Korean J. Plant Res. 30(6):654-661(2017)
https://doi.org/10.7732/kjpr.2017.30.6.654

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

Cytogenetic Mapping of *Carthamus tinctorius* L. with Tandemly Repeated DNA Sequences by Fluorescence *in situ* Hybridization

Franklin Hinosa Mancia[1†], Yoon Ha Ju[2†], Ki-Byung Lim[3], Jung Sun Kim[4], Sang Yong Nam[1] and Yoon-Jung Hwang[1,2]*

[1]Department of Environmental Horticulture, Sahmyook University, Seoul 01795, Korea
[2]Department of Convergence Science, Sahmyook University, Seoul 01795, Korea
[3]Department of Horticultural Science, Kyungpook National University, Daegu 41566, Korea
[4]National Institute of Agricultural Science, Rural Development Administration, Jeonju 54874, Korea

Abstract - Dual-color fluorescence in situ hybridization karyotype analysis was created using repetitive sequences including two types of rDNA repeats (45S and 5S rDNAs) and *Arabidopsis*-type telomere sequence repeats. The somatic metaphase cells of *Carthamus tinctorius* were observed as diploids (2n=2x=24). A symmetrical or slightly asymmetrical karyotype with seven pairs of metacentric and five pairs of submetacentric chromosomes was observed. The lengths of the somatic metaphase chromosomes ranged from 4.18 to 6.53 ㎛, with a total length of 60.71 ㎛. One locus of 45S rDNA was located on the pericentromeric regions of three pairs of chromosomes and the other pair was situated on the terminal regions of the short arms of a single pair of chromosomes. One locus of 5S rDNA was detected on the interstitial regions of the short arms of two pairs of chromosomes. *Arabidopsis*-type telomeric repeats were detected on the terminal regions of all pairs of chromosomes. Co-localization of loci between telomeric repeats and 45S rDNA was observed in a single pair of chromosomes. The results provide additional information for the existing physical mapping project of *C. tinctorius* and will also serve as a benchmark to a more intricate cytogenetic investigation of *C. tinctorius* and its related species.

Key words - FISH, Karyotype, rDNAs, Telomeric repeats

Introduction

Safflower is a thistle-like annual plant belonging to the family Asteraceae. It grows a height of 30-150 cm and displays yellow, orange or red capitula. It is distributed in the Middle East through Iran, Pakistan, and India (Dajue and Mundel, 1996; Sheidai *et al*., 2009). The pappus-less achenes of the plant yields a high-quality edible oil rich in unsaturated fatty acids for cooking and preparation of margarine and salad oil (Ambreen *et al*., 2015; Dajue and Mundel, 1996). The diploid plant with a basic chromosome number of x=12 has an estimated DNA content of 2.4 GB (Ambreen *et al*., 2015; Garnatje *et al*., 2006). Polyploidy is also observed in the genus *Carthamus*, but the accuracy of the number of species within the genus is still debatable, with only a few studies having been done (Chapman and Burke, 2007; Sheidai *et al*., 2009). The relationship between *C. tinctorius* and its wild relatives concerning their genomic constitution is poorly understood (Kumar, 1991).

The scarcity of information regarding crop management and product development or genetic potential of safflower has hindered research efforts and approaches for the conservation and utilization of its genetic resources in the future breeding programs (Dajue and Mundel, 1996; Kumar, 1991; Singh and Nimbkar, 2007). Recent research has been conducted with the goal of crop improvement through concerted molecular breeding programs, which could improve yield and increase resistance to several diseases (Ambreen *et al*., 2015; Varshney *et al*., 2007). Ambreen *et al*. (2015) used next-generation sequencing in generating a set of molecular markers applicable for diversity analysis, synteny studies, construction of linkage maps and marker-assisted selection. Development of trait-associated DNA markers is fundamental in implementing molecular breeding in crops

*Corresponding author. E-mail : hyj@syu.ac.kr
Tel. +82-2-3399-1718

(Collard *et al*., 2005).

