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Chromosomal Locations of a Non-LTR Retrotransposon, *Menolird18*, in *Cucumis melo* and *Cucumis sativus*, and Its Implication on Genome Evolution of *Cucumis* Species

Agus B. Setiawan[a] Chee H. Teo[b] Shinji Kikuchi[c] Hidenori Sassa[c] Kenji Kato[d] Takato Koba[c]

[a]Laboratory of Genetics and Plant Breeding, Faculty of Agriculture, Gadjah Mada University, Yogyakarta, Indonesia; [b]Center for Research in Biotechnology for Agriculture, University of Malaya, Kuala Lumpur, Malaysia; [c]Laboratory of Genetics and Plant Breeding, Graduate School of Horticulture, Chiba University, Matsudo, Chiba, Japan; [d]Graduate School of Environmental and Life Science, Okayama University, Kita-ku, Okayama, Japan

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Cucumis · Genome evolution · *Menolird18* · Non-LTR retrotransposon

Abstract
Mobile elements are major regulators of genome evolution through their effects on genome size and chromosome structure in higher organisms. Non-long terminal repeat (non-LTR) retrotransposons, one of the subclasses of transposons, are specifically inserted into repetitive DNA sequences. While studies on the insertion of non-LTR retrotransposons into ribosomal RNA genes and other repetitive DNA sequences have been reported in the animal kingdom, studies in the plant kingdom are limited. Here, using FISH, we confirmed that *Menolird18*, a member of LINE (long interspersed nuclear element) in non-LTR retrotransposons and found in *Cucumis melo*, was inserted into ITS and ETS (internal and external transcribed spacers) regions of 18S rDNA in melon and cucumber. Beside the 18S rDNA regions, *Menolird18* was also detected in all centromeric regions of melon, while it was located at pericentromeric and sub-telomeric regions in cucumber. The fact that FISH signals of *Menolird18* were found in centromeric and rDNA regions of mitotic chromosomes suggests that *Menolird18* is a rDNA and centromere-specific non-LTR retrotransposon in melon. Our findings are the first report on a non-LTR retrotransposon that is highly conserved in 2 different plant species, melon and cucumber. The clear distinction of chromosomal localization of *Menolird18* in melon and cucumber implies that it might have been involved in the evolutionary processes of the melon (*C. melo*) and cucumber (*C. sativus*) genomes.

Introduction

Transposable elements are mobile genetic sequences found in the genomes of higher organisms. They are classified into 2 major groups based on their mode of transposition. Retrotransposons (class I) are DNA sequences that use RNA as reverse transcription intermediates, while DNA transposons (class II) move directly to another locus. Class I retrotransposons are the most widespread in eukaryotic transposable elements and are fur-

Takato Koba
Laboratory of Genetics and Plant Breeding Graduate School of Horticulture
Chiba University Matsudo
Chiba 271-8510 (Japan)
koba@faculty.chiba-u.jp

ther subdivided into 2 groups: (1) long terminal repeat (LTR) retrotransposons such as the *Ty1-copia* and *Ty3-gypsy* retrotransposons, and (2) non-LTR retrotransposons, which lack LTR. Non-LTR retrotransposons are further categorized as long interspersed nuclear elements (LINE) or short interspersed nuclear elements (SINE) [Schmidt, 1999; Casacuberta and Santiago, 2003].

Plant species have tandem repeat arrays of 45S ribosomal DNA (rDNA) that are located in the nucleolar organizer regions (NOR) of chromosomes. Its coding sequence is conserved in the gourd family (Cucurbitaceae) [Lima-de-Faria, 1976; Ganal and Hemleben, 1986]. 45S rDNAs are separated by intergenic spacer (IGS) regions and provide the codes for 18S, 5.8S, and 25S ribosomal RNA (rRNA), whose coding regions are separated by 2 types of internally transcribed spacers (ITS): ITS1 and ITS2. The amount of 45S rDNA copies and the length of IGS found in plants are highly variable [Hemleben et al., 1982; Rogers and endich, 1987; Layat et al., 2012; Yang et al., 2015; Havlová et al., 2016; Zhang et al., 2016; Huang et al., 2017]. These rDNA clusters achieve mobility via transposable element processes (e.g., insertion) without the involvement of translocations or chromosomal rearrangements [Schubert and Wobus, 1985; Dubcovsky and Dvorak, 1995; Raskina et al., 2008]. Many sequence-specific transposable element insertions (particularly from LINE) congregate in rRNA genes or in repetitive DNA sequences (e.g., telomeric or centromeric repeats) [Kojima and Fujiwara, 2004; Čížková et al, 2013; Fujiwara, 2015; Nagaki et al, 2015].

Many LINEs have been identified in plants. Examples include *Cin4* in corn (*Zea mays*) [Schwarz-Sommer et al., 1987], *ATLN* in thale-cress (*Arabidopsis thaliana*) [Noma et al., 2001], *BLIN* in barley (*Hordeum vulgare*) [Vershinin et al., 2002], *Karma* in rice (*Oryza sativa*) [Komatsu et al., 2003], *LIb* in sweet potato (*Ipomoea batatas*) [Yamashita and Tahara, 2006], *BNR* family in beet (*Beta vulgaris*) [Heitkam and Schmidt, 2009], and BrLINEs family in mustards (*Brassica*) [Nouroz et al., 2017]. Site-specific targeting of non-LTR retrotransposons into the host genome is species specific. For instance, *R1* and *R2* are typically inserted into specific 28S rDNA sites of insects [Jakubczak et al., 1991], but *R2* are inserted into both 28S rDNA and highly conserved regions of 18S rDNA in the startlet sea anemone (*Nematostella vectensis*) and the fresh water polyp (*Hydra magnipapillata*) [Kojima et al, 2006]. LTR retrotransposons found in tomatoes were inserted into 18S rDNA [Jo et al., 2009] and near specific genes in wild rice (*O. brachyantha*) [Gao et al., 2012]. However, information regarding the insertion sites of non-LTR retrotransposons and their chromosomal distribution in plants is still limited.

Transposable elements are major drivers of plant genome evolution due to their ability to modify genome size, shape chromosome structure, and contribute to rapid evolution of heterochromatic states via centromere insertions [Han et al., 2016; Morata et al., 2018]. Transposable elements have increased the size of the melon genome, expanded the pericentromeric regions of melon chromosomes, and have a very low recombination frequency. In comparison, cucumbers have small, transposable element-dense pericentromeric regions and a relatively constant recombination rate. These differences caused nucleotide sequence diversity in the melon and cucumber genomes [Huang et al., 2009; Garcia-Mas et al., 2012; Morata et al., 2018]. It has been reported that non-LTR retrotransposons were randomly distributed but tended to be located or inserted into pericentromeric and/or other heterochromatic chromosome regions [Kojima and Fujiwara, 2004, 2005; Kojima et al., 2006; 2016; Fujiwara, 2015; Morata et al., 2018].

