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Clustered and dispersed chromosomal distribution of the two classes of *Revolver* transposon family in *Secale cereale*

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Genet. Mol. Res. 18 (4): gmr16039970

Received September 20, 2019

Accepted October 4, 2019

Published October 25, 2019

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ABSTRACT. The chromosomal locations of a new class of *Revolver* transposon-like element were analyzed by using FISH method on the metaphase chromosome in somatic cell division of the rye cultivar Petkus. First, *Revolver* standard element probe was weakly hybridized throughout the rye chromosome and a comparatively large signal spotted with a dot-shape was detected. The dot-shape signal was stably detected at one place on the intercalated portion of the center of the short arm in chromosome 1R, two places near the end of the short arm and on the intercalated portion near the kinetochore in chromosome 2R, and one place on the intercalated portion of the center of short arm and two places near kinetochore and on the intercalated portion of the center of the long arm in chromosome 5R. The *Revolver* probe was effective for identification of 1R, 2R, and 5R chromosomes. On the other hand, *Revolver* non-autonomous element specific probe was strongly distributed throughout the rye chromosomes and considerable number and diverse lengths of transcripts were detected by RT-PCR. Although standard elements were found in localized clusters, the non-autonomous elements tended to be dispersed throughout the genome. Clustered nature of *Revolver* is significantly rare case in genomics.

Keywords: Transposon; Chromosomal distribution; Cluster; Dispersed nature; Rye

INTRODUCTION

In higher plants, only a small percentage of the genome is required to maintain life (Flavell et.al.,1977; Grandbastien et al., 1992) and transposable elements and the sequences derived from them are scattered in the other highly repetitive DNA regions (Barakat et al., 1997; Panstruga et al., 1998; San Miguel et al., 1998; Myers et al., 2001). The transposable elements are classified into the class I transposable elements (retrotransposons), which use a transcript as a template and transfer replicatively, and class II transposable elements (transposons), which transfer DNA by a cut-and-paste mechanism. It is believed that class I long terminal repeat (LTR) retrotransposons and class II miniature inverted repeat transposable elements (MITEs) are the major components of the plant genome (Bennetzen et al., 2000; Vicient et al., 2001; Bureau et al., 1996; Zhang et al., 2004).

Such transfer factors can be used as the source of mutations for DNA marker development or gene functional analysis; the transposable elements with a high number of copies can become the entry points for PCR during DNA marker development, and the transposable elements that cause gene disruption enable the tagging of genes and contribute to functional genome research (Kumar and Hirichika, 2001, Lunde et al., 2003). However, the sequences of regions of repetitive DNA other than the known transposable elements (Stein, 2007; Wicker et al., 2008) have not yet been determined, whereas, in the region of repetitive DNA regarded as junk DNA, RNA genes that perform epigenetic regulation of gene expression have been found (Sunker et al., 2005; Feschotte, 2008; Siomi and Siomi., 2008). Investigation of the unknown factors in repetitive DNA regions is an important means to understanding the mechanisms of genome control and phenotypic expression.

The first authors reported a new transposon-like gene, designated as *Revolver*, in Triticeae (Tomita et al., 2008, Tomita, 2008), which has insertion-type consensus sequence (92% homology) with a full-length sequence of 3,041 bp sandwiched between 20 bp of specific terminal inverted repeat (TIR) (Tomita et al., 2008). The TIR at both ends is different from that of the known transposons represented by hAT, CACTA, and Mutator (Flavell et al., 1992; Kumar and Bennetzen, 1999, Kunze and Well, 2002; Bennetzen, 1996; Feschotte et al., 2002a, b; Lisch 2002, Wicker et al., 2007). *Revolver* contains a single gene that codes a single open reading frame (ORF) of a deduced 139 amino acid residue that is actively transcribed into 0.7 kb of mRNA.

In addition to a standard element, the *Revolver* family contain structural variants ranging in length from 2665 to 4269 bp, in which regions of 549–2007 base pairs corresponding to the standard encoding element have been considerably lost or destroyed. These regions were equivalent to the first exon to the first intron of the standard elements. Therefore, these variants were thought to be nonautonomous elements of the *Revolver* family (Tomita et al., 2011).

