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An ortholog of *MIXTA-like2* controls epidermal cell shape in flowers of *Thalictrum*

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Summary

• Here, we investigated the genetic underpinnings of pollination-related floral phenotypes in *Thalictrum*, a ranunculid with apetalous flowers. The variable presence of petaloid features in other floral organs correlates with distinct adaptations to insect vs. wind pollination. Conical cells are present in sepals or stamens of insect-pollinated species, and in stigmas. We characterized a *Thalictrum* ortholog of the *Antirrhinum* majus transcription factor *MIXTA-like2*, responsible for conical cells, from three species with distinct floral morphologies, representing two pollination syndromes.

• Genes were cloned by PCR and analysed phylogenetically. Expression analyses were conducted by quantitative PCR and *in situ* hybridization, followed by functional studies in transgenic tobacco.

• The cloned genes encode R2R3 MYB proteins closely related to *Antirrhinum* AmMYBML2 and *Petunia* hybrida PhMYB1. Spatial expression by *in situ* hybridization overlaps areas of conical cells. Overexpression in tobacco induces cell outgrowths in carpel epidermis and significantly increases the height of petal conical cells.

• We have described the first orthologs of *AmMIXTA-like2* outside the core eudicots, likely ancestral to the *MIXTA/MIXTA-like1* duplication. The conserved role in epidermal cell elongation results in conical cells, micromorphological markers for petaloidy. This adaptation to attract insect pollinators was apparently lost after the evolution of wind pollination in *Thalictrum*.

Introduction

A central goal of evolutionary developmental biology is to investigate the relationship between the evolution of developmental control genes and phenotypic variation. More than a century after Charles Darwin referred to the origin and rapid radiation of the angiosperms as an 'abominable mystery' (Darwin & Seward, 1903), there are still many unanswered questions about the underlying causes of this phenotypic variation. The flower, and the interactions with pollinators that it enhances, is considered one of the most important innovations that have allowed angiosperms to become the dominant element of today's flora (Crepet, 1984). Studies of closely related species whose flowers vary in their pollination syndromes are instrumental in understanding the evolution of the associated adaptive traits.

Since the sequencing of complete genomes from a few model plants, many genes involved in different aspects of floral organ identity and differentiation have been characterized. This knowledge allows for a more informed candidate-gene approach towards understanding adaptive phenotypic diversity throughout the angiosperm phylogeny. Extending our understanding of the role of developmental control genes to taxa that are particularly informative phylogenetically is an important step towards understanding the evolution of floral diversity.

As part of a broader quest to investigate the molecular basis of morphological differences between flowers of related species, we set out to study variation that has evolved as a result of adaptation to different pollination agents: wind or insects. Our work took a comparative approach to the investigation of the expression and function of a MYB family transcription factor, a candidate gene for determining features of the floral epidermis that affect pollination in species of the non core eudicot genus *Thalictrum*.

Thalictrum comprises *c.* 190 species of herbaceous perennials distributed in temperate regions worldwide (Tamura, 1993) with 22 species in North America (Trelease, 1886; Park & Festerling, 1997). It belongs to the family Ranunculaceae,

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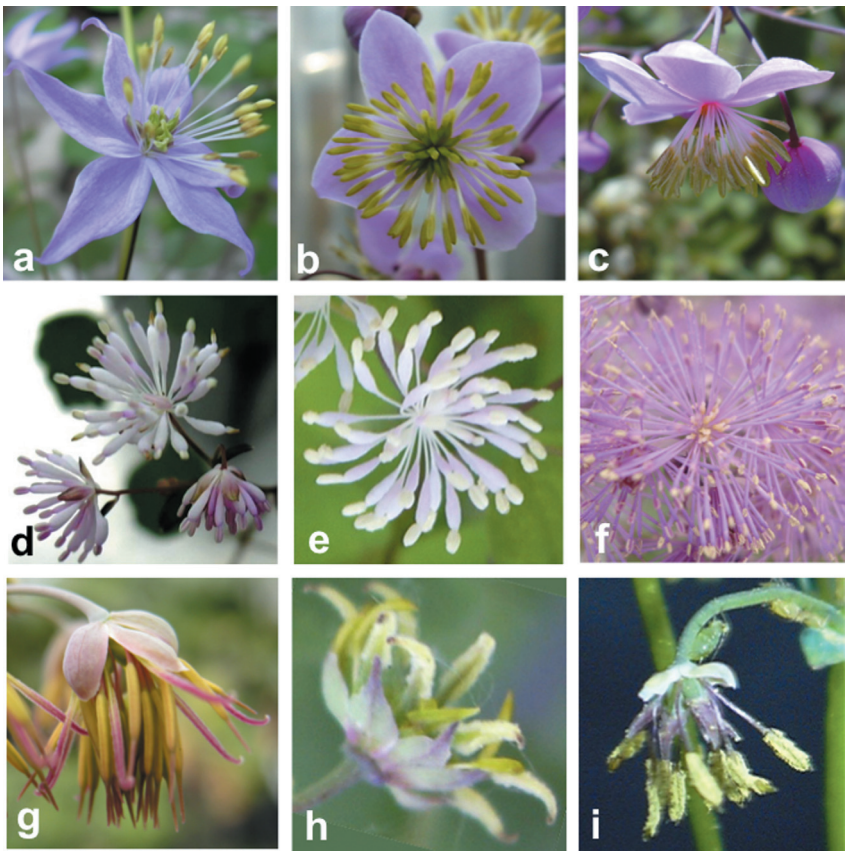

Fig. 1 Floral diversity in the genus *Thalictrum* (Ranunculaceae), representative species. (a–c) Species with petaloid sepals, mostly insect pollinated. (a) *Thalictrum delavayi*; (b) *Thalictrum rochebrunianum;* (c) *Thalictrum uchiyamai*. (d–f) Species with petaloid stamens, mostly insect pollinated. (d) *Thalictrum kiusianum*; (e) *Thalictrum punctatum*; (f) *Thalictrum aquilegifolium* (with pink stamen filaments). (g–i) Species with wind-pollination syndrome (nonshowy, many dioecious). (g) *Thalictrum arsenii*; (h) *Thalictrum fendleri* (carpellate); (i) *Thalictrum alpinum.*

sister to a clade containing *Aquilegia*, the columbines (Hoot, 1995). *Thalictrum* provides a spectrum of variation in floral traits coupled with two pollination mechanisms (reviewed in Pellmyr, 1995) and four breeding systems.

Thalictrum flowers are apetalous, that is, they completely lack petals; however, different species show varying degrees of petaloidy (characters that are usually associated with petals, such as color and conical cells) in sepals or stamens. Ongoing phylogenetic reconstruction and character mapping suggest that insect pollination is the ancestral condition in the genus; wind pollination evolved early followed by the evolution of unisexual breeding systems such as andromonoecy and dioecy (J. Brunet, V. S. Di Stilio & A. Liston, unpublished).

