Journal of Photoscience (2005), Vol. 12(2), pp. 75-82

Application of Chromosome Manipulation, DOP-PCR and AFLP Methods to Isolate Sex-Specific DNAs from *Rumex acetosa* L.

Dong-Chung Jin, Jongsoon Kim, Ji-Young Park, Jae-Wook Bang, and Yoonkang Hur*
School of Bioscience and Biotechnology, Genome Research Center,
Chungnam National University, Daejeon 305-746, Korea

Rumex acetosa L. is a dioecious flowering plant with well developed sex chromosome system: 2n = 12 + XX in the female plants and 2n = 12 + XY1Y2 in the male plants. To isolate sex-linked DNA, we carried out chromosome micromanipulation, followed by DOP-PCR, AFLP of the PCR products, reverse Southern hybridization and sequence analysis. From 500 AFLP specific clones, 13 X-chromosome and 5 Y-chromosome specific clones were obtained. Except one clone *RADAX-239* (*Rumex acetosa* - DOP-PCR – AFLP - Y-chromosome specific), all clones appear to be *R. acetosa* plant-specific sequences and non-coding sequences. Southern blot analysis using these clones could not discriminate genomic DNAs either from male or female plants. Results of this study imply that both autosome-origin and degeneration of sex chromosomes are prevalent in plant systems.

Key words: *Rumex acetosa* L, chromosome manipulation, DOP-PCR, AFLP, X- and Y-chromosome specific sequences

INTRODUCTION

Sexual reproduction gives rise to organisms genetic diversity that is very important to adaptation in the evolutionary process. The major concern in the reproduction is the mechanism for sex (male and female) determination. In animal system, great progress on sex determination at the molecular level has been made recently in *Caenorhabditis elegans* (reviewed by Stothard and Pilgrim [1]), insects (reviewed by Saccone *et al.* [2]) and mammals (reviewed by Cotinot *et al.* [3-4]). However, unraveling the molecular mechanism of sex determination in plant is still infancy.

Most (89%) of the flowering plants on the earth are hermaphroditic (flowers bearing both female and male reproductive organs) and only 11% have unisexual flowers. Among the unisexual flower-bearing plants, 7% and 4% are monoecious and dioecious plants, respectively [5]. In hermaphroditic plants, such as *Antirrhinum sp.* and *Arabidopsis thaliana*, sex differentiation can be understood by ABC model of MADS box genes [6]. However, these genes appear not to have direct roles in sex determination in the monoecious plants, such as cucumber and maize, and dioecious plant [7-9]. Most dioecious plants have sex chromosomes [10], which seem to be recently evolved [11-12].

Sorrel (*Rumex acetosa*) and white campion (*Silene latifolia*) are typical dioecious plants whose sex is determined by sex chromosomes. They have the XY system, in which males are the heterogametic sex (with an X and a Y chromosome) and the females are homogametic with two X chromosomes [13-15]. *R. acetosa* has a highly developed sex chromosome system: 2n = 12 + XX = 14 in the female and 2n = 12+ XY1Y2 = 15 in the male [16-18]. *S. latifolia* is also a well studied dioecious and consists of 2n = 22+XX in female and 2n = 22+XY in male [15]. In *R. acetosa*, the X chromosome contained genes that determined female sex, whereas autosomes contained male sex genes [15,19]. In contrary, male determining factors were located on Y chromosome in *S. latifolia,* which comprised of various genes, for instance, suppressing gynoecium development or stimulating male-development [15,20]. However, Y chromosome of *R. acetosa* contained specific repetitive sequences like *RAYS1* (*R. acetosa* Y chromosome-specific sequence 1) and *RAE180*, which suppress recombination between sex chromosomes [17-18]. These results support the idea that Y chromosomes of *R. acetosa*'s have characteristics of a well-differentiated in comparison to Y chromosome of *S. latifolia*'s [15] this sentence is not clear to me.

Using molecular methods, sex-linked markers have been developed in several dioecious plants: AFLP markers in *Cannabis sativa* [21] and *Rumex nivalis* [22], RAPD markers in *S. latifolia* [23] and *Carica papaya* 24]. Several genes specific to male plants have also been cloned by differential screening of cDNA library [25], Y-chromosome microdissection [26] and fluorescent differential display (FDD) in *S. latifolia* [27]. In *R. acetosa*, seven sequences, *RAYS1*, *RAE180*, *pRA37*, *RAY-1.5*, *RAY-3*, *RAY-7* and *RAY-9*, have been considered to be sex-linked [17-18, 28-30]. However, study on sex specific DNA has been largely carried out in *S. latifolia*, which is recalcitrant to transgenesis, regeneration

*To whom correspondence should be addressed.
E-mail : ykhur@cnu.ac.kr
Received Aug. 5, 2005 Accepted Aug. 12, 2005

and transformation. Although *R. acetosa* has well-differentiated sex chromosomes as well as well-established plant regeneration system [31-32], yet progress in understanding mechanism of sex determination is slow.