These nonautonomic elements have a 370 bp homologous region at the 5’ end, which shows 65% homology with the 5’ part of barley insertion sequence BARE100 (Suoniemi et al., 1996; Vicient et al., 1999; Kalendar et al., 2000). Namely, two kind of *Revolver* exist, the standard element and the nonautonomous element (Figure 1).

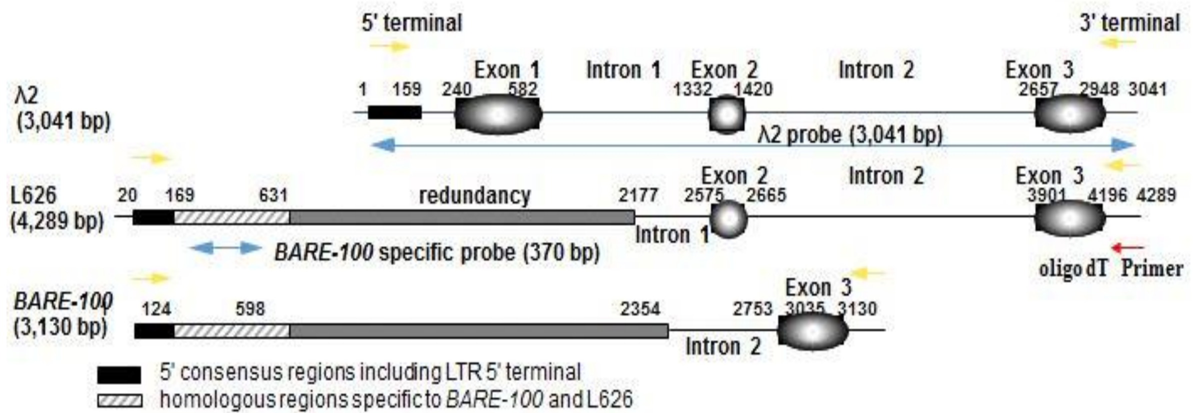

Figure 1. Structural relationship between the *Revolver* consensus element λ2, the Revolver autonomous element L626 and *BARE100.*

Revolver is amplified in some species in the process of Triticeae evolution. An extremely high number of *Revolvers* exist in Secale, with 20,000 copies, and it is found in diploid species, such as Triticum monococcum, which is the ancestor species of bread wheat, and tetraploid species such as emmer wheat (Nevo and Beiles, 1989; Nevo et al., 2002; Peng et al., 2001), with around 10,000 copies; however, it is extremely low in hexaploid bread wheat (Tomita et al., 2008, 2011). In this study we analyzed the chromosomal distribution and amplified nature of two classes of the *Revolver* family, the standard encoding element and the nonautonomous element (Figure 2).

