The Plant Journal (2005) **41**, 755–766 doi: 10.1111/j.1365-313X.2005.02336.x

Floral MADS box genes and homeotic gender dimorphism in *Thalictrum dioicum* (Ranunculaceae) – a new model for the study of dioecy

Verónica S. Di Stilio[1,*,†], Elena M. Kramer[1] and David A. Baum[1,2]
[1]*Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA, and*
[2]*Department of Botany, University of Wisconsin, Madison, WI 53706, USA*

Received 23 September 2004; revised 8 December 2004; accepted 14 December 2004.
[*]For correspondence (fax +206 616 2011; e-mail distilio@u.washington.edu).
[†]Present address: Department of Biology, University of Washington, Seattle, WA 98195, USA.

Summary

In most dioecious angiosperm species, flowers are initially perfect but abort either stamens or carpels during their development, indicating that sex determination occurs after floral organ identity has been established. Dioecious members of the genus *Thalictrum* (meadow-rue), however, produce flowers that lack aborted organs. Examination of early flower development of *T. dioicum* confirms that flowers are male or female from inception, raising the possibility that genetic mechanisms working at or above the level of organ identity promote sex determination through a homeotic-like mechanism. In order to investigate this possibility, we identified homologs of the organ identity genes *PISTILLATA* (*PI*), *APETALA3* (*AP3*) and *AGAMOUS (AG)* from *T. dioicum* and the hermaphroditic species *T. thalictroides*. A combination of early and late duplication events was uncovered in these gene lineages and expression analyses indicate that these events are generally associated with divergence in gene regulation. In light of these findings, we discuss the potential of *T. dioicum* as a new model for the study of sex determination in the basal eudicots.

Keywords: dioecy, homeosis, MADS box genes, *APETALA3, PISTILLATA, AGAMOUS*.

Introduction

Dioecy, the condition of having separate pollen-producing (male) and ovule-producing (female) plants is relatively rare, occurring in only about 6% of angiosperm species. However, the widespread phylogenetic distribution of dioecy across three quarters of all plant families suggests that it has evolved independently multiple times from perfect-flowered (hermaphroditic) ancestors (Charlesworth, 1985; Renner and Ricklefs, 1995). Most dioecious plants go through a hermaphroditic stage early in flower development, followed by differential abortion or arrest of sex organs, which can occur at a variety of stages. Examples of this type of unisexual flower development include the most commonly studied dioecious plants such as *Silene latifolia* (white campion) (Farbos *et al.*, 1997; Grant *et al.*, 1994) and *Rumex acetosa* (sorrel) (Ainsworth *et al.*, 1995; Crossley and Ainsworth, 1994), as well as monoecious species such as maize (Dellaporta and Calderon-Urrea, 1994) and cucumber (Malepszy and Niemirowicz-Szczytt, 1991). However, some dioecious plants like *Spinacia oleracea* (spinach), *Mercurialis annua* (mercury) and *Cannabis sativa* (hemp) have unisexual flowers with no vestiges of organs of the opposite sex (Lebel-Hardenack and Grant, 1997). In cases where organs are initiated and then aborted, the genetic mechanism of sex determination must be acting downstream of organ identity, while in cases that show unisexuality from the earliest stages of development, it is possible that male versus female development is mediated by a homeotic-like mechanism in which all primordia develop as either stamens or carpels.

Analysis of the model species Arabidopsis and *Antirrhinum* has provided a thorough understanding of the genetic pathways that are responsible for floral organ identity. These results are summarized in the ABC model, which posits that three classes of homeotic genes function in overlapping domains to determine the fate of organ primordia as sepals, petals, stamens or carpels (Bowman *et al.*, 1991a; Coen and Meyerowitz, 1991). For the study of sex determination, the genes that control the identity of the sex organs are of particular interest. Stamen identity is promoted by the

combined activity of the B and C class genes, whereas C class genes alone control carpel identity. In *Arabidopsis thaliana*, these classes are represented by *APETALA3* (*AP3*) and *PISTILATA* (*PI*) in the B class and *AGAMOUS* (*AG*) in the C class (Coen and Meyerowitz, 1991). Mutations in B class genes result in flowers that are essentially female due to homeotic conversion of petals into sepals and stamens into carpels (Bowman *et al.*, 1989, 1991a). Alternatively, male flowers can be produced by overexpression of the B class genes, causing stamens to arise in place of carpels (Krizek and Meyerowitz, 1996). Thus, alteration in the regulation of floral organ identity genes allows for the possibility of sex determination via a homeotic-like mechanism.

The potential role of B and C class genes in sex determination has been investigated in two dioecious species, *Silene* and *Rumex* (Ainsworth *et al.*, 1995; Hardenack *et al.*, 1994). In these cases, the early B and C class gene expression patterns were relatively normal, but expression declined once organ arrest began. Although it remains unclear as to whether this reduction in gene expression is responsible for or a product of developmental arrest, it does not appear that sex determination affects the earliest stages of gene regulation in these species. Similar results have been found in dioecious *Asparagus* (Park *et al.*, 2003), monoecious taxa such as *Liquidambar*, *Gerbera* and *Cucumis* (Kater *et al.*, 2001; Liu *et al.*, 1999; Yu *et al.*, 1999), and in a variety of *Daucus* exhibiting a homeotic form of cytoplasmic male sterility (Linke *et al.*, 2003). However, all of the unisexual species studied to date exhibit some degree of hermaphroditism early in floral development, suggesting that sex determination would not be expected to act directly upstream of the organ identity genes. In contrast, in species whose flowers are completely male or female from inception, there is *a priori* greater reason to suspect a homeotic-like mechanism, and hence a role for organ identity genes or their upstream regulators.

The genus *Thalictrum*, in the basal eudicot family Ranunculaceae, contains hermaphroditic, andromonoecious (male and hermaphroditic flowers in the same plant) and dioecious species (Brunet and Liston, 2000). Although some dioecious species are morphologically androdioecious (hermaphrodites produce stamens with non-functional pollen) (Davis, 1997), gynodioecy (females and hermaphrodites), which is a commonly invoked step in the evolution of dioecy (Lloyd, 1980; Ross, 1970), is not represented in the genus. One dioecious member of the genus, *T. dioicum*, is a common wind-pollinated, woodland herb of the eastern and northern regions of North America. In *T. dioicum*, sex is stable throughout the life of an individual and sex ratios are close to 50 : 50 (see Results), suggesting a sex determination factor segregating according to Mendelian expectations. Furthermore, sex determination in related dioecious species of *Thalictrum* has been shown to be under nuclear control (X/Y), with males serving as the heterogametic sex and no heteromorphic sex chromosomes present (Kuhn, 1930, 1939; Westergaard, 1958). Additionally there is evidence for viability of YY males, indicating that *Thalictrum* is at an early stage of sex chromosome evolution, with little differentiation between X and Y chromosomes such that heteromorphic sex chromosomes are not observed and the Y chromosome has not yet accumulated deleterious mutations (Kuhn, 1939, reviewed in Westergaard, 1958).

The overall goal of the current study was to investigate fundamental aspects of the development and genetics of sex determination in *Thalictrum*. More specifically, we evaluate the possibility that sexual dimorphism in *T. dioicum* results from differential regulation of B and C class floral organ identity genes. As a first step in this process, we confirmed that flowers of *T. dioicum* are entirely male or female from the earliest stages, and that sex determination occurs via genetic mechanisms rather than environmental effects. This was followed by characterization and expression analysis of the floral organ identity genes involved with reproductive development, which has revealed that certain paralogs display divergent expression patterns. These findings emphasize the importance of gene duplication and sub/neofunctionalization in the evolution of new floral phenotypes and highlight the potential of *T. dioicum* as a model for the study of the genetic basis of sex determination in the basal eudicots.

Results

Flower development and mature morphology in T. dioicum

Light and scanning electron microscopy (SEM) were used to characterize the development of carpellate and staminate flowers (Figures 1 and 2). In stage 1 (s1) female flowers, spiral initiation of four to five sepal primordia is rapidly followed by the appearance of spirally arranged carpel primordia (Figure 1a). These primordia are clearly distinguishable from those present in the center of s1 male flowers by their size, shape and number (compare Figure 1a–c,i,j). During s2–s3, carpel primordia continue to arise and develop a central depression (Figure 1b,c), taking on what is known as an ascidiate (urn-like) form. This depression is clearly defined by s4 (Figure 1d) and will later become the ovary of the carpel within which a single ovule forms. In contrast, the central primordia of male flowers never exhibit this depression, instead remaining cylindrical (s3–4, Figure 1j,k). During s5 and s6 of female flowers, the adaxial side of the carpel rim extends (Figure 1e,f) and goes on to form the style after carpel closure (Figure 1g,h). In s3–5 male flowers stamen primordia begin to assume their tetrahedral shape and initiate filament development (Figure 1j–l). Late stages of male development primarily involve the elongation of the filament and maturation of the anther (Figure 1m,o,p).

