

## MECHANISMS OF LAMINAR GROWTH IN MORPHOLOGICALLY CONVERGENT LEAVES AND FLOWER PETALS

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Distinct leaf laminar growth dynamics have been found previously in some filicalean ferns and angiosperm seed plants: a discrete zone of marginal growth versus diffuse intercalary growth throughout the leaf without a conspicuous marginal growth zone. The fern pinnules for which marginal growth is known are also morphologically distinct from dicots: one or two orders of dichotomizing veins ending only along the margin versus many orders that include internally directed veins and endings dispersed throughout the leaf. Both of those morphological alternatives have evolved many times among extant and fossil plants, but whether similar developmental processes underlie each recurrence of a particular laminar morphology has not been investigated. Here, marginal growth is demonstrated in two independent examples of fernlike open venation patterns among living seed plants, frond leaflets of the cycad *Ceratozamia* and flower petals of *Ranunculus*. Thus, in these cases, morphological convergence is shown to reflect developmental convergence. Given the preponderance of simple, fernlike laminar morphologies among fossil and extant basal seed plants, angiosperm laminar development is likely to be as highly derived as their unusual laminar venation patterns.

*Keywords:* development, flower, leaf, Ranunculaceae, venation, Zamiaceae.

### Introduction

The existence of a common set of marginal and intercalary meristems that underlie leaf laminar growth across the megaphyllous vascular plants has been a frequent generality (Esau 1953; Bell 1991; and references therein). However, clonal analysis and whole-mount studies of the distribution of cell divisions in a series of dicot angiosperm leaves (*Nicotiana*: Poethig and Sussex 1985*a*, 1985*b*; *Gossypium*: Dolan and Poethig 1998; and references therein) indicate nonmarginal growth dispersed through the lamina without discrete meristematic zones, while whole-mount investigations of fern pinnules indicate a discrete marginal meristem without intercalary growth (*Adiantum* and *Cheilanthes*: Zurakowski and Gifford 1988). With this more recent work, the traditional expectation of a shared set of discrete intercalary and marginal meristems has been recognized as an artifact of reliance on longitudinal leaf sections when paradermal views are needed to discern overall growth dynamics correctly (Poethig and Sussex 1985*a*). Indeed, early uses of paradermal techniques (Foster 1952) today seem prophetic: growth in fern pinnules is marginal (*Nephrolepis*: Pray 1960; *Regnellidium*: Pray 1962), while any marginal growth in angiosperm leaves is early and of minor importance (*Liriodendron*: Pray 1955*a*; *Hosta*: Pray 1955*b*).

That mechanisms of laminar growth in the studied ferns and angiosperms are distinct need not be surprising because the leaf lamina of angiosperms and ferns are independently derived structures as confirmed by the fossil record (Boyce

and Knoll 2002) and discussed sporadically in contexts, ranging from vascular plant systematics, molecular development, angiosperm origins, and leaf evolution (Beck 1970; Doyle and Hickey 1976; Zurakowski and Gifford 1988; Kenrick and Crane 1997; Cronk 2001; Harrison et al. 2005). However, these distinct developmental processes may also account for the broad morphological differences between the groups: the ferns for which marginal growth has been demonstrated have one or two orders of marginally ending dichotomous veins, while the diffuse intercalary laminar growth of angiosperms is accompanied by many hierarchical orders of reticulating, often internally (rather than marginally) directed veins with free-ending internal veinlets (Doyle and Hickey 1976; Wagner 1979; Boyce and Knoll 2002). These characteristics may well reflect causal relationships by which distinct developmental alternatives lead to distinct venation architectures; however, their co-occurrence instead may be only a random association of independent characteristics, given the limited phylogenetic sampling. The nature of this relationship between growth and morphology can be explored across the living plants using the various independent evolutionary derivations of these morphologies for which growth dynamics are unknown (fig. 1).

