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# Male Specific Genes from Dioecious White Campion Identified by Fluorescent Differential Display

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**Fluorescent differential display (FDD) has been used to screen for cDNAs that are differentially up-regulated in male flowers of the dioecious plant *Silene latifolia* in which an X/Y chromosome system of sex determination operates. To adapt FDD to the cloning of large numbers of differential cDNAs, a novel method of confirming the differential expression of these has been devised. FDD gels were Southern electro-blotted and probed with mixtures of individual cDNA clones derived from different FDD product ligation reactions. These Southern blots were then stripped and re-probed with further mixtures of individual cloned FDD products to identify the maximum number of recombinant clones carrying the true differential amplification products. Of 135 differential bands identified by FDD, 56 differential amplification products were confirmed; these represent 23 unique differentially expressed genes as determined by virtual Northern analysis and two genes expressed at or below the level of detection by virtual Northern analysis. These two low expressed genes show bands of hybridization on genomic Southern blots that are specific to male plants, indicating that they are derived from, or closely related to, Y chromosome genes.**

**Key words**: Fluorescent differential display — Sex chromosome — Sex determination — *Silene latifolia* — White campion — Y chromosome.

## Introduction

*Silene latifolia* is one of a small number of dioecious plant species in which the sex of individuals is determined by morphologically distinguishable sex chromosomes. Male plants (2n = 22,XY) possess a Y chromosome that contains dominant genes within two separate regions responsible for the promotion of stamen development and the arrest of pistil development, respectively (Westergaard 1958, Ye et al. 1991, Grant et al. 1994a, Moneger et al. 2000). In female plants (2n = 22,XX), which lack the Y chromosome, stamen primordia do not develop into stamens and pistil development proceeds without arrest. A number of male flower-specific cDNAs from *S. latifolia* have been isolated by subtractive hybridization and differential screening (Matsunaga et al. 1996, Barbacar et al. 1997, Hinnisdaels et al. 1997, Matsunaga et al. 1997, Robertson et al. 1997, Scutt and Gilmartin 1997, Scutt et al. 1997, Scutt and Gilmartin 1998). These previous studies have succeeded in cloning numerous sequences that are up-regulated as a consequence of Y chromosome gene expression with only two reports to date of the identification of Y chromosome-encoded genes (Delichère et al. 1999, Atanassov et al. 2001). The extremely limited success in identification of Y chromosome genes involved in sex determination by differential screening methods indicates a likelihood that these are either not abundantly expressed, or are expressed only transiently during early male flower development.

In this work, the technique of fluorescent differential display (FDD) has been used to clone cDNAs that are up-regulated in male flowers at the early stages of development during which sex-specific differences between male and female flowers first become apparent. The FDD technique, introduced by Bauer et al. (1993) and Ito et al. (1994), is an efficient method designed for high throughput screening of differential transcripts. It uses limited amounts of tissue and is extremely reproducible (Uchida et al. 1998, Kreps et al. 2000, Kuno et al. 2000, Loyall et al. 2000). FDD is a modification of the differential display technique of Liang and Pardee (1992) in which reverse transcriptase polymerase chain reaction (RT-PCR) products are generated from mRNA samples to be compared using combinations of oligo-dT primers and arbitrary decamer primers. In differential display, RT-PCR products are radiolabeled and autoradiographed from DNA sequencing gels for comparison of banding patterns, whereas in FDD, RT-PCR products are detected in a fluorescence DNA sequencer or gel scanner from fluorescent label incorporated into the oligo-dT primer. The male specific sequences from *S. latifolia* identified using this technique include two sequences corresponding to Y chromosome encoded genes.

Though FDD is a more rapid and reproducible technique than conventional differential display, problems may still arise during the cloning of differentially expressed FDD products. Non-differential RT-PCR products frequently co-migrate with differential bands on the gels used to analyse differential display or FDD samples (Callard et al. 1994) and these may

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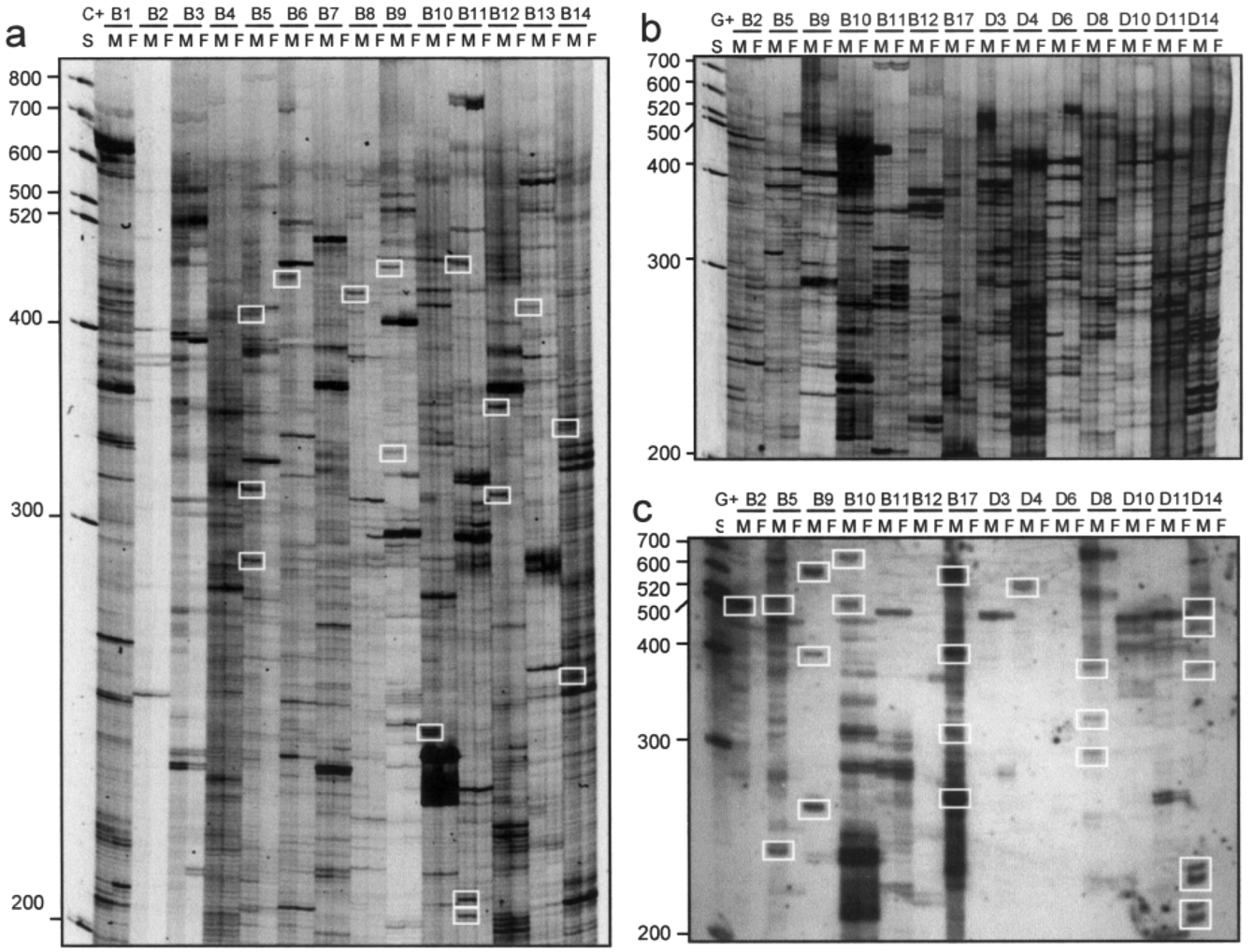