While various types of tandemly organized repetitive DNA sequences in the centromeric regions of melon and their secondary constrictions have been the subject of past research [Koo et al., 2010; Zhang et al., 2016; Setiawan et al., 2018a, 2020], dispersed repetitive DNA locations in melon have not been examined. Our study identifies the species-specific chromosomal distribution of a melon (*C. melo*) and cucumber (*C. sativus*) LINE (*Menolird18*) and discusses its contribution to the evolution of these *Cucumis* species.

Materials and Methods

Plant Materials

We studied 3 melon and 3 cucumber accessions. The melon accessions included: US205 (*C. melo* L. subsp. *agrestis* var. *momordica*), P90 (*C. melo* L. subsp. *agrestis* var. *conomon*), and the Nobel F1 hybrid (*C. melo* L. subsp. *melo* var. *cantalupo* Ser.). The 3 *C. sativus* accessions included: Okute Aodai, RAR 930024, and Shiroibo Fushinari (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/511119). The melon accessions US205 and P90 were obtained from USDA-Genebank, USA, and Institute of Vegetable and Floriculture Science, NARO (National Agriculture and Food Research Organization), Japan, respectively. Nobel F1 hybrid was purchased in the Indonesian market. The cucumber accessions were provided by Genebank of NARO. Seeds were germinated on moistened filter paper in petri dishes and grown in a growth chamber at 25°C.

Data Mining of Non-LTR Retrotransposon

The melon DNA sequence "CM3B0051961 TE" (*Menolird18*, Melon non-LTR retrotransposon in 18S rDNA) from the melon genome database (version 3.5.1: http://melonomics.cragenomica.es) was used for this experiment. The selected sequences were blasted against the melon genome database (http://melonomics.cragenomica.es/tools/blast/run/), and the primers were designed using FastPCR software.

Genomic DNA and Total RNA Isolation

Genomic DNA and total RNA were extracted from leaves using the method described by Setiawan et al. [2020]. Total RNA was treated with Deoxyribonuclease RT Grade (Nippon Gene, Japan) per themanufacturer's instruction to remove genomic DNA.

Cloning of Non-LTR Retrotransposon

Menolird18 was amplified by PCR using the oligonucleotide primer pairs 5′-CAGCGTATCACTAGCTGAATTCGCT-3′ and 5′-CCCTAGGACGAATAGCTTCCCATGA-3′, and a 772-bp PCR product was cloned into a pGEM-T-Easy Vector (Promega) per the manufacturer's instructions.

Semi-Quantitative RT-PCR Amplification of Menolird18 Transcripts

First-strand cDNAs were synthesized from 0.5 μg of the total RNA in 20 μL cDNA synthesis reaction using a ReverTraAce® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). Thirty cycles of semi-quantitative RT-PCR (sqRT-PCR) were performed with a PCR Thermal Cycler Dice™ Touch (Takara, Japan) using *Ex Taq* Hot Start Version (Takara, Japan). A total of 50 ng of the resulting cDNA was used as a template in 30 μL of the PCR reaction solution containing gene-specific primers of *Menolird18*. Expression of the β-actin gene was used as an internal control for determining the sqRT-PCR amplification efficiency in the tissue samples, using the same primer pairs as described by Minamikawa et al. [2013].

Chromosome and Extended DNA Fiber Preparations

Mitotic metaphase and meiotic pachytene chromosome preparations were prepared with Carnoy's solution II [Setiawan et al., 2018b]. Leaf nuclei isolation was completed as described by Jackson et al. [1998] with a slight modification. Briefly, 2 μL of the nuclei suspensions were deposited on one end of a poly-L-lysine slide and semi-dried for 10 min. Next, 8 μL of lysis buffer (0.5% SDS, 5 mM EDTA, 100 mM Tris pH 7.0) was dropped onto the nuclei and the slide was then incubated at RT for 4 min. The DNA fibers were extended by dragging them with a clean coverslip. The slides were air-dried and fixed in 1% paraformaldehyde for 2 min and then baked at 60°C for 30 min.

Probe Preparations and FISH

Cmcent [Koo et al., 2010] and 45S rDNA (pTa71) [Gerlach and Bedbrook, 1979] probes were labeled with biotin-nick translation mix (Roche), while *Menolird18* was labeled with dig-nick translation mix (Roche) per the manufacturer's instructions. The mitotic, meiotic, and fiber FISH tests were conducted according to the method described by Setiawan [2018]. For hybridization onto pachytene chromosomes and extended DNA fibers, the hybridization mixtures were covered with 22 × 40 mm cover slips and sealed with rubber cement. The slides were then denatured on a hot plate at 80°C for 2–3 min and placed in a humidity chamber, where they were incubated at 37°C overnight. Three antibody layers were used to detect the fiber FISH signals. Next, 126 μL of detection solution (125 μL of 1% BSA in 4× SSC +0.5 μL of 0.4 μg/mL anti-digoxigenin rhodamine + 0.5 μL of 0.5 μg/mL biotinylated streptavidin-FITC) was added to the slides and incubated at 37°C for 30 min. The slides were washed in 2× and 0.1× SSC for 3 min to remove any unspecific antibodies. Finally, the slides were counter-stained with 4,6-diamidino-2-phenylindole (DAPI) in a VectaShield antifade solution (Vector Laboratories).

Sequence Comparison and Image Analysis

Menolird18 and melon centromeric satellite DNA sequences were compared to a dot plot using Unipro UGENE software. FISH signals were observed under a fluorescence microscope (Olympus BX53) equipped with a cooled CCD camera (Photometrics CoolSNAP MYO) and then processed using Metamorph, Metavue imaging series version 7.8 and edited with Adobe Photoshop CS 6.

Results

Chromosomal Distribution of Menolird18 in Cucumis Species

Physical mapping of *Menolird18* and 45S rDNA was conducted on melon and cucumber mitotic and meiotic chromosomes. The location of 45S rDNA was previously reported to be on the short arms of chromosomes 4 and 10 [Liu et al., 2010]. We found 45S rDNA to be exclusively co-localized with *Menolird18* on the metaphase chromosomes of melon and cucumber accessions (Fig. 1). The locations of 45S rDNA signals were the same as some of *Menolird18* signals in both plants, except for the signals found in the centromeric regions of melon chromosomes and the other specific signals of cucumber chromosomes. US205 and P90 showed that the 45S rDNA and *Menolird18* signals were co-localized on the short arms of chromosomes 4 and 10 (Fig. 1a–f). Cucumber accessions showed variable amounts and signal strength of 45S rDNA and *Menolird18* signals. Okute Aodai, RAR 930024, and Shiroibo Fushinari showed 9 (6 strong and 3 weak), 10 (6 strong and 4 weak), and 8 (6 strong and 2 weak) signals of 45S rDNA (Fig. 1g–o). In addition, the signals of *Menolird18* were detected on most of the sub-telomeric regions (Fig. 1h, k, n) and some of the pericentromeric regions (Fig. 1i, l, o) in all cucumber accessions.