The genetically mapped markers are not directly related to chromosome structure unless cytogenetic mapping is carried out using fluorescence in situ hybridization (FISH) (Wang *et al*., 2006). Mapping the DNA of interest directly to the chromosomes provides important biological information with respect to genome organization and function, as it correlates genetic distance with cytological structures, which in turn become an invaluable complement to physical sequence data (Harper and Cande, 2000; Wang *et al*., 2006). Hence, FISH is an indispensable molecular technique in integrating genetic and cytological maps (Harper and Cande, 2000). However, the success of FISH relies on the quality of the chromosome slides (Kirov *et al*., 2014).

Repetitive sequence motifs occur as tandem repeats, which are used as molecular markers for chromosome identification and to reveal evolutionary changes (Kubis *et al*., 1997). The highly conserved 45S and 5S ribosomal genes are utilized to depict the rates of speciation by examining the changes of its chromosomal distribution, as what observed in Triticeae (Heslop-Harrison, 2000). Frequently, telomeres and 45S ribosomal genes are closely positioned on the eukaryotic chromosomes. Their dynamic nature is essential to genomic stability and integrity (Dvorackova *et al*., 2015).

In this paper, we analyze the karyotype of *C. tinctorius* and the distribution of tandemly repeated DNA sequences on its somatic chromosomes using FISH.

Materials & Methods

Plant materials and chromosome preparation

Safflower (*Carthamus tinctorius* L.) seeds were obtained from the Korea Rural Development Administration (RDA). The seeds were germinated in a petri dish containing wet filter paper. After a few days, the roots were collected and treated with 2 mM 8-hydroxyquinoline for 5 h in 20℃ water bath. Aceto-ethanol (3:1) was used to fix the roots overnight, and 70% ethanol was used to preserve the roots at 4℃. Two percent Cellulase R-10 (Sigma, USA), cytohelicase (Sigma, USA), and pectolyase γ-23 (Duchefa, Germany) enzymes were used to digest the cellular components for 90 min in a 37℃ chamber, excepting metaphase chromosomes.

Vortex method was employed for the preparation of chromosome slides. The freshly enzyme-treated roots were carefully transferred to a clean petri dish that contained distilled water to remove the unnecessary cellular debris. The roots were transferred to a new microtube and aceto-ethanol was added into it. The roots were homogenized by whirling the tube using the vortex mixer. The tube was placed on ice for 5 min and then was centrifuged for 13,000 x g. The supernatant was discarded and the pellet was immediately reconstituted with 60% acetic acid.

The steam drop method (Kirov *et al*., 2014) was used to spread the chromosomes on a slide thoroughly. The dried slides were mapped for the presence of chromosomes by staining them with the mixture of 4',6-diamidino-2-phenylindole (DAPI) and 2X SCC and detecting them using a fluorescence microscope. The mapped slides were placed in a coplin jar with 2X SSC for 3 min and in a coplin jars with a series ethyl alcohol for dehydration. A moist-free container was used to store the slides at room temperature.

DNA isolation

C. tinctorius seeds were germinated and grown until young plants had developed. The young and fresh leaves were collected and used for genomic DNA extraction (gDNA). A modified method based on cetyltrimethylammonium bromide (CTAB) was adopted from Porebski *et al*. (1997).

Probe preparation

Safflower genomic DNA (gDNA) was used as a template for the amplification of 5S rDNA. The reaction mixture consisted of 10X primer, 10X *exTaq* Buffer, dNTP mix, H_2O, and *exTaq*. The sequences of the primers were 5'-TCC TGG GAA GTC CTC GTG TTG CAT-3' and 5'-GGT CAC CCA TCC TAG TAC TAC TCT-3'. PCR conditions were 3 min of initial denaturation at 95℃ followed by 35 cycles of 1 min denaturation at 94℃, 45 sec of annealing at 66℃, and 1 min of extension for 1 min at 72℃. There was a final 10 min extension at 72℃. The 5S rDNA was labeled with biotin 16-dUTP.

