Research Article

DISTINCTIVE *SIRE1* RETROTRANSPOSON PATTERNS ON BARLEY CHROMOSOMES?

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Abstract: *SIRE1* is an active and relatively high copy-number retroelement belongs to the *Ty1/Copia* long terminal repeat (LTR) retrotransposon superfamily. Distinctive *SIRE1* elements (*ENV* and *GAG*) distributions in barley genome were observed by using fluorescent *in situ* hybridization (FISH). We performed PCR to obtain tetramethylrhodamine-dUTP (TRITC)-labelled probes. Localizations of *SIRE1 ENV* and *GAG* domains were demonstrated under confocal microscope on *Hordeum vulgare* L. cv. Hasat root preparations. Our results revealed the distributions of *SIRE1* elements *ENV* and *GAG* in barley genome. These results may provide to uncover the organization of SIRE retrotransposon pattern in barley genome.

Özet: *SIRE1*, *Ty1/Copia* Uzun Uç Tekrarlı (Long Terminal Repeats- LTR) retrotranspozon üst ailesine ait olan aktif, nispeten yüksek kopyalı bir retroementtir. Arpa genomundaki ayırt edici *SIRE1* elementlerinin (*ENV* ve *GAG*) dağılımları floresan *in situ* hibridizasyonu (FISH) kullanılarak gözlemlendi. Tetramethylrhodamine-dUTP (TRITC)-işaretli probların elde edilmesinde PCR gerçekleştirildi. *SIRE1 ENV* ve *GAG* domainlerinin yerleşimleri, *Hordeum vulgare* L. cv. Hasat kök preparatlarında konfokal mikroskobu altında gösterildi. Sonuçlarımız, arpa genomundaki *SIRE1* elementlerinin *ENV* ve *GAG* dağılımlarını göstermektedir. Bu sonuçlar, SIRE1 elementlerinin arpa genomunun organizasyonunun ortaya çıkarılmasına katkı sağlayacaktır.

Introduction

Transposable elements (TEs) are mobile genetic elements and are distributed throughout the plant genome, over the 14% of *Arabidopsis thaliana* (L.) Heynh. genome, over the 80% maize genome and 80.8% of barley genome consist of TEs (AGI 2000, Schnable *et al.* 2009, Mascher *et al.* 2017). TEs constitute DNA fragments which can move through the genome using a DNA intermediate (DNA transposons) or an RNA intermediate (retrotransposons). Retrotransposons encode their own proteins for replication and integration back into the genome can be subdivided into two main subclasses as LTR retrotransposons and non-LTR retrotransposons. Although both LTR and non-LTR retrotransposons are located on plant genomes, LTR retrotransposons are more abundant than non-LTR retrotransposons (Voytas & Boeke 2002, Sandmeyer *et al.* 2002, Schulman & Wicker 2013) and are divided into two main superfamilies known as *Ty1/copia* and *Ty3/gypsy* groups (Wicker *et al.* 2007).

Ty1/copia (*Pseudoviridae*) elements are prevalent in plants and grouped into three genera: Pseudovirus (*Ty1*), Hemivirus (*Copia*) and the Sirevirus which are closely related to the retroviruses, but have a different *polyprotein* (*pol*) gene order (Kumar & Bennetzen 1999, Llorens *et al.* 2009). Sireviruses are plant specific unique retrotransposons among *Ty1/copia* elements (Gao *et al.* 2003, Bousios *et al.* 2010). Sequence studies revealed that highly conserved sequence motifs of Sireviruses have been found within the extremely divergent non-coding part of the genome (Bousios *et al.* 2010). Recently, Sireviruses are named after the *SIRE1* element from soybean, and they were originally named as Agroviruses according to their host specificity that they were only colonized in plants (Peterson-Burch & Voytas 2002). Two notable features of Sireviruses can be considered as that they consist of an *env*-like gene after *pol* and encode a significantly larger *Gag* protein which differentiate them from other *Ty1/copia* genera (Peterson-Burch & Voytas 2002, Havecker *et al.* 2005). However, no known functions for either of these additional coding regions have been identified (Bousios *et al.* 2010).