Menolird18 Insertion into ITS and ETS of 18S rDNA

High resolution FISH signals of 45S rDNA and *Menolird18* were obtained using pachytene chromosomes and extended melon DNA fibers. The 45S rDNA was located in NOR regions (Fig. 2a). *Menolird18* was precisely localized to the primary constrictions and NORs of pachytene

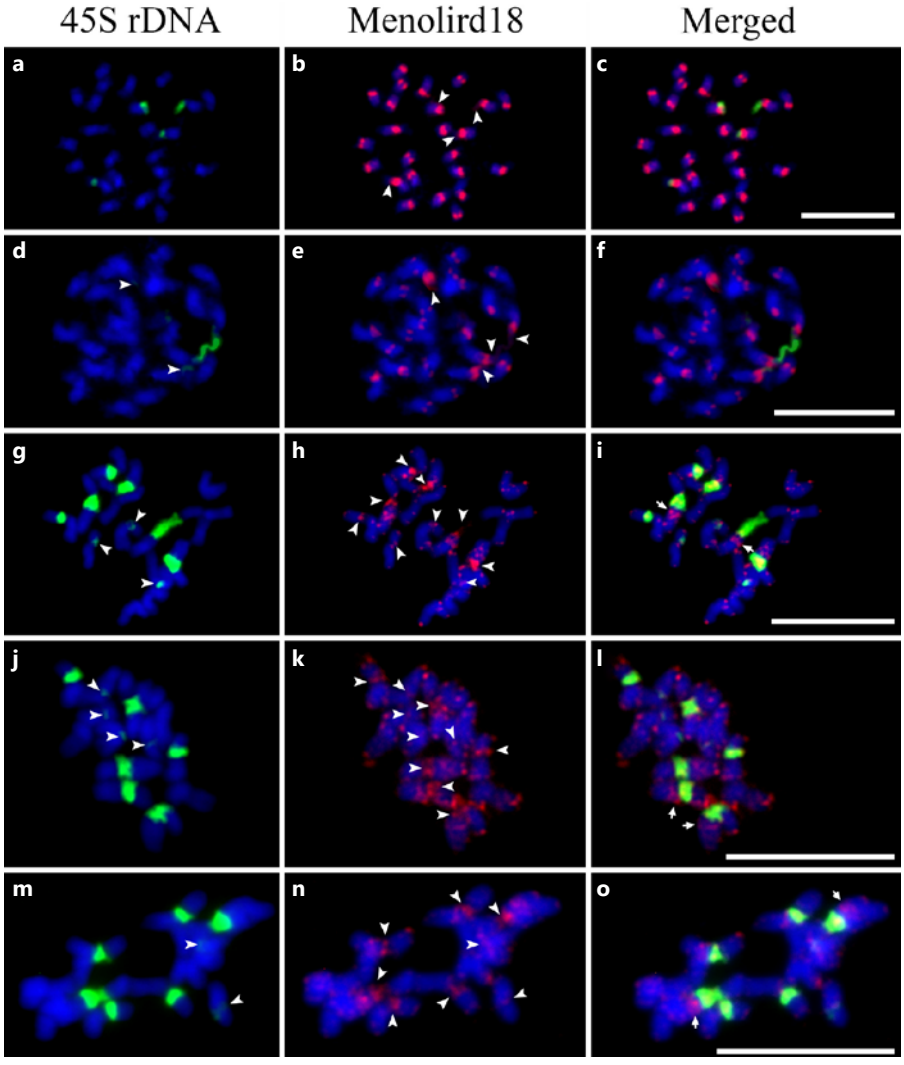


Fig. 1. Physical mapping of 45S rDNA (green: **a**, **d**, **g**, **j**, **m**) and *Menolird18* (red: **b**, **e**, **h**, **k**, **n**), and merged images (**c**, **f**, **i**, **l**, **o**) in somatic chromosomes of melon and cucumber accessions. **a**–**c** US 205. **d**–**f** P90. **g**–**i** Okute Aodai. **j**–**l** RAR 930024. **m**–**o** Shiroibo Fushinari. White arrowheads depict weak signals of 45S rDNA (**d**, **g**, **j**, **m**) and *Menolird18* (**b**, **e**, **h**, **k**, **n**). White arrows (**i**, **l**, **o**) depict the signals of *Menolird18* at pericentromeric regions. Scale bars, 10 μm.

chromosomes (Fig. 2b). The 45S rDNA and *Menolird18* showed co-localized signals at NORs (Fig. 2c). More detailed information regarding *Menolird18* signals was obtained using fiber FISH. We found that *Menolird18* was not only co-localized with 45S rDNA but also intermingled with a 45S rDNA array (Fig. 2d). The detailed structure of the 45S rDNA scheme is shown in Figure 3a. Sequence comparison using a dot plot analysis revealed that *Menolird18* was inserted into the ITS and ETS of 18S rDNA (Fig. 3b). This method also revealed that another type of retrotransposon, the *Ty3/Gypsy*-like LTR retrotransposon, was inserted into 26S rDNA. These results suggest that different types of LTR and non-LTR retrotransposons invaded the 45S rDNA of melon. We observed that cucumber has more 45S rDNA loci than melon. Since melons are affected by *Menolird18* (Fig. 1c, f, i, l, o), we used sqPCR to verify the gene expression of *Menolird18* and found that it was not amplified in melon leaf tissues which maybe is due to being under the detection limit (Fig. 3c).

Menolird18, a rDNA and Centromere-Specific Retrotransposon in Melon

Menolird18 and *Cmcent* were mapped to confirm the precise location of *Menolird18* in melon centromeric regions. We were able to successfully hybridize them to various stages of mitotic chromosomes, namely interphase nuclei, early prophase, prometaphase, metaphase, anaphase, and telophase chromosomes (Fig. 4). We found that they were co-localized on mitotic chromosomes in various stages and that *Menolird18* is restricted to the primary constrictions. *Menolird18* shared low se-

quence homology with melon centromeric satellite DNA, i. e., *Cmcent*, *CmSat162*, and *CmSat189* (online suppl. Fig. 2).

Discussion

Non-LTR retrotransposons lack LTRs, possess a poly(A) tail at the 3′ terminus, and contain 2 open reading frames (ORF), which encode *gag* protein (ORF1) and endonucleases, as well as reverse transcriptase domains (ORF2) [Schmidt, 1999]. We confirmed the structure of the CM3B0051961 TE DNA sequence (*Menolird18*) using a conserved domain database in NCBI for the annotation of functional protein units in this sequence. Our results showed that *Menolird18* is classified as a non-LTR retrotransposon (LINE-like), consisting of a reverse transcriptase (RT) domain commonly found in retrotransposons and a zinc-finger putative reverse transcriptase domain near the N-terminal (online suppl. Fig. 1). We also found that it was similar to *R2* in arthropods, which possess 1–3 zinc finger domains (species-dependent) in addition to the RT domain [Kojima and Fujiwara, 2005].

