Korean J Biol Sci 3: 69-72, 1999

Identification of a Tandemly Repeated DNA Sequence Using Combined RAPD and FISH in Welsh Onion (*Allium fistulosum*)

Bong Bo Seo*, Geum Sook Do, and Seon Hee Lee

Department of Biology, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea

Key Words:
Tandemly repeated DNA sequence
RAPD
FISH
Nucleotide sequence

A tandemly repeated DNA sequence was identified and characterized by the combined RAPD and FISH data from a total genomic DNA of Welsh onion (*Allium fistulosum*). A clone containing this repeating sequence was selected and sequenced. This repeating unit of 314 bp inserted into p*Af* 072 contained 54.1% adenine and thymine residues, and showed the primer sequence used, 5'-GAAACGGGTG-3', in both terminals of the sequence. Fluorescence *in situ* hybridization using this tandemly repeated sequence as a probe indicated that the detected sites were coincident with the major C-banded constitutive heterochromatin in the terminal regions of both arms of all 16 chromosomes.

Random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams et al., 1990) is used to amplify random sequences from a complex genome DNA. Plant molecular biologists have widely applied this technique to design the genetic markers of variation at the interspecific or intraspecific level in plants (Chalmers et al., 1992; Wilkie et al., 1993). This technique can provide genetic markers that are useful in the molecular analysis of the genome (Rafalski and Tigny, 1993; Jiang et al., 1997; Nagaoka and Ogihara, 1997). RAPD markers in plants are usually able to compensate for the G-banding patterns that have been used as chromosome markers for the cytogenetic study in the animal genome (Lee and Seo, 1995).

The fluorescence *in situ* hybridization (FISH) technique using fluochrome allows the visualization of multigene families, such as the 5S and 18S-5.8S-26S ribosomal RNA genes (Mukai et al., 1990, 1991; Castilho and Heslop-Harrison, 1995; Badaeva et al., 1996b) or the repeated DNA sequences (Cabrera et al., 1995; Irifune et al., 1995; Badaeva et al., 1996a; Cuadrado and Jouve, 1997) for their locations on the chromosomes. FISH-detectable occurrences are based on the size of hybridizing-recipient DNA sequences for their complementary probes; the size of probe is not important for signal detection.

Eukaryotic genomes, including those of higher organisms, generally contain many tandemly repeated DNA sequences. The study of these repeated DNA sequences is important in relation to chromosome organization, as these sequences are closely associated with the constitutive heterochromatin (Appels et al., 1978; Bedbrook et al., 1980; Deumling, 1981; Yakura et al., 1987; Iwabuchi et al., 1991; Wu et al., 1991). The constitutive heterochromatin in *Allium fistulosum* is mostly located in the terminal regions of both arms of all eight homologous chromosome pairs (Seo and Kim, 1975; Vosa, 1976). To date, the identification or acquisition of highly repeated DNA sequences, which are considered as components of the constitutive heterochromatin, is generally obtained by digestion with the combinations of several restriction enzymes (Barnes et al., 1985; Irifune et al., 1995).

In this paper, we report the nucleotide sequence of a highly repeated DNA sequence, which is obtained as the constant RAPD using the OPA kit 07 primer. FISH patterns were also detected within the constitutive heterochromatin regions of all 16 chromosomes in *A. fistulosum.*

Materials and Methods

Plant material and chromosome preparation

Allium fistulosum cv. Keumjang single stem was soaked in water, then placed on a moist filter paper in petri dishes at room temperature. After 3-4 d, the root tips were excised and used for chromosome preparation of FISH according to Mukai et al. (1990).