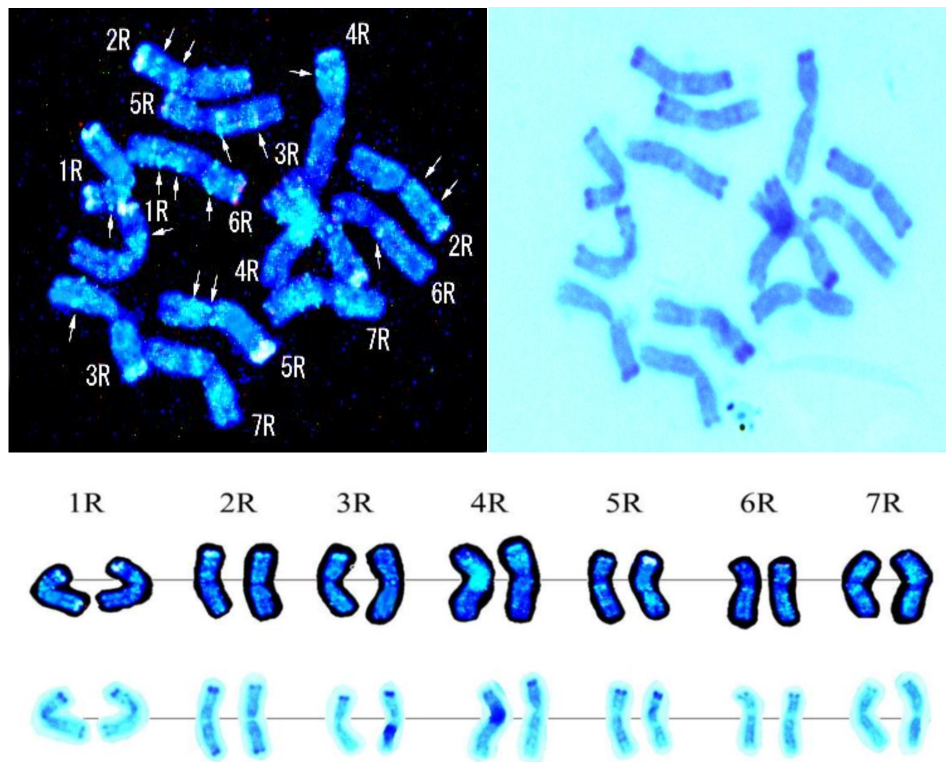

Figure 2. Chromosomal localization of the *Revolver* standard element by FISH and sequential C-banding on rye, *Secale cereale* L.

MATERIALS AND METHODS

Fluorescence *in situ* hybridization

The chromosome position of the full-length standard encoding element of *Revolver* (λ 2 type insertion sequence) and the 370 bp region specific to the nonautonomous element of *Revolver* (Figure 3A), which show homology with the 5' end of BARE-100 was analyzed by using a FISH method. First, the chromosome specimen of the metaphase of somatic cell division of the rye cultivar Petkus was produced and chromosomal preparations were made by using the squash method. Seeds were germinated on a moist filter paper in petri plates at 25°C. The root tips were pretreated with 0.05% (w/v) colchicine at 20°C for 4 h before fixation, softened in a 2% (w/v) cellulase and 1% (w/v) pectolyase solution, and then squashed in 45% acetic acid. Preparations for *in situ* hybridization were then treated with 80 µg/mL ribonuclease (Sigma, St. Louis, USA) in 2×SSC at 37°C for 1 h, and then washed in 2×SSC for 5 min, and a gradient series of 70%, 95%, and 100% ethanol at room temperature; finally, the slides were air dried for 30 min.

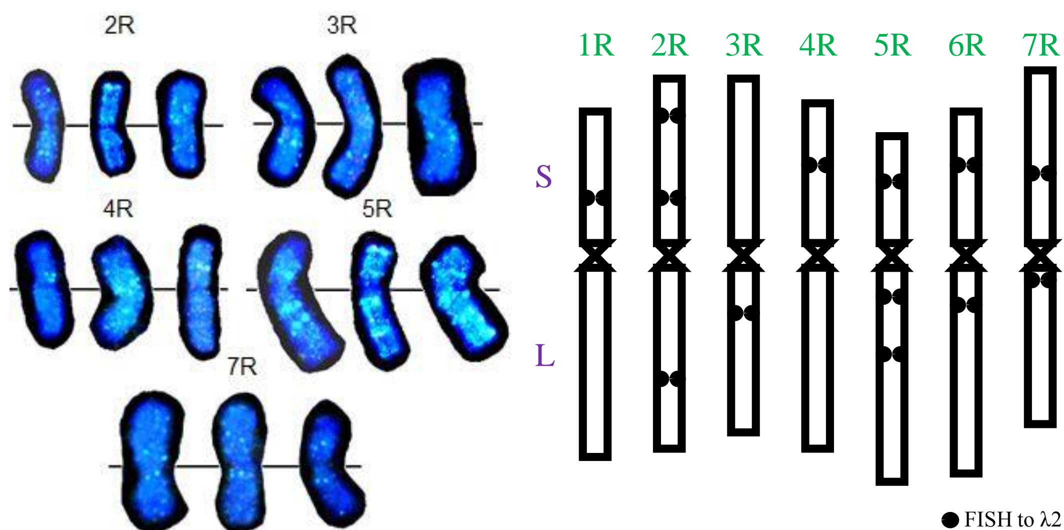

Figure 3. Representative clusters of the *Revolver* standard element on the rye chromosomes.

