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ORIGINAL ARTICLE

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# Analysis of members of the *Silene latifolia* $Cys_2/His_2$ zinc-finger transcription factor family during dioecious flower development and in a novel stamen-defective mutant *ssf1*

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**Abstract** Sex determination in dioecious *Silene latifolia* Poir. is governed by the inheritance of heteromorphic sex chromosomes. In male plants the Y chromosome influences two aspects of male organogenesis, the continued differentiation of stamen primordia and male fertility, and one aspect of female organogenesis, the arrest of development of the pistil. *S. latifolia* is susceptible to infection by the parasitic smut fungus *Ustilago violacea*, which induces stamen development in genetically female plants. Here we describe the identification and characterisation of a novel male mutant, *short stamen filaments 1* (*ssf1*), defective in stamen differentiation. Although several independent studies have identified genes expressed during sex-determination in *S. latifolia*, analyses suggest that none of these encode regulatory proteins involved in the control of sex determination. We therefore isolated six *S. latifolia* cDNAs encoding members of a family of transcriptional regulators, the ZPT-type $Cys_2/His_2$ zinc-finger proteins that had previously shown to be co-ordinately regulated during stamen development in *Petunia* × *hybrida* hort. Vilm.-Andr. We have analysed the genomic organisation of these genes in male and female plants and their expression dynamics in male and female plants, in smut-infected female plants and in the *ssf1* mutant. Our studies reveal expression patterns during development of the androecium that suggest a possible role for *SlZPT2-1* in filament elongation and *SlZPT4-1* in aspects of male fertility during stamen differentiation.

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**Keywords** *Silene* · Dioecy · Stamen development · $Cys_2/His_2$ zinc finger · Transcription factor

**Abbreviations** EMS: Ethyl methanesulfonate · PhZPT: *Petunia* × *hybrida* zinc-finger protein of TFIIIA-type · RACE: Rapid amplification of cDNA ends · SEM: Scanning electron microscopy · SlZPT: *Silene latifolia* zinc-finger protein of TFIIIA-type · ssf1: *short stamen filaments 1*

## Introduction

Dioecy is an outbreeding strategy, used by approximately 4% of angiosperms species (Yampolski and Yampolski 1922), in which individual plants bear either male or female flowers. Sex determination in dioecious *S. latifolia* is controlled by a dominant Y chromosome system (Warmke and Blakeeslee 1940; Westergaard 1946) in a manner reminiscent of that in mammals (Goodfellow and Lovellbadge 1993). Male plants contain 22 autosomes, and an X and a Y chromosome; female plants contain 22 autosomes and a pair of X chromosomes. The sex chromosomes contain a pseudo-autosomal region located on the p-arm of the X and the q-arm of the Y that permits chromosome pairing during meiosis (Lengerova et al. 2003). Early flower development of male and female flowers involves the formation of both stamen and carpel organ primordia. However, subsequent development involves the co-ordinated arrest and suppression of stamen and carpel development in female and male flowers, respectively (Grant et al. 1994b; Farbos et al. 1997; Scutt et al. 1997). Following arrest of stamen development in female flowers, stamen primordia and staminal nectaries persist during female flower development but subsequently degenerate as the flower matures. In male flowers, the presence of a Y chromosome leads to suppression of carpel development with only a thin thread-like structure forming in place

of five fused carpels. (Grant et al. 1994b; Farbos et al. 1997; Scutt et al. 1997).

The sex determination system in *S. latifolia* is robust, resisting perturbation by environmental conditions and exogenous hormone application (Ye et al. 1991; Grant et al. 1994a). However, *S. latifolia* is susceptible to infection by the parasitic smut fungus *Ustilago violacea*, which induces stamen development in genetically female plants. The anthers of these smut-fungus-infected female plants contain little or no tapetal tissue, and are sterile, bearing fungal teliospores of *U. violacea* rather than pollen (Audran and Batcho 1981). Female flowers therefore retain the capacity for production of stamens, and in the absence of a Y chromosome, this developmental pathway is initiated but does not progress. *U. violacea* can therefore provide a signal that mimics Y-chromosome-mediated control of stamen development (Audran and Batcho 1981). The pistils of smut-infected female flowers develop beyond the stage of pistil arrest seen in male flowers; however, they do not reach full size and are infertile.

The active role of the Y chromosome in the control of sex determination in *S. latifolia* was defined using mutant plants containing fragmented Y chromosomes (Westergaard 1946, 1958; Ye et al. 1991; Grant et al. 1994a). Deletions on the p-arm of the Y chromosome resulted in hermaphroditic flowers.

Deletion of a central region of the Y chromosome resulted in asexual male plants lacking both stamens and carpels; morphologically normal male-sterile mutants producing inviable pollen were also observed, which displayed deletions to the q-arm of the Y chromosome (Westergaard 1946). The Y chromosome is therefore implicated in two aspects of stamen development. Recently identified Y chromosome deletion mutants produced through X-irradiation (Grant et al. 1994a; Donnison et al. 1996) and gamma ray irradiation of pollen (Farbos et al. 1999; Lardon et al. 1999), exhibit a similar range of aberrant sexual phenotypes, including bisexual (*bsx*) and asexual (*asx*) mutants, in addition to male-sterile plants. Here we describe the isolation and characterisation of an ethyl methanesulfonate (EMS)-generated mutant of *Silene latifolia*, *short stamen filaments 1* (*ssf1*), which is defective in stamen development.

Although the detailed analysis of the genetic basis of *ssf1* is beyond the scope of this present study, we have used this mutant as tool for comparative gene expression analyses with wild-type flowers.

Several studies to identify genes involved in the control of sex-determination in *S. latifolia* have employed subtractive hybridisation and differential screening approaches. These approaches identified a number of male-specific cDNAs that are expressed during stamen development (Matsunaga et al. 1996, 1997; Barbacar et al. 1997; Hinnisdaels et al. 1997; Robertson et al. 1997; Scutt and Gilmartin 1997, 1998; Scutt et al. 1997).

However, despite acting as useful markers for different stages of male flower development, analyses suggest that none of these genes encode regulatory proteins involved in the control of sex determination. Parallel studies to identify Y-chromosome-encoded sequences have led to the identification of genes located on the male-determining Y chromosome (Delechere et al. 1999; Filatov et al. 2000; Scutt et al. 2002), but as yet key genes controlling carpel arrest and modulating stamen development in male flowers have not been defined.

In *Petunia* × *hybrida* a family of ZPT protein genes has been implicated in a range of developmental processes, including the regulation of stamen development (Kobayashi et al. 1998). These proteins contain $Cys_2/His_2$ zinc-finger proteins in which two cysteine and two histidine residues, within a conserved sequence motif ($CX_{2-4}FX_5LX_2HX_{3-5}H$), tetrahedrally co-ordinate a zinc atom to form a compact structure that interacts with the major groove of DNA in a sequence-specific manner (Paveletich and Pabo 1991). The founding member of this family, EPF1 (subsequently renamed ZPT1 for *Petunia* × *hybrida* Zinc-finger Protein of TFIIIA-type, Kubo et al. 1998), was identified as a nuclear protein in petals that bound to the promoter of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene (Takatsuji et al. 1992). Over 30 related ZPT-type $Cys_2/His_2$ zinc-finger protein genes have subsequently been isolated from plants and these contain between one and four zinc-fingers. Database searches reveal that over 90 such ZPT-type genes may be present in the *Arabidopsis thaliana* (L.) Heynh. genome. A characteristic feature of these predicted and characterised proteins is a highly conserved sequence motif, QALGGH, within the putative DNA-contacting surface of each zinc-finger (Takatsuji and Matsumoto 1996). Regulatory functions for some ZPT family zinc-finger proteins have been identified. The SUPERMAN protein from *A. thaliana* contains a single ZPT-type zinc-finger and is involved in the control of stamen differentiation (Sakai et al. 1995). The *Petunia* ortholog PhSUP1, which plays a distinct role in floral organ morphogenesis, has recently been reported (Nakagawa et al. 2004). Additional roles in floral reproductive development have been proposed for other $Cys_2/His_2$ zinc-finger proteins including seven ZPT-type transcription factors that are activated sequentially during stamen development in *Petunia* (Kobayashi et al. 1998) and one of these proteins PhZPT3-2, renamed as TAZ1, has been shown to be involved in development of the tapetum (Kapoor et al. 2002). Two further *PhZPT* genes, *PhZPT3-3* and *ZPT2-10*, have been shown to be expressed specifically in the pistil (Kubo et al. 2000).