Some non-LTR retrotransposons have specific targets within the genome, particularly rRNA genes or repetitive sequences [Kojima and Fujiwara, 2003]. *R2* is inserted into 28S ribosomal RNA (rRNA) genes in fruit flies (*Drosophila melanogaster*) [Roiha and Glover, 1981] and domestic silkworms (*Bombyx mori*) [Fujiwara et al., 1984], and it is widely distributed in 6 animal phyla [Kojima et al., 2016]. It was also reported that non-LTR retrotransposons had different insertion sites (e.g., in 18S rDNA) dependent upon the host organisms [Kojima et al., 2006, 2016]. Information on its insertion and chromosomal distribution in plant species is still limited, with differing insertion sites of non-LTR and LTR retrotransposons having only been reported in sunflowers [Nagaki et al., 2015], bananas [Čížková et al, 2013], tomatoes [Jo et al., 2009], and wild rice (*O. brachyantha*) [Gao et al., 2012].

Our study confirmed the high conservation of *Menolird18* sequences in both melon and cucumber plants (on-

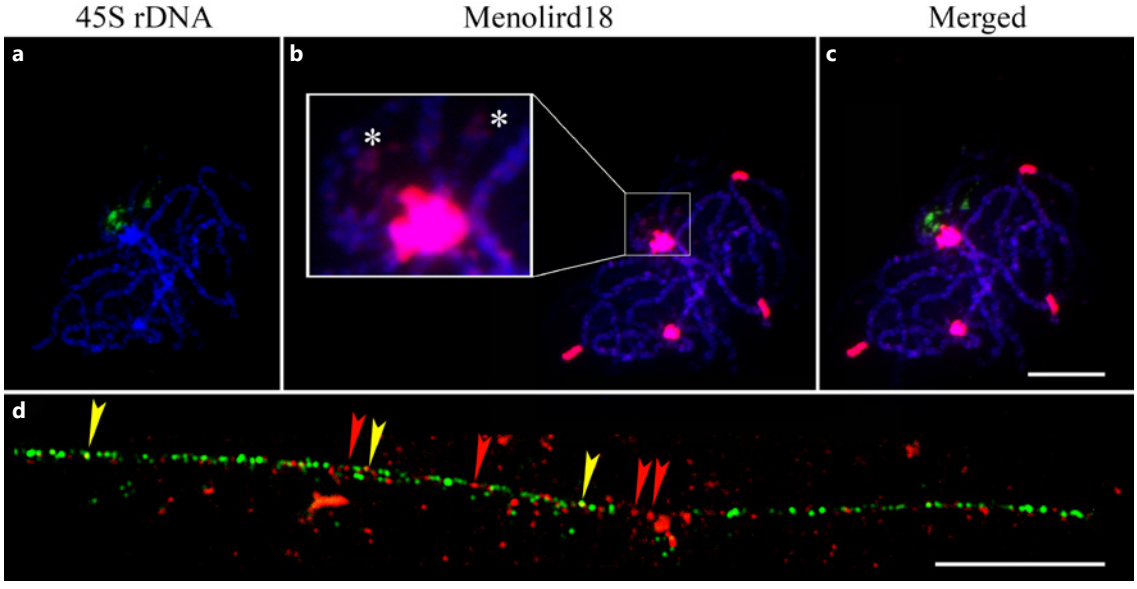
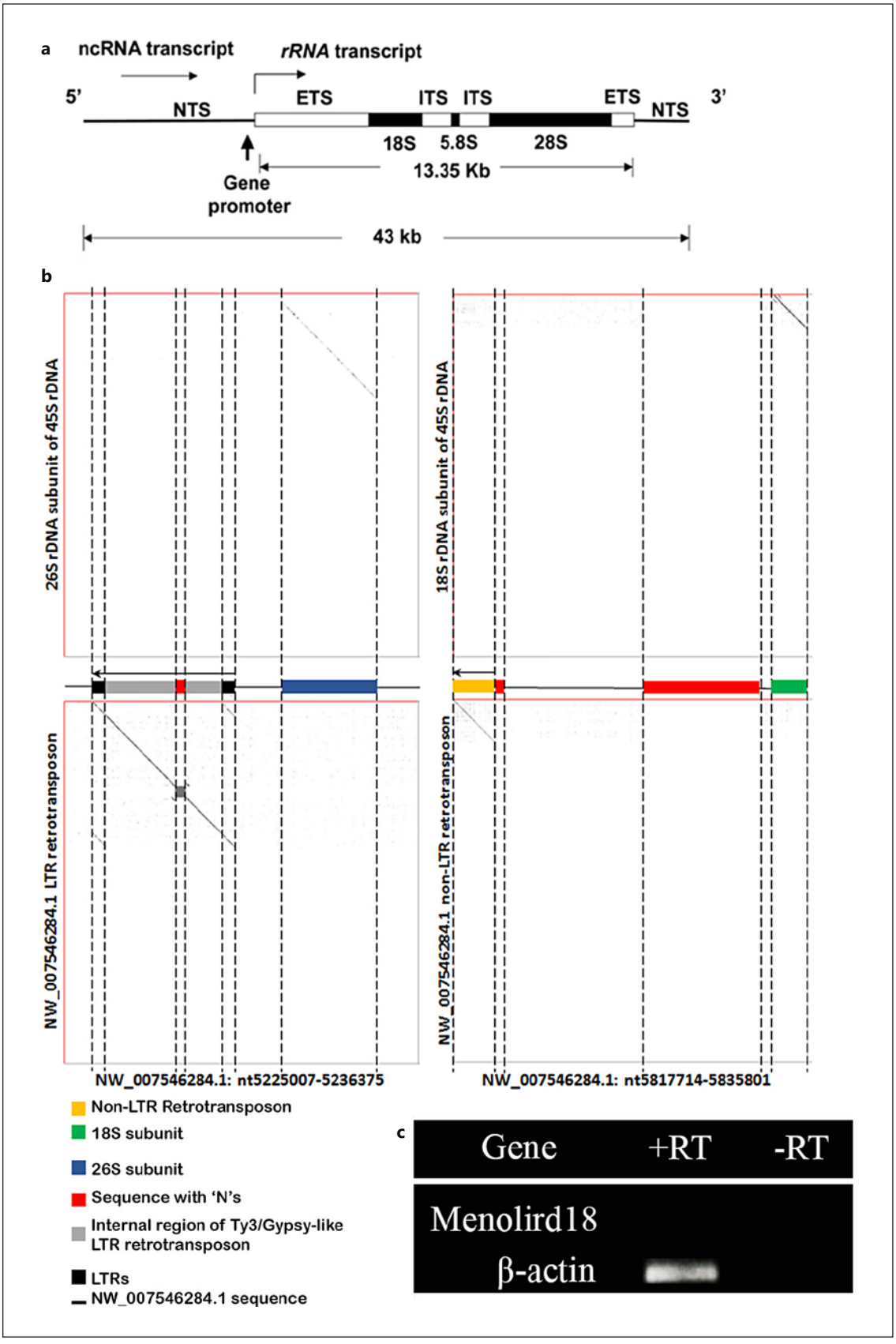


Fig. 2. Fine mapping of 45S rDNA and *Menolird18* on pachytene chromosomes and extended DNA fibers in melon. **a**–**c** Hybridization of 45S rDNA and *Menolird18* on pachytene chromosomes. **b** Asterisks in **inset** show weak signals of *Menolird18* which co-localize with 45S rDNA. **d** Extended DNA fiber of US205. Signals of 45S rDNA and *Menolird18* are shown in green and red, respectively. Yellow and red arrowheads depict co-localized signals and single dots of *Menolird18* signals, respectively. Scale bars, 10 μm.