18S rDNA was amplified by using the gDNA of *Brassica rapa* L. PCR was carried out using the same composition of the mixture mentioned above, except for the primers used.

The primer set 5'-TGA TCC TTC TGC AGG TTC ACC TAC-3' and 5'-AAC CTG GTT GAT CCT GCC AGT-3' was used to amplify the ribosomal gene. PCR conditions were 5 min of initial denaturation at 95℃, followed by 35 cycles of 30 sec denaturation at 94℃, 30 sec of annealing at 60℃, and 1 min of extension at 72℃, with a 10-min final extension at 72℃. The 18S rDNA was labeled with Coumarin 5-dUTP (Perkin Elmer, USA) and dig 11-dUTP (Roche, Germany).

The arabidopsis-type telomeric repeat was amplified without gDNA (Ijdo *et al*., 1991) using the primer set $(TTTAGGG)_n$ and $(CCCTAAA)_n$. PCR conditions were 5 min of initial denaturation, followed by 34 cycles of 5 min denaturation at 95℃, 30 sec of annealing at 55℃, 1 min of extension at 72℃, with a final 10-min extension at 72℃. The telomeric DNA was labeled with dig 11-dUTP (Roche, Germany).

FISH

The method used by Lim *et al*. (2001) was followed with some modification. The hybridization mixture contained formamide, 10% sodium dodecyl sulfate, 50% dextran sulfate, 20X SSC, salmon sperm, and 50-100 ng of DNA probe (hapten and fluorophore-labeled). The mixture was placed in a water bath for DNA denaturation at 90℃ for 10 min and then on ice for 5 min. Each slide received 40 μl of the mixture and hybridization was done using the StatSpin ThermoBrite System (Leica, Biosystem) at 80℃ for 5 min. A container with wetted tissue paper was used to keep the hybridized slide at 37℃ for 16-18 h.

Stringent washing was performed to both slides hybridized with the hapten and fluorophore-labeled probes. The washing conditions for the fluorophore-labeled probe were 2X SCC for 10 min at room temperature (RT), 0.1X SCC for 25 min at 42℃, and 2X SSC for 10 min at RT. This was followed by incubation in an ethanol series. For the hapten-labeled probe, washing conditions were 2X SSC for 15 min at RT, 0.1X SSC for 40 min at 42℃, and 2X SSC for 15 min. The slides were quickly submerged in 1X detection buffer for 5 min at RT. Anti-dig fluorescein isothiocyanate (FITC; Roche, USA) or Streptavidin CY3 (Roche, USA) was used to detect the labeled DNA. Incubation took place in a dark and moistened container for 1 h at 37℃. Three series of 1X detection buffers were carried out for 5 min in a dark chamber at 37℃. This was followed by an ethanol series. The dried slides were counterstained with DAPI/Vectashield (1:100).

The samples were examined by fluorescence microscope using a model BX63 microscope (Olympus, Japan) equipped with a CoolSNAP *cf* camera. Signals were analyzed using ultraviolet excitation filters. Cytovision (Applied Imaging Corp, USA) imaging software was used to measure the chromosomes. The images were edited using Photoshop CS6 (Adobe Systems, USA).

Results & Discussion

Chromosome variants of *C. tinctorious* were observed (Fig. 1). Secondary constrictions were distinct and were only observed in karyotype 1 (Fig. 1A). On the other hand, karyotype 2 (Fig. 2B) that was also observed have coincided with the findings of Anjali and Srivastava (2012), who investigated 12 accessions of *C. tintocrious* that possessed symmetrical or slightly asymmetrical karyotype without secondary constrictions. There are more metacentric chromosomes observed in this study (Table 1).