In order to investigate the role of ZPT-type $Cys_2/His_2$ zinc-finger protein genes in stamen development in *S. latifolia* we identified and classified six members of this gene family. We have analysed the genomic organisation and expression dynamics of these six transcription factor genes in relation to sex determination using wild-type male, female and smut-infected female plants as well as in the novel stamen-development mutant *ssf1*. We have named these genes *SlZPT* for *Silene latifolia* Zinc-finger Protein of TFIIIA-type (Kubo et al. 1998). Here we

present data implicating one of the *S. latifolia* ZPT gene family members, *SLZPT4-1*, in aspects of male fertility. Another family member, *SLZPT2-1*, is differentially regulated between wild-type and *ssf1* plants, suggesting a role in filament elongation during stamen differentiation, a process that is defective in *ssf1* plants.

## Materials and methods

### Plant material and mutant isolation

Seeds of *Silene latifolia* Poir. were obtained from John Chambers Seeds (Barton Seagrove, Northamptonshire, UK) and stocks were maintained through sexual propagation without close inbreeding. *S. latifolia* plants were grown in greenhouses equipped with supplementary lighting to extend the photoperiod to 16 h. Flower tissue from smut-infected *S. latifolia* plants was collected in liquid nitrogen from a wild population growing alongside the A65 York Road, approximately 2 miles outside of York. EMS mutagenesis was performed as described below. Approximately 12,000 seeds were imbibed overnight in 0.1% KCl. They were then mutagenised in 100 mM EMS, 100 mM sodium phosphate (pH 5.0), 5% dimethyl sulfoxide (6,000 seeds/50 ml mutagen solution) for 5 h. Seeds were washed in 100 mM sodium thiosulphate for 15 min twice. All reactions were undertaken in an Atmos bag in a fume hood. All solid waste was treated with 100 mM sodium thiosulphate and liquid waste poured directly onto solid sodium thiosulphate. Seeds were dried on Whatman 3MM paper and then planted at low density in seed trays. 10,000 individual seedlings were pricked out and grown to maturity for screening. *ssf1* was identified as a mutant male plant in which filaments did not elongate during androecium development, resulting in mature anthers that remained at the base of the flower.

### Nucleic acid isolation and identification of an *S. latifolia* ZPT-type family of transcription factors

Genomic DNA was prepared using Nucleon Phytopure plant DNA extraction kits according to the manufacturer's instructions. Primary PCR reactions for the isolation of ZPT family members were performed in a volume of 50 µl containing 200 µM dNTPs, 5 µM of each primer, 1 U of Supertaq DNA polymerase (Stratech) and 1× Supertaq buffer [10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.01% (w/v) gelatin]. 200 ng of genomic DNA was used in the primary PCR reactions. Reactions were performed in 0.2-ml thin-walled tubes (Stratagene) in a thermal cycler (Stratagene) fitted with a heated lid to prevent evaporation. 30 cycles of amplification were performed, consisting of denaturation at 96°C for 30 s, annealing for 30 s and polymerisation at 72°C for 1 min. The sequence of the upstream primer was CA(A/G)GCI(T/C)TIGGIGGICA(T/C) and the downstream primer was (A/G)TGICCICCIA(A/G)IGC(T/C)TGG.

5′ and 3′ RACE–PCR (rapid amplification of cDNA ends–polymerase chain reaction) were performed to isolate full-length cDNA clones representing each *S. latifolia* ZPT family member. Advantage 2 DNA polymerase (Clontech) was used for the 5′ and 3′ RACE–PCR amplifications. RNA was prepared using the Qiagen Plant RNeasy RNA extraction kit according to the manufacturer's protocol. Specific oligonucleotide primers were designed to the 5′ and 3′ regions of candidate ZPT-type genes identified from the primary PCR reactions performed on genomic DNA. Two micrograms of total RNA was DNase-treated prior to reverse transcription, and first-strand cDNA was synthesised using the Clontech SMART cDNA synthesis kit.

### Southern-blot hybridisations

Genomic DNA from *S. latifolia* male and female leaf tissue was prepared as described above and digested with suitable restriction endonucleases. Restriction digests were fractionated by electrophoresis on 0.7% agarose gels, 15 µg per track, in TAE buffer (Sambrook et al. 1989) before processing for Southern blotting onto Hybond-N+ membranes (Amersham-Pharmacia) according to the manufacturer's protocols. Southern blot hybridisations were carried out in 50% formamide at 42°C. Labelling of cDNA probes was prepared by random prime labelling (Feinberg and Vogelstein 1983). All blot hybridisations were washed at high stringency in solutions containing 0.2× SSC and 0.1% (w/v) SDS at 65°C (20× SSC is 3.0 M NaCl, 0.3 M trisodium citrate, pH 7.25).

### Reverse transcription–PCR analysis

First-strand cDNA was synthesised from a template of total cellular RNA using MMLV (H−) reverse transcriptase (Promega) according to the manufacturer's instructions. Two micrograms of total cellular RNA was DNase-treated with RNase-free DNase I (GIBCO–BRL) prior to reverse transcription, according to the manufacturer's instructions.

PCR amplification from first-strand cDNA templates was carried out in 50-µl reaction volumes containing 1/25 of RT reaction, 5 µl 10× *Taq* polymerase buffer, 0.4 µl 25 mM dNTPs, 1 µl 10 mM each primer and 1 U *Taq* DNA polymerase (Promega). PCR cycling was performed with an initial denaturation at 96°C for 2 min followed by denaturation for 30 s, annealing for 30 s and polymerisation at 72°C for 1 min. 6-µl aliquots were taken from each reaction at the end of the extension phase of cycling after 15, 20, 25 and 30 cycles, respectively. 5 µl of each sample, following 15, 20, and 25 cycles of amplification, was fractionated by electrophoresis in TAE buffer (Sambrook et al. 1989) before

processing for Southern blotting onto Hybond-N membranes (Amersham-Pharmacia) according to the manufacturer's instructions. Labelling of cDNA probes and washing conditions after Southern blotting were identical to those used for Southern blotting of genomic DNA.

### Light microscopy

Bright-field photographs of individual flowers were taken using a dissecting microscope (model Orthoplan; Leitz).

### Scanning electron microscopy (SEM) of *S. latifolia* flower buds

Specimens of male flower buds were prepared for SEM as described by Cohen (1979) and examined microscopically (CamScan Series 3; CamScan Analytical, Cambridge, UK).

## Results

### Isolation and characterisation of *short stamen filaments 1* (*ssf1*), a new mutant phenotype in *Silene latifolia* showing defective stamen development.

