A plant Y chromosome-STS marker encoding a degenerate retrotransposon

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The dioecious plant *Silene latifolia* has both X and Y sex chromosomes. Male-specific random amplified polymorphic DNA (RAPD) fragments were analyzed to identify Y-chromosome-linked sequences. One of the RAPD fragments, MS4, was converted into a more reliable and reproducible sequence-tagged site (STS) marker. A set of MS4 STS primers was used to amplify two genomic DNA fragments (MS4a and MS4b) from a male plant and one (MS4a) from a female plant, which indicates that MS4b is located on the Y chromosome. Sequence analysis revealed that MS4a encoded a *gag* protein of a *Ty3-gypsy*-like retrotransposon. A 147-bp region from the middle of MS4a was deleted in MS4b. The MS4b sequence was not detected in the most closely related dioecious species, *S. dioica*. This suggests that a retrotransposon with the MS4b sequence has degenerated recently on the Y chromosome.

INTRODUCTION

Approximately 6% of angiosperm species are dioecious, with separate production of staminate and pistillate flowers (Renner and Ricklefs, 1995). Heteromorphic sex chromosomes have been reported in several dioecious species (Matsunaga and Kawano, 2001, Negrutiu *et al.*, 2001). The dioecious plant *Silene latifolia* has X and Y sex chromosomes, which are the largest and second largest chromosomes in the male genome, respectively (Matsunaga *et al.*, 1994). When fluorescence *in situ* hybridization was performed with microdissected Y chromosome-DNA in the presence of competitor female genomic DNA, the signals on the Y chromosome were no stronger than those on the X chromosome and autosomes (Matsunaga *et al.*, 1999a). This suggests that the Y chromosome of *S. latifolia* has not accumulated chromosome-specific sequences and has not become heterochromatic. An interstitial telomere-adjacent sequence is accumulated in the interstitial region of the Y chromosome, suggesting past telomere-mediated chromosome rearrangements during the evolution of the sex chromo-

somes of *S. latifolia* (Uchida *et al.*, 2002). This primitive characteristic of the sex chromosomes provides empirical data on the initial evolution of sex chromosomes.

The function of the Y chromosome of *S. latifolia* was first revealed by analyzing mutant offspring with aberrant Y chromosomes from crosses that involved experimentally raised tetraploids (Westergaard, 1946). These analyses led to the hypothesis that the regulatory genes for female suppression, stamen promotion, and stamen maturation were located on the Y chromosome. Recently, this hypothesis has gained support from genetic and cytological analyses of hermaphroditic and asexual mutants that were generated by X-ray or γ -ray irradiation of pollen (Donnison *et al.*, 1996; Farbos *et al.*, 1999; Lardon *et al.*, 1999). Male-specific amplified fragment length polymorphism (AFLP) markers were identified using X-ray-induced deletions of the Y chromosome (Lebel-Hardenack *et al.*, 2002). Thus, three different loci of carpel suppression, early stamen development, and late stamen development have been located on the Y chromosome.

The random amplified polymorphic DNA (RAPD) method has provided a number of useful genetic markers in plants (Williams *et al.*, 1990). In the case of dioecious plants, DNA fragments that were amplified only from the male genome were expected to be located on the Y chromosome (Mulcahy *et al.*, 1992; Sakamoto *et al.*, 1995). Several Y-chromosome-linked RAPD markers have been converted into more reliable sequence-tagged site (STS) markers (Zhang *et al.*, 1998; Nakao *et al.*, 2001). We report a novel STS marker that encodes a degenerate

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LTR retrotransposon on the Y chromosome.

The internal domain of the LTR retrotransposon encodes proteins that are homologous to retroviral protease, RNase H, reverse transcriptase and integrase. LTR retrotransposons are divided into two types based on their protein arrangements: the *Ty1-copia*-like and *Ty3-gypsy*-like retrotransposons, which were first reported in yeast (Clare and Farabaugh, 1985) and the fruit fly (Mount and Rubin, 1985), respectively. Both retrotransposon types are found in almost all plants (Grandbastien, 1992). In this paper, we report a degenerate *Ty3-gypsy* retrotransposon sequence that is linked to the Y chromosome.

