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Characterization of *Linaria KNOX* genes suggests a role in petal-spur development

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SUMMARY

Spurs are tubular outgrowths of perianth organs that have evolved iteratively among angiosperms. They typically contain nectar and often strongly influence pollinator specificity, potentially mediating reproductive isolation. The identification of *Antirrhinum majus* mutants with ectopic petal spurs suggested that petal-spur development is dependent on the expression of *KNOTTED 1*-like homeobox (*KNOX*) genes, which are better known for their role in maintaining the shoot apical meristem. Here, we tested the role of *KNOX* genes in petal-spur development by isolating orthologs of the *A. majus KNOX* genes *Hirzina* (*AmHirz*) and *Invaginata* (*AmIna*) from *Linaria vulgaris*, a related species that differs from *A. majus* in possessing long, narrow petal spurs. We name these genes *LvHirz* and *LvIna*, respectively. Using quantitative reverse-transcription PCR, we show that *LvHirz* is expressed at high levels in the developing petals and demonstrate that the expression of petal-associated *KNOX* genes is sufficient to induce sac-like outgrowths on petals in a heterologous host. We propose a model in which *KNOX* gene expression during early petal-spur development promotes and maintains further morphogenetic potential of the petal, as previously described for *KNOX* gene function in compound leaf development. These data indicate that petal spurs could have evolved by changes in regulatory gene expression that cause rapid and potentially saltational phenotypic modifications. Given the morphological similarity of spur ontogeny in distantly related taxa, changes in *KNOX* gene expression patterns could be a shared feature of spur development in angiosperms.

Keywords: evolution, flower development, *Linaria vulgaris*, *KNOX* genes, petal spur, petal shape.

INTRODUCTION

Plants are characterized by indeterminate vegetative growth, which is dependent on the maintenance of a pluripotent stem-cell niche in the shoot apical meristem (SAM). These cells are preserved throughout the lifetime of the plant by expression of the class-I *KNOTTED 1*-like homeobox (*KNOX*) genes (Vollbrecht *et al.*, 1991; Smith *et al.*, 1992; Lincoln *et al.*, 1994; Long *et al.*, 1996; Vollbrecht *et al.*, 2000). By contrast, lateral organs derived from the SAM are determinate, often requiring the suppression of *KNOX* expression (Smith *et al.*, 1992; Jackson *et al.*, 1994).

Studies in an expanding variety of species have revealed surprising additional roles for *KNOX* genes in lateral organ development. The best described of these roles is complex leaf morphogenesis. Ectopic expression of *KNOX* genes in simple-leafed species such as *Arabidopsis thaliana* (L.) Heynh., *Zea mays* L. (maize) and *Nicotiana tabacum* L. (tobacco) produces dramatically lobed and super-compounded leaf morphologies (Vollbrecht *et al.*, 1991; Sinha *et al.*, 1993; Lincoln *et al.*, 1994; Chuck *et al.*, 1996). In contrast, artificially reducing the levels of *KNOX* expression in lobed and compound-leafed species results in plants with simple leaves (Piazza *et al.*, 2010). These observations are supported by a strong positive correlation between *KNOX* re-expression in developing leaf primordia and complex leaf morphology in a broad range of angiosperm taxa (Hareven *et al.*, 1996; Bharathan *et al.*, 2002; Hay and Tsiantis, 2006; Piazza *et al.*, 2010). However, this correlation is not perfect and exceptions have been documented (Hofer *et al.*, 2001; Bharathan *et al.*, 2002). Work on *Solanum lycopersicum* L. (tomato; Shani *et al.*, 2009, 2010) and *Cardamine hirsute* L. (Hay and Tsiantis, 2006; Barkoulas *et al.*, 2008) suggests that the re-activation of *KNOX* expression in leaf primordia

facilitates leaflet formation by maintaining a state of prolonged indeterminacy and morphogenetic activity in a highly context-dependent and dose-dependent manner (reviewed extensively in Hay and Tsiantis, 2009; Canales *et al.*, 2010; Hay and Tsiantis, 2010).

An unexpected role for *KNOX* genes was indicated in two dominant gain-of-function mutants of the common snapdragon, *Antirrhinum majus* L.: namely *Hirz*-d153 (Figure 1a) and *Ina*-d1. A duplicated corolla tube resembling the floral petal spur in closely related genera was caused by the ectopic expression of the *A. majus KNOX* genes *Hirzina* (*AmHirz*) and *Invaginata* (*AmIna*) in the corolla (Golz *et al.*, 2002). Spurs are tubular outgrowths, most commonly of perianth organs (sepals and/or petals); they have evolved independently in a wide range of angiosperm taxa (Weberling, 1992; Hodges and Arnold, 1995; Hodges, 1997; Hodges *et al.*, 2004; Endress and Matthews, 2006), where they function to increase the distance between a perceived (Cozzolino and Widmer, 2005) or real floral reward (nectar) and the reproductive parts of the flower. This increased distance limits the morphologies of animals that can access any food reward, and increases the likelihood of their bodies physically contacting the reproductive structures, thereby improving the chances of pollen transfer. Accordingly, petal-spur morphology is putatively tied to pollinator specificity, and petal spurs are frequently considered to represent a key innovation promoting high species diversity (Hodges and Arnold, 1994, 1995; Hodges, 1997; Whittall and Hodges, 2007).

Identification of the *Hirz*-d153 (Figure 1a) and *Ina*-d1 *A. majus* mutants indicated that petal-spur evolution in Antirrhineae (Plantaginaceae *sensu stricto*; Lamiales) may have occurred via the co-option of *KNOX* activity from the SAM to fulfil a novel biological role in the flower (Golz *et al.*, 2002). Recycling genes from existing developmental pathways in this way is also common among animals, and has become a central concept in our understanding of the evolution of biological novelty (Carroll *et al.*, 1994; Keys *et al.*, 1999; Weatherbee *et al.*, 1999; Monteiro and Podlaha, 2009). Although identifying causative alleles in mutants advances our mechanistic understanding of how new morphologies could arise, such mutants do not constitute definitive evidence that alterations at the same locus are involved in generating new morphologies in nature. However, they do provide a good starting point for comparative studies. Many close relatives of *A. majus* have a ventral petal spur located at the base of the corolla tube (Sutton, 1988; Hodges and Arnold, 1995), including common toadflax, *Linaria vulgaris* (L.) Mill, (Figure 1b).