Sectioned, stained buds confirm the above observations, particularly the lack of staminodes or pistillodes at any stage

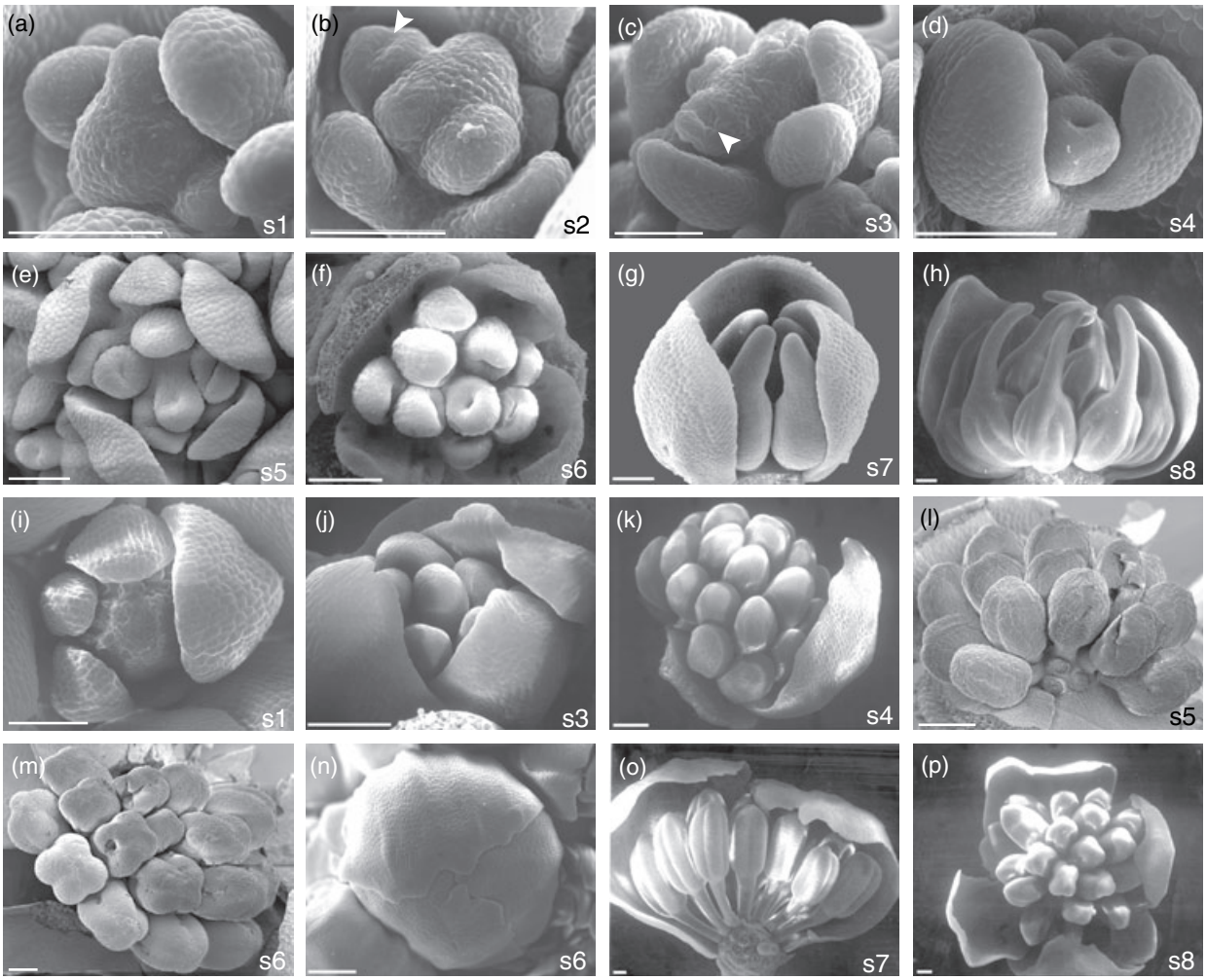

Figure 1. Scanning electron microscopy photos of carpellate (a–h) and staminate (i–p) flowers of *Thalictrum dioicum*. During stages s1–s2, male and female flowers initiate four to five sepal primordia and begin to produce reproductive organs. Subsequent organ differentiation in female flowers is marked by the appearance of an invagination on carpel primordia (s3–s4), followed by the asymmetric initiation of the style (stages s5–s6). Maturation of female flowers (stages s7–s8) primarily involves elongation of the carpels and sepals. In male flowers, stages s2–s4 encompass the differentiation of cylindrical stamen primordia that later (stages s5–s6) become tetrahedral and develop filaments. Male sepals grow to enclose the floral meristem during s5–s7. Stamen maturation is completed with rapid elongation and further differentiation of the anthers (stages s7–s8). Scale bar = 0.1 mm.

(Figure 2A–C,E–G). A longitudinal section through a bud of the hermaphroditic *T. thalictroides* provides a useful comparison, showing sepals, stamens and carpels (Figure 2I).

To address the observation of differing organ numbers in carpellate and staminate buds of *T. dioicum*, stamens and carpels were counted from 27 flowers of each sex from nine plants in two populations. Carpel numbers ranged from five to 17 and stamens from 20–42, sepals were four to five.

In addition to the differences in sex organs, male and female flowers show secondary sexual differences. At maturity, male flowers are pendant but female flowers are erect (Figure 2D,H). The sepals of male flowers grow to tightly enclose the bud (Figure 1n) whereas in the female, sepals remain open (Figure 1g,h). Moreover, female perianth parts seem smaller and slightly more sepaloid than male perianth parts (Figure 2D,H arrows). We closely examined sepal epidermal features in order to determine whether this differentiation is also exhibited in the epidermal cell types. Sepals of the hermaphrodite *T. thalictroides*, which are distinctly petaloid in appearance (Figure 2J arrow), were studied for comparison. The abaxial sepal epidermal cells in carpellate and staminate flowers of *T. dioicum* have similar cell morphologies, being flat and irregularly shaped with slightly sunken stomata and elongated pavement cells overlying the vascular strands (Figure 3a,c). The appearance of the abaxial sepal epidermal cells in *T. thalictroides* is similar, with the exception that the stomata are surrounded by a distinctive ring-like arrangement of subsidiary cells (Figure 3e). Trichomes occur at the edges of the sepals in both sexes of *T. dioicum*, on carpels and occasionally on the abaxial epidermis of sepals in carpellate flowers (Figure 3a inset). These trichomes are