DNA extraction and RAPD analysis

Genomic DNA was extracted from young leaves by the cetyltrimethylammonium bromide (CTAB) extraction buffer (Rogers and Bendich, 1988). Twenty kinds of 10-mer RAPD primers (A01-A20) purchased from Operon Technologies were used for PCR amplification

* To whom correspondence should be addressed.
Tel: 82-53-950-5350, Fax: 82-53-953-3066
E-mail: bbseo@kyungpook.ac.kr

following Williams et al. (1990). Amplification was performed in a 25 µl reaction mixture that contained 1X Ex *Taq* buffer, 200 µM each of dNTP (dATP, dGTP, dCTP, and dTTP), 200 µM of each primer, 0.1 unit of Ex *Taq.* polymerase (Takara) and 10 ng of genomic DNA in sterile distilled water. Initial denaturation was for 5 min at 94℃, followed by 45 cycles of 1 min at 94℃, 1 min at 38℃, 2 min at 72℃, and a final 5 min extension at 72℃. The PCR reaction was run in an automated thermal cycler (Perkin-Elmer Cetus System 9600). Amplification products were analyzed by electrophoresis in a 1.2% agarose gel in 1X TAE buffer and detected by ethidium bromide staining.

Cloning and sequencing of RAPD products

Each RAPD band was isolated from an agarose gel using a Geneclean II kit (Bio 101), and was used to determine whether signal labeled with each isolated DNA was detected or not on chromosomes or nuclei by FISH analysis. The FISH-detectable products were then ligated into the pT7Blue(R) vector using T4 ligase, and introduced into *E. coli* DH5α by transformation (Sambrook et al., 1989). Nucleotide sequencing was done on a PAGE gel with an ABI Prism 377 sequencer (Perkin-Elmer) as an automated fluorescent sequencing system. DNA sequence data were analyzed using the BLAST network service at the National Center for Biotechnology Information (NCBI) (Altschul et al., 1990).

Probe labeling and FISH

The purified RAPD product was labeled with biotin-16-dUTP (Boehringer Mannheim) by the PCR method. Chromosomal DNA on the slides was denatured in 70% formamide at 67℃ for 2 min and dehydrated in 70%, 95%, and 100% ethanol series at -20℃ for 5 min each. The hybridization mixture containing 50% formamide (w/v), 10% dextran sulphate (w/v), 5 ng/µl of autoclaved salmon sperm DNA and probe DNA labeled with biotin-16-dUTP in 2X SSC, was heated at 100℃ for 10 min and then kept on ice for 5 min. Ten µl of the hybridization mixture was applied to each slide preparation denatured in 70% formamide at 67℃ for 2 min, and the slides were covered with a coverslip. Slides were then placed in a humid chamber at 37℃ for 6 h or longer. After hybridization, coverslips were removed in 2X SSC and the slides were washed in 2X SSC for 5 min, 50% formamide at 37℃ for 15 min, 2X SSC for 15 min, 1X SSC for 15 min, and 4X SSC for 15 min, which allowed binding of the probe with minimal homology. The slides were covered with 50 µl of a avidin-FITC conjugate for a biotin-labeled RAPD product dissolved in 1% BSA/4X SSC as a detection buffer and incubated for 1 h at 37 ℃ without coverslips. After that, the slides were washed in 4X SSC for 10 min, 4X SSC/0.1% Triton X-100 for 10 min, 4X SSC for 10 min, and 2X SSC for 5 min. The final concentration (1 µg/µl) of 4, 6-diamidino-2-phenylindole (DAPI) solution containing Vectashield mounting medium (Vector) as a counterstain, was added to an amount of 15 µl on each slide and overlaid with a coverslip. The slides were examined with a Zeiss Axiophot epifluorescence microscope with Zeiss filter sets: 01 for DAPI, and 09 for FITC. Photographs were taken using Kodak G400 color print film.