**Fig. 1** FDD analysis and Southern hybridizations of electro-blotted FDD gels. (a) An FDD gel image showing products derived from male (M) and female (F) *S. latifolia* flower bud RNA using a C-anchor 3′-primer together with Operon Technologies arbitrary decamers B1-B14. Differential bands excised from gels for cloning purposes are boxed. (b) A fluorescence image of an FDD gel showing products derived from male (M) and female (F) *S. latifolia* flower bud RNA using a G-anchor primer and arbitrary decamer primers as shown, prior to Southern electro-blotting. (c) A chemiluminescent detection image of a Southern electro-blot of the FDD gel shown in (b) probed with 20 labeled PCR products derived from cloned, putatively differential FDD products. Signals specific to male tracks, indicated by boxes, identify FDD products in the mixed probes that were derived from differential FDD bands.

amplify or ligate to plasmid vectors more efficiently than the desired differential sequences. If the number of differential bands to be cloned is small, then much effort can be directed to the cloning of each one. However, it was anticipated that the number of differences in gene expression exhibited between male and female flower buds of *S. latifolia*, even at an early developmental stage, would be relatively high. For this reason a novel strategy was designed for the work presented here that enabled the rapid screening in parallel of large numbers of cloned RT-PCR products to distinguish those derived from differential FDD bands from those derived from non-differential FDD bands.

## Results

*FDD analysis of S. latifolia flower bud RNA*

FDD reactions using 140 primer combinations were performed to identify male-specifically-expressed cDNAs from early *S. latifolia* flower buds. In total, 135 bands specific to tracks from male flower FDD products were excised for further study. Fig. 1 shows a typical result of FDD analysis with male-specific bands indicated in Fig. 1a. Following the cloning of FDD products, clones resembling in size the FDD bands from which they were putatively derived were recovered from 98 of the 135 differential FDD bands initially identified. Cloned differential display products have frequently been found to result from non-differential bands of close molecular

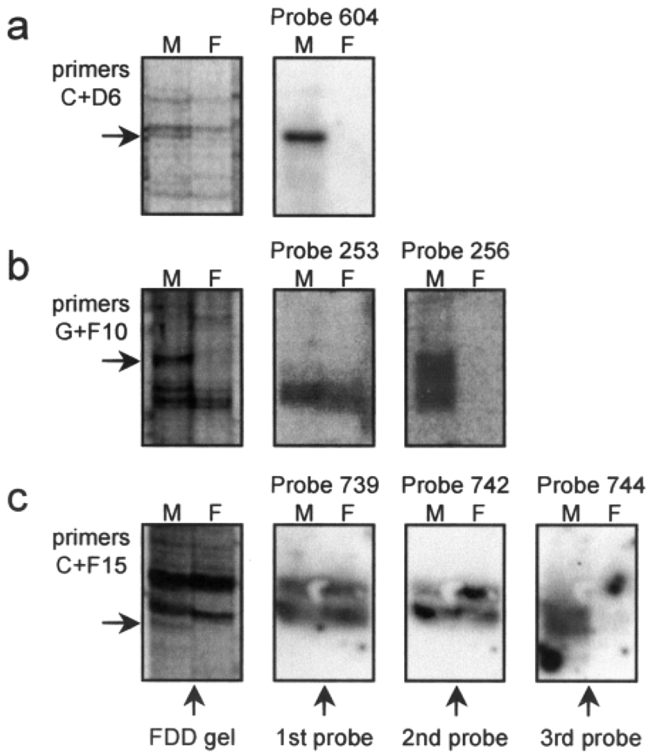


**Fig. 2** Sequential Southern hybridization for the authentication of cloned FDD products. Portions of FDD gels containing differential bands (indicated by horizontal arrows) amplified using the primer combinations shown are aligned with Southern blot chemiluminescent images of (a) 1st, (b) 2nd and (c) 3rd round probes derived from these gels. The Southern blots have been probed sequentially with mixtures of cloned *Men* FDD products containing, amongst other FDD products, those indicated above the blot images. Probes that contained *Men* cDNAs homologous to differential FDD bands are revealed by hybridization signals in male tracks only on Southern blots after one (*Men-604*), two (*Men-256*) and three (*Men-744*) hybridization experiments, respectively.

size to the target differential bands. To identify genuine differential clones, further aliquots of FDD amplification products were run on denaturing polyacrylamide gels and electro-blotted onto nylon hybridization membranes. The resulting blots were then probed with mixtures of cloned *Men* cDNAs. A fluorescence image of an FDD gel prior to electro-blotting and the result of hybridization of a mixture of labeled *Men* probes to that blot are shown in Fig. 1b and 1c, respectively. Male track-specific signals on Fig. 1 at molecular sizes corresponding to excised FDD bands indicate that a genuinely differential FDD product has been cloned.

