Hortic. Environ. Biotechnol. 56(6):793-799. 2015.
DOI 10.1007/s13580-015-1100-3

ISSN (print) : 2211-3452
ISSN (online) : 2211-3460

Research Report

Distribution of Various Types of Repetitive DNAs in *Allium cepa* L. Based on Dual Color FISH

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Received June 23, 2015 / Revised September 1, 2015 / Accepted October 1, 2015

Abstract. Fluorescence in situ hybridization (FISH) is a powerful tool for the detection of DNA sequences in a specific region of a chromosome as well as for integrated physical mapping. The detailed karyotypes of two onion cultivars ('Eumjinara' and 'Sinseonhwang'), which are resources for the onion genome sequencing project were constructed based on dual color FISH using 5S and 45S rDNAs, telomeric tandem repeats, and Cot-1 DNA. All materials showed 2n = 2x = 16. Four loci of 5S rDNAs were located on the interstitial regions of the short arms of one pair of chromosomes in both onion cultivars. One loci of 45S rDNA signal was distally detected on each short arm of the two pairs of chromosomes in 'Eumjinara' and 'Sinseonhwang', but the latter possessed another locus of 45S rDNA on the distal part of the long arm in one homolog of a chromosome pair. Co-localization of telomeric tandem repeats and 45S rDNA signals was observed in 'Eumjinara' and 'Sinseonhwang'. A difference in the distribution of 45S rDNA sites and the co-localization of signals observed between the two cultivars are indicators of recent activities in the nuclear genome that may involve homologous recombination or transposition of certain repeats. Cot-1 DNA signals are distributed throughout the chromosomes and show stronger signals in the terminal regions. The elucidation of Cot-1 DNA through in-situ hybridization would only show a large amount of tandemly repeating, non-coding and dispersedly repetitive DNAs in onion genome.

***Additional key words*:** Cot-1 DNA, in situ hybridization, rDNA, tandem repeats, telomeric repeats

Introduction

Allium is a large and polymorphic genus that comprises 730 spp. with six subgenera and more than 60 sections that are spread throughout the Northern hemisphere (Hanelt, 1990; Pich et al., 1996). The phylogenetic relationships in some variable groups of *Allium* are not yet clear. Nevertheless, comparison of species at the molecular level has been confirmed (Pich et al., 1996) and refined the phylogenetic relationship between the taxa of the genus *Allium* (Bark and Havey, 1994; Jorgensen and Cluster, 1998; Linne Von Berg et al., 1996). The genomic constitution of *Allium* includes large amounts of repetitive DNA sequences (Flavell et al., 1974) that are categorized into tandemly and dispersedly repeats (Kubis et al., 1998). DNA sequences comprised of an array of tandem repeats (e.g., rDNA) may serve as cytogenetic markers that distinguish homologous chromosomes (Ricroch et al., 1992).

A. cepa L. is considered to have one of the largest nuclear genomes among cultivated plants (16,400 Mbp/1C) and is composed of 95% repetitive elements (Flavell et al., 1974; Jakse et al., 2008; Kim et al., 2014). Enormous amount of repetitive DNA sequences in plants play a significant role in augmenting the genome (Vitte and Benetzzen, 2006). The redundancy of these DNA repeats would complicate whole genome sequencing (Yuan et al., 2003).

Two multigene families of rDNA that are composed of tandemly ordered repeats have been investigated for the past several decades in a number of organisms, including plants and animals. A gene family known as 5S rDNA is composed of conserved coding sequence (120 bp) and non-transcribed spacer (NTS) region, but unlike the other gene family it is not associated with a nucleolar organizing region (NOR) (Long and David, 1980; Martins et al., 2000; Shibata and Hizume,

2002b). The second gene family of rDNA, which is associated with NOR, encodes genes for 18S, 5.8S, 26S-28S, and an internal transcribed spacer (ITS) (Gurushidze et al., 2007; Long and David, 1980; Suzuki et al., 1996).

A type of satellite DNA that has a tandem repeat length of approximately 375 bp was isolated and sequenced in *A. cepa* L. (Barnes et al., 1985). This satellite sequence hybridizes on the telomere region of the species (Pich and Schubert, 1998). Do et al. (2001) characterized a 314 bp tandem repeat which is closely associated with constitutive heterochromatin. The tandem repeat has 81% similarity with the 375 bp repeat, suggesting that both are under the same family of repeats. The association between satellite DNAs and C-heterochromatin would only signify a specific role (i.e., structural) they render in the chromosomal and nuclear organization of a genome (Yunis and Yasmineh, 1971).

