechanisms that regulate initial cell activity may be conserved in vascular plants.

The data presented elucidate three novel aspects of SAM function during the adult phase of vegetative growth in the lycopphyte *S. kraussiana*. First, two apical initials cleave to give rise to six merophyte daughters that form distinct lateral, dorsal and ventral domains in the stem.

This observation implies a shoot-patterning role for the division sequence of the apical initials. Second, both left- and right-half shoot sectors can take over the whole shoot. Thus, the two apical initials are developmentally equivalent. Third, leaves initiate from two cells, one of which is derived from a medial merophyte, and one from a lateral merophyte. This suggests that apical cell symmetry may also pattern the M-L leaf axis. The presence of two apical initials thus provides a capacity for regulating shoot symmetry that does not exist in apices with a single apical initial. This illustrates that a seemingly simple transition from a spirally cleaving apical initial to a pair of side-by-side developmentally equivalent initials can generate radical alterations in plant form.

A conspicuous difference in growth form between *S. kraussiana* and flowering plants results from their different modes of branching. In lycopphytes, branching is attained by bifurcation of the meristem, whereas in flowering plants vegetative branches form in the leaf axils. Although it was initially proposed that axillary meristems resulted from the activity of ‘detached’ meristem remnants in the leaf axil (Garrison, 1955), more recent work has shown that, at least in

