The Y chromosome in the liverwort *Marchantia polymorpha* has accumulated unique repeat sequences harboring a male-specific gene

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The haploid liverwort *Marchantia polymorpha* has heteromorphic sex chromosomes, an X chromosome in the female and a Y chromosome in the male. We here report on the repetitive structure of the liverwort Y chromosome through the analysis of male-specific P1-derived artificial chromosome (PAC) clones, pMM4G7 and pMM23-130F12. Several chromosome-specific sequence elements of ≈70 to 400 nt are combined into larger arrangements, which in turn are assembled into extensive Y chromosome-specific stretches. These repeat sequences contribute 2–3 Mb to the Y chromosome based on the observations of three different approaches: fluorescence *in situ* hybridization, dot blot hybridization, and the frequency of clones containing the repeat sequences in the genomic library. A novel Y chromosome-specific gene family was found embedded among these repeat sequences. This gene family encodes a putative protein with a RING finger motif and is expressed specifically in male sexual organs. To our knowledge, there have been no other reports for an active Y chromosome-specific gene in plants. The chromosome-specific repeat sequences possibly contribute to determining the identity of the Y chromosome in *M. polymorpha* as well as to maintaining genes required for male functions, as in mammals such as human.

dioecism | sex chromosome | FISH | RING finger motif | convergent evolution

In contrast to most animals, many plant species generate both male and female organs in the same individual. Only a few plants are unisexual, termed dioecious. Some of these plants have heteromorphic sex chromosomes by which sex is genetically determined. In a liverwort, *Marchantia polymorpha*, a specific Y chromosome is present in the male, and a distinct X chromosome is found only in the female plantlets (1). In a dioecious plant, *Silene latifolia*, the Y chromosome dominantly and positively induces male development by its presence in an XX/XY system similar to the mammalian pair of sex chromosomes (2). In *Rumex acetosa* the presence of the Y_1 and Y_2 chromosomes has no influence on triggering male development, which is, like in *Drosophila melanogaster*, solely determined by the X chromosome dosage (2). However, the primary structure and molecular function of the plant sex chromosomes are much less known than of those in animals.

Several sex chromosome-derived sequences have been isolated in some dioecious flowering plants, e.g., *S. latifolia* (3–7), *Cannabis sativa* (8), and *R. acetosa* (9–11), that suggest a repetitive structure of sex chromosomes in plants. In *S. latifolia,* interspersed and localized repeat sequences were identified on the sex chromosomes by microdissection and degenerate oligonucleotide primed PCR (DOP-PCR; refs. 4, 5, and 7). Some of these sequences are localized at subtelomeric regions, which are near the pseudoautosomal regions of the X and Y chromosomes (5, 7). However, all of these sequences are found on both the sex chromosomes and also on the autosomes, as yet not indicating any exclusive feature for the respective sex chromosomes. Only in *R. acetosa* were repeat sequences unique to the Y chromosomes reported, but they are limited to just a few hundred bases (10).

Several genes have been isolated from the sex chromosomes of *S. latifolia*. A gene specifically expressed in male reproductive organs, *MROS3*, was reported to be X chromosome-linked and to have a degenerate homologous sequence on the Y chromosome (12, 13). Conversely, a Y chromosome-linked gene, *SlY1*, whose homologue, *SlX1*, was identified on the X chromosome, is principally expressed in male flowers. Because the expression pattern of *SlX1* is similar to *SlY1*, these genes are likely to be housekeeping genes (14). So far, no Y chromosome-specific genes have been isolated in plants.

Toward a better understanding of the sex chromosome system in the liverwort *M. polymorpha*, we have initiated detailed structural analyses of the X and Y chromosomes and their gene contents. Sex-specific clone libraries enabled us to identify sequences unique to one of the sexes and therefore, given the X-Y exclusiveness to one sex, unique to the X or Y chromosome (15). We here report that the Y chromosome in *M. polymorpha*, although much smaller than the X chromosome, contains a large number of unique repeat sequences not found on any of the other chromosomes. In one arrangement, ORF162 is encoded; to our knowledge, there has been no previous identification of a Y-specific actively transcribed gene in plants.

Materials and Methods

Plant Materials. Male and female thalli of *M. polymorpha* (E lines; ref. 15) were cultivated on M51C medium (16) at 24°C under continuous light.