Two probes, 3,041 bp of the full-length standard element λ2, and the autonomous-element specific 370 bp region in L626 with homology to BARE100, were labeled with biotin-16-dUTP (Roche Diagnostics, Basel, Switzerland) by PCR. For dual hybridization, the probe pSC74 of the rye tandem repeat 350 bp family (Bedbrook et al., 1980) was labeled with digoxigenin-11-dUTP (Roche Diagnostics, Basel, Switzerland), and L626-370 bp probe were labeled with biotin-16-dUTP, respectively, by PCR. The hybridization mixture contained 50% (v/v) formamide in 2×SSC, 1 µg/mL probe DNA, and 100 µg/mL salmon sperm DNA. The hybridization mixture was denatured at 99°C for 10 min and immediately chilled on ice for 10 min. A 15 µL portion of the mixture was applied for one preparation. The preparations were covered with a 22 × 30 mm cover glass and sealed with a rubber solution. The prepared slides were on the placed in a Thermal Cycler (PHC-3 Techne) and subject to the following conditions: drying the rubber solution at 25°C for 30 min; denaturing DNA at 82°C for 8 min; and *in situ* hybridization at 37°C for 18 h. After the hybridization, the fluorochrome signals were visualized by using avidin-fluorescein isothiocyanate (FITC) (Roche Diagnostics).

Briefly, the sealed rubber was first removed, and the preparations were washed four times at 40°C for 10 min each: three times in 2×SSC, followed by once in 4×SSC. Nonspecific binding was then blocked by incubation with 5% (w/v) bovine serum albumin (BSA) in 0.05% (v/v) Tween 20 and 0.1 M NaHCO3 at 37°C for 5 min. After the blocking solution was decanted from the slides, the probes labeled with biotin-16-dUTP were detected with 10% (v/v) avidin-FITC (Roche Diagnostics, Basel, Switzerland), while the probe labeled with digoxigenin-11-dUTP was

detected with 10% (v/v) antidigoxigenin-rhodamine (Roche Diagnostics, Basel, Switzerland) in a humidity chamber at 38°C for 1 h. After three washes in 0.05% (v/v) Tween 20 and 0.1 M NaHCO3 and one wash in 2×SSC at 40°C (10 min each), the preparations were counter-stained with a mixture of 3 µg/mL DAPI (Roche Diagnostics, Basel) and 10% (v/v) Antifade (Oncor) in glycerol. The preparations were observed by using an Olympus BX40 microscope equipped with the filter sets WBV (blue for DAPI), WIBA (green for FITC) and WIGV (red for rhodamine). Representative cells were photographed by using a Fuji color HG1600 print film. Identification of the rye chromosome was performed by simultaneous FISH method with the tandemly repetitive sequence 350 bp family of terminal region (detected by digoxigenin (DIG)-labelled and rhodamine anti-DIG antibody) and the C-band method after FISH.

Sequential C-banding analysis

Karyotype was determined by sequential C-banding stain. For the sequential C-banding analysis after FISH, the *in situ* probe DNA on a slide was removed by treatment with 45% acetic acid at 82°C for 10 min and the slide was immediately chilled in 10% acetic acid at 4°C for 10 min. The preparations were washed three times in 45% acetic acid, each for 15 min, followed by a series of washings in 70%, 95%, and 100% ethanol at room temperature. The air-dried slides were used for C-banding as described by Gill et al. (1991), with a minor modification: the treatment condition of 5% (W/V) of barium hydroxide solution was changed to 40°C for 10 min from room temperature for 7 min.

