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ORIGINAL ARTICLE

KNOX overexpression in transgenic *Kohleria* (Gesneriaceae) prolongs the activity of proximal leaf blastozones and drastically alters segment fate

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Abstract *KNOX* (*knotted1-like homeobox*) genes have a widely conserved role in the generation of dissected leaves. Ectopic *KNOX* activity in leaves in various angiosperm lineages causes leaf form changes that can elucidate how the configuration of leaf development evolved. We present an analysis of leaf morphology and morphogenesis in transgenic *Kohleria* lines overexpressing a heterologous *KNOX* gene. *Kohleria*, like many members of Gesneriaceae, has simple-serrated leaves with pinnate venation. *KNOX* overexpression causes prolonged segment proliferation in proximal, but not distal, parts of leaf blades. Elaborate dissected segments reiterate the zonation of the whole leaf, with organogenic activity persisting between a distal maturation zone and a proximal intercalary elongation zone. The architecture of vascular bundles is severely altered, with a reduced midvein and a more palmate venation. The initial establishment of organogenically competent primordial margins (marginal blastozones) and the onset of tissue differentiation in early stages of leaf development were similar in wild-type and *KNOX* overexpressing lines. However, leaves overexpressing *KNOX* often failed to fully mature, and persistent marginal blastozones were found at the base of blades in mature portions of the shoot. We conclude that *KNOX*-mediated perpetuation of marginal blastozones in *Kohleria* is sufficient to induce a set of processes that result in highly dissected leaflets, which are unusual in this plant family. Spatial confinement of blastozones between an early maturing tip and a late elongating petiole zone reflects the presence of distinct maturation processes that limit the ability of the leaf margins to respond to ectopic *KNOX* gene expression.

Keywords Blastozone · Gesneriaceae · *KNOX* · *Kohleria* · Leaf dissection · Marginal maturation

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Introduction

Leaves are determinate organs that lose all meristematic activity when mature. However, dissected leaves are indeterminate during a limited period of development when they produce lateral structures. The primordial margins that are capable of initiating lateral growth axes are termed blastozones (Hagemann and Gleissberg 1996). Leaf blade segments forming along the margins of dissected leaves may develop into a range of mature structures, from serrations and lobes connected by common lamina tissue

("simple leaves") to completely individualized leaflets attached to a lamina-less rachis ("compound leaves"). In contrast, simple leaves with entire margins never produce lateral structures, possibly reflecting an early loss of blastozones. In addition to variations in the depth of lamina incision, the diversity in dissected leaf morphology results also from variable arrangement (pinnate or palmate) and sequence of initiation (basipetal or acropetal) of lateral structures.

KNOX1 (*class 1 knotted1-like homeobox*) genes have been important in the elucidation of the developmental pathways underlying the difference between dissected and entire leaves, and in understanding different morphologies of dissected leaves. Available data support a widely conserved requirement of *KNOX* genes for the maintenance of leaf dissection. This is reflected in a general correlation of dissected leaf form with *KNOX* gene expression during development in a range of lineages (Bharathan et al. 2002). Overexpression of *KNOX* genes increases the depth of incision and segment number in species with simple-serrated leaves (Hake et al. 2004), such as *Arabidopsis thaliana* (Lincoln et al. 1994) and *Lactuca sativa* (Frugis et al. 2001). Interestingly, simple leaves with entire margins do not respond in a similar way. *KNOX*-overexpressing tobacco (*Nicotiana tabacum*) leaves fail to produce regularly spaced segments (Sinha et al. 1993). Ectopic expression of the *knotted1* gene in maize (*Zea mays*) results in localized meristematic activity within the blade, but no marginal segments (Sinha and Hake 1994). This suggests that KNOX activity is unable to initiate a leaf dissection pathway, but does enhance an existing patterning program. In species with highly dissected and compound leaves, *KNOX* overexpression results in prolonged production of marginal segments and altered segment fate. Tomato (*Solanum lycopersicon*) plants overexpressing *KNOX* genes exhibit drastic increases in leaf dissection with primary leaflets profusely producing higher-order segments (Hareven et al. 1996). Similarly, *KNOX* overexpression results in supernumerary leaflets in *Cardamine hirsuta* (Hay and Tsiantis 2006). In these cases, the number of primary leaflets along the rachis remains relatively stable, and increase in dissection is mostly achieved through higher-order segments. This indicates that the retention of organogenic competence at leaf margins is limited to certain areas and developmental stages.

The developmental role of *KNOX* genes is linked to various phytohormones. Cytokinins are known to delay tissue maturation and are upregulated in plants overexpressing *KNOX* genes. In lettuce (*L. sativa*), increased lobing of *KNOX* overexpressing plants is accompanied by increased cytokinin levels (Frugis et al. 2001). Thus, *KNOX* gene function may in part be mediated by cytokinins (Hay et al. 2004). Tissue differentiation and maturation and the corresponding loss of organogenic competence are promoted by gibberellin (GA), a function that antagonizes KNOX function. GA biosynthesis is repressed by *KNOX* genes, and external application of GA reduces the excessive leaf dissection seen in *KNOX* overexpressing plants in some species (Tanaka-Ueguchi et al. 1998; Hay et al. 2002).

Another pathway that limits organogenic competence of primordial margins by promoting tissue maturation is enacted by *CINCINNATA*-like (*CIN*) TCP genes. In both, simple-entire and dissected leaves, *CIN* genes establish a basipetal wave of cell proliferation arrest that terminates organogenic competence (Ori et al. 2007). How *KNOX*-mediated maintenance of marginal blastozones interferes with these maturation pathways during leaf development to produce a species-specific leaf architecture is currently not well understood. Comparisons of *KNOX* overexpression phenotypes in morphologically different lineages can help to define how *KNOX* gene activity is integrated in leaf developmental programs, thus shedding light on the evolution of dissected leaves.

