Letter

DNA methylation and heterochromatinization in the male-specific region of the primitive Y chromosome of papaya

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Sex chromosomes evolved from autosomes. Recombination suppression in the sex-determining region and accumulation of deleterious mutations lead to degeneration of the Y chromosomes in many species with heteromorphic X/Y chromosomes. However, how the recombination suppressed domain expands from the sex-determining locus to the entire Y chromosome remains elusive. The Y chromosome of papaya (*Carica papaya*) diverged from the X chromosome approximately 2–3 million years ago and represents one of the most recently emerged Y chromosomes. Here, we report that the male-specific region of the Y chromosome (MSY) spans ~13% of the papaya Y chromosome. Interestingly, the centromere of the Y chromosome is embedded in the MSY. The centromeric domain within the MSY has accumulated significantly more DNA than the corresponding X chromosomal domain, which leads to abnormal chromosome pairing. We observed four knob-like heterochromatin structures specific to the MSY. Fluorescence in situ hybridization and immunofluorescence assay revealed that the DNA sequences associated with the heterochromatic knobs are highly divergent and heavily methylated compared with the sequences in the corresponding X chromosomal domains. These results suggest that DNA methylation and heterochromatinization play an important role in the early stage of sex chromosome evolution.

[Supplemental material is available online at www.genome.org.]

Sex chromosomes are thought to have evolved from autosomes (Ohno 1967; Bull 1983; Charlesworth 1991). Emergence of a sex-determining gene and suppression of recombination in its surrounding region initiates the evolution of sex chromosomes. The recombination-suppressed domain is believed to have expanded through acquiring and selecting sexually antagonistic genes or chromosomal rearrangements (Charlesworth et al. 2005). Absence of recombination led to the accumulation of deleterious mutations and erosion of genes in the Y chromosome of species with the XX/XY sex-determining system. Such a functional degeneration of the Y chromosome can be predicted by several population genetics-based models, including Muller's ratchet (Muller 1964), genetic hitchhiking (Rice 1987a), and background selection (Charlesworth 1994).

The Y chromosomes in *Drosophila melanogaster* and mammalian species are classical examples of highly degenerated Y chromosomes. The Y chromosomes in these species retain only a few functional genes and consist mainly of repetitive DNA sequences (Carvalho 2002; Skaletsky et al. 2003). DNA sequence-based analyses have yielded only limited clues about the evolutionary path of the Y chromosomes in these species (Lahn et al. 2001; Carvalho 2002). Relatively "young" sex chromosomes have been reported in several plant and animal species (Steinemann and Steinemann 1998; Bachtrog and Charlesworth 2002; Griffin et al. 2002; Matsuda et al. 2002; Liu et al. 2004; Peichel et al. 2004; Kondo et al. 2006; Telgmann-Rauber et al. 2007; Yin et al. 2008). Despite extensive studies on evolution of genes and repetitive DNA elements associated with young sex chromosomes in several species (for review, see Charlesworth et al. 2005), cytological analysis of young sex chromosomes has been missing. When and how the primitive sex chromosomes develop into heteromorphic sex chromosomes have remained elusive.

Papaya (*Carica papaya*, $2n = 2x = 18$) is a diploid fruit tree with a relative small genome of 372 Mb (Arumuganathan and Earle 1991). Papaya provides an excellent model to study sex chromosome evolution (Ming et al. 2007a). The sex of this trioecious species is controlled by a recently evolved XY system with two slightly different Y chromosomes—XX for female, XY for male, and XY^h for hermaphrodite (Ming et al. 2007a). The male-specific region of the Y chromosome (MSY) was estimated to account for only 10% of the Y chromosome (Liu et al. 2004). Analysis of X/Y and Y/Y^h gene pairs revealed the estimated divergence at approximately 0.6–2.5 million yr between X and Y and 73,000 yr between Y and Y^h (Yu et al. 2008a,b). The molecular mechanism of sex determination in papaya is not known, but the papaya MSY may contain a female-suppressing gene and a male fertility gene (Ming et al. 2007b). Papaya is also an excellent model species for molecular cytogenetic mapping. The number and sizes of the papaya chromosome provide an ideal system for pachytene chromosome preparation and visualization of euchromatin and heterochromatin in the genome (Ming et al. 2008). We conducted a pachytene chromosome-based cytogenetic mapping of the papaya MSY. We demonstrated that the papaya MSY, which spans ~13% of the Y^h chromosome, includes the Y centromere. We discovered distinct knob-like heterochromatic

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Article published online before print. Article and publication date are at http://www.genome.org/cgi/doi/10.1101/gr.078808.108.