Despite uncertain phylogenetic relationships among genera (Ghebrehiwet *et al.*, 2000; Oyama and Baum, 2004; Vargas *et al.*, 2004; Albach *et al.*, 2005), *Linaria* is consistently placed in phylogenetic analyses as a spur-bearing close relative of *Antirrhinum* (Figure 1c,d) that has been

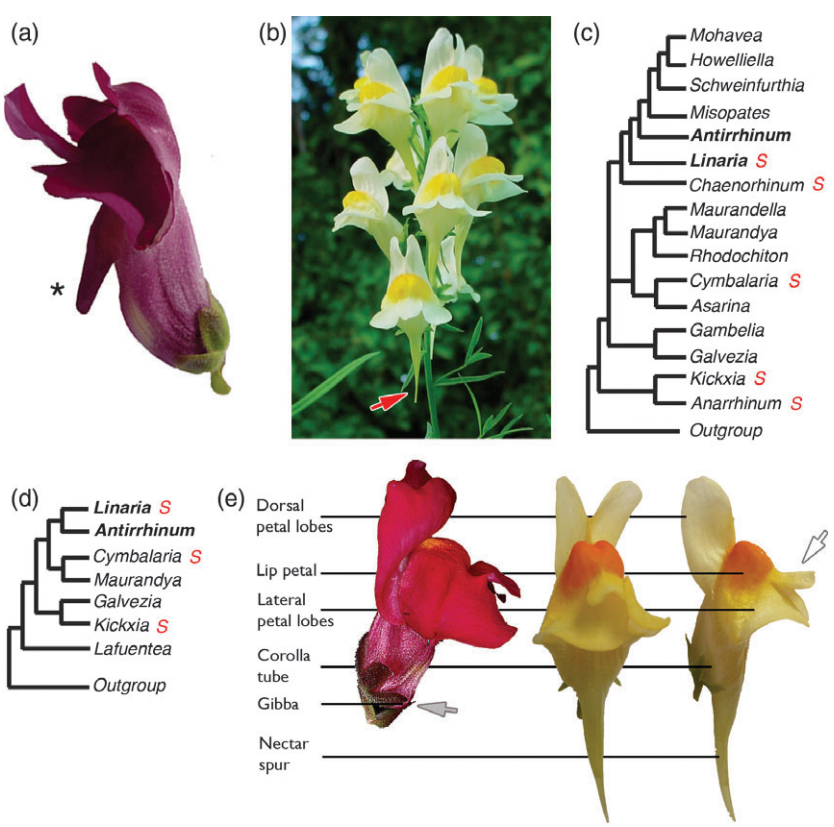

Figure 1. *Antirrhinum majus Hirz*-d153 flowers and the petal spur of *Linaria vulgaris*.
(a) Ectopic petal tube (asterisk) of *A. majus Hirz*-d153.
(b) Petal spurs (red arrow) of *L. vulgaris*.
(c) Combined morphological and molecular *ndh*F analysis of Antirrhineae (Ghebrehiwet *et al.*, 2000).
(d) Antirrhineae *mat*K-*trn*K intron strict consensus (Albach *et al.*, 2005). '*S*' in panels (c) and (d) indicate taxa possessing petal spurs.
(e) Gross floral morphology highlighting the *A. majus* gibba (grey arrow) and *L. vulgaris* petal spur; white arrow indicates the ventral petal lobe.

shown to be amenable to laboratory analysis (Cubas *et al.*, 1999; Hileman and Baum, 2003; Galego and Almeida, 2007). Thus, *L. vulgaris* provides a valuable opportunity to investigate the role of *KNOX* genes in a closely related species with petal spurs. If *KNOX* genes have been co-opted for petal-spur development in Antirrhineae, this event should be reflected in the localization and function of KNOX proteins in *L. vulgaris*.

Here, we describe petal-spur ontogeny and morphology in *L. vulgaris*. We have isolated the *L. vulgaris* orthologs of the *A. majus KNOX* genes *AmHirz* and *AmIna,* which we name *LvHirz* and *LvIna*, respectively. Using quantitative reverse-transcription PCR (qRT-PCR), we describe the pattern of *LvHirz* and *LvIna* expression, and characterize the function of the corresponding proteins (and their orthologs from *A. majus*) by constitutive expression in transgenic tobacco. We conclude that *KNOX* expression is associated with petal-spur development in *L. vulgaris*, and demonstrate that the expression of petal-associated *KNOX* genes is sufficient to induce sac-like outgrowths on petals in a heterologous host.

RESULTS

Petal-spur ontogeny in *L. vulgaris*

Floral morphology in *L. vulgaris* resembles that of *A. majus* (Figure 1e). The corolla consists of five petals that are fused proximally to form a deep tube with distally free lobes that close the entrance to the flower. The most significant morphological difference between the flowers of *A. majus* and *L. vulgaris* is located at the base of the corolla tube, which is distinguished by a sac-like gibba and a long, narrow petal spur, respectively. Both the gibba and spur accumulate a pool of nectar derived from a gynoecial disc nectary.

The early ontogeny of *L. vulgaris* flowers broadly resembles that described for *A. majus* (Vincent and Coen, 2004) and other Lamiales (Bello *et al.*, 2004), allowing us to adopt the phases previously outlined for *A. majus* (Vincent and Coen, 2004). Phase A describes the initiation of the inflorescence apex (Figure 2a). In *L. vulgaris*, floral zygomorphy (bilateral symmetry) is evident during the initiation of the floral apex (phase B), when five sepal primordia are initiated

