Premature arrest of the male flower meristem precedes sexual dimorphism in the dioecious plant *Silene latifolia*

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Most dioecious plant species are believed to derive from hermaphrodite ancestors. The regulatory pathways that have been modified during evolution of the hermaphrodite ancestors and led to the emergence of dioecious species still remain unknown. *Silene latifolia* is a dioecious plant species harboring XY sex chromosomes. To identify the molecular mechanisms involved in female organ suppression in male flowers of *S. latifolia*, we looked for genes potentially involved in the establishment of floral organ and whorl boundaries. We identified homologs of *Arabidopsis thaliana SHOOTMERISTEMLESS* (*STM*) and *CUP SHAPED COTYLEDON* (*CUC*) *1* and *CUC2* genes in *S. latifolia*. Our phylogenetic analyses suggest that we identified true orthologs for both types of genes. Detailed expression analyses showed a conserved expression pattern for these genes between *S. latifolia* and *A. thaliana*, suggesting a conserved function of the corresponding proteins. Comparative *in situ* hybridization experiments between male, female, and hermaphrodite individuals reveal that these genes show a male-specific pattern of expression before any morphological difference become apparent. Our results make *SlSTM* and *SlCUC* strong candidates for being involved in sex determination in *S. latifolia*.

CUC gene | sex determination | *STM* gene

Most Angiosperm species are hermaphrodite and develop bisexual flowers. These include model species such as *Arabidopsis thaliana*, *Antirrhinum majus*, and *Petunia hybrida*, species from which genes involved in different steps of flower development have been identified. An extensive list of genes are known to be involved in processes such as floral meristem identity, floral organ identity, establishment of organ and whorl boundaries, organ polarity, and flower symmetry (see ref. 1 for review).

Dioecious plant species (with separate male and female individuals) represent only a few percent of the angiosperm species but are widely scattered taxonomically. A large proportion of angiosperm families have dioecious members. It is therefore likely that dioecy evolved several times independently in different plant lineages (2, 3). Because most of the dioecious species develop potentially hermaphrodite floral meristems, which subsequently differentiate into male or female flowers, it is assumed that each occurrence of dioecy must have evolved from a hermaphrodite ancestor (2, 4–6). Additionally, in each dioecious species, the sexual dimorphism results from distinct regulatory modifications of the bisexual condition. The molecular mechanisms underlying dioecy, and therefore sex determination in plants, are largely unknown.

In the dioecious species *Silene latifolia*, as in any hermaphrodite species, four whorls of floral organs are observed in both male and female floral meristems: sepals, petals, stamens (male reproductive organs), and carpels (female reproductive organs). At an early stage, when sepal primordia are visible (stage 3; all stages are according to ref. 7), the flower meristem is similar in male and female plants (undifferentiated). As soon as all floral organ primordia are initiated (stage 5), the female territory in the center of the flower meristem is significantly smaller in male compared with female flower buds (Fig. 1). Later, in male flowers, a filament will develop in place of female organs (see Fig. 1). In female flower buds, stamens are initiated but rapidly degenerate, whereas five fused carpels (female organs) develop in the center (7).

In *S. latifolia*, the sexual phenotype is controlled by X and Y sex chromosomes. Two independent loci in the Y chromosome are responsible for sex determination (8, 9). The first locus is responsible for the early arrest of female organs in male flowers (visible at stage 5), and when it is mutated or deleted, hermaphrodite flowers develop (9). The second locus activates the development of male organs in male flowers (Fig. 1). However, the corresponding genes and the regulatory pathways involved in sex determination have yet to be identified. Because the sexual dimorphism is expressed very early during flower organogenesis, reproductive organ identity genes (the B and C functions of the ABC model; see ref. 10 for review) have been believed to play a key role in this process (11). Hardenack *et al.* (11) showed that in both male and female flower buds, these floral organ identity genes have the same expression pattern as that in hermaphrodite species. The authors concluded that sex determination genes must act either downstream from these organ identity genes or in a parallel pathway.