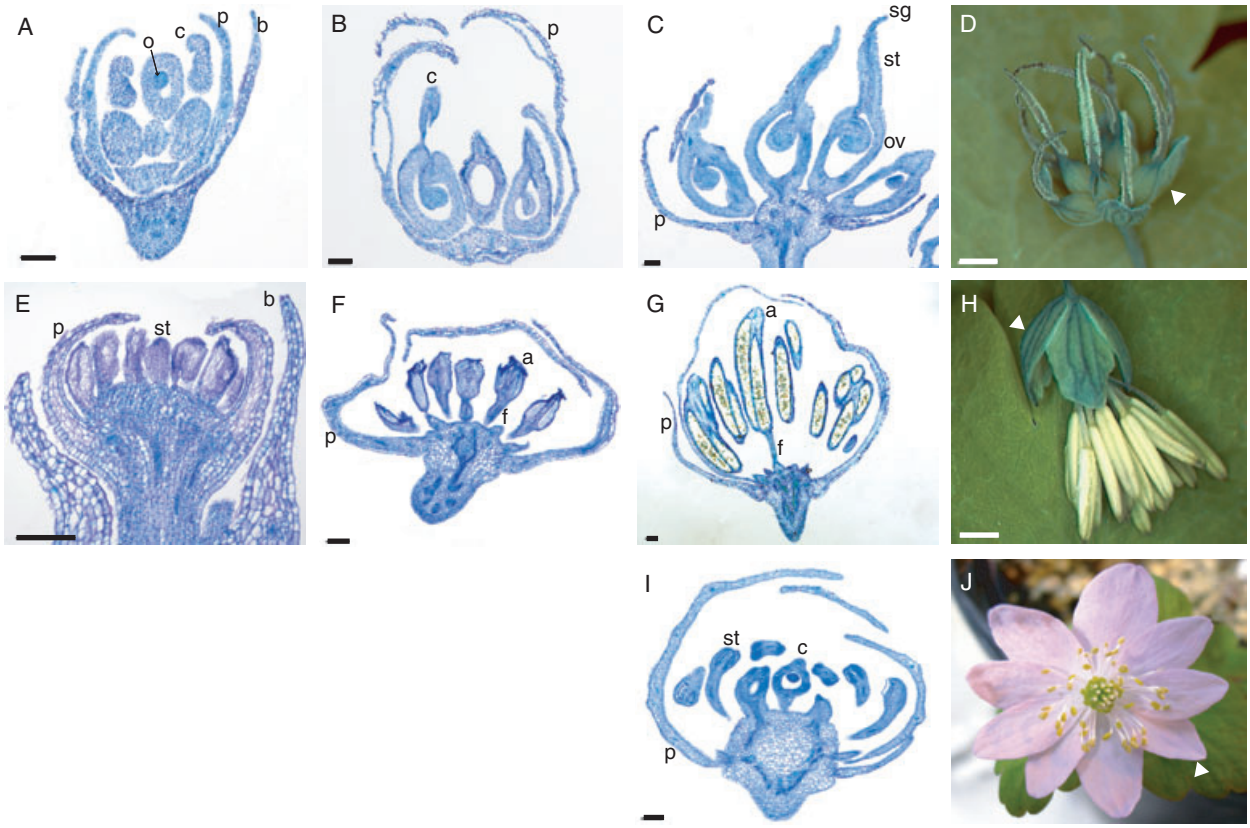

Figure 2. Sectioned, stained sections of carpellate (A–C) and staminate (E–G) flower buds of *Thalictrum dioicum*, and hermaphroditic *T. thalictroides* (I). Mature flowers of carpellate (D) and staminate (H) *T. dioicum* and hermaphroditic *T. thalictroides* (J). Scale bar = 1 mm for (D) and (H); 0.1 mm for others. b, bract; c, carpel, sg, stigma; st, stamen primordium; sy, style; p, perianth; ov, ovule. Arrows in (D), (H) and (J) indicate sepals.

unicellular and glandular as described by Trelease (1886). The adaxial sepal epidermis also appears similar in shape between male and female flowers, with flat cells that become more irregular toward the tip of the organ and which are larger in males (Figure 3b,d). The adaxial side of *T. thalictroides* sepals differs more dramatically in that cells are asymmetrically papillate (Kay *et al.*, 1981) at the base and along the center of the organs (Figure 3f). Nonetheless, although there are subtle differences between male and female sepals of *T. dioicum*, neither closely resembles the more petaloid sepals of *T. thalictroides*.

Characterization of sex determination in T. dioicum

Using plants grown from wild-collected seed, we found 1:1 sex ratios of carpellate to staminate plants in five different controlled crosses (Table 1). A chi-squared test was used to test the hypothesis of equal distribution of males and females in the progeny, all *P*-values were non-significant ($P > 0.1$). Adult plants collected from the field had a constant sex expression throughout 4 years ($n = 16$), with no sex reversal. These two lines of evidence point to a stable genetic sex determination system in this species. The sex ratio data provides evidence for an X/Y system of sex determination similar to that found previously in related species of *Thalictrum* (Kuhn, 1930, 1939; reviewed in Westergaard, 1958), where the male is the heterogametic sex.

Cytogenetics

In order to establish the basic genomic characteristics of *T. dioicum*, we examined chromosome number and nuclear genome content. Previous studies of chromosome numbers across species of *Thalictrum* have revealed a 12-fold variability in ploidy, ranging from a haploid value of 7, considered the base number for the genus, up through 84 chromosomes, with $2n = 14$ and 28 being the most common somatic numbers (Gregory, 1941). Different cytotypes have been reported within species of *Thalictrum*: one previous analysis of *T. dioicum* reported $2n = 14$ (Hill, 1989), another $2n = 42$ (Kaplan and Mulcahy, 1971). Chromosome observations in mitotic root tips stained with toluidine blue showed that the population of *T. dioicum* used in this study has a complement of $2n = 28$ (data not shown), indicating that it is tetraploid. The C-value DNA content of *T. dioicum* as

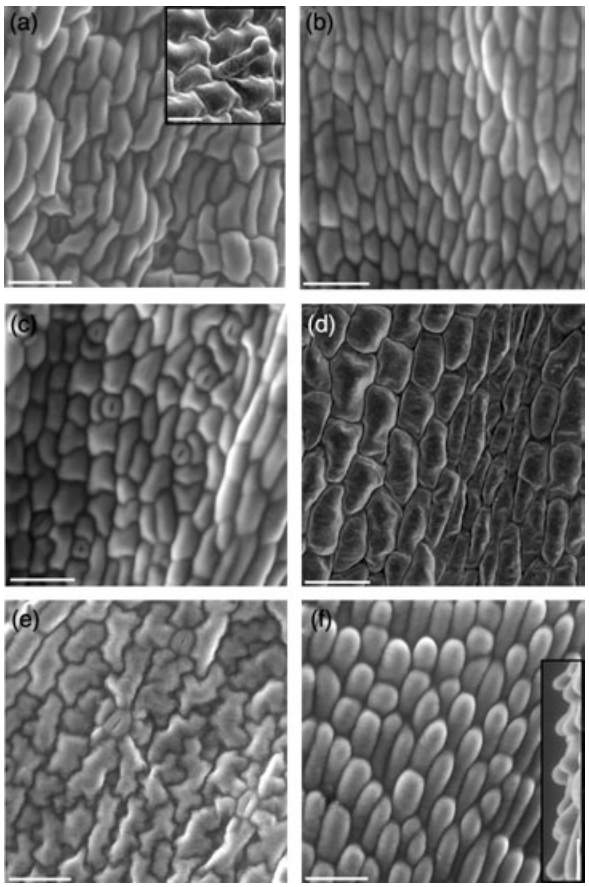

Figure 3. Scanning electron microscopy of the adaxial and abaxial epidermis of perianth organs in carpellate (a, b) and staminate (c, d) flowers of *Thalictrum dioicum* and in hermaphroditic flowers of *T. thalictroides* (e, f). Left panels (a, c, e) show abaxial surfaces, right panels (b, d, f) show adaxial surfaces. Inset in (a) shows a trichome, in (f) cells in profile. Scale bar = 50 μm in (a–d), 20 μm in (e), (f) and insets.

estimated by flow cytometry, was 965 Mbp (average of four independent estimates).

Isolation and characterization of Thalictrum *B and C class genes*

Five transcript classes of B class genes were identified in *T. dioicum* (prefix *Thd*), two *PI* and three *AP3*. One *PI* and three *AP3* homologs have been previously reported from *T. thalictroides* (prefix *Tht*) and were analyzed phylogenetically in the context of a family-wide study of the B class genes (Kramer *et al.*, 2003). It was found that *T. dioicum AP3* homologs follow the same pattern as reported for *T. thalictroides*, with one ancient gene duplication event giving rise to the *AP3-1* and *AP3-2* lineages and a more recent duplication producing *AP3-2a* and *-2b* (Figure 4a and Kramer *et al.*, 2003). The phylogenetic analysis indicates that the *AP3-2a*/*2b* duplication predated the last common ancestor of *Thalictrum* and *Aquilegia*, but no *AP3-2a* ortholog has been detected in *Aquilegia* (Figure 4a and Kramer *et al.*, 2003). For all genes, the *T. thalictroides* and *T. dioicum* loci are placed as sister to one another, indicating that the two species have an identical complement of *AP3* genes. Both *ThtAP3-2a* and *ThdAP3-2a* appear to encode truncated proteins in which the ancestral paleoAP3 motif and half of the PI Motif-derived regions are eliminated (Kramer *et al.*, 1998) (Figure 5). Similar to *T. thalictroides*, orthologs of the *AP3-3* lineage, which is florally expressed in many Ranunculaceae (Kramer *et al.*, 2003), were not detected in *T. dioicum*. In the case of *PI*, we identified two transcript classes in *T. dioicum* when compared with a single class in *T. thalictroides*. The *T. dioicum* cDNA classes are 96% identical at the nucleotide level throughout the coding region with one 9-nucleotide indel, and 93% identical in the 3′ UTR with 7 indels ranging from 1 to 27 nucleotides. Due to this level of difference, particularly the presence of multiple indels, each class has been treated as a distinct locus, termed *ThdPI-1* and *ThdPI-2*. Phylogenetic analysis does not resolve the timing of the *ThdPI-1* and *ThdPI-2* duplication event relative to the *PI* homologs from the closely related genus *Aquilegia* or *T. thalictroides* (Figure 4b), but it is possible that it coincides with the genome duplication that occurred in the *T. dioicum* lineage.