The previous identification of chromosome deletion mutants affected in sex determination (Westergaard 1946; Donnison et al. 1996; Farbos et al. 1997; Lardon et al. 1999) prompted us to instigate a mutant screen for floral mutant phenotypes following EMS mutagenesis of seed. We predicted that by screening plants in the $M_0$ population, dominant mutations, or mutations affecting genes present in a single copy in males on either the X or the Y chromosome would be revealed. Following EMS mutagenesis of seed, 10,000 seedlings were individually potted and grown to maturity in 3 cm×3 cm-cell trays. Several plants exhibiting altered floral phenotypes were identified in this population. Mutant phenotypes included hermaphrodite flowers, where both male and female reproductive structures developed, in addition to flowers defective in aspects of stamen development. Mutant plants defective in non-floral functions were also identified, for example, some EMS-treated plants exhibited variegated leaves, whilst others developed thin, elongated leaves.

One particular mutant male plant with defective stamen development was identified and characterised. This mutant, *short stamen filaments 1* (*ssf1*), is male-sterile and defective in stamen filament elongation. Flower development in *ssf1* was analysed by a combination of SEM and light microscopy techniques, and stamen development compared with wild-type male plants (Fig. 1). Early stages of development are comparable between the wild-type and *ssf1* mutant flowers, as shown by wild-type male flower buds at stages 1, 2 and 3 (Fig. 1a,c,e, respectively; Scutt et al. 1997) and flowers of *ssf1* at equivalent stages of development (Fig. 1b,d,f). In stage-1 flowers (Fig. 1a,b), the lobed anthers can be seen with the central carpel resembling a thin rod-like projection in the centre of the flower. At stage 2 (Fig. 1c,d) anthers development has proceeded to reveal the classical trapezoid shape with no observable difference between wild-type and *ssf1* flowers.

At stage 3, stamen filament formation is clearly evident in both wild-type (Fig. 1e) and *ssf1* flowers (Fig. 1f). It is at this stage of development that the first observable differences become apparent, with somewhat shorter filaments in *ssf1* than in the wild type.

The consequences of short stamen filaments are revealed by comparison between a mature wild-type male flower (Fig. 1g) and a corresponding flower for *ssf1* (Fig. 1h). A montage of SEM images of a mature flower of *ssf1* (Fig. 1i) reveals that stamen filament elongation has not proceeded beyond that observed at stage 3 of flower development (Fig. 1f). Despite the lack of stamen filament elongation in *ssf1*, anther development and maturation occur, with dehisced anthers and pollen grains visible (Fig. 1j). However, despite the production of pollen, the plant is male-sterile; all attempts to use this pollen to fertilise wild-type female flowers have so far been unsuccessful.

### Identification of a family of ZPT zinc-finger protein genes in *Silene latifolia*

The well-documented differential regulation of ZPT family transcription factor genes during stamen development in *Petunia* prompted us to isolate members of this gene family from *Silene latifolia* with the objective of characterizing their organisation and expression in male, female, smut-infected female and *ssf1* flowers of *S. latifolia*. Degenerate oligonucleotide primers designed to the highly conserved QALGGH motif in the $Cys_2$/$His_2$ zinc-finger domain of the plant ZPT family proteins were used in a PCR-based cloning strategy, as originally described by Kubo et al. (1998), to isolate members of the *S. latifolia* ZPT family of transcription factors. This highly conserved sequence motif has not been identified in any of the animal $Cys_2$/$His_2$ zinc-finger proteins. This cloning strategy was predicted to identify sequences encoding proteins with two or more zinc-finger motifs, as the primers were designed to amplify sequences between the QALGGH signature motif present in each zinc-finger.

The ladder of PCR products ranging in size from 0.2 kb to 2 kb, which was obtained by PCR using genomic DNA from male *S. latifolia*, reveals the presence of several genes that contain two or more sequences corresponding to the QALGGH motif (Fig. 2).

These pooled PCR products were cloned into the pCR2.1 vector (Invitrogen) and individual clones sequenced. Based on these sequence data we designed a

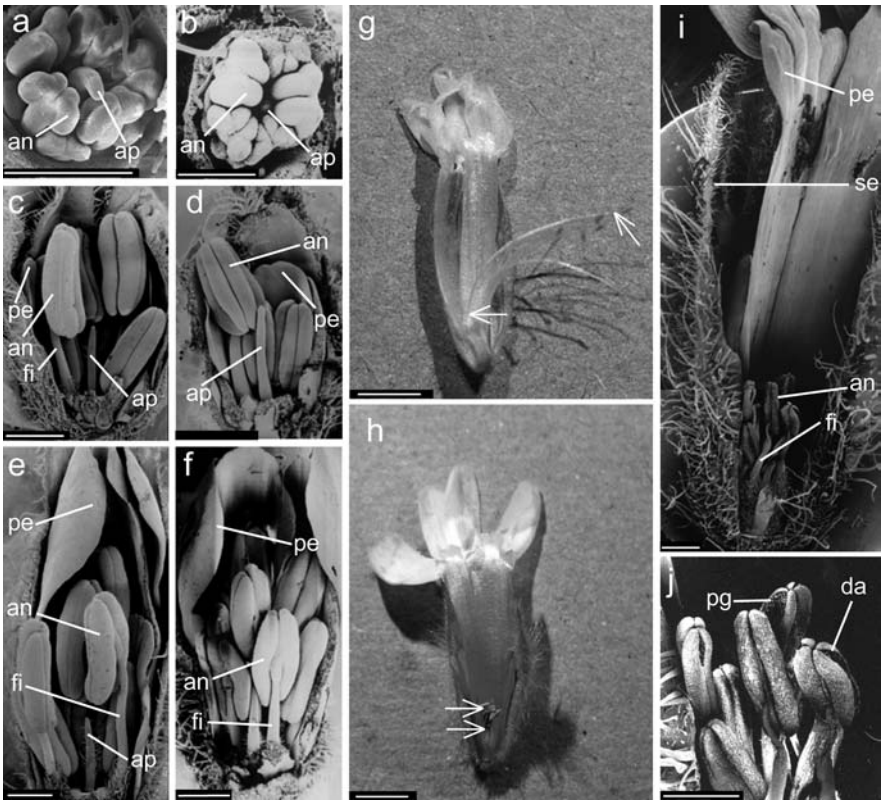


**Fig. 1a–j** SEM and light microscopy of flower development in wild-type male *Silene latifolia* and *ssf1*, a male mutant plant defective in filament elongation. **a** Stage-1 male flower bud with the anther lobes clearly visible and the thin arrested pistil extending in the center of the flower. **b** Stage-1 mutant male flower bud with the anther lobes visible and the arrested pistil located in the center of the flower. **c** Stage-2 male flower bud. The extension of filaments is just apparent. The anthers continue to develop and the arrested pistil can be seen as a healthy filamentous structure in the center of the flower. **d** Stage-2 male mutant flower bud. Filament extension does not occur and the anthers still remain low down within the mutant flower, though are more developed than in **b**; the arrested pistil has extended as a healthy filamentous structure. **e** Stage-3 male flower bud. The anthers continue to develop and there is further extension of the filaments. **f** Stage-3 male mutant flower bud. The anthers are developing and there has been some extension of the filaments so that they resemble the equivalent stage in the wild-type male flower bud. **g** Light-microscope image of a mature wild-type male flower, with *arrows* delineating the extent of the filament. **h** Light-microscope image of a mutant male plant. *Arrows* again delimit the extent of the filament, clearly showing the stunted nature of the filaments in the EMS-generated male mutant plant. **i** SEM image of a mature mutant male flower equivalent to the image in **h**. The very short filaments of the stamens are clearly visible, as are the anthers, which are located very low down in the mature flower, relative to the position occupied in the wild-type male. **j** A magnified image of the anthers in **h**. The anthers in the male mutant produce pollen grains, and a dehiscence zone is clearly visible in the far anther. **a**, **c** and **e**, with permission from Scutt et al. (1997). *an* anther, *fi* filament, *ap* arrested pistil, *pe* petal, *pg* pollen grain, *da* dehiscent anther, *se* sepals. Bars = 1 mm (**a–e,i,j**), 1 cm (**g,h**)