In many cases, the cloned FDD products initially used as probes in the experiments described here hybridized specifically to differential FDD bands derived from male but not female RNA on Southern blotted FDD gels. This observation indicated that the recombinant clone selected at random from the population of cloned products of a specific FDD band corresponded to the originally observed differential product. However, in other cases, hybridization signals in both male and female tracks arose at molecular sizes similar to those of excised FDD bands, indicating that colonies selected from the population of cloned products contained non-differential FDD products that had co-migrated on FDD gels with differential bands. In these cases, further recombinant clones were selected and used to re-probe Southern blots of FDD products. Southern blots were typically probed three or four times with different mixtures of individual *Men* PCR products until differential hybridization signals for as many differential FDD bands as possible had been identified. Fig. 2 illustrates this process of sequential Southern hybridization and shows cases where *Men* clones encoding differential FDD products were identified on the first (Fig. 2a), second (Fig. 2b) and third (Fig. 2c) screenings, respectively. In this way a total of 56 cloned *Men* cDNAs were identified as representing apparently male-specific bands on FDD gels by virtue of their ability to hybridize to the original differential amplification product.

*Sequences of Men cDNAs derived from FDD analysis*

DNA sequence data from the 56 *Men* clones identified by FDD revealed 35 unique sequences and 21 that were duplicated within the cloned population. Clones derived from the same mRNA source were particularly frequent where several differential FDD bands were identified as different sized products derived from the same primer combinations. This finding perhaps reflects the ability of some decamer primers to anneal to different regions of the same cDNA, or alternatively, different lengths of 3′-untranslated regions on allelic mRNA templates. Furthermore, the G-anchor and C-anchor primers yielded cognate differential cDNAs in a number of cases when used with particular arbitrary decamer primers. This may reflect allelic mRNA templates that differed in the nucleotide residue immediately preceding their poly-A tails. Of the remaining 35 unique sequences, seven were found to be equally expressed in both male and female *S. latifolia* flower buds (results not presented). The selection of these non-differential sequences by FDD can also be explained by allelic diversity within the heterogeneous population of plants used in this study. Allelic differences may exist in regions of cDNAs to which arbitrary decamer primers annealed. A further three FDD products showed male-specific expression profiles in blot hybridization experiments, but did not hybridize to *S. latifolia* genomic DNA in Southern analyses (results not presented). It was concluded that these three sequences may have arisen from some virus or other plant pathogen in one or more individuals amongst the population of male plants, but absent from the female plants used for FDD analysis.

Following the elimination of constitutively expressed, duplicated and putatively pathogen-derived FDD products, 23 unique, male-enhanced or male-specific sequences remained for further analysis plus two that were expressed at or below the level of detection by virtual Northern analysis. Sequence