Homologs of the C class gene *AG* were also characterized with the expectation that they would serve as a control, with similar expression in carpellate and staminate flowers. Two main loci, termed *AG1* and *AG2*, were recovered in each species. *ThdAG1* and *ThdAG2* have been previously published as part of a broader study of the *AGAMOUS* lineage in angiosperms (Kramer *et al.*, 2004). For *T. dioicum*, two transcript classes were identified for *AG1*. These cDNA classes, referred to as type 1 (T1) and type 2 (T2), are very similar to each other throughout their length with 97% nucleotide identity and no indels. Phylogenetic analysis (Figure 4c) shows that the *Thalictrum AG1* and *AG2* loci are both members of the C lineage along with *AG* from Arabidopsis and other *AG* functional homologs such as *PLENA* and *ZAG1* (Theissen *et al.*, 2000, and references therein; Kramer *et al.*, 2004). The pairing of *ThdAG1*/*ThtAG1* and *ThdAG2*/*ThtAG2* suggests that the gene duplication that produced these paralogous lineages predated the divergence of the *Thalictrum* species such that each has an identical complement of *AG*-like genes. For *ThdAG1*, the two

Table 1 Sex ratios from controlled crosses of *Thalictrum dioicum*

Cross no.	Female	Male	Total	χ^2	*P*-value
1	14	14	28	0	NS
2	14	10	24	0.67	NS
3	14	16	30	0.13	NS
4	12	7	19	1.31	NS
5	16	10	26	1.38	NS

NS, not significantly different from a 1:1 ratio.

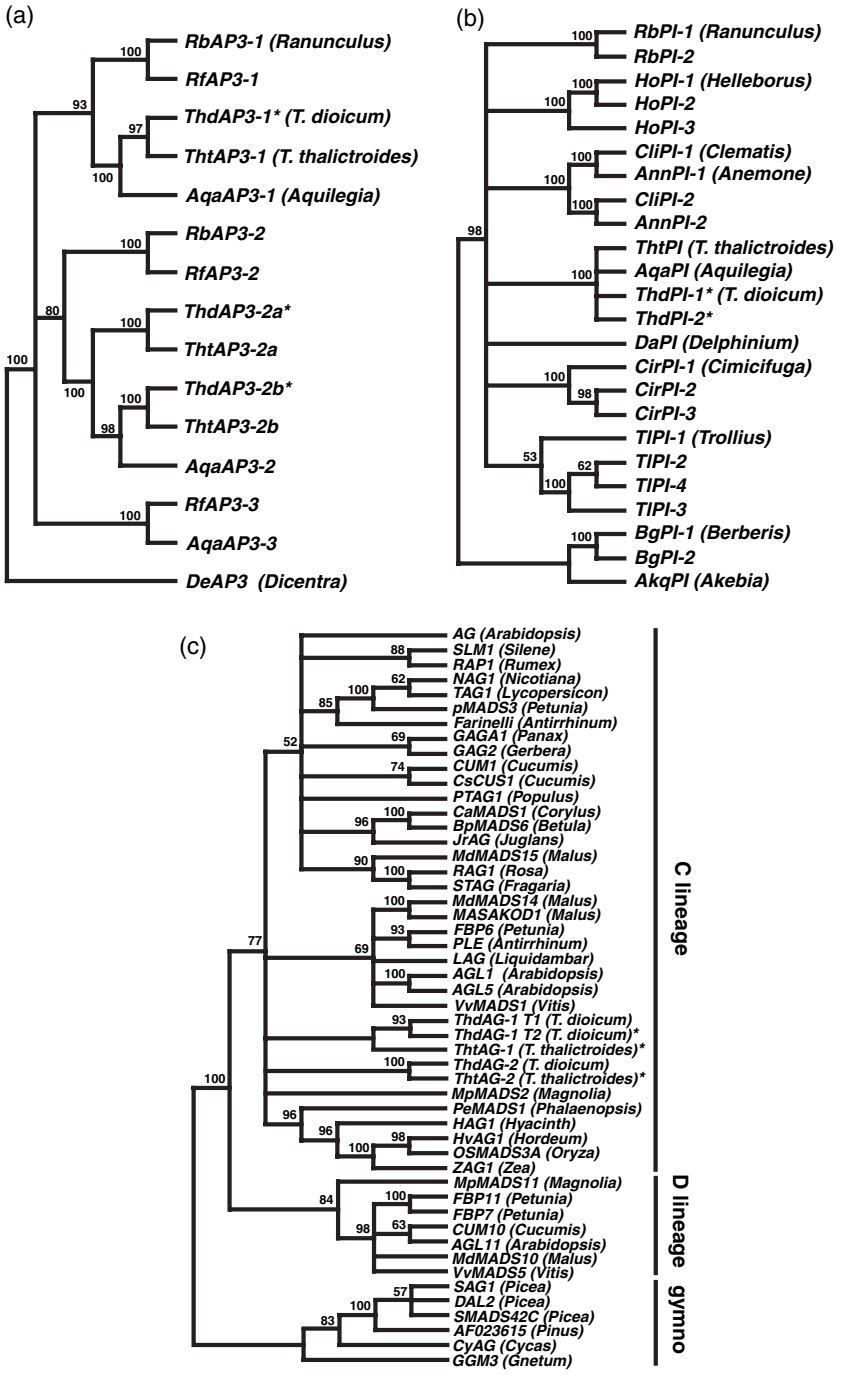

Figure 4. (a) Phylogenetic analysis of *Thalictrum dioicum PI* homologs in the context of other Ranunculaceae *PI* homologs. Consensus tree of three equally parsimonious trees of 1417 steps. (b) Same as (a) for *AP3* homologs. (c) Phylogenetic analysis of *Thalictrum AG* homologs with *AG*-like genes from other angiosperms and gymnosperms. Single most parsimonious tree of 4250 steps. The numbers next to the nodes give bootstrap values from 1000 pseudo replicates. Branches with <50% support are collapsed. Asterisks indicate genes cloned in this study.

types are paired together with strong support, unlike the situation observed for *ThdPI-1* and *-2*. The *ThdAG1* cDNA types may represent homeologous loci derived from the genome-wide duplication in *T. dioicum*.

Expression analysis of Thalictrum *B and C class genes*

Gene-specific semiquantitative RT-PCR was used to assay the expression of the five B and two C class genes in male and female flowers of *T. dioicum*. In the first RT-PCR experiment (Figure 6a), cDNA was prepared from floral organs dissected from young male and female buds. Hermaphroditic flowers of *T. thalictroides* were similarly examined for comparison (Figure 6b). In the second experiment, separate cDNA pools were prepared from young inflorescences of three male and three female individuals, using leaf cDNA pools to control for non-specific amplification (see also Experimental procedures) (Figure 6c).

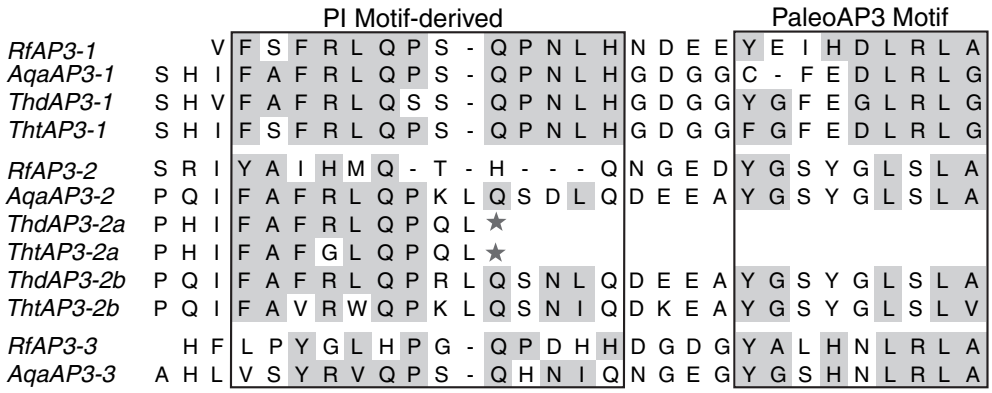

Figure 5. Alignment of C terminal regions of *Thalictrum AP3* homologs with other Ranunculaceae *AP3* homologs. Boxes delimit previously identified conserved motifs (Kramer *et al.*, 1998) and shading indicates conservation with the original motif consensus sequences. Stars indicate protein truncation due to a conserved stop codon at the nucleotide level. *Aqa, Aquilegia alpina*; *Thd, Thalictrum dioicum*; *Tht, Thalictrum thalictroides*; *Rf, Ranunculus ficaria*.