Here, development is investigated in the leaflets of the cycad *Ceratozamia mexicana* and the flower petals of the angiosperm *Ranunculus repens*. Both have a single order of open dichotomous venation running to the distal margin, hypothesized to be the site of marginal growth. Because their frond laminae are not homologous (Boyce and Knoll 2002), marginal growth in cycad leaflets would necessitate a derivation independent from ferns. Ranunculales petals are thought to be stamen derived (Kosuge 1994); therefore, as laminate structures, they are homologous neither to other flower petals for which diffuse intercalary

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growth has been demonstrated (Rolland-Lagan et al. 2003) not to cyad or fern frond leaflets.

### Material and Methods

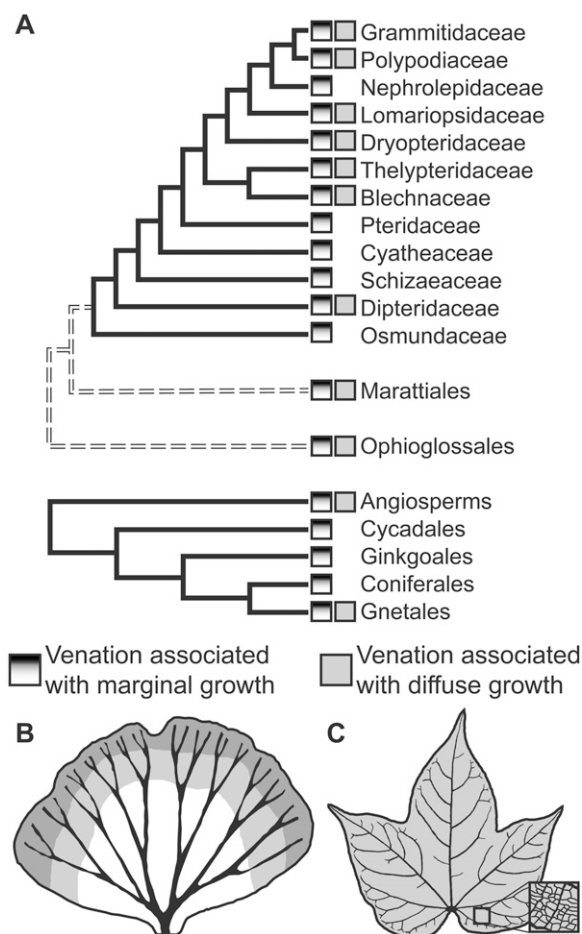
Patterns of cell division were assessed during laminar growth in whole-mount preparations of Feulgen-stained leaflets of greenhouse-grown *Ceratozamia mexicana* fronds and petals of field-collected (Cambridge, MA) *Ranunculus repens* flowers during earlier ontogeny. Individual petals and leaflets were dissected from bud or frond and fixed in Carnoy's solution for at least 2 d in advance of processing. Feulgen staining followed the description of Zurkowski and Gifford (1988), with the duration of HCl treatment during this process amended to reflect the relative robustness of the tissues: *Ceratozamia* leaflets and *Ranunculus* petals received 30 and 12 min, respectively. Sampled *Ceratozamia* leaflets ranged in length from 5.8 to 17.9 mm (full-grown length 35 cm), with leaflets from later ontogeny too large to be effectively stained and therefore unavailable for this study. *Ranunculus* petals analyzed in detail ranged from 1.8 to 3.2 mm (full-grown length 1.0–1.5 cm), with older petals (5.4 mm in length) consisting only of larger, undividing cells. Number of mitotic figures (with clearly distinct chromosomes and spindle orientation, i.e., metaphase through early telophase) per visual field was measured with the aid of a microscope eyepiece reticule along several transects, roughly perpendicular to the distal margin following the proximal-distal vein paths (figs. 2A, 3A). Divisions in both the adaxial epidermal and subepidermal cell layer were included in the count because they often would be difficult to distinguish correctly if counted separately. Density of epidermal cells was also determined along a medial transect of each specimen.

