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SlY1, the first active gene cloned from a plant Y chromosome, encodes a WD-repeat protein

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Unlike the majority of flowering plants, which possess hermaphrodite flowers, white campion (*Silene latifolia*) is dioecious and has flowers of two different sexes. The sex is determined by the combination of heteromorphic sex chromosomes: XX in females and XY in males. The Y chromosome of *S.latifolia* was microdissected to generate a Y-specific probe which was used to screen a young male flower cDNA library. We identified five genes which represent the first active genes to be cloned from a plant Y chromosome. Here we report a detailed analysis of one of these genes, *SlY1* (*S.latifolia* Y-gene 1). *SlY1* is expressed predominantly in male flowers. A closely related gene, *SlX1*, is predicted to be located on the X chromosome and is strongly expressed in both male and female flowers. *SlY1* and *SlX1* encode almost identical proteins containing WD repeats. Immunolocalization experiments showed that these proteins are localized in the nucleus, and that they are most abundant in cells that are actively dividing or beginning to differentiate. Interestingly, they do not accumulate in arrested sexual organs and represent potential targets for sex determination genes. These genes will permit investigation of the origin and evolution of sex chromosomes in plants.

Keywords: dioecy/sex determination/*Silene latifolia*/WD-repeat/X and Y chromosomes

Introduction

Although the majority of plants differ from animals in that they are hermaphrodite, a small proportion have separate sexes; they are dioecious (Renner and Ricklefs, 1995). In some of these dioecious species, sex determination is controlled by heteromorphic sex chromosomes. Sex determination systems based both on the X/autosome ratio (as in *Drosophila*) and on X/Y with a dominant Y chromosome (as in mammals) exist in the plant kingdom (Parker, 1990; Ainsworth *et al*., 1998). This convergent evolution of sex determination mechanisms raises exciting and challenging questions about the structure, function and evolution of sex chromosomes in the two kingdoms. While genetic and molecular mechanisms controlling sex determination in *Drosophila* and mammals have been studied extensively (Nöthiger and Steinmann-Zwicky, 1987; Laurie, 1997; Capel, 1998; Raymond *et al*., 1998), very little is known about such processes in plants. White campion (*Silene latifolia*) is one of the most suitable plants to address such questions since in this species, sex determination is, as in mammals, under the strict genetic control of a dominant Y chromosome (Westergaard, 1958; Van Nigtevecht, 1966). XX individuals develop female flowers, whereas XY individuals develop male flowers. The presence of a single Y chromosome is sufficient to induce the development of male flowers, even in the presence of several X chromosomes (Westergaard, 1958), demonstrating the active nature of this chromosome.

Genetic and cytogenetic studies have shown that the Y chromosome plays a key role in sex determination in this species. At a very early stage, floral meristems from both sexes are potentially hermaphrodite: they contain both male and female reproductive organ primordia, as shown in Figure 1. Sex determination is under the control of two independent functions located on the Y chromosome: the first blocks gynoecium (female organ) development and the second promotes stamen (male organ) development. As a result, in a male XY plant, flowers develop with stamens and an undifferentiated filamentous structure instead of the gynoecium in the center of the flower (Figure 1). Conversely, in a female XX plant, flowers develop with a normal gynoecium but stamens stop developing at an early stage (Farbos *et al*., 1997). The Y chromosome also carries male fertility genes (Figure 1), since male sterile mutants with deleted Y chromosomes have been reported (Westergaard, 1958; Donnison *et al*., 1996). Finally, the X and Y chromosomes share a pseudoautosomal region (PAR) through which the two chromosomes specifically pair during meiosis (Westergaard, 1958; Buzek *et al*., 1997). Our knowledge of the Y chromosome is limited to morphological, genetic and cytogenetic data. Apart from genes involved in sex determination and male fertility, are there other active genes on this chromosome? Does it contain housekeeping genes as has been shown to be the case for the human Y chromosome (Lahn and Page, 1997)? What is the extent of homology between the *S.latifolia* X and Y chromosomes ?

Several attempts to identify active genes located on the Y chromosome have resulted in the isolation of non-coding sequences, the vast majority of which are

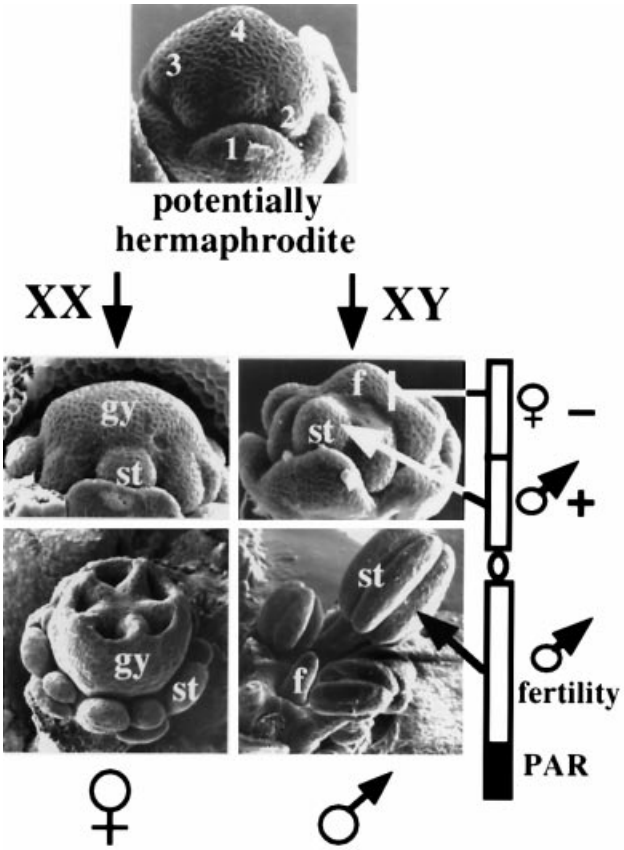

Fig. 1. Sex determination and Y chromosome in *S.latifolia*. At a very early stage of development, the floral meristem is similar to that of a hermaphrodite species and contains four types of organ primordia: sepal (1), petal (2), male organ (3) and female organ (4). The subsequent developmental fate of the floral meristem depends on the presence or absence of the Y chromosome. In an XY background, female organ (gynoecium, gy) development is blocked by the female suppression function on the Y chromosome (♀–) giving rise to a filament (f), and the male organ (stamens, st) development is activated by the male promotion function on the Y chromosome (♂+). Finally, the male fertility genes located on the Y chromosome are involved in pollen development. In an XX background, the gynoecium develops normally, as a default state, and stamens stop developing at a very early stage due to lack of activation. PAR, pseudo-autosomal region.

shared by Y, X and/or autosomes (Donnison *et al*., 1996; Scutt *et al*., 1997; Zhang *et al*., 1998). In this paper, we report the characterization of the first active gene cloned from a plant Y chromosome. We microdissected Y chromosomes from *S.latifolia* and a Y chromosome-specific probe was used to identify five cDNA clones corresponding to Y-linked genes. Here, we describe the detailed analysis of one of these clones, *SlY1* (*S.latifolia* Y-gene 1). We show that it is linked to the Y chromosome and that a closely related gene is most likely located on the X chromosome (*SlX1*). *SlY1* and *SlX1* are highly homologous and both genes are expressed. They encode WD-repeat proteins that are localized in the nucleus and which are most abundant in cells that are actively dividing or beginning to differentiate. Taken together, these results allow us to draw preliminary parallels between the human and a plant Y chromosome. Finally, we envisage the use of

