Dev Genes Evol (2005) 215: 132–142
DOI 10.1007/s00427-004-0459-4

ORIGINAL ARTICLE

Catherine Pfent · Kevin J. Pobursky · D. Noah Sather · Edward M. Golenberg

Characterization of *SpAPETALA3* and *SpPISTILLATA*, B class floral identity genes in *Spinacia oleracea*, and their relationship to sexual dimorphism

Received: 8 September 2004 / Accepted: 25 November 2004 / Published online: 20 January 2005

Abstract Floral organ identity B class genes are generally recognized as being required for development of petals and stamens in angiosperm flowers. Spinach flowers are distinguished in their complete absence of petals in both sexes, and the absence of a developed stamen whorl in female flowers. As such, we hypothesized that differential expression of B class floral identity genes is integral to the sexual dimorphism in spinach flowers. We isolated two spinach orthologs of *Arabidopsis* B class genes by 3′ and 5′ RACE. Homology assignments were tested by comparisons of percent amino acid identities, searches for diagnostic consensus amino acid residues, conserved motifs, and phylogenetic groupings. In situ hybridization studies demonstrate that both spinach B class genes are expressed throughout the male floral meristem in early stages, and continue to be expressed in sepal primordia in reduced amounts at later stages of development. They are also highly expressed in the third whorl primordia when they arise and continue to be expressed in these tissues through the development of mature anthers. In contrast, neither gene can be detected in any stage in female flowers by in situ analyses, although northern blot experiments indicate low levels of *SpAP3* within the inflorescence. The early, strong expressions of both B class floral identity genes in male floral primordia and their absence in female flowers demonstrate that B class gene expression precedes the origination of third whorl primordia (stamen) in males and is associated with the establishment of sexual floral dimorphism as it initiates in the first (sepal) whorl. These observations suggest that regulation of B class floral identity genes has a role in the development of sexual dimorphism and dioecy in spinach rather than being a secondary result of organ abortion.

Keywords Floral homeotic genes · ABC model · Dioecy · Plant sex determination · Evolutionary development

Edited by G. Jürgens

Electronic Supplementary Material Supplementary material is available for this article at http://dx.doi.org/10.1007/s00427-004-0459-4

C. Pfent
School of Veterinary Medicine, Michigan State University,
East Lansing, MI, 48824, USA

K. J. Pobursky
Regeneron Pharmaceuticals,
777 Old Saw Mill River Road,
Tarrytown, NY, 10591, USA

D. N. Sather · E. M. Golenberg (✉)
Department of Biological Sciences, Wayne State University,
Detroit, MI, 48202, USA
e-mail: egolenb@biology.biosci.wayne.edu
Fax: +1-313-5776891

Introduction

Floral organ development is the best studied of the molecular genetic developmental pathways in plants. Characterization of the major genes and functional testing of their products as transcription factors have been established in the two model systems of *Arabidopsis thaliana* and *Antirrhinum majus* and is summarized in the ABC model (Coen et al. 1991; Coen and Meyerowitz 1991; Irish 1999; Jack 2004; Lohmann and Weigel 2002; Weigel and Meyerowitz 1994; Zik and Irish 2003a). However, the expansion of the ABC model to non-model species, as well as studies of variation within species, has been limited. First, comparative studies of the expression patterns of the floral homeotic genes in other species are still relatively few (Ainsworth et al. 1995; Angenent et al. 1993; Chuck et al. 1998; Kater et al. 2001; Kramer et al. 1998; Kramer and Irish 1999; Park et al. 2003; Riechmann and Meyerowitz 1998; Shu et al. 2000; Wang et al. 1999). Second, studies of intraspecific polymorphisms of homeotic genes and expression are almost non-existent (Cubas et al. 1999; Purugganan et al. 2000; Wang et al. 1999). At present, our understanding of the downstream regulatory effects of the master transcription factors is in its infancy. While interactions among some of the transcription factor genes are known (Busch et al. 1999;

Doebley and Lukens 1998; Ito et al. 2004; Krizek and Meyerowitz 1996; Lohmann et al. 2001; Parcy et al. 1998; Samach et al. 1997; Wagner et al. 1999), potential downstream response genes are only beginning to be detected, even in *Arabidopsis* (Bey et al. 2004; Wellmer et al. 2004; Zik and Irish 2003b). Thus, how deviations from the *Arabidopsis* ABC model are related to morphological variation among floral structures in angiosperms, what proximal genes control morphological development in floral organs, and how environmental factors influence the developmental pathway remain largely unknown.

Plants with imperfect flowers provide a powerful tool for studying the floral developmental pathway as the developmental cascade found in hermaphroditic flowers must be altered to produce unisexual flowers. Two basic morphological pathways produce imperfect flowers (Heslop-Harrison 1964). In the first, all organ primordia are initiated and begin differentiation, only to degenerate or abort at a later stage. In the second, organ primordia for particular reproductive organs do not develop at all. In male flowers, carpel primordia do not form and in females stamen primordia are not initiated. Five species with imperfect flowers whose development have been studied on a molecular level, *Zea mays* (Ambrose et al. 2000; Delonget al. 1993; Munster et al. 2001), *Rumex acetosa* (Ainsworth et al. 1995), *Silene latifolia* (Hardenack et al. 1994; Janousek et al. 1996; Viskot et al. 1993), *Cucumis sativus* (Kater et al. 2001), and *Asparagus officinalis* (Park et al. 2003), all develop unisexual flowers following the first pathway.

Cultivated spinach, *Spinacia oleracea*, follows the second developmental pathway (Pobursky 2000; Sherry et al. 1993), and thus provides a novel model for the analysis of floral development. In this dioecious species, both male and female flowers begin organ development with the growth of two opposite sepal primordia. The flowers then differentiate when the male sepal primordia are restricted in growth and two additional sepal primordia form in the space in the outer whorl between the initial sepal primordia. The two female sepal primordia extend laterally around the outer whorl and distally to overcanopy the central organs. Thus, sexual dimorphism is initiated *before* the appearance of reproductive organ primordia. Neither males nor females produce a second (petal) whorl in the perianth. The male flowers develop four stamens opposite the four sepals. The central space (fourth whorl) is flattened in the male flowers and no organs or primordia form (Fig. 1a). The female flowers do not establish organ primordia in the third whorl and only develop a single carpel in the central or fourth whorl. The initial fourth whorl primordia has a ring or girdling rim (Pobursky 2000; Sherry et al. 1993) similar to that found in *Chenopodium album* (Sattler 1973). This structure forms individual lobes that engulf the central region and fuse above it to form the pistil and stigmatic branches while the basal region forms the ovary wall. As the flower matures the central gynoecium protrudes from between the two sepals and a four-lobed stigma extends outward (Fig. 1b).

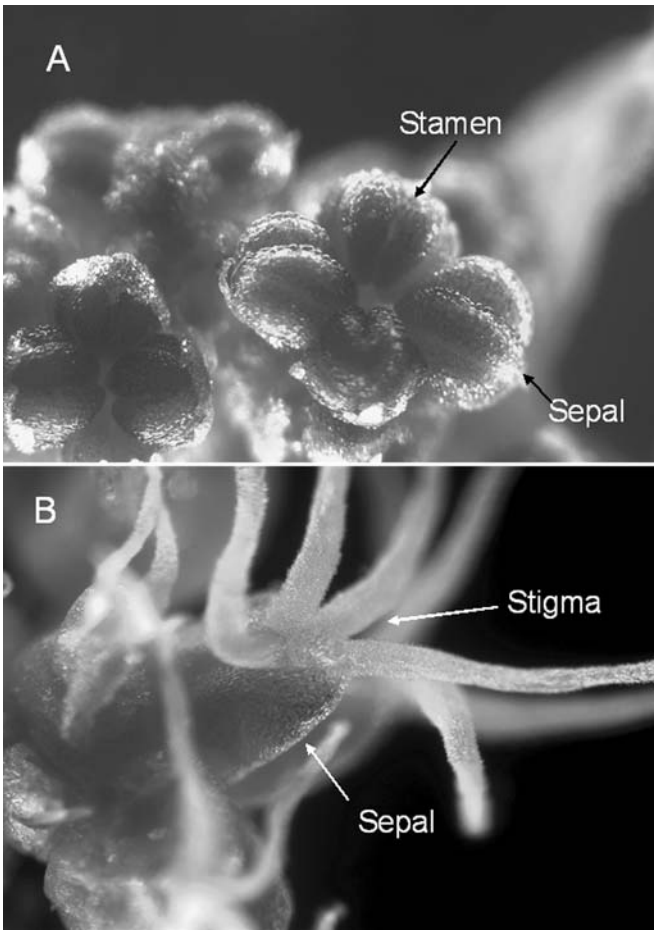

Fig. 1 Male and female spinach flowers. **a** A small cluster of male spinach flowers. On one flower, one of the four stamens and one of the four sepals are indicated. **b** A female mature flower within a cluster. The extending stigma is marked with an *arrow*. One of the two cupping sepals is also indicated