Chromosome Preparation and Fluorescence *in Situ* Hybridization (FISH). Chromosomal preparation and treatments before hybridization were performed as described (15). For FISH, 20 μl of a hybridization mixture containing 30 ng biotin-labeled pMM4G7 (15), 1 μg salmon testis DNA (Sigma-Aldrich), and 15% formamide in 2× SSC were applied to each slide. The mixture was denatured at 85°C for 10 min and transferred to preparations. The preparations were heated at 85°C for 10 min on a thermal

Abbreviations: FISH, fluorescence *in situ* hybridization; PAC, P1-derived artificial chromosome; DAPI, 4′,6-diamidino-2-phenylindole; CDPK, calcium-dependent protein kinase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB062743, AB062742, and AB062741 for the T7 and SP6 portions of pMM4G7 and the 2.2-kb *Bam*HI unit of pMM23-130F12, respectively).

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controller with a slide griddle (PTC-100, MJ Research, Cambridge, MA) and hybridized in a humid chamber at 24°C overnight. The signals were detected with avidin-conjugated fluorescein (Roche Diagnostics) and amplified once with biotinylated anti-avidin D and fluorescein avidin DCS (Vector Laboratories). Chromosomes were stained with 1.0 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories) and observed under a Zeiss Axioplan2 with 01 and 17 filter sets for DAPI and fluorescein, respectively. Fluorescent images were documented by a cooled charge-coupled device (CCD) camera, PentaMax (Princeton Instruments, Trenton, NJ) and analyzed with IPLAB (Scanalytics, Fairfax, VA) and Adobe Photoshop (Adobe Systems, Mountain View, CA).

Restriction Mapping. For mapping with *Bam*HI, plasmid DNA of pMM4G7 was partially digested with *Bam*HI and probed with oligonucleotides designed at the T7 and SP6 ends of the P1-derived artificial chromosome (PAC) vector (15), 5′-GCGGCCGCTAATACGACTCACTATAGGGAGAGGATC-3′ and 5′-GATCCTTCTATAGTGTCACCTAAATGTCGACGGCCAGGCGGCC-3′, respectively. The arrangement of 0.2-kb *Bam*HI units was determined from primary sequence information. For mapping with *Pst*I, pMM4G7 was double-digested with *Not*I and *Pst*I.

DNA Sequence Analysis. For shotgun sequencing, PAC plasmid DNA was sheared by using a point-sink shearer HydroShear (GeneMachines, San Carlos, CA) to average size of 3.5 kb and 7 kb. After end-repair, random fragments were cloned into the *Sma*I site of pUC18. Plasmid DNA for each subclone was isolated by using a 96-well glass-fiber filter protocol as described (17). Nucleotide sequences were determined by automated sequencers ABI PRISM 377XL and ABI PRISM 3700 (Applied Biosystems) by using the BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems) or DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech). Sequence data were assembled by using computer programs PHRED/PHRAP (18, 19), and the assembled sets of overlapping sequence reads were edited by using CONSED 10.0 (20).

Dot Blot Analysis. Copy numbers of the repeat sequence were determined by dot blot hybridization. Dot blots were prepared by using 100 ng to 5 μg of genomic DNA, 12.1 ng to 121 ng of the subcloned 432-bp *Mbo*I element, 4.82 ng to 48.2 ng of the subcloned 2.4-kb *Bam*HI unit, and 9.10 ng to 36.4 ng of pMM4G7. The filter was hybridized with the ^{32}P-labeled *Mbo*I element, and radioactivity was quantified by using a BAS2000 Image Analyzer (Fuji Photo Film). The size of the repetitive region was calculated from the signal intensity of the genomic DNA (GS) compared with those of the control plasmids (CS) as follows: The size of the repetitive region (Mb) = (GS) $\times$ (CS)$^{-1}$ $\times$ (the number of *Mbo*I elements in the repeat unit) $\times$ (the number of *Mbo*I elements in the 2.2-kb *Bam*HI unit)$^{-1}$ $\times$ 2.2 $\times$ 10^{-3}.