18:1938–1943 ©2008 by Cold Spring Harbor Laboratory Press; ISSN 1088-9051/08; www.genome.org

structures specific to MSY. The DNA sequences associated with these knobs are highly divergent and heavily methylated. The cytological data suggest a role of DNA methylation and heterochromatinization in the early stage of evolution of the papaya Y chromosome.

Results

Heterochromatic knob formation and chromosomal pairing in the MSY

Meiotic pachytene chromosomes were prepared using young buds collected from hermaphrodite papaya plants that contain the X and Y^h chromosomes. The pachytene chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI), which preferentially binds to AT-rich regions and stains the heterochromatin domains of eukaryotic chromosomes (Kapuscinski 1995). The nine pachytene bivalents showed consistent and distinct DAPI-staining patterns (Fig. 1a,b). The XY^h pachytene bivalent is largely euchromatic based on its DAPI staining pattern. However, brightly stained and knob-like heterochromatin structures were observed in the MSY (Fig. 1a,c). The identification of this chromosomal region as the MSY was confirmed by fluorescent in situ hybridization (FISH) mapping of MSY-specific bacterial artificial chromosome (BAC) clones (see results below).

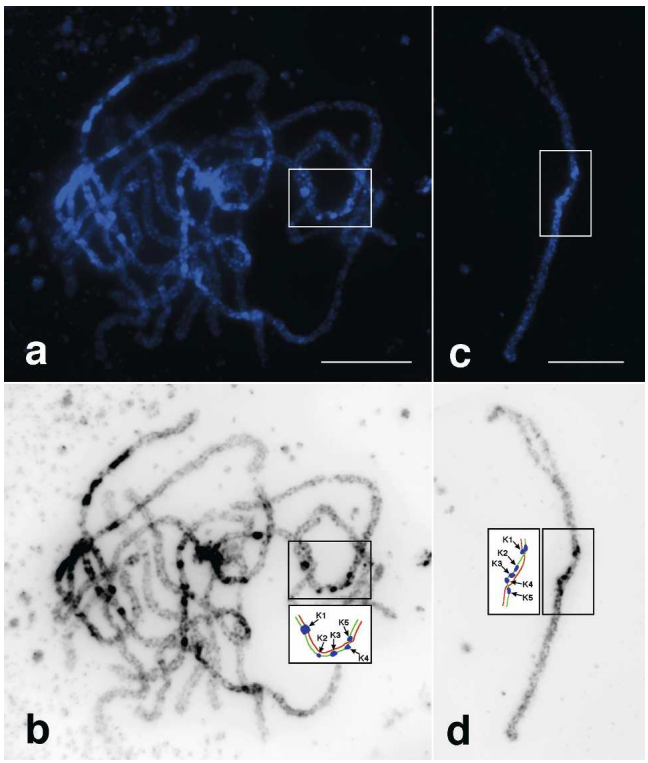

Figure 1. Heterochromatin formation in papaya MSY. (*a*) A complete pachytene cell stained with DAPI. The MSY is included in the square. (*b*) The same pachytene cell was converted into a black-white image. The five knobs in MSY are clearly visible. The diagram in the second square illustrates the (red) X and (green) Y^h chromosomes and the five (blue) knobs. (*c*) A complete pachytene XY^h bivalent stained by DAPI. The MSY is included in the square. (*d*) The DAPI image was converted into a black-white image. The diagram in the second square illustrates the X (red) and Y^h (green) chromosomes and the five knobs (blue). Bars, 10 µm.

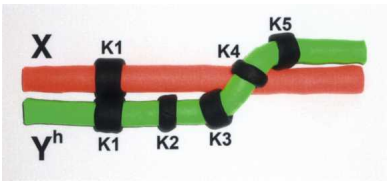

Figure 2. A diagram of X–Y^h chromosomal pairing in the MSY.