DOP-PCR (degenerate oligonucleotide-primed PCR), which employs oligonucleotides of partially degenerate sequence, has been proposed as a rapid, efficient, and species-independent technique for general DNA amplification. Thus, in conjunction with chromosome manipulation, DOP-PCR provided a good cloning method for finding new markers on specific chromosome [33]. Hernould *et al.* [34] isolated chromosome fragments by using microdissection method and amplified chromosome specific regions by DOP-PCR, cloning the transcribed DNA sequences specific for the restored line in alloplasmic male-sterile tobacco. Buzek *et al.* [35] also used the microdissection to isolate X chromosome specific DNA clones. Therefore, it would be the best tool to clone chromosome-specific DNA if AFLP technique is added to DOP-PCR product of isolate chromosome.

R. acetosa plants possess extraordinarily big sex chromosomes that offer convenient manipulation of chromosome. In addition, the relatively recent origin of Y chromosomes, compared with those of most animals, make the dioecious plants particularly suitable for studying the early stages of the degeneration process of sex chromosome. In this study, we applied chromosome manipulation, DOP-PCR and AFLP techniques to isolate sex chromosome specific DNAs. The cloned DNA was subjected to Southern blot and sequence analyses.

MATERIAL AND METHODS

Plant material

Rumex acetosa L. plants were collected from a natural population in Daejon, Chungnam, Korea and grown in greenhouse at The Chungnam National University. Female and male plants were discriminated after flowering and planted in separated pots. Young leaves and roots were harvested for DNA isolation and cytological study, respectively.

Manipulation of chromosome and DOP-PCR of sex-chromosomal DNA

To prepare chromosome sample for micromanipulation, the root tips were treated with 2 mM 8-hydroxyquinoline for 2-3 h at 20°C and fixed in acetic: ethanol (1: 3) at 4°C overnight. The fixed root tips were macerated in 1N HCI (60°C) for 10 min and stained with 1% aceto-carmine and squashed in 45% acetic acid. The cover slips were removed using the dry-ice method.

Chromosome manipulation was performed under the inverted microscope (IM Axiovert 35 Zeiss) with extended micro-needles controlled by an electric micromanipulater (Eppendorf 5170). The micro-needles were prepared from borosilicate glass rods (hilgenberg gmbH) pre-treated in an oven for 2h at 180°C and fashioned with a micropipette puller forming a tip of less then 1 mm in diameter. The tips carrying either X or Y chromosome were broken off and pooled in 1x *Taq* polymerase buffer at 0°C (TaKaRa) according to Telenius *et al.* [33]. The chromosomes were used immediately in PCR amplification reaction. In independent experiments, 1 chromosome was isolated to check the minimum amount of chromosomal DNA required for PCR amplification.

DOP-PCR was performed according to the modified method of Telenius *et al.* [33]. *Taq* polymerase buffer (TaKaRa), 200 mM of each dNTP (TaKaRa), 50 pM primer (5'- CCGAGGT ACCNNNNNNATGTGG-3'), and 1 units of *Taq* polymerase (TaKaRa) were added in a tube containing sex chromosome (one chromosome or two chromosomes per tube). PCR amplification was performed in iCycler (BIO-RAD) with the following program: 5 min at 94°C followed by 5 cycles of 30 sec at 94°C, 30 sec at 30°C, 3 min transition from 30°C to 72 °C, 3 min extension at 72°C, followed by 30 cycles in high-stringency conditions: 1 min at 94°C, 1 min at 50°C, 2 min with an addition of 1 sec per cycle at 72°C, and a final extension step of 10 min at 72°C. Negative control reactions without DNA were processed identically.

To amplify the DOP-PCR products before AFLP analysis, 1 μl of the first round of DOP-PCR product was subjected to a second round of 35 cycles in high-stringency conditions. The PCR products were purified with PCR QuickSpin columns (Qiagen), quantified with Gene Quant spectrophotometer (Pharmacia), and analyzed on 1.8% agarose gel electrophoresis.

AFLP analysis of DOP-PCR products

AFLP analysis of the chromosomal DOP-PCR products was performed according to the method of manufacturer's instruction of the AFLP® Analysis System (GIBCO BRL). The DOP-PCR products were completely digested *Eco*RI and *Mse*I. The digested fragments were purified and ligated with *Eco*RI-adaptor [5'-AATTGGTACGCAGTC (*Eco*RI-oligo 2), 5'-CTC GTAGACTGCGTACC (*Eco*RI-oligo 1)] and *Mse*I-adaptor [5'-TACTCAGGACTCAT (*Mse*I-olig 2), 5'-GACGA TGAGTCCTGAG (*Mse*I-oligo 1)]. The ligation mix was diluted by 10 times and stored at -20°C until use. Preamplification reaction was carried out in the presence of 30 ng of *Eco*RI primer (5'-GACTGCGTACCAATTC+A) and *Mse*I primer (5'-GATGAGTCCTGAGTAA+C) for 20 cycles of 94°C for 30s, 56°C for 60s, and 72°C for 60s. The reaction products was diluted by 50 times and stored at -20°C. *Eco*RI selective primers for AFLP-PCR, which are extended by 1 to 3 bp at its 3', were labeled with T_4 polynucleotide kinase in the presence of γ-^{33}P-dATP at 37°C for 1 h. Selective AFLP amplification was carried out with the labeled *Eco*RI primer and unlabeled *Mse*I primer. The PCR performed with 10 files (1 cycle for file 1 through 9, 33 cycles for file 10): file 1; 94°C for 60s, 65°C for 60s, 72°C for 90s, file 2: 94°C for 60s, 64°C for 60s, 72°C for 90s, file 3; 94°C for 60s, 63°C for 60s, 72°C