Repetitive DNA sequences that are not tandemly organized are localized on the entire regions of chromosomes (Pearce et al., 1996). To elucidate the distribution of these repeats in the genome, a technique (Cot analysis) that is based on renaturation kinetics is used (Peterson et al., 2002). Cot analysis guides a HAP-based fractionation of a genome to create a Cot library for further comprehensive assessment of genomic components (Peterson et al., 2002).

The localization of DNA sequences on the chromosomes was made possible through the in situ hybridization technique. Fluorescence in situ hybridization (FISH) technique allows the visualization of multi-gene families (Castilho and Heslop-Harrison, 1995) and highly repeated DNA sequences (Irifune et al., 1995). The combination of cytogenetics with FISH has augmented the power of karyotype analysis. In this study, we test the presence and chromosomal distribution of two types of ribosomal DNA, telomeric tandem repeats, and Cot-1 DNA on somatic metaphase chromosomes of onion cultivars 'Sinseonhwang' and 'Eumjinara' by using FISH.

Materials and Methods

Plant Materials and Chromosome Preparation

The bulbs and seeds of *A. cepa* L. 'Eumjinara' and 'Sinseonhwang' were obtained from the Rural Development Administration (RDA), Korea. The fresh root tips were excised when they reached 2-3 cm in length and were treated with 2 mM 8-hydroxyquinoline for 5 hr at 20°C. The materials were fixed in aceto-ethanol (1:3, v/v), stored at room temperature for 2 hr, and soaked in 70% ethanol at 25°C until used. The root tips were treated with an enzyme mixture (2% cellulose and 1% pectolyase) in 0.01 M citrate buffer at 37°C for 1 hr and homogenized in 60% acetic acid solution. The prepared slides were dehydrated with an ethyl alcohol series.

DNA Shearing and Isolation for Cot--1 DNA

The young leaves of *A. cepa* L. were used for the extraction of genomic DNA following the protocol of Allen et al. (2006). The genomic DNA of onion was sheared using S220 focused-ultrasonicator (Covaris, Woburn, MA, USA) at the National Instrumentation Center for Environmental Management (NICEM), SNU. The protocol of Zwick et al. (1997) was followed to isolate the Cot-1 DNA from the genomic DNA of *A. cepa* L.

Preparation of Repetitive DNA Probes

The genomic DNA of *A. cepa* L. was used as a template for the generation of PCR products. 45S rDNA (Gerlach and Bedbrook, 1979) was obtained from *Triticum aestivum* and 5S rDNA (Do and Seo, 2000) was amplified using the primer sets 5'-GGATCCCATCAGAACTCC-3' and 5'-GGTGCTT TAGTGCTGGTAT-3'. PCR conditions started with an initial denaturation at 94°C for 5 min and followed by 30 cycles of amplification - denaturation at 94°C for 1 min, annealing of primers at 55°C for 1 min, DNA extension at 72°C for 1 min, and a final extension at 72°C for 7 min. 5S and 45S rDNAs were labeled with Alexa flour 488-5-dUTP (NEN, Eugene, OR, USA) and biotin-16 dUTP (Roche, Penzberg, Balvaria, Germany), respectively. A 375 bp repeat DNA (Barnes et al., 1985) was PCR amplified using the primer sets 5'-GGCCATAACTGTTGCCTGCTT-3' and 5'-CCAC TCTCCAGGGATGGTAA-3'. Its PCR products were labeled with Texas Red 5- dUTP (Perkin Elmer, Boston, MA, USA). Cot-1 DNA was labeled with Alexa flour 488-5-dUTP (NEN, Eugene, OR, USA).