The knob-like heterochromatin is visualized better by converting the DAPI-stained chromosomes into black-and-white images (Fig. 1b,d). In most meiotic cells, five distinct knobs, named from K1 to K5, respectively, were observed in the MSY. These knobs showed different DAPI-staining intensities. K1 is the largest knob and is associated with both X and Y^h chromosomes. The other four knobs were only associated with the MSY and were absent in the corresponding regions on the X chromosome (Fig. 1b,d). The staining of K2 and K4 was lighter than K3 and K5 in most cells. Our recent cytogenetic and mapping data suggest that K1 is likely outside of the MSY boundary. Thus, the four knobs (K2–K5) within MSY are all Y chromosome specific.

A careful examination revealed a unique characteristic of chromosomal pairing in the MSY. The Y^h chromosomal domain associated with K4 consistently protruded away from X and appeared to be in a curved shape (Figs. 1d, 2), which is similar to a loop caused by a duplication/deletion event associated with one of the two chromosomes. A twist of pairing between the X and Y^h chromosomes consistently occurred between K4 and K5 (Figs. 1b,d, 2). These results suggest that the Y^h chromosomal domain around K4 has accumulated significantly more DNA than the corresponding domain on the X chromosome. Several BACs associated with K4 generated multiple FISH signals within the MSY (data not shown), suggesting that the DNA fragments cloned in these BACs have been at least partially duplicated. The larger agglomeration of DNA around K4 than the corresponding X domain may necessitate a twisting of the chromosomes to allow for faithful pairing between the X and the Y^h chromosomes (Fig. 2).

Papaya MSY spans the centromere of the Y chromosome

A BAC library developed from hermaphrodite papaya (Ming et al. 2001) was screened with male-specific DNA markers to develop a physical map of the MSY. The boundary of the MSY is defined by two BAC clones. One border of the MSY is marked by BAC 69A15. This BAC was anchored by a SSR marker CPM1055, which mapped 3 cM from the MSY (Chen et al. 2007). The other border is defined by BAC 86B15, which was isolated from chromosomal walking from a MSY-specific BAC contig. FISH mapping revealed that all five knobs on the XY^h bivalent were localized between these two BAC clones (Fig. 3a,b). The distance between K1 and K5 was measured on 27 X/Y^h pachytene bivalents, and the physical length from K1 to K5 accounted for 13.24% ± 1.02% of the X/Y^h chromosomes.

The papaya MSY was previously mapped to near the centromere of the Y chromosome (Yu et al. 2007). To precisely map the position of the centromere, we used a meiotic metaphase I-based FISH approach to delineate the position of individual knob relative to the centromere of the Y^h chromosome (Fig. 3c–f). Pairs of BAC clones specific to adjacent knobs were labeled in different colors and were mapped on metaphase I bivalent chromosomes. BAC 52H15 associated with K4 was mapped to the most poleward position compared with the other four knobs (Fig. 3c,d). These

