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Display of *Sukkula* distributions on Barley Roots via *in situ* hybridization

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Abstract. Retrotransposon are an abundant and ancient parts of the plant genomes that especially LTR retrotransposons influence the genome size and evolution. *Sukkula* is a non-autonomous and active, relatively high copy-number retroelement. In this study, we performed fluorescence *in situ* hybridization (FISH) to observe the distributions of *Sukkula* elements (*LTRs* and *internal-domain*) by using labelled-PCR products. The localizations of *Sukkula* elements (*LTRs* and *internal-domain*) were observed under confocal microscope on *Hordeum vulgare* L. cv. Hasat root preparations. Our results revealed that *Sukkula* elements is still active and spread through the whole barley chromosomes. Additionally, the re-sequencing analysis of *Sukkula* LTRs demonstrated that LTRs sequences had ~65 bp gain. These analyses represent a valuable resource to reveal genome organization of barley and large sized plants.

Keywords: fluorescence *in situ* hybridization, retrotransposon, *Sukkula*, Barley, *LTRs*, *internal-domain*

INTRODUCTION

Beginning with the pioneering work of Barbara McClinton, transposable elements (TEs) have become to take part a central position in the plant genome studies. TEs consist of DNA fractions capable of chromosomal movement, either *via* replicative or conservative (cut-and-paste) mechanisms (Doolittle and Sapienza 1980; Orgel and Crick 1980; Finnegan 1989). Eukaryotic TEs contain two main classes; Class I elements and Class II elements. Class I elements are also known as retrotransposons move through using an RNA intermediate, while Class II elements move through the genome using a DNA intermediate (Finnegan 1989).

In plants, the vast majority of repetitive DNA in the nuclear genomes is derived from the proliferation of mostly Class I elements called as retrotransposons (SanMiguel *et al.* 1996; Vicient *et al.* 1999; Hawkins *et al.* 2006; Neumann *et al.* 2006; Vitte and Bennetzen 2006) which are subdivided as two major subclasses; Long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons. LTR retrotransposons, which typically comprise GAG and POL protein coding ORFs encoding several enzymes (reverse transcriptase

– RT; protease – PR; RNaseH – RH; integrase – INT) responsible for reverse transcription and integration of daughter sequences into new chromosomal locations, constitute the largest fraction of the TEs (Eickbush and Malik 2002; Havecker *et al.* 2004; Hawkins *et al.* 2006; Neumann *et al.* 2006; Vitte and Bennetzen 2006). Moreover, LTR retrotransposons are found in plants are subdivided in two main superfamilies, *gypsy*-like and *copia*-like (also known as *Metaviridae* and *Pseudoviridae*, respectively), which include the same protein coding domains. However, these domains are rearranged in different order in both LTR retrotransposon types (Eickbush and Malik 2002; Havecker *et al.* 2004).

Sukkula elements were first identified in barley genome at the barley Mlo locus and to an insertion sequence present in the 3' LTR of one BARE1element (Manninen and Schulman 1993). Shirasu *et al.* (2000) later determined two ~5 kb sequence similar this insertion in a 66 kb stretch of barley genome that were also found to be flanked by 5 kb direct repeats. Therefore, these sequences were named as *Sukkula* elements means "shuttle" in Finnish. However, *Sukkula* LTR copies are found to be *gypsy*-like retrotransposons, but non-autonomous elements belonging to a novel group of retroelements, *l*arge *r*etrotransposon *d*erivatives or LARDs (Kemekawa *et al.* 1999; Kalendar *et al.* 2004). Moreover, *Sukkula* elements consist of reverse transcriptase in appx. 3.5 kb central domain which is found to be conserved as in primary sequence and secondary structure, including no open reading frames (ORFs) encodes typical retroelement proteins. According to these features of *Sukkula* elements, they are TRIMs (Terminal-repeat Retrotranposons in Miniature) in their lack of a protein-coding domain (Kalendar *et al.* 2004). Active retrotransposons are important for genome diversification in plants, because of their transposition and accumulation potentials in the genome, thus it can change the overall genome structure (Wessler *et al.* 1995; Vicient *et al.* 1999; Schulman and Kalendar 2005).