Gesneriaceae is a mostly tropical family in the Lamiales (Asterids I) in which simple-serrated leaves with a dense indumentum are common. Here, we investigate a sterile *Kohleria* hybrid that is of horticultural interest. It has serrated, pinnate leaves characteristic of many species in the family. Transgenic *Kohleria* lines overexpressing *KNAP1*, a *KNAT1*-like *KNOX* gene from apple, exhibit moderate to drastic leaf form modifications that were stable after multiple cycles of propagation via cuttings. The main focus of this study is to characterize the morphological responses of *Kohleria* leaves to ectopic *KNOX* gene expression. Therefore, morphological and developmental changes associated with transgene expression are described, focusing on spatial and temporal aspects of the establishment and maintenance of organogenic-competent margins. We infer that *KNOX*-mediated organogenic competence is confined by differentiation processes occurring early in the distal and later in the proximal regions of developing leaves. Our results indicate that simple-serrated leaves may be developmentally more similar to compound leaves than to simple-entire leaves.

Materials and methods

Plant material and cultivation

A sterile *Kohleria* clone designated B29 that resulted from a cross between *K. bogotensis* (G. Nichols.) Fritsch and *K. amabilis* (Planch. and Linden) Fritsch was propagated

via cuttings and grown at ambient light conditions, with shading during sunny days, in a greenhouse at the Geisenheim Research Center (Geisenheim, Germany). The heating was set to provide a minimum temperature of 20°C, and ventilation was done above 23°C. Transgenic plants harboring the *KNAP1* gene from apple (*Malus domestica*; Watillon et al. 1997) under the control of the CaMV-35S promoter were obtained by *Agrobacterium*-mediated transformation using internode explants cut from in vitro-grown microshoots of B29. Explants were first precultured for 1 day on shoot regeneration medium (SRM; Geier and Sangwan 1996) and then infected with *Agrobacterium tumefaciens* LBA 4404 harboring a derivative of plasmid p35S GUS INT (Vancanneyt et al. 1990) in which GUS + intron had been replaced by the *KNAP1* coding sequence (Watillon et al. 1998). After 3 days of co-cultivation on SRM supplemented with 50 μmol l^{-1} acetosyringyl β-glucoside (Joubert et al. 2004), the explants were thoroughly rinsed in deionized water containing 1 g l^{-1} cefotaxime and then transferred to SRM supplemented with 50 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime. Of several independent transformant lines regenerated in vitro and established in the greenhouse, two, designated T22 and T65, were used in this study.

RNA and genomic DNA blot hybridization

DNA blot hybridization was done using 10 μg each of KpnI-digested genomic DNA from WT, T22 and T65 plants per blot. A 1,009 bp probe of the *KNAP1* gene and a 730 bp probe of the *NPTII* gene were DIG-labeled (Roche Diagnostics, Mannheim, Germany) and used for detection following the manufacturer's instructions. RNA blot hybridization was performed using 20 μg samples of total RNA extracted from WT, T22 and T65 leaves and separated on formaldehyde–agarose gels. RNA in the gels was stained with ethidium bromide before transfer to verify that comparable loadings had been achieved. Blotting and hybridization with the *KNAP1* 32P-labeled probe were performed as previously reported (Watillon et al. 1997).

Morphological characterization

Several representative shoots from wild type, T22, and T65 with approximately six mature nodes were harvested for morphological observations and measurements. For each shoot, one leaf per node was detached and numbered from base to tip. To reveal the venation, leaves were briefly boiled in water and then incubated for 60 min in carnoy solution (42% ethanol, 30% acetic acid, 10% chloroform, 2% mercaptoethanol, by volume) under vacuum. For strongly pigmented leaves, carnoy incubation was repeated. Leaves were submerged in 70% ethanol in Petri dishes and their abaxial sides scanned at 600 dpi on a flatbed scanner.

For measurements of segment perimeters, several representative leaves from wild-type, T22, and T65 plants were scanned at 300 dpi. In Photoshop, images were converted to black-and-white silhouettes, and segment outlines were manually drawn on a new layer, leaving small gaps at segment tips and sinuses to separate basiscopic and acroscopic margins. Images containing the black-and-white contours were further processed in NIH image (http://rsb.info.nih.gov/nih-image/about.html). The segment contour lines were skeletonized to one pixel and then measured using the "analyze particles' function.

Scanning electron microscopy

To study leaf development, shoot tips of wild-type and T65 plants were dissected under a dissection microscope and fixed in 70% ethanol. Critical point drying, sputter coating and scanning electron microscopy were performed as described (Gleissberg 2004). Basal margins of older leaves were cut out and processed similarly. The analySIS software (Soft Imaging System, Münster, Germany) was used to measure the length of hair-free margins.

Gibberellin treatment

Wild-type and T65 *Kohleria* plants were grown from cuttings for 23 days before treatment. Ten plants each were sprayed with 50 or 200 μm GA3 solution (with 0.05% Tween 20) in weekly intervals for 6 weeks. Control plants were treated with Tween-containing water. Phenotypes were scored 24 days after the last treatment. Entire shoots were photographed, and series of detached leaves were scanned at 300 dpi.

Results

Leaf morphology in *KNOX*-overexpressing lines

Wild-type *Kohleria* plants have simple leaves covered with a dense indumentum, similar to many members of the Gesneriaceae. Leaves of the wild-type line are elliptical to ovate, up to 140 mm long, with pinnate venation and a conspicuous petiole (Fig. 1, left panel). The margins show a fine regular serration; however, the basal portions of the blade have smooth margins.