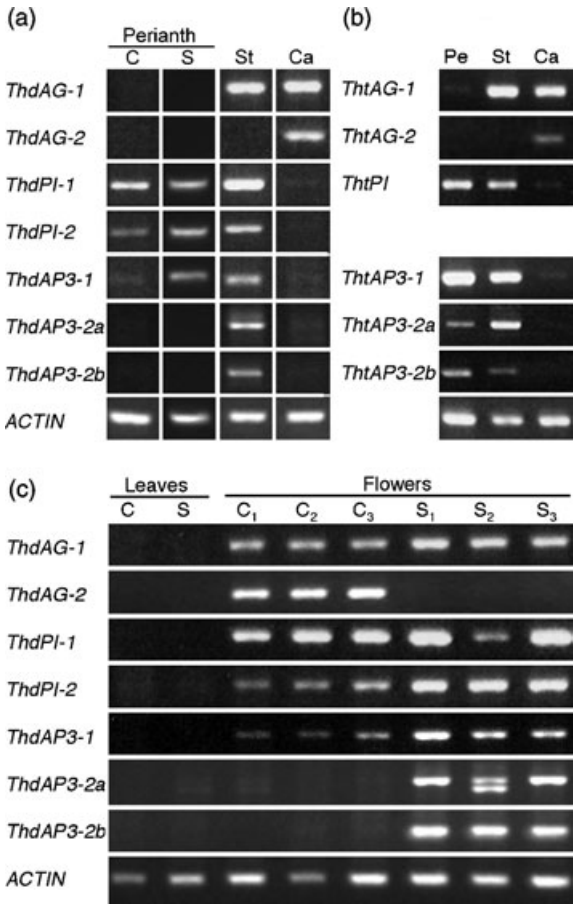

Figure 6. Expression patterns of B and C class gene homologs for dioecious *Thalictrum dioicum* and hermaphroditic *T. thalictroides* as determined by RT-PCR using locus- specific primers.
(a) *Thalictrum dioicum*-dissected organs of carpellate and staminate buds at early stages of development. C, carpellate bud; S, staminate bud.
(b) *Thalictrum thalictroides*-dissected floral organs from buds early stages of development. Pe, perianth; St, stamen, Ca, carpel.
(c) *Thalictrum dioicum* individuals. cDNA prepared from leaves of a carpellate (C) and a staminate (S) plant, and whole inflorescences from three different carpellate (C_1, C_2, C_3) and staminate (S_1, S_2, S_3) plants.

ThdAG1 is expressed, as expected, in the reproductive organs of both male and female flowers (Figure 6a). In contrast, *ThdAG2* and *ThtAG2* are both carpel-specific, with *ThdAG2* expression clearly restricted to female individuals (Figure 6a,c). *ThdAP3-1* and both *ThdPI* loci are expressed in stamens of male flowers and the perianth of both male and female flowers (Figure 6a). This perianth expression accounts for the *ThdAP3-1* and *ThdPI* transcript detected in female individuals (Figure 6c). The *T. thalictroides* orthologs of these genes are expressed in the sepals and stamens in a similar manner (Figure 6b). The *ThdAP3-2a* and *-2b* loci are expressed in stamens but appear to be absent from the sepals of *T. dioicum*. This pattern is distinct from *T. thalictroides* where *ThtAP3-2a* and *-2b* are both expressed in the developing perianth organs. This is consistent with *AP3-2* genes playing a role in the promotion of petaloid perianth features in other Ranunculaceae (Kramer *et al.*, 2003), because *T. thalictroides* has a much more petaloid perianth than *T. dioicum* (compare Figure 2D,J,H, arrows). *ThdAP3-2a* and *-2b* are also detected in carpels of mature flowers (data not shown), most likely reflecting ovule expression, which is common in B class genes (Kramer and Irish, 1999). The double band present for *ThdAP3-2a* in one of the male samples in Figure 6(c) was found to be the result of miss-priming caused by repetitive elements in the 3′ UTR of the cDNA.

In situ *hybridization analysis of* AG *homologs*

The results from the RT-PCR experiments suggest that *ThdAG2* is restricted to female flowers (Figure 6c), which allows for the possibility that this gene could play a specific role in determining carpel, and hence sexual, identity. To determine whether this pattern is stage specific, we also conducted PCR on cDNA made from mature staminate flowers and staminate inflorescences that contained flowers at all stages of development and were unable to detect any

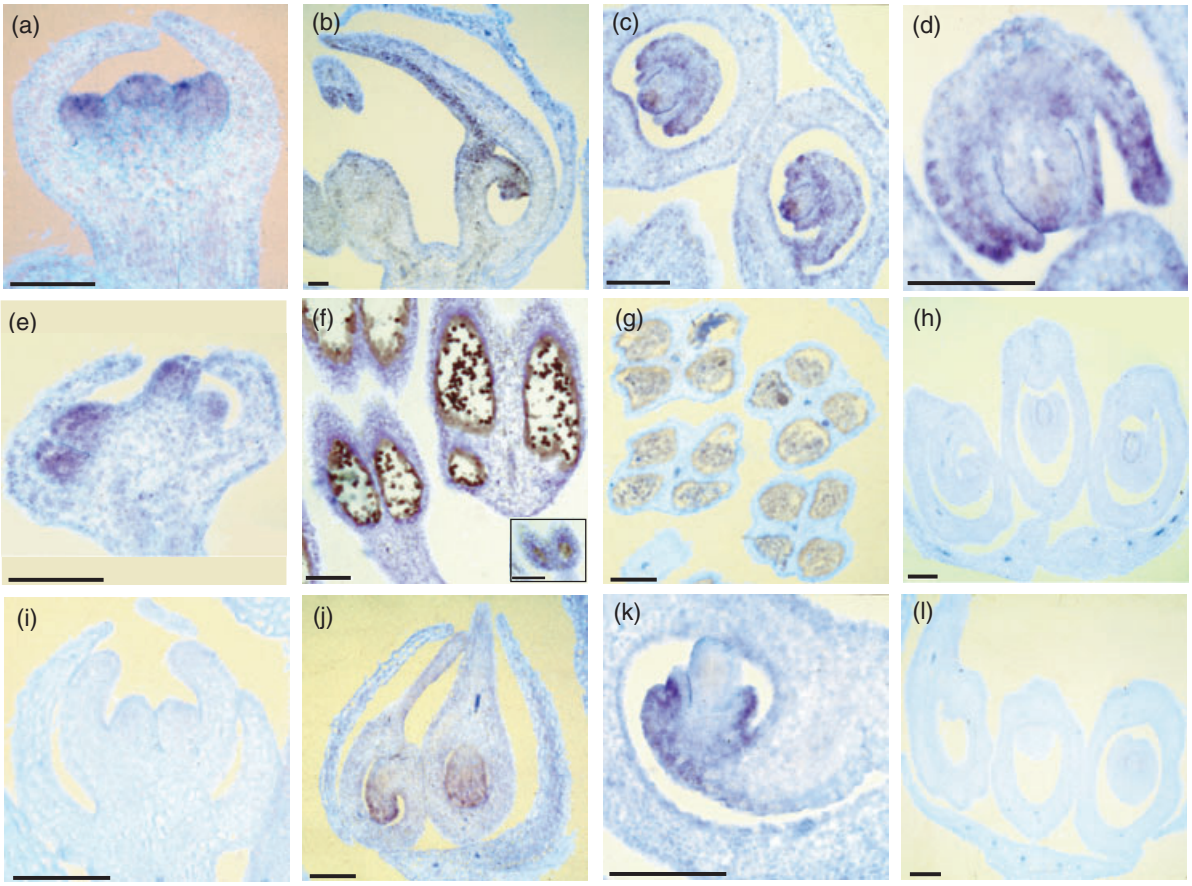

Figure 7. *In situ* hybridization of *AG1* and *AG2* on carpellate and staminate developing buds. (a–h) *ThdAG1*, (i–l) *ThdAG2*. Bar = 100 μm. *ThdAG1*: (a) early carpellate; (b) mature carpel; (c) mature ovules; (d) ovule detail; (e) early staminate; (f) late stamen; (g) male sense control; (h) female sense control. *ThdAG2*: (i) early carpellate; (j) mature carpel; (k) ovule detail; (l) female sense controls.