Fluorescence *in situ* hybridization (FISH) using target-specific DNA probes have become important tool in modern biology and cell research (Hausmann and Cremer 2003). In plants, introducing FISH probes is more difficult, because of the cell wall and the cytoplasm of the plants that they hinder chromosome spreading and low metaphase indices (Salvo-Garrido *et al.* 2001). By using FISH technique, the distribution of retrotransposon families has been reported in various plants such as *Hordeum vulgare, Allium cepa, Aegilops speltoides, Brachypodium distachyon* and *Glycine max* (Vicient *et al.* 1999; Lin *et al.* 2005; Kiseleva *et al.* 2014; Shams and Raskina 2018; Li *et al.* 2018). *BARE1* distributions on barley chromosomes have been demonstrated by using BAC clones a probe *via* FISH (Vicient *et al.* 1999). In barley, FISH technique was also used to reveal gene organization and to integrate the genetic linkage map with a physical map (Stephens *et al.* 2004).

The aim of this study was to present the distributions of *Sukkula* elements (*LTRs* and *internal-domain*) in *Hordeum vulgare* L. cv. Hasat chromosomes using labelled-PCR products via FISH. *Sukkula* localization patterns were observed under confocal microscope on barley root preparations. We also performed sequencing studies and the sequence analysis of *Sukkula* elements (*LTRs* and *internal-domain*) to elucidate *Sukkula* sequence alterations in barley. Our results indicate that *Sukkula* elements (*LTRs* and *internal-domain*) are still active and under genome evolution.

MATERIALS AND METHODS

Plant materials

Barley (*Hordeum vulgare* L.) cv. Hasat was provided from Directorate of Trakya Agricultural Research Institute. The seeds were grown at growth chamber for germination period under controlled conditions (16 h light/8 h dark, 25°C ± 2°C) and relative humidity was kept at 60–75%. The plants were harvested after 72 hours, directly treated with liquid nitrogen and then stored at –80°C until DNA extraction.

gDNA Isolation

gDNA were isolated from 200 mg of the samples by using the cetyltrimethylammonium bromide (CTAB) precipitation method was modified as previously 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). They were then spun down at relative centrifugal force of 16,000 g in a microcentrifuge for 15 min, and the supernatant was isolated twice with chloroform. Then, the aqueous phase was incubated with 2 volumes of CTAB precipitation solution, after which the CTAB protocol was followed as previously described (Mafra *et al.* 2008). DNA yield and purity were measured by UV spectrophotometry at 230, 260 and 280 nm using a NanoDrop 2000c instrument (Thermo Scientific USA). DNA integrity was evaluated by agarose gel electrophoresis, samples were separated on 1% agarose gels containing Ethidium bromide nucleic acid stain in 1X TAE buffer.

Chromosome preparation for FISH analysis

Grains 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. Then, root tips of barley cv. Hasat were harvested, then directly fixed in Carnoy fixative (3:1 ethanol:acetic acid solution) without any chemical pre-treatment, stored roots at 4°C. Chromosome preparations and FISH analysis were performed according to Jenkins and Hasterok (2001, 2007) with modifications. The slides were checked under the light microscope (Olympus U-TVO.5XC-3) and kept in a freezer at -20 °C.

Development of probes and labelling

The FISH probes used in this study were generated from two set of data which is the *Sukkula* (*internal-domain*) gene and *LTR* sequences. To investigate the distribution of *Sukkula*, we amplified *internal-domain* and *LTR* sequences of *Sukkula* using designed specific primer sets Table 1. The probes for *internal-domain* and *LTR* sequences designated by using IDT's PrimerQuest© Tool (2012). GC% and Tm values of probes were around 50 and between 50°C and 55°C, respectively. The sequences of *Sukkula LTR* and *internal-domain* were obtained from barley (AY054376 for *LTR* and *intern-domain*).