Compared to wild-type, T65 plants overexpressing an apple *KNOX* gene have smaller (up to 60 mm), highly dissected leaves (Fig. 1, right panel). Continued growth of basal blade portions results in an overall heart-shaped outline (leaves 4, 5 in Fig. 1, right panel), with further

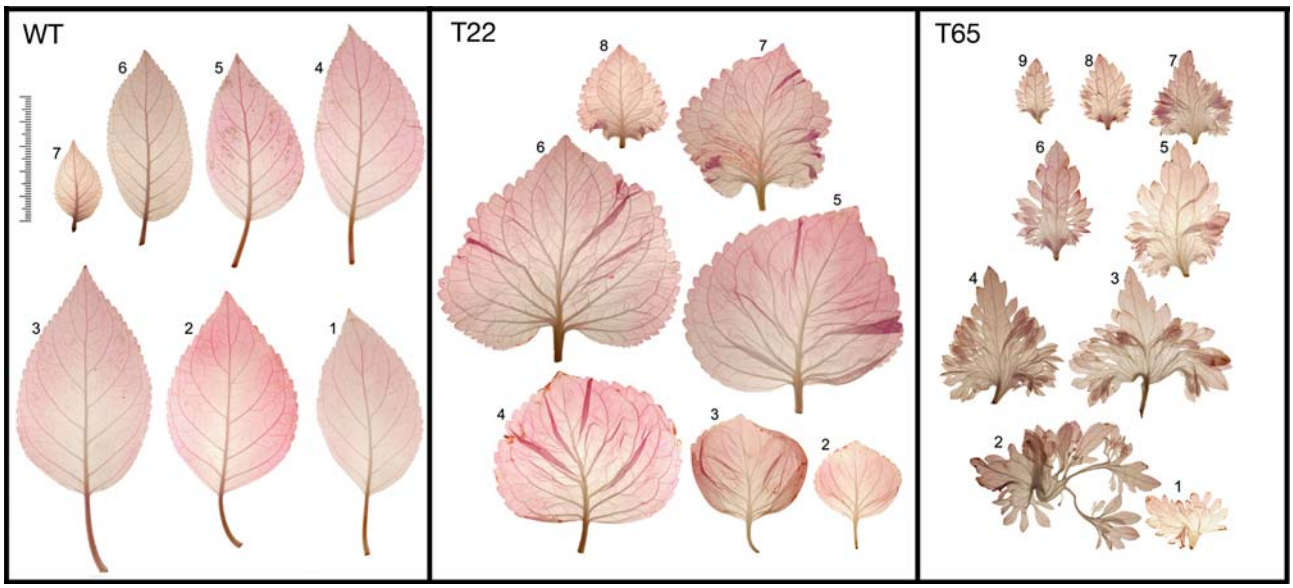

Fig. 1 *Kohleria* leaves at consecutive nodes from wild-type (*WT*, *left panel*), T22 (*center panel*), and T65 (*right panel*) shoots. Lowermost leaves (*number 1*) remain smaller, while upper leaves are still enlarging. *Scale bar* 5 cm

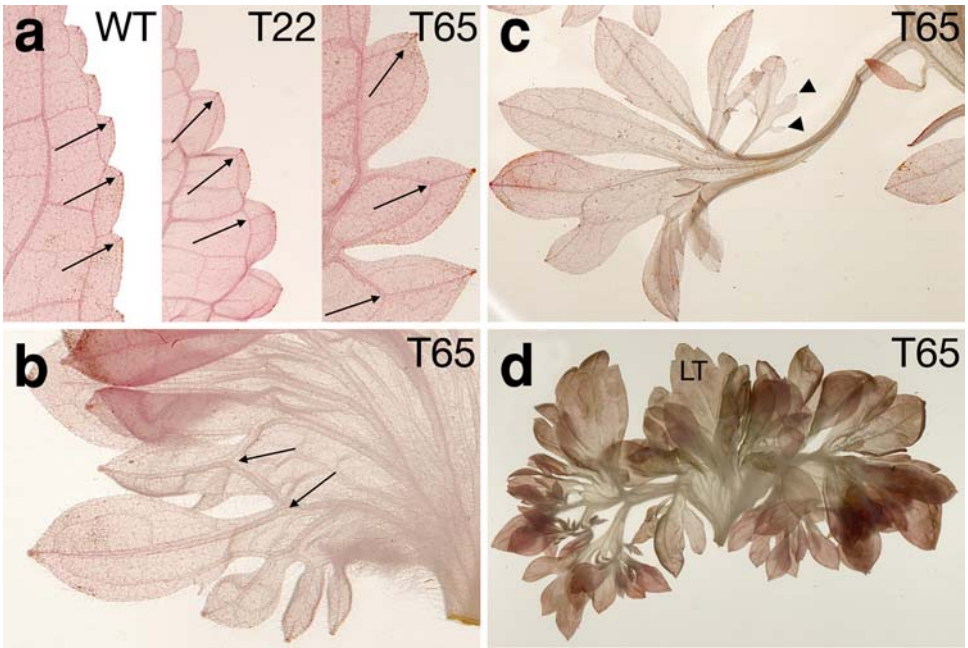

Fig. 2 Morphology of *KNOX*-overexpressing *Kohleria* leaves. **a** Comparison of margins in wild-type, T22 and T65 leaves in distal parts of the lamina. Note differences in incision depth, segment asymmetry and midvein length. **b** Proximal part of a T65 blade with developing leaflet-like segments. **c** Dissected primary segment with secondary and tertiary segments and petiolus. **d** T65 leaf with elaborate, highly dissected lateral portions. *Arrows* in **a** and **b** mark proximal ends of segment midveins, *arrowheads* in **c** mark tertiary segments. LT in **d** marks the leaf tip

growth producing highly elaborate basal parts that in some leaves exceed the length of the leaf. The petiole in T65 leaves remains short. Elaboration of basal blade portions is particularly strong in leaves below a decapitated shoot tip (Fig. 2d). Segments in the proximal blade area that have developed into highly branched complexes exhibit the same zonation along their distal–proximal axis as the entire leaf. Their distal, lobed portion develops into fully mature, broadly laminated, dark green tissue. The proximal part is conspicuously elongated, petiole-like, with strong veins and without conspicuous development of photosynthetic tissue. Marginal serrations do not occur in these proximal parts. In older leaves, this zonation may be established repeatedly, so that several organogenic centers may occur along the margins on each side of the leaf (leaf 2 in Fig. 1, right panel, Fig. 2d).