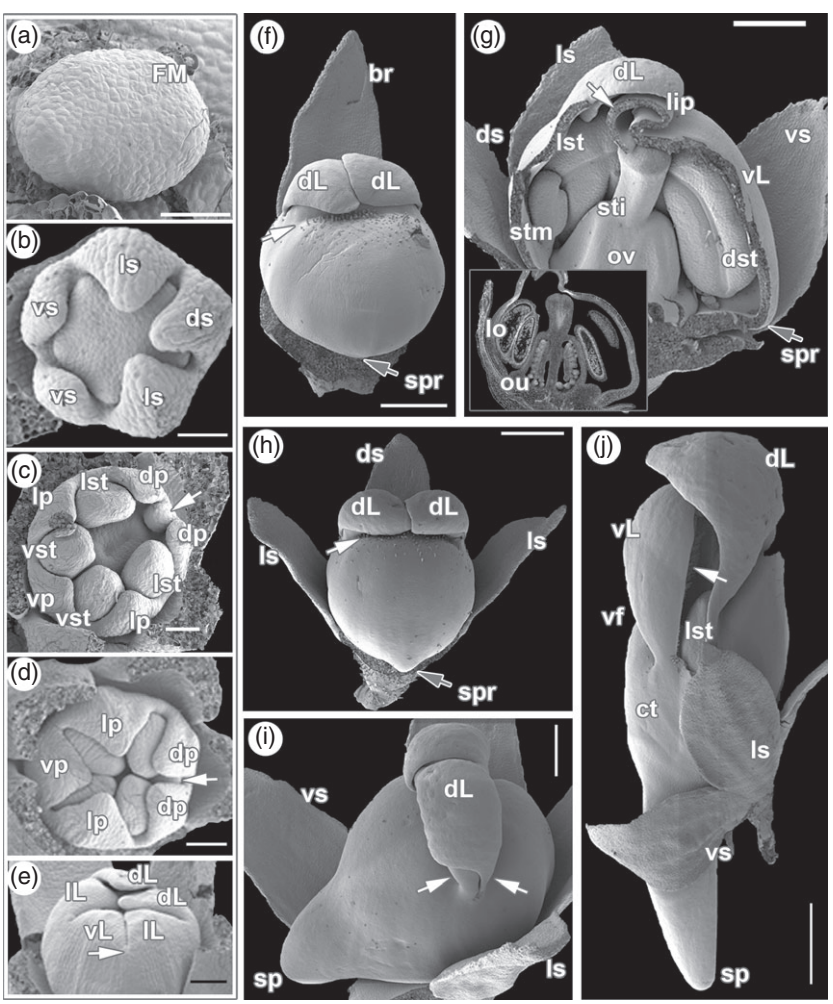

Figure 2. Floral ontogeny in *Linaria vulgaris* can be divided into six phases (Vincent and Coen, 2004).
(a) Phase A, initiation of the inflorescence apex.
(b) Phase B/C, initiation of the floral meristem, sepal and petal whorls.
(c, d) Phase C, initiation of the stamens and gynoecium (arrows indicate staminode).
(e) Phase D, the corolla tube and petal lobes are marked by a tube–lobe boundary (arrow).
(f–h) Phase E, formation of a ventral furrow (white arrows) and outgrowth of the petal-spur primordium (grey arrows).
(i) Phase F, deepening of the ventral furrow (white arrows, i), and elongation of the corolla tube and petal spur.
(j) Phase G, maturation and enlargement of floral organs. Scale bars: (a–e) 50 μm; (f–i) 500 μm; (j) 1 mm. Abbreviations: ct, corolla tube; dL, lL and vL, dorsal, lateral and ventral petal lobes; dp, lp and vp, petals; ds, ls and vs, sepals; lo, anther locule; ov, ovary; ou, ovule; sp, spur; spr, spur primordium; sti, stigma; stm, staminode; vf, ventral furrow; vst and lst, ventral and lateral stamens.

in abaxial to adaxial sequence. The floral apex assumes a pentagonal shape as five petal primordia are initiated, alternating with the sepal primordia (phase C; Figure 2b). The sepals grow and soon enclose the developing petals (Figure 2c). Following the initiation of the petal primordia, four stamen primordia are initiated in two pairs, the lateral pair being initiated shortly before the ventral (adaxial) pair (phase D). The dorsal-most (abaxial), fifth stamen (a sterile staminode) is initiated shortly after the others, but is soon aborted (Figure 2c,d). At the centre of the floral apex, a gynoecial cup primordium forms from two congenitally fused carpels. Coincident with the development of the androecium and gynoecium, the base of the corolla elongates to form a congenitally fused tube with distally free petal lobes that enclose the interior reproductive parts of the flower (Figure 2e). The dorsal petal lobes develop at a greater rate than the ventral and lateral petal lobes, which remain smaller throughout ontogeny. As the corolla tube develops, a ventral trichomatous furrow (Figure 2f) is formed (phase E). At this point a small bulge becomes apparent at the base of the corolla tube on the ventral petal: this is the petal-spur primordium.

Continued growth of the corolla tube and enlargement of the petal-spur primordium mark the remaining phases of development (phases F and G). Yellow pigmentation begins to colour the developing corolla tube, distinguishing it from the green sepals of the calyx. Dissecting the bud longitudinally at this stage demonstrates that outgrowth of the petal-spur primordium is not driven by the enlargement of the androecium and gynoecium (Figure 2g). For much of phase F the bud remains spherical (Figure 2h), undergoing considerable proximo-distal elongation during phase G, particularly in the corolla tube and petal spur (Figure 2i,j).

Cellular development of the petal spur

When initiated (phase E), the cells of the petal-spur primordium (and of the surrounding corolla tissues) are uniformly small and isodiametric, approximately 10 × 10 μm in size in plan view (Figure 3a). Subsequent floral ontogeny is dominated by elongation of the corolla tube and petal-spur primordium (phase F). At this stage the cells of the spur primordium remain small and isodiametric, approximately 10 × 10 μm in size (Figure 3b), whereas the surrounding cells start to increase in size longitudinally, expanding to approximately 20 × 15 μm. During phase F/G cells in the central region of the corolla tube, and at its base, continue to increase in length, attaining sizes of approximately 35 × 25 μm and 30 × 15 μm, respectively (Figure 3c). At the tip of the developing petal spur there is also an apparent longitudinal increase in cell size to approximately 20 × 15 μm. As the bud starts to resemble the mature flower (phase G), cell lengths increase dramatically to approximately 90 × 30 μm in the centre of the corolla tube, approximately 75 × 20 μm at the base of the corolla tube/base of the petal spur and approximately 55 × 20 μm at the tip of the developing spur (Figure 3d). These measurements indicate that much of the length of the petal spur is attained by cell expansion rather than cell division, but that initial outgrowth of the petal spur may incorporate a brief period of cell division.