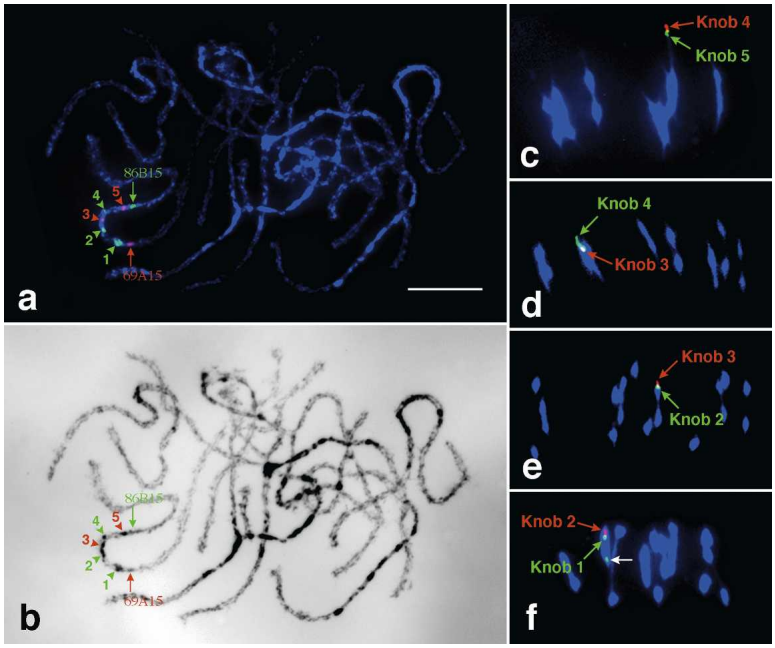

Figure 3. Pachytene FISH mapping of the MSY and the centromere of the Y^h chromosome. (*a*) FISH mapping of seven BAC clones. BACs 69A15 and 86B15, which mark the boundary of MSY, were mapped outside of knobs K1 and K5, respectively. FISH signals pointed to by arrowheads 1–5 were derived from BACs 12I03, 99O03, 95B12, 52H15, and 85B24, respectively. Bar, 10 µm. (*b*) The same pachytene cell image was converted to a black-white image. The locations of the FISH signals derived from the five BACs matched the locations of the five knobs. (*c*) FISH mapping of K4-specific BAC 52H15 (red) and K5-specific 85B24 (green) on metaphase I (MI) XY^h bivalent chromosome. K4 is positioned more poleward than K5. (*d*) MI FISH mapping of K4-specific 52H15 (green) and K3-specific BAC 47B08 (red). K4 is positioned more poleward than K3. (*e*) MI FISH mapping of K3-specific BAC 47B08 (red) and K2-specific 99O03 (green). (*f*) MI FISH mapping of K2-specific 99O03 (red) and K1-specific BAC 12I03 (green). The white arrow points to the K1 on the X chromosome.

results show that the centromere of the Y^h chromosome is either directly associated with K4 or is immediately adjacent to either side of this knob.

Significant divergence and hypermethylation of knob-associated DNA sequences in the MSY

The BAC clones isolated using MSY-specific DNA markers resulted in several BAC contigs. We conducted FISH analysis of more than 50 clones from these BAC contigs. The majority of these BAC clones were mapped to the five knobs (Fig. 3a,b). Most of the MSY-specific BACs associated with K2, K3, K4, and K5 generated strong FISH signals on the MSY, but only faint or no signals on the corresponding region of the X chromosome. We isolated two pairs of BACs (95B12/61H02, 85B24/53E18) using DNA sequences conserved between the MSY and the X chromosome (Yu et al. 2008a). Sequencing analysis of these BACs revealed significant divergence of the DNA fragments derived from the MSY and the corresponding X chromosomal regions (Yu et al. 2008a). FISH analysis showed that these two pairs of BACs are located at K3 and K5 of MSY and the corresponding X chromosomal domains, respectively (Fig. 4a–c). The four BAC clones hybridized almost exclusively to either X or Y chromosome and produced very limited cross hybridization signals (Fig. 4a–c). These results show that the majority of the DNA sequences located within these four MSY-specific knobs have significantly diverged from the sequences located in the corresponding X chromosomal regions. This divergence is in concordance with the fact that most previously isolated MSY-specific DNA markers were derived from the knob-associated DNA sequences.

DNA sequences in the heterochromatic chromosomal domains are often hypermethylated. We conducted immunofluorescence assays using an antibody against 5-methylcytosine (5mC) to investigate whether the knob-related DNA sequences in the MSY are more heavily methylated than the sequences located in the corresponding X chromosomal regions. In interphase nuclei, punctuated immunofluorescence signals, including a prominent signal closely associated with MSY, were associated with most, but not all, regions stained brightly by DAPI (Fig. 4e–g). On pachytene chromosomes, immunofluorescence signals associated with the five knobs in the MSY were similar to the DAPI staining pattern and were significantly brighter than the signals in flanking regions (Fig. 4h,i). We measured the amount of DAPI and 5mC immunofluorescence associated with each knob and its corresponding X chromosome domain on the pachytene chromosomes and then calculated the ratio of each pair of measurements (Table 1). The ratio of 5mC immunofluorescence intensity associated with each knob and its corresponding X domain is consistently higher than the ratio of the DAPI fluorescence (Table 1). These results show that the enhanced 5mC signals associated with the knobs are not a simple correlation with the relative amount of DNA associated with these heterochromatin structures. Thus, the DNA sequences associated with the knobs in MSY are highly methylated compared to those in the non-knob region and those in the corresponding X chromosome.