Wild-type leaves have a prominent midvein from which secondary veins branch off. These secondaries become considerably weaker and arch upward as they approach the margin (Fig. 1, left panel). Some weak tertiary branches near the margin connect to a distal secondary. At the margin, segment sinuses are connected by minor veins, from which a short branch runs into the segment tip (Fig. 2a, WT).

The midvein in T65 blades is shorter and less prominent, and similarly strong lateral veins emerge in a fan-like pattern from the petiole (Fig. 1, right panel, Fig. 2b, d). Secondaries remain strong close to the margin and tend to form prominent loops that are in contact with segment sinuses. Secondaries emerging at the base of the blade are densely arranged and branch halfway to the margins before they reach the segment sinuses. The close spacing of strong veins at the blade base results in only little chlorophyllous intercostal tissue. Cross sections revealed that palisade cells are rounder than in wild type and less well developed (not shown). At segment sinuses, prominent branches form the midveins of the leaflet-like structures (Fig. 2a, T65, b,c). Wild-type segments have very short midveins, similar to those near the leaf tip in T65 leaves. Toward the base of T65 leaves, midveins are longer until they extend into a petiole-like narrowed leaflet base (Fig. 2a, T65, b).

Leaves of an independent transformant line, T22, are intermediate between wild type and T65 (Fig. 1, center panel). The blade is broader than in wild type, with a more heart-shaped base and a shorter petiole. Serrations are similar to wild type, although larger, and no leaflet-like elaborations of the blade base were found. Instead, the basal most margins remain entire as in wild-type leaves, indicating a loss of organogenic activity in this area. Secondary veins arise from the midvein partly in a pinnate fashion, but in closer proximity, often approaching the fan-like pattern seen in T65. Secondary veins tend to form loops, which are less conspicuous than in T65. The length of segment midveins is intermediate between wild-type and T65 leaves (Fig. 2a, T22). Southern hybridization indicated that T65 has at least two insertions of the transgene, which might correspond to the stronger phenotype in T65 (Suppl. Fig. 1). However, Northern hybridization indicated a stronger transcription of the transgene in the milder T22 line (Suppl. Fig. 2).

To characterize changes in segment morphology associated with *KNOX* overexpression, we compared segment perimeter and segment asymmetry in distal, middle and proximal parts of representative leaf blades (Fig. 3). In WT, segments are small and of similar size throughout. T22 segments are slightly larger, while T65 segments are approximately five times larger than wild type in distal and central portions of the blade. Decreased size for T65 proximal segments is attributed to the immature nature of those segments that were not yet highly elaborated at the time of measurements. Segments in transgenic lines are also more symmetrical. In wild-type leaves, the basiscopic segment sides (oriented toward the leaf base) are about three to five times longer than the acroscopic sides (pointing toward the leaf tip) throughout the blade. In T65 and T22 plants, segments were highly asymmetrical only in distal blade parts, and were increasingly symmetrical toward the blade base. Proximal segments in T65 were nearly symmetrical.

Development of wild-type and T65 leaves

To identify developmental changes leading to the altered morphology of *KNOX*-overexpressing plants, we compared

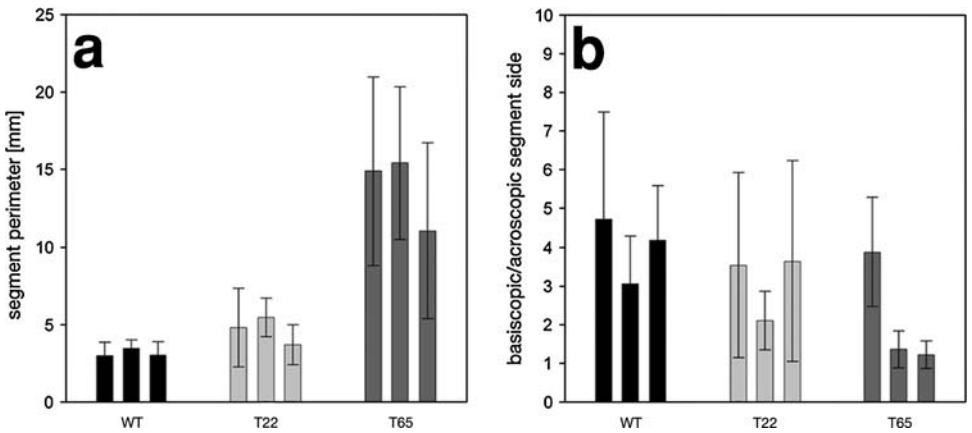

Fig. 3 Segment perimeter and symmetry in *Kohleria* leaves. **a** Segment size in 35S:*KNAP1* lines is slightly (*T22*, *light gray*) or strongly increased (*T65*, *dark gray*) in comparison to wild-type leaves (*WT*, *black*). **b** Segment symmetry is shown as the ratio of basiscopic to acroscopic segment margins. Segments in wild type and T22 are strongly asymmetric. In T65, distal segments resemble WT, but more proximal segments are more symmetrical. For each line, the three columns in **a** and **b** correspond to the distal, middle and proximal blade thirds, from *left to right*. Wild type and T22, $n = 4$; T65, $n = 7$ ±SD