Interestingly, some evidence indicated that individual retrotransposon families demonstrate typical patterns of chromosomal localizations (Presting *et al.* 1998, Friesen *et al.* 2001). However, with the completion of the DNA

sequencing project of *Arabidopsis thaliana* genome and various plant species, including barley, with large genome allowed the analysis of the retroelement distribution, and these studies revealed that TEs demonstrated non-random distribution through chromosomes (AGI 2000, Mascher *et al.* 2017). In barley genome project, Mascher *et al.* (2017) revealed that 3.7 Gb (80.8%) of barley genome consists of transposable elements and that only 10% of them are intact and potentially active. They distinguished the seven barley chromosomes into three fractions as fractions 1, 2 and 3. In the proximal fraction 3 -where older transposable elements are diverged, unique and predominated- contains mostly repetitive 20-mers. Moreover, fraction 3 is favoured by *Gypsy* retrotransposons, while fractions 1 and 2 are populated by *Copia* elements. These differences in the relative abundance of retrotransposon families are considered as distinct distributions of functional domains. Additionally, *Mariner* transposons preferentially occupy within 1 kb up- or downstream of the coding regions of genes. However, *Harbinger* and long interspersed elements are observed to be located further away from genes. Selective pressures are considered to underlie the distribution of different types of transposable elements around genes. As expected, the smallest transposable elements such as Mariner may be more tolerated in regions close to the genes. However, intriguingly, the Harbinger superfamily elements possess a clear preference for promoter regions, while long interspersed elements preferentially possess for downstream regions of genes. Moreover, the large transposable elements such as LTR retrotransposons and CACTA elements are located at greater distances from genes (Mascher *et al.* 2017).

Fluorescence *in situ* hybridization (FISH) using target-specific DNA probes have become a routine technique in modern molecular biology, cell research and medical diagnosis (Hausmann & Cremer 2003). However, application of FISH has been shown to be more difficult, because of the cell wall, the cytoplasm, which prevents chromosome spreading, low metaphase indices and often similar chromosome morphology (Salvo-Garrido *et al.* 2001). The distribution of retrotransposon families has been analysed in a variety of plant genomes including, *Allium cepa*, *Aegilops speltoides*, *Brachypodium distachyon* and *Glycine max* using FISH (Lin *et al.* 2005, Kiseleva *et al.* 2014, Shams and Olga Raskina 2018, Li *et al.* 2018). Li *et al.* (2018) performed FISH analysis on pachytene chromosomes of soybean BAC and three subclones. FISH analysis revealed that a recently inserted *SIRE1*, a solo *SIRE1* LTR and fragments of *Calypso*-like retroelements were located within this BAC. In barley, Acevedo-Garcia *et al.* (2013) used FISH analysis and YAC library to isolate *Ror1* gene, which encodes *Required for mlo-specified resistance* genes and important for basal defence, due to fine mapping and gene synteny strategies. Additionally, BAC clones have also been used to demonstrate *BARE1* patterns on barley chromosomes by FISH (Vicient *et al.* 1999).

The aim of this study was to present the localization of *SIRE1* patterns in *Hordeum vulgare* L. chromosomes using labelled-PCR products via FISH. *SIRE1* patterns were observed under confocal microscope on barley root preparations. Our results indicated the *SIRE1* localizations in barley genome.

Materials and Methods

Plant materials

Hordeum vulgare L.cv. Hasat was provided from the Directorate of Trakya Agricultural Research Institute. The plants were grown in a growth chamber under 16 h light/8 h dark and 25°C ± 2°C conditions. Relative humidity was kept at 60-75%. After 72 hours, plants were harvested, directly treated with liquid nitrogen and then stored at –80°C until DNA extraction.

gDNA Extraction

gDNA were extracted from 200 mg of the samples by using a modified version of the cetyltrimethylammonium bromide (CTAB) precipitation method described in Mafra *et al.* (2008). Specifically, 200 mg homogenized sample was incubated with 1 ml Edward's buffer (0.5% (w/v) SDS, 250 mM NaCl, 25 mM EDTA, 200 mM Tris pH 8.0) at 95ºC for 5 min (Cold Spring Harbor Laboratory, 2005). The mixture was centrifuged at 16,000 g for 15 min. afterwards, the supernatant was extracted twice with chloroform. The aqueous phase was incubated with 2 volumes of CTAB precipitation solution, then the CTAB protocol was followed as previously described (Mafra *et al.* 2008). DNA yield and purity were measured by UV spectrophotometry at 230, 260 & 280 nm using a NanoDrop 2000c instrument (Thermo Scientific, Wilmington, DE, USA). DNA integrity was evaluated by agarose gel electrophoresis with which samples were separated on 1% agarose gels in 1X TAE buffer.

Chromosome preparation for FISH analysis

Seeds were placed randomly in Petri dishes containing filter paper soaked in only water to germinate in an incubator at 18-25°C in the dark for 3 days. Root tips were harvested, directly fixed in Carnoy fixative (3:1 ethanol:acetic acid solution) without any chemical pre-treatment and stored at 4ºC. Chromosome preparations and FISH analysis were performed according to Jenkins & Hasterok (2001, 2007) with modifications. The slides were checked under the light microscope (Olympus U-TVO.5XC-3) and kept in a freezer at -20ºC.