Studies have demonstrated that sexual determination in spinach can be altered by environmental stress (Freeman and Vitale 1985; Vitale and Freeman 1985), hormonal treatment (Chailakhyan 1979; Pobursky 2000), and developmental state (Miglia and Freeman 1996). Additionally, ectopic fourth whorl organs in males can be found at low frequencies in cultivar America (Pobursky 2000). Thus, it appears that genes that can control the development of all sexual organs are present in both male and female spinach, and therefore, altered gene regulation rather than gene presence or absence appears to control sex-specific development in spinach. As the B class genes are responsible for petal and stamen identity in *Arabidopsis* and *Antirrhinum*, and as neither male nor female spinach flowers produce petals, we hypothesized that B class gene expression should be clearly different in the two flower morphs. Here we report on the isolation, characterization, and expression of spinach homologs to the *Arabidopsis* B class genes, *APETALA3* and *PISTILLATA*. We demonstrate that the two genes are expressed at very early stages and have widely overlapping expression patterns in male spinach flowers, while neither is detectable

by in situ hybridization in any stage of female flower development. We relate the regulatory interactions of B class genes with potential downstream targets suggested by studies in *Arabidopsis* to produce a model for the role of spinach B class genes in the development of sexual dimorphism in the first whorl.

Materials and methods

Spinach AP3 isolation and sequencing

DNA was extracted from bagged leaf spinach using a standard CTAB extraction protocol (Doyle and Doyle 1987). PCR primers were designed by comparison of *A. thaliana AP3* (accession no. A42095, Jack et al. 1992, 1994), *AG* (acc. no. S10933, Yanofsky et al. 1990), *A. majus DEF* (acc. no. S12378, Sommer et al. 1990), *PLE* (acc. no. S52900, Bradley et al. 1993), and *Brassica napus Bag1* (acc. no. M99415, Mandel et al. 1992) gene sequences. A degenerate forward primer, *degMADSF*, was based primarily on the *Arabidopsis AP3* gene starting from the start codon (5′ ATG GCI AGR GGI AAR AT 3′). The reverse primer, *AP3-131R*, was derived from the *Arabidopsis AP3* sequence. The 3′ end of the primer falls at position 131 of the coding sequence and is within the MADS box. Three of four amino acids coded at this 3′ end differ between AP3 and AG, thus giving specificity to the primer (5′ TGT TGG AGC TAG AGA ACA TGA TAA TCG 3′). A 25-µl PCR reaction using 3 mM $MgCl_2$ was set up, and Taq polymerase (Gibco BRL, Gaithersburg, Md.) was added to the reaction after incubation at 94°C for 2 min (hot start). The reaction conditions were 40 cycles of 94°C at 40 s, 52°C at 40 s, and 72°C for 1 min. The reaction product was cloned into a TA cloning vector (Invitrogen, Carlsbad, Calif.) and sequenced on an ALF automated sequencing machine following the manufacturer's suggested protocol (Pharmacia, Peapack, N.J.). Sequence comparisons were done using Sequencher 3.0 (Gene Codes, Ann Arbor, Mich.). Spinach *AP3*-specific primers, SPMADS38F (5′ ACA ATA CGA ATC GTC AAG T 3′) and SPMADS77F (5′ AAC GGT CTG TTC AAG AAG G 3′), were designed from the spinach sequence. The last five bases of SPMADS38F and the last seven bases of SPMADS77F are conserved in comparison with *AP3* homologous sequences from *Dianthus caryophyllus CMB2* (acc. no. L40405, Baudinette et al. 2000), *S. latifolia SLM3* (acc. no. X80490, Hardenack et al. 1994), *R. acetosa RaD1* (acc. no. X89113, Ainsworth et al. 1995), and *A. thaliana AP3* (acc. no. A42095, Jack et al. 1992, 1994).

Individual plants of *S. oleracea* cv America were grown from seed in a 3:1 mixture of potting soil and vermiculite. The plants were grown under long-day light conditions (18 h light:6 h dark) at 20°C and were watered as needed. Total RNA was extracted either from anthers or complete bolted inflorescences by grinding the tissue in the presence of Trizol, extracting once with chloroform, and precipitating the RNA with isopropanol, following the supplier's protocol (Gibco BRL, Gaithersburg, Md.). 3′ RACE was executed using 3′ RACE System for Rapid Amplification of cDNA Ends (Gibco BRL, Gaithersburg, Md.). Primary PCR was carried out in 50-µl reactions using the abridged universal adaptor primer (AUAP), the gene-specific SPMADS38F primer, Biolase (ISC Bioexpress, Kaysville, Utah), Biolase buffer (an NH_4-based PCR buffer), and 3 mM $MgCl_2$. The reactions were run in glass capillary tubes in an Idaho Technologies RapidCycler set at 94°C for 2 min followed by 35 cycles of 94°C for 12 s, 47°C for 12 s, and 72°C for 90 s. This was followed by a semi-nested secondary PCR using AUAP and SPMADS77F as primers under similar conditions for 30 cycles. PCR products were cloned into pCRII (Invitrogen, Carlsbad, Calif.) and sequenced using cycle-sequencing (Big Dye; PE Applied Biosystems, Foster City, Calif.). The precipitated sequencing reaction products were read on an ABI Prism Model 377, version 3.0.

Spinach PI isolation and sequencing

The spinach *PI* gene was isolated by 5′ and 3′ RACE as described above. Three nested forward primers were designed from the *S. latifolia SLM2* sequence (acc. no. X80489, Hardenack et al. 1994) in comparison with sequences from *A. majus GLO* (acc. no. S28062, Tröbner et al. 1992), *A. thaliana PI* (acc. no. D30807, Goto and Meyerowitz 1994), and *Petunia hybrida PMADS2* (acc. no. X69947, Kush et al. 1993). The primers were PI.2F (5′ TAA TGG GTA GAG GAA AAA T 3′), PI.59F (5′ CTT ACT CAA AGA GAA GAA ATG G 3′), and PI.100F (5′ GAG ATC ACT GTT CTT TGT GA 3′). PCR products were cloned using TOPO TA Cloning Kit with vector pCR 2.1 (Invitrogen, Carlsbad, Calif.) and sequenced using ABI Prism Model 377, version 3.0. From these sequences, nested primers for 5′ RACE were designed. PI primers used were PI.542R (5′ AAA CCC GTA AGG AAG GTA 3′), PI.308R (5′TCC TCT CCA TTC AAG TGC 3′), and PI.216R (5′ TAG CAT CCC ACA ACC TCT TAC C 3′). 5′ RACE products were cloned and sequenced as above.