In this study, we investigated the possible mechanisms that may lead to female organ arrest in male flowers of *S. latifolia.* Based on data of Fig. 1 (compare the center of the flower meristem in male and female at stage 5), it is clear that there is a whorl-specific arrest in cell proliferation in the early male flower meristem. This reduced proliferation results in the formation of a filament in the center of male flowers at a later stage (Fig. 1). From these observations, we suspected a precocious arrest of the flower meristem in male individuals. We therefore decided to look for the orthologs in *S. latifolia* of two genes central to meristem function in *A. thaliana*: *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*). We also looked for *CUP SHAPED COTYLEDON* (*CUC*) *1* and *CUC2*, which have been shown to participate in meristem homeostasis in concert with *STM* (12). Despite several attempts, we could not identify any *WUS* ortholog in *S. latifolia*. However, our phylogenetic recon-

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The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AM404290 (*SlSTM1*), AM404291 (*SlSTM2*), and AM404292 (*SlCUC*)].

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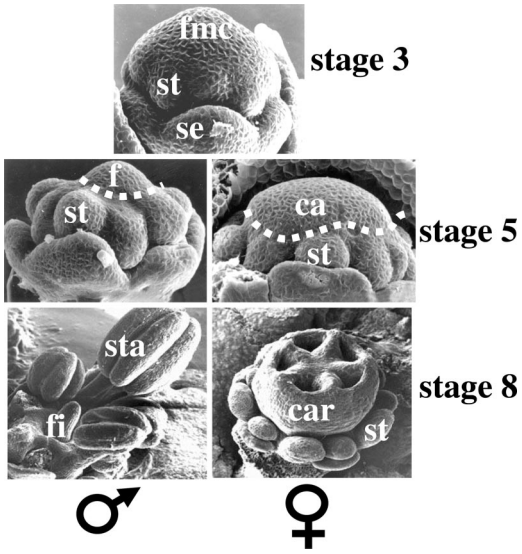

Fig. 1. Sexual dimorphism in *S. latifolia*. Shown are flower meristems observed by scanning electron microscopy. Developmental stages are as defined by Farbos *et al.* (7). At stage 3, two types of primordia are visible: sepal and stamen (petal primordia are initiated later and are not yet visible). The center of the flower meristem has not yet initiated carpel primordia. No difference is observed between flower meristem from male or female plant. At stage 5, all of the primordia are initiated. In flower meristem from female plants, carpel primordia appear as a wide dome. In flower meristem from male plants, the central dome is five times smaller than in the females and corresponds to the filament primordia. At stage 8, the difference between males and females is very clear. The five fused carpels in the center of the female flower are well developed, whereas the center of the male flower exhibits an undifferentiated filament. se, sepal primordia; st, stamen primordia; fmc, flower meristem center; ca, carpel primordia; f, filament primordia; sta, stamen; fi, filament; car, carpel.

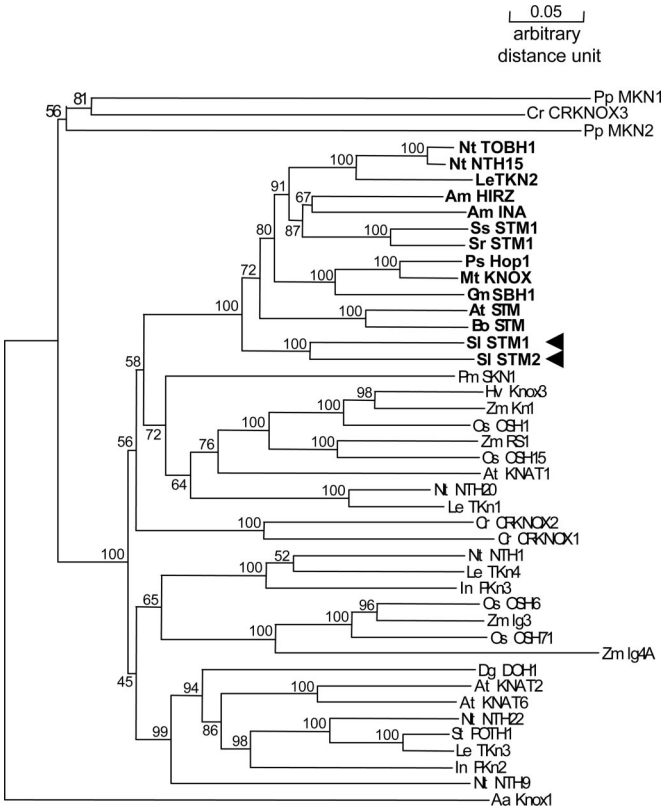

Fig. 2. Phylogenetic tree of *KNOX* gene cDNA sequences. STM-like genes from dicotyls are highlighted in bold, and *SlSTM1* and *SlSTM2* are indicated with arrowheads. *AaKnox1*, *Pp MKN1* and *PpMKN2*, and *CrCRKNOX3* were included as outgroups. The numbers beside the branches represent bootstrap values based on 500 replicates.

structions suggest that we have indeed identified orthologs of *A. thaliana STM* and *CUC1* and *CUC2* in *S. latifolia*. We performed *in situ* hybridization on young flower buds from male, female, and hermaphrodite plants. Both orthologs showed clear differences in their expression pattern between males and females or hermaphrodites, which suggests their possible involvement in the sex determination pathway in *S. latifolia*.