for 90s, file 4; 94°C for 60s, 62°C for 60s, 72°C for 90s, file 5; 94°C for 60s, 61°C for 60s, 72°C for 90s, file 6; 94°C for 60s, 59°C for 60s, 72°C for 90s, file 7; 94°C for 60s, 58°C for 60s, 72°C for 90s, file 8; 94°C for 60s, 57°C for 60s, 72°C for 90s, file 9; 94°C for 60s, 56°C for 60s, 72°C for 90s, and file 10; 94°C for 60s, 64°C for 60s, 72°C for 90s. The PCR product was separated on 6% sequencing gel containing 8 M urea and the gel was dried and exposed to X-ray film.

AFLP specific bands (either X or Y chromosome specific bands) were excised from the dried gel. DNA was eluted by "Crush & Soak" method [36] and PCR-amplified using "Universal AFLP Cloning"-primers (U-EcoRI and U-MseI).

Reverse Southern analysis

The PCR-amplified AFLP DNAs were fractionated on 1.5 % agarose gel, depurinated, denatured, neutralized, and blotted on NYLON TRANSFER MEMBRANE (Schleicher & Schuell) in 20× SSC for 90 min using VacuGene™ XL Vacuum Blotting System (Phamacia Biotech). The blots were prehybridized in a hybridization solution (5x SSC, 5x Denhardt's solution, 0.5% SDS and 100 mg/mL of herring testis DNA) and hybridized in a hybridization solution containing ^{32}P-dCTP-labeled probe prepared from male or female *R. acetosa* genomic DNA using random prime labeling system [Rediprime™ (Aamersham Phamacia)]. The membranes were washed twice in 2x SSC/0.1% SDS for 10 min at room temperature, and twice in 0.1x SSC/0.1% SDS for 10 min at 65°C, and exposed on MEDICAL X-RAY FILM (FUJIFILM).

Cloning of sex specific clones and sequences analysis

Sex-chromosome specific AFLP clones were selected on the basis of the reverse Southern blot analysis. Each DNA was reamplified with "Universal AFLP Cloning"-primers (U-EcoRI and U-MseI) and cloned into pGEM®-T Easy Vector (Promega). The recombinant plasmid DNA was sequenced using ABI Prism 377 automated sequencer (Applied Biosystem Division of Perkin Elmer) with four fluorescent dyes. The similarity of DNA sequences was determined with known sequences in the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov) with the BLAST search program.

Genomic Southern hybridization

Rumex genomic DNA was extracted from leaves by the CTAB precipitation method [37]. Twenty micrograms of the genomic DNA were digested with *Pst*I, *Eco*RI, and *Hin*dIII, and fractionated on 0.7% agarose gel. The DNA was transferred to Nylon (Schleicher & Schuell) using VacuGene™ XL Vacuum Blotting System (Phamacia Biotech) and membrane fixed by UV crosslinker. The DNA on the filter was hybridized at 42°C overnight with ^{32}P-labeled probe (4×10^6 cpm/ml) in hybridization solution containing 6× SSPE, 5× Denhardt's reagent, 0.1% SDS, 50% formamide and 100 μg/ml salmon sperm DNA. The filter was washed twice with 6× SSPE and 0.1% SDS for 5 min at room temperature, followed by two washings 1× SSPE and 0.1% SDS at 60°C for 30 min. The filter was then exposed on Screen of Personal FX (BIO-RAD).

RESULTS

Micromanipulation of sex chromosomes from R. acetosa

To isolate sex chromosome specific DNA from *Rumex acetosa*, sex chromosomes were first isolated from mitotic chromosome spreads using a micromanipulator. After aceto-carmine staining of metaphase chromosomes from male and female plant roots, X and Y chromosomes were removed by extended micro-needles under the inverted microscope (IM Axiovert 35 Zeiss)(Fig. 1). Since Y1 and Y2 chromosomes were hardly distinguishable in common staining, we collected two chromosomes in a same tube.

DOP-PCR amplification of isolated chromosomal DNA

To clone coding sequences as many as possible, the primer for DOP-PCR was designed in which ATG start codon was located at the 3': 5'- CCGAGGTACCNNNNNNATGTGG-3'. As shown in Figure 2A, DOP-PCR product from either X or Y chromosomes ranged from 500 to 5,000 bp. The maximum yield was obtained from the PCR with 2 chromosomes each (data not shown). Before AFLP analysis, the DOP-PCR products were enriched by amplification of the second round