ThdAG2 transcripts. This led us to wonder whether staminate plants might lack the *TdAG2* locus. However, we were able to amplify a 1.6-kb fragment of *ThdAG2* from genomic DNA of both males and females (data not shown).

In order to determine whether *ThdAG2* might be an essential determinant of carpel identity, *in situ* hybridization of both *ThdAG1* and *ThdAG2* was performed on developing male and female *T. dioicum* flowers. At the earliest stages of development, *ThdAG1* is expressed in the developing inner primordia of both male and female flowers (Figure 7a,e). These early sections also highlight the morphological differences between carpel and stamen primordia at inception. At later stages, as the morphology of the organs diverges, the expression patterns also come to differ. In stamens, *ThdAG1* expression becomes restricted to specific tissues surrounding the tapetum within the developing stamen (Figure 7f and inset). In carpels, expression continues through intermediate stages, and in later carpels, *ThdAG1* transcripts are present in the transmitting tract of the style, placenta and within the developing ovule (Figure 7b). Ovule expression includes the integuments and nucellus (Figure 7c,d). This expression pattern is consistent with the findings for *AG* in Arabidopsis (Bowman *et al.*, 1991b). In contrast, *ThdAG2* is never detected in male flowers (data not shown) or early stage female flowers (Figure 7i). In mature carpels, expression of *ThdAG2* is restricted to the ovule (Figure 7j) where it is strongly expressed in the initiating outer integument and the abaxial side of the inner integument (Figure 7k). This expression persists through maturation of the ovule. In summary, whereas *ThdAG1* shows an expression pattern typical of a C class gene promoting stamen and carpel identity, *ThdAG2* expression is strictly limited to the developing ovules of female flowers.

Discussion

Developmental analysis of *T. dioicum* has demonstrated that the two types of floral meristems diverge at the earliest stages: female meristems initiate only large, ascidiate primordia that go on to develop into functional carpels and male meristems produce smaller primordia that go on to form fertile stamens. This type of flower development differs from that of sorrel in that, although male sorrel flowers do

not develop carpels, a carpel area is still present in the center of the flower (Ainsworth *et al.*, 1995). We do not observe this in *T. dioicum*, where sections through the central area of the staminate flower meristem clearly show that it contains additional stamen primordia (Figure 6e). Female flowers of sorrel do initiate stamens that arrest (Ainsworth *et al.*, 1995), while *T. dioicum* carpellate flowers never initiate observable stamen primordia (Figure 1b–d). C class homologs in sorrel were found to be markers for the initial placement of primordia, their expression fading as the 'wrong' primordia arrest (e.g. stamens in female flowers) or fail to develop altogether (e.g. carpels in male flowers) (Ainsworth, 2000; Ainsworth *et al.*, 1995). In *Silene*, the B class expression domain is expanded towards the center of the male flower, resulting in a smaller fourth whorl that correlates with repressed gynoecium development (Hardenack *et al.*, 1994). Our expression studies in *Thalictrum* show that, in contrast, some B gene paralogs are largely male specific, while the C gene homolog *AG1* is maintained in all developing internal organs. Therefore, flower development in *T. dioicum* suggests that sex determination is more likely to act upstream of organ identity in this species and, thus, more directly implicates the reproductive organ identity genes and their upstream regulators.

Our data on stamen and carpel number in *T. dioicum* show that there are always more stamens than carpels and that there is variability in organ number. This observation represents a potential conflict for a 1:1 homeotic conversion of organs. However, in Arabidopsis *ap3* and *pi* mutants there are fewer carpels in the third whorl than the usual number of stamens (Bowman *et al.*, 1989, 1991a; Jack *et al.*, 1992), while in *AP3/PI* overexpression lines, there are many more stamens in the inner whorl than the usual number of carpels (Krizek and Meyerowitz, 1996). This evidence suggests that there is a feedback relationship between organ identity and primordium number such that within a given space, the smaller stamen primordia will result in more organs than when carpel primordia are initiated. As to the issue of variability in organ number, which is seen both among individual flowers and between the *Thalictrum* species, this is commonly observed across the family Ranunculaceae and appears primarily to be a result of variation in floral meristem size (Tamura, 1993).

Given the floral phenotype observed in *T. dioicum*, one possibility is that differentially regulated alleles of a B class gene are actually linked to the sex-determining factor. Under this hypothesis, we would expect to find at least one B locus that is not expressed at all in females or which expresses male- and female-specific alleles. *ThdAP3-2 a/b* are possible candidates for this type of locus given that they are not detected in early female inflorescences and are only observed in late carpels (most likely due to ovule expression). An analysis of genomic sequence variation in full-sib families will be needed in order to test for sex linkage. An alternative hypothesis is that the homeotic mechanism acts through upstream regulators of the B class genes.

In the C class genes, the restriction of *ThdAG2* to carpels raised the possibility that this gene could play a role in sexual identity. Whereas within Arabidopsis and *Antirrhinum* only the B genes act to distinguish between types of sexual organ, *ThdAG2* could play such a role in *Thalictrum* if the gene was critical to carpel identity. However, the late and ovule-limited expression of *ThdAG2* in the *in situ* hybridizations suggests that while it may be involved in ovule development, it does not appear to play a role in carpel identity. As a consequence, *ThdAG2* orthologs display what appears to be either neo- or sub-functionalization (Force *et al.*, 1999). Although the ovule-specific expression pattern is reminiscent of that found for the *AG*-like *SHATTERPROOF* or *FBP11* loci from Arabidopsis and *Petunia*, respectively (Colombo *et al.*, 1995; Liljegren *et al.*, 2000), phylogenetic analysis indicates that Ranunculaceae *AG2* lineage is not orthologous to either of these genes (this study and Kramer *et al.*, 2004). Furthermore, while the *ThdAG2* ortholog from the closely related *Aquilegia* also shows carpel-specific expression, that from the more distantly related Ranunculid *Clematis* does not (E.M. Kramer, unpublished data). This may suggest that the ovule-specific expression of *AG2* in *Aquilegia* and *Thalictrum* is recently derived within the family.

Our analysis of *T. dioicum* has also revealed some novel aspects of B gene paralog expression (compared with the model systems). The perianth organs of *T. thalictroides* are large and petaloid in comparison with those of *T. dioicum* which are smaller, green to purple, and less petaloid (Figure 1j), closer to the sepals of model species such as Arabidopsis. Despite this similarity, the organization of the *Thalictrum* perianth, with only one type of organ, is not easily comparable to the two-whorl perianth of Arabidopsis or *Antirrhinum*. Likewise, the B class genes in *Thalictrum* are not directly equivalent to those in Arabidopsis due to intervening gene duplications. Botanists refer to the perianth organs of *Thalictrum* species as sepals, in spite of their representing a range of petalloidy. The similar epidermal morphology observed between the perianth organs of the two species is consistent with the botanical viewpoint that they are equivalent. However, *T. thalictroides* sepals have asymmetrically papillate cells on their adaxial surface, which are commonly found on petalloid organs and may be responsible for light refractive properties that attract pollinators (Glover and Martin, 2000). Interestingly, the gene expression profiles of *T. dioicum* and *T. thalictroides* differ in the expression of *AP3-2a/b* orthologs in the perianth. This may suggest that the expression of *AP3-1* and *PI* is sufficient to promote the similar aspects of the perianth epidermal morphology between *T. dioicum* and *T. thalictroides* (and even a minor degree of petaloidy), but that expression of *AP3-2a/b* is necessary for the presence of papillate cells and other petaloid characteristics of *T. thalictroides* sepals. Broader studies of B gene

homologs across the Ranunculaceae have also indicated that the paralogous *AP3* lineages common to the family may have evolved distinct roles in the production of different types of perianth organs (Kramer *et al.*, 2003).

Although the *T. dioicum* plants we used in this analysis appear to be recent tetraploids, closely related paralogs were only detected for the *PI* and *AG1* loci. This may be due to the fact that RT-PCR was used to survey the species for B and C gene homologs. Homoeolog-specific gene silencing is a common occurrence (Adams *et al.*, 2003) in polyploids such as *Gossypium* (cotton). This epigenetic process results in only one of the two recent copies being expressed in specific tissues. Further analysis of the genomic complement of *AP3*, *PI* and *AG* homologs will help us to understand whether this phenomenon is at work in *T. dioicum*.