In this study, we compare three representative species: *Thalictrum thalictroides* has the ancestral features of insect pollination syndrome (Kaplan & Mulcahy, 1971), consisting of expanded, 'showy' sepals that are white or purple and contain conical cells in the adaxial epidermis (Figs 1a–c, 2a–d); closely related *Thalictrum filamentosum* is a representative of a different type of insect pollination syndrome, consisting of petaloid stamens containing conical cells and/or pigments (Figs 1d–f, 2e–h) and *Thalictrum dioicum*, with derived wind pollination features (Kaplan & Mulcahy, 1971) of inconspicuous, small, green flowers without conical cells, with extended stigmatic surfaces, trichomes, many pendulous stamens, and often dioecious or monoecious (Figs 1g–i, 2i–l).

The presence of conical cells in the adaxial petal epidermis of most angiosperms is an important component of petal appearance that enhances color and brightness (Noda *et al.*, 1994). In *Antirrhinum majus* (snapdragon), the *MYB* transcription factor *MIXTA* and its relatives play a role in petal coloration by promoting the formation of conical epidermal cell shape (rather than by affecting pigment concentration), resulting in conical cells that affect light reflection, making them more attractive to insect pollinators (Noda *et al.*, 1994; Glover & Martin, 1998). MIXTA and MIXTA-like are members of the R2R3-MYB family of transcription factors consisting of a conserved DNA-binding (MYB) domain comprised of two repeats (R2 and R3). A motif in the *C*-terminal domain characterizes them as members of Subgroup 9 (Kranz *et al.*, 1998; Stracke *et al.*, 2001).

Three other *MIXTA* relatives have been identified in snapdragon, known as *AmMYBMIXTA-like* (*ML*) 1–3, involved in aspects of epidermal cell fate that relate to adaptation to pollinators: conical cells that intensify petal color, trichomes that collect pollen, and mesophyll cell expansion that provides a grasping zone for pollinators (Martin *et al.*, 2002; Perez-Rodriguez *et al.*, 2005).

While *MIXTA* and *MYBML1* have so far been found only in *Antirrhinum*, other *MIXTA-like* genes have been isolated from *Petunia* (*PhMYB1*; van Houwelingen *et al.*, 1998), and *Arabidopsis* (Romero *et al.*, 1998; Baumann *et al.*, 2007).

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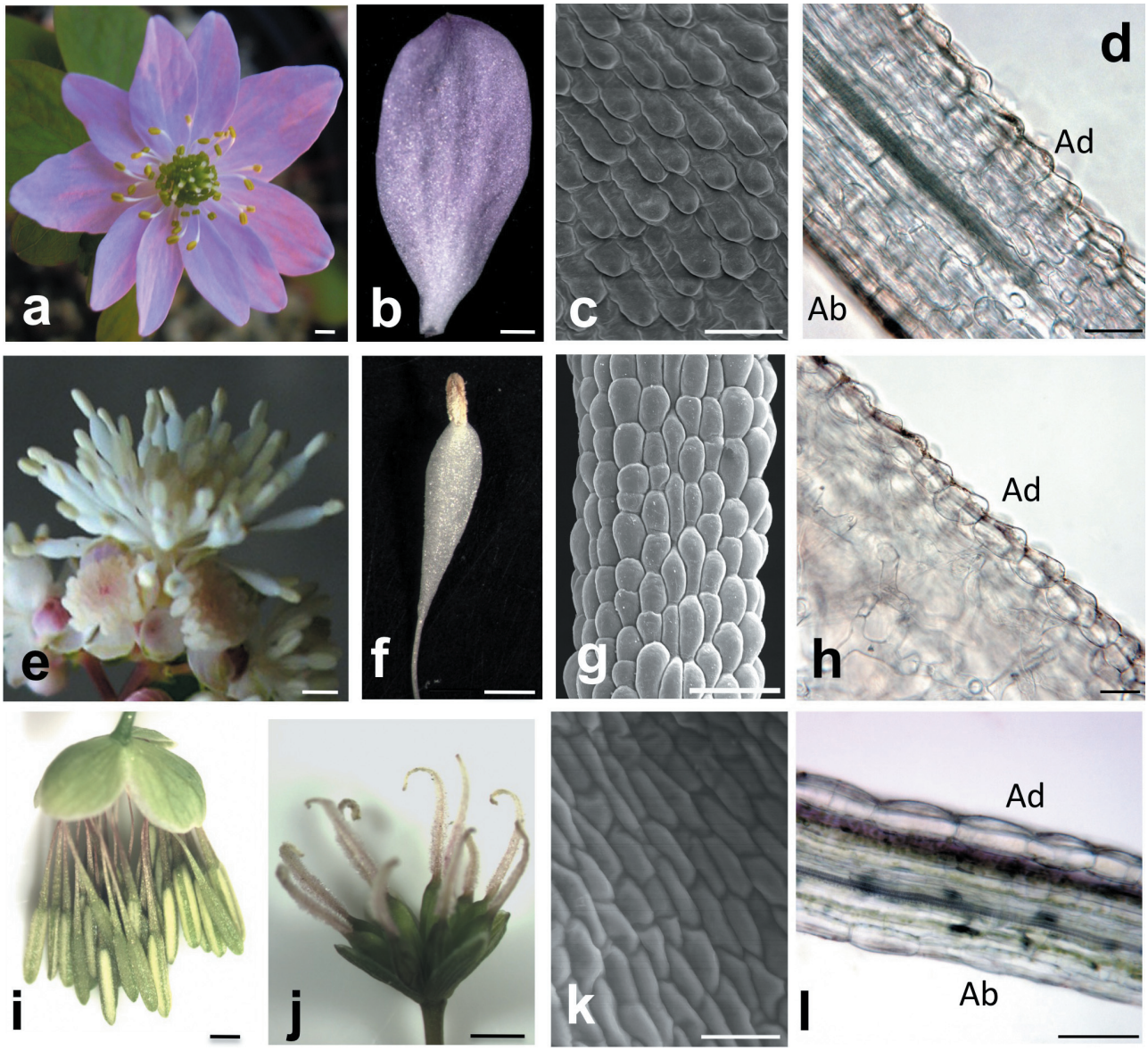

Fig. 2 Characterization of flowers and their epidermal cell types in three representative species of *Thalictrum* used in this study. (a–d) *Thalictrum thalictroides*. (a) Showy insect-pollinated hermaphroditic flowers; (b) detail of petaloid sepal; (c) Scanning electron microscopy (SEM) of adaxial sepal epidermis showing conical-papillate cells; (d) transverse section of fresh sepal showing conical cells on adaxial surface. (e–h) *Thalictrum filamentosum*. (e) Showy hermaphroditic flower; (f) detail of stamen with expanded petaloid filament; (g) SEM of stamen filament epidermis showing slightly conical cells; (h) transverse section through stamen filament showing conical cells. (i–l) *Thalictrum dioicum*. (i,j) Green, small and inconspicuous wind pollinated staminate and pistillate flowers of this dioecious species; (k) SEM of adaxial sepal showing flat epidermal cells; (l) transverse section of sepal showing flat cells on adaxial surface. Bars, (a,b,e,f,i,j) 1 mm, (c,d,g,h,k,l) 50 μm. Ad, adaxial; Ab, abaxial.