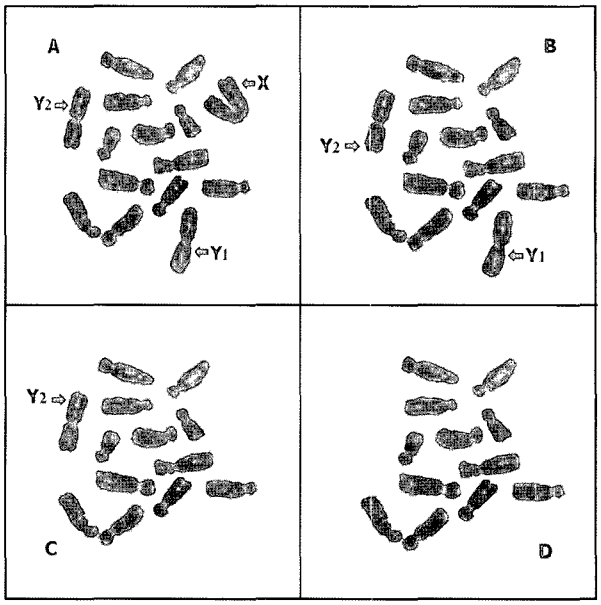

Figure 1. A serial process of chromosome micromanipulation. A, Mitotic chromosome spreads from root tip cells of a male *R. acetosa* plant. B, Chromosome set after X chromosome removed. C, Chromosome set after Y1 chromosome removed. D, Chromosome set after Y2 chromosome removed.

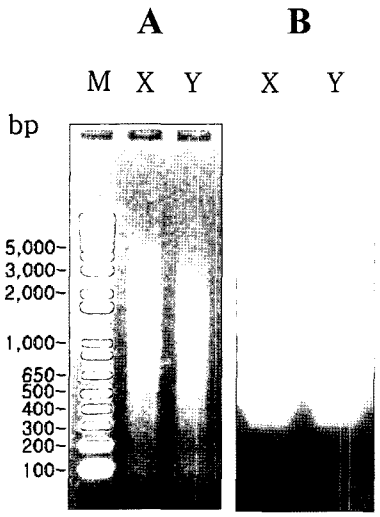

Figure 2. Electrophoresis of DOP-PCR products from X and Y chromosomes (A), and its reamplified products (B). DOP-PCR was performed with 2 chromosomes from either X or Y chromosome. M, 1kb DNA ladder marker. Numbers at left indicate mobilities of markers with lengths in base pair.

of 35 cycles in high stringency (Fig. 2B). Most of DNAs reamplified ranged from 500 to 1,000 bp.

AFLP analysis of DOP-PCR products

To clone sex chromosome specific DNA efficiently, DOP-PCR products were subsequently subjected to AFLP analysis with *Eco*RI and *Mes*I primers. After selective PCR, AFLP products were resolved on 6% sequencing gel. As shown in Figure 3, each lane (X or Y chromosome products) consists of

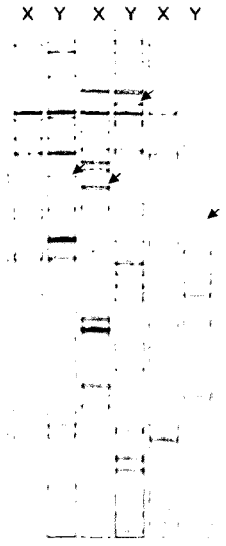

Figure 3. AFLP pattern of X- and Y-chromosome DOP-PCR products. X, AFLP bands from X-chromosome DOP-PCR product; Y, AFLP bands from Y-chromosome DOP-PCR product. Arrowhead indicates chromo-some specific band.

average 76 bands. Different combination of the selective primers produced different AFLP patterns. From 64 primer pairs, a total of 1,749 AFLP bands specific to either X or Y chromosome was obtained. Among them, 658 bands were X specific, while 1,091 Y specific (data not shown).

Reverse Southern hybridization, cloning of sex specific clone and sequence analysis

AFLP specific bands for either X or Y chromosome were excised, eluted and reamplified with 'Universal AFLP Cloning' primers. The size of the PCR products ranged from 200 to 600 bp (Fig. 4). Blots were prepared from 1.5% agarose gel electrophoresis using AFLP specific bands and hybridized with ^{32}P-dCTP-labeled probe prepared from either male or female genomic DNA (Fig. 4). As a result of the reverse Southern hybridization using 500 AFLP specific bands, 13 female- and 5 male-specific clones were obtained, ranging from 72 to 593 bp. We designated them *RADAX* (*Rumex acetosa* DOP-PCR AFLP X-chromosome specific) and *RADAY* (*Rumex acetosa* DOP-PCR AFLP Y-chromosome specific), respectively. These putative sex-chromosome specific AFLP DNAs were subsequently cloned into pGEM®-T Easy Vector, subjected to nucleotide sequencing and sequence analysis using NCBI Blast search program.