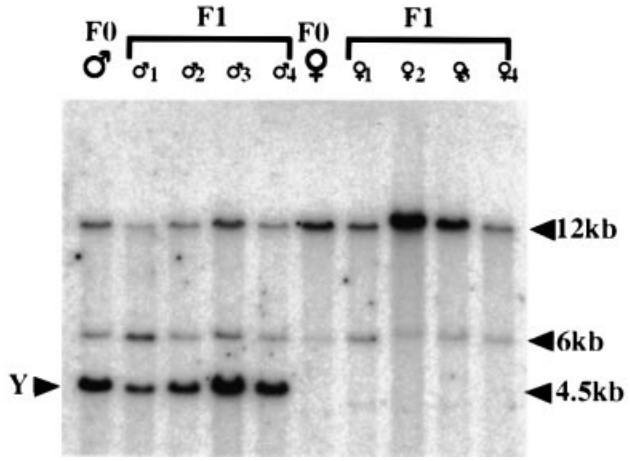

Fig. 2. Segregation analysis of a male-specific fragment and characterization of *SlY1*. Genomic DNA isolated from two parent plants (F_0 male and F_0 female) and from their male (F_1 male 1–4) and female (F_1 female 1–4) progeny was digested with *Hin*dIII and analysed by Southern blot. Genomic DNA was hybridized with a restriction fragment derived from the *SlY1* cDNA (probe B, Figure 3A). The size of the fragments which hybridize to the probe are indicated. The 4.5 kb band which segregates with the male sex is labelled with a Y (left).

SlY1 and *SlX1* in the cloning of the sex determination genes in white campion.

Results

Screening of an early male flower cDNA library with a Y-derived probe

In order to characterize expressed genes located on the Y chromosome, Y chromosomes were microdissected on metaphase spreads from root cultures. Two pools of 10 microdissected Y chromosomes were used as template for Degenerate Oligonucleotide Primed–PCR amplification (DOP–PCR). Each pool was amplified with one of two primers (Materials and methods) in two successive PCR amplifications. The sizes of the amplicons ranged from ~0.2 to 4 kb (data not shown). The PCR products were pooled to produce the Y-derived probe (Materials and methods) and used to screen a premeiotic male flower cDNA library (Barbacar *et al*., 1997). We selected 115 positive clones showing different signal intensities. In order to test for Y-linkage, segregation analysis was performed: each insert was hybridized with restricted genomic DNA from male and female individuals as well as their male and female progeny. Three kinds of profiles were obtained: (i) clones showing no difference between males and females; (ii) clones showing restriction fragment length polymorphism; and (iii) clones showing sex-linked polymorphism. In total, we identified five partial cDNA clones hybridizing to one or more male-specific fragment(s). In this paper, we report the detailed analysis of one of these cDNAs named *SlY1*.

A genomic DNA fragment located on the Y chromosome contains the gene encoding the SlY1 cDNA

In order to confirm the Y-linkage of *SlY1*, segregation analysis was performed by genomic Southern blot analysis. *Hin*dIII-restricted genomic DNA from two parents (F_0)

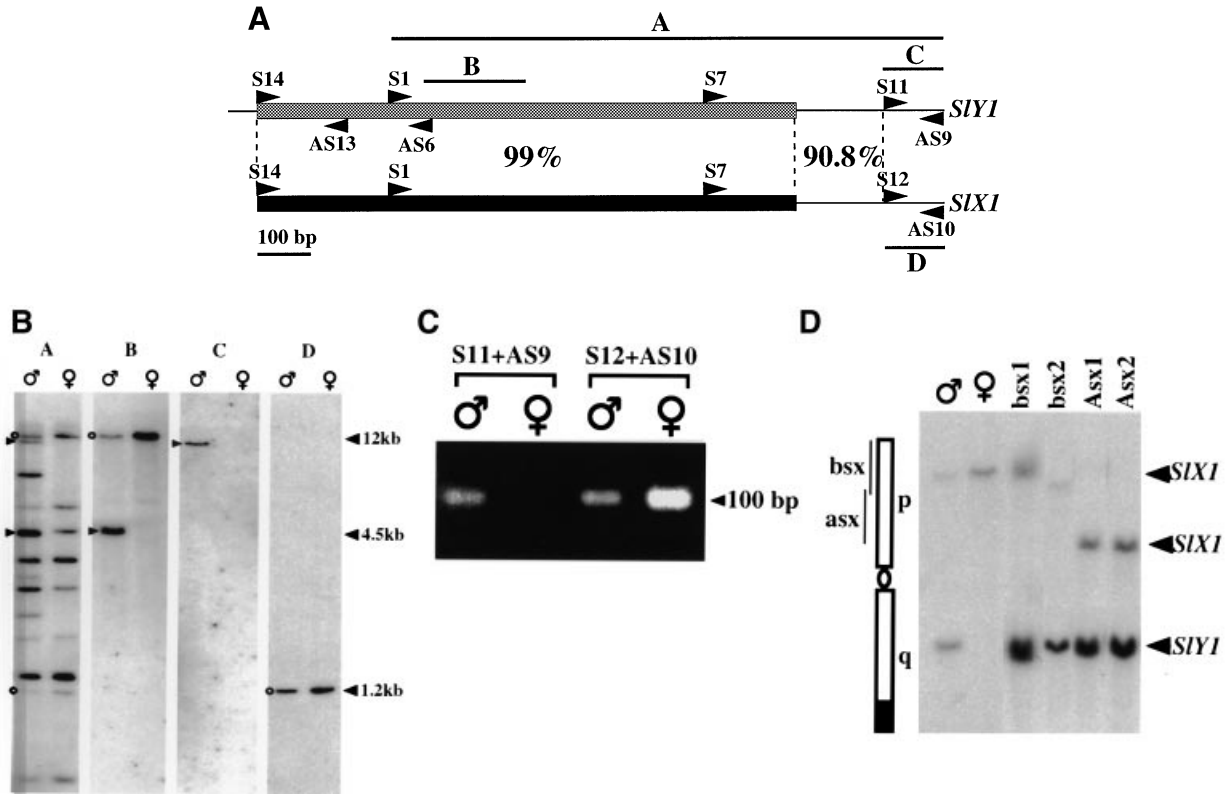

Fig. 3. Characterization of gene-specific probes for *SlY1* and *SlX1* respectively. (**A**) The cDNA sequences of *SlY1* and *SlX1* are represented with their coding regions boxed in grey and black respectively. The primers used for PCR are shown by arrowheads. The percentage of homology between the coding regions and part of the 3′ untranslated regions of the two genes are indicated. The four fragments (A, B, C, D) used as probes in the Southern blot analysis in panel (B) are represented by dark lines. Probe A is a 1192 bp RT–PCR product amplified from *SlY1* cDNA using primers S1 and AS9, probe B corresponds to a 273 bp *Sau*3AI subfragment from the *SlY1* cDNA, probe C is a 107 bp RT–PCR product amplified from *SlY1* cDNA using the primers S11 and AS9 primers, probe D is a 117 bp RT–PCR product amplified from *SlX1* cDNA using the primers S12 and AS10 primers. The scale is indicated by a bar (bottom left) representing 100 bp. *SlY1* and *SlX1* sequences are available in the DDBJ/EMBL/GenBank databases under accession Nos Y18517 and Y18519 respectively. (**B**) Genomic DNA isolated from a male (♂) or a female (♀) plant was digested with *Hin*dIII and analyzed by Southern blotting. The same DNA samples were hybridized with four different probes [A–D, panel (A)] as indicated at the top of each blot. The fragments labelled with a triangle correspond to the *SlY1* locus. The fragments labelled with a circle correspond to the *SlX1* locus. The size of some fragments are indicated. (**C**) Genomic DNA from male or female individuals was used as template in PCR amplification using either the S11 and AS9 primers specific for *SlY1*, or the S12 and AS10 primers specific for *SlX1* (panel A). The PCR products were loaded on an agarose gel and separated by electrophoresis in the presence of ethidium bromide. DNA fragments were visualized under UV light. (**D**) Genomic DNA isolated from male (♂) or female (♀) plants, or from four Y chromosome deletion mutants exhibiting hermaphrodite (*bsx*) or asexual (*asx*) phenotypes, was digested with *Hin*dIII and analyzed by Southern blot using probe B (panel A). The origin of the fragments are indicated on the right. *SlX1* exhibits an allelic polymorphism in the asexual mutants. On the left, the Y chromosome is represented with its p and q arms. The black box represents the pseudo-autosomal region. Vertical bars represent the regions covered by the deletions in each type of mutant.