Slide Pretreatment and Fluorescence in situ Hybridization

Slides were pre-treated with 100 $\mu g \cdot mL^{-1}$ RNase A (Sigma, St. Louis, MO, USA) for 1 hr at 37°C, washed in 2× SSC several times, then incubated in 0.01 N HCL and 0.1% pepsin for 2 min and 10 min, respectively. 4% paraformaldehyde was applied to slides after a few minutes of 2× SSC treatments. Slides were washed with 2× SSC and dehydrated with an ethanol series. The probe mixture was denatured at 70°C for 10 min and placed on ice for 5 min. After the hybridization mixture (50% formamide, 10% dextran sulfate, 4 μL 20× SSC, 5 $ng \cdot \mu L^{-1}$ salmon sperm DNA, 500 $ng \cdot \mu L^{-1}$ of each probe, and water to a total volume of 40 μL per slide) was added cover slips were placed on the slides, the latter were heated at 80°C for 5 min and incubated overnight at 37°C in a humid chamber. The slides were washed in 2× SSC for 5 min, 0.1× SSC at 42°C for 40 min, 2× SSC for 5 min, and 1× detection buffer for 5 min. The biotin-labeled probe was detected by streptavidin-Cy3. The chromosomes were counterstained with 2 $\mu L \cdot mL^{-1}$ of 4′, 6-diamidino-2-phenylindole

(DAPI) in a Vectashield (Vector Lab, Inc., Burlingame, CA, USA).

Microscopy and Image Processing

The prepared slides were examined with an Olympus BX63 fluorescence microscope equipped with a CCD camera (CoolSNAP ^{TM}cf). Images were captured, analyzed, and measured using UV excitation filters and Cytovision$^{©}$/ Genus^{TM} version 7.2 "Probe Measurements" tool (Applied Imaging Corporation, CA, USA). Karyotype analysis was confirmed based on the analysis of at least ten metaphase cells using their morphological characters and FISH results. Chromosome formula was made based on the arm ratio values (Levan et al., 1964).

Results and Discussion

Characterization of chromosomes of 'Eumjinara' and 'Sinseonhwang'

The chromosomes were arranged according to their sizes in decreasing order and paired by their sizes, position of centromeres, and FISH markers (5S and 45S rDNA) (Table 1). The chromosome count for 'Eumjinara' was determined to be 2n = 2x = 16 with a basic chromosome number of x = 8. The lengths of the shortest and longest chromosomes were 11.41 ± 0.42 μm and 17.96 ± 0.07 μm, respectively (Table 1). Six pairs of chromosomes were metacentric (chromosome 1, 2, 3, 4, 5, and 7), one pair was subtelocentric (chromosome 6) and another pair was submetacentric (chromosome 8). Its chromosomal formula is 2n = 2x = 16 = 2m + 2m + 2m + 2m + 2m + 2st^{sat} + 2m + 2sm. The somatic chromosomal images of 'Sinseonhwang' showed 2n = 2x = 16 with a basic chromosome number of x = 8. The longest chromosomes measured 15.61 ± 0.22 μm, while the shortest was 10.68 ± 0.09 μm. There were seven pairs of metacentric chromosomes (chromosome 1, 2, 3, 4, 5, 7, and 8) and one pair was subtelocentric chromosome (chromosome 6). Its chromosomal formula is 2n = 2x = 16 = 2m + 2m + 2m + 2m + 2m+ 2st^{sat} + 2m + 2m.

Chromosomal indices such as arm ratios or the number of

Table 1. Karyotype analyses of *Allium cepa* L. 'Eumjinara' and 'Sinseonhwang'

Chr. No.	Chromosome length (μm)				Karyotype formula[x]	FISH signals[w]			
	Short arm (S)	Long arm (L)	Total length (S + L)	Arm ratio[z]		5S rDNA	45S rDNA	Telomeric tandem repeat	Cot-1 DNA
'Eumjinara'									
1	8.37 ± 0.15[y]	9.52 ± 0.09	17.96 ± 0.07	1.14	2m + 2m + 2m + 2m + 2m + 2st + 2m + 2sm	-	-	+	+
2	6.57 ± 0.20	10.40 ± 0.05	16.96 ± 0.14	1.58		-	-	+	+
3	6.19 ± 0.11	10.21 ± 0.12	16.38 ± 0.22	1.65		-	-	+	+
4	6.11 ± 0.19	8.89 ± 0.03	15.13 ± 0.13	1.48		-	-	+	+
5	6.82 ± 0.33	7.49 ± 0.04	14.49 ± 0.22	1.10		-	-	+	+
6	3.35 ± 0.06	10.69 ± 0.25	14.27 ± 0.03	3.19		-	S	+	+
7	6.46 ± 0.25	6.71 ± 0.06	13.39 ± 0.12	1.04		S	-	+	+
8	4.09 ± 0.34	7.36 ± 0.11	11.41 ± 0.42	1.81		-	S	+	+
Total	47.96	71.27	119.99						
'Sinseonhwang'									
1	7.61 ± 0.23	8.00 ± 0.03	15.61 ± 0.22	1.05	2m + 2m + 2m + 2m + 2m + 2st + 2m + 2m	-	-	+	+
2	6.04 ± 0.20	9.08 ± 0.19	15.23 ± 0.14	1.50		-	-	+	+
3	5.38 ± 0.20	8.76 ± 0.23	14.14 ± 0.28	1.63		-	-	+	+
4	6.64 ± 0.18	7.30 ± 0.25	13.94 ± 0.41	1.10		-	-	+	+
5	5.11 ± 0.13	8.12 ± 0.26	13.22 ± 0.19	1.62		-	-	+	+
6	2.83 ± 0.05	9.3 ± 0.12	12.18 ± 0.15	3.30		-	S	+	+
7	4.90 ± 0.05	6.6 ± 0.20	11.53 ± 0.23	1.36		S	-	+	+
8	3.96 ± 0.14	6.52 ± 0.22	10.68 ± 0.09	1.65		-	S	+	+
Total	42.47	63.76	106.53						