Intraspecific variation in chromosome morphology has been observed in several species including *Crinum latifolium* L., *Lilium lancofolium* Thunb., and *Rutidosis leptorrhynchoides* F. Muell. (Akhter *et al*., 1992; Murray and Young, 2001; Noda, 1978). Uozu *et al*. (1997) asserted that the amplification of repetitive DNA sequences causes the variation in the chromosome morphology among species in

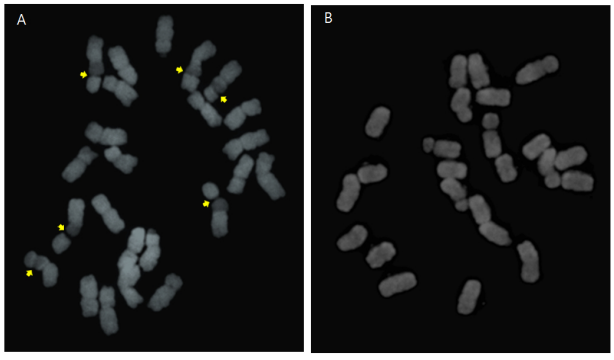

Fig. 1. Somatic chromosomes of *Carthamus tinctorius* L., 2n=24. A: Karyotype 1, B: Karyotype 2. Arrows indicate secondary constrictions of the chromosomes.

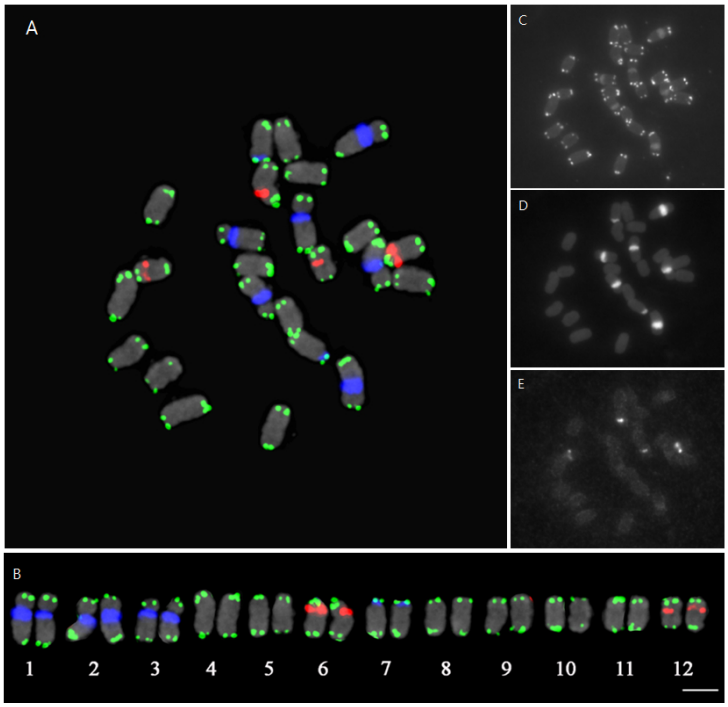

Fig. 2. Tri-color FISH of *Arabidopsis*-type telomeric repeat, 45S rDNA, and 5S rDNA on the metaphase chromosomes of *Carthamus tinctorius* L. (A), Karyotype of *C. tinctorius* (B), Raw images of telomeric (C), 45S rDNA (D), and 5S rDNA signals (E). Dig- labeled *Arabidopsis*-type telomeric repeat was detected by Anti-dig FITC (green), Coumarin 5-dUTP revealed as blue, and Biotin-labeled 55 rDNA was detected by Streptavidin CY3 (red). Bar= 10 ㎛.

Table 1. Analyses of somatic metaphase chromosomes of *Carthamus tinctorious* L.