Discussion

Although the mechanism through which the proto-sex chromosomes diverged into heteromorphic sex chromosomes is not fully understood, it is widely agreed that suppression of genetic recombination in the sex-determining region is the prerequisite for sex chromosome evolution. However, it has been unclear as to how the recombination-suppressed region expands during the evolution, which leads to degeneration of the entire Y chromosome. Chromosome inversion spanning the sex-determining region has been used as a ready explanation because such a chromosomal rearrangement would result in complete suppression of recombination in the inverted region (Charlesworth et al. 2005). Four chromosomal inversions were proposed to explain the evolution of the human Y chromosome (Lahn and Page 1999). Despite the DNA sequence divergence associated with the papaya MSY (Yu et al. 2007, 2008a), the synteny of several genes and single copy sequences is well conserved between the MSY and its corresponding X chromosomal region based on both molecular

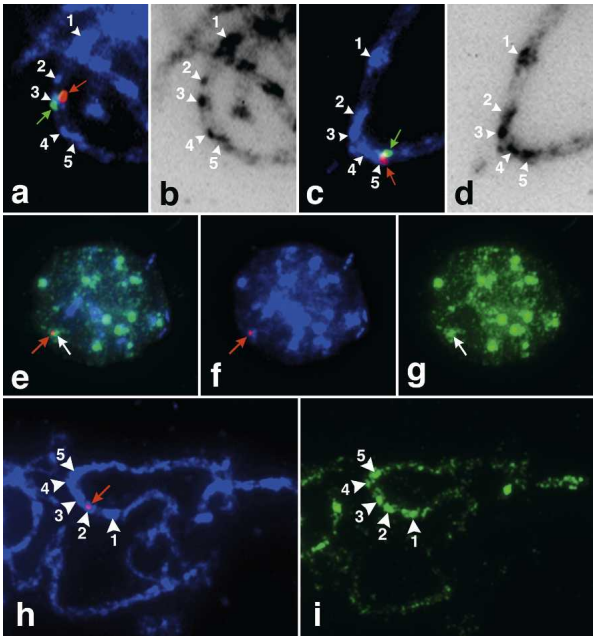

Figure 4. Divergence and hypermethylation of knob-associated DNA sequences in papaya MSY. (*a*) BACs 95B12 (green) and 61H02 (red) were selected by a pair of conserved DNA sequences specific to the MSY and the X chromosome, respectively. These two BACs hybridized only to their respective chromosomes. (*b*) The same pachytene chromosome was converted into a black-white image. BAC 95B12 can be located on K3. (*c*) BACs 85B24 (green) and 53E18 (red) selected by a pair of conserved DNA sequences specific to the MSY and the X chromosome, respectively. These two BACs hybridized only to the respective chromosomes. (*d*) The same pachytene chromosome was converted into a black-white image. BAC 85B24 can be located on K5. (*e*) Detection of 5mC (green signals) on an interphase nucleus. The MSY region is identified by a MSY-specific BAC 99O03 (red arrow), which is adjacent to a major punctuated 5mC signal (white arrow). The nucleus was stained by DAPI (blue). (*f*) Digitally separated BAC (red arrow) and DAPI signals. (*g*) Digitally separated 5mC signals. (*h*) A partial pachytene cell stained with DAPI. The MSY region was identified by the MSY-specific BAC 99O03 (red arrow). (*i*) Immunofluorescence assay using an anti-5mC antibody on the same pachytene cell. Bright signals are clearly visible on K1, K2, K3, and K5. The signal associated with K4 is relatively weaker. The five arrowheads in images *a*, *b*, *c*, *d*, *h*, and *i* point to the five knobs.

and cytological analyses (Fig. 4a–d) (Yu et al. 2008a). These results show that the MSY of papaya does not appear to contain a microscopic-sized chromosomal inversion that spans the complete or a major part of the MSY. The abnormal pairing around K4 in the MSY appears to be caused by the larger agglomeration of DNA around this knob.