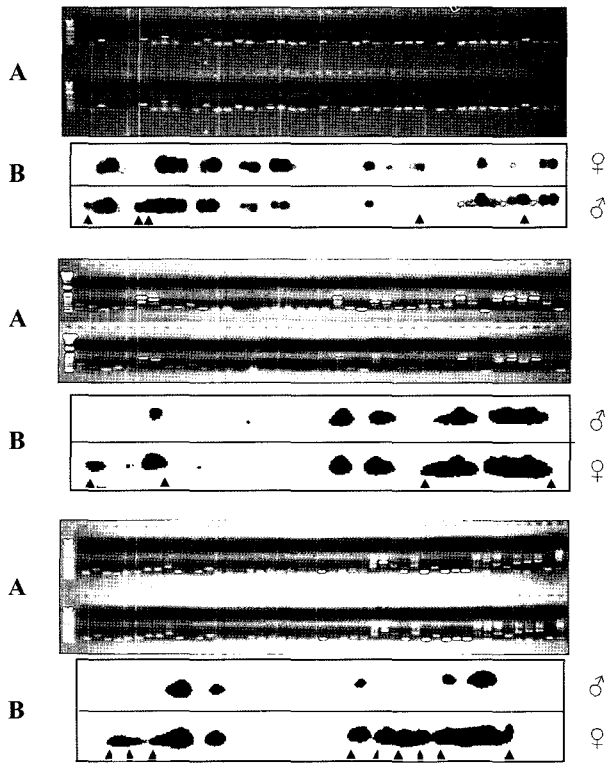

Figure 4. AFLP fragments (A) and reverse Southern hybridization pattern (B). The fragments were separated on 1.5% agarose gels (duplicated), blotted onto nylon membrane and hybridized with probes from either sex genomic DNA (symbol on right). Arrows indicate sex-specific bands.

As summarized in Table 1, all clones have no high similarity with any known DNA up to date, except *RADAX-239*. Even though we designed DOP-PCR primer capable to amplify coding sequence, non of them besides *RADAX-239* (Figure 5) seemed to contain a part of open reading frame (ORF). RADAX-239 contains a conserved domain for putative ABC-ATPase and it shows identities of 65% and 50% with bacterial hemolysin secretory transport system ATP-binding protein (GenBank Accession No, AH09491 = [38]) and rice homolog (GenBank Accession No, CAD59588) at the amino acid sequence level, respectively. However, *RADAX-239* has no good match to any known DNA at nucleotide sequence level.

Genomic Southern hybridization

To examine whether clones isolated from each sex chromosome are sex specific, genomic Southern blot analysis was carried out. Unexpectedly all clones showed similar patterns as shown in Figure 6 as two selected clones; Southern hybridization patterns were similar in male and female plants, implying that degeneration is not completed in sex chromosomes or sequences are redundant.

DISCUSSION

R. acetosa (sorrel) is a dioecious plants that have well developed sex chromosome system, XY system, that is analogous to those of many animals [14]. The fact that Y chromsome of *R. acetosa* is constitutive heterochromatic while *S. latifolia* not [5] can lead that the sex chromosome of *R. acetosa* are older than those of those of *S. latifolia,* the best

```
TCACTGCGTACCAATTCAGGGTCATGTGGAGTGGTTATGCAGGAAGGTTAT
S  L  R  T  N  S  G  S  C  G  V  V  M  Q  E  G  Y

GTTTTCAATGATACGATTGCGGAAAATATTGCTGTAGGTGAAGATTATGTT
V  F  N  D  T  I  A  E  N  I  A  V  G  E  D  Y  V

GACAAACAAAAACTTAGAAAAGCCGTTGAGATTGCAAACATCAAAGATTTT
D  K  Q  K  L  R  K  A  V  E  I  A  N  I  K  D  F

GTAGAAAGTCTTCCGTTGAGCTATAACACGAAAATTGGCAATGAAGAAGTG
V  E  S  L  P  L  S  Y  N  T  K  I  G  N  E  E  V

GGTGTTAGCGGAGGTCAAAAACAGCGTTTATTTATTGCAAGAGCCGTTTAT
G  V  S  G  G  Q  K  Q  R  L  F  I  A  R  A  V  Y

AAATCTCCTGATTACATATTTTTTGATGAAGCAACTTCTGCTTTAGACGCG
K  S  P  D  Y  I  F  F  D  E  A  T  S  A  L  D  A

AATAATGAGAAAATCATTATGGAAAATCTGGAACAATTCTTTGAAGGTAAA
N  N  E  K  I  I  M  E  N  L  E  Q  F  F  E  G  K

ACAGCCATTGTCATTGCACACCGGTTGTCCACAGTAAAACACGCGGACAAA
T  A  I  V  I  A  H  R  L  S  T  V  K  H  A  D  K

ATTATTGTTTTGGATCAGGGGAAGGTTGTAGAAGAAGGAAACCATCAAGAG
I  I  V  L  D  Q  G  K  V  V  E  E  G  N  H  Q  E

CTTGTAACTTTGAAAGGCGAATATTACCGTTTAGTTACTCAGGACTCATC
L  V  T  L  K  G  E  Y  Y  R  L  V  T  Q  D  S
```

Figure 5. The nucleotide and deduced amino acid sequences of *RADAX-239*.

Table 1. Summary of sex chromosome specific clones, *RADAX* and *RADAY*

DNA Origin	Sequence Designation	Size (bp)	Result of BLAST Search	Remarks
X-chromosome	RADAX-146	415	no hit found	AY520580
	RADAX-229	316	no hit found	
	RADAX-230	337	no hit found	
	RADAX-232	200	no hit found	
	RADAX-239	509	ABC-ATPase ORF*	
	RADAX-241	298	no hit found	
	RADAX-248	142	no hit found	
	RADAX-251	278	no hit found	
	RADAX-263	217	no hit found	
	RADAX-264	246	no hit found	
	RADAX-266	333	no hit found	
	RADAX-271	427	no hit found	
	RADAX-272	301	no hit found	
Y-chromosome	RADAY-426	593	no hit found	AY522655
	RADAY-429	195	no hit found	AY522656
	RADAY-431	74	no hit found	AY522658
	RADAY-433	197	no hit found	AY522660
	RADAY-434	72	no hit found	AY522661

*putative hemolysin secretion transport system ATP-binding protein [*Bacteroides fragilis* NCTC 9343].