Results

Identification of *STM* and *CUC1 and CUC2* Orthologs. Degenerate primers (see *Materials and Methods*) used to amplify orthologs of *STM* were designed against the region spanning the MEINOX domain and the homeodomain, both of which are highly conserved among all of the *STM* orthologs examined to date in different species. We performed RT-PCR on *S. latifolia* total RNA extracted from shoot apices of germinating seedlings. Among the clones analyzed, we found two 900-bp-long *STM*-like cDNAs. Alignment with the available *KNOX* gene sequences already used by Harrison *et al.* (13) showed that both sequences contain all typical features of class I *KNOX* genes, including regions encoding the MEINOX domain, the ELK domain, and the homeodomain. The alignment was used to construct a phylogenetic tree (Fig. 2). Both cDNA sequences group with the *STM* clade (class I *KNOX* genes), and the branch is supported by a strong bootstrap value, which suggests that we indeed cloned two *STM* orthologs. We called these *SlSTM1* and *SlSTM2*.

Primers used to amplify orthologs of *CUC1* and *CUC2* were designed by using the CODEHOP method (14) against the region spanning the conserved NAC domain and the miR164 binding site. Among the amplified sequences potentially encoding NAC domain, we identified a 500-bp-long *CUC*-like sequence that we aligned with NAC genes, including *CUC* orthologs, to construct a phylogenetic tree (Fig. 3). The clone from *S. latifolia* groups with *NAM* (*NO APICAL MERISTEM*) from *P. hybrida*, with *CUP* (*CUPULIFORMIS*) from *Antirrhinum majus*, and with both *CUC1* and *CUC2* (but not *CUC3*), from *A. thaliana*. The branch is supported by a strong bootstrap value, suggesting that we indeed cloned a *CUC1* and *CUC2* ortholog, which we named *SlCUC*.

RT-PCR Analysis. The expression patterns of *SlSTM1*, *SlSTM2*, and *SlCUC* were initially analyzed by RT-PCR on different plant tissues from male and female individuals. Specific primers were designed for each gene, and transcript accumulation was normalized according to eIF4A transcript levels (15). Results are presented in Fig. 4. Both *SlSTM1* and *SlSTM2* are expressed in shoot apical meristems (meristems), young and medium flower buds from both males and females, petals from male flowers, both young and old gynoecium, and young anthers. Some differences were observed between *SlSTM1* and *SlSTM2*, because *SlSTM1* is also expressed in middle anthers and in stems from both males and females, whereas *SlSTM2* is expressed in roots. Apart from its expression in stems, *SlSTM1* shows an expression pattern very close to its *A. thaliana* ortholog *STM* (16).

SlCUC is expressed in shoot apical meristems and in flower buds from both males and females (Fig. 4). It is also expressed in the gynoecium and in young anther. This expression pattern is very similar to that of *CUC1* and *CUC2* from *A. thaliana* (17, 18).

***In Situ* Hybridization Analyses.** To investigate in more detail the expression pattern of *SlSTM1*, *SlSTM2*, and *SlCUC* and evaluate

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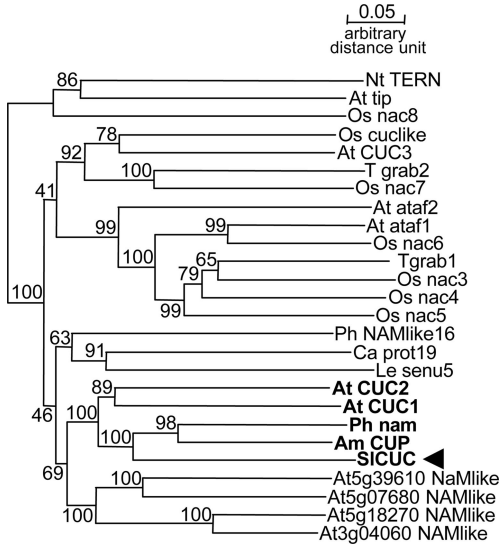

Fig. 3. Phylogenetic tree of NAC gene cDNA sequences. The members of the *CUC* clade are highlighted in bold, and the *S. latifolia* gene is indicated with an arrowhead. The numbers beside the branches represent bootstrap values based on 500 replicates.