DNA Hybridization and PCR. For the genomic library screening, high density filters containing the complete male genomic library (15) were probed with the ^{32}P-labeled 2.4-kb *Bam*HI unit (see Fig. 2*C*) subcloned from pMM4G7. For verification, plasmid DNAs of positive clones were rescreened with the same probe. DNA blot hybridization was basically performed as described (15). Ten micrograms of genomic DNA from *M. polymorpha* or 2.5–5 ng of PAC plasmid DNAs were hybridized with ^{32}P-labeled probes. The same 2.4-kb *Bam*HI fragment and the 275-bp fragment of ORF162 with the RING finger motif were used as probes for repeat organization analysis and Southern analysis of ORF162, respectively.

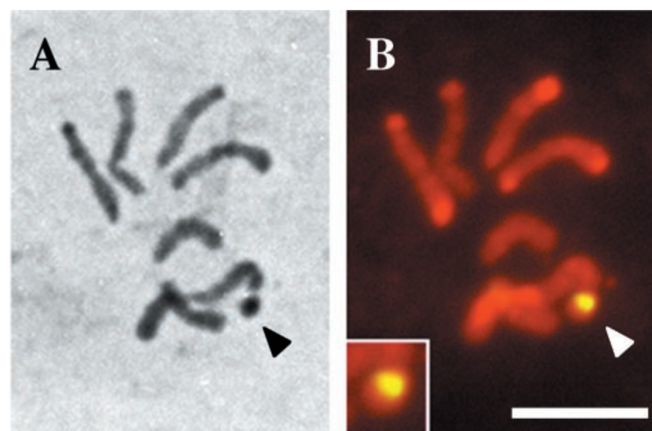

Fig. 1. FISH analysis of a chromosome preparation from male *M. polymorpha*. (*A*) Giemsa-stained prometaphase chromosomes are shown with the Y chromosome indicated by an arrowhead. (*B*) The false color image of the DAPI stain (red) is superimposed onto the FITC image of the biotin-labeled PAC clone pMM4G7 (yellow). The magnified Y chromosome is presented in the lower left corner. Bar = 5 μm.

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PCR for male-specificity tests was done as described (15), except for different pairs of primers and templates. Male and female genomic DNAs of *M. polymorpha* (E lines; ref. 15) were used as templates. Primers 5′-CAAGAGACGACTGACTCGACTG-3′ and 5′-TCTCCATCCACGCATTGAAGAG-3′ were used for the 2.2-kb *Bam*HI unit. Primers 5′-CTTGGTCAGATATGCCCACATG-3′ and 5′-CATGTGGATTGTCATCCTGACC-3′ were used for ORF162. Primers designed from the calcium-dependent protein kinase (CDPK) gene (15) were used for quality evaluation of genomic DNAs.

Northern Blot Analysis. Poly(A)$^+$ RNA from male thalli and male sexual organs were prepared as described (21). Northern blots containing 10 μg of poly(A)$^+$ RNA were hybridized with the probe described for the DNA hybridization. Hybridization was performed with ExpressHyb Hybridization Solution (CLONTECH) according to the manufacturer's instructions.

Results

Chromosomal Location of the Male-Specific Sequences in a Clone pMM4G7. We previously reported that a PAC clone pMM4G7 isolated from the *M. polymorpha* male genomic library maps to the Y chromosome and that closely related sequences have accumulated in the Y chromosome (15). To map pMM4G7 more precisely, FISH analysis with prometaphase chromosomes was performed (Fig. 1). The observed signal was concentrated in only one half of the Y chromosome, whereas the other half remains largely free. This observation suggests that the sequences cloned in pMM4G7 are located in one part of the Y chromosome only. The intensity of the signal and the area of the chromosome covered indicate that these sequences are heavily amplified in the Y chromosome.

Organization of the Y Chromosome-Specific Repeat Sequences in a Clone pMM4G7. To analyze the structure of pMM4G7, we constructed restriction maps with *Bam*HI, which cuts pMM4G7 into mainly convergent multiple 2.4-kb fragments. The restriction map of *Bam*HI is consistent with the FISH data that pMM4G7 mostly contains repeat sequences (Fig. 2*A*).