Sequence alignment and analysis

Sequences were imported into Sequencher 3.0 (Gene Codes, Ann Arbor, Mich.) and multiply aligned. Alignments were manually adjusted. Amino acid sequences were projected using the universal code. Amino acid sequences of the spinach AP3 and PI proteins were first aligned with SLM3 (accession number X80490), SLM2 (acc. no. X80489), AP3 (acc. no. D21125), PI (acc. no. D30807), GLO (acc. no. X68831), and DEFA (acc. no. X52023) using the nucleotide sequence as a guide. Following this initial alignment, the spinach sequences were aligned with additional proteins according to the alignment in Kramer et al. (1998). The initial alignments based

on nucleotide sequences and those of Kramer et al. (1998) differed substantially at the carboxyl end. As such, the sequences were truncated to position 201 of Appendix 2 in Kramer et al. (1998) approximately 20 positions before the PI motif regions. Phylogenetic analysis of the amino acid sequences were completed using PAUP 3.0 (Swofford 1993) and Protdist and Neighbor programs of PHYLIP 3.573c (Felsenstein 1993). The parsimony searches used ten replicates with the TBR and MULPARS options in effect. Strength of parsimony branching inferences were tested in 1,000 bootstrap resamplings using the same options.

Northern blot

Total RNA was extracted from floral, and vegetative tissues by grinding the tissue in the presence of Trizol, extracting once with chloroform, and precipitating the RNA with isopropanol, following the supplier's protocol (Gibco BRL, Gaithersburg, Md.). The RNA was size fractionated by electrophoresis and transferred to a nylon membrane using the Northen Max-Gly protocols (Ambion, Austin, Tex.). Truncated clones of *SpAP3* and *SpPI*, which included only the C regions of each gene, were subcloned into pBluescript SK. Antisense RNA probes were generated for both *SpAP3* and *SpPI* by transcription using the T7 and T3 promoters of pBluescript, respectively, and labeled by incorporation of digoxigenin-11-UTP. A portion of the spinach *G6pdh* gene was cloned using information from the published sequence AJ000182. An antisense probe of *G6pdh* was used as a positive control. The probes were hybridized with ULTRAhyb hybridization buffer following the manufacturer's protocol (Ambion, Austin, Tex.) and detected by conjugation with anti-digoxigenin-AP and exposure to CDP-star following standard protocols.

In situ hybridization

In situ hybridizations were carried out following a modification of the mRNAlocator-Hyb system (Ambion, Austin, Tex.). Male and female inflorescences were dissected from cv America plants and incubated in fixative at 2–8°C for 8–12 h. They were then dehydrated in an ethanol-xylene series and imbedded in paraffin. The paraffin block was cooled on ice during sectioning. Sections were floated in nuclease-free water and positioned on Fisherbrand Superfrost/Plus microscope slides. Slides were incubated at 60°C overnight. The sections were deparaffinized and rehydrated in a reverse xylene-ethanol series. Tissues were treated with proteinase K for 20–30 min. Initial hybridizations using biotin-labeled probes resulted in non-specific background staining. Use of digoxygenin-labeled probes produced highly defined and sex-specific hybridization as detected by dark blue NBT staining with low background. *SpAP3* expression was first tested using a probe containing the *SpAP3* I, K, and C regions. Non-specific hybridization occurred throughout the floral tissues which may reflect cross hybridization to paralogous genes. A truncated probe containing just the unique C region (from coding position 432–834) generated tissue-specific hybridization patterns. Based on these results, *SpPI* expression was detected using an *SpPI* C region (from position 526 to the poly-A site) probe only. Sections were hybridized with digoxigenin RNA probes at 50–55°C. Hybridization was detected by incubation with anti-digoxigenin-AP followed by an NBT/BCIP solution. Slides were subsequently dehydrated, cleared, mounted, and stored at −20°C under desiccant.

Results

Spinach AP3 and PI isolation and sequence analysis

Utilizing a 3′ RACE, we isolated a 930-bp fragment, which was cloned and sequenced. The majority of clones gave identical sequences (shown in the Electronic Supplementary Material Fig. 1) in comparison with the *S. latifolia AP3* ortholog *SLM3* (X80490, Hardenack et al. 1994). A second cloned sequence was identical except for a 14-bp addition (AATGCTTGCTGGGT) at the 3′ UTR before the poly-A tail. This sequence has been deposited in GenBank as accession AY604514.

A second spinach B class sequence was differentially amplified by using *PI* consensus primers. The spinach *PI*-sequence was isolated in two overlapping fragments from independent 3′ and 5′ RACE reactions. The nucleotide sequence was aligned with *Arabidopsis PI* and *Silene SLM2* (Electronic Supplementary Material Fig. 2). The spinach *PI* sequence has been deposited in GenBank as accession AY604515.

To infer homology of these sequences to published B class genes, we first compared nucleotide and translated amino acid sequences to known genes for general similarities and presence of diagnostic motifs. The *SpAP3* (spinach *APETALA3*) sequence was aligned with *A. thaliana APETALA3* (D21125, Thomas et al. 1992), and *S. latifolia SLM3* (Hardenack et al. 1994). The percent amino acid identities between the spinach and *Arabidopsis*, spinach and *Silene*, and *Arabidopsis* and *Silene* are 76%, 82%, and 82% within the MADS domain, 53%, 53%, and 42% within the I region, and 44%, 65%, and 52% within the K box. Theißen et al. (1996) identified residues that are particularly characteristic of the different paralogs in the MADS gene family (marked in red in Electronic Supplementary Material Fig. 1). Specifically, the SpAP3 protein has a conserved phenylalanine at position 29 and a conserved methionine at position 47. It differs from the consensus residues at position 36 where it has a serine instead of a threonine (as does *Antirrhinum* DEF) and at position 42 where it has a threonine instead of a lysine (as does *Silene* SLM3; Electronic Supplementary Material Fig. 1).

Kramer et al. (1998, 2000) identified two conserved C terminal regions in *AP3* homologs. The first region is

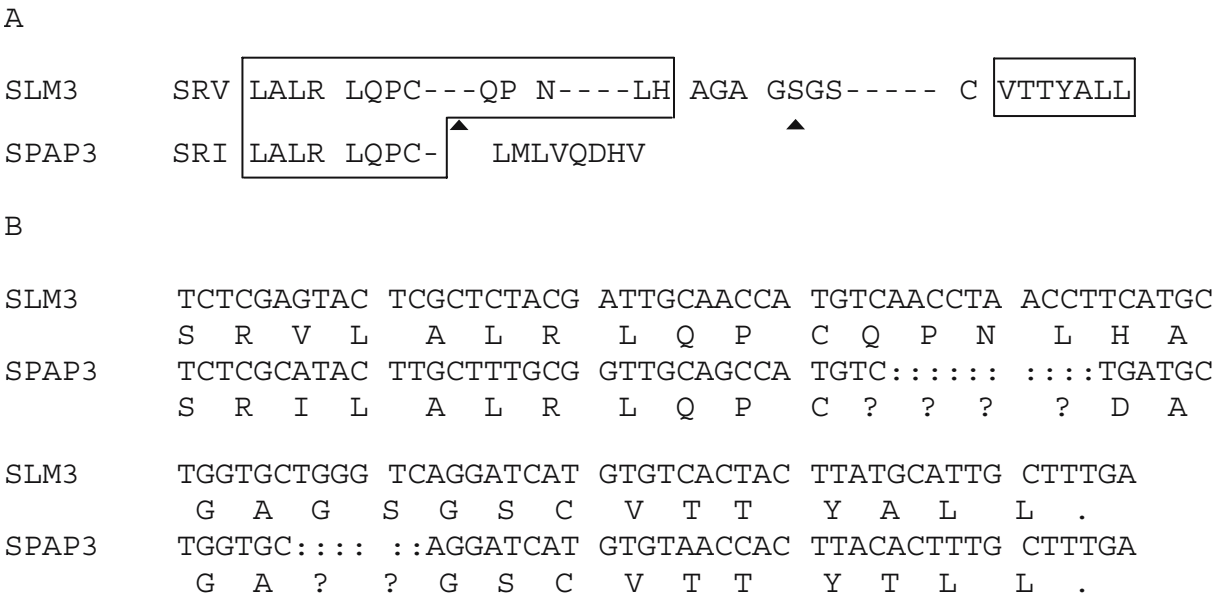

Fig. 2 Comparisons of euAP3 motifs and PI motifs between SpAP3 and SLM3. **a** Comparison of the amino acid sequences at the carboxyl ends of SLM3 and SpAP3. The euAP3-motif and PI-motif regions are *boxed* for comparison. The *black triangles* under the SLM3 sequence indicate the positions of the *SpAP3* deletions on the nucleotide level. **b** Aligned nucleotide sequences of the ends of the coding regions of both *SLM3* and *SpAP3*. Gaps are introduced into the *SpAP3* sequence to improve sequence similarity and are marked with a *colon*. The *SpAP3* sequence is then translated with the gaps to demonstrate the conserved coding sequence of this putative carboxyl end in comparison with SLM3. The first gap is a 10-bp deletion whereas the second gap is an in-frame 6-bp deletion