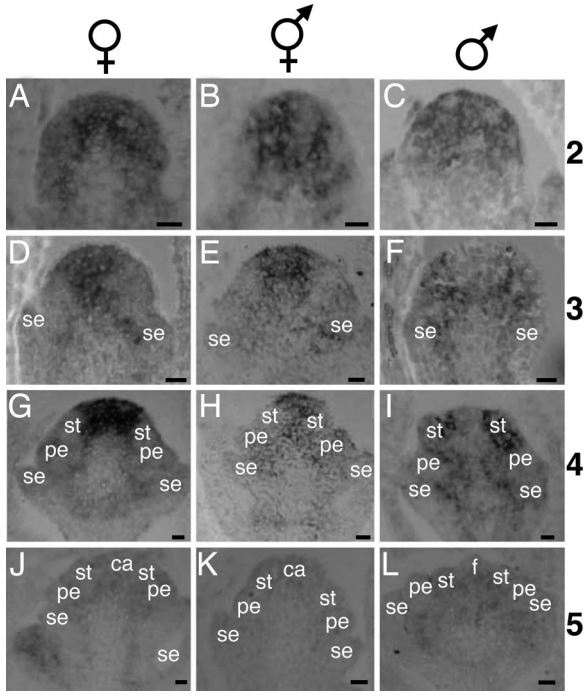

Fig. 5. *In situ* hybridization with a *SlSTM1* and *SlSTM2* probe. Sections of *S. latifolia* flower buds hybridized with a *SlSTM1* and *SlSTM2*-specific probe. The signal appears in gray or black. The sex of the individuals is indicated above each column, and the stages of development are indicated on the right of each row. se, sepal primordia; pe, petal primordia; st, stamen primordia; ca, carpel primordia; f, filament primordia. (Scale bars: 20 μm.)

their potential implication in sex determination, we performed *in situ* hybridization experiments on young flower meristems from male, female, and hermaphrodite (*bsx11* mutant) individuals. The *bsx11* hermaphrodite mutant lost the portion of the Y chromosome responsible for the arrest of carpel development (9) and therefore develops a functional gynoecium. We have included this $Y_{deleted}$ hermaphrodite mutant in the analysis to make sure that the differences observed between males and females were linked to the absence of carpels in males and not to pleiotropic, unrelated differences between males and females. Developmental stages of *S. latifolia* flower meristems correspond to those described by Farbos *et al.* (7). At stage 2, the flower meristem is round, and no primordia are yet visible. At stage 3, the sepal primordia are initiated. During stage 4, petal and stamen primordia are successively initiated, and at stage 5, all organ primordia are formed (Fig. 1). At stage 6, floral organs start to differentiate (7).

***SlSTM1* and *SlSTM2*.** We first performed *in situ* hybridization using probes specific for SlSTM1 or SlSTM2. The pattern of expression was similar for the two genes (data not shown). Because a stronger signal was observed with the 900-bp SlSTM1 cDNA probe that hybridized to both types of transcripts, this probe was used for further analyses. The pattern of accumulation of both *SlSTM1* and *SlSTM2* transcripts is shown in Fig. 5. A strong signal was detected at stage 2 (Fig. 5 *A–C*) in all of the cells of the male, female, and hermaphrodite flower meristems. In flower meristems from female and hermaphrodite, from stages 3 to 5 (Fig. 5 *D*, *E*, *G*, *H*, *J*, and *K*), we observed a progressive restriction of *SlSTM1* and *SlSTM2* transcripts toward the inner part of the flower meristem. No signal was detected in organ primordia. When carpel primordia are initiated at stage 5, *SlSTM1* and *SlSTM2* transcripts were no longer detected (Fig. 5 *J* and *K*). Strikingly, no transcripts were detected in the center of the male flower meristem from stage 3 onwards (Fig. 5 *F*, *I*, and *L*). This absence of signal was particularly evident at stages 3 (Fig. 5*F*) and 4 (Fig. 5*I*), whereas *SlSTM1* and *SlSTM2* transcripts were still present in regions where future primordia would develop. Depending on the plane of the sections, some signal could also be detected around the primordia and in vascular tissues (Fig. 5 *F*, *H*, and *I*).