[z]Long arm length/short arm length.
[y]Mean ± standard deviation.
[x]m, metacentric; sm, submetacentric; st, subtelocentric.
[w]S, +, and –indicate the short arm of chromosome, present, and absent signals, respectively.

metacentric and subtelocentric chromosomes of the two studied cultivars were similar with those of the several onion species studied by Mukherjee and Roy (2012), except that a pair of submetacentric chromosomes were found only in 'Eumjinara'.

Chromosomal Localization of rDNAs, Telomeric Tandem Repeats, and Cot-1 DNA

The two cultivars possessed four loci of 5S rDNA on the interstitial regions of the short arms of each chromosome 7 (Figs. 1, 2, and 3). Shibata and Hizume (2002b) have reported that *A. cepa* L. possessed two loci of 5S rDNA (four loci in duplicated chromosomes). This results were also confirmed by Do et al. (2001) and Seo et al. (2007). It could be deduced from the results that the distribution of 5S rDNA loci between the two cultivars did not vary compared with the 45S rDNA. This could be explained by looking at the chromosomal position of 5S rDNA, which is not prone to the reassignment of its loci, since it is interstitially located along the chromosomes that protect it from the dispersion in the genome that is observed in the other rDNA family (Martins and Wasko, 2004).

On the other hand, a minor difference of the 45S rDNA signal distributions between the two onion cultivars were observed. In 'Eumjinara', four loci of 45S rDNA were detected: one on each NOR of chromosome 6 and on the terminal regions of the short arms of chromosome 8 (Fig. 2A). A distinct 45S rDNA locus found on the terminal region of the long arm of chromosome 8 caused 'Sinseonhwang' to have five loci (Fig. 2B). However, Ricroch et al. (1992) reported that *A. cepa* L. possessed 5 loci of 45S rDNA: 3 signals are on the short arms of subtelocentric satellite chromosomes