and 28 F_1 progeny plants (14 males and 14 females) were tested. The results obtained for 10 representative plants are shown in Figure 2. A portion of the *SlY1* cDNA (probe B, Figure 3A) recognized two bands of 12 and 6 kb, respectively, which were present in both male and female DNA, but also a band of 4.5 kb which was only detected in DNA from the male plants (Figure 2). The probability that this strict segregation could be a random event was extremely low: one chance in 3.7×10^9 [$P = (1/2)^n$, where n is the number of segregating male and female individuals for the fragment of interest]. These results demonstrate that the 4.5 kb *Hin*dIII fragment is linked to the Y chromosome.

Identification of two highly homologous cDNAs, SlY1 and SlX1

The initial *SlY1* cDNA was 706 bp long and was truncated at both the 5′ and 3′ ends. In order to obtain a complete *SlY1* cDNA, we performed rapid amplification of cDNA ends (RACE–PCR). The primers used for the RACE–PCR experiments are shown in Figure 3A. The sense primer S7 was used to amplify two distinct 3′ cDNA sequences. The 5′ region of the *SlY1* cDNA, including the putative initiation codon of a 1416 bp open reading frame, was amplified with the antisense primers AS6 and AS13. We used RT–PCR amplifications with primers S14 and AS9 to verify the continuity of the *SlY1* cDNA clone. Similarly, RT–PCR with primers S14 and AS10 confirmed the existence of a related cDNA named *SlX1*. The sequences of each of these two cDNAs were determined from PCR products obtained from at least two independent RT–PCR experiments. These two cDNAs are represented in Figure 3A and were respectively 1785 (*SlY1*) and 1739 (*SlX1*) bp long (not including their polyA tails). The two putative coding sequences are 99% homologous at the DNA level. In the 3′ untranslated region, the two cDNAs

are 90.8% homologous, with the exception of a region of 100 bp at the extreme 3′ end where they diverge completely.

In order to determine the chromosomal origin of each of these two cDNAs, Southern blot analysis was performed using different DNA fragments as probes against *Hin*dIII-restricted genomic DNA. The probes are shown in Figure 3A, and corresponding Southern blots are shown in Figure 3B. Probe B hybridized to a male-specific, 4.5 kb fragment and, more weakly to 12- and 6-kb fragments present in DNA from both males and females. Hybridization to the 12 kb fragment was twice as strong in DNA from females compared with DNA from males (probes A and B) as would be expected for a X-linked gene. Probe A includes the probe B region and extends into the 3′ untranslated region of the *SlY1* cDNA. It hybridized to more fragments than probe B. One of the male-specific fragments was 11 kb long and also hybridized to the *SlY1*-specific probe C (Figure 3A and B). The male-specific fragments corresponding to *SlY1* (4.5 and 11 kb) are indicated with an arrowhead. The *SlX1*-specific probe D hybridized twice as strongly to a 1.2 kb fragment in DNA from females compared with DNA from males (Figure 3A and B). This supports the hypothesis that *SlX1* is linked to the X chromosome. Therefore, we named this gene *SlX1* (for *S.latifolia* X-gene 1). The fragments corresponding to *SlX1* (12 and 1.2 kb) are indicated with a circle.

Finally, we noted a complex hybridization pattern with probe A, indicating the existence of additional homologous genes within the genome. Nine restriction fragments were revealed by probe A in DNA from both males and females (Figure 3B, panel A). Among these, two correspond to *SlX1* and are labelled with a circle. Three others showed a double intensity in the DNA from females suggesting they could correspond either to *SlX1* (since the locus has not yet been sequenced completely) or to other homologues located on the X chromosome. The other four (one of them had the same size as the 4.5 kb fragment corresponding to *SlY1*) probably corresponded to homologues located on autosomes. Five fragments were detected only in DNA from the males. Based on the gene sequence (data not shown), two of them were assigned to *SlY1* and are indicated with an arrowhead. The other three could correspond to homologue(s) also located on the Y chromosome. Their analysis is underway.

The specificity of the primers, S11 and AS9 for *SlY1*, and S12 and AS10 for *SlX1* (Figure 3A), was confirmed by PCR amplification of genomic DNA from male and female individuals. The results are shown in Figure 3C: primers S11 and AS9 amplified a 107 bp fragment only when DNA from a male plant was used as a template, whereas S12 and AS10 amplified a 117 bp fragment with DNA from both males and females. The intensity of the band with the DNA from the female was stronger than with the DNA from the male. This is in agreement with X-linkage of *SlX1*.

In order to establish whether *SlY1* maps to regions of the Y chromosome known to contain sex determination loci, we performed genomic Southern blot analysis on sexual mutants obtained by γ-irradiation of pollen grains. These mutants have been shown to have deleted Y chromosomes (Farbos *et al*., 1999; Lardon *et al*., 1999). The hermaphrodite mutants have lost the female suppressing function, whereas the asexual mutants have lost the male promoting function. Both of these functions are located on the Y chromosome and are responsible for sex determination (Figure 1). We chose to test four mutants with the largest reported Y chromosome deletions: two hermaphrodite mutants *bsx1* (51% of the differential arm deleted) and *bsx2* (38% of the differential arm deleted) (Lardon *et al*., 1999), and two asexual mutants *asx1* (40% of the differential arm deleted) and *asx2* (24% of the differential arm deleted) (Farbos *et al*., 1999). Total *Hin*dIII-restricted genomic DNA from male, female and mutant plants was hybridized with probe B (Figure 3A and B) and the results are shown in Figure 3D. As expected, the 4.5 kb fragment corresponding to the *SlY1* gene was present in the DNA from the male plant but not from the female plant. All the mutants tested contained this 4.5 kb fragment, indicating that *SlY1* is not deleted in any of them. We concluded that *SlY1* does not map to the regions covered by these deletions. Note that *SlX1* seemed to exhibit allelic polymorphism in the two asexual mutants, probably due to the different origin of the female parent compared with hermaphrodite mutants. All Y chromosome deletion mutants received the X chromosome from the non-irradiated mother.