Table 1. RAPD products amplified from genomic DNA in *Allium fistulosum*

Lane No.	Primer	Nucleotide sequence (5' to 3')	Products (ca. bp)
1	OPA01	CAGGCCCTTC	800, 600, 400
2	OPA04	AATCGGGCTG	900, 500, 350
3	OPA05	AGGGGTCTTG	1000, 800, 500, 400
4	OPA07	GAAACGGGTG	300[a]
5	OPA08	GTGACGTAGG	700, 300
6	OPA09	GGGTAACGCC	1300, 400, 300
7	OPA10	GTGATCGCAG	800
8	OPA11	CAATCGCCGT	1300, 800
9	OPA12	TCGGCGATAG	400, 300
10	OPA13	CAGCACCCAC	1200, 850, 700, 400
11	OPA14	TCTGTGCTGG	300
12	OPA15	TTCCGAACCC	1000, 800, 600
13	OPA16	AGCCAGCGAA	1500, 900
14	OPA18	AGGTGACCGT	700

[a] underlined DNA fragments are FISH-detectable products.

Results

To obtain the RAPDs that can be detected with FISH technique, all 20 kinds of 10-mer primers (OPA kit) were applied to the total genomic DNA in *A. fistulosum.* Thirty-two kinds of different reproducible DNA bands were obtained from 14 kinds of primers (Fig. 1 and Table 1). The number of bands in the profile varied depending on the primer sequences. To determine which of these RAPD products should be used as a potential genetic marker in the FISH, all RAPD bands showing reproducibility were isolated from the agarose gel, labeled with biotin-16-dUTP by PCR amplification, and detected by FISH, respectively. As a result, four kinds of RAPD products from three primers (A07, A11, and A14) were detected on metaphase chromosomes. Among these FISH-detectable products, the RAPD product amplified by primer A07 was cloned and examined again by FISH. As a result of FISH procedures, the cloned sequence was detected in the regions of constitutive heterochromatin of all chromosomes. We designated this recombinant plasmid as p*Af*072.

The 314-bp RAPD product inserted into p*Af*072 is practically hybridized to the terminal regions in both arms of all 16 metaphase chromosomes and interphase nuclei in *A. fistulosum* (Fig. 2). Fig. 3 shows the nucleotide sequence of the 314-bp fragment inserted into the p*Af*072 plasmid. This repeating unit contains 54.1% adenine and thymine residues, and shows the primer sequence used, 5'-GAAACGGGTG-3', in both terminals of the sequence. This sequence

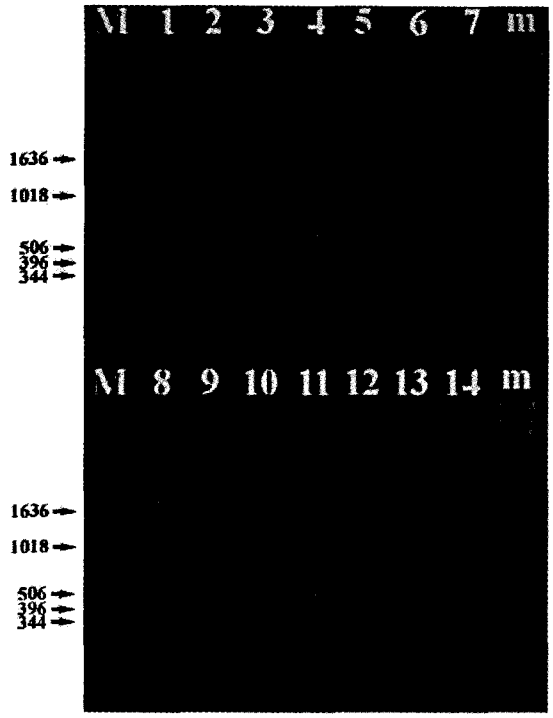

Fig. 1. RAPD fragment amplified by the OPA kit in *Allium fistulosum*. M; molecular weight marker (1-kb ladder), 1-14; electrophoretic pattern of each RAPD product, m; molecular weight marker (λ DNA/*Hin*dIII). Sizes of bands are indicated in base pairs.