Chr. no.	Chromosome length (㎛)			Chromosome Type	Arm ratio[w]	Centromere index[v]	FISH results	
	Short arm (S)	Long arm (L)	Total (S+L)				45S	5S
1	2.38±0.31[z]	4.19±0.46	6.53±0.43	sm[y]	1.76	36.17	+[u]	-[t]
2	2.17±0.38	4.16±0.82	6.20±0.77	sm[x]	1.91	34.32	+	-
3	1.79±0.40	3.81±0.71	5.47±0.59	sm	2.13	31.96	+	-
4	2.27±0.76	3.15±0.71	5.44±0.87	m	1.39	41.91	-	-
5	1.91±0.85	3.21±0.79	5.02±0.72	m	1.68	37.34	-	-
6	1.77±0.71	3.32±0.72	4.89±0.81	sm	1.88	34.77	-	+
7	1.79±0.39	3.13±0.68	4.89±0.10	sm	1.75	36.39	+	-
8	2.06±0.85	2.83±0.72	4.74±0.54	m	1.37	42.15	-	-
9	1.70±0.87	2.98±0.76	4.59±0.53	m	1.76	36.28	-	-
10	2.10±0.72	2.34±0.66	4.41±0.75	m	1.11	47.31	-	-
11	1.95±0.92	2.43±0.43	4.35±0.52	m	1.25	44.54	-	-
12	1.58±0.61	2.46±0.95	4.18±0.63	m	1.56	39.10	-	+

[z]mean±SD.
[y]submetacentric.
[x]metacentric.
[w]short arm length: long arm length.
[v]short arm length/(short arm length + long arm length) × 100.
[u]present.
[t]absent.

the genus *Oryza*.

The probe constructed of *Arabidopsis*-type telomeric repeats hybridized mostly on the subtelomeric regions of all chromosomes (Fig. 2 and 3). Interestingly, this pattern of hybridization was also observed by Raina *et al*. (2005) when *pCtKpnI-1*, a novel repetitive DNA isolated from *C. tintocrious* gDNA, was mapped on the metaphase chromosomes of that species. The present and previous observations suggest the presence of conserved motifs, such as dinucleotide, pentanucleotide, between the two families of tandem repeats in the safflower and *Arabidopsis thaliana* genomes. Low throughput genome sequencing of safflower revealed a high amount of dinucleotide repeats followed by tri-, tetra-, penta- and hexanucleotides (Ambreen *et al*., 2015).

These motifs could be important in the amplification and maintenance of tandem repeats in a genome and in the determination of specific chromatin properties of loci containing the repetitive DNA (Mehrotra and Goyal, 2014; Meszaros and Nouzova, 2002). One locus of 45S rDNA co-localized with telomeric repeats in chromosome 7 (Fig. 2B-7). This phenomenon was also previously observed in several crops, including *Allium cepa* L., *Chrysanthemum boreale* L., *Brassica rapa* L., *Raphanus sativus* L. and *Chrysanthemum segetum* L. (Belandres *et al*., 2015; Cuyacot *et al*., 2016; Li *et al*., 2012; Mancia *et al*., 2015).

Telomeric repeats are vital components in the genome of all plants and animals because they stabilize the chromosome ends and enable replication through the enzymatic activity of telomerase (Zakian, 1995). However, repetitive sequences of 45S rDNA can also protect the telomeres by substituting the telomeric DNA at some chromosomal termini. This has been observed in *Allium cepa*, where 45S rDNA locus was detected on the chromosomal termini instead of the telomeric repeats (Mancia *et al*., 2015; Pich *et al*., 1996).

The localization of 45S RNA genes on the metaphase chromosomes of *C. tinctorious* revealed their centromeric positions in chromosomes 1, 2, and 3 (Figs. 2B-1, 2, 3). In this study, the number of loci detected is similar with the findings of Agrawal *et al*. (2013). The previous study reported six 45S rDNA loci located in the interstitial regions of chromosomes 1, 2, and 4, while another pair of loci was located in the subterminal regions of chromosome 3. Presently, we observed six sites of paracentromeric 45S rDNA loci in chromosomes 1, 2, and 3 (Figs. 2B-1, 2, 3). The other two sites are positioned in the terminal regions of chromosome 7 (Fig. 2B-7). The differences in observation between the previous and present studies may have resulted from the ambiguity of the chromosomes morphology.