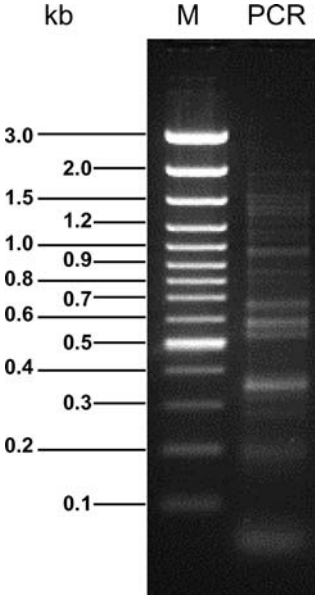


**Fig. 2** PCR amplification of ZPT-type sequences from *S. latifolia* male genomic DNA. PCR was performed on male genomic DNA using degenerate oligonucleotide primers designed to the conserved QALGGH motif, which amplify genomic sequences between two adjacent zinc-finger motifs. PCR products were run on a 2% agarose gel in 1% TAE buffer and DNA visualised by ethidium bromide staining. Lanes are DNA size markers (*M*) and the products of PCR (*PCR*)

series of gene-specific primers that were used in 5′- and 3′-RACE experiments using male flower bud cDNA as the template. These analyses resulted in the identification of six full-length *S. latifolia* ZPT-type cDNA sequences encoding different $Cys_2/His_2$ zinc-finger proteins. Analysis of these six cDNA clones revealed that they encode proteins with two, three and four zinc-finger

motifs. The number of zinc-finger motifs present in each ZPT protein is represented by a number, together with a unique gene identifier number. For example SlZPT2-1 contains two zinc-fingers and was the first *S. latifolia* family member of this structure to be identified. Three of the six *S. latifolia* ZPT family proteins, SlZPT2-1, SlZPT2-2 and SlZPT2-3, contain two zinc-fingers; SlZPT3-1 and SlZPT3-2 contain three zinc-finger motifs; and SlZPT4-1 contains four zinc-finger domains.

## Analysis and comparison of *S. latifolia* ZPT proteins

The proposed classification system for ZPT family zinc-fingers (Kubo et al. 1998) reveals a distinction between the N- and C-terminal fingers of two-finger proteins, such that the peptide sequence surrounding the conserved QALGGH motif of the N-terminal finger of a pair is classified as an A-type finger, and the C-terminal finger is classified as a B-type finger. In two-finger proteins, an A-type zinc-finger is always paired with a B-type (Kubo et al. 1998). Proteins containing more than two zinc-finger motifs contain A- and B-type fingers in addition to a modified (M-type) zinc-finger. The additional M-type zinc-fingers are located towards the N-terminus of the protein and do not typically contain the conserved QALGGH motif but instead contain a modification of this sequence. These types of zinc-finger were accordingly classified as modified or M-type zinc-fingers in *S. latifolia*. Although a number of ZPT-type zinc-finger proteins have been identified and characterised from a range of plant species, many are only as predicted protein sequences. Furthermore, database searches reveal that five out of the six *S. latifolia* proteins show greatest similarity to *Petunia* proteins (Table 1). We have therefore compared our *S. latifolia* ZPT-type zinc finger proteins with those of *Petunia* that have been well defined and characterised in terms of their expression and DNA-binding properties (Takatsuji and Matsumoto 1996; Kobayashi et al. 1998; Kubo et al. 1998).

Alignment of the zinc-finger domains of the six *S. latifolia* $Cys_2/His_2$ zinc-finger proteins against typical A-type and B-type zinc-fingers from the *Petunia* protein ZPT2-1 reveals a surprising finding. Five of the six proteins contain A-type zinc-fingers, but SlZPT3-2, a three-fingered protein does not contain an A-type finger (Fig. 3a); it contains two M-type fingers and a B-type finger. This finding is in contrast to the observations in *Petunia* and *Arabidopsis* that were used to formulate the model for ZPT family zinc-finger structure (Kubo et al. 1998). The remaining five proteins, SlZPT2-1, SlZPT2-2, SlZPT2-3, SlZPT3-1 and SlZPT4-1 all contain an A-type zinc-finger. These sequences are shown in Fig. 3a, with the residues C, F QALGGH and H that define the ZPT family zinc-finger motif highlighted in white with a black background. These amino acid residues are conserved in five of the six *S. latifolia* ZPT proteins, with the exception of SlZPT2-1, in which the histidine at position +11 is replaced by a cysteine residue; other key residues involved in the definition of A-type fingers are highlighted with a grey background.

All six of the characterised *S. latifolia* ZPT proteins contain a predicted B-type zinc-finger domain. The amino acid sequence of these domains is shown in Fig. 3a, again with the key C, F QALGGH and H residues that define the $Cys_2/His_2$ zinc-finger highlighted in white with a black background, and other key residues that define B-type zinc-fingers highlighted with a grey background. As predicted by the classification system of Kubo (Kubo et al. 1998) the B-type finger is the most C-terminally located in the two-, three- and four-fingered proteins. The alignment of M-type zinc-finger domains

**Table 1** Database accessions showing homology to ZST proteins. Similarities between *Silene latifolia* ZST proteins and database accessions are indicated. The top three scores for each ZST are shown, revealing similarities to proteins from *Medicago sativa*, *Arabidopsis thaliana* and *Petunia* × *hybrida*

| ZST protein | Related protein | Organism | E value | % Identity | % Similarity | No. of residues | Accession No. |
|---|---|---|---|---|---|---|---|
| ZST2-1 | $Cys_2/His_2$-type ZF | *M. sativa* | 3e-12 | 38 | 50 | 123 | Y18788 |
| | ZAT11 | *A. thaliana* | 4e-11 | 43 | 59 | 91 | X98672 |
| | ZAT1 | *A. thaliana* | 4e-11 | 45 | 56 | 86 | X98669 |
| ZST2-2 | ZPT2-11 | *P.* × *hybrida* | 6e-34 | 38 | 45 | 252 | AB006598 |
| | ZAT5 | *A. thaliana* | 9e-32 | 39 | 48 | 256 | X98678 |
| | ZPT2-1 | *P.* × *hybrida* | 8e-31 | 36 | 43 | 276 | X60700 |
| ZST2-3 | ZPT2-1 | *P.* × *hybrida* | 1e-20 | 32 | 36 | 240 | X60700 |
| | ZAT5 | *A. thaliana* | 2e-18 | 343 | 45 | 185 | X98676 |
| | ZPT2-11 | *P.* × *hybrida* | 2e-17 | 38 | 43 | 142 | AB006598 |
| ZST3-1 | ZPT3-3 | *P.* × *hybrida* | 8e-56 | 42 | 56 | 331 | AB006505 |
| | $Cys_2/His_2$-type ZF | *A. thaliana* | 5e-53 | 42 | 57 | 295 | AL138646 |
| | $Cys_2/His_2$-type ZF | *A. thaliana* | 5e-45 | 38 | 51 | 312 | AC007659 |
| ZST3-2 | ZPT3-3 | *P.* × *hybrida* | 2e-41 | 34 | 48 | 344 | AB006605 |
| | $Cys_2/His_2$-type ZF | *A. thaliana* | 6e-39 | 32 | 49 | 349 | AL138646 |
| | $Cys_2/His_2$-type ZF | *A. thaliana* | 9e-38 | 34 | 50 | 345 | AC007659 |
| ZST4-1 | ZPT4-1 | *P.* × *hybrida* | e-109 | 46 | 55 | 546 | AB000455 |
| | ZPT3-1 | *P.* × *hybrida* | 7e-50 | 30 | 41 | 536 | AB000453 |
| | ZPT4-4 | *P.* × *hybrida* | 2e-18 | 23 | 31 | 462 | AB006606 |