shows about 95% homology in only the overlapping region with those of the highly repeated DNA sequences obtained from *A. fistulosum*, which was registered in the EMBL data bank.

```
GAAACGGGTG CAGGGATTTG AAATGGACCA GTACCATGGT GCACCACTCT   50
CCAGGGATGG TAAATCGTAG ACCAGCCACG GTGTTCCCTT TTTAACGTAA  100
AAATTCACGT GATGGAAAAA CGAAGGCCAA AAAACTTCAG AGACCCGATT  150
GAGGTTCGGA TAGGCTTTTT TGGGTATGAA ACTAACCGTT GACGGCTTAC  200
AGCGGTCAAA GCCGTAGAAC TCACTCAGAC CTTCGTTTTG ACTGGTTATG  250
GCTCCCGTAA CTCTAAACGA GGCAACAGTT ATGGCCGTCC GAATAATCGA  300
TGTCCACCCG TTTC 314
```

Fig. 3. Nucleotide sequence of tandemly repeated DNA inserted into p*Af*072. Primer sequence used, 5'-GAAACGGGTG-3', are underlined in both terminals. The nucleotide sequence is registered in the EMBL-GenBank-DDBJ Nucleotide Sequence Data Base under accession No. AF074873.

This FISH-detectable RAPD product existing in both terminal regions of all chromosomes in this study was characterized by a highly repeated DNA sequence for the following reasons. First, the probe of this product was detected in large amounts of chromosomal regions, and second, the detected regions of this probe were located in the terminal regions, constitutive heterochromatin, which is darkly stained by the C-banding technique in *A. fistulosum*.

Discussion

The present study of the combined RAPD and FISH data shows that signal regions detected by FISH using a tandemly repeated DNA sequence as a probe are clustered in the terminal regions of all 16 chromosomes, and are coincident with the regions of constitutive heterochromatin observed by the C-banding technique. Similar locations of highly repeated DNA sequences in terminal regions of chromosomes have been reported, including *Allium fistulosum* (Irifune et al., 1995), *Scilla sibirica* (Deumling, 1981) and