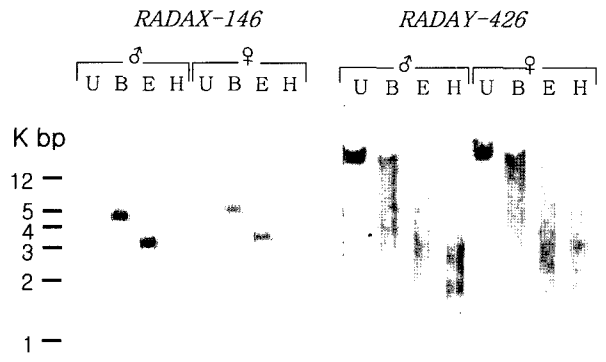

Figure 6. Southern hybridization analysis of *RADAX-146* and *RADAY-426* clones. Genomic DNA from male and female plants of *R. acetosa* was digested with three restriction endonucleases, fractionated on 0.7% agarose gel, transferred to Nylon membrane and hybridized with probes prepared from each DNA clone. ♂, male plant; ♀, female plant; M, 1 kb marker; U, undigested DNA; B, *Bam*HI-digested DNA; E, *Eco*RI-digested DNA; H, *Hin*III-digested DNA. Numbers at left indicate mobilities of markers with lengths in kilobase pair.

studied dioecy [39]. It is generally accepted that the sex determination in sorrel is controlled by the activities of genes present in X chromosome and autosomes, while Y chromosome only possess gene required for the production of fertile pollen [13]. However, neither sex determination-associated gene nor molecular mechanism underlined in the sex determination has been elucidated yet.

The well-established regeneration system [31-32] and its extraordinarily big sex chromosomes of *R. acetosa* make the plant a model system for chromosome micromanipulation and cloning of sex-determination genes. We isolated sex choromosomes (X, Y1 and Y2) by micromanipulation, followed by DOP-PCR and AFLP experiment. As a result of reverse Southern blot analysis for AFLP fragments, 18 clones were selected (13 specific for X and 5 Y chromosome) and subjected to sequence analysis. Most clones possess unique sequences for *R. acetosa*, while even one clone, RADAX-239, shows a conserved domain at the amino acid level. Although DOP-PCR primer was designed to amplify possible ORF sequence, only one clone obtained from this study was protein coding genes whatever they are degenerated or not. *RADAX-239* clone showed the highest identity to a bacterial ABC-ATPase transporter, while the lowest to the rice homolog. However, it is uncertain whether the *RADAX-239* clone is involved in the sex determination in *R. acetosa*. In plants, the first active gene, *MOROS*, on Y chromosome has been identified in *S. latifolia* by differential screening of cDNA library [25]. Male reproductive organ specific (*MOROS*: *1-4*) cDNAs were expressed only in male reproductive organs. Among them, *MROS3* was expressed in the early buds and its products showed no similarity to known proteins. Genomic Southern blot analysis showed that male and female genomes included the gene [25]. Further analysis revealed that *MOROS3* gene was X-linked and its degenerate locus was present on Y chromosome, showing that a degenerate Y-linked homologue exists for an X-linked locus [11]. Similar situation is true in human. Human Y chromosome contains the testis determining factor, *SRY*, and several genes necessary for spermatogenesis. Y chromosome seemed to be homologous with X, but has been progressively degraded, and now composed of largely repeated sequences as well as degraded copies of X-linked genes [40].

The result of Southern blot analysis with clones that are isolated from different sex chromosome did not discriminate between two sexes. Matsunaga *et al.* [25] also obtained similar result in *S. latifolia*. This phenomenone may provide an idea on the evolution and degeneration of sex chromosomes. Over the time, evolutionary process of sex chromosomes may cause the Y chromosome to degenerate and to diverge from the X chromosome over much of its length; for example, only 5% of the human Y chromosome still shows X-Y recombination [41]. Liu et al. [42] reported that papaya has a primitive Y chromosome, with a male specific region that accounts for only about 10% of the chromosome but undergone severe recombination suppression and DNA sequence degeneration. From this finding, they concluded that sex chromosomes were originated from autosomes. In addition to the origin of autosomes, the homology of certain clones between two sex chromosomes also supports a suggestion by Gutttman and Charlesworth [11] who insisted the common ancestry for two loci if there is homology between the active X-linked gene and its degenerate Y-linked locus.

To confirm the Southern blot data by proving the localization of clones on chromosomes, we tried to carry out fluorescence *in situ* hybridization (FISH), but failed to obtain information because of its short DNA sequence as probes. However, *R. acetosa* plant may be still good material for chromosome micromanipulation, study on the evolution of sex chromosomes and cloning of sex-determination genes because it contain a primitive and extraordinarily big sex chromosomes and well-established regeneration system.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Korea Research Foundation (KRF-2000-DP0401) to Y. Hur.