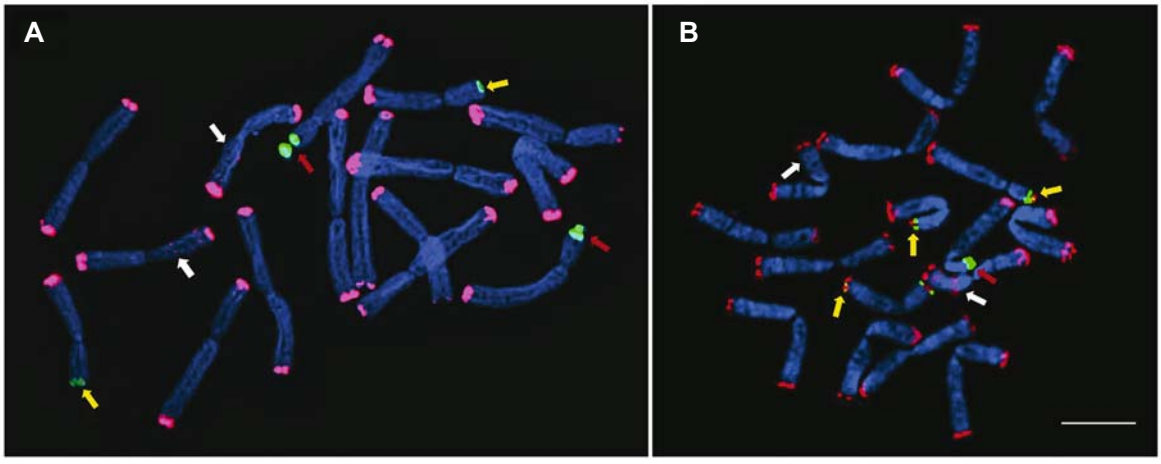

Fig. 1. FISH images of *A. cepa* L. 'Eumjinara' (A) and 'Sinseonhwang' (B) probed with 5S rDNA (red), 45S rDNA (green), and telomeric tandem repeats (red). White arrows indicate weak signals of 5S rDNA. Red arrows point out 45S rDNA. Yellow arrows show co-localization of telomeric tandem repeats and 45S rDNA. Bar = 10 μm.

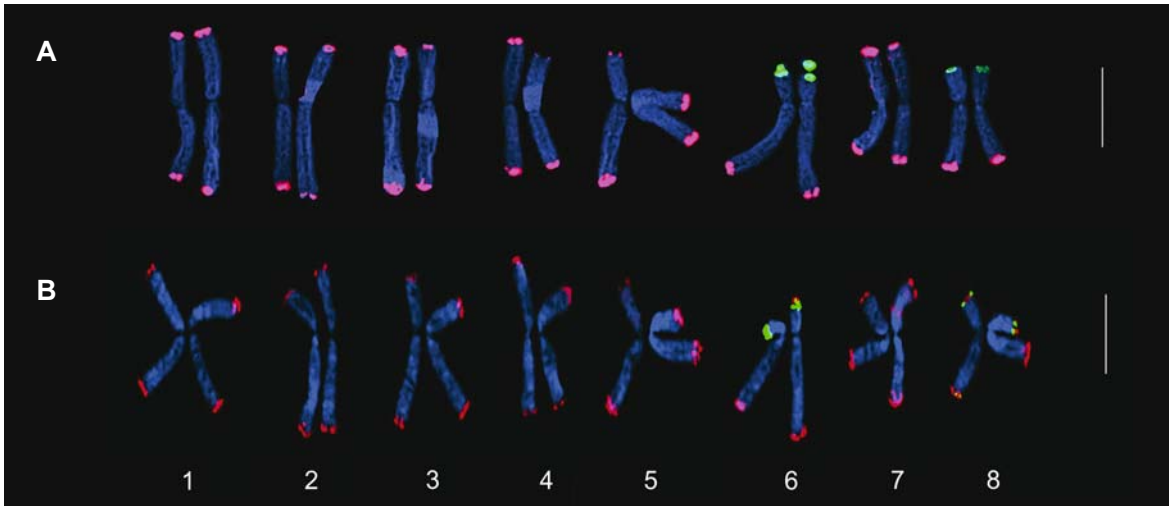

Fig. 2. FISH mapping with three markers of *A. cepa* L. 'Eumjinara' (A) and 'Sinseonhwang' (B). Telomeric tandem repeats and 5S rDNA are shown in red while 45S has green signals. Bars = 10 μm.