MATERIALS AND METHODS

Plant materials and DNA isolation. Seeds of *Silene latifolia* Poir. ssp. *alba* (P. Mill.) Greuter and Burdet were obtained from an inbred line in our laboratory. Seeds of *S. dioica* (L.) Clairville were collected from an herb garden near Lake Kawaguchi (Yamanashi, Japan). Plants were grown in flowerpots at 22°C in a temperature-regulated chamber. Genomic DNA was isolated from young male and female leaves using a Nucleon PhytoPure Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Isolation of RAPD and sequence-tagged site (STS)

PCR analysis. The PCR reaction mixture contained 200 µM of each dNTP, 0.4 µM each of two arbitrary 10-mer primers (Wako Chemicals, Japan), 50 ng of genomic DNA as the template, 1 U of Takara ExTaq DNA polymerase (Takara Shuzo, Otsu, Japan), and 1 × ExTaq buffer (Takara Shuzo), in a total volume of 50 µl. After an initial denaturation step at 94°C for 3 min, the samples were subjected to 35 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min. An aliquot of the PCR reaction (5 µl) was electrophoresed on a 4% polyacrylamide gel and stained with SYBR Green I (Molecular Probes, OR, USA).

Two 18-mer STS primers, MS4-R (5'-GGACACGATGACACCAAC-3') and MS4-F (5'-AGGCGTTGACCAGTTCAG-3'), were used for the STS-PCR analysis. The PCR reaction mixture contained 200 µM of each dNTP, 0.4 µM of each primer, 50 ng of genomic DNA as the template, 1 U of Takara ExTaq DNA polymerase (Takara Shuzo), and 1 × ExTaq buffer (Takara Shuzo), in a total volume of 50 µl. After an initial denaturation step at 94°C for 3 min, the samples were amplified for 35 cycles of 94°C for 1 min, 1 min at 58°C for *S. latifolia* or 54°C for *S. dioica*, and 72°C for 1 min (for *S. latifolia*). An aliquot of the PCR reaction (5 µl) was electrophoresed on a 2% agarose gel.

Southern hybridization analysis. Genomic DNA samples from *S. latifolia* male and female tissues were

digested with *Hind*III. Samples (4 µg) were electrophoresed on 1.5% agarose gels, transferred to a Biotodyne B nylon membrane (Pall Biosupport Division, NY, USA) using a G capillary blotter (TETEC, Nagoya, Japan), and fixed with a Spectrolinker XL-1500 UV cross-linker (Spectronics). Probe preparation, hybridization and detection were performed using an ECL Direct Nucleic Acid Labeling and Detection System (Amersham). When MS4a and MS4b were used as probes, the primary washes consisted of 2 × SSC at 42°C and 0.1 × SSC at 42°C, respectively. The hybridization membrane was exposed to X-OMAT AR film (Kodak, USA) at room temperature. The MS4a and MS4b hybridization membranes were exposed for 45 min and 16 h, respectively.

Sequence analyses. The nucleotide sequences were determined by the dideoxy chain termination method using a 373S DNA Sequence System and the THERMO Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham) or the Dynamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). The BLASTN and BLASTX programs (Altschul *et al.*, 1990) were used for similarity searches of the DDBJ/EMBL/GenBank databases. The sequences were analyzed using the DNA Space software (Hitachi Software Engineering Co., Ltd., Hitachi, Japan).

Accession numbers. The DDBJ/EMBL/GenBank accession numbers of MS4a and MS4b are AB072492 and AB072494, respectively.

RESULTS AND DISCUSSION

We performed RAPD analysis using male and female genomic DNA samples in order to isolate male-specific DNA sequences from the Y chromosome. Using a total of 94 combinations of arbitrary 10-mer primers, 29 male-specific RAPDs were obtained. The male-specific RAPDs ranged in size from approximately 0.1 to 1.2 kb, and three of these sequences were converted successfully to STS markers for the Y chromosome. In this paper, we report the detailed analysis of one of these STS markers. Amplification with the CT63 (5'-AGGCGTTGAC-3') and CT65 (5'-GGACACGATG-3') primers revealed that a male-specific RAPD, which we named MS4, was ubiquitous in males and absent in females (Fig. 1a). The RAPD fragment was cloned and sequenced, and based on the sequencing results, we designed the 18-mer primers MS4-R (5'-GGACACGATGACACCAAC-3') and MS4-F (5'-AGGCGTTGACCAGTTCAG-3'). As shown in Fig. 1a, the MS4 primer set amplified two types of fragment from the male and female genomic DNAs of four individuals; we named the longer fragment MS4a, and the shorter fragment MS4b. MS4a was amplified from both male and female genomic DNAs, whereas MS4b was amplified