Fig. 3. Structure of 45S rDNA, insertion site of *Menolird18*, and gene expression of *Menolird18* in melon. **a** Full length scheme of 45S rDNA. **b***Menolird18* inserted into 18S rDNA visualized by dot plot analysis. **c** Transcription of *Menolird18* amplified from melon leaves.

(For figure see next page.)



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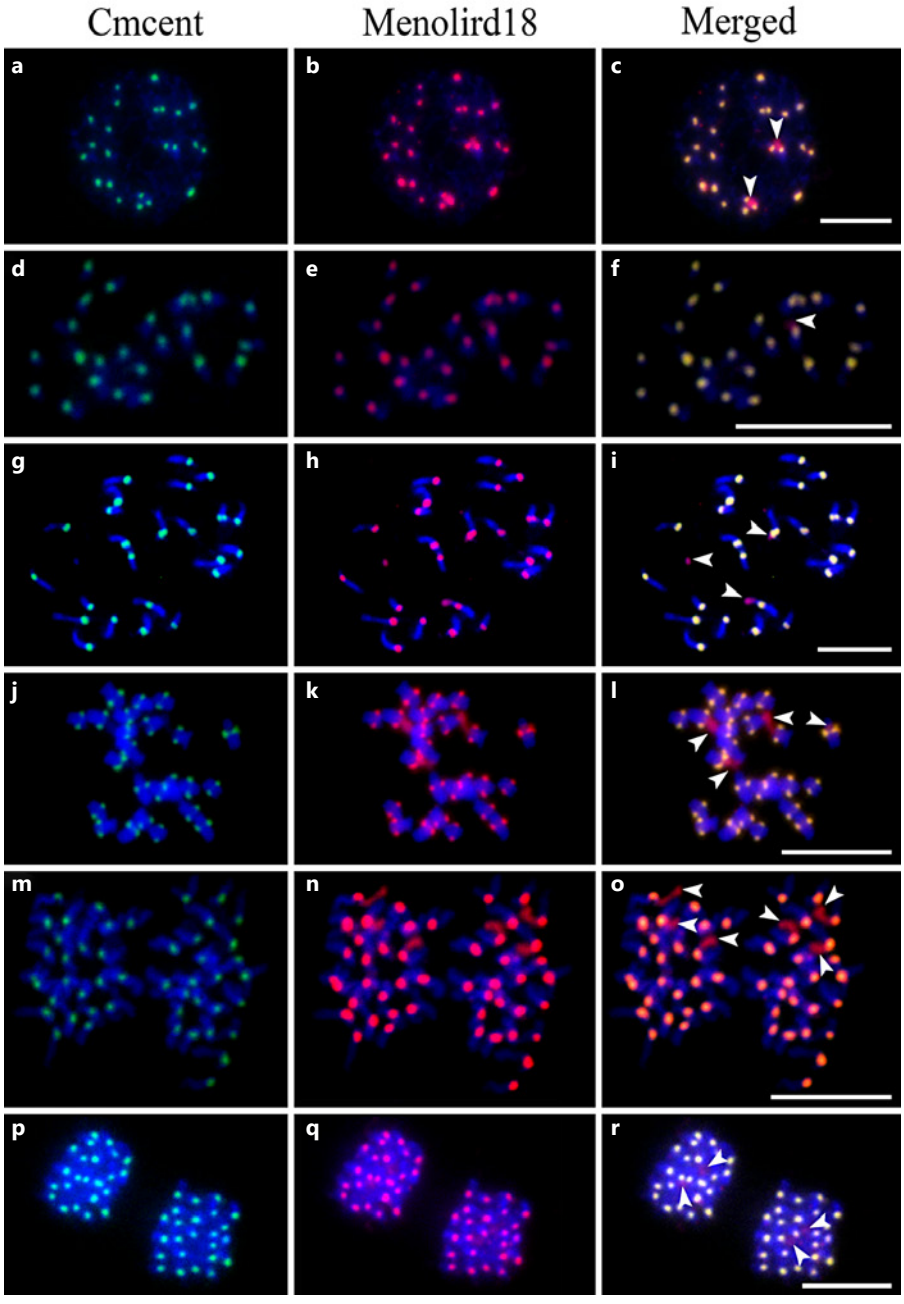

Fig. 4. Co-localization of *Cmcent* and *Menolird18* signals on various stages of mitotic chromosomes in melon, Nobel F1 hybrid. **a**–**c** Interphase nuclei. **d**–**f** Early prophase. **g**–**i** Prometaphase. **j**–**l** Metaphase. **m**–**o** Anaphase. **p**–**r** Telophase. Arrowheads depict *Menolird18* signals at NORs. Scale bars, 10 μm.

line suppl. Table 2). FISH results revealed that *Menolird18* was inserted into 2 specific regions: 18S rDNA and melon centromeres. This non-LTR retrotransposon showed different preferential insertion sites in comparison to those of animals, where it was mostly located at only one specific gene locus [Roiha and Glover, 1981; Fujiwara et al., 1984; Kubo et al., 2001; Kojima and Fujiwara, 2004; Kojima et al., 2006, 2016].

Menolird18 was consistently localized at 45S rDNA and centromeric regions of metaphase and pachytene chromosomes and at extended DNA fibers of melon. The organization of *Menolird18* was intermingled with its 45S rDNA array. This result suggests that *Menolird18* inserts into 45S rDNA, which is supported by our results of this element in the 18S rDNA of melon. The sequence comparison of 45S rDNA indicated that another type of retrotransposon, a *Ty3/Gypsy*-like LTR retrotransposon, in-

serts into the 26S rDNA of melon. These results suggest that different types of retrotransposons, both LTR and non-LTR, insert into the 45S rDNA sequence of melon, which is similar to the reports by Jo et al. [2009] and Gao et al. [2012], who both found LTR retrotransposons inserted into the rDNA of tomato plants.

The number of 45S rDNA signals detected in cucumber accessions varied, which is consistent with previous reports [Han et al., 2008; Tagashira et al., 2009; Wibowo et al., 2018]. Five pairs of 45S rDNA signals, 3 major and 2 minor ones, were found in RAR 930024, however, 2 cucumber accessions showed different numbers of 45S rDNA loci. Okute Aodai possessed 9, while Shiroibo Fushinari possessed 8. RAR 930024 originated from the USSR (Uzbekistan, Kazakhstan, and Kyrgyzstan area), while Okute Aodai and Shiroibo Fushinari are historically domesticated Japanese landraces (online suppl. Table 1).