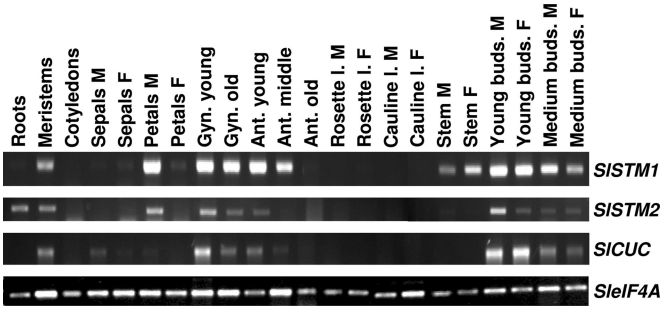

Fig. 4. RT-PCR analyses of *SlSTM1*, *SlSTM2*, and *SlCUC* in *S. latifolia* tissues. The expression patterns of *SlSTM1*, *SlSTM2*, and *SlCUC* were investigated by RT-PCR analysis on the tissues indicated above each lane. The genes amplified are indicated on the right. *SleIF4A* was used as an internal reference. PCR products were analyzed on agarose gels and visualized under UV light in the presence of ethidium bromide. M, male; F, female; Gyn, gynoecium; Ant, anther; l, leaf.

***SlCUC*.** The pattern of accumulation of the *SlCUC* transcripts is shown in Fig. 6. *SlCUC* was detected at the boundaries between whorls before each type of primordia became visible from stage 3 to stage 5. This sequence was observed in flower meristems of male, female, and hermaphrodite. An enlarged signal could be observed in some sections (Fig. 6 *B* and *G*) and probably corresponds to the future interprimordia boundary within the same whorl because of the plane of the section. Remarkably, at stage 3 and in male meristems only, *SlCUC* was detected in a wide region in the center of the meristem (Fig. 6*C*). This expression was transient; it was not observed at later stages. Serial sections of a male flower meristem at stage 3 are shown in Fig. 6*J* (sections 1–8 from the periphery toward the center).

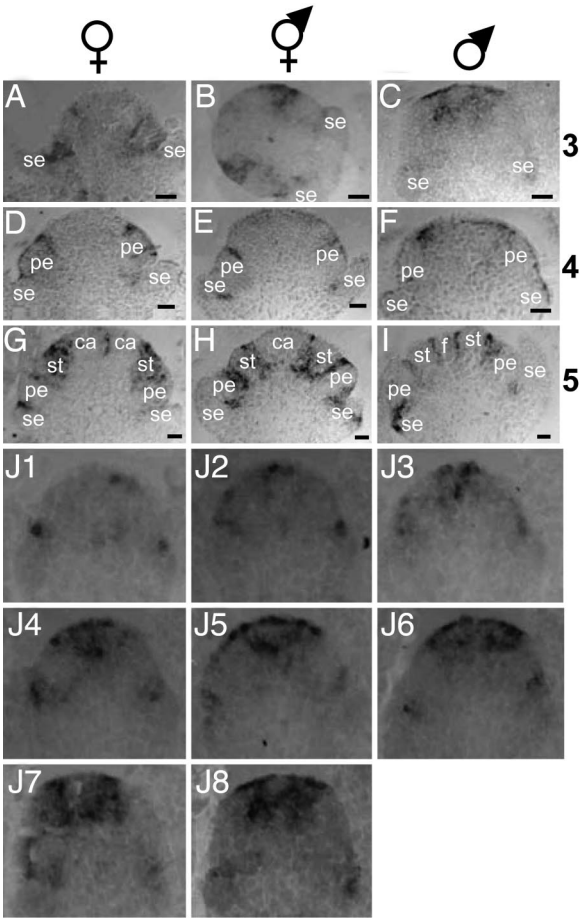

Fig. 6. *In situ* hybridization with *SlCUC* probe. Sections of *S. latifolia* flower buds hybridized with a *SlCUC*-specific probe. The signal appears in gray or black. The sex of the individuals is indicated above each column, and the stages of development are indicated on the right of each line. se, sepal primordia; pe, petal primordia; st, stamen primordia; ca, carpel primordia; f, filament. (Scale bars: 20 μm.) (*J*) Serial sections (J1–J8 from outside to inside) of a male flower meristem at stage 3.