Fig. 1. RAPD and STS-PCR profiles of four male and four female individuals. PCR amplification was performed using genomic DNA from four male (♂) and four female (♀) *S. latifolia* plants. (a) RAPD profile of *S. latifolia* genomic DNA. Fragments were amplified with the CT63 and CT65 primers, separated on a 4% polyacrylamide gel, and stained with SYBR Green I. The solid arrowhead indicates a male-specific fragment, MS4. (b) Fragments were amplified with the MS4 primer set, separated on a 3% agarose gel, and stained with ethidium bromide. The open and solid arrowheads indicate the 596-bp common fragment (MS4a) and the 443-bp male-specific fragment (MS4b), respectively. Lane M contains *HincII*-digested ϕ X174 DNA as the molecular weight marker.

only from male genomic DNA. The PCR results obtained with the MS4 primer set were reproduced with the DNA from ten male and female individuals from five different strains (data not shown). This suggests that MS4a is localized on the autosome, the X chromosome, or pseudoautosomal regions of the sex chromosomes, while MS4b is located only on the Y chromosome.

In order to analyze the relationship between MS4a and MS4b, the two fragments were cloned and sequenced. The MS4a fragment contained a complete open reading frame of 198 amino acids, and the nucleotide sequences of MS4a were perfectly identical in males and

females. Database searching revealed that MS4a shared homology with several gag proteins of *Ty3-gypsy*-like retrotransposons in *Arabidopsis thaliana* and *Oryza sativa* (Fig. 2). Most of the gag proteins in *O. sativa* have significant homology to the *Ty3-gypsy*-like retrotransposon, RIRE2 (Ohtsubo *et al.*, 1999). These results indicate that MS4a encodes a gag protein in a *Ty3-gypsy*-like retrotransposon. Retrotransposons are commonly found in plant species (Kumekawa *et al.*, 1999; Noma *et al.*, 1999). Southern blots and fluorescence *in situ* hybridization analysis revealed that *Ty3-gypsy*-like retrotransposons were distributed throughout the genome and did

Fig. 2. Comparison of MS4a amino acid sequences. The solid and shaded boxes indicate similar and identical amino acids, respectively. Capital letters and asterisks indicate perfect and imperfect consensus sequences, respectively. The 'At' designation signifies a hypothetical gag-pol protein of *Arabidopsis thaliana* (accession no. AC006085), and 'Os' represents the gag-pol protein that is encoded by a retrotransposon in *Oryza sativa* (accession no. AF111709).

not accumulate preferentially on the Y chromosome of *S. latifolia* (Matsunaga *et al.*, 2002). In order to determine the genomic organization of MS4a, Southern hybridization analysis was performed using *Hind*III-digested genomic DNA from four males and four females. MS4a hybridized to several fragments, and gave identical hybridization patterns in the male and female genomes. This result suggests that MS4a is one of the repetitive sequences that are dispersed over the male and female genomes (Fig. 3a). The genomic distribution of MS4a (Fig. 3a) is consistent with previous analyses of *Ty3*-

gypsy-like retrotransposons (Matsunaga *et al.*, 2002).

On the other hand, comparison of the nucleotide sequences of MS4a and MS4b suggests that MS4b originated mainly through deletion of the middle region of MS4a, as shown in Fig. 5. A 147-bp central region of MS4a is deleted in MS4b. Moreover, the putative MS4b ORF has three stop codons, whereas MS4a encompasses a retrotransposon that lacks stop codons. This indicates that the retrotransposon with the MS4b sequence is inactive due to degeneration. When the hybridization experiment was performed at high stringency, MS4b hybridized specifically to an approximately 8-kb fragment in males (Fig. 3b). This finding supports our contention that MS4b is located on the Y chromosome. In fact, a set of these STS primers, MS4-R and MS4-F, is very useful for genetical determination of sex for two reasons as follows. One reason is that MS4b is a stable STS marker for males because the *gypsy*-type retrotransposon including MS4b is inactive on the Y chromosome. The other is that false negatives are eliminated from PCR samples because this primer set can simultaneously amplify a non-sex linked fragment MS4a.