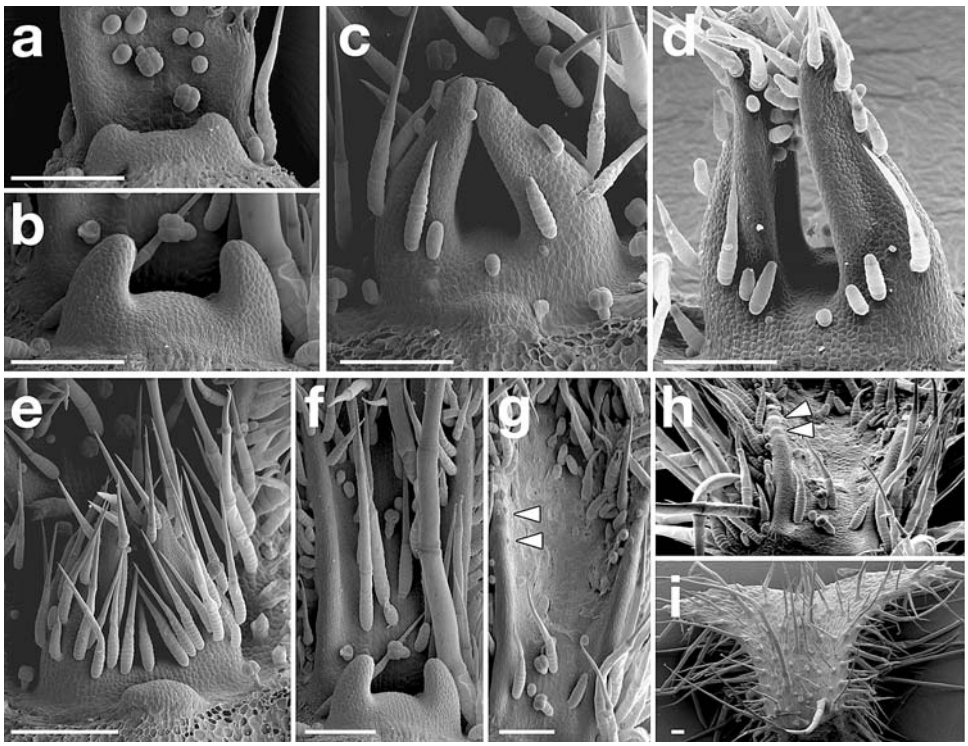

Fig. 4 Leaf development in wild-type *Kohleria* revealed by scanning electron microscopy. Note the progression of trichome development (**c**–**e**) soon after primordium emergence (**a**–**b**) and the persistence of trichome-free margins seen in adaxial view (**f**–**g**) from which serration primordia form (**g**–**h**). **i** Adaxial view of mature leaf lacking hair-free basal margins. *White arrowheads* in **g** and **h** mark segment primordia. *Bars* 100 μm

wild-type and T65 leaf development using scanning electron microscopy. Early stages of leaf development were found to be very similar (Figs. 4, 5). Leaf primordia arise in pairs in a decussate pattern from an almost flat shoot apical meristem. Ad-abaxial polarity is evident early from acrovergent curvature (Figs. 4a, b, 5a, b). Trichomes are indicative of tissue maturation and arise in a particular order. Trichome formation is first evident on the abaxial side, but soon trichomes appear also on the adaxial side, at the margins near the leaf tip and near the leaf base (Figs. 4c, d, 5c, d). Trichome formation then becomes concentrated in the distal portion of the leaf primordia, indicating the onset of a basipetal maturation process. The ultimate leaf primordium tip is marked by enlarged cells that do not develop trichomes. Trichomes near the leaf primordium bases form a line on the shoot axis that connects the two primordia (Fig. 4d, e), indicating early maturation at the leaf bases. Hair-free ridges are evident in both wild-type and T65 leaves at this time that are curved inwards (Figs. 4f, g, 5e). These ridges represent organogenic competent margins from which basipetal segment formation is initiated soon after (arrowheads in Figs. 4g, h, 5e–i). Basipetal segment initiation in this zone is accompanied by intercalary elongation of the leaf. As older segments are displaced toward the tip, they enter the histogenic zone, where marginal trichome buds mark the loss of organogenic competence.

To see if the increased size of mature segments in T65 plants and prolonged dissection corresponded to the size of the marginal blastozones, the length of trichome-free margins in primordia up to 5 mm was measured (Fig. 6). Between 0.5 and 5 mm length, blastozones in both lines range between about 200 and 450 μm in length. Therefore, different segment morphology in transgenic T65 leaves did not correspond to changes in the size of the organogenic zone or of emerging segments at this stage of leaf development. A dense cover of trichomes in leaves larger than 5 mm prevented the further observations of the leaf margins by scanning electron microscopy. However, inspection of basal blade portions of highly elaborate leaves in the mature portion of shoots revealed small-celled, trichome free blastozones, which actively produced segments in T65 leaves (Fig. 5i). These looked similar to those seen in leaf primordia close to the shoot apical meristem. Similar zones were not found in equivalent wild-type leaves (Fig. 4i).