The practically complete sequence analysis of the 35-kb insert in pMM4G7 by shotgun sequencing revealed a complex arrangement of various repeat sequences (Fig. 2 *A* and *B*). The smallest repeat elements are 69 and 432 nt long, characterized by

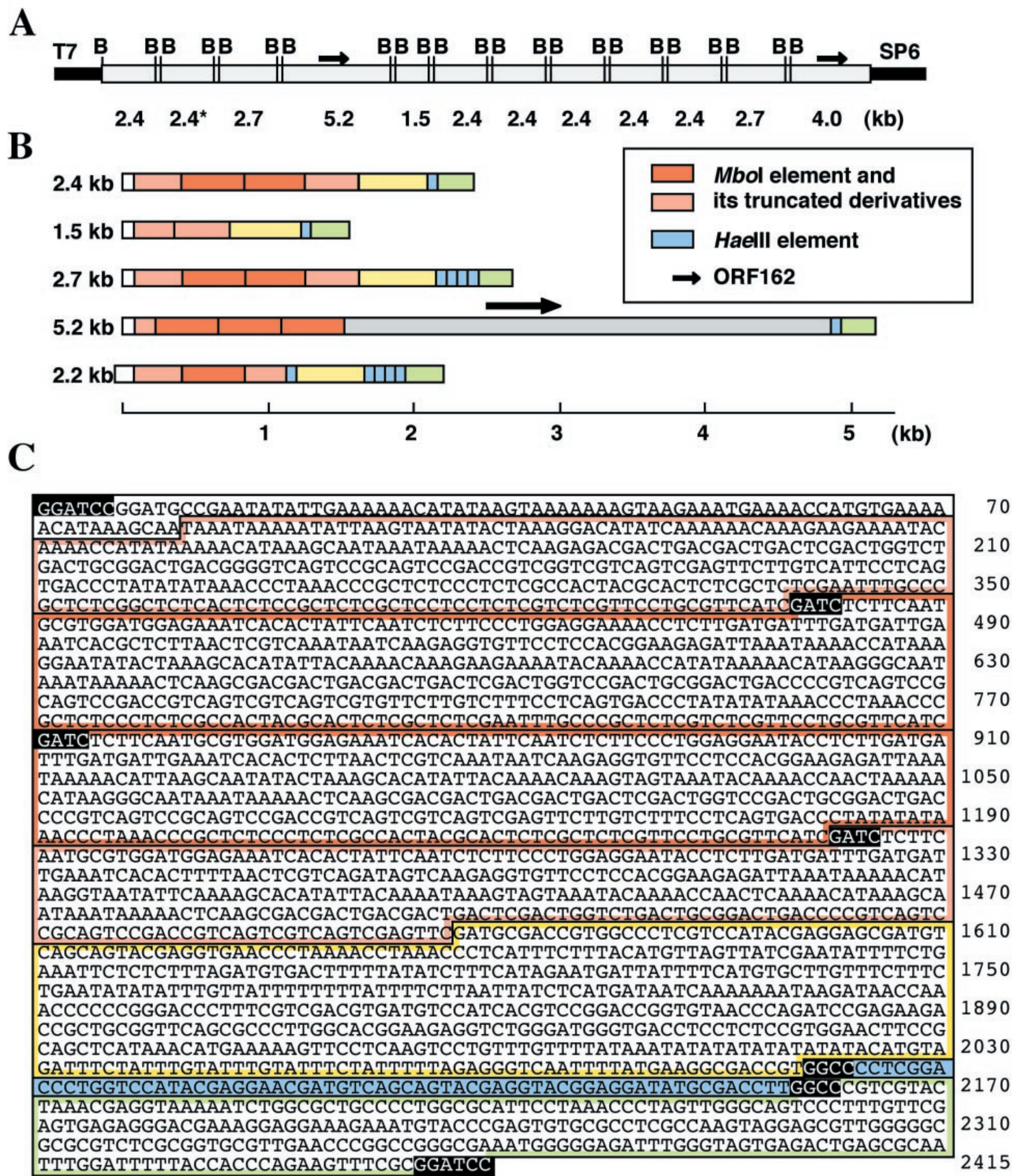

Fig. 2. Schematic diagram of the repeat unit organization in the Y chromosome. (*A*) Structure of pMM4G7. *Bam*HI sites are indicated by vertical lines with letters B. Various 0.2-kb *Bam*HI units are inserted between the other *Bam*HI repeat units. (*B*) The 1.5-, 2.4-, 2.7-, and 5.2-kb *Bam*HI units of pMM4G7 and the 2.2-kb *Bam*HI unit of pMM23-130F12 consist of common subrepeats, mostly *Mbo*I (indicated in red) and *Hae*III elements (blue). Other sequences are color-coded according to their respective similarities. (*C*) The nucleotide sequence of one of the 2.4-kb *Bam*HI units in pMM4G7 marked with an asterisk in *A*. Each colored box corresponds to the region of the same color shown in *B*. Nucleotide residues of signature restriction sites (*Bam*HI, *Mbo*I, and *Hae*III) are highlighted.