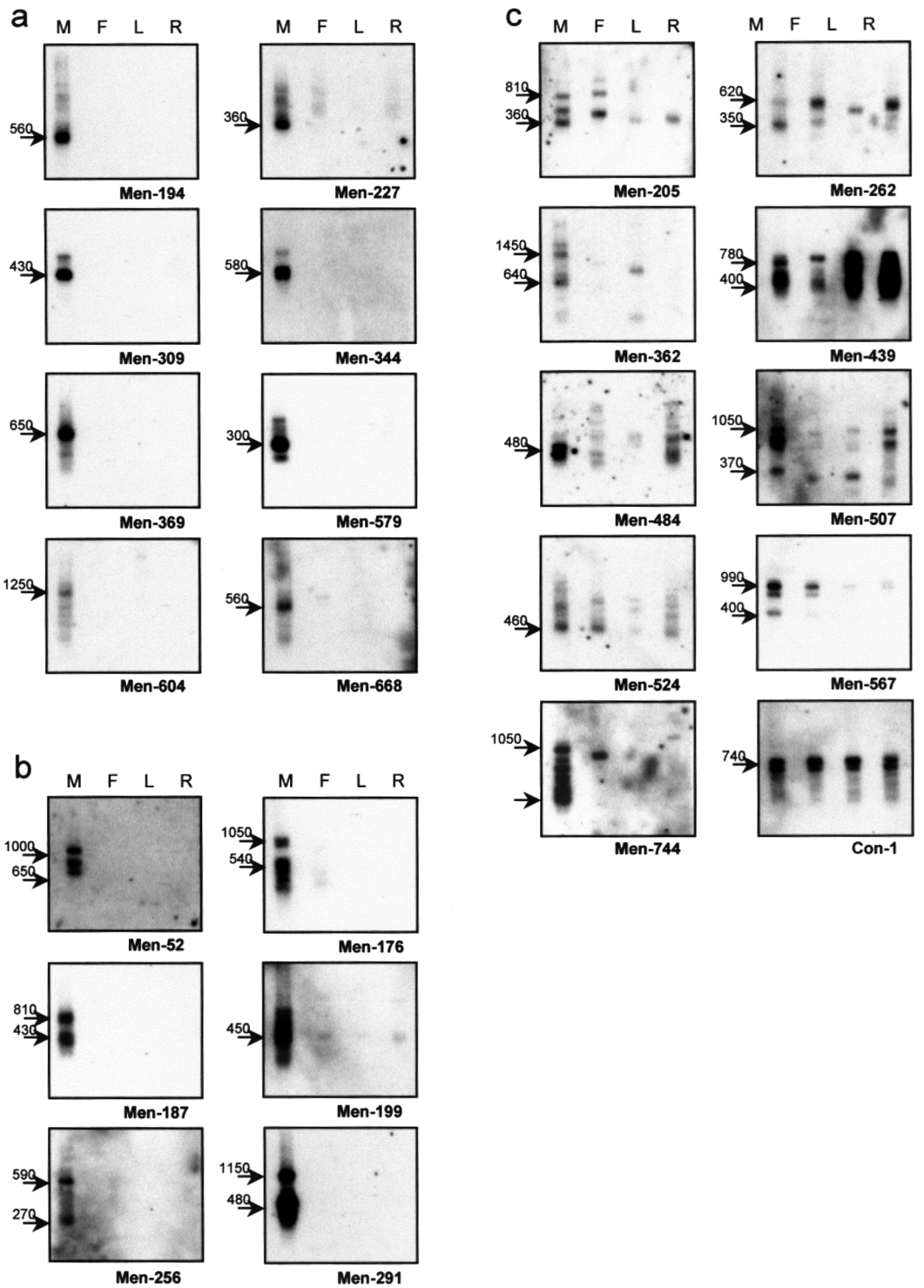


**Fig. 3** Virtual Northern hybridizations of twenty-five Men cDNA probes derived from FDD analyses. The Virtual Northern blots shown contain tracks of RT-PCR products derived from *S. latifolia* RNA of young male (M) and young female (F) flower buds, leaves (L) and roots (R). (a) Sequences showing predominantly single male specific hybridization bands, (b) multiple male specific hybridization signals and (c) distinct hybridization signals with male and female samples. Relative loading of tracks is demonstrated using a nominally constitutive FDD probe, *Con-1* shown in (c). Molecular sizes, or size ranges, of prominent hybridization signals are indicated.

**Table 1** Expression dynamics of *Men* genes as defined by virtual Northern analyses

| Expression profile | *Men* genes with this expression profile |
|---|---|
| Single male flower-specific hybridization signal | *Men-194, Men-227, Men-309, Men-344, Men-369, Men-579, Men-604, Men-668* |
| Multiple male flower-specific hybridization signal | *Men-52, Men-176, Men-187, Men-199, Men-256, Men-291* |
| Male flower specific-signal with different hybridization signals in other tissues | *Men-205, Men-262, Men-362, Men-439, Men-484, Men-507, Men-524, Men-567, Men 744* |
| Expression below detectable levels | *Men-153, Men-470* |

Expression of the *Men* genes characterized here fall into four different categories as shown. Database accession numbers are as follows: *Men-52*, AJ430354; *Men-153*, AJ430356; *Men-176,* AJ430357; *Men-187*, AJ430358; *Men-194*, AJ430359; *Men-199*, AJ430360; *Men-205*, AJ430361; *Men-227*, AJ430362; *Men-256*, AJ430363; *Men-262*, AJ430364; *Men-291*, AJ430365; *Men-309*, AJ430366; *Men-344*, AJ430367; *Men-362*, AJ430368; *Men-369*, AJ430369; *Men-439*, AJ430370; *Men-470*, AJ430371; *Men-484*, AJ430372; *Men-507*, AJ430373; *Men-524*, AJ430374; *Men-567*, AJ430375; *Men-579*, AJ430376; *Men-604*, AJ430377; *Men-668*, AJ430378; *Men-744*, AJ430379; *Con-1*, AJ430355.

analysis of these 25 cDNAs was performed and homologies with sequences in protein and nucleic acid databases were investigated. As the FDD products represented the 3′ ends of cDNAs, many did not show a long enough open reading frame to confidently predict a translation product. However, all open reading frames longer than ten amino acid residues continuous with the 5′ ends of FDD products were used in peptide sequence searches and complete nucleotide sequences were also used to search nucleic acid sequence databases. Seven *Men* cDNAs showed clear homology to database accessions. The following description of these homologies gives the EMBL database accessions to which *Men* sequences matched, followed in parentheses by the percentages of predicted amino acid sequence identities and the lengths of matched predicted peptide regions.

The Men-176 and Men-604 predicted peptides showed homology to those of two different *Arabidopsis thaliana* genomic sequences of unknown function: respectively accessions AC003680 (52%, 32 residues) and AC000098 (64%, 71 residues). The Men-194 predicted peptide showed highest homology to coumarate acetyl co-enzyme A ligase from soybean: accession X69954 (65%, 79 residues), and also is homologous to the same enzyme from numerous other species. The Men-369 predicted peptide was identical to that predicted from the previously published *S. latifolia Men-8* cDNA: accession Y08780 (100%, 37 residues) and homologous to tapetum-specific proteins from a number of plant species. The Men-439 predicted peptide showed homology to those of three tissue-specifically expressed sequences as follows, tomato predominantly leaf-expressed mRNA: accession U19886 (74%, 63 residues); banana ripening-associated mRNA: accession AF001530 (57%, 47 residues) and asparagus post-harvest accumulating mRNA: accession X77320 (62%, 59 residues). The Men-484 predicted protein was homologous to phosphoglycerate kinases from many species and most similar to that from wheat: accession X15232 (97%, 41 residues). The Men-524 predicted peptide showed homology to malonyl co-enzyme A acyl carrier protein transacylase from many prokaryotes and is most homologous to an *A. thaliana* genomic sequence that may encode a similar function: accession AC0004165 (85%, 64 residues). The Men-744 predicted protein was homologous to numerous cysteine proteases and showed greatest similarity to the vignain precursor protein from *Vigna mungo*: accession X15732 (66%, 39 residues). Many of these male-specific sequences show similarity to sequences not previously implicated in male-specific flower development.