RT-PCR

The primers for amplification of the Revolver –BARE100 common DNA sequence were designed from the 5' end of the common sequence. Total RNA for RT-PCR extracted from seedlings was treated with DNase I. First-strand cDNAs were synthesized by AMV reverse transcriptase (Life Science) using an oligo(dT) primer. Reaction mixtures contained 10 ng of template cDNA, 50 pmol of forward primer (5'-GGCACGAGGGTACGAGTCCGAG-3'), and 5' oligo(dT) primer including the common 3'-sequence of the Revolver family (5'-TTTTTTTTTTTTTTTGGCACAACTCATGTAAAAGAGGG-3'), 0.4 mM dNTPs, 1× LA PCR buffer II, 2.5 mM $MgCl_2$, and 0.5 U of LA Taq polymerase (Takara) in a total volume of 25 µL. The PCR reaction program consisted of 30 cycles of 30 s at 95˚C, 2 min at 63˚C, and 1 min at 72˚C. For the FISH probe, the primers for amplification of *Revolver* –BARE100 common DNA sequence were designed from both ends of the common sequence (5'-GGCACGAGGGTACGAGTCCGAG-3', 5'-GGCACAACTCATGTAAAAGAGGG-3'). The 370 bp region specific to nonautonomous element of Revolver, was obtained by PCR in the following conditions, 30 cycles of 30 s at 95˚C, 30 s at 63˚C, and 1 min at 72˚C. by using clone L626 as a template, and used for biotin labeling.

Gel blot analysis

RT-PCR products were subjected to electrophoresis in a 1% agarose gel, then transferred and crosslinked to nylon membranes. The membranes were pre-hybridized at 65˚C for 3 h in hybridization solution containing 5×SSC, 2% blocking reagent (Roche), 0.1% N-lauroylsarcosine and 0.02% SDS. A Revolver-BARE100 common sequence probe (25 ng) labeled with digoxigenin-11-dUTP was added to the pre-hybridization solution and allowed to hybridize at 65˚C for 14 h. Blots were washed twice in 2×SSC, 0.1% SDS at room temperature for 5 min, and twice in 0.1×SSC, 0.1% SDS at 65˚C for 15 min, and then exposed to Kodak X-OMAT film by using CDP-star.

RESULTS

The probes used for FISH were *Revolver* (3041 bp) labeled with biotin-16-dUTP. After hybridization, biotin-labeled probe was detected by avidin-FITC with signal amplification using a biotinylated anti-avidin secondary antibody and fluorescein-avidin DCS. The images were captured by using a cooled CCD camera. The lambda 2 probe was weakly hybridized throughout the rye chromosome and a comparatively large signal interspersed with dots was detected (Figures 2 and 3). Reproducible signals were observed at several chromosomal locations. FISH with a *Revolver* probe showed dot-like FITC signals at one site on chromosome (chr.) 1R (middle part of the interstitial region of the short arm), two sites on chr. 2R (distal part of the interstitial region and adjacent to the centromere on the short arm) and three sites on chr. 5R (middle part of the interstitial region of the short arm, middle part of the interstitial region, and adjacent to the centromere on the long arm) (Figures 2 and 3). In addition,

specific signals were observed at two sites on chr. 6R (middle part of the interstitial region of the short arm and adjacent to the centromere on the long arm), one site on chr. 3R (adjacent to the centromere on the long arm), and one site on chr. 4R (middle part of the interstitial region of the short arm) (Figures 2 and 3). The *Revolver* multigene probe, which detected distinct clusters across several chromosomes, can serve as a useful chromosome marker.

In contrast, a 370 bp region specific to BARE-100 was comparatively strongly hybridized throughout the rye chromosome and it is scattered with the number of copies higher than the lambda 2 type insertion sequence (Fig. 3C). *Revolver* is strongly hybridized throughout the chromosome when using the FISH method, and it is scattered throughout the genome (Figure 4A).