SlY1 and SlX1 encode WD-repeat proteins

Both *SlY1* and *SlX1* cDNAs contained open reading frames encoding putative polypeptides of 472 amino acids (52 kDa). The sequences of these two polypeptides are 99.6% identical and differ at only two amino acid positions: position 154, residue Trp for SlY1 and Leu for SlX1, and position 441, Gly for SlY1 and Arg for SlX1. The sequences were established from independent PCR products in order to rule out PCR errors. These differences may or may not have consequences on the specificity of interaction of each protein with potential partners. When the SlY1 protein sequence was compared with the database, homology with members of the WD-repeat protein family was revealed. A typical core element of a WD-repeat is (G,V,A,N,S,I)H-X_{28}-(W,F)(D,S), where X stands for any amino acid (Neer *et al*., 1994). Such proteins are found almost exclusively in eukaryotes, where they are involved in a variety of processes including regulation of transcription, control of cell growth and differentiation, and chromatin structure (Neer *et al*., 1994; Neer and Smith, 1996; Mulligan and Jacks, 1998; Parkhurst, 1998). Significant similarities ranging from 30 to 42% were found with proteins from different organisms: RbAp46 and RbAp48 from human (Qian *et al*., 1993; Qian and Lee, 1995); MSI1 and Hat2p from yeast (Ruggieri *et al*., 1989; Parthun *et al*., 1996); the p55 subunit of dCAF-1 from *Drosophila* (Kamakaka *et al*., 1996); and MSI1-like proteins recently reported in plants: LeMSI1 from tomato and AtMSI1, AtMSI2 and AtMSI3 from *Arabidopsis thaliana* (Ach *et al*., 1997). Another MSI1-like protein from *A.thaliana*, named AtMSI4 (Kenzior and Folk, 1998), is very homologous to the SlY1 protein, sharing 82% similarity (77% identity) with it. In Figure 4A, we show an amino acid alignment between the SlY1 and AtMSI4 proteins. The five highly conserved WD-repeats are boxed as well as the two amino acids flanking the core element.

We selected 11 members of the WD-repeat family from

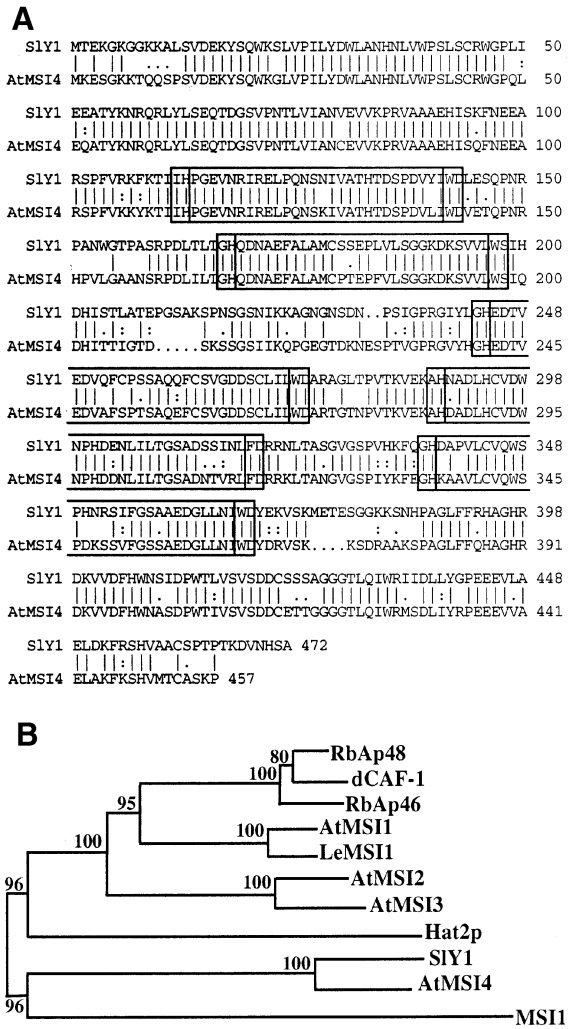

Fig. 4. *SlY1* encodes a WD-repeat protein. (**A**) Alignment of the protein sequences of SlY1 with that of AtMSI4 from *A.thaliana*. The two proteins are 77% identical. Vertical bars represent identical amino acids, single and double dots represent similar amino acids. The five WD-repeat domains are boxed, as well as the two amino acids flanking this core: GH, IH or AH at the beginning, and WD, WS or FD at the end (Neer *et al*., 1994). (**B**) Phylogenetic tree based on an alignment of *SlY1* and 10 related genes. The 10 SlY1-related proteins are members of the WD-repeat family and include proteins from tomato (LeMSI) (Ach *et al*., 1997), *A.thaliana* (AtMSI1, AtMSI2, AtMSI3 (Ach *et al*., 1997) and AtMSI4 (Kenzior and Folk, 1998), human RbAp48, RbAp46 (Qian *et al*., 1993; Qian and Lee, 1995), *Drosophila* p55 subunit of dCAF-1 (Kamakaka *et al*., 1996) and yeast MSI1 (Ruggieri *et al*., 1989), Hat2 (Parthun *et al*., 1996). Protein sequences were aligned using the MUST package (Philippe, 1993). Distance trees were calculated using the neighbour-joining method. The length of the branches are proportional to the degree of divergence and thus correspond to the statistical significance of the phylogeny between the protein sequences. The bootstrap values supporting the branches are indicated and were calculated using 1000 replicates.

plants, human, *Drosophila* and yeast with which SlY1 shares the highest levels of homology. The deduced protein sequences were aligned and Figure 4B shows the corresponding phylogenetic tree. Among these proteins, SlY1 and AtMSI4 define a separate group. The phylogenetic tree indicates a common origin for *SlY1* and *AtMSI4*. Interestingly, proteins that have been shown to bind the Rb (Retinoblastoma protein), namely RbAp48, RbAp46, LeMSI1, and their close homologues, AtMSI1 and dCAF-1, form a phylogenetic branch distinct from the other members of the family. The alignment of these 11 proteins not only identified strong homologies in the first three WD-repeat domains (for example 65% homology between SlY1 and RbAp48), but also in the N- and C-terminal regions (yeast genes excluded), suggesting that in addition to the WD-repeats, the conserved N- and C-terminal regions may be important for the activity of these proteins in higher eukaryotes.

Transcripts of both SlY1 and SlX1 accumulate in young flowers

In order to investigate the expression profile of each of these two genes, the gene-specific probes C and D (Figure 3A and B) were used to perform Northern blot analysis on total RNA from different tissues. The developmental stages of the flowers are described in Materials and methods. The results are shown in Figure 5A. The *SlY1* transcript (~2.2 kb) was detected in male flowers at all stages of development tested but not in the vegetative tissues tested (leaves, stems and seedlings). Stamens harvested at stage 3 also contain relatively abundant *SlY1* transcripts. As expected, no transcript was detected in tissues from females since the gene is absent. The *SlX1* transcript (~2.2 kb) was detected in male and female flowers at all stages of development tested, including gynoecium tissues at pre-meiotic stages where it was most abundant. A small amount of *SlX1* transcript was detected in leaves and stems of female plants as well as in seedlings. As a control, a 1.9 kb transcript was detected by a fragment corresponding to the *actin* gene from white campion, confirming the presence of hybridizable RNA in all samples. These results were confirmed by RT–PCR amplification which allows a more sensitive detection, and are shown in Figure 5B. The primers used for the RT–PCR amplifications are shown in Figure 3A. The sizes of the amplification products were as expected: 1.2 kb for primers S1 and AS9 (*SlY1*), and 1.21 kb for primers S1 and AS10 (*SlX1*). The results were in agreement with the Northern blot analysis: *SlY1* was only expressed in tissues from male plants and the transcript was mainly detected in male flowers and in stamens. A very small amount of *SlY1* transcript was detected in leaves and stems, whereas it was not detected in seedlings (a mixture of male and female seedlings). *SlX1* was expressed in both male and female tissues, with preferential expression in flowers of both sexes. However, the transcript was more abundant in female flowers. A smaller amount was detected in non-floral tissues of both sexes (leaves, stems, seedlings). As a control, two primers specific for *actin* genes were used and amplified a 0.7 kb fragment from all the samples.