Interestingly, the very inner zone of the region expressing *SlCUC* was devoid of signal and may correspond to the area where the filament will develop.

***SlSTM1*, *SlSTM2*, and *SlCUC* Are Not Sex-Linked.** To determine whether *SlSTM1* and *SlSTM2* and *SlCUC* were located on sex chromosomes (and more precisely on the Y chromosome), we performed Southern blot experiments using these genes as probes against genomic DNA from a segregating family of male and female individuals. We found no evidence of sex linkage for these genes (Fig. 7, which is published as supporting information on the PNAS web site).

Discussion

Sexual dimorphism in the dioecious species *S. latifolia* occurs very early during flower development. As soon as all of the floral primordia are initiated, the central zone of the floral meristem, which corresponds to the female territory, is five times smaller in males than in females, revealing a whorl-specific restriction of cell proliferation (7). Genes involved in meristem function are essential to determine the patterns of proliferation that lead to the correct position of organ primordia and boundaries (19, 20). Such genes have been extensively characterized in *A. thaliana*, and we identified the corresponding genes in *S. latifolia* to investigate their possible involvement in sex determination. In this study, we report the cloning of *STM* and *CUC1* and *CUC2* orthologs in the dioecious species *S. latifolia* and show that they are likely involved in the molecular mechanisms underlying sex determination in *S. latifolia*.

Conserved Roles of *STM* and *CUC* Orthologs in Eudicots. In *A. thaliana*, *STM* is required not only for the establishment of the shoot meristem but also for the maintenance of undifferentiated cells in the shoot meristem and for proper proliferation of cells in the floral meristem (16, 21–23). It is expressed in the shoot apical meristem, the inflorescence meristem, and the floral meristem. The transcript is also detected in vascular tissues and in boundaries between floral whorls (16). *STM* transcripts are down-regulated in incipient floral primordia (16). Our results show a similar pattern of expression of *SlSTM1* and *SlSTM2* in flower meristems of *S. latifolia*, although the expression pattern is not strictly identical between *SlSTM1* and *SlSTM2* (see RT-PCR results in Fig. 3). Similar patterns of expression have also been observed for *HIRZINA* (*HIRZ*) and *INVAGINATA* (*INA*), two *STM* orthologs in *Antirrhinum majus* (24). More recently, *STM* orthologs have been characterized in two Papaveraceae species, *Chelidonium majus* and *Eschscholzia californica*, and have been shown to be down-regulated in organ primordia, including floral primordia (25). It is therefore likely that in *S. latifolia* and *Antirrhinum majus* species, the two paralogs are at least partially redundant. The presence of two *STM* paralog genes has also been noted in the genus *Streptocarpus* (13). Our phylogenetic analyses suggest that the duplication events in *S. latifolia* and *Antirrhinum* species are recent and independent in the two lineages. There are not enough data to draw any conclusions on the origin of the duplication events in *Streptocarpus*.

In *A. thaliana*, three *CUC* genes are present. All of them and their orthologs, the *NO APICAL MERISTEM* (*NAM*) gene in *P. hybrida* (26) and the *CUPULIFORMIS* (*CUP*) gene in *Antirrhinum majus* (27), are involved in the establishment and maintenance of organ boundaries in the shoot apical meristem, the inflorescence meristem, and the floral meristem (17, 28, 29). These genes are expressed in one or two rows of cells that correspond to the boundaries around each organ primordia (18). Our *in situ* data support an involvement of *SlCUC* in the control of organ boundaries in *S. latifolia.*

All of the above-mentioned results suggest that regulatory mechanisms controlling meristem homeostasis are presumably conserved among eudicots. *SlSTM1* and *SlSTM2* and *SlCUC* are therefore likely to control apical meristem functions in *S. latifolia* in a way similar to how *STM* and *CUC1* and *CUC2* control functions in *A. thaliana.*

Identification of a Candidate Regulatory Pathway for Sex Determination. In the model plant *A. thaliana*, *CUC1* and *CUC2* are required for *STM* activation during shoot apical meristem (SAM) establishment in the embryo (17, 30). However, *STM* is required for proper spatial expression of *CUC2*: in the *stm-1* mutant, *CUC2* is misexpressed, because it is detected in the central region between the cotyledon primordia (where *STM* transcripts are normally expressed), whereas in the presence of *STM*, *CUC2* is restricted to the boundaries between meristem and primordia (30). *STM* also promotes *CUC1* activity (31). All these data support the existence of a regulatory feedback loop between *STM* and *CUC1* and *CUC2* in apical meristems.