To explore whether basipetal auxin flow within the shoot affected axillary meristem and leaf growth, we released growing shoots from apical dominance by decapitation. *Kohleria* grows with horizontal subterranean rhizomes that develop into erect, above-ground, elongated and leafy shoots. Following decapitation of above-ground shoots, T65 plants showed a delayed or inhibited development of axillary shoots below the decapitation site, while axillary buds of wild-type shoots were activated (Suppl. Fig. 3). Compared to wild-type, T65 shoots from underground rhizomes developed more frequently and grew more rapidly to establish new above-ground shoots following decapitation. Interestingly, leaves of decapitated

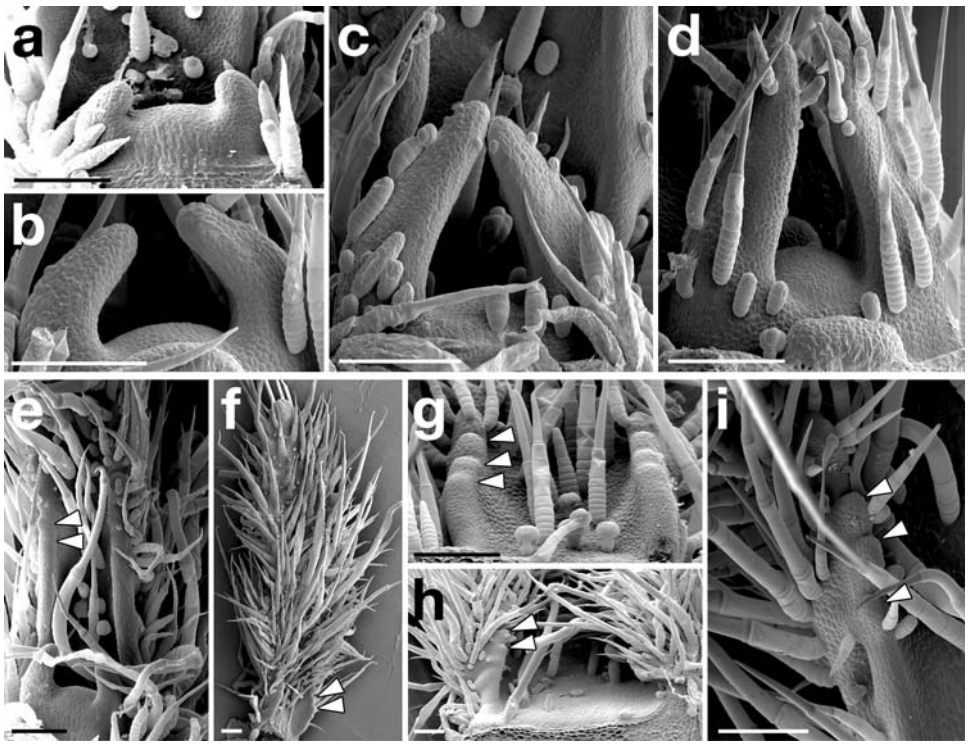

Fig. 5 Leaf development in *Kohleria* line T65 overexpressing a *KNOX* gene under the 35S promoter. After initiation (**a**–**b**), trichome-free margins are evident (**c**–**h**) from which segment primordia emerge (**f**–**h**). **i** Basal blade margin of an older leaf in which continued segment production is seen at the persistent trichome-free portion. *White arrowheads* in (**e**–**i**) mark segment primordia. *Bars* 100 μm

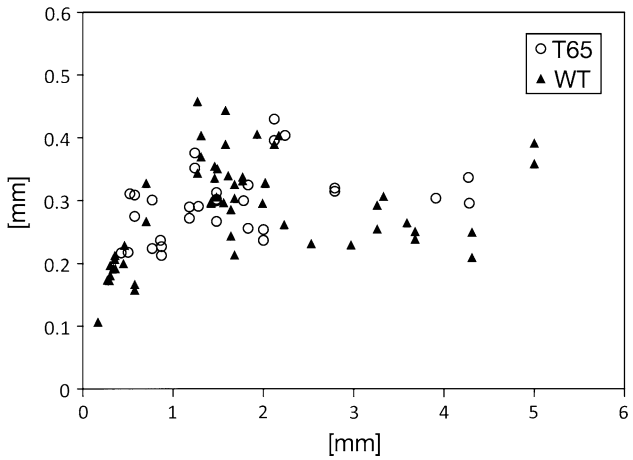

Fig. 6 Length of trichome-free margins during earlier stages of leaf development in growing wild type (*black triangles*) and T65 (*empty dots*). Between 1 and 5 mm, trichome-free margins in most leaves range between 200 and 400 μm

T65 shoots reacted with excessive elaboration of basal segments (Fig. 2d), indicating that the response of *KNOX*-maintained marginal blastozones to decapitation is similar to that of axillary meristems.

To investigate whether external application of GA, an antagonist to *KNOX* in other species, can rescue the wild-type leaf in *Kohleria*, we studied morphological effects after spray application of GA3 to wild-type and T65 plants. In both wild-type and T65 plants, internode elongation was significantly enhanced by GA3 treatment, demonstrating the sensitivity to this type of GA (not shown). Developing wild-type leaves examined 24 days after a six-week exposure were similar in shape to control plants (Suppl. Fig. 4, lower panels). T65 leaves exposed to exogenous GA did not have noticeably fewer segments and therefore did not appear to rescue the determinate nature of the wild-type leaf. However, basal segments in younger T65 leaves developed conspicuous, elongated petiolules that were not seen in control plants (Suppl. Fig. 4, upper panels). These petiole-like segment bases were similar to those developed in older, extremely elaborate T65 leaves in which extensive proliferation of the blade had taken place (Fig. 2c, d). This suggests that GA signaling may be activated at the base of T65 segments undergoing prolonged development, resulting in a leaflet petiolule. Similar signals may not be available in determinate wild-type segments.