In situ hybridization experiments were performed using the gene-specific probes but the use of such short probes on low abundance transcripts did not provide good quality data. Probe B (Figure 3A and B) was also used and gave very clear results (data not shown). This probe, which does not distinguish *SlY1* from *SlX1*, revealed an expression pattern at the transcript level very similar to that obtained by immunolocalization of SlY1/SlX1 protein (see below).

SlY1 and SlX1 accumulate preferentially in flowers

In order to characterize the pattern of expression of the proteins encoded by *SlY1* and *SlX1*, antibodies were

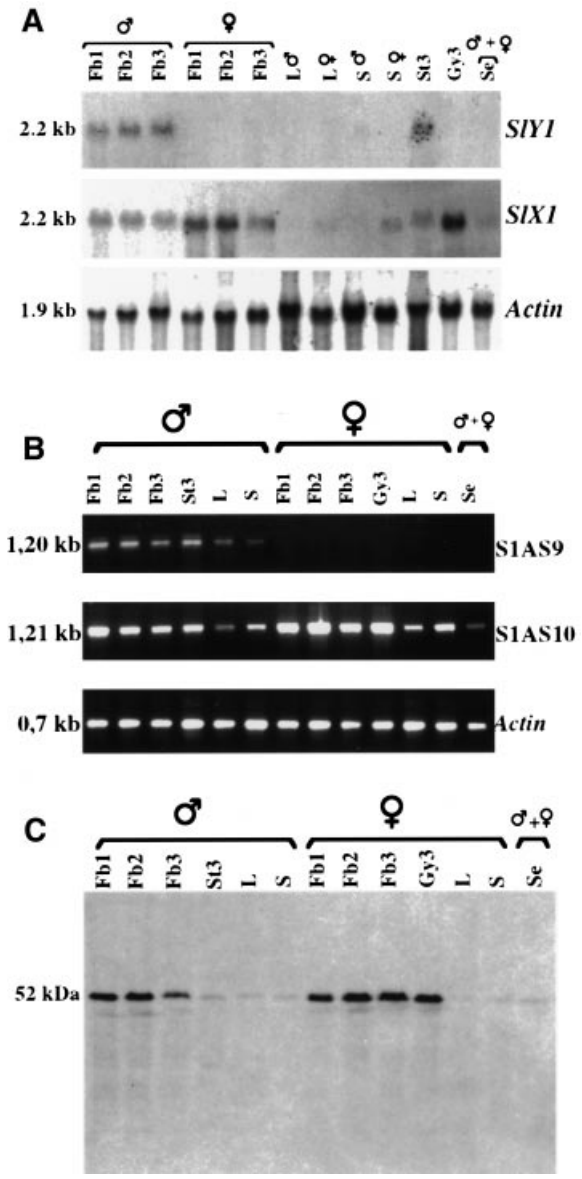

Fig. 5. Expression of *SlY1* and *SlX1*. (**A**) Northern blot analysis. Flower developmental stages are as described in Materials and methods. Fifteen microgrammes of total RNA from different tissues of male (♂) or female (♀) plants were loaded in each lane: flowers at three successive stages of development (Fb1, Fb2 and Fb3), leaves (L), stems (S), isolated male organs at stage 3 (St3), isolated female organs at stage 3 (Gy3) and seedlings (Se). The RNAs were hybridized with the three different probes indicated on the right. *SlY1* corresponds to the *SlY1*-specific fragment (probe C, Figure 3A). *SlX1* corresponds to the *SlX1*-specific fragment (probe D, Figure 3A). *Actin* corresponds to a fragment of *actin* cDNA amplified from *S.latifolia* with the *actin*-specific primers (Materials and methods). On the left, the sizes of the transcripts are indicated in kb. (**B**) RT–PCR analysis. Total RNA from the same tissues as in (A) were used as template for reverse transcription from an oligo-dT primer and subsequent PCR amplification using three different pairs of primers: S1 and AS9 to detect *SlY1* transcripts, S1 and AS10 to detect *SlX1* transcripts (Figure 3A) and *actin*-specific primers as a control (Materials and methods). The PCR products were separated on an agarose gel and stained with ethidium bromide. Their sizes are indicated on the left in kb. (**C**) Western blot analysis. Purified anti-SlY1/SlX1 antibody was used for immunodetection on 10 µg of total protein extracted from the same tissues as in (A), separated by SDS–PAGE and transferred onto nitrocellulose. The size of the band detected is indicated on the left.

raised against a mixture of three synthetic peptides corresponding to three antigenic regions of SlY1, as deduced from the analysis of the protein sequence (Materials and methods). Since gene-specific sequences were identified in untranscribed regions only, and because of the high degree of homology between the two proteins, it was not possible to raise antibodies specific for SlY1. In male tissues, the antibody should reveal both SlY1 and SlX1 if present, whereas in the female tissues, the antibody should detect only SlX1. Affinity-purified antibodies were used in a Western blot analysis performed on total protein extracts from different tissues (the same as for the Northern blot analysis, Figure 5C). A single band of ~52 kDa was detected in all extracts. The mass of this band corresponded to the predicted molecular mass of SlY1 and SlX1. SlY1/SlX1 protein was principally detected in flowers of both sexes at relatively high levels, including the isolated gynoecium. Very low levels of protein were observed in seedlings, leaves or stems. Overall, the abundance and localization of transcripts and proteins corresponding to *SlY1* and *SlX1* are very similar. However, very low levels of protein were detected in stamens at a stage when the corresponding transcripts were fairly abundant (Figure 5, compare C with A and B). This observation suggests the existence of post-transcriptional regulation in stamens after meiosis.

SlY1 and SlX1 are localized in the nuclei of cells actively dividing or beginning to differentiate

In order to determine the precise localization of SlY1 and SlX1, immunolocalization experiments were performed with the purified antibodies used above for the Western blot analysis. The results obtained with male flowers are shown in Figure 6A–E. SlY1/SlX1 protein was detected in very young floral meristem (fm, Figure 6A) before any organ primordia arise, and was more abundant in the external cell layers. At a later stage when organ primordia emerge (Figure 6B), protein was detected throughout the floral meristem, again being more abundant in the outer cell layers. Later, SlY1/SlX1 protein was very abundant in stamen (st) before and during meiosis (Figure 6C). Interestingly, no antigen was detected in the filamentous structure (f) in the centre of the flower, which corresponds to the arrested female organ. Figure 6E represents a longitudinal section of a male inflorescence which contains four flowers at different developmental stages: the flower labelled 1 has a sepal primordium on the right, the flower labelled 2 (a stage similar to the one in Figure 6B) has sepal and stamen primordia. SlY1/SlX1 protein was abundant in both of these two flower buds. On the right side, the section passes through a flower bud (labelled 3) with a strongly labelled stamen at the bilobal stage. Stamens (st) after meiosis appear in the upper part of the section and the antigen was detected in the microspores (m) and in the tapetum (t). The petal (p) tip was labelled as well as the vascular tissues of the flower. Figure 6P represents a detail of a petal (p) tip from a male flower and showed strong accumulation of SlY1/SlX1 in the cell nuclei. As a control, a longitudinal section of a male inflorescence was incubated with the pre-immune serum (Figure 6D). The results obtained with female