### Results

For both taxa, rates of cell division are highest along the distal margin and progressively decrease in more proximal tissues (fig. 4). A steep drop-off to near zero rates of cell division away from the margin is particularly pronounced in *Ranunculus* (fig. 2B). An immediate decline in division rate with distance from the margin also is seen in the smaller *Ceratozamia* leaflets, but a broader distal zone of meristematic activity is present in all *Ceratozamia* leaflets before declining toward zero more proximally (fig. 3B). Despite these differences in structural detail, the organs of both taxa demonstrate clear proximal/distal gradients and greatest growth along the distal margin. Medially and marginally placed transects equally support distal growth, indicating that the meristematic area is specifically the distal portion of the margin along which veins end, rather than a broader characteristic of the entire margin. Neither organ shows indication of the basipetal growth exhibited by most angiosperm leaf laminae with early termination of distal growth in favor of more proximal internal growth. Although maximum distal rates of cell division do decline through ontogeny, proximal rates in these larger structures also decline, so that the overall structure of elevated division rates in distal relative to proximal areas is maintained for both taxa (figs. 2B, 3B).

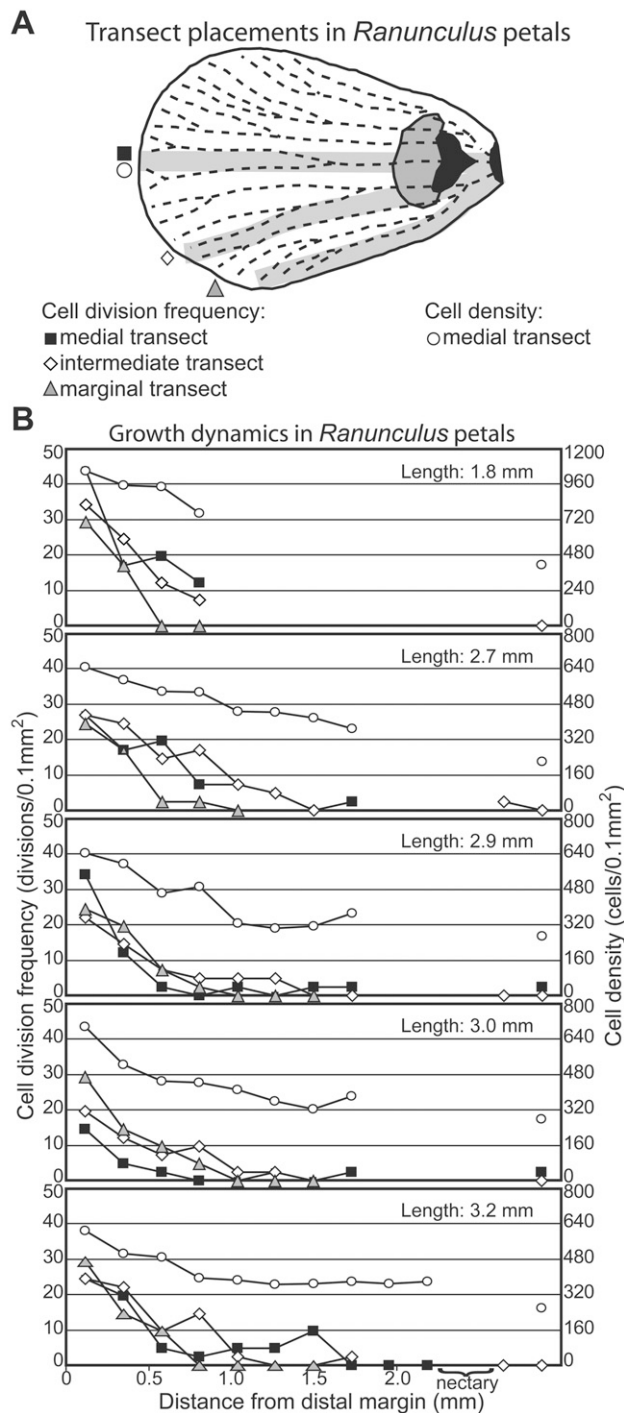
Epidermal cell size in *Ranunculus* increases markedly with distance from the distal margin (figs. 2B, 4A, 5A). *Ceratozamia*

does not show any clear gradient in overall cell density (except a zone of consistently smaller cells near the proximal point of attachment regardless of leaflet length, perhaps related to the imbricate vernation of young fronds that is not straightened out until later in ontogeny) (figs. 3B, 4B); however, this lack of