*Expression patterns of Men cDNAs*

The expression patterns of *Men* cDNAs cloned from FDD gels were investigated by virtual Northern blotting (Clontech). Seven of the 35 unique sequences characterized by sequence analysis gave signals of equal intensity from male and female flower tracks on virtual Northern blots (data not shown). Two sequences, *Men-153* and *Men-470* were expressed at such low levels that expression could barely be detected after extended exposure of X-ray film (data not shown). Further analysis of the expression of these sequences was determined by RT-PCR (see below). Virtual Northern blots for the remaining 23 male-enhanced or male-specific *Men* sequences identified in this work are presented in Fig. 3. The constitutively expressed sequence (*Con-1*) (Fig. 3c) was used to re-probe one of the virtual Northern blots to indicate relative loading of gel tracks. All of the virtual Northern blots from *Men* clones presented show male-specific or male-enhanced banding patterns. However, the hybridization patterns shown can be divided into three different groups as summarized in Table 1. Eight probes show a single hybridizing band specific to early male flower RNA, with no signals in tracks from young female flowers, leaves or roots (Fig. 3a). A further six *Men* cDNAs also show entirely male-specific banding patterns, though hybridize to more than one size of PCR amplification product derived from male-specific transcripts (Fig. 3b). The third category of nine *Men* cDNAs

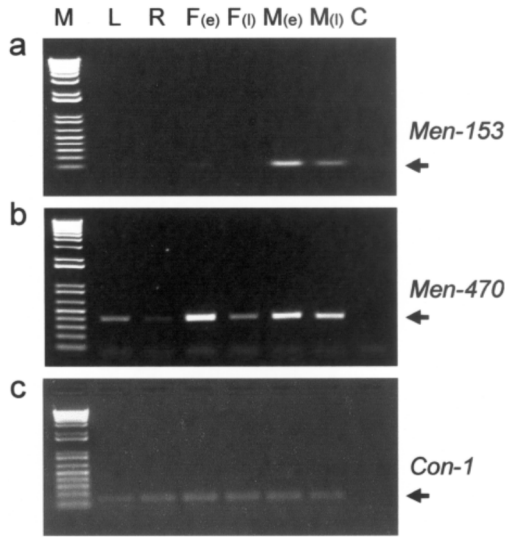


**Fig. 4** RT-PCR expression analysis of *Men-153*. RT-PCR reactions from leaf (L), root (R) and early female ($F_{(e)}$), late female ($F_{(l)}$), early male ($M_{(e)}$) and late male ($M_{(l)}$) flowers were carried out with primers designed to either (a) *Men-153,* (b) *Men-470*, or (c) *Con-1*. PCR products were fractionated on 1.2% agarose TAE gels using 1kb ladder markers (M).

show male flower-specific or male flower-enhanced bands, though also show additional bands of lower signal intensity or different molecular size in tracks derived from female flowers, leaves or roots (Fig. 3c). We were unable to reliably detect transcripts corresponding to *Men-153* and *Men-470* on virtual Northern blots, and could only detect these by RT-PCR.

*Analysis of Men-153 and Men-470 expression by RT-PCR*

The absence of a signal on virtual Northern blots for *Men-153* and extremely weak signal observed from *Men-470* (data not shown) prompted us to investigate their expression profiles using RT-PCR. Two primers were designed to each cDNA sequence that would generate 150 bp and 400 bp PCR products for *Men-153* and *Men-470* respectively. In addition, two primers were designed to the nominally constitutive control *Con-1* that would produce a product of approximately 150 bp. RT-PCR analysis was undertaken using RNA derived from leaves and roots, as well as two stages of male and female flower buds. Early flower buds at stage 1 and late flower buds after stage 3 (Scutt et al. 1997) were compared. Fig. 4 shows the results of these analyses. Transcripts corresponding to *Men-153* (Fig. 4a) can only be detected in male flower buds, and not in leaf, root and female flower buds. It is of interest that the intensity of the amplification product from early male flowers is greater than that from late male flowers, although it should be noted that this is a non-quantitative analysis. Surprisingly we were able to detect *Men-470* (Fig. 4b) in all tissues analysed despite the fact that this sequence was identified and confirmed as male specific by FDD analysis. The potential significance of these two sequences with regard to sex determination is highlighted by data obtained from Southern analysis of male and female genomic DNA (Fig. 5) in which both *Men-153* and *Men-470* identify Y chromosome located DNA sequences (see below). The RT-PCR control reactions monitoring the nominally constitutive *Con-1* transcript are shown in Fig. 4c.

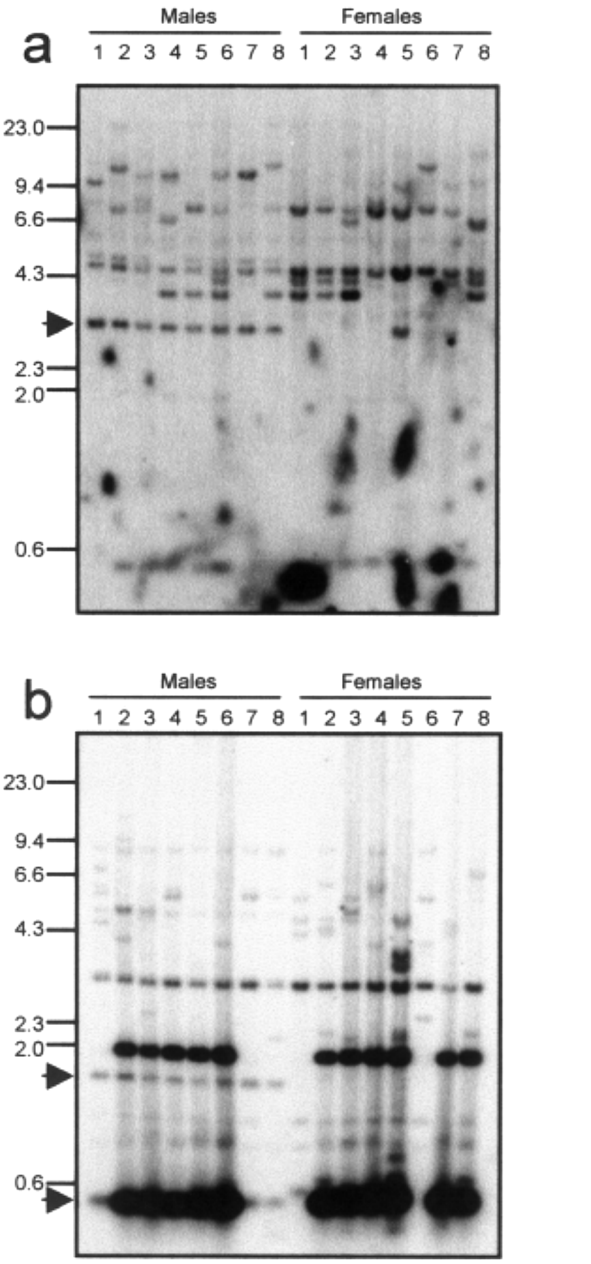


**Fig. 5** Genomic Southern hybridization of cDNAs homologous to Y chromosome sequences. Hybridizations are shown of (a) *Men-153* and (b) *Men-470* probes to *Hin*dIII digests of genomic DNA digests from eight male (M1 to M8) and eight female (F1 to F8) plants from a heterozygous population of *S. latifolia*. Positions of size markers are shown at the left. Arrows indicate the Y chromosome and asterisks indicate X chromosome bands referred to in the text.