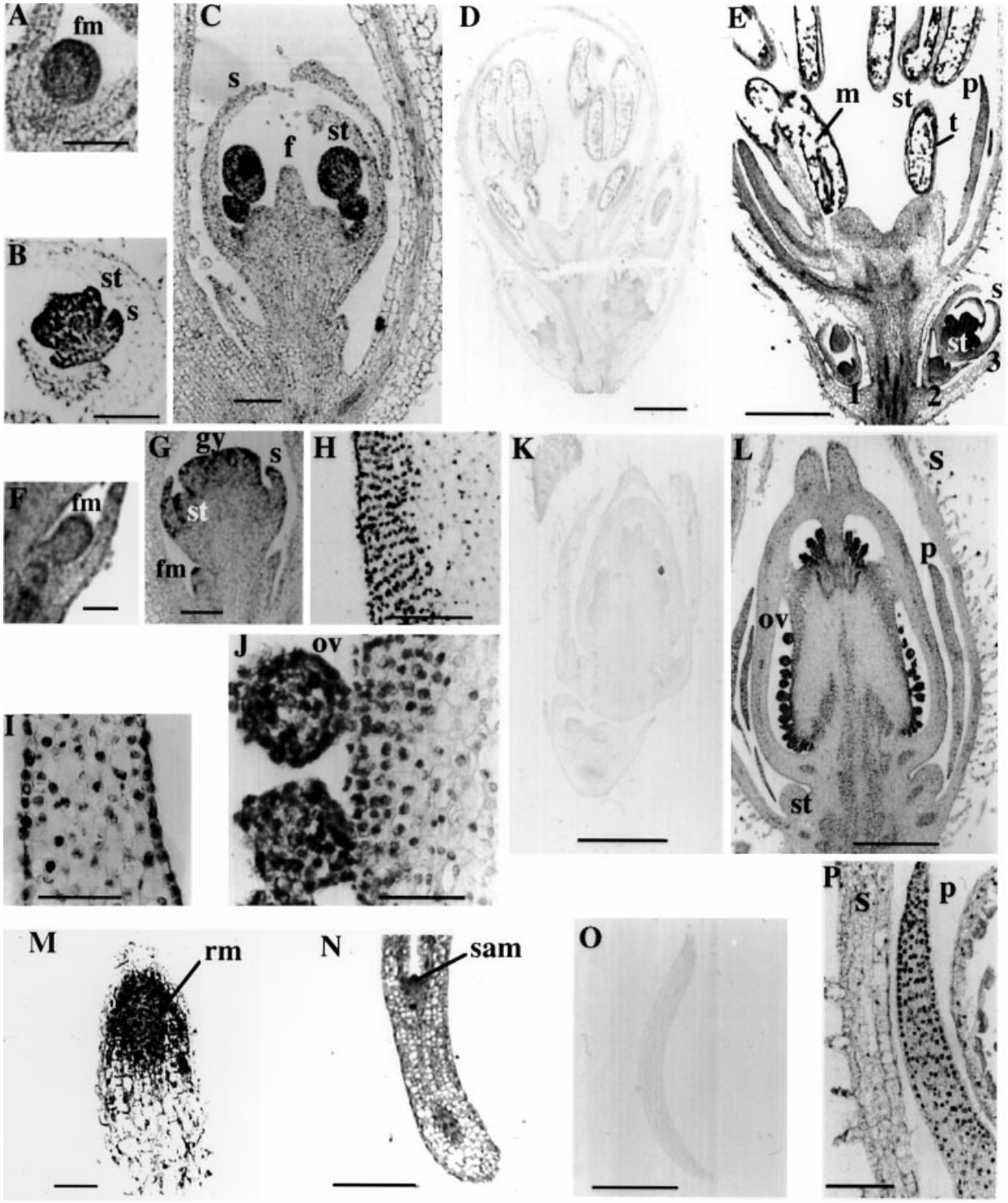

Fig. 6. Expression of SlY1 and SlX1 at the cellular level. Longitudinal sections from different tissues were incubated with the purified antibodies (Figure 5C; Materials and methods) recognizing SlY1 and SlX1 proteins, except for (D), (K) and (O) which were incubated with the pre-immune serum. (**A**–**E**) Male flowers at different developmental stages. (**F**–**L**) Female flowers at different stages. (**M**–**O**) Germinating seedlings and (**P**) detail of a male petal tip. Abbreviations are as follows: fm, floral meristem; st, stamen; s, sepal; f, filament; m, microspore; t, tapetum; p, petal; gy, gynoecium; ov, ovule; rm, root meristem; sam, shoot apical meristem. Bars, 50 μm for (I) and (J), 100 μm for (A), (B), (C), (F), (G), (H), (M) and (P), and 500 μm for (D), (E), (K), (L), (N) and (O).

flowers are shown in Figure 6F–L. As for male flowers, SlX1 was detected in very young floral meristems (fm, Figure 6F), as well as in floral meristems at a stage where organ primordia arise (Figure 6G). Again, SlX1 was more abundant in the outer cell layers. Figure 6L shows a longitudinal section of a female flower. SlX1 was abundant in the ovules (ov) and was also detected in the petal (p) tips and in the vascular tissue. Interestingly, no antigen was detected in the arrested stamens (st) at the base of the female flower. As a

control, a longitudinal section of a female inflorescence was incubated with the pre-immune serum (Figure 6K). Figure 6H represents a detail of the placenta and showed a strong accumulation of SlX1 in the nuclei of the cells which give rise to the ovules. These cells are actively dividing in a periclinal division pattern generating aligned cells. Figure 6I represents a detail of the ovary wall and again, SlX1 is localized in the nucleus. Figure 6J represents a detail of ovules (ov) and clearly shows that SlX1 is concentrated in the nuclei of the cells. The results obtained with vegetative tissues are shown in Figure 6M–O. Seedlings representing a mixture of males and females exhibited reproducible accumulation of antigen in the root meristem (rm, Figure 6M) and in the shoot apical meristem (sam, Figure 6N). The vascular tissue was weakly labelled. As a control, a longitudinal section of a seedling was incubated with the pre-immune serum (Figure 6O). In summary, SlY1 and SlX1 showed a nuclear localization and were most abundant in cells that were dividing actively or beginning to differentiate.

Discussion

While sex chromosomes in animals have been extensively studied at the molecular level, very little is known about such chromosomes in plants. We report here the characterization of *SlY1*, the first active gene to be cloned from a plant Y chromosome. We report also the characterization of a close homologue, *SlX1*, putatively located on the X chromosome. Such genes represent essential tools to investigate structural and functional organization of plant sex chromosomes. Genetic and cytogenetic studies reveal similarities between sex chromosomes in animals and in plants. In particular, the Y chromosome from mammals and from *Silene* both contain at least one PAR that pairs with the X during meiosis, as well as a nonrecombining region (NRY) which contains the sex determination gene(s) (Westergaard, 1958). Recently, Lahn and Page (1997) have cloned several new human Y-linked genes. Genes located in the NRY fall into three classes. *SRY* from the first group is involved in sex determination. The second group consists of housekeeping genes which have X homologues that escape X inactivation. Genes from the third group belong to gene families and are expressed specifically in the testis (Lahn and Page, 1997). It is known from genetic data that the Y chromosome from *S.latifolia* contains at least two genes involved in sex determination which could be equivalent to the first group (Westergaard, 1958; Van Nigtevecht, 1966), as well as genes involved in male fertility, which could be equivalent to the third group (Westergaard, 1958; Donnison *et al*., 1996). However, these genes remain to be characterized at the molecular level. Our results suggest that *SlY1* could be equivalent to genes of the second group characterized on the human Y chromosome. Interestingly, another gene from the five that we have identified on the Y chromosome may belong to the same group (I.Atanassov, F.Monéger, C.Delichère and I.Negrutiu, manuscript in preparation).