Previous studies reported that rDNA clusters are mobile due to their transposable activities [Schubert and Wobus, 1985; Dubcovsky and Dvorak, 1995; Raskina et al., 2008]. The different number of 45S rDNA loci in these 3 cucumber accessions may be due to the insertion of non-LTR retrotransposons and ectopic recombination during their domestication. Similar observations were reported in common bean plant (*Phaseolus vulgaris*) accessions, which showed variation in the numbers of 45S rDNA loci (3–9 loci) [Pedrosa-Harand et al., 2006]. Transposable elements were able to transfer rDNA sequences that were capable of causing evolutionary changes to rDNA chromosomal distribution [Raskina et al., 2008]. Our results showed that both non-LTR and LTR retrotransposons insert into 45S rDNA (Fig. 3b), which indirectly supports the hypothesis that the variation of rDNA loci amounts are regulated by mobile element activities [Schubert and Wobus, 1985; Dubcovsky and Dvorak, 1995; Raskina et al., 2004a, b, 2008].

Menolird18 was localized at the centromeric regions of melon (Fig. 4). This result suggests that melon centromeres consist of a centromere-specific non-LTR retrotransposon besides centromeric repetitive sequences, *CmSat162*, *CmSat189*, and *Cmcent* [Koo et al., 2010; Setiawan et al., 2020]. Moreover, *Menolird18* did not share the sequence homology with these centromeric satellite DNAs of melon (online suppl. Fig. 2). Thus, *Menolird18* preferably inserted into centromeric regions of melon other than 45S rDNA loci. This finding is similar to those reported in *O. sativa*, *Z. mays*, sorghum, *Beta* species, barley, and sunflower in which their centromeres were composed of satellite DNAs and retrotransposons [Miller et al., 1998a, b; Presting et al., 1998; Gindullis et al., 2001; Hudakova et al., 2001; Cheng et al., 2002; Nagaki et al., 2003, 2015].

The insertion of *Menolird18* into 18S rDNA and other specific regions might be caused by the function of retrotransposons. This process is known to be initiated by nicking one strand of DNA at the target site and creating a 3′-hydroxyl end under the control of endonuclease activity. Then, it can be used as a primer for reversing the transcription of the retrotransposon mRNA onto the DNA target. This unique process is called target-primed reverse transcription (TPRT) [Luan et al., 1993; Fujiwara, 2015]. Thus, the selection of a sequence-specific target by non-LTR retrotransposons is primarily regulated by endonucleases [Zingler et al., 2005].

It was found that *Menolird18* is highly conserved in 2 different species, melon and cucumber, and this was clarified by a BLAST result of *Menolird18* in the cucumber genome (online suppl. Table 2). Based on FISH stringency calculation by Schwarzacher and Heslop-Harrison [2000], the hybridization stringencies of the probes were 77% (online suppl. Fig. 3). High stringency means that only high homologues sequences were hybridized during the hybridization process and it does not allow imperfect probes and targets to hybridize.

Both melon and cucumber are members of the genus *Cucumis*. They derived from a common ancestor and diverged approximately 10 Mya [Sebastian et al., 2010]. It was considered that the genome size of melon has been increased due to a recent higher accumulation of transposable elements [Garcia-Mas et al., 2012]. *Menolird18* showed contrast hybridization patterns between melon and cucumber. Although *Menolird18* was consistently found in the NORs of the 2 species, the evolutionary directions of the chromosomes might be different. *Menolird18* occurred at pericentromeric and subtelomeric regions of the chromosomes in cucumber. On the other hand, in melon it was found at heterochromatic blocks of primary constrictions. Moreover, the more loci of 45S rDNA were found, the more signals of *Menolird18* were seen at NORs. This might be the evidence for the hypothesis that the amount of rDNA is correlated with those of mobile elements, especially non-LTR retrotransposons, as previously reported [Schubert, 1984; Dubcovsky and Dvorak, 1995; Raskina et al., 2008; Jo et al., 2009]. These results suggest that *Menolird18* might have been involved in the evolution of rDNA and centromeres in both melon and cucumber. We hypothesize that before the divergence of melon and cucumber from the same common ancestor around 10 Mya [Sebastian et al., 2010], *Meno-*

lird18 was inserted into 2 locations: NORs and pericentromeric regions. After the divergence, *Menolird18* would have remained in NORs and expanded into centromeric regions in melon, while in cucumber, it would have remained in NORs and pericentromeric regions and then expanded into subtelomeric regions.

The sqRT-PCR revealed that *Menolird18* was not expressed in leaf tissues of melon. This suggests that *Menolird18* might be inactive under normal growing conditions. Retrotransposons are mostly silent and can be activated under stress conditions [Ramallo et al., 2008; Cavrak et al., 2014; Makarevitch et al., 2015]. Further gene expression studies using other tissue samples treated with various abiotic stresses, such as salinity and drought, would be needed to confirm the expression of *Menolird18* in melon.

In conclusion, *Menolird18*, a non-LTR retrotransposons, possesses 2 different patterns of hybridization signals in *Cucumis* species: (1) it was inserted into 18S rDNA in melon and cucumber, and (2) it was restricted to centromeric regions in all melon chromosomes, whereas it was located at pericentromeric and subtelomeric regions in cucumber. The clear distinction in chromosomal locations of *Menolird18* in melon and cucumber allows us to study evolutionary effects of non-LTR retrotransposon in *Cucumis* species. The differences in the chromosomal locations of *Menolird18* clearly showed its involvement in the expansion/reduction of the melon and cucumber genome during the evolutionary process which subsequently gave rise to the present *Cucumis* taxa with different genome sizes. These results provide an initial step for future cytogenetic studies, such as chromosomal evolution and cytogenetic mapping, in the related species of the genus *Cucumis* by utilizing retrotransposons.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

A.B.S. designed the study, performed the research, analyzed the data, and wrote the manuscript. C.H.T. performed the research and wrote the manuscript. S.K. and H.S. interpreted the data and reviewed the manuscript. K.K. provided the melon seed materials and reviewed the manuscript. T.K. designed the study, interpreted the data, and wrote and reviewed the manuscript. All authors read and approved the final manuscript.

References

Casacuberta JM, Santiago N. Plant LTR-retrotransposons and MITEs: Control of transposition and impact on the evolution of plant genes and genomes. Gene. 2003;311:1–11.

Cavrak VV, Lettner N, Jamge S, Kosarewicz A, Bayer LM, Mittelsten Scheid O. How a retrotransposon exploits the plant's heat stress response for its activation. PLoS Genet. 2014;10(1):e1004115.

Cheng Z, Dong F, Langdon T, Ouyang S, Buell CR, Gu M, et al. Functional rice centromeres are marked by a satellite repeat and a centromere-specific retrotransposon. Plant Cell. 2002;14(8):1691–704.