Development of probes and labelling

The FISH probes used in this study were generated from two sets of data which are the *ENV* (*envelope-like*) and *GAG* (encoding a structural protein) genes of *SIRE1*. To investigate the distribution of *SIRE1*, we amplified *ENV* and *GAG* domains of *SIRE1* using designed specific primer sets (Table 1). The probes for *ENV* and *GAG* domains were designated by using IDT's PrimerQuest© Tool (2012). GC% and Tm values of the probes were around 62 and between 47°and 55°C, respectively. The

sequences of *SIRE1 ENV and GAG* were obtained from barley (KP420209 for *GAG* and KP420210 for *ENV*).

Probe synthesis was carried out individually by using *SIRE1 ENV* and *GAG* primers. The reactions were performed in a total volume of 50 µl including 18.25 µl nuclease-free dH$_2$O, 25 µl of HotStart PCR Master Mix (Bio-Rad), 1.5 µl of each primer (10 µM/µl), 1.75 µl of tetramethylrhodamine-dUTP (TRITC) (1 mM), and 2 µl template DNA (40 ng/µl). PCR conditions were as follows: 94°C for 5 min followed by 40 cycles of 94°C for 25 s, annealing 50°C for 25 s and 72°C for 30 s. The reaction was completed by a final extension step at 72°C for 5 min.

Table 1. Primers used in this study.

No	Primer Name	Sequence (5'→3')
1	*SIRE1 ENV F*	CGACAACACCAGAGGAGAATG
2	*SIRE1 ENV R*	CGCCTTGGTGGCCAATTA
3	*SIRE1 GAG F*	AACCGAGATGGAGGTAGTACA
4	*SIRE1 GAG R*	GAAACGGCACACGCTAGA

Fluorescence in situ hybridization (FISH) analysis

The FISH analysis protocol was adopted from Jenkins and Hasterok (2001, 2007) protocol with modifications. Chromosome spreads were scanned under light microscope with ×40 objective to determine the number and quality of well-spread metaphase plates, and then they were treated with 100 µg/ml of RNase at 37°C for 1 h. The hybridization mixture contains 20 µl of deionised formamide (50%), 8 µl of dextran sulphate (10%), 4 µl of 20X SSC (2X SSC), 2 µl of 10% SDS (0.5%), 10 µl of probe (75-200ng/slide), 1 µl of blocking DNA (sonicated salmon sperm DNA) (25-100X probe) and added sterile dH$_2$O to bring final volume 40 µl. Final concentrations were indicated in parenthesis. The mixture was denatured at 85°C for 10 min and kept on ice for 10 min. A 38 µl aliquot of the hybridization mixture was applied onto each slide, covered with a coverslip and sealed with paper bond. Both chromosomal DNA and probe DNA on the slides were denatured together in a thermal cycler at 70°C for 6 min and hybridized with each other at 37°C overnight in a humid dark box. Afterwards, hybridized the chromosome spreads were washed three times in 2X SSC: once 2X SSC to float coverslips off, once in 15% formamide/0.1X SSC and again once in 15% formamide/0.1X SSC, each for 10 min at 42°C. Then, the slides were washed in 2X SSC for 3 min at 42°C. This step was repeated twice with fresh 2X SSC at 42°C. Ultimately, the slides were washed three times in 2X SSC for 3 min at RT. Then, the slides were dehydrated in alcohol series (70, 90 and 100%), each for 1 min at RT and waited in the dark for 15-20 min. Vectashield-DAPI mounting-staining medium (7-10 µL) was dropped onto the chromosome spreads, which were then stored at 4°C until used.

Image acquisition

To image the slides, 551-575 nm wavelengths for probes labelled with TRITC and 420-480 nm wavelengths for DAPI were used for fluorescence detection in the Leica DM5500 confocal microscope. The different fluorescent images were acquired separately. Afterwards, they were merged into single composite images. The signal images were analysed by Adobe Photoshop CC 2014.