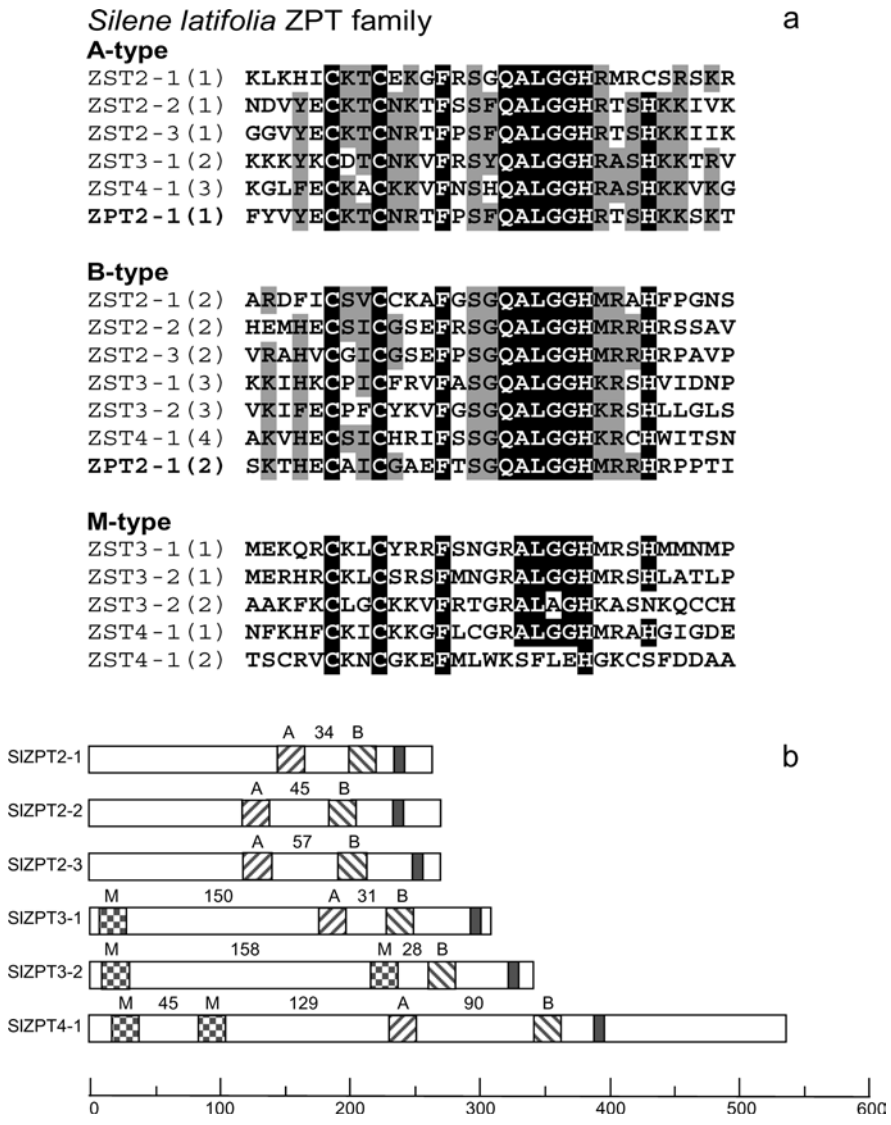


**Fig. 3** **a** Alignment of *S. latifolia* ZPT-type zinc-finger motifs. A- and B-type zinc-fingers are aligned separately. Modified type (*M-type*) represents the zinc-fingers in which some amino acids in the QALGGH sequence were replaced. Consensus amino acids of the ZPT-type zinc-fingers are indicated by *black boxes*, and the amino acids characteristic of the A- and B-type zinc-fingers by *grey boxes*. *Numbers in parenthesis* after protein names represent the position of zinc-fingers in the respective protein, the most N-terminal being *1*. The groupings and classification of finger types are according to Kubo et al. (1998). **b** Representation of the deduced protein sequences for the *S. latifolia* ZPT family. The zinc-finger motifs are represented by *chequered and hatched boxes* and the type (A, B or M) indicated above the proteins. The hydrophobic regions (including the DLNL box) are shown as *solid gray boxes*

is also presented in Fig. 3a, with residues common to A-type and B-type zinc-fingers highlighted with a black background.

Figure 3b shows the predicted configuration of the six *S. latifolia* ZPT proteins. In addition to the zinc-finger motif the presence of a hydrophobic region, including a highly conserved DLNL motif, the DLNL box (Kubo et al. 1998), was identified towards the C-terminal portions of the *S. latifolia* ZPT proteins described here. The DLNL motif is found in five of the six *S. latifolia* proteins, the exception being SlZPT2-1, which contained a hydrophobic region at the C-terminus of the protein but lacked the DLNL sequence. The DLNL motif has been shown to be part of the ERF-associated amphiphilic repressor (EAR) motif found in a number of ERF transcription-factor proteins as well as several ZPT transcription factors (Ohta et al. 2001). As observed with ZPT-type zinc-finger proteins from *Arabidopsis* and *Petunia* (Tague and Goodman 1995; Tague et al. 1996; Kubo et al. 1998), the spacing between individual zinc-finger motifs and the overall size of individual proteins are also variable in the *S. latifolia* ZPT proteins. The paired arrangements of A-type and B-type zinc-fingers, and the presence of additional M-type fingers, are shown in Fig. 3b, which also highlights the unexpected pairing of an M-type finger with a B-type finger in SlZPT3-2.

Comparison of the predicted protein sequences of the six *S. latifolia* ZPT proteins with available databases revealed some strong sequence similarities, particularly to the *P. hybrida* ZPT family of zinc-finger proteins and the $Cys_2$-$His_2$ proteins from *Arabidopsis* (Table 1). SlZPT4-1 was most similar to PhZPT4-1 over each of the four zinc-finger motifs, showing 100% sequence identity over the A-type zinc-finger motif. PhZPT4-1 is expressed in *P. hybrida* stamens during late anther development (Kobayashi et al. 1998). The A-type zinc-finger of SlZPT3-1 is equally similar to the A-type zinc-fingers of PhZPT2-10 and PhZPT3-3 from *Petunia*, and this similarity is also maintained over the B-type zinc-fingers.

The unexpected pairing of an M-type zinc-finger with the B-type zinc-finger in SlZPT3-2 prompted us to fur-

ther analyse the different types of zinc-finger motif in the six ZPT proteins from *S. latifolia*. A phylogenetic tree (Fig. 4) was produced using the individual zinc-finger domains of the six predicted *S. latifolia* ZPT proteins (underlined) and the zinc-finger motifs from the *P. hybrida* ZPT proteins (Kubo et al. 1998; Takatsuji 1998). The predicted A-type, B-type and M-type zinc-fingers cluster into the three clades as predicted by comparison to *Petunia* ZPT-type zinc-finger proteins. The only exception to the predicted clustering is the second M-type zinc-finger motif SlZPT3-2 (2) (Fig. 4, shown in bold and underlined), which clusters with the A-type zinc-fingers. The N-terminal M-type zinc-finger in this protein SlZPT3-2 (1) clusters as expected within the M-type zinc-finger clade and the B-type zinc-finger motif SlZPT3-2 (3) clusters as expected in the B-type clade. The zinc-finger motif SlZPT3-2 (2) does not contain the conserved QALGGH motif (Fig. 3a) and for this reason was classified as an M-type finger. However, this motif is paired with the B-type zinc-finger SlZPT3-2 (3) in SlZPT3-2 and also contains conserved secondary A-type amino acid residues outside the core QALGGH motif, features that are normally observed in A-type fingers, and may explain its clustering in the A-type zinc-finger clade.