However, no related genes have yet been isolated or characterized functionally outside of the core eudicots. Functional characterization of *PhMYB1* and *AmMYBML2* showed that they promote directional cell expansion and have functions overlapping with that of *MIXTA* (Baumann *et al.*, 2007). A detrimental effect of mutations in these genes on pollination visitation and fitness has been shown in the field for *Antirrhinum* (Glover & Martin, 1998) and has been implied, because of effects on flower shape and apparent size, in *Petunia* (Baumann *et al.*, 2007).

Our goal is to identify a tractable floral adaptive trait, characterize the gene responsible for it and compare its expression and function across species representative of the different morphologies. To that end, our study takes the following approach: (1) identification of a micromorphological trait that correlates with petaloidy (conical cells), given that petals are absent but other organs take over the attraction role (sepals and stamens); (2) cloning of the gene responsible for making conical cells, taking a candidate gene approach (*ThalictrumMYBML2*);

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(3) comparative expression of the candidate gene among species with different placement of conical cells or lack thereof; and (4) characterization of gene function using a transgenic approach in a model plant system (tobacco).

Thalictrum provides an opportunity to study the genes underlying changes in floral morphologies associated with adaptation to different pollination agents. In this study, we have identified and characterized a gene encoding a floral transcription factor responsible for petaloid features associated with insect pollination. Importantly, this system also allows the dissection of organ identity from organ differentiation and function: in this case, a feature that is typical of petals (conical cells), is expressed ectopically in stamens and sepals, conferring on them the function (but not the identity) of petals.

Materials and Methods

Plant materials

Plants were grown at the University of Washington (UW; Seattle, WA, USA) glasshouses from seed collected in the wild for *T. dioicum* and from nursery-bought plants (Heronswood, WA, USA) for *T. thalictroides* [vouchers previously deposited at the Arnold Herbarium, V. Di Stilio 101-102 (A)] and *T. filamentosum* [voucher deposited at the University of Washington Herbarium, V. Di Stilio 104 (WTU)]. Flowers were photographed using a hand-held digital camera and a dissecting microscope (Nikon SMZ800; Nikon Instruments Inc.) equipped with a QImaging MicroPublisher 3.3 RTV digital camera (QImaging Corporation, Surrey, BC, Canada). Free-hand sections of sepals and stamens were observed and photographed using a Leitz Orthoplan 2 compound scope and the same camera described above.

Scanning electron microscopy (SEM) and freeze fracture

Floral tissue was dissected, fixed overnight in formaldehyde-acetic acid-alcohol (FAA), dehydrated for 30 min through an alcohol series (50, 60, 70, 80, 95 and 100%), then critical-point dried, mounted and sputter coated. Observations were made in a JEOL JSM-840A scanning electron microscope at the UW microscopy facility. For freeze fractures, plant tissue was frozen in a nitrogen slush at −190°C, warmed to −100°C, and then fractured. Samples were sputter-coated with platinum and examined in a Philips XL 30 FEG scanning electron microscope fitted with a cold stage as specified in Perez-Rodriguez *et al*. (2005).

Cloning of a MIXTA-like ortholog from *Thalictrum*

We initially identified the complete coding sequence of two R2R3 MYB transcription factors having the subgroup 9 motif by searching with *Antirrhinum MIXTA* using tblastx against the *Aquilegia formosa* × *pubescens* TIGR (The Institute for Genomic Research, http://www.jcvi.org/) expressed sequence tag (EST) database. *Aquilegia*, the columbine, belongs in a sister clade to *Thalictrum*, with over 90% sequence similarity in the coding region of floral MADs box genes (Kramer *et al.*, 2003, 2004). After performing a diagnostic phylogenetic analysis of aligned MIXTA-like (ML) proteins from several taxa, one of the *Aquilegia* ESTs, TC15922 (here referred to as *AqMYBML*), appeared to encode a protein most closely related to MIXTA-like proteins AmMYBML2 from snapdragon and PhMYB1 from petunia.

After designing primers to the *Aquilegia* sequence: Aq15922_for2, 5′-ATGGGTCGATCTCCTTGTTGTGAC-3′ and Aq15922_rev, 5′-AAATGTGGGTGAATCGGA-TGGAGA-3′, we amplified an approx. 1200 bp fragment of a related gene from floral cDNA of *T. thalictroides*, *T. dioicum* and *T. filamentosum* (GenBank FJ487606, GQ324997 and GQ324998). The PCR products were cloned into pCRII plasmid using a TA cloning kit (Invitrogen) followed by 3′-rapid amplification of cDNA ends (RACE) with poly-T primer. A 5′-RACE system (Invitrogen) was used to obtain the complete coding region for *T. thalictroides*. Sequencing of over a dozen clones from different floral tissues always recovered the same gene. No additional genes were recovered after using degenerate primers to the MYB domain (before the start of R2) and subgroup 9 motif, designed to recover both *MIXTA* and *MIXTA-like* related loci: AmMIXTA/ML_F (5′-ATGGKBMGRTCYCCATKYTGYGATAAR-3′) and Sbgrp9_R (5′-TTSKARNCGAGCGCTYTCCCAY-TGMGC-3′).

The amino acid sequence for a representative of the *Thalictrum MYB MIXTA-like2* genes (TtMYBML2) was aligned with related proteins obtained from GenBank using CLUSTALX, manually adjusted in MACCLADE 4.05 (Maddison & Maddison, 2001) and prepared for presentation using BOXSHADE 3.21. A neighbor-joining tree was generated using PAUP*4.0b10 (Swofford, 2001) from a fragment of the alignment comprising the first 169 amino acids, including the diagnostic motifs up to the subgroup 9 domain (Baumann *et al.*, 2007; Bailey *et al.*, 2008). GenBank accessions used were: CAA55725 (MIXTA), AY661653 (AmMYBML1), AY821655 (AmMYBML2), AY661654 (AmMYBML3), CAA78386 (PhMYB1), X99809 (AtMYB16), NM_115989 (AtMYB17), NM_110979 (AtMYB106) and BAA86879 (AtGL1).

Gene expression analyses

Reverse transcriptase (RT) PCR Total RNA was extracted from 50 to 100 mg of frozen dissected floral tissue of *T. thalictroides* (sepals, stamens and carpels) using TRIzol (Invitrogen); then treated with amplification grade DNaseI (Invitrogen) according to the manufacturer's instructions. cDNA was prepared from 1 μg of the resulting DNase-treated RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

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The gene-specific primers for *TtMYBML2* were TthMixta_for4 (5′-TACCCTTAAATACAGGCCTTCAAGATATCC-3′) and TthMixta_rev2 (5′-CATAAACTTTGCCAGACAATTT-CAGAATCA-3′) resulting in a PCR product of *c*. 400 bp after 25 cycles.