In this paper, we show a clear difference in the expression pattern of *SlSTM1* and *SlSTM2* and *SlCUC* in males compared with females and hermaphrodites. *SlCUC* is expressed in the central region of the male floral meristem at stage 3, concomitantly with the disappearance of *SlSTM1* and *SlSTM2* from the

same region. Interestingly, *CUC* genes have been shown to be associated with the absence of cell proliferation, particularly in whorl boundaries (18). In addition, Weir *et al.* (27) have shown a direct interaction between *CUPULIFORMIS* (*CUP*) (a *CUC* ortholog in *Antirrhinum majus*) and a TCP transcription factor known to be associated with inhibition of proliferation. In *S. latifolia*, such an inhibition of proliferation could result in the reduced number of cells observed in the center of male flower meristems.

The disappearance of *SlSTM1* and *SlSTM2* in male buds from stage 3 onwards presumably reflects an early arrest in meristem function and could therefore be the cause of the lack of cell divisions observed. It is likely that the central region of the meristem loses its meristematic identity and therefore behaves as it does in a weak *stm* mutant, where meristematic cells are rapidly consumed and differentiated. The filament would then be the product of a precocious determinacy of the male floral meristem. The formation of a filament associated with a defect in meristem function has indeed already been reported. In *hairy meristem* (*ham*) flower meristems of *P. hybrida*, *PhWUS* and *PhSTM* disappear precociously, and the meristematic cells are consumed to form a filament instead of carpels (32). In the *A. thaliana ago1-11 stm-2* double mutant, flowers are replaced by filaments (33). Consistent with an early loss of meristematic activity in male flower meristem of *S. latifolia*, Matsunaga *et al.* (34) observed a strong reduction in cell division in the central region of male flower meristem. The reduced proliferative activity, however, was observed at stage 5 and could be a direct consequence of the lack of *SlSTM* expression at stage 3, as described here.

Relationship Between *SlSTM1* and *SlSTM2* and *SlCUC* in Flower Meristems. The fact that *SlSTM1* and *SlSTM2* and *SlCUC* show a misexpression pattern in the same place and at the same time (in male flower meristems at stage 3, compared with females and hermaphrodites) could indicate that a regulatory feedback loop also exists between these genes in the dioecious species *S. latifolia*. Because the expression pattern of *SlCUC* in the male flower meristem resembles the expression pattern of *CUC2* in the *stm-1* mutant (31), it is tempting to correlate the lack of *SlSTM1* and *SlSTM2* at stage 3 (in the center of the male flower meristem) with the concomitant expansion of *SlCUC*. We therefore propose that in male flower meristems of *S. latifolia*, the disappearance of *SlSTM1* and *SlSTM2* would then lead to the expansion of *SlCUC* in the center of the meristem. This expansion being only transient, it is likely that a mechanism controlling this expansion would subsequently be turned on. Interestingly, Laufs *et al.* (20) have proposed that the microRNA miR164 constrains the expansion of the boundary domain by degrading *CUC1* and *CUC2* transcripts. The miR164 target site is present in *SlCUC* because it has been used as a primer to amplify the gene. In addition, because of the good conservation of microRNA during plant evolution (35), it is possible that *SlCUC* expansion is controlled by miR164 in flower meristems from *S. latifolia*.

In summary, the concomitant extinction of *SlSTM1* and *SlSTM2* and expression of *SlCUC* in the center of the male flower meristem at stage 3 are completely consistent with the arrest of proliferation observed in later stages. Interestingly, this male-specific pattern of expression is observed before any morphological differences between males and females and could therefore well be responsible for it. Consequently, the Y-linked locus responsible for gynoecium arrest in male flowers from *S. latifolia* presumably acts upstream from the regulatory pathway involving *SlSTM1*, *SlSTM2*, and *SlCUC*. This regulatory pathway could be subjected to epigenetic regulation, because treatment of male plants with 5-azacytidin, a demethylating agent, has been shown to induce hermaphrodite flower development (36). However, the number of carpels (from 0.5 to 3) in hermaphrodite mutants obtained by deletions in the Y chromosome has been shown to increase at each cycle of self-fertilization, showing an incomplete penetrance of the phenotype (9). These data suggest an epigenetic regulation of carpel suppression and a role for methylation in particular.