Thalictrum dioicum appears to be a promising model for elucidating mechanisms of gender dimorphism, as it represents an example of genetic sex determination mechanisms acting upstream of the floral homeotic pathways. It provides complementary insights into sex determination to those obtained from the established dioecious systems (e.g., *Silene* and *Rumex*). *Thalictrum* is distinct from the above systems in having free carpels (apocarpy) and by showing developmental evidence of a homeotic sex determination mechanism. Apocarpy, which is common among the basal eudicots, allows one to more easily envision a homeotic conversion of primordia without the confounding effects of carpel fusion. Additionally, *Thalictrum* is unusual due to the absence of closely related gynodioecious species and the presence of andromonoecy and morphological androdioecy, raising the possibility that negative regulation of female development may have been an early step on the path to dioecy. Hybrids between dioecious and hermaphroditic species are even possible (Kuhn, 1930), which will further facilitate the analysis of sex determination mechanisms. In spite of being polyploid, the very small chromosome size typical of this genus results in a *C*-value for *T. dioicum* of 965 Mbp (<1 pg), a workable genome size for molecular biology endeavors. The presence of berberine alkaloids of medicinal value in the genus *Thalictrum* has prompted the development of transgenic techniques in the hermaphroditic species *T. flavum*, which will be very useful as we try to assess gene function in this system (Samanami *et al.*, 2002). The addition of *Thalictrum dioicum* to the set of dioecious model species will enhance our understanding of the genetics and evolution of this interesting phenomenon in a basal eudicot.

Experimental procedures

Plant material

Plants used in this study were collected from the wild in South Hadley, MA, and kept in growth chambers at Harvard University. Additional *T. thalictroides* plants were obtained from the New England Wildflower Society. Voucher specimens have been deposited with the Arnold Herbarium at the Harvard University Herbaria [*V. Di Stilio* 101 (A) and *V. Di Stilio* 102 (A)].

Microscopy

Fixed tissue was embedded in Paraplast, sectioned and stained with 0.05% toluidine blue. Light microscopy was conducted with a Leica microscope (DMRB, Northvale, NJ, USA) with digital camera (Harvard Imaging facility). SEM and ESEM observations of fixed and fresh material, respectively, were conducted in a Quanta 200 (FEi, Hillsboro, OR, USA) electron microscope (Harvard University).

Cytogenetics

Nuclear DNA content was estimated from fresh leaves' cells with a FACScan analyser (UW Cell Analysis Facility, Department of Immunology) using chicken erythrocyte nuclei as the internal standard and propidium iodide as stain.

For chromosome counts, fresh root tips were collected from seedlings, treated with 0.05% colchicine solution for 4 h at RT, fixed in 3:1 (ethanol : acetic acid), hydrolyzed in 1 N HCl for 10 min at 65°C and stained with toluidine blue.

Transcript isolation and characterization

Total RNA was extracted separately from male and female floral buds, dissected floral parts from young buds or leaves using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 5 μg of total RNA with Superscript™ II reverse transcriptase (Invitrogen).

Amplification of *AP3* and *PI* homologs was performed on floral cDNA with a forward degenerate primer designed to anneal in the MADS domain, 5′- AAY MGR CAR GTN ACN TAY TCR AAR MG-3′, and an anchored reverse primer to the poly A tail, 5′-CCG GAT CCT CTA GAG CGC GGC CGC $(T)_{17}$-3′. A nested PCR approach was used to amplify *AG* homologs. The first round of amplification was performed with the forward primer 5′-GGI MGI GGI AAR ATI GAR ATI AAR RGI AT-3′ and the poly-T primer described above. The second round used forward primers designed to the *AG1* or *AG2* paralogs based on the sequences found in the close relative *Aquilegia alpina* (*AqaAG1* and *AqaAG2*; E.M. Kramer, unpublished data): 5′-AAY RGI CAR GTI ACI TTY TGY AAR RGI RG-3′ or 5′- CAY TTR ATG GGI GAR GCI CTI AGY TG-3′ and the poly T primer. Cycling conditions were: 95°C for 10 min, followed by 25–30 cycles of 94°C for 30 sec, 45°–55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 min. Reactions contained 2 μl of cDNA template (10-fold dilution), 0.5 U Platinum Taq® DNA polymerase (Invitrogen) in 25 μl of PCR buffer (20 mM Tris–HCL pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.4 μM each forward and reverse primer, 2 μl cDNA. PCR products were cloned using the TOPO TA® cloning system (Invitrogen) and plasmid DNA was prepared with Perfectprep® (Eppendorf, Westbury, NY, USA). Twenty to 200 PCR products were screened by restriction analysis and/or sequencing, six to 60 were sequenced for each locus (ABI Prism® 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). The 5′ ends of each cDNA were obtained using the 5′RACE System for Rapid Amplification of cDNA Ends (Gibco BRL, Gaithersburg, MD, USA).

The nucleotide sequences of the eight new loci cloned in this study have been deposited in GenBank (accession numbers AY867872–AY867879). Sequences for *T. thalictroides PI* and *AP3* and *T. dioicum AG* have been reported previously (Kramer *et al.*, 2003, 2004).

Phylogenetic analysis

Initial amino acid alignments were generated with CLUSTALW, then adjusted by hand in MacClade 4.0. Phylogenetic analyses were performed with PAUP 4.0b1 (Swofford, 2001). Fitch parsimony (MP) trees were generated with TBR searches (1000 RANDOM addition replicates) with gaps treated as missing data. Bootstrap support for nodes was estimated with 1000 pseudo replicates and subject to SIMPLE addition sequence, TBR searches. *T. dioicum PI* homologs were analyzed in the context of sequences from other Ranunculaceae genera and rooted with *PI* homologs from *Berberis gilgiana* (Berberidaceae) and *Akebia quinata* (Lardizabalaceae) (see Kramer *et al.*, 2003 for accession numbers). Putative C-class homologs were analyzed in the context of published sequences from representative angiosperm *AG*-like genes and rooted with gymnosperm *AG* homologs (Kramer *et al.*, 2004).

RT-PCR characterization of expression

Locus-specific forward and reverse primers (Table S1) were designed and used to amplify cDNA from whole inflorescences, dissected floral organs or leaves. Primers to actin were used as a positive control (Table S1). Reaction conditions were as described above, with 25 cycles and annealing temperatures that varied with the primer pair (46–60°C). Reactions were run in a 1.2% agarose gel and digitally photographed. The identity of the amplified fragments was confirmed either based on size and restriction pattern or with cloning and sequencing.

In situ *hybridization*

In situ hybridizations were performed as previously described (Long *et al.*, 1996) using gene-specific probes which excluded regions of high similarity between *ThdAG1* and *ThdAG2*.

Acknowledgements

Thanks to Karen Searcy of University of Massachusetts at Amherst for locating *T. dioicum* and *T. thalictroides* populations in the field. Chen Xuening for participation in generating the cytogenetic, sex ratio and flower organ number data. Richard Schalek at the Center for Imaging and Mesoscale Structures for technical help in the use of the SEM and ESEM, which were funded by National Science Foundation Division of Biological Infrastructure grant 0099916. This work was supported by the Harvard Milton Fund to E. M. Kramer and the Mercer fellowship of the Arnold Arboretum to V. S. Di Stilio.

Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2336/TPJ2336sm.htm

Table S1 Gene-specific primers used for RT-PCR

References

Adams, K.L., Cronn, R., Percifield, R. and Wendel, J.F. (2003) Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. *Proc. Natl Acad. Sci. USA*, **100**, 4649–4654.

Ainsworth, C. (2000) Boys and girls come out to play: the molecular biology of dioecious plants. *Ann. Bot.* **86**, 211–221.

Ainsworth, C., Crossley, S., Buchanan-Wollaston, V., Thangavelu, M. and Parker, J. (1995) Male and female flowers of the dioecious plant sorrel show different patterns of MADS box gene expression. *Plant Cell*, **7**, 1583–1598.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1989) Genes directing flower development in *Arabidopsis*. *Plant Cell*, **1**, 37–52.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1991a) Genetic interactions among floral homeotic genes of *Arabidopsis*. *Development*, **112**, 1–20.