The constitutively expressed gene encoding ACTIN was cloned from *T. thalictroides* (GenBank GQ324996) and used as a loading control. Specific *Thalictrum ACTIN* primers were designed giving a product of approx. 155 bp: TthActin_for1 (5′-TGACTATGAGCAAGAGTTGGAGACCG-3′) and TthActin_rev2 (5′-CCTGCAGCTTCCATTCCGATCA-3′).

Real-time PCR cDNA was synthesized from 1 μg of DNase-treated total RNA obtained from dissected floral tissue – sepals (from carpelate flowers in *T. dioicum*), stamens and carpels from pooled individuals of each of three species (*T. thalictroides*, *T. filamentosum* and *T. dioicum*) – as described earlier. Each amplification reaction was performed with 1 μl of template cDNA, 25 μl of SYBR Green PCR Master Mix (Bio-Rad), and 500 nM of the gene-specific primers described above (or *ACTIN* control, as above). Samples were amplified in triplicate for 40 cycles of 95°C for 15 s and 60°C for 30 s and run on the MJ Research Chromo4 PCR system (Waltham, MA, USA) at the UW, Comparative Genome Center. Melting curve analysis was used to test whether a single amplification product had been amplified. Since primers had been designed based on sequence information for *T. thalictroides*, PCR products were sequenced in the other species to confirm their identity. The melting curve was measured from 60°C to 95°C after the final cycle of PCR. Absence of genomic DNA contamination was verified by control cDNA reactions lacking reverse transcriptase, which resulted in no PCR product. Reactions were performed in triplicate in two separate experiments and normalized to *ACTIN* levels using the two-delta CT relative quantification method (Livak & Schmittgen, 2001). Average values and standard deviations were graphed for each species relative to the tissue expressing the most (carpels for all three species).

In situ hybridization *In situ* hybridization was performed on young flowers of *T. thalictroides* and *T. filamentosum* as described in Kramer (2005), with minor modifications. The probe consisted of a 400 nucleotide fragment of the *TtMYBML2* gene described earlier for the RT-PCR, comprising the end of the *C*-terminus and part of the 3′UTR, for specificity. Given the high level of nucleotide similarity among the species, this probe was also used on *T. filamentosum*.

Biochemical characterization of a *Thalictrum* MIXTA-like in *Nicotiana* (tobacco bioassay)

To test whether the *Thalictrum MIXTA*-like gene product plays a role in epidermal cell elongation, the complete cDNA of TtMYBML2 was used in a tobacco bioassay (Baumann *et al*., 2007). The assay consists of overexpressing the query gene under strong, constitutive double CaMV35S promoter for *Agrobacterium*-mediated transformation of tobacco (Glover *et al*., 1998), then analysing the epidermis of the transgenic plants by SEM to look for changes in cell shape, in particular cell elongation (conical cells) and the presence of ectopic trichomes. Transgenic seed were grown on selective Kanamycin (100 μg/ml) plates; survivors were transplanted to soil and grown in the glasshouse until flowering. The presence of the transgene in Kan-resistant seedlings was confirmed by PCR using the locus-specific RT-PCR primers detailed earlier. Nontransgenic segregants grown on soil and tested by PCR were used as controls (wt). All floral organs and leaves were fixed in FAA and processed for SEM analysis.

The height of petal conical cells was measured from SEM images of wt and transgenic tobacco plants using ImageJ (Abramoff *et al*., 2004). Measurements were made from the tip of the cell to the 'waist', or constriction point (see graph inset, Fig. 6k). Averages were based on a total of 880 cells measured from four individuals for the control wild type plants and 776 cells measured from eight transgenic individuals. A two-tailed unpaired *t*-test was used to evaluate the statistical significance of variation in average cell heights for the control and treatment groups.

Results

Comparative characterization of epidermal cell types in flowers of *Thalictrum*

Differences in the suites of characters that comprise the insect and wind pollination syndromes can be striking (compare Fig. 1a–c with g–i). Insect pollination is often associated with hermaphroditism and petaloid sepals (Fig. 1a–c) or petaloid stamens (Fig. 1d–f). Wind pollination is often associated with monoecy or dioecy, leafy sepals, trichomes and pendulous stamens (Fig. 1g–i).

We conducted a SEM survey of representative species and found that asymmetrically papillate cells are present in the sepal epidermis of the insect-pollinated species *T. thalictroides*, which has a petaloid appearance (Fig. 2a–d; see also Di Stilio *et al*., 2005), and in the petaloid filaments of *T. filamentosum* stamens (Fig. 2e–h); these two species belong in the same clade in the phylogeny. This contrasts with the findings for the more distantly related wind-pollinated species, *T. dioicum*, which has flat or only slightly raised cells in the epidermis (Fig. 2i–l).

Cloning and characterization of *Thalictrum* MYBML2

In order to obtain candidate genes for the control of papillate cell production in *Thalictrum*, we first identified a *MIXTA-like* sequence in the publicly available EST database of closely related *Aquilegia* (TC15922). This sequence was then used to design primers, and the full-length cDNA was first obtained by PCR from the species *T. thalictroides*. The *Thalictrum MIXTA-like* gene, *TtMYBML2*, is 1623 bp long, of which

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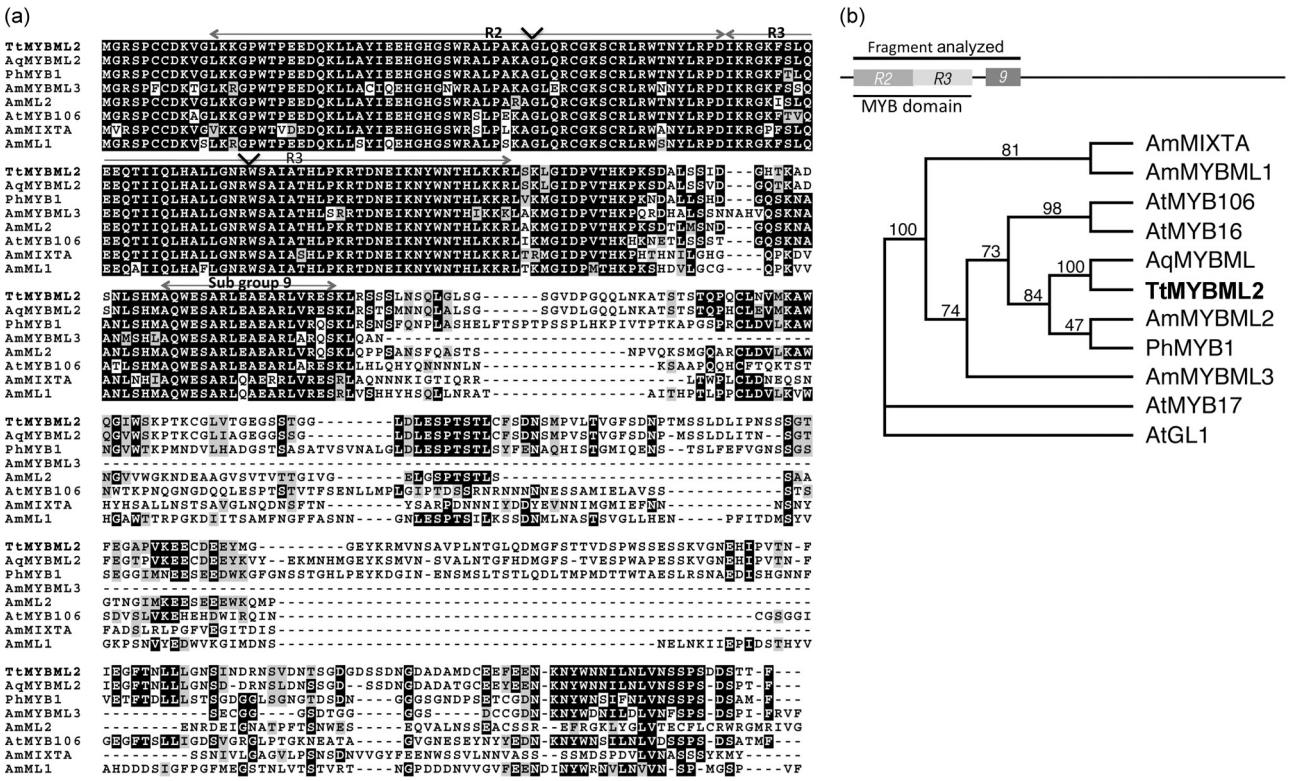