In conclusion, we have identified a regulatory pathway that is a strong candidate for being involved in sex determination in *S. latifolia*. Our results open perspectives for future research in the field.

Materials and Methods

Plant Material. To obtain *S. latifolia* seedlings, seeds were surface-sterilized and germinated for 6 days in sterile conditions. *S. latifolia* plants were grown in a greenhouse. The stages of the flower buds were assessed according to ref. 7: young buds were less than 2.5 mm long, medium buds were between 2.5 and 10 mm long, and open flowers were longer than 10 mm. Young gynoecia were collected from young flower buds and old gynoecia from open flowers. Young anthers were collected from young flower buds, middle anthers from medium flower buds, and old anthers from open flowers. *S. latifolia* hermaphrodite mutants with the Y chromosome deleted have been produced in our group by x-irradiation of pollen grains and have been described in ref. 9. The *bsx11* mutant used in this study is one of them.

Ortholog Identification. Total RNA was extracted from *S. latifolia* shoot apices from 6-day-old seedlings by using TRIzol Reagent according to the manufacturer's instructions (Invitrogen, Cergy Pontoise, France). The oligo(dT) primer T_{11}VN was used to synthesize the cDNA. To amplify *STM* orthologs, RT-PCR was done by using STM primers AAGATYATGGCTCATCCTCACTA (forward) and TCCATBACHACAAACTGCAT (reverse). The PCR conditions were as follows: 3 min at 94°C, followed by 20 cycles of 20 sec at 94°C; 1 min at 40°C (minus 1°C at each cycle) and 1 min at 72°C, followed by 15 cycles of 15 sec at 94°C; 1 min at 20°C and 1 min at 72°C, followed by a final extension of 7 min at 72°C. To amplify *CUC* orthologs, RT-PCR was performed by using CUC primers TTGGGAACTTCCTTGGAAGGCTCCRATGGGNGARAA (forward) and TYGGAGAARCAGGDCACGT (reverse). The PCRs were performed in two steps. Step one, with the CUC-reverse primer only, was performed as follows: 3 min at 94°C followed by 18 cycles of 20 sec at 94°C, 1 min at 65°C (minus 0.3°C at each cycle), and 1 min at 72°C. Step two, with both primers, was performed as follows: 18 cycles of 15 sec at 94°C, 1 min at 55°C (minus 0.5°C at each cycle), and 1 min at 72°C, followed by 18 cycles of 15 sec at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a final extension of 7 min at 72°C. PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI). The complete 3′ sequences of *SlSTM1* and *SlSTM2* were obtained by using STM-forward in a 3′ RACE-PCR experiment. Nucleotide sequences were determined by using the ABI Big Dye Terminator V1.1 DNA sequencing kit on a 3100 DNA Sequencer (Applied Biosystems, Foster City, CA).

Phylogenetic Analysis. The sequences of the published orthologs of *STM* and *CUC* were obtained from GenBank. Nucleic acid sequences were aligned with the ClustalW program (37) and refined by hand by using the graphical multiple-sequence alignment editor SeaView (38). Ambiguous positions in the alignment were excluded from the analysis. *AaKNOX1*, *MKN1*, and *CrKNOX3* were used as outgroups to root the KNOX tree, as described in ref. 13. The NAC tree was rooted by using *NtTERN* and *AtTIP*. We used the graphical color interface Phylo_win (38) to construct and bootstrap phylo-

genetic trees, using the neighbor-joining method, with 500 bootstrap replicates.

Expression Analyses. RT-PCRs using specific primers for *SlSTM1, SlSTM2*, *SlCUC*, and for a constitutively expressed *S. latifolia* gene [*eIF4A* (15)] were performed according to the instructions in ref. 15. *In situ* hybridization with *SlSTM1* and *SlCUC* antisense strand riboprobes was performed as described in ref. 39. *In situ* hybridization images were captured under bright-field illumination with a Nikon Optiphot-2 (Champigny-sur-Marne, France) Axiovert 125 inverted microscope (Zeiss, Thornwood, NY).

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