It is interesting that the centromere of the papaya Y chromosome is located within the MSY. Recombination suppression is a common characteristic associated with all eukaryotic centromeres. It has been well demonstrated in rice, a plant species with a similar genome size as papaya, that the recombination-suppressed domains associated with the centromeres span several megabases of DNA and contain active genes (Yan and Jiang 2007). It is noteworthy that the centromere of the Y^h chromosome is located very close to K4, a region showing more divergence between X and Y^h than the rest of the MSY. Thus, the first sex-determining gene of papaya was possibly located within the centromeric region where recombination is severely or completely suppressed. Such a location would be favorable to the survival of the sex-determining gene. In parallel, several plant genes responsible for self-incompatibility (SI) have been mapped very close to centromeres (Entani et al. 1999; Wheeler et al. 2003). Entani et al. (1999) proposed that the centromeric location may suppress recombination and preserve the sequential uniqueness of the SI-responsible loci. Acquiring and/or selecting genes favorable to male functions near the original sex-determining gene, rather than chromosomal inversions, may be responsible for the expansion of the papaya MSY. Natural selection of such genes may result in a selective advantage to recombination suppression between these genes and the sex-determining region on the proto-sex chromosome (Rice 1987b; Charlesworth 1991; Charlesworth et al. 2005).

We observed multiple knob-like heterochromatin structures in papaya MSY but not in the corresponding X chromosomal region (Fig. 1). Heterochromatic knobs were first described by Barbara McClintock in maize (McClintock 1929, 1930). These heterochromatin features can be best visualized on meiotic pachytene chromosomes because of the differential condensation of euchromatin and heterochromatin at this stage of meiosis. Satellite DNA and transposons are the main DNA components of knobs characterized in several plant species (Peacock et al. 1981; McCombie et al. 2000; Cheng et al. 2001). The DNA sequences associated with the knobs in papaya MSY appear to be more extensively methylated compared with the sequences in the corresponding X chromosome domains (Fig. 4; Table 1). Complete sequencing of the MSY and its corresponding X chromosomal region and application of a more precise 5mC mapping method will reveal if this differential methylation is directly associated with the characteristics of the DNA sequence composition of the MSY.

We propose that the formation of the heterochromatic knobs in the MSY was initially induced by transposon invasion. One of the most common features associated with the "young" Y chromosome in several different species is the accumulation of transposon or transposon-derived repetitive DNA sequences (Steinemann and Steinemann 1992, 1998; Bachtrog 2003; Peichel et al. 2004). Sequencing several of the MSY-specific BAC clones of papaya revealed a high density of retroelements (Yu et al. 2007). It has been demonstrated that insertion of a tandem array of a transposon or amplification of transposon-derived satellite repeat can give rise to de novo formation of heterochromatin (Dorer and Henikoff 1994; Tek et al. 2005).

The impact of heterochromatinization on sex chromosome evolution was proposed previously based on research in animal species (Griffin et al. 2002; Steinemann and Steinemann 2005).

Table 1. Ratio of DAPI staining and 5mC immunofluorescence intensities between the knobs and their corresponding X chromosome region

Knob in MSY	Relative DAPI intensity[a]	Relative 5mC immuofluorescence intensity[b]	*n*
K2	1.82 ± 0.32	2.53 ± 0.73	20
K3	1.86 ± 0.56	2.41 ± 0.49	25
K4	1.79 ± 0.25	2.32 ± 0.45	23
K5	1.71 ± 0.31	2.24 ± 0.42	25

[a]The amount of DAPI fluorescence from a specific knob and its corresponding X chromosomal domain was measured, and the ratio was calculated from each pair of measurements.
[b]The amount of 5mC immunofluorescence from a specific knob and its corresponding X chromosomal domain was measured, and the ratio was calculated from each pair of measurements.