Fig. 3 Amino acid alignment and phylogeny of *Thalictrum* MYB MIXTA-like2 (TtMYBML2) and related proteins in other taxa, identified from GenBank. The *Thalictrum* gene and its protein product (in bold) are closely related to other MIXTA-like proteins that have been shown to affect cell shape. (a) Amino acid alignment showing conserved R2 R3 MYB domain and subgroup 9 motifs with arrows, black shading indicates identity, gray similarity. The two introns are indicated by arrowheads: the first in the R2 domain (105 bp), the second in the R3 domain (400 bp). (b) Schematic of gene structure showing the fragment used for the alignment. Phylogenetic relationship (neighbor-joining tree) of proteins shown in (a). *Antirrhinum majus* (Am), *Petunia hybrida* (Ph), *Arabidopsis thaliana* (At), *Aquilegia formosa* × *pubescens* (Aq) and *Thalictrum thalictroides* (Tt) (in bold). AtGL1 (GLABROUS1), a subgroup 15 R2 R3 MYB, as outgroup. Bootstrap values on main nodes.

1218 bp encode a protein of 406 amino acids; the coding sequence is 87% identical at the nucleotide level to the *Aquilegia* EST. An amino acid alignment to related proteins from other taxa identified high sequence conservation at the *N*-terminus of all proteins with the characteristic R2R3 MYB DNA-binding domain and subgroup 9 motif, and a more variable *C*-terminus (Fig. 3a). Sequencing of genomic clones revealed the presence of two introns within the MYB domain, the first 105 bp long in the R2 domain, the second 396 bp separating the sequences encoding the R3 domain (Fig. 3a, arrowheads).

The complete sequence of *TtMYBML2* was used to design primers spanning most of the coding region and hence obtain near-complete predicted amino acid sequences for the MYBML2 protein from each of the other two species by PCR, followed by 3′-RACE with a polyT primer. Overall identity values for predicted amino acid sequence were 99% between MYBML2 from *T. thalictroides* and *T. filamentosum* (TfMYBML2), 92% to *T. dioicum* (TdMYBML2) and 93% between the two new sequences. The amino acid alignment (see the Supporting Information Fig. S1) showed complete identity between the protein from *T. filamentosum* and that from *T. thalictroides* over the MYB DNA-binding domain and a single, conservative difference (A compared with S) between the DNA-binding domain of the protein from *T. thalictroides* and that from *T. dioicum*. Comparison of the amino acid sequences of the DNA-binding domain of other R2R3MYB proteins (Jackson *et al.*, 1991; Kranz *et al.*, 1998; Stracke *et al.*, 2001) suggested that this amino acid difference would not affect the ability of these proteins to bind their specific DNA-binding sites. The motif specific to members of R2R3MYB subgroup 9 proteins (AQWESARxxAExRLxRES; Stracke *et al.*, 2001, see also Fig. 3a) is completely conserved in the MYBML2 proteins from all three species (AQWESARLEAEARLVRES). In the *C*-terminal region the proteins are more diverged, with four amino acid differences between the protein from *T. filamentosum* and that from *T. thalictroides* (N vs S; S inserted; A vs S; Y vs C) and 25 differences between the protein from *T. dioicum* and that from *T. thalictroides.* Six of the amino acid differences in the *C*-terminal domain between *T. thalictroides* and *T. dioicum* are conservative substitutions. Although there are eight indels that distinguish MYBML2 from *T. thalictroides* and *T. dioicum*, all maintain the open reading frame of the genes and the sequences of the proteins they encode. Considering the variation in amino acid sequences of the *C*-terminal domain of orthologous R2R3MYB proteins

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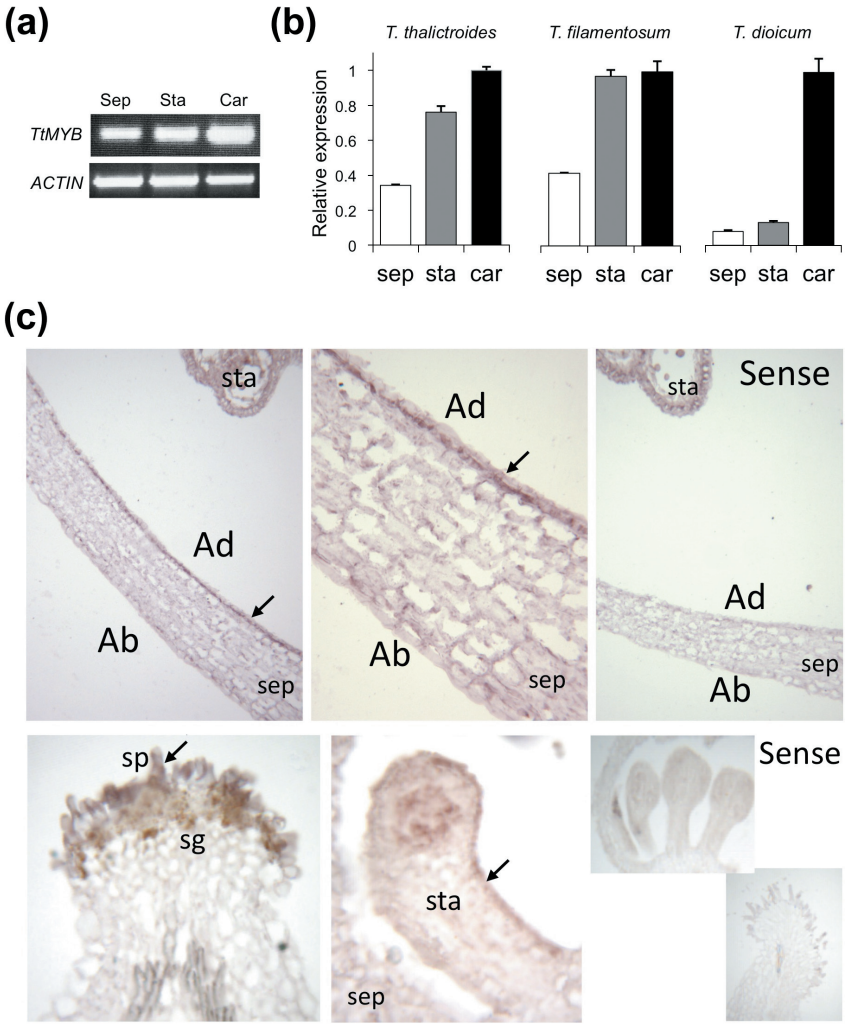