In the case of human sex chromosomes, X and Y are believed to have evolved from a pair of autosomes through suppression of recombination and subsequent divergence between the sex chromosomes (Ellis, 1998). As a consequence, most NRY-located genes have a tendency to degenerate during evolution. This resulted in females having two copies but males having only one copy of many genes. This inequality was addressed by inactivating the genes on one of the two X chromosomes in females to achieve dosage compensation (Charlesworth, 1996; Ellis, 1998). Interestingly, X inactivation has been shown to occur in *S.latifolia* (Vyskot *et al*., 1999 and references therein). The existence of active Y copies with X homologues can be interpreted as an alternative mechanism operating to maintain comparable expression of certain housekeeping functions in males and females. The fact that *SlY1* and *SlX1* encode almost identical proteins indicates that these genes are subject to selection pressure and most likely encode functional proteins. The fact that *SlY1* segregates consistently to male plants suggests it is probably located in the non-recombining region of the Y chromosome (although additional experiments are needed to validate this interpretation). If this were the case our results, together with those of Guttman and Charlesworth (1998) who identified a degenerate form of an X-linked gene (*MROS3*) on the Y chromosome, support the idea that the Y chromosome from *Silene* contains both active and inactive copies of X-linked genes. In this context, the analysis of the other Y-linked clones we have identified will be particularly interesting. Localization of these genes will be essential to determine the extent of structural and functional homologies between the Y and X chromosomes. In a context where such problems have not yet been addressed in plants, our genes provide essential tools to investigate the similarities and the specificities of plant sex chromosomes compared with sex chromosomes in animals.

SlY1 and SlX1 belong to the WD-repeat family. WD (Trp-Asp)-repeats are thought to be involved in protein–protein interactions (Neer *et al*., 1994). The WD-repeat (also called WD-40 repeat or β transducin-like) protein family contains at least 50 known eukaryotic proteins involved in various functions (Neer *et al*., 1994; Neer and Smith, 1996). Among these proteins, RbAp46, RbAp48 and LeMSI are able to bind the Retinoblastoma (Rb) protein (Neer *et al*., 1994; Ach *et al*., 1997; Parkhurst, 1998), which acts as a major negative regulator of the cell cycle in mammals. Rb has a dual role in the regulation of E2F-target gene expression: inhibition of E2F-transactivation and local modification of the chromatin resulting in transcriptional repression of the target genes (Magnaghi-Jaulin *et al*., 1998). Some Rb-binding proteins are also involved in chromatin assembly and in the regulation of acetylation and deacetylation of histones (Brehm *et al*., 1998; Luo *et al*., 1998; Magnaghi-Jaulin *et al*., 1998; Mulligan and Jacks, 1998). Other WD-repeat proteins regulate growth and differentiation as trancriptional co-repressors (Parkhurst, 1998).

No defined function has so far been assigned to the few MSI-like proteins reported recently in plants. The best studied at present is LeMSI1 from tomato, which was shown to complement mutations of the MSI1 gene in yeast and to bind the Rb proteins from human and maize *in vitro* (Ach *et al*., 1997). Like SlY1 and SlX1, LeMSI1 is localized in the nucleus. In contrast with SlY1 and SlX1, LeMSI1 is expressed in nearly all cells and its expression is apparently not correlated with the state of

differentiation or cell division activity. This difference could be due to the fact that LeMSI1 is a single-copy gene. The existence of several members of the gene family in the genomes of *S.latifolia* and *A.thaliana* could allow some genes to specialize in certain functions and to exhibit a more specific pattern of expression.

We demonstrate that both *SlY1* and *SlX1* are active and present evidence that, apart from minor differences in expression in vegetative tissues, *SlY1* and *SlX1* have similar temporal and spatial patterns of expression (although *SlY1* is absent from female plants) and therefore behave as alleles of the same gene. The pattern of expression of *SlY1* and *SlX1* suggests that these genes might be part of a major regulatory pathway promoting cell division and early differentiation in the plant. SlY1 and SlX1 could interact with the plant Rb homologue (*in vitro* binding assays are underway), or interact with other WD-repeat proteins that control cell cycle and early differentiation. Concerning the functional analysis of SlY1 and SlX1, we are facing a technical problem which is the inability to transform *S.latifolia.* As an alternative, we are currently investigating the function of *AtMSI4* from *A.thaliana*, which is most likely an ortholog of *SlY1*.

The immunolocalization experiments show that the SlY1/SlX1 are not detected in the arrested sexual organs: the undifferentiated filament in the male flowers (f, Figure 6C) and the arrested stamens in the female flowers (st, Figure 6L). Thus, while these genes are expressed in sexual organs during cell division and early differentiation, they are not expressed at equivalent developmental stages in sexual organs undergoing developmental arrest. This absence of expression could either be the cause or the consequence of the developmental arrest. In other words, these genes, or upstream genes of the pathway they belong to, could be under the control of the sex determination genes in reproductive organs. Promoter analysis and two hybrid approaches should allow the identification of proteins interacting with SlY1 and SlX1, and possibly the sex determination genes. *SlY1* and *SlX1* may provide faster access to sex determination genes in *S.latifolia*, since tagging approaches are not yet available in this species and chromosomal strategies would require microdissection of more specific regions of the Y chromosome.

Materials and methods

Plant material

The original *S.latifolia* plants were harvested in the Fontainebleau forest (France) in 1987. The wild-type male (RAF63) and the wild-type female (MR1) were crossed for five generations (only two parents for each generation) in order to obtain genetically homogeneous populations. Flowers were harvested at different stages according to the length of the flower: stage 1 are <1 mm long, stage 2 are 1–2.5 mm long and stage 3 are 2.5–10 mm long. Corresponding developmental stages were deduced from the length of the flowers as described by Farbos *et al*. (1997); male flowers at stage 1 (floral meristems to anthers at the tetralobal stage), male flowers at stage 2 (anthers before and during meiosis) and male flowers at stage 3 (anthers after meiosis to binucleate pollen); female flowers at stage 1 (floral meristems to gynoecium with five distinct locules), female flowers at stage 2 (carpels with differentiated tissues and vascularization established) and female flowers at stage 3 (developing ovules to embryo sac formation).

Scanning electron microscopy

Male and female flowers were prepared as described by Ye *et al*. (1991).

Chromosome microdissection

Y chromosomes were microdissected from metaphase spreads obtained from root cultures transformed with *Agrobacterium rhizogenes*, according to the protocol described by Hernould *et al*. (1997).

Screening of the library

The Y-specific probe was used to screen 35 000 phages from a cDNA library of male flowers at pre-meiotic and meiotic stages (Barbacar *et al*., 1997). Two microgrammes of Y-specific DNA amplified by DOP–PCR were radiolabelled by random-priming using 500 μCi of [α-^{32}P]dCTP following the instructions from the Random Priming DNA Labelling kit from Boehringer Mannheim. Hybridization was performed at 65°C in 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 5× Denhardt's (Sambrook *et al*., 1989), 1% sodium dodecylsulfate (SDS) and 0.1 mg/ml of sperm DNA. Washes were as follows: 15 min at 65°C in 2× standard saline citrate (SSC) (Sambrook *et al*., 1989), 0.1% SDS, 15 min at 65°C in 1× SSC, 0.1% SDS, 15 min at 65°C in 0.5× SSC, 0.1% SDS and 15 min at 65°C in 0.1× SSC, 0.1% SDS.