**Fig. 1** Phylogenetic distribution of laminar venation characteristics among filicalean fern families, Ophioglossales and Marattiales, and among seed plants (A). Several families of Filicales that do not influence the overall pattern have been omitted. Leaf laminae are independently derived in the seed plants and ferns. Whether the common ancestor of Marattiales and Filicales had laminate leaves is ambiguous, as is the case with these ferns and the Ophioglossales, which have a poor fossil record. Marginal growth has been demonstrated in several ferns with simple, marginally directed, dichotomous venation (B). In addition to many ferns, strictly marginal vein endings are found in cycads, *Ginkgo*, and at least two independent evolutions of laminate leaves among conifers: *Agathis* and *Aracauria* of the Araucariaceae and *Nageia* of Podocarpaceae. Such venation is also found among reproductive structures, including various flower petals and the fertile bracts of *Welwitschia*. Diffuse intercalary growth is found in angiosperm leaves, where it is associated with many hierarchical orders of reticulating veins and vein endings dispersed throughout the leaf (C). More angiosperm-like venation has evolved in the Gnetales among extant seed plants, in the eusporangiate ferns *Ophioglossum* and *Christensenia*, and at least eight times among leptosporangiate ferns. Shading in B and C indicates location of growth. Phylogenetic distribution of venation characteristics drawn from a previous study (Boyce 2005).

significant change in cell size belies distinct changes in cell shape: near isodiametric at the distal margin versus strongly elongated along the proximal/distal axis, with increasing distance from the distal margin (fig. 5B).



**Fig. 2** Transect paths (A) and division frequency/epidermal cell density (B) for individual *Ranunculus* petals. Left Y-axes indicate division rate for medial (squares), lateral (triangles), and intermediate (diamonds) proximal-distal transects. Right Y-axes indicate epidermal cell density along medial transects (circles). Measurements along medial and intermediate transects are interrupted by the nectary, as indicated.

## Discussion

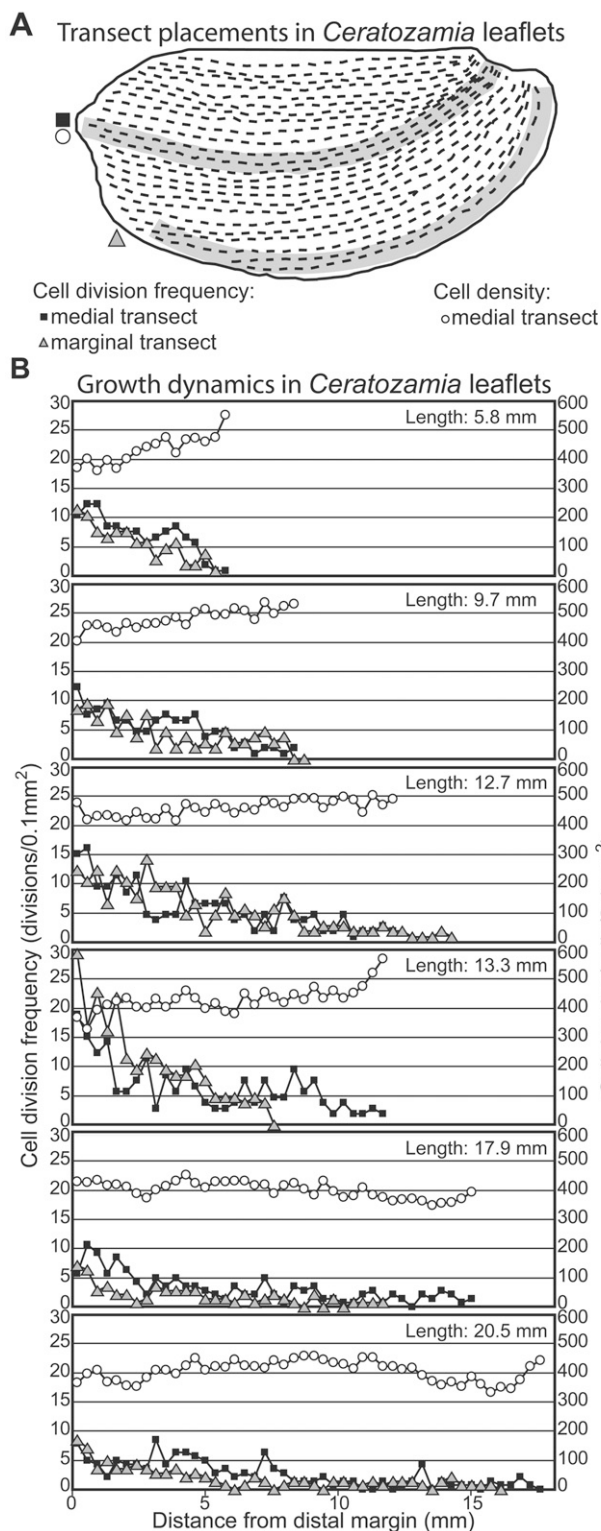
### *Comparisons of Marginal Laminar Growth in Ferns and Seed Plants*