Discussion

We demonstrate that overexpression of a heterologous *KNOX* gene in *Kohleria* is sufficient to drastically alter the developmental fate of leaf lateral segments from small, asymmetric, entire and determinate serrations to elaborate, symmetrical, dissected and petiolated leaflets supplied with a midvein. Developmental observations show that all these diverse lateral structures arise from initially similar marginal primordia that form in a basipetal sequence in a proximal area of growing leaves. Serrations and leaflets can

therefore be viewed as variants of a homologous pathway, in contrast to the usual distinction between simple (including simple-serrated and lobed) and compound leaves (Bharathan et al. 2002). One distinguishing feature separating simple-entire leaves and simple-serrated leaves may be that the former lack the activity of *CUP SHAPED COTYLEDON* (*CUC*) genes that are important in segment-sinus patterning in both compound and serrated leaves (Nikovics et al. 2006). The presence of *CUC* genes in the sinuses in Gesneriaceae leaves has not been demonstrated so far.

In *KNOX*-overexpressing *Kohleria*, prolongation of marginal organogenesis is confined to the proximal parts of the leaf blade. Although we cannot exclude the possibility that the 35S promoter drives expression of the *KNOX* transgene in a tissue-specific way, we suggest that the observed phenotype is due to the presence of a maturation-promoting pathway that limits the ability of the leaf margins to respond to KNOX in distal parts. We used the progression of trichome formation to mark tissue differentiation and maturation, and observed marginal differentiation beginning near the leaf tip and progressing basipetally in both wild-type and *KNOX* overexpressing plants. *CINCINNATA* (*CIN*)-like TCP genes are known to install a basipetal wave of maturation in *Antirrhinum majus* leaves and result in the delimitation of basipetal leaflet formation to proximal leaf parts in tomato (Nath et al. 2003; Ori et al. 2007). Hence, it is possible that this CIN-controlled pathway is conserved in other asterids and also controls the basipetal maturation and associated loss of organogenic activity observed in *Kohleria* (Efroni et al. 2008). However, 35S-directed expression of *KNAP1* in one transgenic line (T65) prevented this maturation process to extend to the basal portion of leaf blades where continued segment proliferation was observed. This suggests that tissue maturation can be overridden by excessive KNOX activity.

We also noted that leaf segments near the leaf tip in T65 plants and all segments in the milder transgenic line T22 grow to larger sizes than in wild type, but remain entire and undissected. Therefore, a milder effect of *KNOX* activity here is the promotion of segment growth, possibly by prolongation of cell proliferation, in the absence of organogenic capacity at the growing segment margins. Taken together, serrations in wild-type and dissected leaflets in T65 are connected by intermediate forms. Along this morphocline, increasing elaboration first results in a larger segment perimeter, increased segment symmetry and development of a segment midvein. These changes indicate a stronger growth axis of the lateral segments relative to growth along the main leaf axis. Further elaboration is associated with segment dissection and the establishment of a longitudinal zonation between the mature distal and a petiole-like basal part.

Auxin accumulates at the leaf tip as well as at leaflet and segment tips in *C. hirsuta* (Barkoulas et al. 2008) and *Pisum sativum* (DeMason and Polowick 2009), and is known to negatively regulate *KNOX* genes. Thus, this phytohormone may also contribute to the establishment of mature leaf tips in wild-type *Kohleria*, and also to mature leaflet tips in T65 plants. Further investigations are needed to clarify if, and to what degree, CIN and auxin promote maturation in the distal parts, thereby restricting KNOX activity to more proximal areas.

We observed that removal of the shoot tip enhanced segment proliferation in T65 leaves, while activation of axillary buds was reduced in comparison to decapitated wild-type plants. Shoot decapitation is known to disrupt basipetal auxin flow and to enable cytokinin-promoted outgrowth of axillary buds (McSteen 2009). This suggests that in the presence of KNOX, marginal blastozones respond to shoot-borne signals such as auxin and cytokinin that usually control the development of axillary meristems. A more systematic study of this aspect of KNOX activity is needed to corroborate this assumption.

Organogenic activity in *KNOX*-overexpressing *Kohleria* leaves becomes confined to a few segments at the very base of the leaf blade that produce higher-order segments. The growth axes of these elaborately dissected segments establish the same longitudinal zonation characteristic of the main leaf axis, with a broad, serrated, early maturing tip region and a proximal blastozone region with continued segment production. In addition to early maturation of distal portions of the leaflet-like segments in T65 leaves, a narrowed, elongated, petiole-like zone develops proximal of the blastozone. Intercalary elongation in this zone is not accompanied by segment formation, indicating a loss of organogenic capacity. These observations led us to suggest that segment production may be spatially restricted by two distinct processes: maturation starting early at the leaflet tip, and intercalary elongation that occurs later during ontogenesis in the most proximal part. We speculate that both processes also act in wild-type *Kohleria* to limit the basipetal production of simple serrations, and that *KNOX* overexpression is capable of maintaining organogenic centers in between the two zones.

It is notable that elaborate segments form a narrowed, petiole-like base, similar to leaflet petiolules in species from other families, but unusual for Gesneriaceae. How this zone of intercalary growth is established is generally not understood. We observed that these narrow segment bases in T65 plants formed earlier in plants subjected to GA3, suggesting that gibberellin promotes petiolule elongation in the highly elaborate T65 leaflets. Gibberellins promote cell elongation and tissue maturation and antagonize the organogenic activity of *KNOX* gene activity in tomato and other species (Hay et al. 2002; Jasinski et al.