*Genomic organization of Men sequences*

The genomic organization of *Men* sequences was examined to determine whether any of these could be located on the Y chromosome and potentially involved in sex determination.

Genomic Southern hybridizations were performed using probes derived from the 25 different *Men* cDNAs identified in the present study using *Hin*dIII- and *Eco*RI-digested genomic DNA isolated from two male and two female plants (data not shown). Hybridization data for some clones suggested that they were single copy genes, while others were members of small multigene families. All 25 *Men* cDNAs revealed polymorphisms within the group of four plants investigated for one or both of the restriction endonucleases used. In some cases, these polymorphisms were slight and affected the positions of only one or two hybridizing bands. For other probes, the degree of restriction fragment length polymorphism was high, yielding different banding patterns for each individual plant investigated and with both restriction enzymes used.

No probe hybridized uniquely to genomic DNA from male plants: a pattern that would clearly indicate a Y chromosome-gene. However, the two probes *Men-153* and *Men-470* showed sufficient differences in banding patterns or signal intensity between male and female plants to warrant further investigation. For these two clones, further Southern analyses on larger numbers of plants were performed. For each of the cDNAs *Men-153* and *Men-470*, male-specific bands corresponding to a Y chromosome sequences were detected. Southern blots of *Hin*dIII genomic digests from eight male and eight female plants probed with the *Men-153* and *Men-470* cDNAs presented in Fig. 5. Hybridization with *Men-153* reveals a 2.9 kb *Hin*dIII fragment present in all male plants and absent from all female plants (Fig. 5a). Several further *Men-153* bands are present, which show polymorphisms that are independent of the sex of the plants tested. In addition to these male-specific and polymorphic bands, a *Hin*dIII fragment of 4.5 kb is revealed by the *Men-153* probe in digests from all the plants tested. A Southern blot of *Eco*RI-digested DNA (not presented) showed a male-specific *Men-153* band at 3.0 kb, in addition to some non-sex-specific bands, similar to those observed by *Hin*dIII digestion. These non-sex-specific *Hin*dIII and *Eco*RI bands revealed by the *Men-153* probe show approximately twofold higher signal strength in female plants than in males. This result suggests that these *Men-153*-related genomic fragments may be X-linked, as female possess two copies of the X chromosome per diploid genome, whereas male possess a single X copy.

The *Men-470* probe (Fig. 5b) reveals a male-specific *Hin*dIII fragment at 1.5 kb. A striking feature of *Men-470* Southern hybridization is that in some plants (males 2, 3, 4, 5 and 6, and females 2, 3, 4, 5, 7 and 8), two very strong bands are revealed at 2.0 kb and 0.53 kb, whereas in the remaining plants these bands are absent. In those males that lack these strong bands (males 1, 7 and 8), a second male specific hybridization signal is visible at 0.5 kb. This band is not present in the two female plants (females 1 and 6), which lack the high copy-number band at that same molecular size. The presence of the male specific genomic fragments indicates that *Men-470* or a related sequence is located on the Y chromosome. In addition, a non-sex-specific *Hin*dIII fragment, which appears slightly stronger in females, is present in all plants at 3.2 kb. As for *Men-153*, these results suggest that a second *Men-470*-related sequence is present on the X chromosome.

## Discussion

Two cDNAs have been identified in the present work that both appear to encode members of gene families that each identify Y chromosome sequences of *S. latifolia*. The only other genes so far identified on the Y chromosome of *S. latifolia*, *SlY1* (Delichère et al. 1999, Filatov et al. 2000) and *SlY4* (Atanassov et al. 2001) are not flower specifically-expressed, suggesting that they do not play a key role in the process of sex-determination. Southern blotting with either of the *Men-153* and *Men-470* cDNAs as probes reveal one genomic DNA fragment present in all male plants, but absent from all female plants, indicating the presence of this fragment on the Y chromosome. In addition to the Y chromosome copy, both of the *Men-153* and *Men-470* cDNAs reveal further bands of hybridization that are present in all male and female plants, but with approximately twofold greater intensity in females. These results suggest the presence of a second copy homologous to each of the two genes that is located on the X chromosome. It is not known at present whether the sequenced *Men-153* and *Men-470* cDNAs identified by FDD represent transcripts of the identified Y chromosome genomic fragments, or of the other, possibly X-linked genomic DNA fragments.

The Southern hybridization pattern for *Men-470* is markedly different from that of *Men-153* in one important respect. Unlike *Men-153*, *Men-470* hybridizes to two *Hin*dIII bands of very high signal intensity, representing some tens to hundreds of genomic copies of a DNA fragment. These two *Hin*dIII bands are always co-inherited, suggesting that they are derived from a single repeated element that is cleaved by *Hin*dIII over that part of its sequence that is homologous to the *Men-470* cDNA probe. One of these amplified bands occurs at a restriction fragment size (2 kb) that is different from those of the Y chromosome *Men-470*-related band (1.5 kb) and the second, possibly X-linked fragments (3.2 kb). The highly iterated repeats of *Men-470*-like sequences are present in nearly 70% of the plants analyzed, and absent from the rest. When present, the copy-number of this amplified genomic fragment appears to be consistent between plants, suggesting that these repeats occur within one genomic region that is inherited, or not, in its entirety. The presence or absence of this amplified region is not related to the sex of the plant: in the analysis presented in this work, five out of eight males, and six out of eight females possessed an amplified *Men-470*-related genomic region. The chromosomal location of these *Men-470*-related repeats is not

known, but cannot be uniquely on the Y chromosomes due to the presence of these repeats in a proportion of female plants.