We wished to confirm that MS4a and MS4b were located on the Y chromosome of a closely related dioecious species, *S. dioica*. The genomic DNA of *S. dioica* was subjected to PCR with the MS4F and MS4R primers. As shown in Figure 4, the primers amplified MS4a sequences in both male and female *S. dioica* individuals. The MS4a fragments in *S. dioica* were cloned and sequenced, to reveal a nucleotide sequence identity of 99.7% between male and female individuals. The discrepancy in sequence identity is due to polymorphisms, since *S. dioica* was derived from a wild population rather than an experimental line. MS4a was 95.7% identical in *S. latifolia* and *S. dioica* (Fig. 5). In contrast, MS4b could not be detected in male *S. dioica*. This pattern was reproduc-

Fig. 3. Genomic distribution of MS4a and MS4b in male and female genomes. Southern hybridization was performed using *Hind*III-digested genomic DNA from four male (♂) and four female (♀) plants. *Hind*III-digested genomic DNAs were separated on a 1.5% agarose gel. Labeling, hybridization and detection were performed using the ECL Direct System (Amersham). (a) The blotted membrane was hybridized with the MS4a probe. (b) The blotted membrane was hybridized with the MS4b probe at high stringency. The arrowhead indicates an MS4b-hybridizing fragment.

Fig. 4. Analysis of MS4 homologues in *S. dioica*. MS4 homologues were amplified using the MS4F and MS4R primers. PCR products were separated on a 3% agarose gel and stained with ethidium bromide. Lanes 1, 2, 3 and 4 show the PCR products from the genomic DNA of male *S. dioica*, female *S. dioica*, male *S. latifolia* and female *S. latifolia* plants, respectively.

dio-MS4a♀	GGACACGATG	ACACCAAC	CANTGTGAG	TATTACCAG	GCAAGAAC	AGCACCAG	GCAAGTCAT	JACAGGTT	TACGCAAC	ACCTTGCCA	GGTATTATC	110
dio-MS4a♂	GGACACGATG	ACACCAAC	CANTGTGAG	TATTACCAG	GCAAGAAC	AGCACCAG	GCAAGTCAT	JACAGGTT	TACGCAAC	ACCTTGCCA	GGTATTATC	110
lat-MS4a	GGACACGATG	ACACCAAC	CANTGTGAG	TATTACCAG	GCAAGAAC	AGCACCAG	GCAAGTCAT	JACAGGTT	TACGCAAC	ACCTTGCCA	GGTATTATC	110
lat-MS4b	GGACACGATG	ACACCAAC	CAG	TATTACCAG	GCAAGAAC	TAGACCAG	GCAAGTCAT	TACAGGCTG	TACGCAAC	ACCTTGCCA	GGTATTATC	104
dio-MS4a♀	AGGCCTTGGG	AGC GCAACA	CATGTGCTC	CACAACA	CTGTCTGGG	ACA TGTGGA	CTG GGG AA	TATACA GGG	TC CAGCAIT	TATACACAA	TCAATCGCG	220
dio-MS4a♂	AGGCCTTGGG	AGC GCAACA	CATGTGCTC	CACAACA	CTGTCTGGG	ACA TGTGGA	CTG GGG AA	TATACA GGG	TC CAGCAIT	TATACACAA	TCAATCGCG	220
lat-MS4a	AGGCCTTGGG	AGC GCAACA	CATGTGCTC	CACAACA	CTGTCTGGG	ACA TGTGGA	CTG GGG AA	TATACA GGG	TC CAGCAIT	TATACACAA	TCAATCGCG	220
lat-MS4b	AGGCCTTGGG	AGC GCAACA	CTGTGCTC	CACAACA	CTGTCTGGG	ACA TGTGGA	CTG GGG AA	TATACA GGG	TC CAGCAIT	TATACACAA	TCAATCGCG	214
dio-MS4a♀	GGCTA AACG	CTATCTGGA	GUACAATAC	CA GAATGG	GTCTCTATA	TAT AAACA	GAT CCGGG	ATGCCATCC	CTATGAGAG	AGCAACCAC	GACAGCTATG	330
dio-MS4a♂	GGCTA AACG	CTATCTGGA	GUACAATAC	CA GAATGG	GTCTCTATA	TAT AAACA	GAT CCGGG	ATGCCATCC	CTATGAGAG	AGCAACCAC	GACAGCTATG	330
lat-MS4a	GGCTA AACG	CTATCTGGA	GUACAATAC	CA GAATGG	GTCTCTATA	TAT AAACA	GAT CCGGG	ATGCCATCC	CTATGAGAG	AGCAACCAC	GACAGCTATG	326
lat-MS4b	GGCTA AACG	CTATCTGGA	GUACAATAC	CA GAATGG	GTCTCTATA	TAT AAACA	GAT CCGGG	ATGCCATCC	CTATG			295
dio-MS4a♀	CAGACTCCCT	GTT TGTGGAC	CAGATATCAC	TGATAACGGT	TCCCAAGGGA	TTACGGCAT	CTACGATGAC	GCTCTACGAG	GGGACAACAG	ATCCCTACGA	TCATATAAGC	440
dio-MS4a♂	CAGACTCCCT	GTT TGTGGAC	CAGATATCAC	TGATAACGGT	TCCCAAGGGA	TTACGGCAT	CTACGATGAC	GCTCTACGAG	GGGACAACAG	ATCCCTACGA	TCATATAAGC	440
lat-MS4a	CAGACTCCCT	GTT TGTGGAC	CAGATATCAC	TGATAACGGT	TCCCAAGGGA	TTACGGCAT	CTACGATGAC	GCTCTACGAT	GGGACAACAG	ATCCCTACGA	TCATATAAGC	436
lat-MS4b	CAGACTCCCT	GTT TGTGGAC	CAGATATCAC	TGATAACGGT	TCCCAAGGGA	TTACGGCAT	CTACGATGAC	GCTCTACGAT	GGGACAACAG	ATCCCTACGA	TCATATAAGC	295
dio-MS4a♀	TATTTAAAGC	AAAAGATGAT	GGTATAAATA	CAGTTGGAG	COITAAAGGA	AGCCTCATG	TATAAGGGGT	TTGCTCAAC	TGTGTAGT	GCCCTTGC	AATGTTCT	550
dio-MS4a♂	TATTTAAAGC	AAAAGATGAT	GGTATAAATA	CAGTTGGAG	COITAAAGGA	AGCCTCATG	TATAAGGGGT	TTGCTCAAC	TGTGTAGT	GCCCTTGC	AATGTTCT	550
lat-MS4a	TATTTAAAGC	AAAAGATGAT	GGTATAAATA	CAGTTGGAG	COITAAAGGA	AGCCTCATG	TATAAGGGGT	TTGCTCAAC	TGTGTAGT	GCCCTTGC	AATGTTCT	546
lat-MS4b	TATTTAAAGC	AAAAGATGAT	GGTATAAATA	CAGTTGGAG	COITAAAGGA	AGCCTCATG	TATAAGGGGT	TTGCTCAAC	TGTGTAGT	GCCCTTGC	AATGTTCT	393
dio-MS4a♀	GAGT TGGCA	AACAAATCA	TATCTCCTT	GCTGAACCTG	GTCAACGCCCT							600
dio-MS4a♂	GAGT TGGCA	AACAAATCA	TATCTCCTT	GCTGAACCTG	GTCAACGCCCT							600
lat-MS4a	GAGT TGGCA	AACAAATCA	TATCTCCTT	GCTGAACCTG	GTCAACGCCCT							596
lat-MS4b	GAGT TGGCA	AACAAATCA	TATCTCCTT	GCTGAACCTG	GTCAACGCCCT							443