For both cycad leaflets and buttercup flower petals, rates of cell division are highest at the distal margin and decline in more proximal tissues. Since cell division is a necessary part of cell differentiation (Sachs 1991), the low but persistent rate of division in proximal tissues is to be expected. Occasional concentrations of dividing cells in proximal tissues (more common in *Ceratozamia* than *Ranunculus*) show no regularity in their distribution, often represent a patch of closely adjacent cells, and appear to be no more than a part of the differentiation process. Such areas can lead to individual values within a transect that are elevated over the low background rate of proximal tissues but remain much lower than distal rates in the same organ. The *Ceratozamia* marginal meristem is a broader zone and possesses a larger population of dividing cells than with either the *Ranunculus* petals studied here or the clear marginal initials of a fern pinnule (Zurkowski and Gifford 1988). This may simply reflect the formation of a more robust, sclerified structure, due to either the differentiation of a more complex diversity of cell types or just the greater thickness and number of cell layers in the cycad than in the thin fern leaflets and ephemeral flower petals.

Cell division measurements along the lateral margins show similar patterns to medial transects with decreasing division rates in more proximal tissues, indicating a zone of growth specifically only in the distal fraction of the margin along which veins end. The lateral margins, like the rest of the leaf tissue, are a product left behind by distal growth. However, many ferns (and extinct seed plants) possess dichotomous veins ending along both distal and lateral margins, suggesting growth along all or most of the margins. Such morphologies probably derive from differential growth and dilation along a marginal meristem and often are associated with arched vein paths, asymmetric rates of vein dichotomy, or other hallmarks, suggesting such progressive modification of portions of the marginal meristem through ontogeny (Boyce and Knoll 2002). Understanding this diversity is not possible with the taxa chosen here specifically for their extreme simplicity. Regardless of exact morphology, however, this and other studies (Pray 1960, 1962; Zurkowski and Gifford 1988) support the location of vein endings as an indicator of the marginal fraction along which growth occurred.

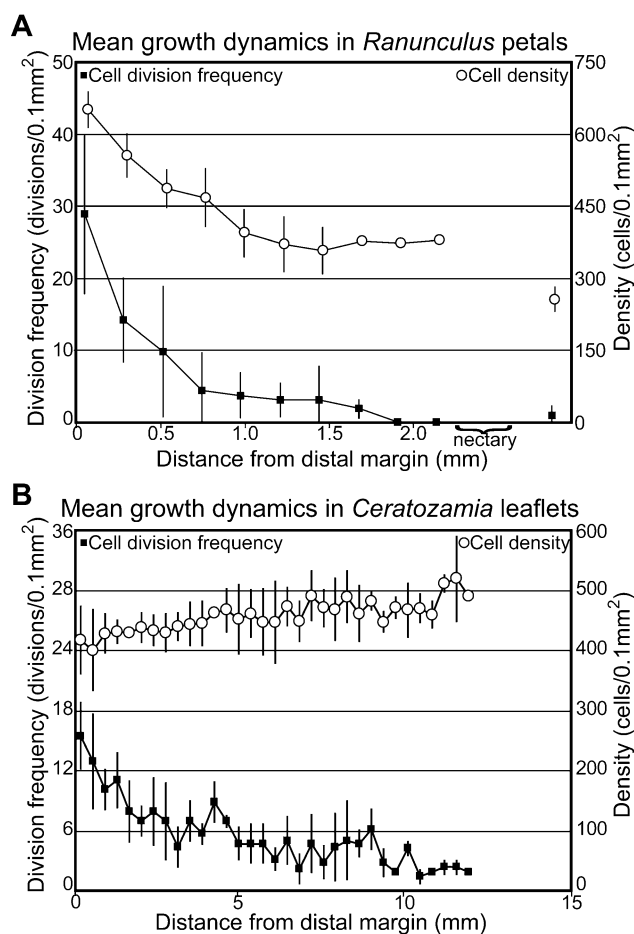
### *The Relationship between Growth Dynamics and Laminar Venation*