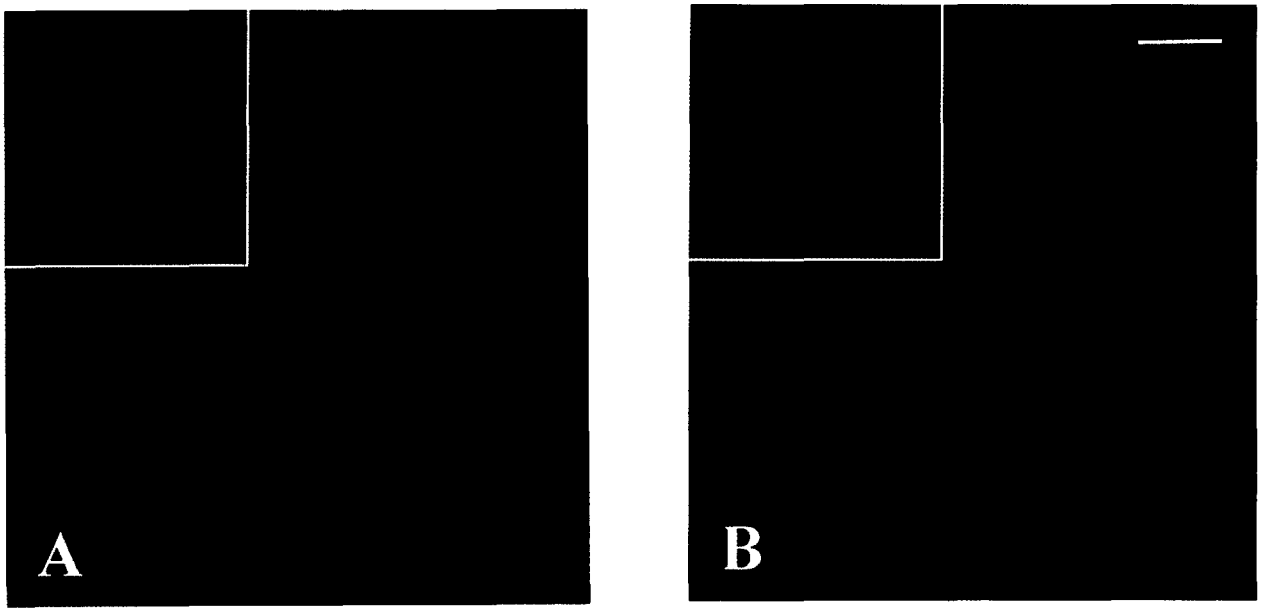

Fig. 2. Fluorescence *in situ* hybridization using a probe labeled with biotinylated p*Af*072 at interphase and metaphase of *Allium fistulosum*. A. The signals were detected with FITC (green) in the terminal regions of all chromosomes. B. Double-exposure photographs showing *in situ* hybridization with biotin-labeled p*Af*072 and simultaneous staining with DAPI (blue). Scale bar=10 μm (B).

Secale cereale (Appels et al., 1978).

It has been reported that the *Bam*HI repeated DNA sequence of 375 bp in satellites of *A. cepa* (Barnes et al., 1985), and the *Eco*RV repeated DNA sequence of 378 bp which is confirmed by the FISH technique in *A. fistulosum* (Irifune et al., 1995). Irifune et al. (1995) confirmed that both sequences between *A. fistulosum* and *A. cepa* have high similarity in sequence length, nucleotide composition, genomic content, and chromosomal localization. The nucleotide sequence of the 314 bp RAPD product inserted into p*Af*072 obtained in this study is also coincident with the 378-bp fragment of *A. fistulosum* obtained by restriction enzyme digestion (Irifune et al., 1995), with about 95% of homology of nucleotide sequence between only the overlapping regions of both sequences.

The content of constitutive heterochromatin in *A. fistulosum* was reported to involve about 12% of the genome, which was estimated from the darkly stained chromosome length to total chromosome length (Seo and Kim, 1975). Irifune et al., (1995) reported that the 380 bp sequence covered almost half of the constitutive heterochromatin because approximately 4.5% of the total genome was estimated by quantitative results of a Southern-blot analysis. Any of these repeated sequences obtained by *Bam*HI, *Eco*RV, or RAPD, may be part of actual tandemly repeated unit in the genome, because signals using a partial fragment in multicopy repeating families will be detectable. To identify and characterize the actual repeating unit in the genome of *A. fistulosum*, we are planning to undertake sequence walking from additional analysis, including application of many OPA primers or of some arbitrary primers within this sequence.

Acknowledgements

This is a part of a study supported by the academic research fund of the Ministry of Education, GE 97-11, Republic of Korea.

References

Altschul SF, Gish W, Miller W, Meyers EW, and Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-410.

Appels R, Driscoll C, and Peacock WJ (1978) Heterochromatin and highly repeated DNA sequences in rye (*Secale cereale*). *Chromosoma* 70: 67-89.

Badaeva ED, Friebe B, and Gill BS (1996a) Genome differentiation in *Aegilops*. 1. Distribution of highly repetitive DNA sequences on chromosomes of diploid species. *Genome* 39: 293-306.

Badaeva ED, Friebe B, and Gill BS (1996b) Genome differentiation in *Aegilops*. 2. Physical mapping of 5S and 18S-26S ribosomal RNA gene families in diploid species. *Genome* 39: 1150-1158.

Barnes SR, James AM, and Jamieson G (1985) The organization, nucleotide sequence, and chromosomal distribution of a satellite DNA from *Allium cepa*. *Chromosoma* 92: 185-192.

Bedbrook JR, Jones J, ODell M, Thompson RD, and Flavell RB (1980) A molecular description of telomeric heterochromatin in *Secale* species. *Cell* 19: 545-560.