reminiscent of a PI motif and is found in nearly all angiosperms listed. The second is an AP3-specific motif that occurs either as a euAP3 (derived) or paleoAP3 (ancestral) sequence. The spinach AP3 C terminal sequence is shown in Fig. 2a in comparison with that of *S. latifolia*. The PI Motif LALRLQPC is conserved between *S. latifolia* and spinach. The remaining QPNLH and the subsequent EuAP3 motif are absent in spinach and are replaced by eight residues, LMLVQDHV, before terminating. On the nucleotide level, this abrupt divergence in sequence is due to a 10-bp deletion that results in a frameshift (Fig. 2b). A second 6-bp deletion in the spinach relative to the *Silene* sequence is further downstream and occurs in the intervening, variable region between the PI motif and the euAP3 motif. If the spinach sequence were to be translated in the original reading frame, an euAP3 motif (GS) CVTTYTLL would be present, indicating that the euAP3 motif was conserved on the nucleotide level following the 6-bp deletion. This conservation of a translated sequence suggests that the upstream 10-bp deletion is the more recent of the two deletions, and that the spinach sequence shares remnants of the expected motif consistent with the inferred homology.

The SpPI (spinach PISTILLATA) translated amino acid sequences demonstrate a high degree of identity in the MADS box with the spinach sequence identity ranging from 88% compared to the *Silene* MADS box and 74% compared to the *Arabidopsis* sequence. The *PI* I region is more highly conserved than the comparable *AP3* I region with 76% identity between spinach and *Silene*, and 59% identity between spinach and *Arabidopsis*, whereas the K region is comparable at 64% and 50% identities between spinach and *Silene* and spinach and *Arabidopsis*. Also, following the Theißen et al. (1996) prediction, the translated spinach sequence has the conserved PI serine residue at position 14 (marked in red in Electronic Supplementary Material Fig. 2).

The carboxyl terminus of the translated protein is highly variable and requires the placement of indels in the cDNA sequence to align the sequences. However, as opposed to the *AP3* sequences, all indels are in frame. The first 13 amino acids following the K region are identical in *Silene* and spinach, but the sequences are then marked by extensive variation including indels until reaching the PI motif. The PI motif (Kramer et al. 1998; Kramer and Irish 2000) is present in the spinach sequence (PYGFRGQPNQ QG) two residues before the carboxyl end of the protein, but differs from the consensus by a deletion of a PNL motif (dotted underline in Electronic Supplementary Material Fig. 2). Similar internal deletions are reported in other species by Kramer et al. (2000). Therefore, both general sequence similarity and presence of diagnostic motifs support the homology of the two isolated spinach sequences to previously characterized B class genes.

Phylogenetic analysis of spinach B class genes

To test for orthology of the spinach sequences, we carried out phylogenetic analyses to assess systematically consistent grouping of the spinach sequences to those of other species. Parsimony analysis produced two equally parsimonious trees (Fig. 3a). The two maximum parsimony

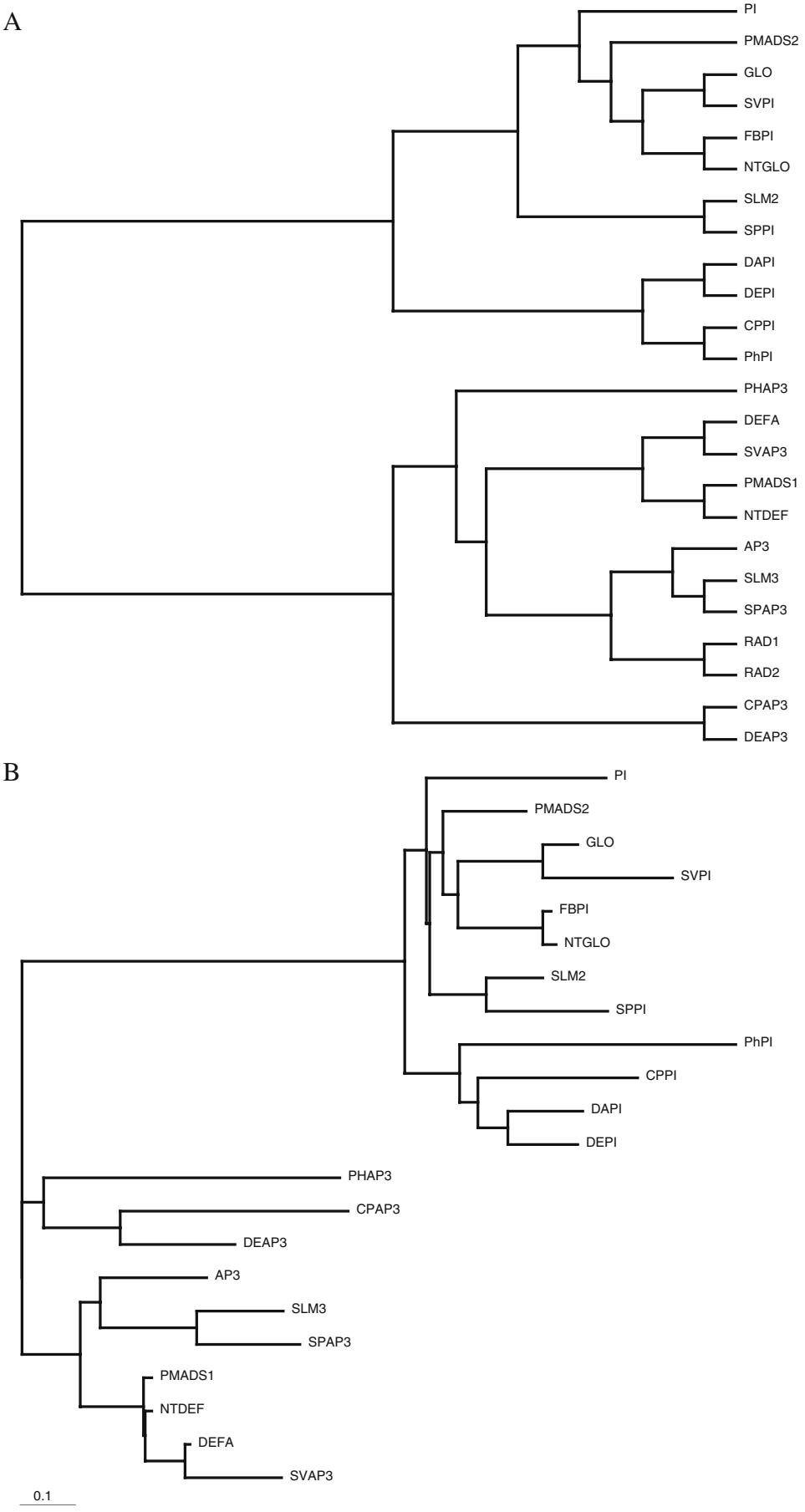

Fig. 3 Phylogenetic analysis of amino acid sequences of AP3 and PI homologs. **a** One of two maximum parsimony trees. The trees were generated using heuristic search settings with random taxon additions in PAUP. Optimal trees were search using TBR branch-swapping using the MULPARS option. The shortest trees required 1,013 steps. **b** Neighbor-joining tree. Distances were generated using the Dayhoff PAM matrix. The tree was generated using the Neighbor program of PHYLIP 3.5