## Genomic organisation of *S. latifolia ZPT* genes

In order to investigate the organisation of the genes encoding the six ZPT proteins within the *S. latifolia* genome we carried out Southern analysis using genomic DNA from male and female plants digested with *Eco* RI, *Hin* dIII and *Xba* I and the six cDNA sequences as probes. Any genes located on the male-determining Y chromosome would be expected to show unique and distinct profiles between male and female plants. Any autosomal or X-linked genes would give rise to similar hybridisation profiles between male and female plants. These data are presented in Fig. 5. Hybridisation patterns obtained from *SlZPT2-1*, *SlZPT3-2* and *SlZPT4-1* show hybridisation predominantly to single genomic DNA fragments, observations that are consistent with the presence of single-copy genes. Hybridisation patterns obtained for *SlZPT2-2*, *SlZPT2-3* and *SlZPT3-1* are more complex possibly due to the presence of restriction-enzyme cleavage sites within the gene sequence. The significant finding from these analyses is the absence of any suggestion that any of the six *ZPT* genes are located on the Y chromosome. Subtle differences between male and female hybridisation profiles are consistent with what would be expected for restriction fragment length polymorphism between individuals in an obligate outbreeding population, such as that used in this genomic analysis.

The presence of all six genes in both male and female genomes and the implication from studies in *Petunia* for a role in stamen development prompted us to investigate the expression dynamics of these members of the gene family during flower development.

## Expression analysis of *S. latifolia ZPT* genes

Preliminary Northern blot analysis of the expression of the six *S. latifolia ZPT* genes revealed that they were expressed at extremely low levels (data not shown), the expression levels were also below the level of detection by in situ hybridisation (data not shown). We therefore used Southern analysis of RT–PCR products to quantify the expression dynamics of these six genes. RNA was isolated from male and female flower buds at five stages of development, as described by Scutt et al. (1997). RNA was also extracted from flower buds at the equivalent five developmental stages from female plants infected with *Ustilago violacea* as well as from flower buds from *ssf1*, the new short stamen filament mutant. Aliquots were removed from the PCR reactions following 15, 20, 25 and 30 cycles of amplification, prior to transfer to nitrocellulose filters, in order to perform a semi-quantitative analysis of the expression of *ZPT* genes. Hybridisation was performed with probes specific to each of the *S. latifolia ZPT* cDNA clones.

All six *SlZPT* genes are expressed throughout male flower development with *SlZPT2-1*, *SlZPT3-1* and *SlZPT3-2* showing elevated transcript levels in the earliest stages of development (Fig. 6). In contrast, *SlZPT4-1* expression was not detected during stages 1 and 2 of male flower development but showed maximal expression at stage 5 (Fig. 6). *SlZPT2-3* shows expression throughout male flower development with a slight increase in expression detected at stage 5. *SlZPT2-2* exhibits constitutive expression, at a constant low level, through all developmental stages of male flower bud development analysed (Fig. 6). The expression dynamics of these ZPT-type zinc-finger genes reveal dynamic changes in expression during androecium development in male *S. latifolia* flowers.

Expression of five of the six *ZPT* genes was detected during female flower development; the exception was *SlZPT4-1* for which no expression was detected at any stage of development (Fig. 6). The only significant changes in expression observed during female flower development were for *SlZPT2-1*, *SlZPT3-1* and *SlZPT3-2*, the three genes that showed the highest levels of expression during stage 1 of male flower bud development (Fig. 6), which showed elevated levels of expression during stage 4 of female flower development (Fig. 6); the anthers in stage-4 female flowers are developmentally equivalent to those of much earlier male flower buds at stage 1 (Scutt et al. 1997). The expression dynamics of *SlZPT2-1*, *SlZPT3-1* and *SlZPT3-2* correlate with the developmental stage of stamens in stage-1 male flowers and stage-4 female flowers, and suggest their involvement in aspects of development occurring during the initial stages of androecium morphogenesis.

Analysis of *ZPT* transcript abundance in female *S. latifolia* flowers infected with the smut fungus *U. violacea* reveals expression of all the *SlZPT* genes, with the exception of *SlZPT4-1*, throughout flower