Probe synthesis was carried out individually by using *Sukkula LTR* and *internal-domain* primers. The reactions were carried out in total volume of 50 µl including 18.25 µl nuclease-free water, 25 µl of Hot-Start 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	*Sukkula LTR* F	CCCTCCTTCCCTCTTCTCTAAT
2	*Sukkula LTR* R	CCATACTCTGAACCTGATCCTAAAC
3	*Sukkula LTR sequencing* F	AACCAGTCAACCAGCATAGG
4	*Sukkula LTR sequencing* R	GGAGAGGGAGAGATAAGAGGAA
5	*Sukkula internal-domain* F	CCTTGCACTTGATGGCTACT
6	*Sukkula internal-domain* R	CGGATGAGACACGGAAGAAA

Fluorescence in situ hybridization (FISH) analysis

The FISH analysis procedure was performed based on Jenkins and Hasterok protocol (2001, 2007) with modifications. Chromosome spreads were scanned under ×40 objective light microscopes to define the number and quality of well-spread metaphase plates, and they were treated with 100 µg/ml of RNase at 37°C for 1 h. The hybridization mixture consist of 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_2O 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, hybridization 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, slides were washed in 2X SSC for 3 min at 42°. This step was repeated twice with fresh 2X SSC at 42°. Ultimately, slides were washed three times in 2X SSC for 3 min at RT. After, 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

For imaging the slides, the following wavelengths were utilized for fluorescence detection: 551-575 nm for probes labelled with TRITC and 420-480 nm for DAPI in 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.

Sequence analysis

For sequence analysis of *Sukkula LTRs*, we performed PCR reaction. The PCR products were re-sequenced. The sequence homology search was conducted in barley genome by using BLASTN in the Ensembl website (http://plants.ensembl.org/barley). However, the re-sequencing results of *Sukkula LTRs* were compared the original *Sukkula LTR* sequences using Clustalomega (Altschul *et al.* 1990).

RESULTS AND DISCUSSION

Total copy number of TEs in plant genomes expands from as little as a few hundred in those with smaller genome sizes, including *Arabidopsis*, to hundreds of thousands in their larger genome counterparts (e.g. maize, *Triticum*, *Hordeum*) (Bennett and Leitch 2005). Comparison studies suggest that the same general TE types are found in all plant species, however the relative proportions of diverse classes and subclasses can differ dramati-

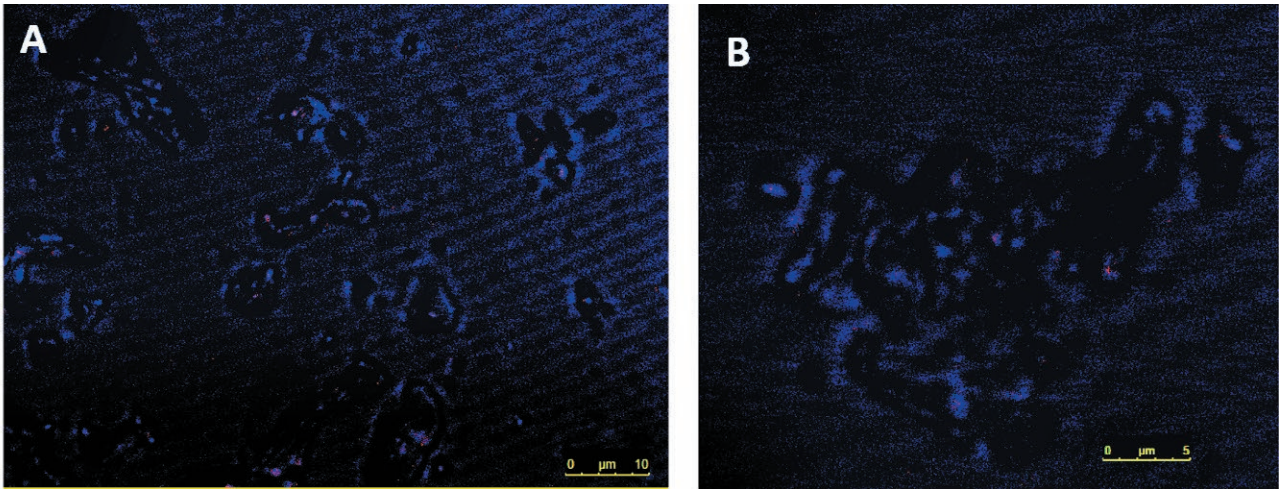

Figure 1. Display of *Sukkula internal-domain* distributions in barley root preparations *via* FISH.