trees support the separation of AP3/DEF orthologs and PI/GLO orthologs into two distinct clades. They differ only in the placement of the AP3 root, resulting in the placement of the PhAP3 sequence as either a sister taxon to the CpAP3/DeAP3 clade or to the remaining AP3 orthologs. The consensus tree of 1,000 bootstrap resamplings using parsimony shows 100% support for the separate AP3 and PI clades. Furthermore, a number of other hierarchical groupings are also strongly supported. Of particular interest in this study, SLM2 and SpPI form a clade with 90% bootstrap support, and SLM3 and SpAP3 form a clade with 95% bootstrap support which would be predicted given accepted phylogenetic relationships of the species. The Neighbor-joining tree does not differ significantly from that of the parsimony analysis and again shows the placement of SpAP3 with SLM3 in the AP3 lineage, and SpPI with SLM2 in the PI lineage (Fig. 3b). Thus, phylogenetic analyses support the assignment of SpAP3 and SpPI as AP3 and PI orthologs.

Northern hybridization of *SpAP3* and *SpPI*

Northern hybridization analysis was carried out to determine whether our sequences hybridized to unique or multiple mRNA molecules and to determine in which organs the genes are expressed. Antisense probes of the 3′ ends of both genes were used to challenge two independent total RNA blots The blots included extracts from male inflorescences, female inflorescences, stem, and leaf tissues. Both probes generated a single band in the male inflorescence RNA (Fig. 4). In the case of *SpAP3*, a weak signal was detected in the female inflorescence RNA, but not in the stem or leaf extracts. *SpPI* was not detected in any additional tissue extracts. Positive controls using a spinach *G6pdh* antisense probe indicate hybridization in all RNA extracts. These results indicate that each probe hybridizes to a unique transcript and that the gene expression patterns are broadly floral and gender specific.

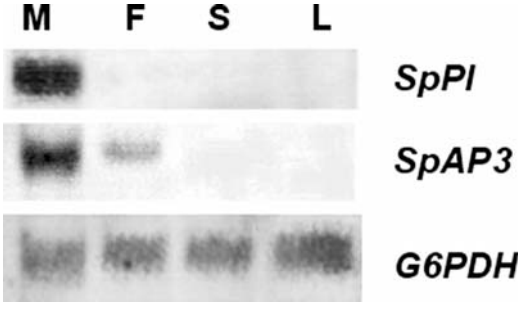

Fig. 4 Northern blot hybridizations of *SpAP3* and *SpPI*. Hybridizations using *SpPI* and *SpAP3* 3′-end probes. The *first lane* is male inflorescence RNA; the *second lane* is female inflorescence RNA; the *third lane* is stem RNA; the *fourth lane* is leaf RNA. The control hybridization using a spinach *G6pdh* probe of the filter is shown *below*

In situ hybridization analyses of *SpAP3* and *SpPI*

Fine scale spatial and temporal expression patterns were determined by in situ hybridization of *SpAP3* and *SpPI* on thin sections of male and female inflorescences. *SpAP3* and *SpPI* demonstrated similar expression patterns. *SpAP3* was detected through all stages of male flower development. At the earliest developmental stage when no organ primordia can be distinguished, *SpAP3* was detected throughout the floral meristem (Fig. 5a). As the sepal primordia form, *SpAP3* expression could be seen in the sepal primordia, mainly in the central cells. Once the stamen primordia formed, *SpAP3* expression became more concentrated in the developing stamen (Fig. 5e). Expression in the sepals was reduced at this stage, though was still detectable in cells in the central layer. As the locules differentiate within the developing anthers and the microsporangia form by meiosis, *SpAP3* transcripts were detected in the tapetum of the locule walls as well as in the microsporangia (Fig. 5I). There was no detectable signal in the male flowers at any stage using the sense probe of the *SpAP3* C region (Fig. 5m).

SpPI is also expressed throughout all stages of male flower development. *SpPI* transcripts were first detected in the earliest stages (before the establishment of stamen primordia) of the male flower (Fig. 5c). *SpPI* was expressed at high levels in the stamen primordia as they developed (Fig. 5g). At this stage, *SpPI* transcripts were also detected in the sepals, though at lower levels. As the stamen developed further, *SpPI* became more strongly expressed in the anthers, eventually with stronger signals within the locule (Fig. 5k). As with *SpAP3*, there is no detectable signal at any stage using the sense strand probe (Fig. 5o).

Figure 5b, f and j shows sections of female flowers in progressive stages of development. There was no detectable level of *SpAP3* hybridization in any female section. These results are in contrast to the northern blot in which a faint *SpAP3* signal was detected. The lack of detection in the in situ hybridizations may be the result of low and/or transient signal per cell. Figure 5d, h, and l shows sections of female flowers in comparable developmental stages to the males hybridized with *SpPI* and stained following the conditions used on male tissue. As with *SpAP3*, there is no detectable level of *SpPI* expression in any of the female sections. Negative controls of sense probes for *SpAP3* and *SpPI* are shown in Fig. 5n and p, respectively. In positive controls using a spinach *G6pdh* probe, hybridization was detected throughout the female inflorescence using anti-sense probes and no hybridization was detected using sense probes (Fig. 5q–t). This indicates that the lack of B class signal in female tissue is a gene-specific effect and not a result of the inability to hybridize to female tissue preparations.

In summary, the in situ hybridization experiments demonstrate that B class genes are exclusively expressed together in developing male flowers in spinach and not in female flowers. This gender-specific pattern differs from the *Arabidopsis* expression pattern in its early expression