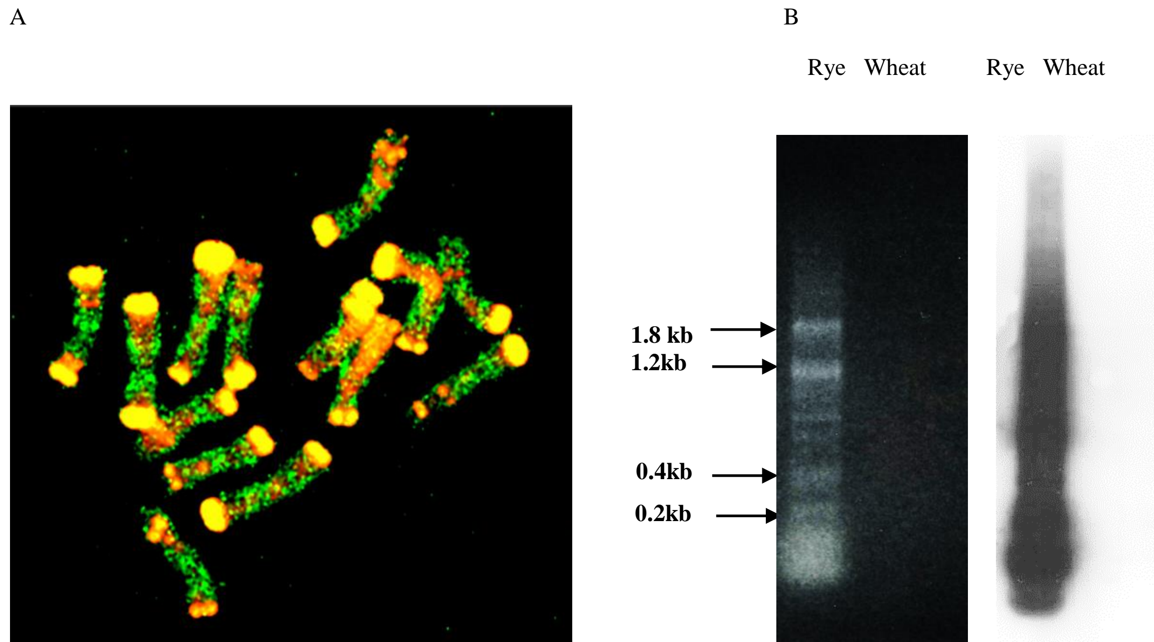

Figure 4. Chromosomal distribution and transcription of the common sequence between *Revolver* and *BARE-100*. (A) Chromosomal distribution of the *Revolver-BARE-100* common sequence (green) and telocentric 350-tandem family (orange) , (B) RT-PCR, using primers designed from the 5' end of *Revolver-BARE-100* common sequence.

Then, we performed RT-PCR using the 5' end primer synthesized from the Revolver-BARE100 common sequence and the oligo dt reverse primer. These primers amplified multi-length fragments ranging from small fragments with less than 100 bp to 1.8 kb (Figure 4B). Next, we conducted gel blot hybridization of southern-transferred RT-PCR products. Revolver-BARE100 common probes hybridized to multi-length RT-PCR fragments showed divergent length-polymorphism in rye, but no signal in wheat (Figure 4B).

DISCUSSION

In our previous, a part of the reiterated sequence (89 bp) specific to the rye genome was cloned by the genome subtraction technique, which removes the sequence common with bread wheat from the rye genome (Tomita et al., 2008, 2009). To determine the entire structure of this reiterated sequence, the λ FixII genomic library of the rye inbred line was screened by using 89 bp of the repetitive clone as a probe, and the base sequence of a region of approximately 21.6 kb was decoded in three lambda clones. As a result, the author found the new transposon-like gene *Revolver* in Triticeae, with a length is 3,041 bp and with 20 bp of specific TIR at both ends, which contains one gene that codes for an ORF of the deduced 139 amino acid residues actively transcribed into

mRNA [Tomita et al 2008]. The *Revolver* family was found to contain inverted repeat sequences at both ends along with a destroyed portion of the first exon at the 5' end. Instead, it contained a 370 bp region homologous to BARE-100, which is thought to belong to the class of non-autonomous elements (Tomita et al., 2011). When we used 726 bp-cDNA probe of *Revolver* as FISH probe in our previous study, FISH signal were simply distributed on rye chromosomes. In this study, we investigated the distribution of two kinds of elements, namely λ2 type insertion sequence and a 370 bp sequence homologous to BARE-100, which were not overlapped in the sequences.