diagnostic restriction sites for *Hae*III and *Mbo*I, respectively (Fig. 2*C*). They are assembled in varying stoichiometries into 1.5-kb to 5.2-kb units that in turn can be resolved by unique *Bam*HI sites. The nucleotide sequences of these Y chromosome-specific repeat units showed no similarity to sequences in the public databases.

Various Arrangements of the Y Chromosome-Specific Repeat Sequences. To investigate the organization of these Y chromosome-specific repeat sequences in the Y chromosome, genomic Southern blot analysis was performed (Fig. 3). The hybridization signals of the *Bam*HI-digested male DNA were reproducibly of quite asymmetric intensities. The most intensive hybridization

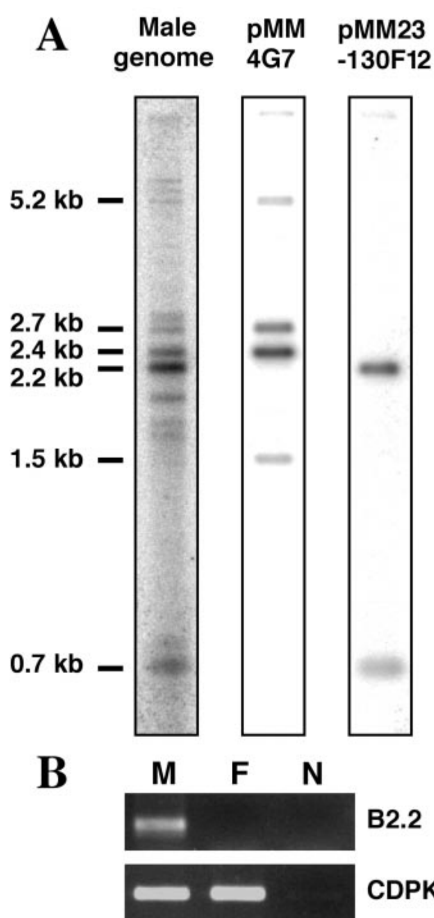

Fig. 3. Genomic distribution of repeat sequences related to pMM4G7. (*A*) *Bam*HI digests of total genomic DNA from male plantlets and of PAC clones pMM4G7 and pMM23-130F12 were blotted and hybridized with the radiolabeled 2.4-kb *Bam*HI unit subcloned from pMM4G7. The sizes of *Bam*HI fragments in pMM4G7 and pMM23-130F12 are indicated on the left. (*B*) Analytical PCR with genomic DNAs of male (M) and female (F) plants. Primers derived from the 2.2-kb *Bam*HI unit showed amplification only in the male DNA. Control PCR with CDPKEx1-F and CDPKEx1-R (15) was performed to detect a single-copy sequence present in both genomes. Negative control PCR was done without DNA (N).

signal was observed at ≈2.2 kb, distinct from the less prominent 2.4-kb repeat unit of pMM4G7 (Fig. 3). The other *Bam*HI repeat units of pMM4G7, 1.5, 2.7, and 5.2 kb, likewise appeared as only minor signals. The most prominent 2.2-kb *Bam*HI fragment was indeed found as the major repeat unit in other PAC clones such as pMM23-130F12 for instance. In this 100-kb cloned fragment, ≈40 copies of the 2.2-kb *Bam*HI unit were repeated as well as a 0.7-kb *Bam*HI unit (Fig. 3 and data not shown). The 2.4-, 1.5-, 2.7-, 5.2-, and 2.2-kb repeat units contain the different numbers of the *Hae*III and *Mbo*I elements (Fig. 2*B*). The *Hae*III elements were >92% identical to each other, and similarly >92% identities were observed among the *Mbo*I elements. The presence of additional hybridization signals in Fig. 3*A* suggest further variants of these repeat sequences in the Y chromosome, albeit mostly at lower copy numbers and/or a lower degree of similarity.