Čížková J, Hřibová E, Humplíková L, Christelová P, Suchánková P, Doležel J. Molecular analysis and genomic organization of major DNA satellites in banana (Musa spp.). PLoS One. 2013;8:e54808.

Dubcovsky J, Dvorák J. Ribosomal RNA multigene loci: Nomads of the triticeae genomes. Genetics. 1995;140(4):1367–77.

Fujiwara H. Site-specific non-LTR retrotransposons. Microbiol Spectr. 2015;3:25–32.

Fujiwara H, Ogura T, Takada N, Miyajima N, Ishikawa H, Maekawa H. Introns and their flanking sequences of Bombyx mori rDNA. Nucleic Acids Res. 1984;12(17):6861–9.

Ganal M, Hemleben V. Comparison of the ribosomal RNA genes in four closely related Cucurbitaceae. Plant Syst Evol. 1986;154(1-2):63–77.

Gao D, Chen J, Chen M, Meyers BC, Jackson S. A highly conserved, small LTR retrotransposon that preferentially targets genes in grass genomes. PLoS One. 2012;7(2):e32010.

Garcia-Mas J, Benjak A, Sanseverino W, Bourgeois M, Mir G, González VM, et al. The genome of melon (Cucumis melo L.). Proc Natl Acad Sci USA. 2012;109(29):11872–7.

Gerlach WL, Bedbrook JR. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. 1979;7(7):1869–85.

Gindullis F, Desel C, Galasso I, Schmidt T. The large-scale organization of the centromeric region in Beta species. Genome Res. 2001;11(2):253–65.

Han J, Masonbrink RE, Shan W, Song F, Zhang J, Yu W, et al. Rapid proliferation and nucleolar organizer targeting centromeric retrotransposons in cotton. Plant J. 2016;88(6):992–1005.
Han YH, Zhang ZH, Liu JH, LuHuang JYSW, Huang SW, Jin WW. Distribution of the tandem repeat sequences and karyotyping in cucumber (Cucumis sativus L.) by fluorescence in situ hybridization. Cytogenet Genome Res. 2008;122(1):80–8.
Havlová K, Dvořáčková M, Peiro R, Abia D, Mozgová I, Vansáčová L, et al. Variation of 45S rDNA intergenic spacers in Arabidopsis thaliana. Plant Mol Biol. 2016;92(4-5):457–71.
Heitkam T, Schmidt T. BNR - a LINE family from Beta vulgaris - contains a RRM domain in open reading frame 1 and defines a L1 subclade present in diverse plant genomes. Plant J. 2009;59(6):872–82.
Hemleben V, Leweke B, Roth A, Stadler J. Organization of highly repetitive satellite DNA of two Cucurbitaceae species (Cucumis melo and Cucumis sativus). Nucleic Acids Res. 1982;10(2):631–44.
Huang S, Li R, Zhang Z, LiGu LX, Gu X, Fan W, et al. The genome of the cucumber, Cucumis sativus L. Nat Genet. 2009;41(12):1275–81.
Huang Y, Yu F, Li X, Luo L, Wu J, Yang Y, et al. Comparative genetic analysis of the 45S rDNA intergenic spacers from three Saccharum species. PLoS One. 2017;12(8):e0183447–20.
Hudakova S, Michalek W, Presting GG, ten Hoopen R, dos Santos K, Jasencakova Z, et al. Sequence organization of barley centromeres. Nucleic Acids Res. 2001;29(24):5029–35.
Jackson SA, Wang ML, Goodman HM, Jiang J. Application of fiber-FISH in physical mapping of Arabidopsis thaliana. Genome. 1998;41(4):566–72.
Jakubczak JL, Burke WD, Eickbush TH. Retrotransposable elements R1 and R2 interrupt the rRNA genes of most insects. Proc Natl Acad Sci USA. 1991;88(8):3295–9.
Jo SH, Koo DH, Kim JF, HurLee CGS, Lee S, Yang TJ, et al. Evolution of ribosomal DNA-derived satellite repeat in tomato genome. BMC Plant Biol. 2009;9:42.
Kojima KK, Fujiwara H. Evolution of target specificity in R1 clade non-LTR retrotransposons. Mol Biol Evol. 2003;20(3):351–61.
Kojima KK, Fujiwara H. Cross-genome screening of novel sequence-specific non-LTR retrotransposons: Various multicopy RNA genes and microsatellites are selected as targets. Mol Biol Evol. 2004;21(2):207–17.
Kojima KK, Fujiwara H. Long-term inheritance of the 28S rDNA-specific retrotransposon R2. Mol Biol Evol. 2005;22(11):2157–65.
Kojima KK, Kuma K, Toh H, Fujiwara H. Identification of rDNA-specific non-LTR retrotransposons in Cnidaria. Mol Biol Evol. 2006;23(10):1984–93.
Kojima KK, Seto Y, Fujiwara H. The wide distribution and change of target specificity of R2 non-LTR retrotransposons in animals. PLoS One. 2016;11(9):e0163496.
Komatsu M, Shimamoto K, Kyozuka J. Two-step regulation and continuous retrotransposition of the rice LINE-type retrotransposon Karma. Plant Cell. 2003;15(8):1934–44.
Koo DH, Nam YW, Choi D, Bang JW, de Jong H, Hur Y. Molecular cytogenetic mapping of Cucumis sativus and C. melo using highly repetitive DNA sequences. Chromosome Res. 2010;18(3):325–36.
Kubo Y, Okazaki S, Anzai T, Fujiwara H. Structural and phylogenetic analysis of TRAS, telomeric repeat-specific non-LTR retrotransposon families in Lepidopteran insects. Mol Biol Evol. 2001;18(5):848–57.
Layat E, Sáez-Vásquez J, Tourmente S. Regulation of pol I-transcribed 45S rDNA and pol III-transcribed 5S rDNA in Arabidopsis. Plant Cell Physiol. 2012;53(2):267–76.
Lima-de-Faria A. The chromosome field: I. Prediction of the location of ribosomal cistrons. Hereditas. 1976;83:1–22.
Liu C, Liu J, Li H, Zhang Z, Han Y, Huang S, et al. Karyotyping in melon (Cucumis melo L.) by cross-species fosmid fluorescence in situ hybridization. Cytogenet Genome Res. 2010;129(1-3):241–9.
Luan DD, Korman MH, Jakubczak JL, Eickbush TH. Reverse transcription of R2Bm RNA is primed by a nick at the chromosomal target site: a mechanism for non-LTR retrotransposition. Cell. 1993;72(4):595–605.
Makarevitch I, Waters AJ, West PT, Stitzer M, Hirsch CN, Ross-Ibarra J, et al. Transposable elements contribute to activation of maize genes in response to abiotic stress. PLoS Genet. 2015;11(1):e1004915.
Miller JT, Dong F, Jackson SA, Song J, Jiang J. Retrotransposon-related DNA sequences in the centromeres of grass chromosomes. Genetics. 1998a;150(4):1615–23.
Miller JT, Jackson SA, Nasuda S, Gill BS, Jiang J. Cloning and characterization of a centromere-specific repetitive DNA element from Sorghum bicolor. Theor Appl Genet. 1998b;96(6-7):832–9.
Minamikawa MF, Fujii D, Kakui H, Kotoda N, Sassa H. Identifcation of an S-RNase binding protein1 (SBP1) homolog of apple (Malus domestica). Plant Biotechnol. 2013;30:119–23.
Morata J, Tormo M, Alexiou KG, Vives C, Ramos-Onsins SE, Garcia-Mas J, et al. The evolutionary consequences of transposon-related pericentromer expansion in melon. Genome Biol Evol. 2018;10(6):1584–95.
Nagaki K, Song J, Stupar RM, Parokonny AS, Yuan Q, Ouyang S, et al. Molecular and cytological analyses of large tracks of centromeric DNA reveal the structure and evolutionary dynamics of maize centromeres. Genetics. 2003;163(2):759–70.
Nagaki K, Tanaka K, Yamaji N, Kobayashi H, Murata M. Sunflower centromeres consist of a centromere-specific LINE and a chromosome-specific tandem repeat. Front Plant Sci. 2015;6:912–2.
Noma K, Ohtsubo H, Ohtsubo E. A new class of LINEs (ATLN-L) from Arabidopsis thaliana with extraordinary structural features. DNA Res. 2001;8:291–9.
Nouroz F, Noreen S, Khan MF, Ahmed S, Heslop-Harrison JSP. Identification and characterization of mobile genetic elements LINEs from Brassica genome. Gene. 2017;627:94–105.
Pedrosa-Harand A, de Almeida CC, Mosiolek M, Blair MW, Schweizer D, Guerra M. Extensive ribosomal DNA amplification during Andean common bean (Phaseolus vulgaris L.) evolution. Theor Appl Genet. 2006;112(5):924–33.
Presting GG, Malysheva L, Fuchs J, Schubert I. A TY3/GYPSY retrotransposon-like sequence localizes to the centromeric regions of cereal chromosomes. Plant J. 1998;16(6):721–8.
Ramallo E, Kalendar R, Schulman AH, Martínez-Izquierdo JA. Reme1, a Copia retrotransposon in melon, is transcriptionally induced by UV light. Plant Mol Biol. 2008;66(1-2):137–50.
Raskina O, Barber JC, Nevo E, Belyayev A. Repetitive DNA and chromosomal rearrangements: speciation-related events in plant genomes. Cytogenet Genome Res. 2008;120(3-4):351–7.
Raskina O, Belyayev A, Nevo E. Quantum speciation in Aegilops: molecular cytogenetic evidence from rDNA cluster variability in natural populations. Proc Natl Acad Sci USA. 2004a;101(41):14818–23.
Raskina O, Belyayev A, Nevo E. Activity of the En/Spm-like transposons in meiosis as a base for chromosome repatterning in a small, isolated, peripheral population of Aegilops speltoides Tausch. Chromosome Res. 2004b;12(2):153–61.
Rogers SO, Bendich AJ. Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol Biol. 1987;9(5):509–20.
Roiha H, Glover DM. Duplicated rDNA sequences of variable lengths flanking the short type I insertions in the rDNA of Drosophila melanogaster. Nucleic Acids Res. 1981;9(21):5521–32.
Schmidt T. LINEs, SINEs and repetitive DNA: Non-LTR retrotransposons in plant genomes. Plant Mol Biol. 1999;40(6):903–10.
Schubert I. Mobile nucleolus organizing regions (NORs) in Allium (Liliaceae s. lat.)? -- Inferences from the specifity of silver staining. Plant Syst Evol. 1984;144:291–305.
Schubert I, Wobus U. In situ hybridization confirms jumping nucleolus organizing regions in Allium. Chromosoma. 1985;92(2):143–8.
Schwarz-Sommer Z, Leclercq L, Göbel E, Saedler H. Cin4, an insert altering the structure of the A1 gene in Zea mays, exhibits properties of nonviral retrotransposons. EMBO J. 1987;6(13):3873–80.