Genomic Southern blot analysis

Genomic DNA was extracted as described by Vallejos *et al*. (1992) and then treated with RNase A (100 μg/ml) for 30 min at 37°C. DNA was then purified by phenol/chloroform extraction and ethanol precipitated. Ten microgrammes of restricted genomic DNA were separated by electrophoresis on a 0.8% agarose gel and transferred by capillary blotting onto Hybond N+ (Amersham) in 0.4 N NaOH. Probes were radiolabelled following the instructions from the Random Priming DNA Labelling kit from Boehringer Mannheim. For small fragments, specific priming was used by replacing the random hexamers by specific primers at a final concentration of 5 μM. Hybridization and washes were as for the screening of the library.

Northern blot analysis

Total RNA was extracted from different tissues as described by Ausubel *et al*. (1990). Twenty microgrammes of total RNA was separated by electrophoresis in a 1% agarose gel containing formaldehyde as described by Sambrook *et al*. (1989). The RNA was then transferred onto Hybond N (Amersham) in 10× SSC. Probes were radiolabelled using a random priming DNA labelling kit (Boehringer Mannheim). Hybridization was performed at 42°C in 50% formamide, 6× SSC, 5× Denhardt's, 0.5% SDS, 0.1 mg/ml sperm DNA. Washes were as follows: 20 min at 50°C in 2× SSC, 0.1% SDS, 20 min at 50°C in 1× SSC, 0.1% SDS, 30 min at 50°C in 0.1× SSC, 0.1% SDS.

Western blot analysis

A mixture of three synthetic peptides CKKAGNGNSDNP (aa 222–233), CVDWPHDENL (aa 295–304), and CVAELDKFRS (aa 445–454) was used to immunize two rabbits (CovalAB, Oullins, France). A fragment of *SlY1* cDNA (from the S1 primer to the stop codon) was subcloned into the expression vector pQE-30 (Qiagen). The corresponding protein was expressed in *Escherichia coli* and subsequently used to affinity-purify the polyserum. The preimmune serum did not recognize this protein confirming the specificity of the antibodies. These purified antibodies were used in Western blot analysis. Total protein was extracted from different tissues by grinding in liquid nitrogen. The extracts were resuspended in the SDS–PAGE loading buffer, boiled for 5 min and centrifuged for 20 min at 18 000 *g* at 4°C to remove cellular debris. Protein from the supernatant was precipitated with acetone and the protein concentration was measured as described by Lowry *et al*. (1951). Ten microgrammes of protein was separated by SDS–PAGE electrophoresis following the method of Laemmli (1970). Proteins were subsequently transferred onto nitrocellulose with a semi-dry Milliblot SDE (Millipore) at 2 mA/cm^2 for 30 min in 10 mM CAPS pH 11, 10% (v/v) methanol. Proteins were revealed with Ponceau-S red 10% (v/v) (Sigma). Immunodetection was performed using an ECL detection reagent (Amersham).

PCR

DOP–PCR was performed using two degenerate primers 5′-CCGACTCGAGNNNNNNATGTGG-3′ and 5′-CCGACTCGAGNNNNAANNATGG-3′) on pools of 10 microdissected Y chromosomes. Each pool of chromosomes was PCR-amplified with one of the primers as described by Hernould *et al*. (1997). A second round of amplification with the same primer was performed on a 1/20th of the volume of the first reaction as template in order to accumulate sufficient DNA. PCR products obtained with the two primers were pooled to make the Y-specific probe.

For the RACE–PCR experiment, RNA was extracted from male flowers at stage 1. The synthesis of cDNA and RACE–PCR were performed with the Marathon kit from Clontech according to the manufacturer's instructions. The primer S7 5′-AAATTCAGATCCCATGTAGCCGCTTGCT-3′ (Figure 3A) was used to amplify

the 3′ region of *SlY1* and *SlX1* cDNA. The antisense primer AS6 5′-TGACTTATCCTTTCCTCCAGAAAGCACC-3′ (Figure 3A) was used to amplify part of the 5′ region of the *SlY1* cDNA and a second RACE–PCR amplification with a new antisense primer AS13 5′-GAGGGTATTAGGAACACTCCCATCAGTC-3′ (Figure 3A) was used to amplify an upstream fragment containing the putative initiation codon as well as 74 bp of putative 5′ untranslated region.

For RT–PCR experiments, total RNA was extracted as for Northern blot analysis. RT–PCR was performed as described by Frohman *et al*. (1988). For reverse transcription, the primer 5′-GACTCGAG-TCGACATCGATTTTTTTTTTTTTTTT-3′ was used (Frohman *et al*., 1988). The primers were as follows: S1, 5′-ATTCGCTCTTGC-AATGTGCTCTTCTGAA-3′; S14, 5′-AGAGATGACGGAGAAAG-GGAAAGGAGGC-3′; S11, 5′-ATTTCAATATCGCAGCAGTA-3′; AS9, 5′-GACCTCTCAGTGAATCCGTCCACCTCAA-3′; S12, 5′-GATAATAATGCTCAAGGGTT-3′; and AS10, 5′-GATAAATA-GGAGGGTGCCATTTCATTAC-3′. Two primers 5′-GATTTGGC-ATCACACTTTCTACA-3′ and 5′-GTTCCACCACTGAGCACAC-AATG-3′ specific for the *actin* gene from *A.thaliana* and which also hybridized to the gene from *Silene*, were used as an internal control and confirmed that an equal amount of amplifiable cDNA was available in all the samples. PCR amplification was then performed from 0.5 µl of the reverse transcription reaction using 0.6 U Taq polymerase from Pharmacia, 0.4 µM final of each specific primer and 200 µM final of each dNTP. PCR cycles were as follows: 5 cycles of 30 s at 94°C, 1 min at 65°C, 2 min at 74°C, followed by 25 cycles of 30 s at 94°C, 1 min at 55°C, 2 min at 72°C, and were performed in a GeneAmp PCR System 2400 (Perkin Elmer).

Cloning and sequencing

For the cloning of DNA fragments, we used pBlueScript (Stratagene) except for PCR products where we used the vector pGEM-T Easy (Promega). Nucleotide sequences were determined by the dideoxynucleotide chain termination method using a 373DNA sequencer from Applied Biosystems and ABI PRISM dye primer or dye terminator cycle sequencing kit from Perkin Elmer. Sequencing data were analysed using BLASTX and FASTA (GCG 7.3 version). Multiple sequence alignments were performed using the program MUST (Philippe, 1993). Distance trees were calculated using the neighbour-joining method with 1000 bootstrap replicates.

Immunolocalization

Fixation, embedding, sectioning, dewaxing and rehydration of the tissues were as described previously (Barbacar *et al*., 1997). Tissue sections on the slides were then treated twice for 1 h with blocking solution (1% BSA, 0.3% Triton X-100 in TBS) and rinsed for 1 min in TBS (10 mM Tris pH 8, 150 mM NaCl). Sections were then incubated with 500 µl of the purified antibody (as for Western blot analysis) in the blocking solution for 2 h at room temperature and then rinsed five times in TBS. Sections were incubated for 1 h at room temperature with 500 µl of the secondary antibody conjugated to alkaline phosphatase diluted in the blocking solution. Sections were rinsed five times in TBS and incubated for 2 min in the detection buffer (100 mM Tris pH 9.5, 5 mM $MgCl_2$, 100 mM NaCl) at room temperature before incubation for 4–24 h at room temperature in the dark in the detection buffer containing 0.225 mg/ml of nitroblue tetrazolium and 0.175 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate.

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