Fig. 4 Expression analysis of *Thalictrum MYB MIXTA-like2*. (a) Expression of a *Thalictrum MIXTA-like* gene (*TtMYBML2*) on floral parts by reverse transcriptase polymerase chain reaction (RT-PCR). Sep, sepals; sta, stamens; car, carpels. *ACTIN* loading control shown below. (b) Relative quantitative expression of *TtMYBML2* by real time PCR in dissected organs of three species of *Thalictrum*: *T. thalictroides*, *T. filamentosum* and *T. dioicum* (all samples normalized to *ACTIN*). Expression is relative to the highest expression organ in each species (carpel). (c) *In situ* hybridization in young flowers. First row, *T. thalictroides* showing expression in the inner (adaxial) epidermis of the sepals (sep), followed by a detail, compared with a sense control (third panel). Second row: expression in stigmatic papillae (sp) of a *T. thalictroides* stigma (sg) and adaxial epidermis of stamen (sta) filament in *T. filamentosum* bud, sense controls in third panel. Ad, adaxial (inner); Ab, abaxial (outer), arrow indicates areas of gene expression.

from different species (Baumann *et al.*, 2007), the predicted sequences derived from the cDNAs suggest that all three species contain *MYBML2* genes that encode active proteins.

The neighbor-joining tree confirms that the *Thalictrum* and *Aquilegia* proteins are closely related and that they are members of the *Antirrhinum* AmMYBML2 and the *Petunia* PhMYB1 lineage (92% identical over the first 169 diagnostic amino acids), which is sister to AmMIXTA and AmMYBML1 (Fig. 3b). AmMYBML2 and PhMYB1 have been shown to have similar functions in the elongation of conical cells in the petal epidermis.

Expression analysis of a *MIXTA-like* gene from *Thalictrum*

Reverse transcriptase PCR with locus-specific primers in *T. thalictroides* showed expression throughout the flower, consistent with related genes in *Petunia* and *Antirrhinum* (Baumann *et al.*, 2007). The ubiquitous expression pattern (including low levels in leaves; data not shown) apparently increasing towards the center of the flower (Fig. 4a), prompted us to pursue quantitative and spatial expression analyses.

Quantitative expression analyses by real-time PCR in dissected floral organs confirmed a trend of increasing expression levels from sepals to stamens to carpels for the three species (Fig. 4b, relative to the highest-expressing tissue in each species, carpels). In the case of stamens, the highest proportional expression was observed in *T. filamentosum* (Fig. 4b), which has petaloid stamen filaments with slightly conical epidermal cells (Fig. 2f–h) that show signal by *in situ* hybridization (Fig 4c, fifth panel, arrow). The wind-pollinated species, *T. dioicum*, showed the same overall pattern of lowest expression in sepals, followed by stamens and carpels, and had overall low expression. However, because the primers used were designed based on the *T. thalictroides* sequence, and this species was found *a posteriori* to have a deletion in the reverse primer sequence, this may have affected the effectiveness of the PCR reaction and therefore the data for *T. dioicum* cannot be directly compared with the other two species.

In situ hybridization showed localization of expression to the upper epidermis of sepals in recently opened floral buds of

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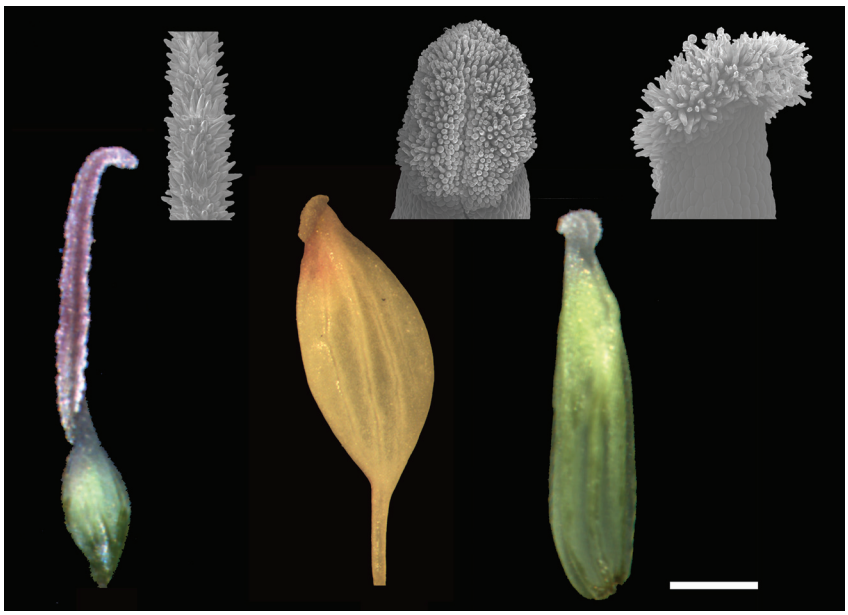

Fig. 5 Carpel and stigma morphology in the three species of *Thalictrum* studied. From left to right: *T. dioicum*, *T. filamentosum* and *T. thalictroides*, each followed by an SEM detail of the stigma showing papillae. Bar, 1 mm.

T. thalictroides, coincident with the area of differentiation of conical cells (Fig. 4c, first row, arrow). Expression was also detected in stigmatic papillae of *T. thalictroides* and the epidermis of stamen filaments in *T. filamentosum* (Fig. 4c, fifth panel, arrow). Stigmas typically have secretions that produce background, but this is a different color (brown-yellow, in the foreground) and is therefore distinct from the signal inside the papillae (arrow).

The high levels of *Thalictrum MYBML2* in carpels may be related to the development of abundant papillate cells in the stigmas of the three species (Fig. 5); this notion is supported by the *in situ* hybridization data which shows strong expression of *TtMYBML2* in these cells in *T. thalictroides* (Fig. 4c, fourth panel, arrow).