Cabrera A, Friebe B, Jiang J, and Gill BS (1995) Characterization of *Hordeum chilense* chromosomes by C-banding and *in situ* hybridization using highly repeated DNA probes. *Genome* 38: 435-442.

Castilho A and Heslop-Harrison JS (1995) Physical mapping of 5S and 18S-25S rDNA and repetitive DNA sequences in *Aegilops umbellulata*. *Genome* 38: 91-96.

Chalmers KJ, Waugh R, Sprent JI, Simons AJ, and Powel W (1992) Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers. *Heredity* 69: 465-472.

Cuadrado A and Jouve N (1997) Distribution of highly repeated DNA sequences in species of the genus *Secale*. *Genome* 40: 309-317.

Deumling B (1981) Sequence arrangement of highly methylated satellite DNA of plant, *Scilla*: a tandemly repeated inverted repeat. *Proc Natl Acad Sci USA* 78: 338-342.

Irifune K, Hirai K, Zheng J, Tanaka R, and Morikawa H (1995) Nucleotide sequence of a highly repeated DNA sequence and its chromosomal localization in *Allium fistulosum*. *Theor Appl Genet* 90: 312-316.

Iwabuchi M, Itoh K, and Shimamoto K (1991) Molecular and cytological characterization of repetitive DNA sequences in *Brassica*. *Theor Appl Genet* 81: 349-355.

Jiang C, Lewis ME, and Sink KC (1997) Combined RAPD and RFLP molecular linkage map of asparagus. *Genome* 40: 69-76.

Lee SH and Seo BB (1995) Partial mitotic synchronization and Giemsa G-banding in *Allium wakegi*. *J Plant Biol* 38: 33-38.

Mukai Y, Endo TR, and Gill BS (1990) Physical mapping of the 5S rRNA multigene family in common wheat. *J Hered* 81: 290-295.

Mukai Y, Endo TR, and Gill BS (1991) Physical mapping of the 18S-26S rRNA multigene family in common wheat: identification of a new locus. *Chromosoma* 100: 71-78.

Nagaoka T and Ogihara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. *Theor Appl Genet* 94: 597-602.

Rafalski JA and Tingy SV (1993) Genetic diagnostic in plant bleeding: RAPDs, microsatellite and machines. *Trends Genet* 9: 275-280.

Rogers SO and Bendich AJ (1988) Extraction of milligram amounts of DNA from fresh, herbarium and mummified plant tissues. *Plant Mol Biol* 5: 69-76.

Sambrook J, Fritsch EF, and Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, New York, pp 1.82-1.84.

Seo BB and Kim, JH (1975) Karyotypic analyses based on the heterochromatin distribution in *Allium fistulosum* and *A. ascalonicum*. *Korean J Bot* 18: 92-100.

Vosa CG (1976) Heterochromatic patterns in *Allium*. 1. The relationship between the species of the *cepa* group and its allies. *Heredity* 36: 383-392.

Welsh J and McClelland M (1990) Fingerprinting genomes using arbitrary primers. *Nucleic Acids Res* 18: 7213-7218.

Wilkie SE, Issac PG, and Slater RJ (1993) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. *Theor Appl Genet* 83: 871-877.

Williams JGK, Kubeik AR, Levak KJ, Rafalski JA, and Tingey SV (1990) DNA polymorphism amplification by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18: 6531-6535.

Wu HK, Chung MC, Ning CN, and Wu R (1991) Localization of specific repetitive DNA sequences in individual rice chromosomes. *Chromosoma* 100: 330-338.

Yakura K, Kato A, and Tanifuji S (1987) Cytological localization of highly repeated sequences, the *Fok*I sequence family and *Bam*HI sequence families, in *Vicia fava*. *Jpn J Genet* 62: 325-332.

[Received December 14, 1998; accepted January 12, 1999]