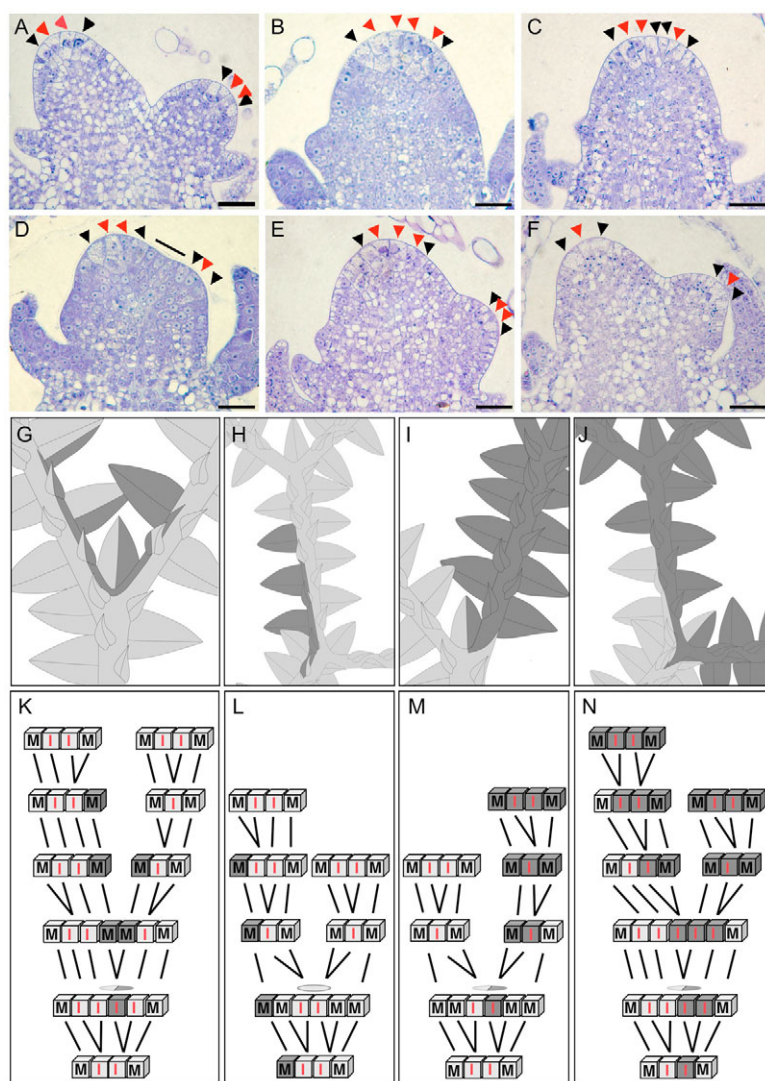


Fig. 6. Apical initial number varies during shoot growth and branching. (A-F) Frontal longitudinal sections through recently bifurcated meristems (A,E,F), the meristems of major branches that have initiated at least two (B) or three (C) leaf pairs, and an apex that has visibly begun to split into two (D). Arrowheads point to large epidermal cells that are deduced from sector analysis and *KNOX* and *ARP* in situ hybridisation patterns (Harrison et al., 2005) to be apical initials (red) or merophytes (black). Scale bars: A, 32 µm; B, 20 µm; C, 27 µm; D, 24 µm; E, 32 µm; F, 27.5 µm. (G-J) Schematic representation of sectors that span branch points. (K-N) Deduced cell division patterns giving rise to the sectors in G-J. The boxes represent the presumed number of initials and merophytes present in the surface layer. Light grey represents *au*+ tissue and dark grey represents *+*/*-* tissue. M, merophyte; I, initial. The ventral branch-point leaf is represented at the six-cell stage.

eudicots, axillary meristems form from the adaxial leaf surface (McConnell and Barton, 1998). Axillary meristems may be clonally related to the subtending leaf and internode as in *Arabidopsis thaliana* (Furner and Pumfrey, 1992; Irish and Sussex, 1992), or to the internode and leaf above as in maize (Johri and Coe, 1983; McDaniel and Poethig, 1988). Previous histological studies of *Selaginella* have similarly disputed whether apical initials contribute directly (Hagemann, 1980) or indirectly (Von Guttenberg, 1966) to branch meristem formation. In contrast to the mode of establishment of axillary meristems, and to suggestions from histological studies, we conclude that initials in both the major and minor branch SAMs are direct descendants of those in the parent SAM. This observation negates the need to invoke *de novo* initial specification during bifurcation, and highlights an important distinction between bifurcation and axillary branching.

Branching and theories of megaphyll evolution

The dominant theory of leaf evolution is the telome theory (Zimmermann, 1952), which suggests that megaphylls arose by overtopping, planation and webbing of subsets of shoots within a dichotomously branched shoot system (Kenrick, 2002; Zimmermann, 1952). As yet, few mechanisms have been proposed to explain how these processes may have operated. In *S. kraussiana*,

the bifurcating shoot system is unequal, with one branch overtopping the other and becoming the major axis. Within the context of the telome theory, the minor branch of the *S. kraussiana* shoot is therefore equivalent to a 'proto' megaphyll. The data presented here suggest that these inequalities in shoot growth result from differences in the number of apical initials inherited by daughter branches: major branches initiate growth from two apical initials, whereas minor branches originate from just one. Thus, overtopping, and hence megaphyll evolution, may have arisen from a simple mechanism whereby apical cell partitioning during branching resulted in fewer apical initials and reduced growth in side branches, relative to main branches.

Leaf initiation and axis formation

The data presented here demonstrate that the leaves of *S. kraussiana* initiate from two adjacent epidermal cells. These two cells first divide to extend the M-L axis, then to initiate the Ad-Ab axis and finally to establish the P-D axis, yielding a founder population of twelve or sixteen epidermal cells. One conspicuous distinction between *S. kraussiana* and seed plant leaves is therefore the number of founder cells recruited to form the primordium. In all seed plants in which clonal analyses have been performed, the number of leaf founder cells ranges from 100-200 (Dolan and Poethig, 1998; Korn,

2002; Poethig, 1984; Poethig and Szymkowiak, 1995). The 12-16 leaf founder cells in *S. kraussiana* are therefore more comparable to the number recruited to form floral organs in *Arabidopsis* (Bossinger and Smyth, 1996). This similarity may reflect the relatively small size of the *S. kraussiana* SAM and *Arabidopsis* floral meristems.