2008). Hence, GA-mediated petiole elongation may also prevent further organogenesis at the elongating petiolule margins. Interestingly, GA application did not induce petiolule development in wild-type leaves, suggesting that sufficient prolongation of segment development, mediated by ectopic KNOX activity, is required to establish this intercalary elongation zone in *Kohleria*. Since *KNOX* genes are known to negatively regulate GA biosynthesis, it is not known how constitutive *KNOX* activity would permit GA-mediated petiolule elongation. We speculate that some KNOX functions, such as the repression of GA biosynthesis in blastozones, may require the expression of other factors, such as BEL-like homeobox proteins that may not be available in petiolus-forming areas of the growing leaf (Chen et al. 2004; Rutjens et al. 2009). The significance of protein multimerization in region-specific KNOX function is currently not well understood.

Indeterminate leaf development by means of a basal meristem has evolved in some *Streptocarpus* species and possibly independently in other Gesneriaceae, such as *Monophyllaea*. It has been proposed that *KNOX* genes are implicated in these evolutionary novelties (Harrison et al. 2005). In *Streptocarpus wendlandii*, trichome-free blastozones are found at the base of the phyllomorphs that indeterminately produce serrations in a basipetal manner (S.Gleissberg, unpublished observations). Wild-type *Kohleria* leaves are similar in that marginal serrations are produced basipetally. Interestingly, prolonged segment production in proximal parts of *Kohleria* T65 leaf blades did not prolong serration formation in a way that would result in indeterminate, pinnately veined leaves as in *S. wendlandii*.

The dramatic transformation of simple serrations into dissected leaflets observed here suggests that Gesneriaceae may have retained developmental programs necessary to make highly dissected leaves. On the whole, serrated leaf margins are very common in Gesneriaceae, but more deeply dissected leaves occasionally also occur (A. Weber, personal communication). Some species in *Ridleyandra*, such as *R. quercifolia* and *R. morganii* and *Henckelia pectinata* have deeply lobed leaves that resemble enlarged serrations (Weber and Burtt 1998; Weber and Skog 2007 onward). Although secondary serrations also occur in other Gesneriaceae species, highly dissected and petiolated leaflets as in *Kohleria* T65 plants are, to our knowledge, not found in extant species of this family.

In addition to leaf dissection, *KNOX* overexpression affects venation architecture and overall leaf shape. T65 *Kohleria* leaves have a reduced main leaf axis with a shorter midvein and petiole. Equally strong lateral veins arise from the short petiole in a fan-like pattern. Examination of early stages of leaf development (Figs. 4, 5) indicated that such a transition from median to lateral growth is not initiated at early stages before leaves reach a length of 5 mm. Frugis et al. (2001) have suggested that similar changes in venation architecture in *L. sativa* may be due to an antagonistic interaction in the developing leaf vein system between basipetal auxin fluxes and cytokinin.

Leaf axis reduction, elaborate lateral parts and a dense, fan-like arrangement of veins at the blade base have been observed in leaves of other species with *KNOX* overexpression. For example, ectopic expression of the *C. hirsuta BREVIPEDICELLUS* (*BP*) gene at the base of developing leaves in *ASYMMETRIC LEAVES 1* (*AS1*) silenced plants correlates with a strong repression of growth of the rachis, resulting in the formation of leaflets from the base (Hay and Tsiantis 2006). In dandelion (*Taraxacum officinale*), the basal leaflet-like segments of plants overexpressing a *KNOX* gene are the largest, in contrast to wild-type leaves (Müller et al. 2006). Potato (*Solanum tuberosum*) leaves overexpressing the endogenous *POTH1* gene exhibit a switch from pinnate to palmate vein architecture (Rosin et al. 2003).

Reduction of the leaf axis and broadening of the blade base also occurs in species with entire leaf margins in which *KNOX* overexpression does not elicit segmentation. Examples include *N. tabacum* (Suppl. Fig. 5) and *A. majus* (Golz et al. 2002). We conclude that enhancement of leaf dissection and the shift from axial to lateral growth are two distinct processes mediated by KNOX overexpression. It is possible that palmate venation, seen in species with both dissected and entire leaves, evolved through enhanced *KNOX* gene activity at the blade base.

Enhancement of leaf segmentation through constitutive KNOX activity appears independent of whether or not *KNOX* genes participate in serration formation in wild-type leaves. Serration formation in *Streptocarpus*, another member of Gesneriaceae, is accompanied by marginal activation of an *STM*-like *KNOX* gene (Harrison et al. 2005; Mantegazza et al. 2007). Therefore, it is possible that endogenous *KNOX* genes may regulate serration formation in wild-type *Kohleria* leaves as well. In contrast, in *Arabidopsis*, *KNOX* genes are repressed during serration formation, in part by activation of BEL1-like homeobox proteins in leaves (Kumar et al. 2007). Still, ectopic KNOX activity leads to enhanced leaf lobing in this species (Lincoln et al. 1994). Future studies should particularly address possible lineage-specific requirement of *KNOX* expression during leaf serration formation.

The various phenotypic facets of *KNOX* overexpression in different species underline the central role of these genes in leaf development. Lineage-specific effects reflect evolutionary changes in interactions between KNOX and other pathways. As these interactions become better characterized in well-studied systems, the morphological evolution in Gesneriaceae and other families can be better assessed. From

the developmental analyses presented here, we propose that two distinct processes, distal maturation and proximal petiolule elongation, may restrict KNOX-mediated organogenic activity to marginal blastozones at the proximal ends of the leaf blade. As segments in this zone develop into elaborate leaflets, they establish the same distal/proximal zonation seen in the entire leaf. This indicates that constitutive expression of *KNOX* genes cannot override these maturation-promoting processes, except at the blade base. Studies from other species point to *CIN*-like TCP genes, auxin and gibberellins as likely components of *KNOX* antagonizing processes. Further studies in species with differing leaf architecture are needed to better define these maturation processes, and how they contribute to the spatial and temporal restriction of leaf margin organogenesis.

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