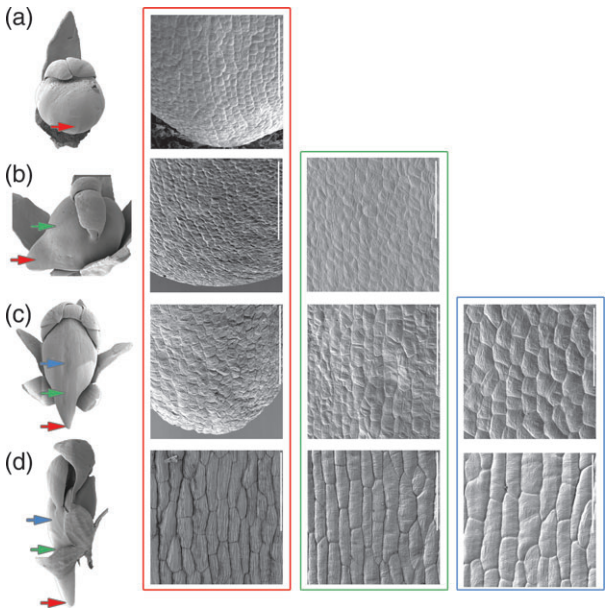

Figure 3. Cellular dimensions during *Linaria vulgaris* petal-spur ontogeny.
(a) Cells of the petal-spur primordium are small and isodiametric (phase E), approximately 10 × 10 μm.
(b) Throughout phase F, cells remain small at the tip of the petal spur (red, approximately 10 × 10 μm), whereas the surrounding cells of the corolla tube increase in length (green, approximately 20 × 15 μm).
(c) Subsequent floral development (phases F/G) is characterized by significant longitudinal increases in cell size. Cells at the tip of the petal spur are approximately 20 × 15 μm, spur base approximately 30 × 15 μm, corolla tube approximately 35 × 25 μm.
(d) Cells during phase G continue to lengthen along the axis of the corolla tube and petal spur. Cells at the spur tip are approximately 55 × 20 μm, the spur base approximately 75 × 20 μm and the corolla tube approximately 90 × 30 μm. Red arrows and borders indicate the spur tip, green the spur base and blue the corolla tube. Scale bars: 100 μm.

LvHirz and *LvIna* are orthologues of *AmHirz* and *AmIna*

Sequences resembling *AmHirz* and *AmIna* isolated from *L. vulgaris* by degenerate RT-PCR and RACE were named *LvHirz* and *LvIna*, respectively (GenBank ID – JN005930 and JN005931; Figure 4a). Translating each coding sequence generated proteins of 367 and 343 amino acids in length, respectively, including the MEINOX, GSE box, ELK and homeobox motifs typical of other published KNOX transcription factors. The LvHirz protein is 73% identical and 79% similar to AmHirz, whereas LvIna is 82% identical and 89% similar to AmIna (Figure 4b). Phylogenetic analyses of LvHirz and LvIna proteins in the context of ten other landmark protein sequences from *Arabidopsis thaliana*, *A. majus* and *Oryza sativa* L. (rice) support the homologous

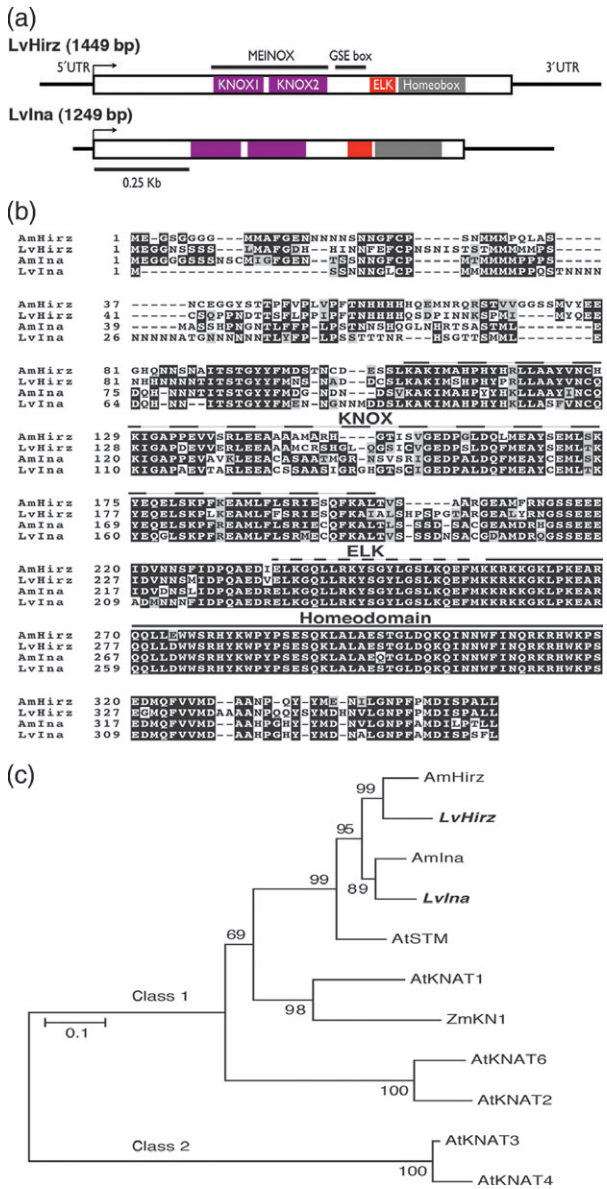

Figure 4. Sequence analysis of *LvHirz* and *LvIna*.
(a) Schematic representation of *LvHirz* and *LvIna* showing the conserved MEINOX (KNOX1 and KNOX2, silver), ELK (red) and Homeobox (purple) encoding domains. The total size of the clone is indicated in parentheses.
(b) Protein alignment of *Linaria vulgaris* and *Antirrhinum majus KNOX* genes.
(c) Neighbour-joining tree of LvHirz and LvIna proteins compared with those from Antirrhinum (AmHirz; AmIna), Arabidopsis (AtSTM; AtKNAT1, NP_192555; AtKNAT2, NP_177208; AtKNAT6, NP_173752; AtKNAT3, NP_001031938; AtKNAT4, NP_196667) and maize (ZmKN1). Numbers below the branches represent percentage bootstrap support.

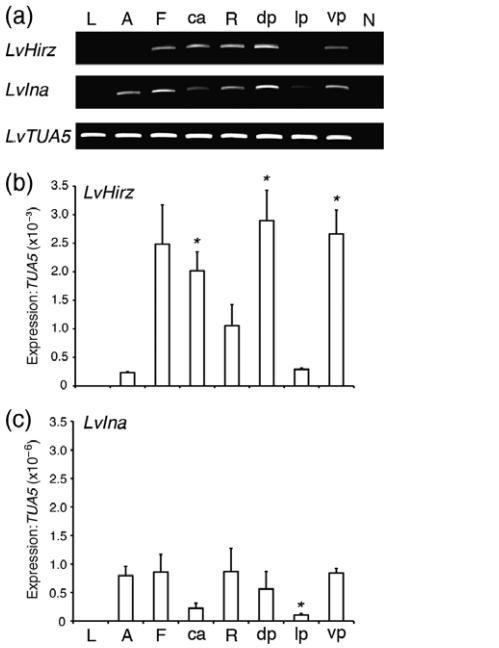

Figure 5. Expression of *LvHirz* and *LvIna* in developing floral buds.
(a) Gene-specific RT-PCR of *LvHirz* (305 bp) and *LvIna* (301 bp) relative to *LvTUA5* (358 bp).
(b) Quantitative expression of *LvHirz* ($\times 10^{-3}$) and (c) *LvIna* ($\times 10^{-6}$) in developing floral buds. Each bar represents the geometric mean from three biological replicates, each consisting of a minimum of three technical replicates. Error bars were calculated from the log (base 2) of the data. Expression was measured relative to *LvTUA5*. Statistical significance is indicated by an asterisk. Note the difference in scale. Abbreviations: A, apex; ca, calyx; dp, lp and vp, dorsal, lateral and ventral petals; F, whole floral bud; L, leaf; N, negative control; R, reproductive tissues; spr petal-spur primordium.

status of LvHirz and LvIna relative to their respective proteins from *A. majus* (Figure 4c). Furthermore, only one KNOX protein with homology to Am/LvHirz and Am/LvIna is present in the recently sequenced genome of *Mimulus guttatus* DC. (http://www.phytozome.net; transcript mgv1a008484m), which is sister to Plantaginaceae, indicating that Hirz and Ina arose by a recent duplication event within the family Plantaginaceae.