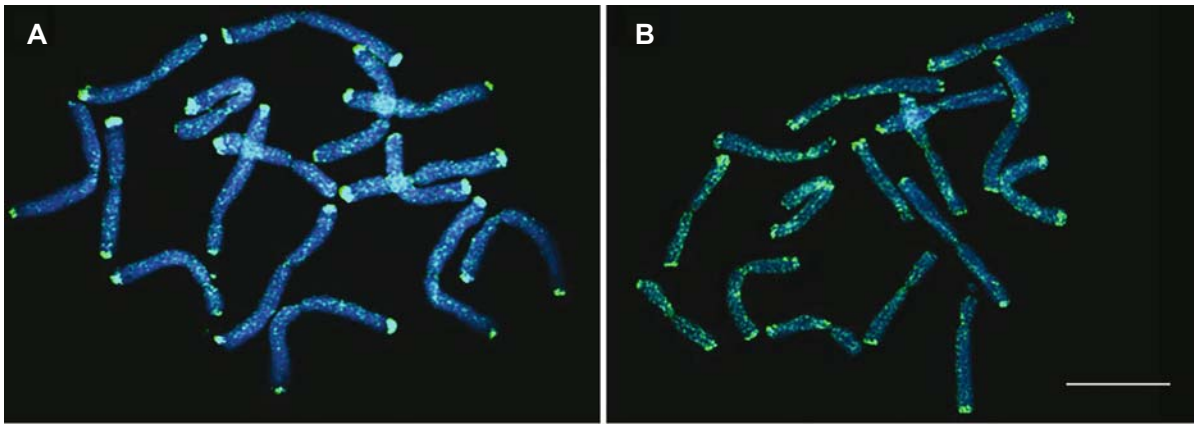

Fig. 3. Localization of Cot-1 DNA (green signals) in *A. cepa* L. 'Eumjinara' (A) and 'Sinseonhwang' (B) chromosomes. Note the intense signal at the telomeric region. Bar = 10 μm.

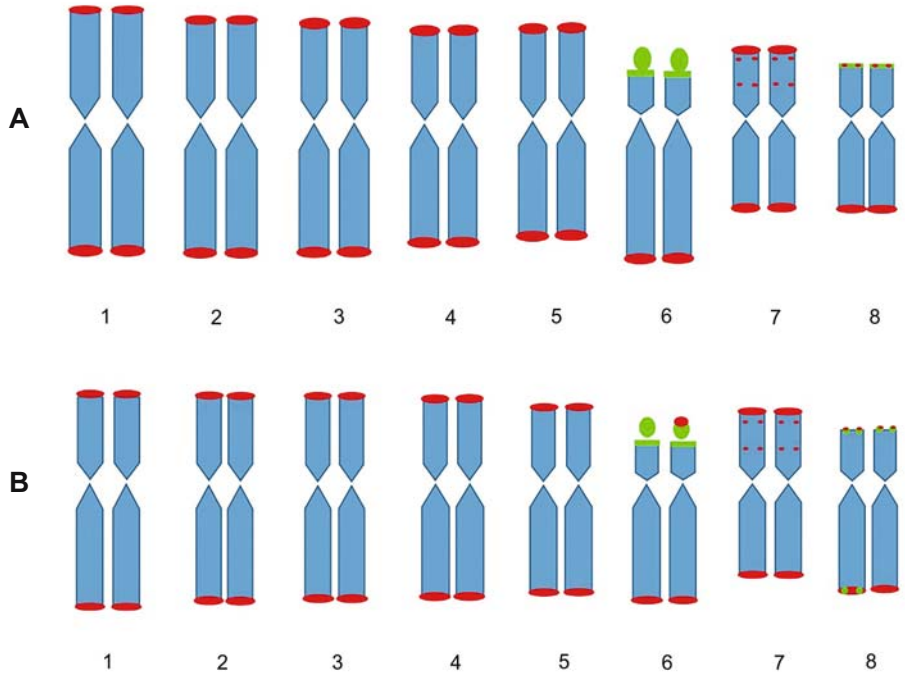

Fig. 4. Dual color FISH idiograms of *A. cepa* L. 'Eumjinara' (A) and 'Sinseonhwang' (B); showing telomeric tandem repeats and 5S rDNA in red signals and 45S rDNA in green signals. The NOR region of one chromosome of pair 6 in 'Sinseonhwang' is localized with 45S rDNA together with a telomeric tandem repeat. This can be observed also in chromosome 8.

and the other 2 are on the short arms of the shortest chromosomes pairs. Nevertheless, Shibata and Hizume (2002a) reported signal distribution of 45S rDNA in *A. cepa* L., which coincide with the results of our study.

The localization of 45S rDNA locus on the distal region of the long arm in one chromosome of the smallest pair in 'Sinseonhwang' would imply that the nucleolus organizing region (NOR) could also reside in that region. Several studies have documented that the 8th pair of chromosome in *A. cepa* L. contained an NOR other than on the satellite chromosomes (Maggini et al., 1978; Ricroch et al., 1992), which could be attributed to the localization of rDNA sequences on the terminal ends of the short and long arms of chromosome 8. Another possible explanation is that this rDNA tandem repeats in the NOR may have undergone a concerted evolution which is driven by extrachromosomal excision-amplification–reintegration mechanisms (i.e., excision and amplification of DNA sequences and integration to a new region of the chromosome) (Do et al., 2001; Ricroch et al., 1992; Stupar et al., 2002). A similar view from Schubert and Wobus (1985) illustrated that the rDNA in *A. cepa* L. has its means of moving from one locus to another region of the chromosomes. Apparently, all 45S rDNA loci were positioned in the telomeric region as shown in this study, suggesting

that repeats from the rRNA gene may have undergone an excision-reintegration process that is mediated by sequence recognition, taking into account its original location and relocation site that resided in the same chromatin domain (i.e., heterochromatin) (Paolozzi et al., 1999). Nevertheless, Paolozzi et al., (1999) elaborated that reintegration occurs without sequence recognition; these findings may not generalize the reintegration mechanisms since they focused on prokaryotic DNA, contrary with this study.