Although the sites are considered “centromeric”, they are not really located in the functional regions of the centromeres (Roa and Guerra, 2012). Nevertheless, 45S rDNA sites are preferentially located in the short arms and in the terminal regions of chromosomes in flowering plants. Roa and Guerra (2012) described that out of 729 karyotypes of angiosperms, 69.9% possessed sites located in the short arms while 3.5% and 26.6% were located in the centromeres and the long arms, respectively.

Sousa *et al*. (2011), asserted that the predominant terminal position of the 45S rDNA sites does not appear to be influenced by the centromere-telomere polarization as suggested by the “chromosome field” hypothesis. The localization and distribution of rDNA sites in the different regions of the chromosomes may have resulted from homologous recombination and complex chromosome rearrangements (Wendel *et al*., 1995; Zhang *et al*., 2016). The instability of the genomic region harboring rDNA has been noted. Nevertheless, its molecular mechanism is still not well understood (Kobayashi, 2008). Kobayashi and Ganley (2005) asserted that rDNA loci are predominant sites for repeated recombination, which could be the reason for this instability.

Four loci of 5S rDNA were detected in the paracentromeric regions of the short arms of chromosomes 6 and 12 (Fig. 2B-6 and 12). On the contrary, Agrawal *et al*., (2013) showed that chromosomes 8 and 11 harbor the 5S rDNA genes in *C. tinctorius* var. *tinctorius*. A survey of the distribution of 5S RNA gene in 784 plant species revealed its preferential position in the proximal region of the short arm of the chromosome (Roa and Guerra, 2015). It was also shown that the number of loci of 5S rDNA is conserved among 10 species of *Carthamus* compared to 45S rDNA (Agrawal *et al*., 2013).

The site of 5S rDNA is considered to be stable, unlike 45S rDNA that corresponds to a region of chromosome fragility

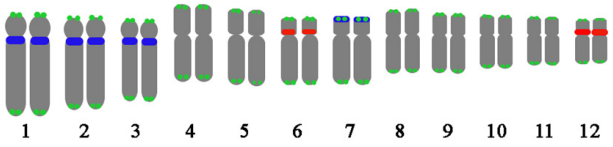

Fig. 3. Idiogram of karyotype showing the physical location of *Arabidopsis*-type telomeric repeats (green), 45S rDNA (blue), and 5S rDNA (red) on the somatic metaphase chromosomes of *Carthamus tinctorius* L.

due to its failure of condensation, which in turn could lead into a wider range of loci number variation in seed plants (Bustamante *et al*., 2014; Galián *et al*., 2014; Huang *et al*., 2012; Vaio *et al*., 2005). The parental 5S rDNA units show no or little change in allopolyploid tobacco, which was interpreted as evidence that gene conversion did not take place in the former (Fulneček *et al*., 2002). Some of the hypotheses that could explain why this rDNA site seems less vulnerable to this type of genetic modification promulgated that the sub-repeats of 5S non-coding region which facilitates interlocus genetic recombination at meiosis or interphase are less abundant, and that the location of 5S RNA genes in the interstitial and centromeric regions saves them from genetic recombination (Cronn *et al*., 1996; Fulneček *et al*., 2002). Not all blocks of tandemly repetitive sequences, such as in 5S RNA gene can be observed as heterochromatin blocks (Cabral *et al*., 2006), suggesting its exclusion from DNA methylation, which in turn could influence its stability in the genome.

The physical mapping of *Arabidopsis*-type telomeric repeat, 45S rDNA, and 5S rDNA markers has contributed to the identification of homologous chromosomes and has provided insights about the recent molecular activities in the genome of safflower. The present data will be utilized as a benchmark for future investigations of *C. tinctorius* and its relative species

Acknowledgement

This work was carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01095307)" Rural Development Administration, Republic of Korea.

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(Received 2 August 2017 ; Revised 21 November 2017 ; Accepted 30 November 2017)