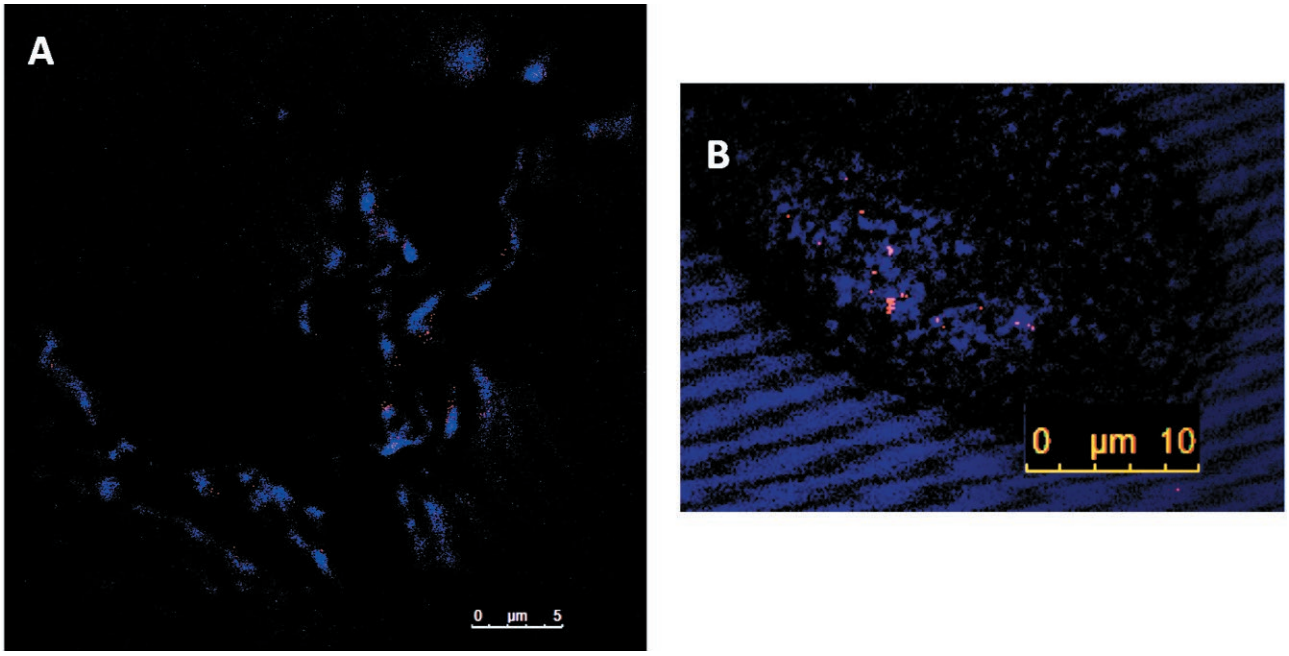

Figure 2. Display of *Sukkula LTRs* distributions in barley root preparations *via* FISH.