The labeled telomeric tandem repeats hybridized extensively on the terminal regions of the chromosomes, except on the short arms of chromosome 6 in 'Eumjinara' and on one satellite chromosome in 'Sinseonhwang' (Figs. 2 and 4). Do et al. (2001) have also shown a similar pattern of hybridization of telomeric tandem repeat probes with an absence of signals on the short arm of chromosome 6. It is interesting to note the co-localization of signals between telomeric tandem repeats and 45S rDNA probes in both cultivars (Fig. 4). A bi-color chromosome fiber FISH on *Chrysanthemum segetum* L. conducted by Li et al. (2012) shows that telomere sequences are structurally connected or interspersed into the rDNA sequences. Further investigation of a DNA fragment obtained by using a combined primer of telomere and rDNA suggested that telomere sequences have invaded the conserved rDNA sequences (Li et al., 2012). However, Do et al. (2001) have only documented integration of satellite DNA on the rDNA sequences in chromosome 8 of *A. cepa* L., which is also evident from this study. On the other hand, 2D8 tandem repeat-DNA elements in potato, with high homology to rDNA intergenic spacer (IGS) sequences, have cross-hybridized with the NOR, which contains the 45S rDNA unit (Stupar et al., 2002). These rDNA related sequences may have invaded the satellite DNA during evolution (Falquet et al., 1997). Transposition and amplification of DNA repeats could occur in a genome, although the mechanism remains ambiguous (Gernand et al., 2007). The absence of the telomeric sequence, TTTAGGG, has promoted the repetition of satellite and rDNA sequences on the telomere during the evolution of *A. cepa* L. (Do et al., 2001). The use of bi-color FISH with satellite and rDNA sequence probes may reveal signals of satellite or rDNA sequences, since these two repeats may substitute each other in a specific position of the telomere (Do et al., 2001), or they may yield signals simultaneously as observed in this study. It is also evident that the two onion cultivars differ in the distribution of 45S rDNA and telomeric tandem repeats. Putatively, modification of a genetic makeup is steered by the occurrence of nuclear mutation (Scitable by nature education, 2014). Hence, the species differentiation between these cultivars may have resulted in an accumulation of mutations in the satellite or rDNA sequences during the union of two or more genomes.

Cot analysis has been proven to be an efficient way of elucidating the major fraction of most plant nuclear genomes (e.g., repetitive DNA) (Kubis et al., 1998; Peterson at al., 2002). Repetitive elements, (e.g., satellite sequences, transposable elements) are congregated in the heterochromatic region, particularly at the telomere and centromere (Shiv et al., 2007). In *A. cepa* L., Shibata and Hizume (2002a) reported a chromosomal hybridization of Cot-1 DNA with more prominent probe signals at the terminal ends of the chromosomes. Similarly, the results of this study show intense telomeric signals and weak dispersed signals along the chromosome (Fig. 3). The concentration of signals at the telomere clearly shows the heterochromatic regions, which comprise a large amount of repetitive DNAs (tandem repeats), while the dispersed signals on the chromosomes show transposable elements, such as long terminal repeat retrotransposons, the major components of *A. cepa* L. genomes (Barnes et al., 1985; Vitte et al., 2013).

Wei et al. (2007), who reported Cot-1 DNA localization in *Brassica oleracea* L. on its pericentromeric region, have insisted that Cot-1 DNA contains rDNA. This could be possible in *A. cepa* L. since these results show weak signals in the NOR (Fig. 3). Cot-1 DNA constituted various classes of repetitive DNA together with some repeats that are normally found in the NOR. A thorough investigation of these dispersed repetitive DNAs, together with rDNA and telomeric tandem repeat markers would be a valuable means of understanding the genome complexity of *A. cepa* L. and provide adequate information for the future breeding program and whole genome sequencing.

Acknowledgement: This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project title: National Agricultural Genome Program, Project No. PJ010448)" Rural Development Administration, Republic of Korea.

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