Biochemical characterization of a *Thalictrum* MIXTA-like gene product in a tobacco bioassay

Twenty independent transgenic lines were obtained, and their floral epidermis analysed by SEM and freeze-fracture. Eleven independent T1 lines showed very strong or strong phenotypes consisting of elongated epidermal cells in carpels compared with wt (Fig. 6a–c). These phenotypes persisted in the next transgenic generation (T2, Fig. 6d–f).

Petals showed a more subtle phenotype, consisting of increased conical cell height in the adaxial epidermis (tobacco wt petal cells are already conical) (Fig. 6g–i). Extended conical cells on the adaxial petal lobe were abundant in all transgenic individuals examined (Fig. 6h). Freeze fracture highlighted the difference in conical cell height of the adaxial petal lobe between wt and transgenic lines (Fig. 6i,j).

Quantification of the height of the conical cells confirmed that the difference between wt and transgenic lines was statistically highly significant ($P < 0.003$). Interestingly, the ectopic conical cells generated by overexpression of *TtMYBML2* in tobacco carpel epidermis were asymmetric, like those in *T. thalictroides* sepals (compare Fig. 2c,d with Fig. 6c,f), while the tobacco wt petal cells were symmetrically conical (Fig. 6g). Leaves and stamens showed no phenotype and no ectopic trichomes were found, indicating that the phenotype induced by *TtMYBML* is flower-specific, as also reported for *AmMYBML2*, *AtMYB16* and *PhMYB1* (Baumann *et al.*, 2007). Based on the high amino acid sequence similarity (Fig. S1), we predict that the proteins from the other two species are likely to have similar functions.

Discussion

Conical cells as micromorphological markers of petaloidy in *Thalictrum*

Our findings in three distinct species of *Thalictrum*, comprising both wind and insect pollination adapted suites of characters, leads us to hypothesize that the presence of conical cells on the epidermis of sepals or stamens may be a reliable micromorphological marker for the ancestral condition of insect-pollinated flowers in this genus.

Evidence from closely related genera in the phylogeny of Ranunculaceae suggests that loss of petals occurred before the diversification of *Thalictrum* (Wang & Chen, 2007). Following this event, petaloidy of the sepals, which is found throughout the family, was further lost in association with transitions to wind pollination. In other cases, insect-pollinated species shifted attractive functions from the sepals to the stamens. Slightly conical cells are found on the petaloid stamens of one such species, *T. filamentosum* (Fig. 2g,h).

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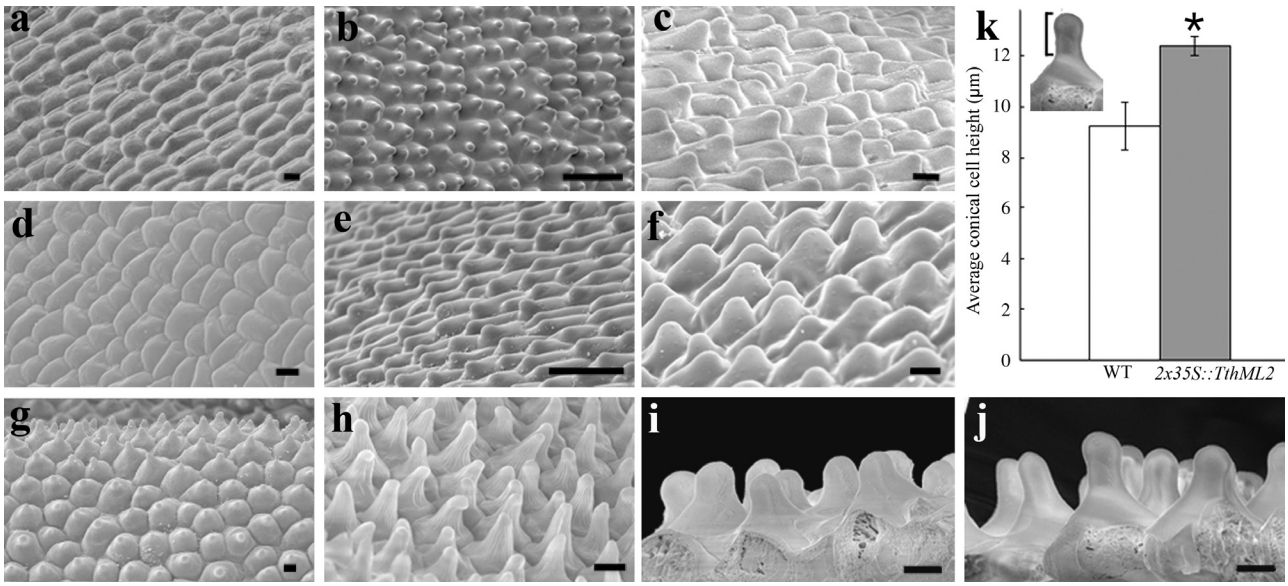

Fig. 6 *Thalictrum Mixta-like2* induces ectopic conical cells in tobacco. Scanning electron microscopy (SEM) and freeze-fracture images of floral organs of three representative tobacco lines over-expressing *TtMYBML2*. (a–c) T1 generation. (a) Flat epidermal cells of wild-type tobacco; (b) overview of conical cells on ovary epidermis of tobacco overexpressing *Thalictrum Mixta-like2*; (c) close up of the ectopic conical cells. (d–f) T2 generation. (d) Flat epidermal cells in wild-type tobacco carpel epidermis; (e,f) conical cell growth on the carpel epidermis of transgenic plants expressing *TtMYBML2* under the control of the CaMV35S promoter; (g) normal conical cells on adaxial petal lobe of wild-type tobacco; (h) extended conical cells on the adaxial petal lobe of transgenic line. Freeze fracture highlights the difference in conical cell height of the adaxial petal lobe between wild-type (i) and transgenic (j) individuals. (k) Quantitative difference in adaxial petal cell heights, measured as diagramed on inset cell. Averages ± SE, asterisk indicates statistical significance between the means (P = 0.00355). Bars, (b,e) 50 μm, (a,c,d,f–k) 10 μm.

Insect pollination is the ancestral condition in the genus; wind pollination evolved early, followed by the evolution of unisexual flowers (monoecy and dioecy). Some species appear to have secondarily evolved insect pollination features without conical cells, mainly with showy stamens that have pigmented and/or flattened filaments (Fig. 1d–f; J. Brunet *et al.*, unpublished) or showy sepals (Fig. 1a–c).

Thalictrum MIXTA-like predates the *MIXTA/ML1* duplication

PhMYB1 has been shown to be orthologous to AmMYBML2 rather than MIXTA (Baumann *et al.*, 2007). These proteins are more widespread than the *AmMIXTA/AmML1* duplication, which so far appears specific to the snapdragon family (Baumann *et al.*, 2007). Indeed, in the same study, Southern blot analysis and cDNA library screening showed that *PhMYB1* is the only *MIXTA*-like gene in the *Petunia* genome. Similarly, our cloning efforts involving several independent RT-PCR reactions and primer combinations in three species, always recovered the same gene, suggesting that this is the only *MIXTA-like* gene in *Thalictrum*.