Both *Men-153* and *Men-470* are expressed at very low levels: *Men-153* proved undetectable by Northern blotting, and *Men-470* was at the limit of detection by this technique. Using RT-PCR we have shown that *Men-153* is expressed specifically in male flowers. Though *Men-153* is male-specifically expressed (Fig. 4a) and homologous to at least one Y chromosome-linked sequence (Fig. 5a), it is possible that the expressed *Men-153* gene may be situated on the X chromosome or on one of the autosomes. Results from RT-PCR analysis of *Men-470* expression are surprising and show that transcripts are present in all tissues analyzed, which include both male and female flowers. This finding suggests that the expression of *Men-470* is not derived from the Y chromosome gene copy alone. It is therefore possible that the expressed *Men-470* gene is located on the 3.2 kb *Hin*dIII restriction fragment presumed to be located on the X chromosome, or from one of the highly repeated copies present in both males and females. Discovery of this sequence by FDD as a male specific transcript could have occurred through allelic differences between male and female plants. The *Men-153* and *Men-470* cDNAs show no homology to known gene sequences, though at present only the 3′-ends of these cDNAs have been cloned.

The identification of two expressed cDNA clones with homology to Y chromosome sequences presented here was achieved by a large-scale screen for male-flower-specific cDNAs by FDD. This screen was followed by the analysis of all of the identified male flower-specific cDNAs on Southern blots, to reveal those with homology to genes located on the Y chromosome of male plants. In the course of the large scale FDD analysis undertaken, 56 male flower-specific cDNAs were identified from a total of 98 FDD bands cut from acrylamide gels. To facilitate this scale of experimentation, the present work introduces a novel and efficient method for the identification of cloned cDNAs derived from differential FDD bands, based on the sequential probing of Southern blots of FDD products with mixture of candidate cDNAs.

From the 56 differential cDNAs cloned in the present study, 25 different classes of male-specific or male-enhanced sequences were found to be present. Most of the redundancy in the sequences cloned resulted from near identical FDD products amplified using the C-anchor and G-anchor PCR primers in combination with particular arbitrary decamer primers. As the 3′-terminal base of primers must be of correct match to allow DNA polymerization to commence in PCR reactions (Newton and Graham 1994), it is probable that the duplication arose due to allelic diversity in the population of plants used.

The 25 *Men* cDNAs presented in this work include only one sequence previously identified from studies of *S. latifolia* sex determination that used subtractive hybridization techniques: *Men-369* is identical to *Men-8* (Scutt et al. 1997), which showed expression specifically in early male flower buds. The various subtractive hybridization studies previously undertaken (Matsunaga et al. 1996, Barbacar et al. 1997, Robertson et al. 1997, Scutt and Gilmartin 1997, Scutt et al. 1997) resulted in the cloning of overlapping sets of cDNA sequences, largely representing highly abundant mRNAs specific to male flower buds. The lack of previously-cloned sequences in the population of cDNAs identified in the present study confirms that the FDD technique is capable of identifying different classes of sequences from those obtained by subtractive hybridization, including less abundant and earlier-expressed mRNAs.

Future work will aim to define the expression patterns of *Men-153* and *Men-470* and to determine which of the genomic sequences identified by Southern blotting are transcribed to encode the identified cDNAs. Further studies will provide full-length cDNA of *Men-153* and *Men-470*, enable characterization of genomic sequences, and also map the Y chromosome-specific bands identified on Southern blots to particular regions of the Y chromosome. This will be performed using a series of plants containing deletions of regions of the Y chromosome and which show altered sex determination phenotypes (Farbos et al. 1999, Lardon et al. 1999).

## Materials and Methods

*Plant material*

Seed of *S. latifolia*, originally from a highly heterozygous wild population, was obtained from Chambers' Seeds, 15 Westleigh Road, Barton Seagrove, Northamptonshire, U.K. *S. latifolia* plants were grown from seed under glasshouse conditions and induced to flower with supplementary lighting to increase the photoperiod to 16 h.

*Fluorescent differential display*

RNA was prepared from young male and female flower buds of *S. latifolia* as described previously (Scutt 1997). Flower buds used for FDD analysis included material up to stage 5 in flower development, as defined previously (Grant et al. 1994b), at which distinct anther and carpel organs have formed in males and females, respectively. Aliquots of 20 µg of RNA were treated by incubation with 2 units of RNase-free DNase I (Stratagene) for 20 min at 37°C, extracted with phenol/chloroform and recovered by precipitation in ethanol (Sambrook et al. 1989).

Aliquots of 2.5 µg of RNA from early male and female *S. latifolia* flower buds were used as templates for first-strand cDNA synthesis primed from primers that contained a 3′-terminal dG or dC residue, fifteen dT residues and a Texas Red fluorophor attached to a 5′-terminal dG residue (Yukigoseikagaku). These primers are termed G-anchor or C-anchor, depending on their 3′-terminal base. Synthesis of cDNA by the action of reverse transcriptase was performed using a SuperScript Preamplification System (Gibco-BRL) according to the manufacturers instructions. Newly synthesized cDNA samples were diluted to 200 µl with distilled water. PCR was carried out in 20 µl volumes containing 2 µl of diluted cDNA samples and the following additional components: 1× PCR buffer (Nippon Gene), 1 mM each of the four deoxynucleotides, 250 nM Texas Red-labeled G- or C-anchor primer, 500 nM arbitrary decamer primer, 0.5 units GeneTaq (Nippon Gene) and 0.5 units AmpliTaq (Perkin Elmer). Eighty arbitrary decamer primers (primers sets B, D, F and X, Operon Technologies) were used in conjunction with the G-anchor primer and sixty arbitrary decamers (primer sets B, D and F) were used with the C-anchor primer. Amplifications were performed under mineral oil in a 96-well thermal cycler

(GeneAmp PCR System 9600, Perkin Elmer) for one cycle of 94°C for 15 s, 40°C for 5 min and 72°C for 1 min and then 24 cycles of 94°C for 15 s, 40°C for 2 min and 72°C for 1 min. An additional incubation for 5 min at 72°C was carried out after the final thermal cycle.