The full length of λ2 sequence was distributed throughout the rye chromosome, which accompanied with several scattered distribution of the dot signals. The characteristic dot signals were detected on the 1R, 2R, and 5R chromosomes, with high reproducibility. Namely, the dot-shape signal was stably detected from one place on the intercalated portion of the center of short arm in chr. 1R, two places near the end of short arm and on the intercalated portion near the kinetochore in chr. 2R, and one place on the intercalated portion of the center of the short arm and two places near the kinetochore and on the intercalated portion of the center of the long arm in chr. 5R. The lambda 2 probe is effective for the identification of the 1R, 2R, and 5R chromosomes. The λ2 insertion sequences were clustered at different places on each chromosome; hence, the localization differs from the already-known tandem repeats, which can be considered as a new chromosomal marker.

In contrast, the 370 bp element homologous to BARE-100 that includes the L626 insertion repeat contains several copies throughout the rye chromosomes. There is a comparatively large dot signal on the 1R arm near the centromere. Considerable number of fragments with extremely diverse length were amplified by RT-PCR, using the Revolver-BARE-100 common sequence at the 5' end as the forward primer and the sequences common to the *Revolver* family at the 3' end as a reverse primer. However, no transcripts from common wheat. Revolver-BARE-100 sequences were distributed throughout rye chromosomes, resulting in many transcripts of varying lengths. These Revolver-BARE-100 sequences are thought to be those that have been mainly dispersed elements, which can mozable in the genome. In contrast, the 3041 bp *Revolver* standard element, which containing the intact coding region, may exists forming cluster-like localization and they may regulate the Revolver-BARE-100 mobile element.

There have been a few reports on the clustered sequences described as follows in transposons and micro RNAs. Ac-like elements (hAT family) were concentrated preferentially in chromosomal proximal regions. High percentage of clusters on the border between euchromatin and heterochromatin in ecologically contrasted populations of three Triticeae species - Aegilops speltoides, Triticum urartu, and Hordeum spontaneum (Altinkut et al., 2006). The majority of the Isaac-TD markers was determined to have been distributed throughout the ten maize chromosomes [Lee et al 200638]. The full TE element of D. buzzatii, named PERI, which shares several structures in common with DINE-1, is an abundant TE found abundantly along the Drosophila genus. PERI accumulates near or at heterochromatic regions of all six pairs of chromosomes, especially on the sex chromosomes, with some clustering (Locke et al., 1999). MicroRNAs (miRNAs) are an important class of posttranscriptional gene expression regulators. In the course of mapping novel marsupial-specific miRNAs in the genome of the gray short-tailed opossum, Monodelphis domestica, a cluster of 39 actual and potential miRNAs spanning 102 kb of the X chromosome was identified (Devor et al., 2011). In rodent, most piRNA sequences were present in a small number of genomic regions referred to as clusters, which range from one kilobase to hundreds of kilobases (Assis & Kondrashov, 2009). Above all, clustered sequence family of *Revolver* is significantly rare case in biological world.

CONCLUSION

In conclusion, *Revolver* standard element probe was weakly distributed throughout the rye chromosome and a comparatively large signal spotted with a dot-shape was detected by FISH. The *Revolver* probe was effective for identification of 1R, 2R, and 5R chromosomes. On the other hand, *Revolver* non-autonomous element specific sequence was strongly distributed throughout the rye chromosomes and considerable number and diverse lengths of transcripts were detected by RT-PCR. Although standard elements were found in localized clusters, the non-autonomous elements tended to be dispersed throughout the genome. Clustered nature of *Revolver* is significantly rare case in genomics.

ACKNOWLEDGMENTS

This work is founded by the Grant-in-Aid for Scientific Research No. 1360006 and No. 04760006 of the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) to Motonori Tomita.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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