Copy Number Estimation of the Cloned Y Chromosome-Specific Repeat Sequences. To determine the size of the region of the Y chromosome covered by the Y chromosome-specific repeat sequences, the number of these sequences in the Y chromosome was estimated by calibrated dot blot hybridization using the *Mbo*I element as a probe (data not shown). These estimates indicated that $1.4–3.6 \times 10^3$ copies of the *Mbo*I element are present in the male genome. There is a stretch of the repeat sequence equivalent to 2 1/2 copies of the *Mbo*I elements in the major 2.2-kb *Bam*HI unit, which is the predominant form of this repeat family (see Fig. 2*B*). Therefore, the copy number of the 2.2-kb *Bam*HI unit was estimated to be $0.6–1.5 \times 10^3$, which *in toto* accounts for 1.3–3.3 million base pairs (Mb). Consequently, these Y chromosome-specific repeat sequences contribute approximately one quarter of the 10-Mb long Y chromosome (15).

For another independent estimation of the copy number of these repeat sequences in the Y chromosome, we analyzed their representation in the library of male PAC clones. This library, with 33,000 clones and an average insert size of 90 kb, covers the total 280-Mb male genome of *M. polymorpha* ≈10 times (15). The number of the PAC clones identified with the 2.4-kb *Bam*HI unit, altogether 429 clones, suggests that about one third of the Y chromosome to harbor sequences related to the 2.4-kb *Bam*HI unit. This result is consistent with the dot blot data and the visual impression obtained by the FISH analysis (Fig. 1).

A RING Finger Protein is Encoded in the Cloned Y Chromosome-Specific Repeat Sequences. Analysis of the unique sequence stretch embedded in the 5.2-kb *Bam*HI unit revealed an ORF potentially coding for a protein of 162 aa (ORF162) from the first AUG codon to the next translational stop (Fig. 4). Another copy of ORF162 was found near the SP6 end of pMM4G7 (Fig. 2*A*). For this ORF, an uninterrupted coding region without introns was predicted. BLAST searches for similar genes in the databases detected several putative proteins of the flowering plant, *Arabidopsis thaliana*. Similarities are concentrated around the C-terminal region of ORF162, where all of the signature amino acid residues of a RING finger motif are conserved (Fig. 4). An increasing number of studies indicate that RING finger proteins participate in the ubiquitin-mediated protein turnover processes (22). Genomic Southern and PCR analyses demonstrated that

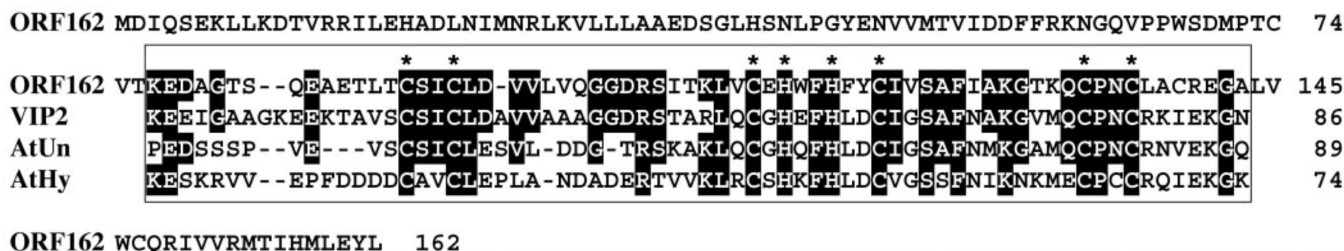

Fig. 4. Predicted amino acid sequence of ORF162. Local alignment of RING finger motifs from ORF162 and several homologs is given in a box with positions on the right. VIP2 is a protein from *Avena fatua,* AtUn is an ORF of unknown function in *A. thaliana*, and AtHy is a hypothetical protein in *A. thaliana* (accession nos: AJ251051, AC005309, and AL049656, respectively). Amino acids identical to those of *M. polymorpha* are highlighted. The conserved cysteine and histidine residues of the RING finger motif are indicated by asterisks.