PCR products were mixed with equal volumes of a solution containing 95% (v/v) formamide, 5 mM EDTA and 0.5% (w/v) blue dextran. Ten µl aliquots of the resulting solutions were concentrated by evaporation at 60°C for 25 min and denatured by heating to 80°C for 3 min. Samples were electrophoresed on 6% (w/v) polyacrylamide sequencing gels in 1× TBE buffer and 8 M urea (Sambrook et al. 1989) poured between quartz gel plates (TaKaRa). Following electrophoresis, one gel plate was removed from each gel assembly and gels were soaked briefly in distilled water to remove the remaining urea. Gels were scanned using a FM Bio II Multi-Vian Fluorescence Image Analyzer (TaKaRa) and same-size gel images were printed onto paper. Gels were aligned with their printed images and differential bands were excised. FDD gels were then re-scanned to confirm that alignment had been correct.

*Cloning and screening for PCR products derived from differential FDD bands*

Fragments of acrylamide containing differential FDD bands were frozen and thawed three times in 20 µl distilled water to release the DNA. Aliquots of 1 µl from these solutions were then used as templates in further PCR amplification reactions of 50 µl volume using PCR components as specified above, including Amplitaq polymerase, though omitting GeneTaq polymerase, to generate sufficient quantities of PCR products for ligation into plasmid vectors. Amplified products were precipitated in ethanol and redissolved in 5 µl of distilled water. Aliquots of 2 µl from these samples were ligated into the pGEM-T vector (Promega) using a rapid ligation kit (TaKaRa). These ligation reactions were used to transform *E. coli* JM 109 competent cells (TaKaRa). Six bacterial transformants derived from each ligation were selected and FDD products were amplified from these using cloning vector primers in standard PCR reactions (Kid and Ruano 1995). The resulting cloned PCR products were numbered sequentially and given the prefix *Men* (for *Male-enhanced*), as with previously published cDNAs from *S. latifolia* flower buds (Robertson et al. 1997, Scutt et al. 1997). *Men* PCR products were analyzed on 1% agarose gels to confirm that their molecular sizes were similar to those of the differential FDD bands from which they were cloned.

Further samples of FDD PCR amplification reactions were analyzed on denaturing acrylamide gels and imaged on a fluorescence scanner as described above. These gels were lifted onto 3MM paper (Whatman) and electro-blotted onto Hybond N+ membranes (Amersham) using a transfer buffer containing 80 mM Tris/HCl, 118 mM sodium borate and 2.4 mM EDTA (pH 8.3). Blotting was performed on a semi-dry electro-blotting apparatus as described by (Trnovsky 1992). Southern blots containing tracks of FDD products were probed with mixtures of labeled *Men* clones and hybridization signals were detected by exposure to film using a chemiluminescent detection system (AlkPhos Direct, Amersham, U.K.). The mixed probes used for this contained one clone putatively derived from each excised FDD band under investigation. Differential cDNAs were identified from hybridization signals of expected molecular sizes in male tracks only. Where signals were observed in both male and female tracks, blots were stripped of probes by washing at 60°C in 0.5% (w/v) SDS for 1 h and probed with further mixtures of cloned FDD products.

*Virtual Northern and genomic Southern blot analyses*

Virtual Northern blots, which are Southern blots of PCR-amplified cDNA samples, were used to confirm the male-specificity of cloned FDD products. First-strand cDNA was prepared using a Smart cDNA Synthesis Kit (Clontech), such that PCR primer sites were incorporated at both ends of all cDNA molecules. These products were then used in PCR amplifications of entire cDNA populations, as directed in the instructions accompanying the Smart cDNA Synthesis Kit. Amplified cDNA samples were concentrated by ethanol precipitation, resuspended in alkaline gel-loading buffer and analyzed on 1.5% alkaline agarose gels (2 µg per track) together with DNA molecular size markers, as specified by (Sambrook et al. 1989). Alkaline agarose gels were then neutralized and capillary blotted onto Hybond N+ membranes (Amersham), according to the manufacturer's instructions. Probes of *Men* cDNAs and molecular size markers were radiolabeled by random priming (Feinberg and Vogelstein 1983) and hybridizations of these to virtual Northern blots were performed as previously described for Southern hybridizations (Scutt et al. 1997).

Genomic DNA was extracted from leaves of *S. latifolia* plants using a Phytopure DNA extraction kit (Scotlab). Genomic DNA samples were digested with restriction endonucleases and analyzed on 0.7% agarose gels prior to denaturation and capillary blotting onto Hybond N+ membrane (Amersham) as directed by the manufacturers. Probe labelling and hybridizations were performed as for virtual Northern hybridizations.

*RT-PCR analysis of transcript expression levels*

Reverse transcription (RT) used SMART cDNA (Clontech) as template, synthesized as described above for virtual Northern blot analysis. PCR was performed using of one tenth of the RT reaction as template in 50 µl reaction mixtures using 1× buffer containing 1.5. mM $MgCl_2$ (Clontech), 0.2 mM each dNTP, 5 µM primers, 1 u Taq Polymerase (Clontech). Samples were denatured for 5 min at 95°C followed by 35 cycles of 96°C for 15 s, 60°C for 30 s, and extension at 68°C for 60 s. Products were fractionated on 1.2% agarose 1× TAE gels (Sambrook et al. 1989).

*Sequence analysis of Men cDNAs*

Cloned FDD products were sequenced from both strands using an Applied Biosystems automated DNA sequencer. Sequence homologies were investigated with GCG Computer software to search the EMBL, GenBank and Swissprot databases using Fasta (Pearson and Lipman 1988) and Blast (Altschul et al. 1990) search methods.

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