Results

To characterize the abundance and localization patterns of soybean *SIRE1 ENV* and *GAG* domains in barley, we used root tip cells and *SIRE1 ENV* and *GAG* probes were labelled with TRITC. Specifically, we observed TRITC labelled *SIRE1 ENV* exhibited characteristic patterns at prophase in nucleus (Fig. 1). TRITC labelled *SIRE1 GAG* probes also demonstrated distinctive patterns in prophase phase in barley chromosomes (Fig. 2).
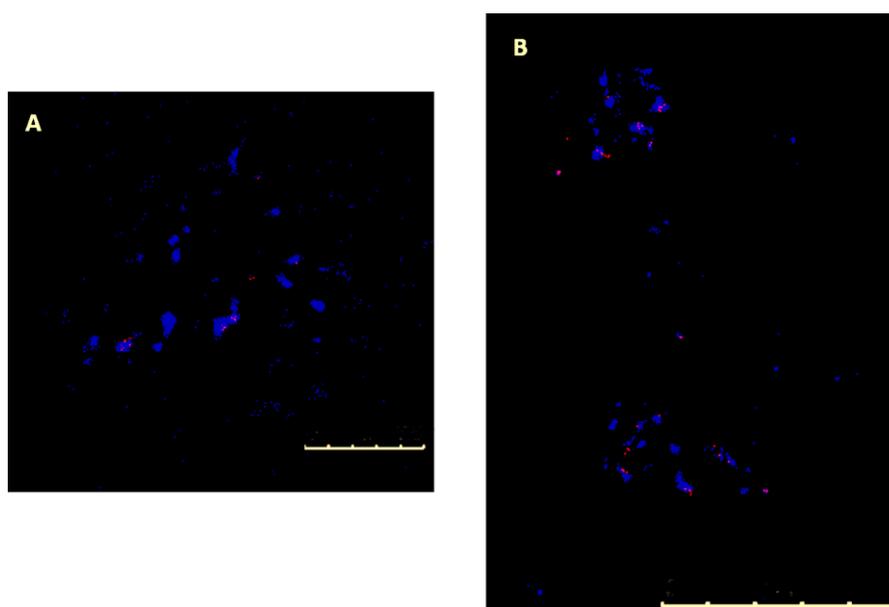
Fig. 1. Display of SIRE1 ENV distributions in barley root preparations *via* FISH. According to FISH results, characteristic feature of *SIRE1 ENVs* are localized in centred regions. Scale bar=7.5 and 25 µm for A and B, respectively.
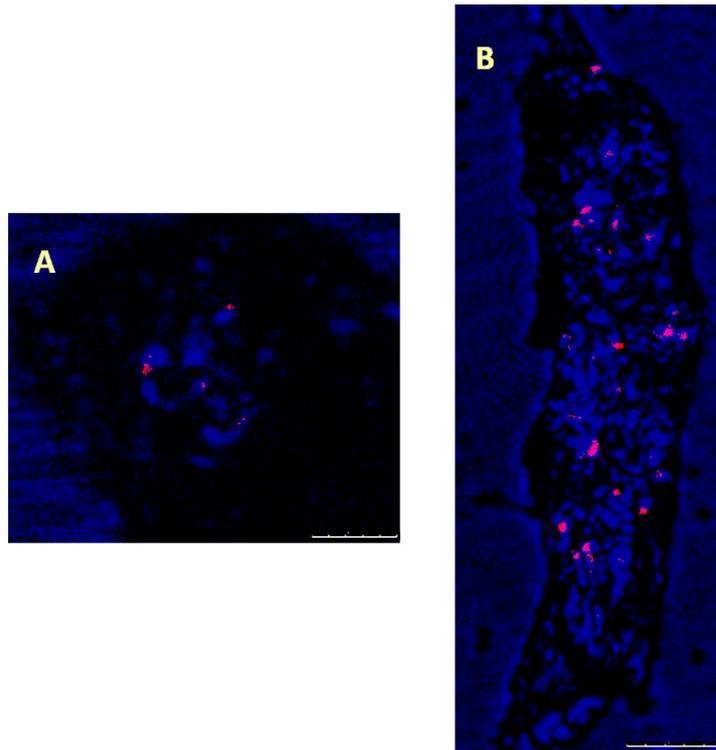
Fig. 2. Demonstrations of *SIRE1 GAG* localizations in barley root preparations *via* FISH. According to FISH results, *SIRE GAGs* are distinctively localized close to centred regions, similarly *SIRE1 ENV.* Scale bar=5 and 10 μm for A and B, respectively.