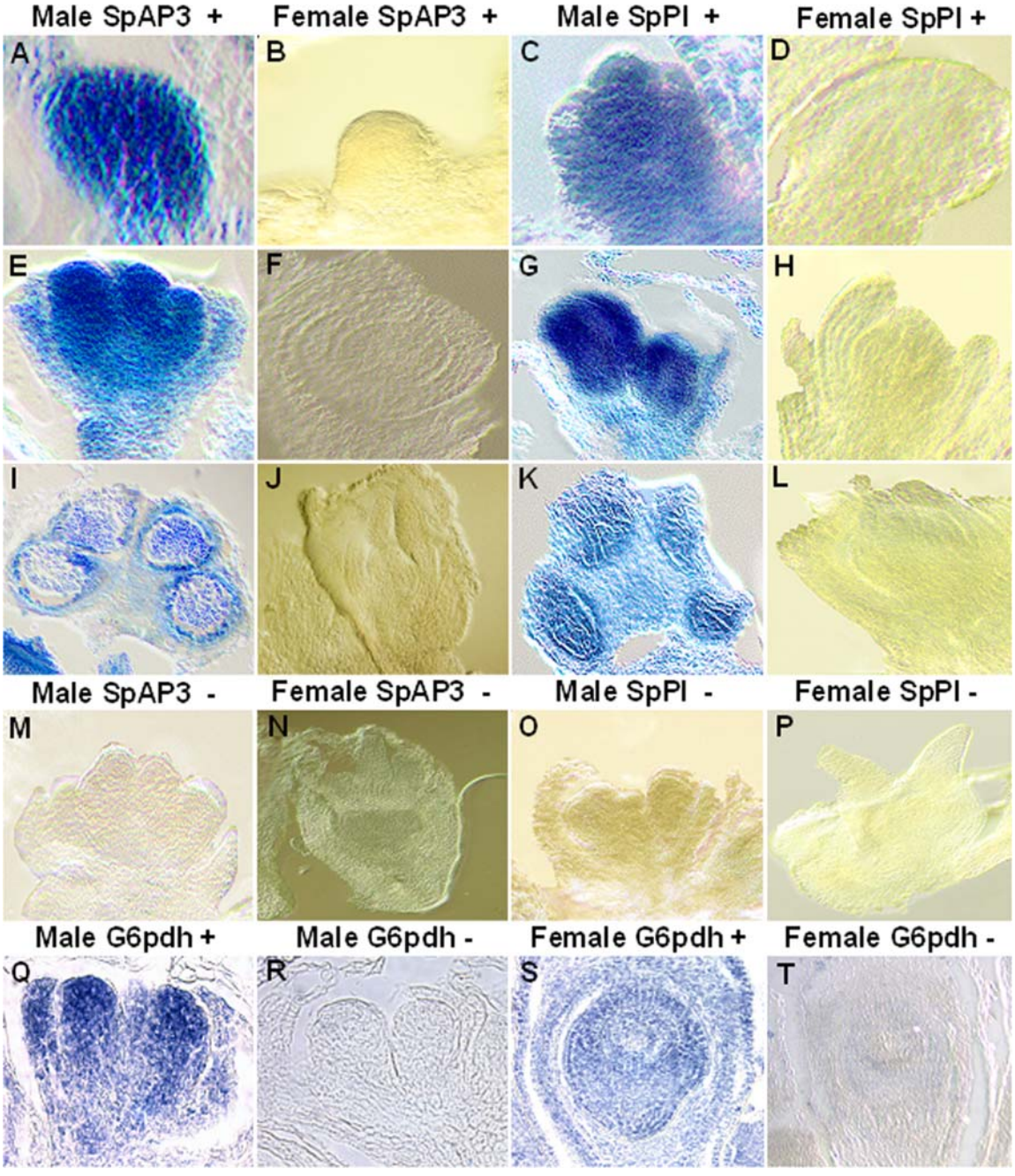

Fig. 5 In situ hybridization of spinach *SpAP3* and *SpPI* in male and female flowers. Male flowers are in **a**, **e**, **I**, **m** of the *first column* , **c**, **g**, **k**, **o** of the *third column* , and **q** and **r**. Female flowers are in **b**, **f**, **j**, **n** of the *second column* , **d**, **h**, **l**, **p** of the *fourth column* , and **s** and **t**. Stages 1 and 2 male flowers are shown in **a** and **c**. Stages 4 and 5 are shown in **e**, **g**, **m**, **o**, **q**, and **r**. Transverse sections of stage 6 anthers showing locules and microsporangia are seen in **I** and **k**. Female stages 1 and 2 flowers are seen in **b** and **d**. Stages 3 and 4 are seen in **f**, **h**, **j**, **l**, **n**, and **p**. **f**, **l** Transverse sections through female flowers. *SpAP3* antisense hybridization is shown in **a**, **b**, **e**, **f**, **j**, and **l**. **m**, **n** Male and female negative controls using an *SpAP3* sense probes. *SpPI* antisense hybridization is shown in **c**, **d**, **g**, **h**, **k**, and **l**. **o**, **p** Male and female *SpPI* negative controls using an *SpPI* sense probe. **q**, **s** Spinach *G6pdh* positive controls using an antisense probe for males and females, respectively. **s**, **t** *G6pdh* negative controls. Additional time for NBT signal development was given to the female samples in order to detect faint signal should it be present

throughout the male floral primordium prior to the appearance of sepal primordia and its persistent, although declining, expression in the sepals after the stamen primordia form.

Discussion

Our results indicate that spinach flower sexual differentiation is initiated at a very early stage and is accompanied by early dimorphism. The prediction that spinach homologs of the *Arabidopsis* B class genes, *AP3* and *PI*, would

be differentially expressed in males versus females has been verified by in situ hybridization. In addition to the expected expression patterns, however, further deviations from the *Arabidopsis* model indicate that spinach B class gene expression is temporally correlated with morphological differentiation in terms of organ number and placement outside of their expected effects in whorls two and three.

Spinach B class genes follow the *Arabidopsis/Antirrhinum* expression patterns in the overlapping expression of *SpAP3* and *SpPI*, and their dominant concentration in the third whorl by stage 4 and following stages. The absence of detectable expression in female flowers that do not have second or third whorl primordia is consistent with this pattern. It should be noted that the homologous genes *SLM3* and *SLM2* of *S. latifolia* are initially expressed in petals and stamens of both male and female flowers (Hardenack et al. 1994). Similar to its spinach ortholog in males, *SLM3* appears to have a more diffuse expression pattern in the initial floral meristem but then becomes concentrated in the petal and sepal primordia. This is consistent with B class gene expression being spatially fixed in second and third whorls in higher eudicots, regardless of the ultimate fate of the organs. However, in contrast, the spinach expression patterns differ in the very early expression of both genes in stage 1, and the expression in sepals beginning in stage 2 in males and persisting at lower intensities even after the stamen primordia form and develop. In the case of the first whorl primordia, flowers of both sexes physically retain the organs. Thus, in spinach, there is a novel region of expression that is sex-specific and independent of the presence/absence of the organs involved.

In *Arabidopsis*, primordia are initiated and subsequently differentiate; i.e. organ identity is separate from and occurs subsequent to organ primordia initiation. Many molecular studies have demonstrated that the organ fate of floral organ primordia is determined by organ identity genes (Bowman et al. 1989, 1991, 1993; Bowman and Smyth 1998; Chuang et al. 1999; Hill and Lord 1989; Irish and Sussex 1990; Jack et al. 1992; Komaki et al. 1988; Kunst et al. 1989; Weigel and Meyerowitz 1994). Therefore, the order and placement of organ primordia are considered to occur independently of the function of floral identity genes. Genes that control the number and placement of organ primordia primarily do so by altering cell division and differentiation in meristem growth (Chuang et al. 1999; Clark et al. 1993; Fletcher 2001; Running et al. 1998; Running and Meyerowitz 1996). Larger meristems, particularly larger areas in the circumference where cell differentiation begins, often results in the development of additional primordia and ultimately additional organs.

The development of spinach sexual dimorphism may deviate from this strict model of distinction between organ identity genes and organ number genes. The sex of a spinach flower can be accurately predicted by sepal number, which is determined before either stamen or carpel primordia form. In contrast to the studies of mutants in *Arabidopsis* in which organ number is modified by an increase in meristem size or rate of cell differentiation, the increased number of sepals in males appears to be a result of the restriction of the lateral field of sepal growth around the first whorl found in females. The additional male sepal primordia then develop in the spaces in the circumference of the first whorl. Several genes that establish organ boundaries in *Arabidopsis*, *Petunia*, and *Pisum* belong to the NAC gene family (Aida et al. 1997, 1999; Liu et al. 1999) which differs from gene families or systems that control meristem growth and differentiation. Similarly, an unrelated gene *SUPERMAN* controls organ number in both *Arabidopsis* and *Petunia* by regulating cell division in regions around primordia and between whorls (Bowman et al. 1992; Nakagawa et al. 2004; Sakai et al. 1995; Schultz et al. 1991). It is noteworthy that both *NAP*, an *Arabidopsis* NAC family gene, and *SUPERMAN* expressions have been reported to be positively regulated by *AP3* and *PI* (Sablowski and Meyerowitz 1998; Sakai et al. 2000). Thus, B class gene expression can potentially affect organ number through the regulation of such genes. In *Arabidopsis*, this control is not exclusive, as *NAP* is expressed in tissues in the absence of B class expression (Sablowski and Meyerowitz 1998). Our observations show that spinach floral sexual dimorphism of non-reproductive organs is tightly associated with sex-specific B class gene expression patterns. This suggests that, in spinach, the regulation of organ identity genes may be associated with developmental features in addition to organ identity. The involvement of B class organ identity genes on organ number through regulation of downstream organ boundary genes would be a likely mechanism.