LvHirz is highly expressed in floral tissues, particularly in the dorsal and ventral petals

Reverse transcription PCR (RT-PCR) revealed that *LvHirz* and *LvIna* transcripts accumulate to readily detectable levels in a variety of floral and vegetative tissues. To precisely localize transcript accumulation in floral buds, developing flowers at the earliest stages of petal-spur development were dissected into the following groups of floral organs: sepals, fertile organs (androecium plus gynoecium), dorsal petals, lateral petals and ventral petal (which bears the petal-spur primordium). *LvHirz* transcripts were readily detectable in developing floral buds with early initiating petal-spur primordia but, unusually, they could not be detected in the vegetative shoot apex containing the SAM. Specifically, *LvHirz* transcripts were detectable in the calyx, the combined fertile organs, and in the dorsal and ventral petals of the fused corolla tube, but not in the lateral petals (Figure 5a). As expected, *LvHirz* does not appear to be expressed in leaves. In the same cDNA, expression of *LvIna* was apparent in the

vegetative shoot apex and developing floral buds, specifically in the calyx, the combined fertile organs (androecium and gynoecium), and in the dorsal and ventral petals of the fused corolla tube. By contrast, transcript accumulation was barely detectable in the lateral petals. *LvIna* transcripts were also not detectable in leaves. For both *LvHirz* and *LvIna*, DNA sequencing analysis of PCR products from all sampled tissues confirmed the specific amplification of products, and did not indicate any alternative splicing.

The expression of *LvHirz* and *LvIna* was measured relative to the housekeeping gene *LvTUA5* by qRT-PCR from three biologically independent experiments using the same tissues and developmental stages assayed by RT-PCR. Expression of neither *LvHirz* nor *LvIna* was detectable in the leaf. Compared with transcript levels in the apex, *LvHirz* expression was increased 10-fold in developing floral buds with early initiating petal-spur primordia (Figure 5b). Expression of *LvHirz* is ninefold greater in the calyx than in the apex, is fivefold greater in the combined fertile organs (androecium plus gynoecium), and is approximately 12-fold greater in the dorsal and ventral petals. However, expression of *LvHirz* in the lateral petals remained equivalent to that in the apex. A Students' *t*-test was performed on these data. Elevated levels of *LvHirz* expression in the calyx, dorsal and ventral petals were found to be statistically significant ($P \leq 0.05$). Despite a 10-fold increase in expression of *LvHirz* in developing floral buds relative to the apex, this increase was not found to be statistically significant. However, data for floral-bud expression exhibited the greatest biological variation, and *KNOX* gene expression has not previously been reported at all in the developing buds of wild-type flowers. By contrast, *LvIna* was expressed to a much lower level than *LvHirz* in all tissues including the apex (Figure 5c). There was a marked reduction in *LvIna* transcript accumulation in the calyx and statistically significantly reduced expression in the lateral petal ($P \leq 0.05$), in which *LvIna* expression was <20% relative to that of the apex.

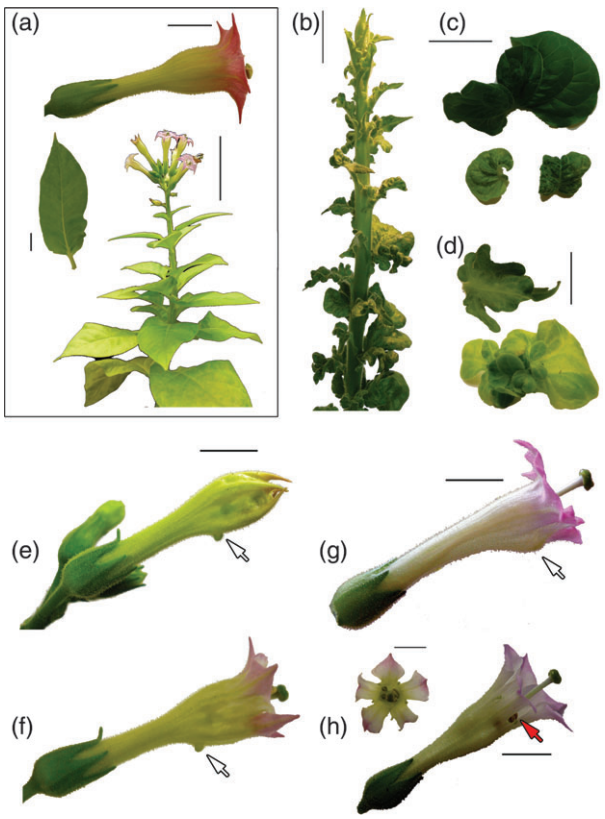

Figure 6. Transgenic analysis of *Antirrhinum majus* and *Linaria vulgaris* KNOX proteins in tobacco.
(a) Empty-vector tobacco control (boxed).
(b) *35S::AmHirz* plants have typical *KNOX* constitutive expression phenotypes.
(c) Vegetative phenotypes of *35S:AmIna* plants.
(d) Vegetative phenotypes of *35S:LvHirz* plants.
(e, f) *35S:AmHirz* flowers are near-normal, with ectopic sac-like outgrowths of tissue (white arrow) formed at the fused petal margins.
(g) *35S:AmIna* flowers have a reduced corolla tube with ectopic bulges (white arrow) and an exerted style.
(h) *35S:LvHirz* flowers have shortened stamens and a distally dissected corolla tube (red arrow) with less pronounced bulges. Scale bars: 5 cm (whole plant); 1 cm (leaf/flower) in (a); 5 cm in (b); 3 cm in (c, d); 1 cm in (e–h).