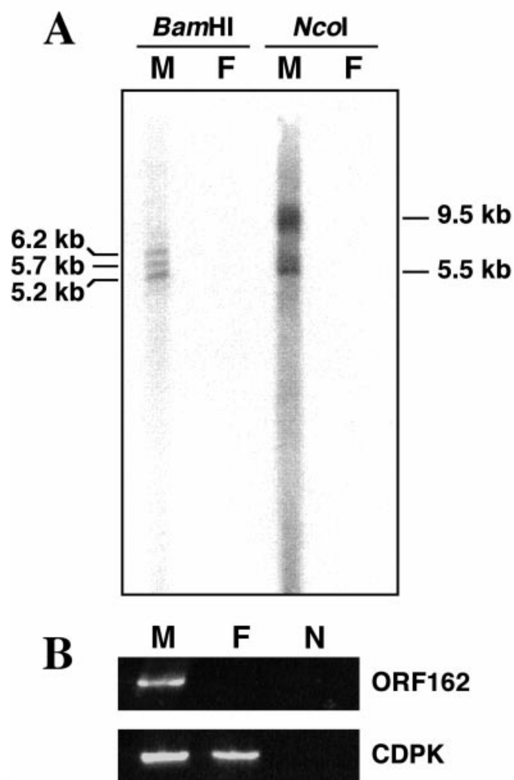

Fig. 5. Male specificity of ORF162. (*A*) Genomic Southern hybridization analysis. Total DNAs of male (M) and female (F) plants were digested with *Bam*HI and *Nco*I and probed with a DNA fragment covering the RING finger motif of ORF162. (*B*) Analytical PCR with genomic DNAs of male (M) and female (F) plants. Primers specific to ORF162 showed amplification only in the male DNA. Negative control PCR was done without DNA (N).

ORF162 is specific to the male genome and thus to the Y chromosome (Fig. 5 *A* and *B*). In the male genomic Southern blot, the probe for ORF162 hybridized with 5.2-, 5.7-, and 6.7-kb *Bam*HI fragments and produced broad signals at ≈5.5 kb and 9.5 kb when digested with *Nco*I. The multiple hybridization signals indicate the presence of several identical or similar copies of this gene in different sequence contexts in the Y chromosome. Northern blot analysis detected a 2.3-kb RNA transcript for ORF162 in male sexual organs but not in thalli, suggesting an intriguing tissue-specific expression (Fig. 6).

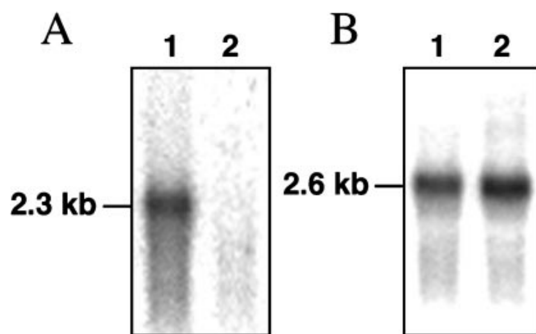

Fig. 6. Northern blot analysis of ORF162. (*A*) Ten micrograms of poly(A)$^+$ RNA from male sexual organs (lane 1) and male thalli (lane 2) were blotted and probed with ^{32}P-labeled DNA encoding ORF162. (*B*) The same membrane was reprobed with the CDPK gene, which is constitutively expressed in male sexual organs (lane 1) and male thalli (lane 2).

Discussion

In this study, we have characterized a 35-kb region of the liverwort Y chromosome by determining the structure of a Y-originated PAC clone, pMM4G7, isolated from the male PAC genomic library (15). Despite many attempts by various techniques (3–11), only fragmentary information on the primary structure of plant sex chromosomes has been obtained because of intrinsic limitations of the methodologies. The male and female PAC genomic libraries of *M. polymorpha* (15) made it possible to isolate and analyze such long regions of plant sex chromosomes.

The 35-kb-long fragment cloned in pMM4G7 is mostly composed of Y chromosome-specific repeat sequences. The mosaic structure of repeats-within-repeats, with several levels of repeats and subrepeats (Fig. 2), is unique to *M. polymorpha*. These repeat sequences are unique not only in their organization, but are also in their primary structure distinct from those in all other plant species investigated so far. The nucleotide sequences of the Y chromosome-specific repeat elements in the liverwort show no similarity to those amplified in the Y chromosomes of flowering plants such as *S. latifolia* or, as to be expected, in animals. The Y chromosome-specific repeat sequences in the liverwort thus most likely originate from a unique evolutionary root and are derived from amplification events unique to the liverwort lineage.