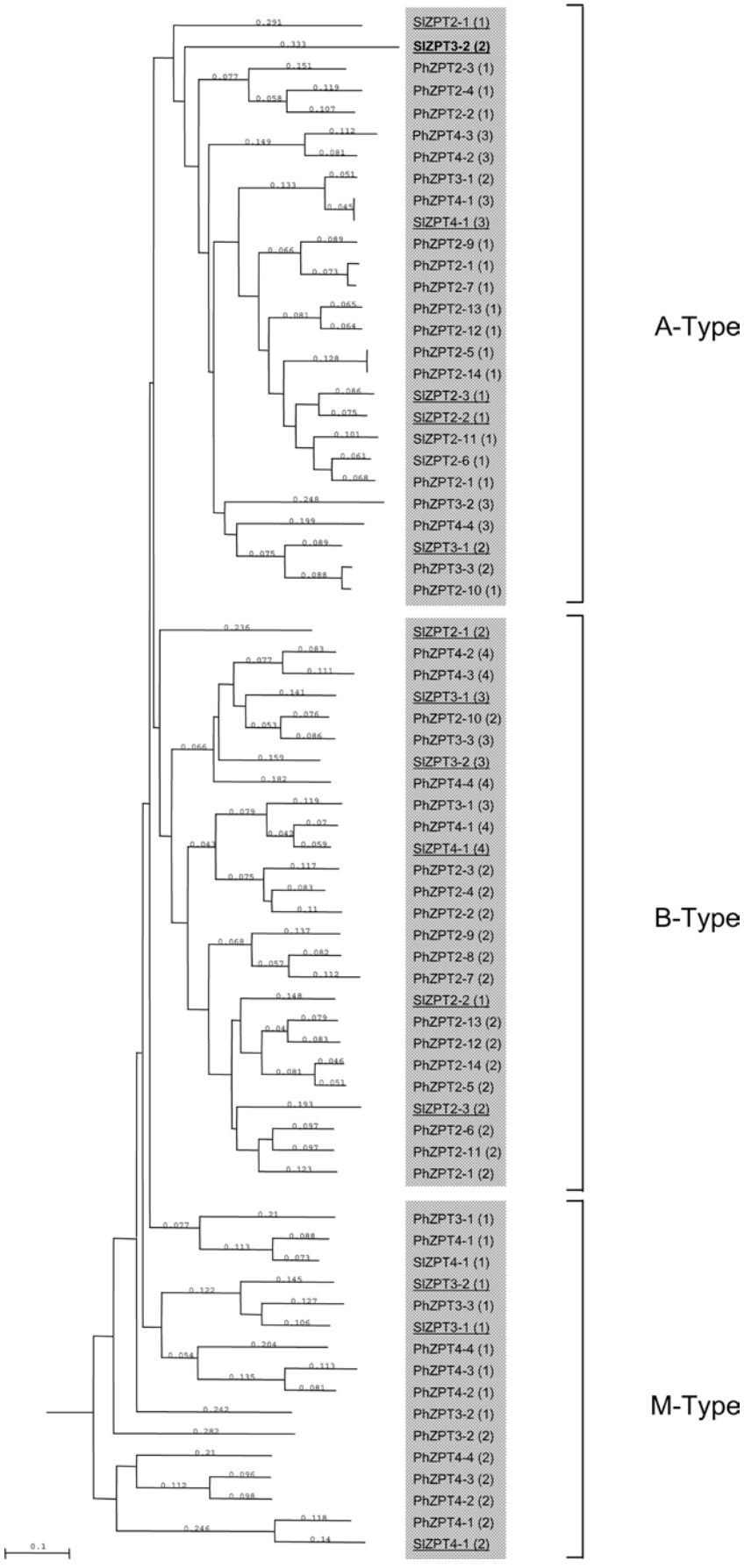


**Fig. 4** Phylogram of the *Silene latifolia* and *Petunia* × *hybrida* ZPT-type zinc-finger motifs. Multiple alignments of the zinc-finger motifs were generated using the ClustalW algorithm. Alignments were performed using the Blosum scoring matrix and default alignment parameters. The phylogenetic tree was constructed in the besttree mode, using the neighbour-joining tree-building method. The second zinc-finger motif of SlZPT3-2 is in *bold and underlined*, whilst all other *S. latifolia* zinc-finger motifs are *underlined*. *SlZPT*, *S. latifolia* Zinc-finger Protein of TFIIIA type; *PhZPT*, *Petunia* × *hybrida* Zinc-finger Protein of TFIIIA type. Numbers of tree branches represent the number of zinc-finger motifs followed by a specific identifier for each protein sequence, for example SlZPT2-1 represents the first two-fingered ZPT-type protein identified in *S. latifolia*

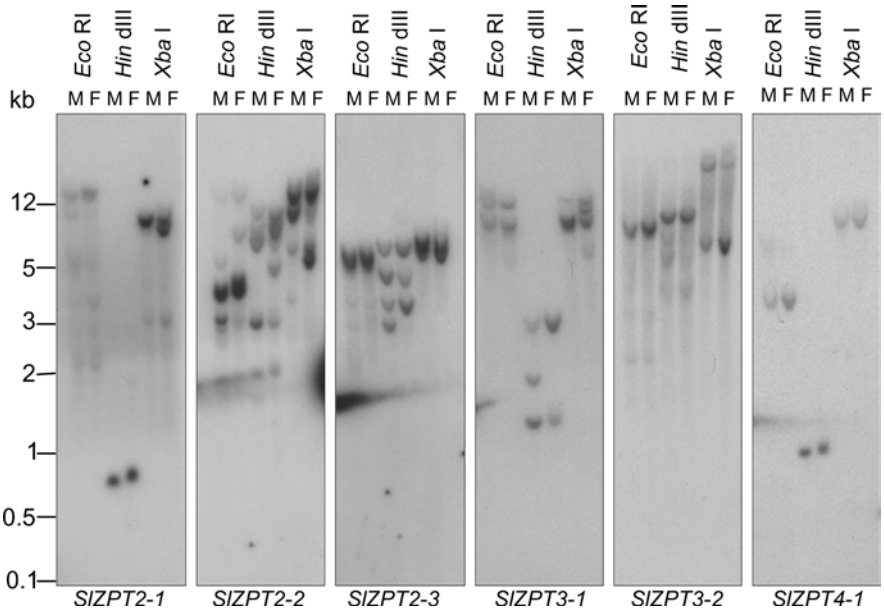


**Fig. 5** Southern analysis of the *S. latifolia ZPT* genes. Radiolabelled *S. latifolia ZPT* cDNA probes were hybridised to Southern blots containing 15-mg aliquots of male (*M*) and female (*F*) genomic DNA, digested with one of the three restriction enzymes, as indicated. Following high-stringency washing, the membrane was exposed to X-ray film for 3 days

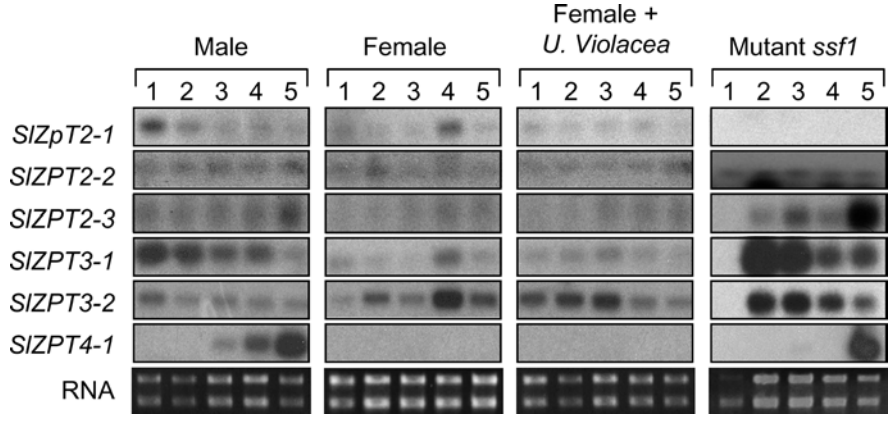


**Fig. 6** RT–PCR analysis of *S. latifolia ZPT* genes during stages 1–5 of flower bud development in female, smutted-female, and male plants and in an EMS-generated male mutant plant defective in filament elongation. Primers were designed to specifically amplify regions of the cDNA corresponding to each of the six *S. latifolia* ZPT sequences. The hybridisations shown represent 25 cycles of amplification for all *ZPT* genes, except *SlZPT3-1*, which is 20 cycles. An RNA gel is also included to show the relative amounts of RNA used in the RT–PCR reactions

development (Fig. 6). In contrast to normal female flower development there was no peak of expression for *SlZPT2-1*, *SlZPT3-1* and *SlZPT3-2* at stage 4 (Fig. 6). However, *SlZPT3-2* expression was elevated during stages 1, 2 and 3 of development in flowers infected with *U. violacea* (Fig. 6), whereas the high levels of expression of *SlZPT3-1* seen in early male flowers was not observed in female flowers that developed anthers in response to *U. violacea*. The RNA loading controls show consistent and equivalent signals for all five developmental stages (Figure 6). The lack of *SlZPT4-1* expression at any stage during development of *U. violacea*-infected female flowers is particularly significant in relation to third-whorl development in these smut-infected female flowers. The stamens that develop in smut-infected female flowers are normal in appearance but fail to produce a tapetum or pollen, and anther dehiscence releases fungal spores rather than pollen grains (Antonvics and Alexander 1992). This phenomenon suggests that either the smut fungus triggers the activation of all genes required for male fertility but the process of infection impairs the ability to produce viable pollen, or that two independent pathways exist for androecium development in *S. latifolia*, one for stamen development and one for fertility. The lack of *SlZPT4-1* transcripts at any stage of development in the smut-infected female flowers suggests that SlZPT4-1 is required for some aspect of stamen development related to fertility.

Expression analysis of the six *SlZPT* genes in the *ssf1* mutant that fails to undergo stamen filament development reveals differences in gene expression as compared to wild-type male flower buds. The most significant difference is the total absence of detectable transcripts for *SlZPT2-1*. This gene is not expressed at any stage of flower development in the *ssf1* mutant (Fig. 6).

*SlZPT2-2* is expressed at a constant and low level from stage 1 through stage 5 (Fig. 6) and, as in previous analyses, shows no developmental regulation of expression. Expression of *SlZPT2-3*, *SlZPT3-1* and *SlZPT3-2* during stage-1 flower development appears to be reduced as compared to wild-type male flowers (Fig. 6). Significantly, however, the trend towards maximal expression at stage 5 in wild-type male flowers for *SlZPT2-3* and *SlZPT4-1* is mirrored in *ssf1* (Fig. 6). Similarly, the decrease in expression of *SlZPT3-1* and *SlZPT3-2* during progression of development seen in wild-type male flowers is maintained in *ssf1* flowers (Fig. 6). The higher specific activity of the probe used in these blots resulted in increased signals as compared to analyses of male, female and smut-infected female samples.