The gender-specific B class expression patterns in spinach differ in significant ways from those found in other species with unisexual flowers that have been previously studied (reviews in Ainsworth et al. 1998; Grant et al. 1994; Juarez and Banks 1998; Tanurdzic and Banks 2004). In *Z. mays* and *C. sativus*, hormone levels, especially of gibberellic acid, play a role in determination of the ultimate sex of the flower (Delong et al. 1993; Hansen 1976; Kahana et al. 1999; Perl-Treves 1999; Yin and Quinn 1995). In comparison, regulation of floral organ identity genes has not been causally related to sex determination. In *R. acetosa*, *S. latifolia*, *A. officinalis*, and *C. sativus*, B class genes are initially expressed in primordia of the second and third whorls following the *Arabidopsis* pattern. The expression becomes non-detectable upon degeneration of the respective organs (Ainsworth et al. 1995; Hardenack et al. 1994; Kater et al. 2001; Park et al. 2003). Similarly, in *Z. mays*, the B class homolog *SILKY1* is initially identically expressed in both male and female flowers (Ambrose et al. 2000). There is evidence in *Silene* that differential methylation is correlated with sexual phenotypes and methylation inhibition results in the production of hermaphroditic plants with male karyotypes (Janousek et al. 1996; Viskot et al. 1993). Yet, in general, these studies suggest that sex determination is not a result of initial regulation of floral identity genes in species that develop imperfect flowers through abortion

of existing or developing organs. The unique pattern of early suppression of B class expression in spinach female floral primordia may, therefore, reflect the unique development of unisexual flowers without organ abortion.

Acknowledgements We are indebted to D. Carl Freeman for his introduction to and insight into the sexual dimorphism and lability of spinach. We thank John M. Lopes, Aleksandar Popadic, Markus Friedrich, and two anonymous reviewers for their insightful comments on a previous version of this manuscript.

References

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in *Arabidopsis*: an analysis of the *cup-shaped cotyledon* mutant. Plant Cell 9:841–857

Aida M, Ishida T, Tasaka M (1999) Shoot apical meristem and cotyledon formation during arabidopsis embryogenesis: interaction among the cup-shaped cotyledon and shoot meristemless genes. Development 126:1563–1570

Ainsworth C, Crossley S, Buchanan Wollaston V, Thangavelu M, 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

Ainsworth C, Parker J, Buchanan Wollaston V (1998) Sex determination in plants. Curr Top Dev Biol 38:167–223

Ambrose BA, Lerner DR, Ciceri P, Padilla CM, Yanofsky M, Schmidt RJ (2000) Molecular and genetic analysis of the *silky1* gene reveal conservation in floral organ specification between eudicots and monocots. Mol Cell 5:569–579

Angenent GC, Franken J, Busscher M, Colombo L, van Tunen AJ (1993) Petal and stamen formation in petunia is regulated by the homeotic gene *fbp1*. Plant J 4:101–112

Baudinette SC, Stevenson TW, Savin KW (2000) Isolation and characterisation of the carnation floral-specific MADS box gene, *cmb2*. Plant Sci 155:123–131

Bey M, Stuber K, Fellenberg K, Schwarz-Sommer Z, Sommer H, Saedler H, Zachgo S (2004) Characterization of antirrhinum petal development and identification of target genes of the class B MADS box gene *deficiens*. Plant Cell 16(12):3197–3215

Bowman JL, Smyth DR (1998) Patterns of petal and stamen reduction in Australian species of *Lepidium* l. (Brassicaceae). Int J Plant Sci 159:65–74

Bowman JL, Smyth DR, Meyerowitz EM (1989) Genes directing flower development in *Arabidopsis*. Plant Cell 1:37–52

Bowman JL, Smyth DR, Meyerowitz EM (1991) Genetic interactions among floral homeotic genes of *Arabidopsis*. Development 112:1–20

Bowman JL, Sakai H, Jack T, Weigel D, Mayer U, Meyerowitz EM (1992) Superman, a regulator of floral homeotic genes in *Arabidopsis*. Development 114:599–615

Bowman JL, Alvarez J, Weigel D, Meyerowitz EM, Smyth DR (1993) Control of flower development in *Arabidopsis thaliana* by *apetala1* and interacting genes. Development 119:721–743

Bradley D, Carpenter R, Sommer H, Hartley N, Coen E (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of antirrhinum. Cell 72:85–95

Busch MA, Bomblies K, Weigel D (1999) Activation of a floral homeotic gene in *Arabidopsis*. Science 285:585–587

Chailakhyan MK (1979) Genetic and hormonal regulation of growth, flowering, and sex expression in plants. Am J Bot 66:717–736

Chuang CF, Running MP, Williams RW, Meyerowitz EM (1999) The *perianthia* gene encodes a bzip protein involved in the determination of floral organ number in *Arabidopsis thaliana*. Genes Dev 13:334–344

Chuck G, Meeley RB, Hake S (1998) The control of maize spikelet meristem fate by the apetala2-like gene *indeterminate spikelet1*. Genes Dev 12:1145–1154

Clark SE, Running MP, Meyerowitz EM (1993) Clavata1, a regulator of meristem and flower development in *Arabidopsis*. Development 119:397–418

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353:31–37

Coen ES, Doyle S, Romero JM, Elliot R, Murphy G, Carpenter R (1991) Homeotic genes controlling flower development in *Antirrhinum*. Development Suppl 1:149–155

Cubas P, Vincent C, Coen E (1999) An epigenetic mutation responsible for natural variation in floral symmetry. Nature 401:157–160

Delong A, Calderon-Urrea A, Dellaporta S (1993) Sex-determination gene *tasselseed2* of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. Cell 74:757–768

Doebley J, Lukens L (1998) Transcriptional regulators and the evolution of plant form. Plant Cell 10:1075–1082

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11

Felsenstein J (1993) Phylip (phylogeny inference package). Department of Genetics, University of Washington, Seattle, Wash.

Fletcher JC (2001) The *ultrapetala* gene controls shoot and floral meristem size in *Arabidopsis*. Development 128:1323–1333

Freeman DC, Vitale JJ (1985) The influence of environment on the sex ratio and fitness of spinach. Bot Gazette 146:137–142

Goto K, Meyerowitz EM (1994) Function and regulation of the *Arabidopsis* floral homeotic gene *pistillata*. Genes Dev 8:1548–1560

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

Hansen DJ (1976) Gibberellic acid-controlled sex expression of corn tassels. Crop Sci 16:371–374

Hardenack S, Ye D, Saedler H, 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

Heslop-Harrison J (1964) Sex expression in flowering plants. In: Brookhaven National Laboratory (eds) Brookhaven symposia in biology. Brookhaven National Laboratory, Upton, N.Y., pp 109–125

Hill JP, Lord EM (1989) Floral development in *Arabidopsis thaliana*: comparison of the wild type and the homeotic *pistillata* mutant. Can J Bot 67:2922–2936

Irish VF (1999) Patterning the flower. Dev Biol 209:211–220

Irish VF, Sussex IM (1990) Function of the *apetala-1* gene during *Arabidopsis* floral development. Plant Cell 2:741–753

Ito T, Wellmer F, Yu H, Das P, Ito N, Alves-Ferreira M, Riechmann JL, Meyerowitz EM (2004) The homeotic protein agamous controls microsporogenesis by regulation of sporocyteless. Nature 430:356–360

Jack T (2004) Molecular and genetic mechanisms of floral control. Plant Cell 16:S1–S17

Jack T, Brockman LL, Meyerowitz EM (1992) The homeotic gene *apetala3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell 68:683–697

Jack T, Fox GL, Meyerowitz EM (1994) *Arabidopsis* homeotic gene *apetala3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell 76:703–716

Janousek B, Siroky J, Vyskot B (1996) Epigenetic control of sexual phenotype in a dioecious plant, *Melandrium album*. Mol Gen Genet 250:483–490

Juarez C, Banks JA (1998) Sex determination in plants. Curr Opin Plant Biol 1:68–72