Transgenic analysis of KNOX function in tobacco

To understand their potential functions, *LvHirz* and *LvIna* transcripts were constitutively expressed in tobacco under the cauliflower mosaic virus (CaMV) *35S* promoter. For comparison, transgenic tobacco plants constitutively expressing *AmHirz* and *AmIna* were also created. Vegetative and floral phenotypes were recorded from multiple independent transgenic tobacco lines. The presence of the T-DNA was confirmed by PCR using genomic DNA template and constitutive expression assayed by RT-PCR. Only phenotypes of lines with confirmed expression of *AmHirz*, *AmIna* and *LvHirz* genes are presented here, as transgenic plants constitutively expressing *LvIna* could not be regenerated. Although transgenic 35S::*LvIna* callus formed shoots, these failed to root. To ensure that any phenotype observed resulted from the T-DNA, multiple tobacco plants carrying the empty CaMV *35S* vector were generated and found to closely resemble wild-type tobacco (Figure S1). These empty vector lines were used as controls for phenotypic comparison.

Tobacco transformants constitutively expressing *AmHirz*, *AmIna* and *LvHirz* differed significantly from wild-type tobacco and empty vector transgenic control plants (Figure 6a). Both within and between transgenic lines the phenotype varied in severity, but was consistent within a single plant (Figures S2–S5). Transgenic plants constitutively expressing *AmHirz*, *AmIna* and *LvHirz* all exhibited typical vegetative *KNOX* constitutive expression phenotypes, including shortened internodes and thickened leaves of reduced size that were mildly to deeply lobed, and showed disrupted symmetry across the less prominent,

and shorter, midvein (Figure 6b–d). As phenotypic severity increased, plants became dwarfed and leaves became increasingly lobed and divided, the reduced petiole being almost indistinguishable from the leaf blade. In some instances, additional shoots and meristems formed on adaxial leaf surfaces. All transgenic lines showed decreased leaf senescence and continual branching from axillary buds (a pattern normally suppressed by strong apical dominance in wild-type tobacco), resulting in prolonged vegetative growth and a bushy habit.

A grade of morphological abnormalities was also observable in flowers and inflorescences. However, the number of floral organs was never affected in any of the transformants. Constitutive expression of *AmHirz*, *AmIna* and *LvHirz* often resulted in plants that flowered early and for an extended duration. However, more severely affected dwarf plants never flowered (Figure S2). In 35S::*AmHirz* plants, numerous distinct sac-like outgrowths of tissue formed on the margins of the fused petals of the corolla tube (Figure 6e,f). A single outgrowth often formed between a pair of petals, most commonly on the ventral side of the corolla tube. Closer examination of these sac-like structures demonstrated that they are not merely folds of tissue. They retain their shape throughout manipulation and longitudinal dissection, suggesting that they are discrete proximal–distal outgrowths of the corolla tube, often reaching several millimetres in size. These sac-like structures were also present in 35S::*AmIna* (Figure 6g) and 35S::*LvHirz* (Figure 6h) flowers, but were on average less distinct. Furthermore, 35S::*LvHirz* plants had a short, divided corolla tube with a stigma extending far beyond the end of the corolla tube, together with short anther filaments that disrupted self-pollination.

DISCUSSION

The *L. vulgaris* petal spur is homologous with the gibba of *A. majus*

Early floral ontogeny in *L. vulgaris* has been discussed previously in relation to understanding floral zygomorphy (Almeida *et al.*, 1997; Cubas *et al.*, 1999; Luo *et al.*, 1999). These studies necessarily focused on early ontogenetic events, but many key floral features that distinguish species arise much later during ontogeny (Vincent and Coen, 2004). This is certainly true when comparing flowers of *A. majus* and *L. vulgaris*, which are primarily distinguished by the initiation of a long, narrow spur on the ventral petal at the end of an otherwise highly conserved ontogenetic series (Vincent and Coen, 2004).

The spatio-temporal coordinates of petal-spur initiation in *L. vulgaris* are identical to a small sac that forms at the base of the ventral petal of the corolla tube in *A. majus*, termed the gibba (Sutton, 1988). In these genera, both structures store nectar secreted from the base of the shared gynoecial nectary to attract bee pollinators (Elisens and Freeman, 1988; Sutton, 1988). Furthermore, our data indicate that the *L. vulgaris* petal spur is derived only from the ventral petal, and is not derived from the synorganized corolla and androecium (Endress and Matthews, 2006). Taken together, these observations indicate that the petal spur of *L. vulgaris* and the gibba of *A. majus* are homologous; this insight offers a critical first step in exploring the evolution of corolla tube elaborations in the Antirrhineae. The relatively poorly resolved phylogenetic framework for Lamiales (Ghebrehiwet *et al.*, 2000; Oyama and Baum, 2004; Vargas *et al.*, 2004; Albach *et al.*, 2005) makes the interpretation of evolutionary polarity problematic, although it seems likely that a relatively long spur is a derived feature of *L. vulgaris*.

Petal-spur ontogeny is fundamentally similar in evolutionarily disparate taxa

On a much broader evolutionary scale, petal-spur ontogeny in *L. vulgaris* is remarkably similar to that described in distantly related angiosperm taxa such as *Aquilegia* (Ranunculaceae) (Gottlieb, 1984; Tucker and Hodges, 2005) and orchids (Orchidaceae) (Rudall and Bateman, 2002; Bateman and Sexton, 2008; Box *et al.*, 2008; Bell *et al.*, 2009). These observations suggest that, despite multiple independent origins, petal spurs are under similar genetic control, perhaps as a result of parallel or convergent evolution driven by shared developmental constraints. As there is no clear morphological difference between petal spurs and sepal spurs, and some families have both (Weberling, 1992), these conclusions may also be applicable to non-petal-derived spurs.

Petal-spur length in *L. vulgaris* is predominantly associated with longitudinal cell expansion

In *A. majus* it is clear that much of the petal growth observed during very early phases of ontogeny is associated with cell division, whereas growth during subsequent stages of floral ontogeny is more strongly influenced by cell expansion (Rolland-Lagan *et al.*, 2003). As many of the morphological features that distinguish the flowers of *L. vulgaris* and *A. majus* arise during late stages of floral ontogeny, it is likely that these differences are largely the result of differential patterns of cell expansion. In *L. vulgaris*, cells undergo significant increases in longitudinal dimensions during late stages of floral ontogeny, generating much of the length of the corolla tube and petal spur. A similar phenomenon has also been observed in the petal spur of the orchid genus *Platanthera*, which continues to elongate after the other perianth segments have ceased to do so (Bateman and Sexton, 2008). However, when the petal spur is initiated, it is composed of a large number of small isodiametric cells, suggesting that early growth and development of the petal spur may incorporate a brief period of cell division. It is likely that morphological variation in the gibbous and spurred

corolla tubes of Antirrhineae result from differences in the relative importance and/or timing of cell division and cell expansion, presumably operating through heterochronic shifts (Box *et al.*, 2008; Box and Glover, 2010).