In combination, these assays reveal differences in *SlZPT* gene expression during development of male and female flowers, as well as during development of *U. violacea*-infected female flowers and flowers of the new mutant phenotype *ssf1*, and implicate a regulatory role for this family of zinc-finger transcription factors in the coordination of stamen development in wild-type male flowers.

## Discussion

Here we report the identification and characterisation of a novel *S. latifolia* mutant, *ssf1* from a population of plants derived from EMS-mutagenised seed. Previous studies to identify mutations affecting flower development in *S. latifolia* have employed X-ray irradiation of seed (Grant et al. 1994a; Donnison et al. 1996) or gamma ray irradiation of pollen (Farbos et al. 1999; Lardon et al. 1999). We selected EMS as the mutagen to create small genetic lesions rather than induce deletions or chromosome breaks. A number of flower development mutants were identified, including hermaphrodites and male-sterile mutants, including *ssf1* described here; analysis and detailed descriptions of these other mutant phenotypes will be presented elsewhere. The mutant population also included variegated and narrow-leaved plants, reminiscent of the observations of Winge (1931) who demonstrated that the *variegated leaves* gene was located on the Y chromosome and the narrow-leaved *angustifolia* mutant inherited as an X chromosome locus. By screening individuals from the $M_0$ population we anticipated that we would identify either dominant mutant phenotypes, or recessive phenotypes in male plants caused by mutations in genes located on either the X or Y chromosome; however, the genetic basis of the *ssf1* phenotype is not yet known.

Through the identification and characterisation of the *ZPT* gene family in *S. latifolia*, insight into their potential involvement in androecium development has been gained through the comparative analysis of male, female, smut-infected female and *ssf1* mutant flowers. Five of the six proteins encoded by the six *S. latifolia* *ZPT* genes described here contain DLNL motifs characteristic of the EAR repressor domain (Ohta et al. 2001), suggesting that they could act as transcriptional repressors. *SlZPT4-1* is expressed in late-stage male flowers, but is not expressed at any stage of female flower development.

The absence of expression of this gene in smut-infected female flowers suggests a role for this gene in an aspect of male flower development that is not under the influence of *U. violacea*. Although the fungus can promote stamen development in female flowers in the absence of a Y chromosome, the stamens produced do not contain a tapetum, do not produce pollen, and contain only fungal spores (Audran and Batcho 1981). The lack of *SlZPT4-1* expression in smut-infected female plants (Fig. 6) would therefore suggest that *SlZPT4-1* plays a role in aspects of androecium development relating to male fertility. Indeed, *SlZPT4-1* expression is detected first in stage-3 male flowers, when the external (antisepalous, W3.1) anthers produce free microspores and the tapetum starts to degenerate. At this stage the internal (anti-petalous, W3.2) whorl exists as tetrads (Farbos et al. 1997). By stage 4, *SlZPT4-1* expression increases and is maximal in stage-5 male flowers. These stages are characterised by tetrad formation and microspore maturation.

The *P. hybrida* ZPT protein most closely related to SlZPT4-1 is PhZPT4-1. These proteins share 46% sequence identity over their entire lengths. *ZPT4-1* is expressed in later-stage *Petunia* anthers (stages 6–10) at the binucleate stage (Kobayashi et al. 1998). This mirrors the expression pattern seen for *SlZPT4-1*. The timing of *SlZPT4-1* expression in *S. latifolia* in relation to the developmental events occurring in the anther of the male plants, together with the lack of *SlZPT4-1* expression in smut-infected female plants, strongly implicates *SlZPT4-1* in an aspect of male fertility relating to pollen development. The similarity between *P. hybrida ZPT4-1* and *S. latifolia SlZPT4-1* suggests that the two genes may be performing similar functions in these two different plants. Genetic evidence provided by Westergaard (1958), and more recently by Lardon et al. (1999) and Farbos et al. (1999), indicates that a region of the q-arm of the Y chromosome is necessary for male fertility. However, Southern analysis demonstrates that *SlZPT4-1* is not a Y-chromosome-encoded sequence. This observation would imply that *SlZPT4-1* is expressed in response to a Y-chromosome-mediated signal required for normal fertility in wild-type male flowers, a signal that is not replicated by *U. violacea* infection of female flowers.

The patterns of expression of *SlZPT2-2*, *SlZPT2-3*, *SlZPT3-1* and *SlZPT3-2* in male, female and smut-infected female flowers are not significantly different, suggesting that these genes are involved in processes common to all these types of flower. This could indicate either that they are not involved in the control of sexual differentiation or, if as in *Petunia* members of this gene family are involved in a developmental

cascade modulating stamen development, that they function during the stages of stamen primordia development common to male and female flowers. The same conclusion could be drawn for *SlZPT2-1*, were it not for the fact that in the *ssf1* mutant the gene encoding this protein is not expressed. This finding is particularly important as it suggests that *SlZPT2-1* expression is required for some aspect of development that is perturbed in the mutant. SlZPT2-1 is the only one of the proteins encoded by the six *S. latifolia* ZPT genes characterised here that does not contain the DLNL motif characteristic of the EAR repressor domain (Ohta et al. 2001). *SlZPT2-1* is maximally expressed in early stage-1 and -2 male flower buds, which corresponds to aspects of male flower development preceding the rapid elongation of filaments seen in later stage-4 and -5 flower buds.

SEM analysis of a mature *ssf1* flower (Fig. 1i,j) indicates that the filaments have not developed significantly from stage 3, whilst other aspects of floral development, such as sepal and petal development, appear to have continued similarly to wild-type male flowers. Given the complete absence of expression of *SlZPT2-1* in *ssf1* it is possible that this function could relate to filament development. The observation that *SlZPT2-1* is not encoded on either sex chromosome (Fig. 5) suggests that the lack of *SlZPT2-1* expression is a downstream consequence of the *ssf1* mutation. In *Petunia*, two of the characterised *PhZPT* genes, *PhZPT2-10* and *ZPT3-3*, are expressed in the pistil (Kubo et al. 2000). The most similar gene from *S. latifolia* is *PhZPT3-1*, which encodes a three-finger protein; all three zinc-fingers show greatest similarity to those of PhZPT3-3, with strong similarity also to the zinc-fingers of PhZPT2-10. *PhZPT3-3* and *PhZPT2-10* are specifically expressed in the pistil of *Petunia* (Kubo et al. 2000) and it has been proposed that *PhZPT2* evolved following duplication of *PhZPT3-3* and subsequent loss of the first zinc-finger (Kubo et al. 1998). Despite the similarity among the proteins encoded by *SlZPT3-1*, *PhZPT3-3* and *PhZPT2-10*, there does not seem to be any conservation in the expression between *SlZPT3-1* and the related *Petunia* genes as *SlZPT3-1* is most highly expressed in early male flower buds and shows only low levels of expression in female flowers.

The identification of *ssf1* has provided a valuable tool, which, together with male, female and smut-infected female plants, has enabled us to characterise the expression dynamics of the ZPT-type family in *S. latifolia* and equate differential expression patterns with specific aspects of dioecious flower development. This mutant also represents a novel phenotype that could provide further insight into the control mechanisms underlying stamen development in *S. latifolia*. A number of male-sterile mutants have been well characterised in *Arabidopsis*, revealing aspects of the regulation of stamen development in this hermaphrodite. Although it is premature to speculate on the basis of the *ssf1* mutant and the specific role that the *ssf1* gene plays in stamen development in *S. latifolia*, experiments are underway to determine whether any of the regulatory cascades involved in stamen development in *Arabidopsis* are perturbed in *ssf1*.

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