REFERENCES

1. Stothard, P. and D. Pilgrim (2003) Sex-determination gene and pathway evolution in nematodes. *Bioessays* **25,** 221-31.
2. Saccone, G., A. Pane and L. C. Polito (2002) Sex determination in flies, fruitflies and butterflies. *Genetica* **116,** 15-23.
3. Cotinot, C., E. Pailhoux, F. Jaubert and M. Fellous (2002)

Molecular genetics of sex determination. *Semin. Reprod. Med.* **20,** 157-68.
4. Morrish, B. C. and A. H. Sinclair (2002) Vertebrate sex determination: many means to an end. *Reproduction* **124,** 447-57.
5. Grant, S., A. Houben, R. Vyskot, J. Siroky, W.-H. Pan, J. Macas and H. Saedler (1994) Genetics of sex determination in flowering plants. *Dev. Genet.* **15,** 214-230.
6. Theissen, G., A. Becker, A. D. Rosa, A. Kanno, J. T. Kim, T. Münster, K.-U. Winter, and H. Saedler (2000) A short history of MADS-box genes in plants. *Plant Mol. Biol.* **42,** 115-149.
7. Hardenack, S., H. Saedler, D. Ye and S. Grant (1994) Comparison of MADS box gene expression in developing male and female flowers of the dioecious plants white campion. *Plant Cell* **6,** 1775-1787.
8. Ainsworth, C., S. Crossley, V. Buchanan-Wollaston, M. Thangavelu and J. Parker (1995) Male and female flowers of the dioecious plant sorrel show different patterns of MADS box gene expression. *Plant Cell* **7,** 1583-1598.
9. Janousek, B., J. Siroky and B. Vyskot (1996) Epigenetic control of sexual phenotype in a dioecious plant, *Melandrium album*. *Mol. Gen. Genet.* **250,** 483-490.
10. Charlesworth, D., and P. M. Gilmartin (1998) Lily or Billy-Y the difference? *Trends Genet.* **14,** 261-262.
11. Guttman, D. S. and D. Charlesworth (1998) An X-linked gene with a degenerate Y-linked homologue in a dioecious plant. *Nature* **393**, 263-266.
12. Filatove, D. A., F. Monéger, I. Negrutiu and D. Charlesworth (2000) Low variability in a Y-linked plant gene and its implications for Y-chromosome evolution. *Nature* **404,** 388-390.
13. Ainsworth, C., Parker, J. S., and Buchanan-Wollaston, V. (1998) Sex determination in plants. *Curr. Top. Dev. Biol.* 38, 167-223.
14. Charlesworth, D. (2002) Plant sex determination and sex chromosomes. *Heredity* **88**, 94-101.
15. Matsunaga, S. and S. Kawano (2001) Sex determination by sex chromosomes in dioecious plants. *Plant Biol.* **3**, 481-488.
16. Jin, D.-C., D. Koo, Y. Hur and J.-W. Bang (2003) Variation of RAPD patterns between male and female genomic DNAs in dioecious *Rumex acetosa L. Korean J. Plant Res.* **16**, 55-60.
17. Shibata, F., M. Hizume and Y. Kuroki (1999) Chromosome painting of Y chromosomes and isolation of a Y chromosome-specific repetitive sequence in the dioecious plant *Rumex acetosa. Chromosoma* **108**, 266-270.
18. Shibata, F., M. Hizume and Y. Kuroki (2000) Differentiation and the polymorphic nature of the Y chromosomes revealed by repetitive sequences in the dioecious plant, *Rumex acetosa. Chromosome Res.* **8**, 229-236.
19. Ono, T. (1930) Chromosome morphologie von *Rumex acetosa. Sci. Repts. Tohoku Imp. Univ. Fourth Ser.* 5, 158-182.
20. Ono, T. (1939) Polyploidy and sex determination in *Melandrium*. 1: colchicines-induced polyploids of *Melandrium album*. *Bot. Mag.* **53,** 549-556.
21. Peil, A., H. Flachowsky, E. Schumann and W. E. Wever (2003) Sex-linked AFLP markers indicate a pseudoautosomal region in hemp (Cannabis sativa L.). *Theor. Appl. Genet.* **107,** 102-109.
22. Stehlik, I., and Blattner, F. R. (2004) Sex-specific SCAR markers in the dioecious plant *Rumex nivalis* (Polygonaceae) and implications for the evolution of sex chromosomes. *Theor. Appl. Genet.* 108: 238-242.
23. Nakao, S., S. Matsunaga, A. Sakai, T. Kuroiwa and S. Kawano (2002) RAPD isolation of a Y chromosome specific ORF in a dioecious plant, *Silene latifolia. Genome* **45,** 413-420.
24. Deputy, J. C., R. Ming, H. Ma, Z. Liu, M. M. M. Fitch, M. Wang, R. Manshardt and J. I. Stiles (2002) Molecular markers for sex determination in papaya (*Carica papaya* L.). *Theor. Appl. Genet.* **106,** 107-111.
25. Matsunaga, S., S. Kawano, H. Takano, H. Uchida, A. Sakai and T. Kuroiwa (1996) Isolation and developmenta expression of male reproductive organ-specific genes in a dioecious campion, *Melandrium album* (*Silene latifolia*). *Plant J.* **10,** 679-689.
26. Delichère, C., J. Veuskens, M. Hernould, N. Barbacar, A. Mouras, I. Negrutiu and F. Monégers (1999) SlY1, the first active gene cloned from a plant Y chromosome, encodes a WD-repeat protein. *EMBO J.* **18,** 4169-4179.
27. Scutt, C. P., T. Jenkins, M. Furuya and P. M. Gilmarthin (2002) Male specific genes from dioecious white campion identified by fluorescent differential display. *Plant Cell Physiol.* **43,** 563-572.
28. Ruiz Rejón, C., M. Jamilena, M. Garrido Ramos, J. S. Parker and M. Ruiz Rejón (1994) Cytologenetic and molecular analysis of the multiple sex chromosome system of *Rumex acetosa. Heredity* 72, 209-215.
29. Park, J.-Y., D.-H. Koo, D.-C. Jin, J.-W. Bang and Y. Hur (2004) Identification of a Long Y-chromesome enriched repetitive sequence in *Rumex acetosa* L. *Mkor. J. Genet.* 26, 163-170.
30. Jin, D.-C., J. Y. Park, J.-W. Bang and Y. Hur (2004) Isolation and characterization of male-specific DNAs by genomic AFLP from the dioecious plant *Rumex acetosa* L. *Kor. J. Genet.* **26**, 171-178.
31. Lee, M. K. and J.-W. Bang (1991) Plant regeneration and somaclonal variation from the leaf segment cultures of *Rumex acetosa. Kor. J. Plant Tissue Culture* **18**: 377-382.
32. Lee, M. K., Choi, H.-W., and Bang, J.-W. (1991) Karyotype and chromosomal polymorphism in *Rumex acetosa* L. *Kor. J. Genet.* **13,** 271-280.
33. Telenius, H., N. P. Carter, C. Bebb, M. Nordenskjold, B. J. Ponder and A. Tunnacliffe (1992) Degenerate oligonucleotide-Primed PCR; General Amplification of Target DNA by a single degenerate primer. *Genomics* **13,** 718-725.
34. Hernould, M., K. Glimelius, J. Veuskens, P. Bergman and A. Mouras (1997) Microdissection and amplification of coding sequences from a chromosome fragment restoring male fertility in alloplasmic male-sterile tobacco. *Plant J.* **12,** 703-709.
35. Buzek, J., H. Koutnikova, A. Houben, K. Riha, B. Janousek,