Linaria vulgaris KNOX genes are expressed outside the SAM in a similar pattern to those of *A. majus* mutants with ectopic petal tubes

LvHirz and *LvIna* are a paralogous gene pair that are orthologous with the *STM*-like class-1 *KNOX* genes *AmHirz* and *AmIna.* In *A. majus*, expression of these two genes is confined to the SAM, where the proteins function redundantly to maintain a pluripotent stem cell niche (Golz *et al.*, 2002). In *L. vulgaris*, both *LvHirz* and *LvIna* transcripts are readily detectable in the SAM, and are absent from vegetative tissues such as leaves. Given the close phylogenetic relationship between *L. vulgaris* and *A. majus* (Ghebrehiwet *et al.*, 2000; Oyama and Baum, 2004; Vargas *et al.*, 2004; Albach *et al.*, 2005), it is likely that *LvHirz* and *LvIna* have an ancestral role in maintaining the SAM. Such a role may be common for orthologs of these genes among other genera in Antirrhineae.

In addition to their role in the SAM, wild-type transcripts of *LvHirz* and *LvIna*, identical in sequence to those expressed in the SAM, have much broader patterns of expression than their wild-type orthologues from *A. majus* (Golz *et al.*, 2002). Both *LvHirz* and *LvIna* are detectable in late developmental stages of floral organs such as the calyx, androecium, gynoecium, and the dorsal and ventral petals of the corolla, which is reminiscent of the pattern of ectopic *KNOX* gene expression in the *Hirz*-d153 and *Ina*-d1 mutants (Golz *et al.*, 2002). However, *LvHirz* and *LvIna* are never detected in the leaves. Quantitative RT-PCR results clearly indicate that, although transcripts of both *L. vulgaris KNOX* genes are detectable outside the SAM, *LvHirz* is expressed to a significantly higher level in floral tissues. In particular, *LvHirz* transcripts accumulate predominantly in the dorsal and ventral parts of the corolla. By contrast, *LvIna* has much lower levels of expression in floral organs, equivalent to that observed in the SAM.

Although a role in SAM maintenance is likely for both genes, the broad and high-level expression of *LvHirz* reported here suggests additional roles for this gene in one or more processes related to the development of floral organs such as carpels (Endrizzi *et al.*, 1996; Foster *et al.*, 1999; Pautot *et al.*, 2001; Scofield *et al.*, 2007) and petal spurs (Golz *et al.*, 2002). Ectopic *KNOX* expression in the *A. majus* mutants *Hirz*-d153 and *Ina*-d1 resulted in a range of pleiotropic phenotypes, including altered leaf shape, excessive trichome formation and, most notably, an additional ectopic petal tube on the ventral part of the corolla (Golz *et al.*, 2002). Consistent with this observation, floral *KNOX* expression was considered important in the development of the petal tube of closely related species (Golz *et al.*, 2002). The high level of *LvHirz* expression in the spur-bearing ventral petal during early petal-spur ontogeny supports this hypothesis, and implies that it may apply to other spur-bearing Antirrhineae.

In the *Hirz*-d153 and *Ina*-d1 *A. majus* mutants, ectopic *KNOX* expression in petals and leaves is a result of transposon insertions in putative *cis*-regulatory regions in the 5′ untranslated region (5′-UTR) and first intron (Golz *et al.*, 2002). Changes in *cis*-regulatory elements are a common source of morphological variation (Carroll, 2005; Gompel *et al.*, 2005; McGregor *et al.*, 2007; Cretekos *et al.*, 2008; Jeong *et al.*, 2008; Frankel *et al.*, 2010): floral *KNOX* expression in *L. vulgaris* may have evolved through similar changes in *cis*-regulatory elements, and could have played a larger role in the evolution of the diverse gibbous and spurred corolla tube morphologies of other Antirrhineae. Remarkably, several *KNOX*-related mutations are attributable to *cis*-regulatory change, such as the barley *Hooded* mutant (Inada *et al.*, 2003; Santi *et al.*, 2003). Furthermore, *cis*-regulatory changes in *KNOX* genes have also been demonstrated in the evolution of diverse leaf morphologies in Arabidopsis and its close relatives (Hay and Tsiantis, 2006; Uchida *et al.*, 2007; Piazza *et al.*, 2010). Given the similarity of petal-spur ontogeny in evolutionarily-disparate taxa (Gottlieb, 1984; Tucker and Hodges, 2005; Box *et al.*, 2008; Bell *et al.*, 2009), changes in *KNOX cis*-regulatory elements could have played a wider role in the evolution of the petal spur.

KNOX protein can induce novel outgrowths on the petals of transgenic tobacco

The introduction of *AmHirz*, *AmIna* and *LvHirz* into transgenic tobacco significantly affects the morphology and determinacy of tobacco shoots, leaves and flowers. Many of the resultant vegetative and floral phenotypes have previously been described for a broad range of class-1 *KNOX* genes that have been constitutively expressed in Arabidopsis and tobacco, and their causes are now relatively well understood (Hake *et al.*, 2004; Hay *et al.*, 2004; Shani *et al.*, 2006). Interestingly, reduced corolla-tube length was also noted in the *Hirz*-d153 and *Ina*-d1 mutants (Golz *et al.*, 2002).

Unique among these phenotypes are the numerous sac-like protrusions on the corolla tube. This phenotype was observed in several independent 35S::*AmHirz* transgenic lines and, to a lesser extent, on the flowers of 35S::*AmIna* and 35S::*LvHirz* tobacco plants. These phenotypes have not previously been reported in the literature, perhaps indicating that they represent a specific property of the KNOX proteins tested here. An alternative possibility is that the phenotype is highly context dependent, related to the developmental status of the tissue at the time of *KNOX* misexpression. A similar explanation has been offered for the variable leaf phenotypes observed in transgenic tobacco plants constitutively expressing the tobacco *KNOX* genes *TKN1* and *TKN2* (Shani *et al.*, 2009).

The production of ectopic sac-like structures on the corolla tube of tobacco transformants suggests that ectopic *KNOX* gene expression is sufficient to promote the outgrowth of petal tissue, providing support for the hypothesis that misexpression of class-1 *KNOX* genes was an important factor in the evolution of petal spurs in Antirrhineae (Golz *et al.*, 2002). However, the floral outgrowths described in this paper are by no means bona fide petal spurs: they are not homologous in terms of location, and more closely resemble the 'knots' observed in maize constitutively expressing *KNOTTED 1* (Lincoln *et al.*, 1994). This observation indicates that additional factors are essential for the development of a true petal spur. In order to better understand the sufficiency of *KNOX* genes to induce petal-spur development, transgenic experiments should be conducted on *L. vulgaris* itself or on a non-spurred close relative such as *A. majus*.