Schwarzacher T, Heslop-Harrison P. Practical in situ Hyridization. Oxford: BIOS Scientific Publishers Ltd; 2000. p. 203.
Sebastian P, Schaefer H, Telford IR, Renner SS. Cucumber (Cucumis sativus) and melon (C. melo) have numerous wild relatives in Asia and Australia, and the sister species of melon is from Australia. Proc Natl Acad Sci USA. 2010;107(32):14269–73.
Setiawan AB. Molecular cytogenetic studies on satellite DNA and retrotransposon in Cucumis species. PhD Thesis, Chiba University; 2018.
Setiawan AB, Teo CH, Kikuchi S, Sassa H, Kato K, Koba T. Cytogenetic variation in Cucumis accessions revealed by fluorescence in situ hybridization using ribosomal RNAs genes as the probes. Chromosome Sci. 2018a;21:67–73.
Setiawan AB, Teo CH, Kikuchi S, Sassa H, Koba T. An improved method for inducing prometaphase chromosomes in plants. Mol Cytogenet. 2018b;11:32.
Setiawan AB, Teo CH, Kikuchi S, Sassa H, Kato K, Koba T. Centromeres of Cucumis melo L. comprise Cmcent and two novel repeats, CmSat162 and CmSat189. PLoS One. 2020; 15(1):e0227578.
Tagashira N, Hoshi Y, Yagi K, Plader W, Malepszy S. Cytogenetic comparison among three cultivars of cucumber (Cucumis sativus L.) by using post-heated DAPI band, 45S and 5S rDNA site. Chromosome Bot. 2009;4:19–23.
Vershinin AV, Druka A, Alkhimova AG, Kleinhofs A, Heslop-Harrison JS. LINEs and gypsy-like retrotransposons in Hordeum species. Plant Mol Biol. 2002;49(1):1–14.
Wibowo A, Setiawan AB, Purwantoro A, Kikuchi S, Koba T. Cytological variation of rRNA genes and subtelomeric repeat sequences in Indonesian and Japanese cucumber accessions. Chromosome Sci. 2018; 21:81–7.
Yamashita H, Tahara M. A LINE-type retrotransposon active in meristem stem cells causes heritable transpositions in the sweet potato genome. Plant Mol Biol. 2006;61(1-2):79–94.
Yang K, Robin AH, Yi GE, Lee J, Chung MY, Yang TJ, et al. Diversity and inheritance of intergenic spacer sequences of 45S ribosomal DNA among accessions of Brassica oleracea L. var. capitata. Int J Mol Sci. 2015;16(12): 28783–99.
Zhang ZT, Yang SQ, Li ZA, Zhang YX, Wang YZ, Cheng CY, et al. Comparative chromosomal localization of 45S and 5S rDNAs and implications for genome evolution in Cucumis. Genome. 2016;59(7):449–57.
Zingler N, Weichenrieder O, Schumann GG. APE-type non-LTR retrotransposons: determinants involved in target site recognition. Cytogenet Genome Res. 2005;110(1-4):250–68.