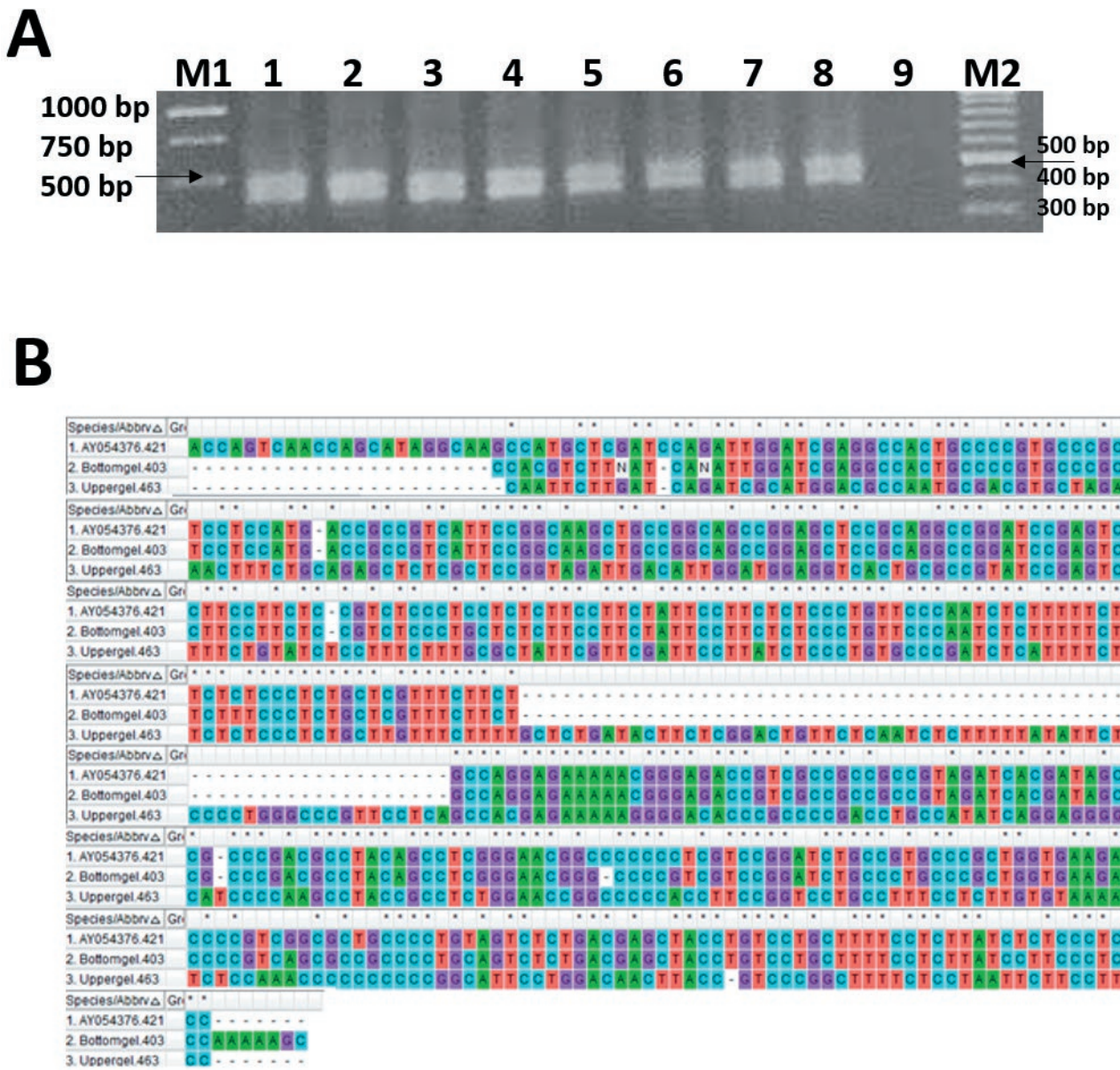

Figure 3. A) Gel figuration of sequencing primers results. **B)** Demonstration of sequencing analysis results. Original Genbank sequence AY054376 compared with the sequences recovered from bottom gel band and upper gel band displayed in Figure 3A.