A further difference between *S. kraussiana* and seed plant leaves is seen in the order of axis development within the organ. The pattern deduced here for *S. kraussiana* differs from that previously suggested from histological analyses where the Ad-Ab axis (Wardlaw, 1957) or the P-D axis (Dengler, 1983) was thought to be established first. Interestingly, the pattern of *S. kraussiana* leaf initiation inferred here is strikingly similar to moss and liverwort gametophyte leaf development, where one and then two side-by-side cells undergo M-L and P-D divisions to give rise to a leaf with a single cell layer (Janzen, 1929). Fern scales reportedly form in the same way (Bower, 1935). The main developmental difference between moss gametophyte leaves, fern scales and *S. kraussiana* leaves, however, is the capacity of leaf founder cells in *S. kraussiana* to divide in a third plane that initiates the Ad-Ab axis, and then ultimately gives rise to the inner leaf layer (Dengler, 1983). Notably, in seed plant leaves the Ad-Ab layers are patterned from inception because leaf founder cells are recruited from both epidermal and sub-epidermal layers of the meristem. Thus, key differences between microphylls of *S. kraussiana* and megaphylls of seed plants are the number of meristematic cell layers recruited into the primordium and the consequent mode of axis establishment.

Theories of microphyll evolution

Because the lycophyte leaf cannot be easily interpreted in terms of the telome theory, three further theories have been advocated to explain their origin (Kenrick, 2002). The reduction theory suggests that lycophyte leaves were derived by reduction of flattened lateral branches or megaphylls (Zimmermann, 1959), whereas the enation theory states that leaves arose spontaneously from the stem as epidermal outgrowths (Bower, 1908), and the sterilisation theory suggests that leaves are modified sporangia (Crane and Kenrick, 1997; Kenrick, 2002; Kenrick and Crane, 1997). From a developmental perspective, the reduction theory implies a switch from indeterminate to determinate growth in a lateral branch, whereas the enation theory invokes the de novo introduction of a determinate developmental pathway in stem epidermal tissue, and the sterilisation theory implies a switch in branch fate within the context of an already determinate lateral organ (Kenrick, 2002).

Recent developmental studies have attempted to resolve which of the three theories of lycophyte leaf evolution is most plausible by examining expression patterns of genes that are orthologous to those involved in megaphyll development. *KNOX* and *ARP* gene expression patterns in the *S. kraussiana* apex support the reduction theory in that concomitant *KNOX* gene downregulation and *ARP* gene upregulation occurs in leaves of both microphyllous and megaphyllous species (Harrison et al., 2005). However, this observation may simply reflect the fact that both microphylls and megaphylls are determinate structures, and the *KNOX-ARP* interaction can be interpreted as regulating indeterminacy versus determinacy. Support for the enation theory has been argued from analysis of *S. kraussiana* HD-Zip gene expression patterns (Floyd and Bowman, 2006). There are two HD-Zip genes in *S. kraussiana*, one of which is expressed in the leaf and one in the stem vasculature (Floyd and Bowman, 2006; Floyd et al., 2006; Prigge and Clarke, 2006). Since the gymnosperm orthologue of the *Selaginella* 'stem

vasculature' gene is expressed in both the leaves and stems of representative seed plants, the authors favour the hypothesis that microphylls and megaphylls evolved through co-option of distinct developmental mechanisms, or at least distinct HD-Zip gene functions (Floyd and Bowman, 2006). However, this inference assumes that both gene paralogues have retained the function that they originally performed in the lineage that gave rise to *S. kraussiana*, yet there are many examples of paralogues switching function in other systems (Wray and Abouheif, 1998). With respect to the sterilisation theory, the genetic basis of sporangial development is unexplored. However, sporangia in leptosporangiate ferns initiate from single superficial cells in a manner reminiscent of leaf initiation in *S. kraussiana* (Gifford and Foster, 1989). It therefore remains a possibility that mechanistic support for the sterilisation theory may be forthcoming in the future. In combination, these observations suggest that despite the recent elucidation of aspects of leaf development in *S. kraussiana* (Floyd and Bowman, 2006; Harrison et al., 2005; Prigge and Clarke, 2006), the data obtained do not allow distinction between the existing theories of lycophyte leaf evolution. It might therefore be more beneficial to determine what distinguishes bifurcating shoot systems and megaphylls from epidermal protrusions such as lycophyte microphylls, moss gametophyte leaves and fern scales.

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