Fig. 5. Alignment of the MS4 genes of *S. latifolia* and *S. dioica*.

Dio-MS4a and dio-MS4a indicate the MS4a nucleotide sequences in male and female *S. dioica* individuals, respectively. Lat-MS4a and lat-MS4b represent the MS4b nucleotide sequences in male and female *S. latifolia* individuals, respectively. The solid and shaded boxes represent fully and partially identical nucleotides, respectively.

ible in two other strains of *S. dioica* (data not shown). These results suggest that at least regions of MS4b are not conserved on the Y chromosomes of *S. latifolia* and *S. dioica*. It is possible that the MS4b sequence emerged in *S. latifolia* after divergence from *S. dioica*.

It seems likely that the retrotransposon with MS4b lost function after moving to the Y chromosome of *S. latifolia*. A male reproductive organ-specific gene, *MROS3*, is located on the X chromosome (Matsunaga *et al.*, 1996; Guttman and Charlesworth, 1998; Matsunaga *et al.*, 1999b; Kejnovsky *et al.*, 2001). A paralogous sequence on the Y chromosome, *MROS3Y*, is highly homologous to the 3'-region of the expressed *MROS3* sequence (Guttman and Charlesworth, 1998). *MROS3Y* appears to have become non-functional following degeneration of the Y chromosome. The analysis of MS4b also offers evidence of Y chromosome degeneration in *S. latifolia*. More refined analyses of MS4b will provide interesting data regarding the process of differentiation in plant sex chromosomes.

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