cally (Kejnovsky *et al.* 2012). Moreover, LTR retrotransposon turnover in monocots have been demonstrated to be extremely rapid with both gains and losses of TEs comparing with eudicots (Ma *et al.* 2004; Vitte and Bennetzen 2006). In the present study, distribution of *Sukkula LTRs* and *intern-domain* were observed on *Hordeum vulgare* L. cv. Hasat root tips preparations using FISH analysis (see Figure 1 and 2). Because of the large genome size of barley, single-copy probes are mostly designed from BAC or YAC contigs (Vicient *et al.* 1999; Acevedo-Garcia *et al.* 2013; Bustamante *et al.* 2017). In the current study, we used direct PCR products derived from barley genomik DNA to generate single-stranded probes were short, ~ 421 bp for *LTRs* and 451 bp for *internal-domain* which were highly specific and stable for hybridization, therefore it leads to very good amplification of the signals. In addition to display of *Sukkula* elements in barley chromosomes, our group were able to observe the distributions of *SIRE1 ENV* and *GAG* by using barley root tips via FISH (Karlik and Gozukirmizi unpublished data). Nowadays, it is

also possible to use short direct labelled-PCR products to observe lncRNAs on barley chromosomes by using FISH (Karlik *et al.* 2018).

The impact of retrotransposons especially LTR-retrotransposon proliferations and loss on genome structure and evolution of plant species have been studied in species with small- or medium sized genomes. However, large sized genomes have been reported in monocotyledonous species, including maize (2.3 gigabase pairs) and barley (5.1 gigabase pairs) (Schnable *et al.* 2009; Mascher *et al.* 2017), thus we studied with barley in the present study. *Sukkula* element is known as non-autonomous LTR retrotransposons which use other retrotransposons proteins for transposition, thus we observed that *Sukkula* elements (*LTRs* and *internal-domain*) distributed on whole barley genome (see Figure 1 and 2). However, some studies demonstrated the prevalence or eventual decay of TEs in the different genomic regions depends on the process of selection and "host control" in a very long evolutionary time (Rebollo *et al.* 2012; Vitte *et al.* 2014). Kartal-Alacam *et al.* (2014) has investigated *Sukkula* polymorphism rates in non-cultured mature embryos, 40- and 80-days old callus materials by using IRAP and iPBS techniques that *Sukkula* is the second most active retrotransposon in barley genome. Moreover, our study indicated that transposition of *Sukkula* elements does not depend on the process of selection and "host control".

Kalendar *et al.* (2004) suggested that *Sukkula LTRs* are rarer than *BARE1* and not distributed in the barley genome. However, their FISH results demonstrated *Sukkula LTRs* with a high copy number. Their labelled-LTR probes were hybridized with the chromosome arms except the telomeres, nucleolar organizing regions, and centromeres, where the signals are blocking. Interestingly, we also observed a high copy number, in addition with our *LTR* and *internal-domain* probes labelled the regions in the whole chromosomes, including telomeres, nucleolar organizing regions, and centromeres (see Figure 1 and 2). However, our FISH results are consistent with the chromosome hybridization data were confirmed by PCR reaction that both *Sukkula* segments (*LTRs* and *internal-domain*) were found to be present on all barley chromosomal segments.

The abundance of repetitive DNA is mostly responsible for genome size variations in species or interspecies that especially in LTR-retrotransposons, these differences in abundance may be originated from extreme amplification through retro-transposition or from DNA loss by unequal homologous recombination, which produced solo-LTRs (Flavell 1986; Lisch 2013). During the probe synthesis, we noticed the two bands in agarose gel electrophoresis analysis (see Figure 3A). Then, we performed sequencing analysis to reveal the difference between two bands. Therefore, re-sequencing analysis of *Sukkula LTRs* has revealed that *Sukkula LTRs* had some gains, especially ~ 65 bp during the evolutionary time, indicating that this event may depend on DNA gain by unequal homologous recombination (see Figure 3B). Additionally, *Sukkula LTRs* sequences (AY054376) demonstrated 95.20% sequence identity to bottom gel band and 58.81% identity to upper gel band.

In conclusion, we were able to observe the distributions of the *Sukkula LTRs* and *internal-domain* elements via FISH by using labelled-PCR products in barley root preparations. *Sukkula* is a non-autonomous LTR retrotransposon which is still active. However, how these elements function or organize the genome is still a mystery, thus FISH analysis of TEs has important potentials to uncover the organization of large sized plant genomes.

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