Towards a model of *KNOX* gene involvement in petal-spur development and evolution

In the *Hirz*-d153 and *Ina*-d1 *A. majus* mutants, ectopic expression of *KNOX* genes in the corolla is thought to induce a novel axis of growth by generating an additional ectopic organizer that can direct altered cell division and growth, resulting in the petal tubes that characterize these mutants (Golz *et al.*, 2002). A similar explanation was offered for the *Hooded* florets observed in barley when ectopically expressing the barley orthologue of *KNOTTED 1* (Muller *et al.*, 1995; Williams-Carrier *et al.*, 1997). We find no evidence to support or refute the organizer concept, but favour a model in which *KNOX* expression during later stages of petal ontogeny functions to promote and maintain further morphogenetic potential of the petal, analogous to the role of *KNOX* in the development of compound leaves (Hay and Tsiantis, 2006; Shani *et al.*, 2009, 2010).

Our results suggest a likely role for *KNOX* expression in petal-spur development. However, it is clear that *KNOX* expression does not set the petal-spur fate. Any gene or genes that do set petal-spur fate presumably operate downstream of canonical ABC (Kramer *et al.*, 2007) and floral symmetry-breaking genes (Cubas *et al.*, 1999; Golz *et al.*, 2002), and most likely require *KNOX* gene re-expression during early petal ontogeny. Several additional genes controlling the balance between cell division and expansion are also likely candidates for petal-spur development, including members of the TCP family of transcription factors, such as *CINCINNATA* (Crawford *et al.*, 2004), *JAGGED* (Dinneny *et al.*, 2004; Ohno, 2004) and the *AP2*-like factor *AINTEGUMENTA* (Krizek, 1999; Mizukami and Fischer, 2000). Further understanding of the genetic mechanisms of petal-spur development and evolution is likely to emerge from exploring floral *KNOX* expression in other spur-bearing taxa, most notably the emerging model system *Aquilegia* (Kramer, 2009; Kramer and Hodges, 2010).

EXPERIMENTAL PROCEDURES

Plant growth conditions

All plants were grown from seed in a controlled glasshouse environment at 26°C with a 16-h light regime.

Scanning electron microscopy (SEM) and X-ray tomography (XRT)

Fresh plant material was fixed immediately using formalin-acetic-alcohol for a minimum of 72 h and dissected in 70% ethanol. Dissected samples were dehydrated using an ethanol series, critical-point dried using a Tousimis Supercritical Autosamdri 815B critical-point drier (Tousimis, http://tousimis.com), mounted onto SEM stubs and coated with platinum using an Emitech K550 sputter coater (Emitech, http://www.emitechinc.com). Samples were imaged using a Hitachi S-4700-II cold-field emission scanning electron microscope (FE-SEM) at 2.0 kV. Tissue was prepared for X-ray tomography using the same protocol.

Nucleic acid isolation, amplification and analysis

RNA was extracted using a standard phenol:chloroform-LiCl method (Sambrook and MacCallum, 2001), and cDNA was prepared from 1 μg of DNase-treated RNA using SUPERSCRIPT III (Invitrogen, http://www.invitrogen.com). *LvHirz* and *LvIna* were amplified from 1:100 diluted cDNA using degenerate primers designed using the CODEHOP algorithm (Rose *et al.*, 1998, 2003) to anneal to the highly conserved 'DQFM' and 'WFIN' motifs of the KNOX and Homeodomain encoding regions. The full-length coding sequence was obtained by 5′ and 3′ RACE using the GENERACER Kit (Invitrogen), re-sequenced and deposited in GenBank (*LvHirz* JN005930; *LvIna* JN005931). A full set of primer sequences is presented in Table S1.

Gene-specific primers were designed for expression analysis by RT-PCR and qRT-PCR. The primers were designed to amplify across predicted introns (Czechowski *et al.*, 2005). RT and qRT-PCR were performed using 4 μl of 1:100 diluted cDNA template. RT-PCR was carried out over 35 cycles. qRT-PCR was conducted using the SYBR GREEN-ER qRT-PCR kit (Invitrogen) on a CHROMO4 Real Time Detector and DNA ENGINE Peltier Thermocycler (BioRad, http://www.bio-rad.com), following the manufacturer's instructions. The resulting qRT-PCR data were analyzed using LINREGPCR (Ruijter *et al.*, 2009). In each case, expression was measured relative to the housekeeping gene *Tubulin* alpha5-chain (*LvTUA5*) obtained using primers based on the sequence of the Arabidopsis orthologue (AT5G19780).

Protein alignment was performed using the MAFFT server (Katoh and Toh, 2008), with default settings. Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al.*, 2007) using the neighbour-joining method (Saitou and Nei, 1987) obtained via a JTT distance matrix (Jones *et al.*, 1992). The resulting tree was subject to 1000 bootstrap replicates (Felsenstein, 1985).

Recombinant DNA constructs and transgenic analysis

The full-length coding sequences of *AmHirz* (AY072736), *AmIna* (AY072735) and *LvHirz* were cloned directly from cDNA into a modified pGreenII0029 vector between a double CaMV *35S* promoter and a single CaMV *35S* terminator (Hellens *et al.*, 2000) using gene-specific primers containing the *Hin*dIII and *Bam*HI restriction sites. *LvIna* was cloned in the same way but using primers containing *Pst*I and *Bam*HI restriction sites (Table S1). Constructs were transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation (Mattanovich *et al.*, 1989) at 1.8 kV, which was then used to transform leaf segments of tobacco var.

Samsun (Horsch *et al.*, 1985). Successful transformants were selected with kanamycin and confirmed by gene-specific PCR.

ACCESSION NUMBERS

LvHirz, JN005930; *LvIna*, JN005931.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Empty vector transgenic tobacco controls are identical to wild-type tobacco.

Figure S2. 35S::*AmHIRZ* transformants exhibit a range of vegetative phenotypes from weak, intermediate to severe.

Figure S3. 35S::*AmHIRZ* floral phenotypes also range in severity. As the severity increases, flowers are less darkly pigmented.

Figure S4. Vegetative and floral phenotypes of 35S::*AmINA* transformants.

Figure S5. Vegetative and floral phenotypes of 35S::*LvHIRZ* transformants.

Table S1. Primers used in this work.

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