In axial systems, vascular differentiation and vein orientation occur along auxin gradients (Sachs 1991; Berleth et al. 2000) between distal auxin sources in growing areas and proximal auxin sinks that are actively maintained by polar auxin transport (Gälweiler et al. 1998; Blakeslee et al. 2005). Auxin also is a determinant of vasculature morphology in leaves (Sieburth 1999; Dengler and Kang 2001; Aloni et al. 2003; Scarpella et al. 2006), although work has been confined to angiosperms and no leaf with marginal growth has been investigated. In the dichotomously veined laminae studied here, the orientation of the vascular network toward and confinement of vein endings

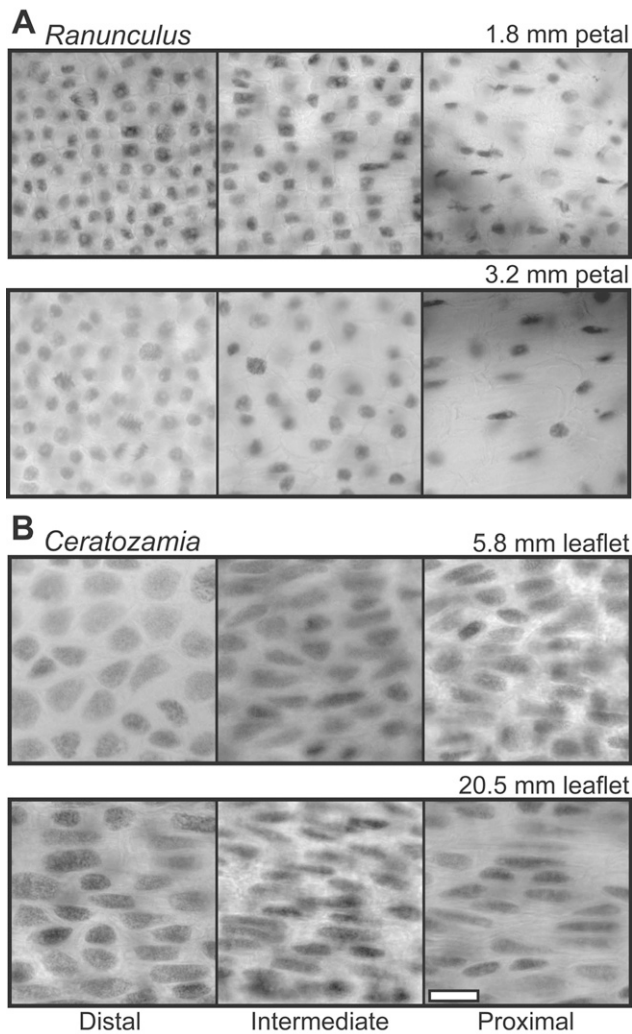


**Fig. 3** Transect paths (A) and division frequency/epidermal cell density (B) for individual *Ceratozamia* leaflets. Left Y-axes of graphs indicate division rate for medial (squares) and lateral (triangles) proximal-distal transects. Right Y-axes indicate epidermal cell density along medial transects (circles). The longer lateral margin was damaged during processing of the 13.3-mm leaflet, for which a transect of the shorter lateral margin is here substituted.