In *M. polymorpha*, the repeat elements identified cover approximately one quarter of the Y chromosome and are unique to this one chromosome. This estimate was confirmed by three different methods: FISH, dot blot analysis, and representation in the library. Highly amplified repeat sequences are one of the hallmarks of plant chromosomes in general (23), but are usually found on many or even all chromosomes of a given genome, including the X and Y chromosomes when present. In the dioecious flowering plants investigated for repeat sequences on the Y chromosome, e.g., *S. latifolia* (3–7), *C. sativa* (8), and *R. acetosa* (9–11), similar if not identical sequences are also found on the X chromosome and the autosomes. Only in *R. acetosa* was one of the isolated repeat sequences unique to the Y chromosome, *RAYS1* (10). In animals, notably mammalia, analogous small genome-wide repeat elements constitute the bulk of the chromosomal DNA (24–26). In animals, in contrast to plants, numerous repeat sequences unique to the Y chromosome have been found, one of the largest such loci identified to date being a 2.4-Mb region in the chinook salmon Y chromosome (27). From the evolutionary point of view, sex chromosomes in animals are believed to gradually accumulate sex-specific sequences through suppression of recombination between X and Y chromosomes (28). In *S. latifolia*, the paucity of sex chromosome-specific sequences suggests a recent acquisition of the sex chromosomes (4). On the other hand, the accumulation of the Y chromosome-specific repeat sequences in *M. polymorpha* presumably reflects an earlier acquisition of the sex chromosomes than in *S. latifolia*.

We have identified a male-specific gene family, ORF162, embedded among the Y chromosome-specific repeat sequence in *M. polymorpha.* Our preliminary estimation by dot blot using a part of the ORF162 coding sequence as a probe suggests that a few hundred copies of ORF162 have accumulated in the male genome (data not shown). Consistently, partial cDNA sequencing also showed the presence of other copies of ORF162 with sequence variations (data not shown). In humans, a multitude of male-specific gene families are encoded within Y chromosome-specific repeat sequences (29), which are concentrated in the nonrecombining portion of the Y chromosome (NRY; ref. 24). The human NRY has consequently been proposed to possess a unique tendency to accumulate male-specific genes that is driven by the selective advantage conferred by suppressed recombina-

tion with the X chromosome and the amplification of male-fertility factors (29, 30). This analogy suggests that a similar mechanism could operate in *M. polymorpha*.

Up to now, *SlY1* was the only gene known to be linked to a plant Y chromosome (14). However, because *SlY1* has a homologue, *SlX1,* on the X chromosome with an expression pattern similar to *SlY1*, this gene was concluded to be a housekeeping gene rather than to encode a sex-specific function. The putative gene, ORF162, described in this paper, has specifically accumulated in the Y chromosome; to our knowledge, there have been no other examples of a Y chromosome-specific multicopy gene in plants. In humans, most Y chromosome-linked genes are categorized into two groups: X-homologous housekeeping genes, which are mostly single-copy genes, and testis-specific gene families with no apparent homologues on the X chromosome (29). This observation and the intriguing specific expression of ORF162 in male sexual organs suggest that ORF162 is most likely involved in a male-specific function, such as spermatogenesis. Indeed, a RING finger protein involved in spermatogenesis has been identified in mouse (31). Further in-depth analysis of the ORF162 transcription pattern and protein analyses are required to identify its function in *M. polymorpha*.

Although the Y chromosome-specific sequences amplified in *M. polymorpha* are unique to this plant, the basic structural feature of the Y chromosome organization, i.e., a high percentage of repeat elements, is shared by bryophytes, higher plants, and animals. This common structural feature could be a consequence of convergent evolution in response to the pressure to avoid frequent crossovers between the X and Y chromosomes in the region characterized by these repeat sequences (28, 32, 33). Physical isolation from these recombination processes thus appears to be most easily achieved and maintained by specific amplification of repeat sequences between and around the sex-specific genes presumably present on the sex chromosomes in all of these dioecious systems.

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