Discussion

In plants, retroelements cover a large zone of genomes and are considered to have participated in the genome organization and evolution (McCarthy *et al.* 2002). Several studies have focused to detecting the chromosomal position of *Ty1-copia* retroelements, including *SIRE1* and anticipating their copy number (Pearce *et al.* 1996, Lin *et al.* 2005, Alipour *et al.* 2013, Kolano *et al.* 2013, Lee *et al.* 2013). In the current study, localizations of *SIRE1 ENV* and *GAG* domains were indicated on barley chromosomes (see Fig. 1 and 2). The results were obtained from *Hordeum vulgare* L. cv. Hasat root tips preparations using FISH analysis. Due to the large genome size of barley, researchers mostly prefer to use single-copy probes derived from BAC or YAC contigs (Vicient *et al.* 1999, Acevedo-Garcia *et al.* 2013, Bustamante *et al.* 2017). In our study, we used direct PCR products derived from barley DNA to produce single-stranded probes were small, appx. 233 bp for *ENV* and 120 bp for *GAG.* These small PCR probes were highly specific and stable for hybridization thus, it leads to very good amplification of the signals. By using short size of probes, we were able to visualize the first time *SIRE1 ENV* and *GAG* distribution patterns on barley chromosomes, indicating they intend to demonstrate different patterns. Additionally, it is possible to observe localization of lncRNAs on chromosomes and in the cells using direct labelled-PCR products *via* FISH techniques (Karlik *et al.* 2018).

In plant genomes, most of the TEs are amplified to thousands of copies and present as low- or moderate-number copies (Baucom *et al.* 2009). Besides increasing genome sizes, TEs can impact on genome structure and gene expression at a global scale. Moreover, deletions of the interleaving genome sequences or creations of new chromosomal rearrangements can be generated by recombination between two TEs (Weil & Wessle 1993, Hughes *et al.* 2003, Vicient & Casacuberta 2017). However, TE karyotype differences may be significant tool contributing to reproductive isolations of TEs and crop domestication, to discriminate species diversification in plants (Vicient & Casacuberta 2017). Also, the abundance and size of LTR retrotransposons in plant genomes exert interesting questions on how they affect genes and how they are regulated so that their insertions do not negatively influence the host genome (Galindo-González *et al.* 2017). Mascher *et al.* (2017) identified 3.7 Gb (80.8%) of the assembled barley genome sequence as derived from TEs, most of them were present as truncated and degenerated copies. Interestingly, only 10% of mobile elements are intact and potentially active. According to the sequencing results of Mascher *et al.* (2017), TEs presented notable variation in their insertion site preferences. The distribution of *SIRE1 ENV* and *GAG* barley were consistent with each other that they were mostly characterized close to the centromere regions (see Fig. 1 and 2). Presting *et al.* (1998) demonstrated the centromere of barley is enriched with *cereba* is a member of the *Ty3/Gypsy* class, that ~200 copies of *cereba* have been determined in each barley centromere (Hudakova *et al.* 2001, Houben & Schubert 2003). Two conserved centromere-specific repeats [Cereal Centromeric Sequence1 (CCS1) and Sau3A9] were reported, later is

revealed that they are related to *Ty3/Gypsy*-like retrotransposons. In barley, parts of the LTRs of *cereba* element and parts of integrase region of its polygene correspond to CCS1 and Sau3A9, respectively (Hudakova *et al*. 2001). Our results also indicated that presence of *SIRE1* is favoured mostly in regions close to centre locations that as we know *SIRE1* is active and belongs to the *Tyl/Copia* retrotransposon superfamily (Lin *et al*. 2005). However, *SIRE1* may be related to the centromeric repeats BCS1/CCS1 (barley cereal centromeric sequence), thus these elements may also contribute to *SIRE1* elements movements. Lin et al. (2005) reported the presence of a 102-bp tandem repeat (STR102) which was a *SIRE1* element and a *SIRE1* solo LTR in soybean. However, the flanking sequences of 3 of 10 *SIRE1* insertions were found to be repetitive, belonging to either *Ty3/gypsy* or other repetitive families (Laten *et al.* 1998). Moreover, these fragments have been demonstrated for maize and rice (Ananiev *et al.* 1998, Cheng *et al.* 2002) suggesting that they are common motifs of higher plants of centromeric/pericentromeric regions (Martinez-Zapater *et al.* 1986, Ananiev *et al.* 1998, Cheng *et al.* 2002, Lin et al. 2005), which is also consistent with our results. In soybean, Lin et al. (2005) also considered that *SIRE1* may preferentially get in heterochromatic and/or pericentromeric regions which may alternatively be selected against the gene-rich euchromatic regions. In barley, we also observed that *SIRE1* elements were mostly

concentrated in centred regions. FISH analysis of barley *SIRE1* elements provided us to allow cursory insight into the barley genome organization.

In conclusion, we were able to observe the distinctive distributions of the *SIRE1 ENV* and *GAG* elements via FISH analysis by using labelled-PCR products as probes in barley root preparations. How these elements function to organize genome is still a mystery, however the applications of FISH analysis of TEs may have an important potential to uncover the organization of higher plant genomes.

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