J. Siroky, S. Grant and B. Vyskot (1997) Isolation and characterization of X chromosome-derived DNA sequences from a dioecious plant *Melandrium album*. *Chromosome Res.* **5,** 57-65.

36. Sambrook, J., R. Maniatis and F. F. Fritsch (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor, *New York.*
37. Murray, M. G. and W. F. Thompson (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acid Res.* **8,** 4321-4325.
38. Cerdeño-Tárraga, A. M., S. Patrick, L. C. Crossman, G. Blakely, V. Abratt, N. Lennard, I. Poxton, B. Duerden, B. Harris, M. A. Quail, A. Barron, L. Clark, C. Corton, J. Doggett, M. T. G. Holden, N. Larke, A. Line, A. Lord, H. Norbertczak, D. Ormond, C. Price, E. Rabbinowitsch, J. Woodward, B. Barrell and J. Parkhill. 2005. Extensive DNA inversions in the *B. fragilis* genome control variable gene expression. *Science* **307**, 1463-1465.
39. Negrutiu, I., B. Vyskot, N. Barbacar, S. Georgiev and F. Moneger (2001) Dioecious plants, a key to the early events of sex chromosome evolution. *Plant Physiol.* **127,** 1418-1424.
40. Graves, J. A. M. (1995) The origin and function of the mammalian Y chromosome and Y-borne genes-an evolving understanding. *BioEssays* **17,** 311-321.
41. Skaletsky, H., T. Kuroda-Kawaguchi, P. J. Minx, H. S. Cordum, L. Hillier, L. G. Brown, S. Repping, T. Pyntikova, J. Ali, T. Bieri, A. Chinwalla, A. Delehaunty, K. Delehaunty, H. Du, G. Fewell, L. Fulton, R. Fulton, T. Graves, S. F. Hou, P. atrielle, S. Leonard, E. Mardis, R. Maupin, J. McPherson, T. Miner, W. Nash, C. Nguyen, P. Ozersky, K. Pepin, S. Rock, T. Rohlfing, K. Scott, B. Schultz, C. Strong, A. Tin-Wollam, S. P. Yang, R. H. Waterston, R. K. Wilson, S. Rozen and D. C. Page (2003) The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*, **423,** 825-837.
42. Liu, Z., P. H. Moore, H. Ma, C. M. Ackerman, M. Ragiba, Q. Yu, H. M. Pearl, M. S. Kim, J. W. Charlton, J. I. Stiles, F. T. Zee, A. H. Paterson and R. Ming (2004) A primitive Y chromosome in papaya marks incipient sex chromosome evolution. *Nature* **427,** 348-352.