Kahana A, Silberstein L, Kessler N, Goldstein RS, Perl-Treves R (1999) Expression of *acc* oxidase genes differs among sex genotypes and sex phases in cucumber. Plant Mol Biol 41:517–528

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

Komaki MK, Okada K, Nishino E, Shimura Y (1988) Isolation and characterization of novel mutants of *Arabidopsis thaliana* defective in flower development. Development 104:195–203

Kramer EM, Irish VF (1999) Evolution of genetic mechanisms controlling petal development. Nature 399:144–148

Kramer EM, Irish VF (2000) Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. Int J Plant Sci 161: S29-S40

Kramer EM, Dorit RL, Irish VF (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

Krizek BA, Meyerowitz EM (1996) Mapping the protein regions responsible for the functional specificities of the *Arabidopsis* MADS domain organ-identity proteins. Proc Natl Acad Sci USA 93:4063–4070

Kunst L, Klenz JE, Martinez-Zapater J, Haughn GW (1989) *Ap2* gene determines the identity of perianth organs in flowers of *Arabidopsis thaliana*. Plant Cell 1:1195–1208

Kush A, Brunelle A, Shevell D, Chua NH (1993) The cDNA sequence of two MADS box proteins in petunia. Plant Physiol 102:1051–1052

Liu C-M, Johnson S, di Gregorio S, Wang T (1999) *Single cotyledon* (*sic*) mutants of pea and their significance in understanding plant embryo development. Dev Genet 25:11–22

Lohmann JU, Weigel D (2002) Building beauty: the genetic control of floral patterning. Dev Cell 2:135–142

Lohmann JU, Hong RL, Hobe M, Busch MA, Parcy F, Simon R, Weigel D (2001) A molecular link between stem cell regulation and floral patterning in *Arabidopsis*. Cell 105:793–803

Mandel MA, Bowman JL, Kempin SA, Ma H, Meyerowitz EM, Yanofsky MF (1992) Manipulation of flower structure in transgenic tobacco. Cell 71:133–143

Miglia K, Freeman DC (1996) The effect of delayed pollination on stigma length, sex expression, and progeny sex ration of spinach: a test of Fisher's prediction. Am J Bot 83:326–332

Munster T, Wingen LU, Faigl W, Werth S, Saedler H, Theissen G (2001) Characterization of three globosa-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. Gene 262:1–13

Nakagawa H, Ferrario S, Angenent GC, Kobayashi A, Takatsuji H (2004) The petunia ortholog of *Arabidopsis superman* plays a distinct role in floral organ morphogenesis. Plant Cell 16:920–932

Parcy F, Nilsson O, Busch MA, Lee I, Weigel D (1998) A genetic framework for floral patterning. Nature 395:561–566

Park JH, Ishikawa Y, Yoshida R, Kanno A, 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

Perl-Treves R (1999) Male to female conversion along the cucumber shoot: approaches to studying sex genes and floral development in *Cucumis sativus*. In: Ainsworth C (ed) Sex determination in plants. Bios, Oxford, pp 189–216

Pobursky KJ (2000) Early flower development and the influence of gibberellic acid on sex expression in *Spinacia oleracea*. MSc, Wayne State University, USA

Purugganan MD, Boyles AL, Suddith JI (2000) Variation and selection at the cauliflower floral homeotic gene accompanying the evolution of domesticated *Brassica oleracea*. Genetics 155:855–862

Riechmann JL, Meyerowitz EM (1998) The *ap2/erebp* family of plant transcription factors. Biol Chem 379:633–646

Running MP, Meyerowitz EM (1996) Mutations in the *perianthia* gene of *Arabidopsis* specifically alter floral organ number and initiation pattern. Development 122:1261–1269

Running MP, Fletcher JC, Meyerowitz EM (1998) The *wiggum* gene is required for proper regulation of floral meristem size in *Arabidopsis*. Development 125:2545–2553

Sablowski RWM, Meyerowitz EM (1998) A homolog of *no apical meristem* is an immediate target of the floral homeotic genes *apetala3/pistillata*. Cell 92:93–103

Sakai H, Medrano LJ, Meyerowitz EM (1995) Role of *superman* in maintaining *Arabidopsis* floral whorl boundaries. Nature 378:199–202

Sakai H, Krizek BA, Jacobsen SE, Meyerowitz EM (2000) Regulation of *sup* expression identifies multiple regulators involved in *Arabidopsis* floral meristem development. Plant Cell 12:1607–1618

Samach A, Kohalmi SE, Motte P, Datla R, Haughn G (1997) Divergence of function and regulation of class B floral organ identity genes. Plant Cell 9:559–570

Sattler R (1973) Organogenesis of flowers. University of Toronto Press, Toronto

Schultz EA, Pickett FB, Haughn GW (1991) The *flo10* gene product regulates the expression domain of homeotic genes *ap3* and *pi* in *Arabidopsis* flowers. Plant Cell 3:1221–1237

Sherry RA, Eckard KJ, Lord EM (1993) Flower development in dioecious *Spinacia oleracea* (Chenopodiaceae). Am J Bot 80:283–291

Shu GP, Amaral W, Hileman LC, Baum DA (2000) Leafy and the evolution of rosette flowering in violet cress (*Jonopsidium acaule*, Brassicaceae). Am J Bot 87:634–641

Sommer H, Beltran J, Huijser P, Pape H, Lonnig W, Saedler H, Schwarz-Sommer Z (1990) *Deficiens*, a homeotic gene involved in the control of flower morphogenesis. EMBO J 9:605–613

Swofford DL (1993) PAUP: phylogenetic analysis using parsimony. Illinois Natural History Survey, Champaign, Ill.

Tanurdzic M, Banks JA (2004) Sex-determining mechanisms in land plants. Plant Cell 16:S61–S71

Theißen G, Kim JT, Saedler H (1996) Classification and phylogeny of the MADS-box multigene family suggest defined roles of MADS-box gene subfamilies in morphological evolution of eukaryotes. J Mol Evol 43:484–516

Thomas J, Laura BL, Meyerowitz EM (1992) The homeotic gene *apetala3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell 683–697

Tröbner W, Ramirez L, Motte P, Hue I, Huijser P, Lonnig WE, Saedler H, Sommer H, Schwarz-Sommer Z (1992) *Globosa*: a homeotic gene which interacts with *deficiens* in the control of antirrhinum floral organogenesis. EMBO J 11:4693–4704

Viskot B, Araya A, Veuskens J, Negrutiu I, Mouras A (1993) DNA methylation of sex chromosomes in a dioecious plant, *Melandrium album*. Mol Gen Genet 239:219–224

Vitale JJ, Freeman DC (1985) Secondary sex characteristics in *Spinacia oleraceae* l.: quantitative evidence for the existence of at least three sexual morphs. Am J Bot 72:1061–1066

Wagner D, Sablowski RWM, Meyerowitz EM (1999) Transcriptional activation of *apetala1* by *leafy.* Science 285:582–584

Wang R-L, Stec A, Hey J, Lukens L, Doebley J (1999) The limits of selection during maize domestication. Nature 398:236–239

Weigel D, Meyerowitz EM (1994) The abcs of floral homeotic genes. Cell 78:203–209

Wellmer F, Riechmann JL, Alves-Ferreira M, Meyerowitz EM (2004) Genome-wide analysis of spatial gene expression in *Arabidopsis* flowers. Plant Cell 16:1314–1326

Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM (1990) The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Nature 346:35–39

Yin TJ, Quinn JA (1995) Tests of a mechanistic model of one hormone regulating both sexes in *Cucumis sativus* (Cucurbitaceae). Am J Bot 82:1537–1546

Zik M, Irish VF (2003a) Flower development: initiation, differentiation, and diversification. Annu Rev Cell Dev Biol 19:119–140

Zik M, Irish VF (2003b) Global identification of target genes regulated by *apetala3* and *pistillata* floral homeotic gene action. Plant Cell 15:207–222