to the site of marginal growth presumably reflects vascular differentiation along a simple auxin gradient from the distal marginal growth zone, corresponding to the simpler arrangement of axial systems rather than to the more complex distribution of auxin sources and of growth in angiosperm leaves (Aloni et al. 2003; Scarpella et al. 2006) and perhaps the leaf laminae of other taxa with nonmarginal growth. Although most of the relevant taxa are phylogenetically distant from the advantages of model system plants, verification that marginal auxin production provides the organizing source for marginally ending vein networks may be available through studies involving auxin



**Fig. 4** Mean cell division frequencies (left Y-axes) and cell densities (right Y-axes) along medial transects for *Ranunculus* petals (A) and *Ceratozamia* leaflets (B). Bars indicate  $\pm 1$  SD. Since overall division rates change through ontogeny (figs. 2, 3), if standard deviations were applied to transect averages from structures of substantially different sizes, then the appropriate variance of multiple samples from the same underlying distribution of division rates from a single developmental stage would be conflated with the ontogenetic variance between organs of different ages. For example, the smallest *Ranunculus* petal analyzed conforms to the patterns seen in the larger petals (see fig. 2) but with much higher division rates and cell densities that would artificially inflate the variance between the similarly sized larger petals. For this reason, only the four *Ranunculus* petals between 2.7 and 3.2 mm in length and the three *Ceratozamia* leaflets between 9.7 and 13.3 mm in length are included here. *Ranunculus* transects are interrupted by the nectary, as indicated.



**Fig. 5** Representative images of epidermal cells of smallest and largest *Ranunculus* petals (A) and *Ceratozamia* leaflets (B) showing distal/proximal changes in cell size and shape. All images to same scale; bar = 20  $\mu$ .

antibodies (Aloni et al. 2003) or exogenous application of auxin or its transport inhibitors (Sieburth 1999; Poli et al. 2003).

Investigation of marginally growing organs must also be balanced with the study of *Gnetum* and appropriate ferns (fig. 1) that possess more angiosperm-like venation patterns to verify nonmarginal growth. Unlike the discrete end member case of strictly marginal growth, taxa with more angiosperm-like

venation may represent a more complex spectrum of growth dynamics. For example, qualitative observation of *Dipteris*, *Platycerium*, and several other ferns suggests an early phase of distal marginal growth, which leads to the dissected, dichotomizing morphology of these laminae and which is consistent with their marginally directed primary veins, followed by a period of intercalary growth creating a more angiosperm-like network of secondary veins. The single apical initial typical of fern axes (and frond primordia) prevents stable genetic chimeras (Bierhorst 1977) and limits the potential of clonal analysis investigations beyond the seed plants; however, the methods employed here can be applied broadly to test these suppositions.

#### *The Evolutionary History of Leaf Development*

With this study and the previous work with ferns (Pray 1960, 1962; Zurakowski and Gifford 1988), three independent evolutions of laminar structures with a marginally directed vein system have been found to involve marginal growth. This vascular architecture was the only known vein pattern for the first hundred million years of leaf evolution through the Devonian and Carboniferous, which includes at least four independent derivations of leaf laminae: the seed plants, progymnosperms, sphenophylls, and ferns (with the possibility of independent origins in the Filicales and Marattiales and perhaps additional instances in groups of obscure affinity such as the extinct Noeggerathiales). The results here suggest that each of these evolutions of a leaf lamina, including that of the seed plants, represents the evolution of marginal laminar growth. Thus, angiosperm laminar development is probably a highly derived condition distinct from that of ancestral seed plants, just as with so many other aspects of angiosperm biology. However, the distribution of angiosperm-like laminar venation among other taxa suggests that the diffuse intercalary growth of angiosperm leaf laminae is a derived state for which there may also be multiple independent evolutionary origins: in addition to several extant fern lineages and the Gnetales, similar venation characteristics evolved in the Permian gigantopterids and some Triassic taxa among fossil seed plants (Boyce 2005).

#### **Acknowledgments**

I thank A. H. Knoll, N. M. Holbrook, and M. A. Zwieniecki for helpful discussion and A. Zwieniecka for some sample preparation. Funding was provided by the National Science Foundation (EAR 0106816 to A. H. Knoll and N. M. Holbrook) and the Block Fund of the University of Chicago.

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