In cichlid fish (*Oreochromis niloticus*), the terminal portion of the X/Y bivalents show an absence of synapsis (Carrasco et al. 1999), which is caused by a delay in chromosome pairing in this region (Griffin et al. 2002). Cytological analysis showed no evidence of chromosomal rearrangements associated with this unpaired region (Griffin et al. 2002). Griffin et al. (2002) proposed that de novo accumulation of heterochromatin may cause the delay of sex chromosome pairing in meiosis, resulting in a decrease in recombination. Steinemann and Steinemann (2005) postulated that the massive invasion of retrotransposons in the neo-Y of *Drosophila miranda* can lead to a switch from euchromatin to heterochromatin in this chromosome. However, there was no cytological evidence showing heterochromatin formation specifically associated with the young Y chromosomes in these species.

We propose that DNA methylation plays a key role in heterochromatinization and gene silencing in Y chromosome evolution. DNA methylation and histone modification pathways are mechanistically linked, and the cross-talk of these pathways leads to heterochromatin formation and gene silencing (Esteve et al. 2006; Vire et al. 2006). The initial DNA methylation in papaya MSY was likely a defense response against invasion of transposon or transposon-derived sequences. DNA methylation can then accelerate the heterochromatinization of MSY. DNA methylation could also directly alter or silence genes in the MSY. Bachtrog (2006) analyzed the allelic expression of genes located in the neo-X and neo-Y chromosomes in male *D. miranda*. Of the 58 genes analyzed, 22 of them were nonfunctional on the neo-Y, and the majority of the remaining genes from the neo-Y were expressed at lower levels than those from the neo-X (Bachtrog 2006). The down-regulation of these genes appeared to be random (Bachtrog 2006). This random and variable transcription reduction can be explained by a non-uniform DNA methylation across the entire neo-Y chromosome, which diverged from the neo-X approximately 1 million yr ago (Bachtrog and Charlesworth 2002). A different degree of cytosine methylation in the promoter regions can result in such a variable degree of transcription reduction.

Methods

Fluorescence in situ hybridization (FISH)

Two Hawaiian gynodioecious papaya cultivars, Kapoho and SunUp, were used for chromosome preparations. Young hermaphrodite flower buds containing anthers at various stages of meiosis were collected and fixed in 3:1 (100% ethanol:glacial acetic acid) Cannoy's solution and kept at −20°C until use. Microsporocytes at the pachytene stage were squashed in acetocarmine solution and slides were stored at −80°C until use. FISH was conducted according to published procedures (Jiang et al. 1995). DNA probes were labeled with digoxigenin-dUTP or biotin-dUTP and detected with anti-digoxigenin antibody coupled with Rhodamin (Roche) or anti-avidin antibody conjugated with FITC (Vector Laboratories), respectively. The chromosomes were counterstained with DAPI in an antifade solution VectorShield (Vector Laboratories), and FISH images were captured digitally with a Sensys cold CCD camera (Roper Scientific).

Immunodetection of 5-methylcytosine

Chromosome preparations were the same as those used in the above FISH procedure. After denaturing in 70% formamide containing 2× SSC for 3 min at 80°C, the slides were washed in ice-cold 70% ethanol for 5 min and incubated in the blocking reaction (1× PBS containing 1% bovine serum albumin and 0.5% Tween 20) for 30 min at 37°C in a wet chamber. The mouse antiserum raised against 5-methylcytosine (Aviva Systems Biology) was diluted by 1:250 in 1× TNB (100 mM Tris HCl at pH 7.5, 150 mM NaCl, 0.5% blocking reagent) and applied to the slides and kept in a humid chamber for 5 h at 37°C. After washing in 1× PBS three times for 5 min, FITC-labeled goat anti-mouse IgG (Jackson Immunoresearch Lab) was applied as the secondary antibody. Chromosomes were counterstained with DAPI. After recording the 5mC signals, the slides were dipped in 1× PBS buffer to remove the coverglasses, and dehydrated in an ethanol series. The MSY region was identified by probing with an MSY-specific BAC clone 99O03. Quantification of fluorescence from DAPI staining and 5mC immunofluorescence was performed following published protocols (Cheng et al. 2002).

Acknowledgments

We thank Weiwei Jin for technical assistance and input at the early stage of this project. This research was supported by grant DBI-0553417 from the National Science Foundation.

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Received March 24, 2008; accepted in revised form June 17, 2008.

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Genome Res. 2008 18: 1938-1943 originally published online July 1, 2008
Access the most recent version at doi:10.1101/gr.078808.108