The evidence to date indicates that Am*MIXTA* and Am*MYBML1* have resulted from a recent duplication that has not yet been found outside of *Antirrhinum.* The *MIXTA*-related gene that we were able to recover from *Thalictrum* is most closely related to *AmMYBML2* and *PhMYB1* (Fig. 3b). Therefore, it is likely that the duplication that gave *MIXTA* and *ML1* occurred after the diversification of *Thalictrum*, so that no simple orthology can be assessed between these loci.

With regard to related proteins in other taxa that were used in our alignment and gene tree, AtMYB106 represents the *Arabidopsis* member of the AmML2/PhMYB1 clade and is encoded by the *NOECK* (*NOK*) gene (Jakoby *et al.*, 2008) which functions in trichome formation in *Arabidopsis*. It may also function redundantly with AtMYB16 in conical cell formation in petals (Baumann *et al.*, 2007; Jakoby *et al.*, 2008). AmMYBML3 is another snapdragon-related protein with functions similar to those of the other MIXTA-like proteins (Jaffe *et al.*, 2007).

The main points emerging from the evolution of these gene lineages are: the MYB family is difficult to analyse phylogenetically because of the presence of short, highly conserved domains combined with highly divergent domains (Fig. 3a); the MYBML2 clade appears to have representatives from across the eudicots whereas MIXTA itself and its close paralog MYBML1 have only been identified in *Antirrhinum*, leading us to conclude that the MIXTA lineage is not deeply conserved but may be quite recently evolved (Fig. 3b and Baumann *et al.*, 2007); and *MYBML2*-genes are much more broadly distributed and appear to serve similar cellular functions in promoting cell elongation (Baumann *et al.*, 2007).

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Thalictrum MIXTA-like2 functions in conical cell differentiation, not trichomes

One of the three genes isolated, *TtMYBML2*, was expressed under double 35S promoter in tobacco to observe the overexpression phenotype. Given the high sequence identity (93–99%) among the *Thalictrum* loci, we assume that all three proteins are most likely functionally equivalent. The results confirmed that the biochemical function of the *Thalictrum MIXTA-like* gene in tobacco is similar to *AmMYBML2*. Our hypothesis was that if *TtMYBML2* triggered epidermal cell elongation (conical cells or trichomes) in tobacco epidermis, this would indicate conservation of biochemical function in relation to the other closely related genes *PhMYB1* and *AmMYBML2*. Combined with the epidermis-specific expression of *TtMYBML2* in sepals and *TfMYBML2* in stamens (Fig. 4c), we believe that this finding strongly supports a role for these loci in papillate cell production in *Thalictrum*. Moreover, expression of *TtMYBML2* in stigmas is suggestive of a role in stigmatic papillae in carpels (Fig. 4c) not reported for other related genes. This observation needs to be confirmed in additional species, especially given the variation in stigma morphologies represented in these three species (Fig. 5).

Interestingly, it appears that the *Thalictrum* MYBML2 can only promote conical cell growth in the petal and carpel epidermal cells of tobacco (Fig. 6) rather than development of both conical cells and trichomes, as in *AmMYBML1* (Perez-Rodriguez *et al.*, 2005). This finding is consistent with a closer relation to *AmMYBML2* and *PhMYB1*, both of which promote conical cells, not trichomes. It remains possible that this difference results from the phylogenetic distance between *Thalictrum* and tobacco rather than an actual difference in the endogenous function of *TtMYBML2*. Alternatively, this may reflect the independent evolution and elaboration of trichomes via separate genetic pathways (Serna & Martin, 2006). Interestingly, a recent study identified a gene in the trichome pathway of *Arabidopsis* (*NOECK*) as encoding the MIXTA-like transcription factor AtMYB106 (Jakoby *et al.*, 2008).

Expression levels of *MIXTA-like* genes correlate with pollination syndromes in *Thalictrum*

The hypothesis being tested with the comparative gene expression analyses was that *TtMYBML2* is a cell-shape regulator that promotes elongation in the perianth epidermis, resulting in conical cells and/or trichomes. The implication is that having a petaloid perianth is the ancestral condition (*T. thalictroides* and *T. filamentosum*), which was lost (or downregulated) after the transition to wind pollination early in the evolutionary history of the genus and was then regained in the instances in which insect specialization has secondarily evolved (e.g. *Thalictrum delavayi* and *Thalictrum aquilegifolium*) by a mechanism that utilizes pigment rather than surface cell-shape regulators. Under this scenario, we expected and found a correlation between spatial expression of the *Thalictrum MYBML2* and the presence of conical cells in sepals, stamen filaments and stigmatic papillae in species with the ancestral insect pollination features. Expression in wind-pollinated *T. dioicum* was almost negligible in sepals and stamens compared with carpels; we hypothesize that this could be because of an extended stigmatic surface in carpels and a lack of conical cells in sepals nor stamens (this remains to be tested by *in situ* hybridization).

Conical cells are often used as markers for petal cell identity. Our study in the genus *Thalictrum* shows that conical cells can be formed on petaloid organs that do not have petal identity, representing an ancestral character linked to insect pollination. Conical cells therefore fulfill the criterion of a tractable marker for petaloidy in *Thalictrum* species – their presence is closely correlated with the expression of the subgroup 9 MYB gene *TtMYBML2*, which may well be selected by enhancing the attractiveness of sepals and stamens to insect pollinators.

Conclusions

In our study system, conical cells do not provide a marker for petal identity (in the developmental sense), instead they are useful indicators of petal functionality (for example, in organs involved in pollinator attraction). Our work defines the gene that is responsible for conical cell formation in *Thalictrum*.

Our molecular analyses identified the gene encoding the transcription factor that can induce conical cell formation in *Thalictrum*. The expression of this gene in different floral organs of the three *Thalictrum* species studied is correlated to whether or not these organs are petaloid (with the exception of carpels, where it correlates with the presence of stigmatic papillae). Therefore, the development of petaloidy in different organs of species with the ancestral insect pollination traits is associated with redeployment of conical cells, which probably involves changes in expression pattern of *TtMYBML2* and its orthologues.

The fact that TtMYBML2 is structurally and functionally closest to AmMYBML2 and PhMYB1 suggests that this represents the original clade of R2R3 MYB subgroup 9 regulators and that MIXTA and AmMYBML1 diverged following subsequent gene duplications. Ours is the first characterization of a member of this gene family outside of the core eudicots.

Given the high level of sequence identity among the three predicted proteins for MYBML2 from *Thalictrum* (over 93%, with identical DNA-binding domains), it seems likely that these genes will be functionally equivalent. Evolution may therefore, once again, have worked through changes in regulatory sequences that affect expression (Jeong *et al.*, 2008), in this instance of a gene encoding a floral transcription factor.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Amino acid alignment of MYBML2 predicted proteins from *Thalictrum thalictroides*, *Thalictrum filamentosum* and *Thalictrum dioicum*.

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