Bowman, J.L., Drews, G.N. and Meyerowitz, E.M. (1991b) Expression of the *Arabidopsis* floral homeotic gene *agamous* is restricted to specific cell types late in flower development. *Plant Cell*, **3**, 749–758.

Brunet, J. and Liston, A. (2000) Breeding system evolution in the genus *Thalictrum* (meadow rue) Ranunculaceae. In *Abstracts of the Botanical Society of America Meetings*, Portland, OR.

Charlesworth, D. (1985) Distribution of dioecy and self-incompatibility in angiosperms. In *Evolution – Essays in Honour of John Maynard Smith* (Greenwood, P.J. and Slatkin, M., eds). Cambridge: Cambridge University Press, pp. 237–268.

Coen, E.S. and Meyerowitz, E.M. (1991) The war of the whorls: genetic interactions controlling flower development. *Nature*, **353**, 31–37.

Colombo, L., Franken, J., Koetje, E., Van Went, J., Dons, H.J.M., Angenent, G.C. and Van Tunen, A.J. (1995) The petunia MADS box gene FBP11 determines ovule identity. *Plant Cell*, **7**, 1859–1868.

Crossley, S.J. and Ainsworth, C.C. (1994) Structural and molecular aspects of dioecy in *Rumex acetosa*. *Biochem. Soc. Trans.* **22**, 79S.

Davis, S.L. (1997) Stamens are not essential as an attractant for pollinators in females of cryptically dioecious *Thalictrum pubescens* Pursch. (Ranunculaceae). *Sex. Plant Reprod.* **10**, 293–299.

Dellaporta, S.L. and Calderon-Urrea, A. (1994) The sex determination process in maize. *Science*, **266**, 1501–1505.

Farbos, I., Oliveira, M. and Negrutiu, I. (1997) Sex organ determination and differentiation in the dioecious plant *Melandrium album* (*Silene latifolia*): a cytological and histological analysis. *Sex. Plant Reprod.* **10**, 155–167.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.-L. and Postlethwait, J. (1999) Preservation of duplicate genes by complementary, degenerative mutations. *Genetics*, **151**, 1531–1545.

Glover, B.J. and Martin, C. (2000) Specification of epidermal cell morphology. In *Advances in Botanical Research Incorporating Advances in Plant Pathology* (Callow, J.A., ed). San Diego, CA: Academic Press, Vol. 31. pp. 193–217.

Grant, S., Houben, A., Vyskot, B., Siroky, J., Pan, W.H., Macas, J. and Saedler, H. (1994) Genetics of sex determination in flowering plants. *Dev. Genet.* **15**, 214–230.

Gregory, W.C. (1941) Phylogenetic and cytological studies in the Ranunculaceae. *Trans. Am. Philos. Soc.* **31**, 446–497.

Hardenack, S., Ye, D., Saedler, H. and Grant, S. (1994) Comparison of Mads box gene expression in developing male and female flowers of the dioecious plant white campion. *Plant Cell*, **6**, 1775–1787.

Hill, L.M. (1989) IOPB chromosome data 1. *Int. Org. Plant Biosyst. Newslett.* **13**, 17–19.

Jack, T., Brockman, L.L. and Meyerowitz, E.M. (1992) The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. *Cell*, **68**, 683–697.

Kaplan, S.M. and Mulcahy, D.L. (1971) Mode of pollination and floral sexuality in *Thalictrum*. *Evolution*, **25**, 659–668.

Kater, M.M., Franken, J., Carney, K.J., Colombo, L. and Angenent, G.C. (2001) Sex determination in the monoecious species cucumber is confined to specific floral whorls. *Plant Cell*, **13**, 481–493.

Kay, Q.O.N., Daoud, H.S. and Stirto, C.H. (1981) Pigment distribution, light reflection and cell structure in petals. *Bot. J. Linn. Soc.* **83**, 37–84.

Kramer, E.M. and Irish, V.F. (1999) Evolution of genetic mechanisms controlling petal development. *Nature*, **399**, 144–148.

Kramer, E.M., Dorit, R.L. and Irish, V.F. (1998) Molecular evolution of genes controlling petal and stamen development: Duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. *Genetics*, **149**, 765–783.

Kramer, E.M., Di Stilio, V.S. and Schluter, P. (2003) Complex patterns of gene duplication in the APETALA3 and PISTILLATA lineages of the Ranunculaceae. *IJPS*, **164**, 1–11.

Kramer, E.M., Jaramillo, M.A. and Di Stilio, V.S. (2004) Patterns of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS box genes in angiosperms. *Genetics*, **166**, 1011–1023.

Krizek, B.A. and Meyerowitz, E.M. (1996) The Arabidopsis homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. *Development*, **122**, 11–22.

Kuhn, E. (1930) Uber Kreuzungen des getrenntgeschlechtigen Thallictrum fendleri mit gemischtgeschlechtigen. *Arten. Biol. Zentr.* **50**, 79–102.

Kuhn, E. (1939) Selbstbestäubungen subdiözischer Blütenpflanzen, ein neuer Beweis für die genetische Theorie der Geschlechtsbestimmung. *Planta*, **30**, 457–470.

Lebel-Hardenack and Grant, S. (1997) Genetics of sex determination in flowering plants. *Trends Plant Sci.* **2**, 130–136.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L. and Yanofsky, M.F. (2000) *SHATTERPROOF* MADS-box genes control seed dispersal in *Arabidopsis*. *Nature*, **404**, 766–770.

Linke, B., Nothnagel, T. and Borner, T. (2003) Flower development in carrot CMS plants: mitochondria affect the expression of MADS box genes homologous to GLOBOSA and DEFICIENS. *Plant J.* **34**, 27–37.

Liu, J.Y., Huang, Y.H., Ding, B. and Tauer, C.G. (1999) cDNA cloning and expression of a sweetgum gene that shows homology with Arabidopsis AGAMOUS. *Plant Sci.* **142**, 73–82.

Lloyd, D.G. (1980) The distribution of gender in four angiosperm species illustrating two evolutionary pathways to dioecy. *Evolution*, **34**, 123–134.

Long, J.A., Moan, E.I., Medford, J.I. and Barton, M.K. (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature*, **379**, 66–69.

Malepszy, S. and Niemirowicz-Szczytt, K. (1991) Sex determination in cucumber (*Cucumis sativus*) as a model system for molecular biology. *Plant Sci.* **80**, 39–47.

Park, J.H., Ishijawa Y., Yoshida, R., Kanno, A. and Kameya, T. (2003) Expression of AODEF, a B-functional MADS-box gene, in stamens and inner tepals of the dioecious species *Asparagus officinalis* L. *Plant Mol. Biol.* **51**, 867–875.

Renner, S.S. and Ricklefs, R.E. (1995) Dioecy and its correlates in the flowering plants. *Am. J. Bot.* **82**, 596–606.

Ross, M.D. (1970) Evolution of dioecy from gynodioecy. *Evolution*, **24**, 827–828.

Samanami, N., Park, S.-U. and Facchini, P.J. (2002) In vitro regeneration and genetic transformation of the berberine-producing plant, *Thalictrum flavum* ssp. *glaucum*. *Physiol. Plant.* **116**, 79–86.

Swofford, D.L. (2001) *Phylogenetic Analysis Using Parsimony (*and other methods)*.* Sunderland, MA: Sinauer Associates.

Tamura, M. (1993) Ranunculaceae. In *The Families and Genera of Vascular Plants. 2. Flowering plants. Dicotyledons. Magnoliid, Hamamelid and Caryophyllid Families*, (Kubitzki, K., Rohwer, J.G. and Bittrich, V., eds). Berlin: Springer, pp. 563–583.

Theissen, G., Becker, A., Di Rosa, A., Kanno, A., Kim, J.T., Munster, T., Winter, K.-U. and Saedler, H. (2000) A short history of MADS-box genes in plants. *Plant Mol. Biol.* **42**, 115–149.

Trelease, W. (1886) North American species of *Thalictrum*. *Proc. Boston Soc. Nat. Hist.* **23**, 293–304.

Westergaard, M. (1958) The mechanism of sex determination in dioecious flowering plants. *Adv. Genet.* **9**, 217–281.

Yu, D., Kotilainen, M., Pöllänen, E., Mehto, M., Elomaa, P., Helariutta, Y., Albert, V.A. and Teeri, T.H. (1999) Organ identity and modified patterns of flower development in *Gerbera hybrida* (Asteraceae). *Plant J.* **17**, 51–62.

Accession numbers: AY867872–AY867879.