Identification and Chromosomal Location of Tandemly Repeated DNA Sequences in *Allium cepa*

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A 314-bp tandemly repeated DNA sequence, named pAc074, was characterized in *Allium cepa* by fluorescence *in situ* hybridization (FISH) analyses using random amplified fragment as probe. The nucleotide sequences of the clone pAc074 is partially homologous to the satellite DNA sequences, ACSAT1, ACSAT2, and ACSAT3, of *A. cepa* with 81%, 81% and 78% similarity, respectively. Our sequential C-banding and FISH with pAc074 probe also clearly showed a close relation between C-heterochromatin at telomeric region and pAc074 sequences on all the chromosomes except on chromosome 6. On the long arm of chromosome 7, pAc074 sequences appeared as interstitial band which did not correspond to C-heterochromatin bands. Instead, the C-heterochromatin bands corresponded with the 5S rDNA signals. This is the first evidence of simultaneous banding of the 5S rDNA and C-band in *A. cepa*.

INTRODUCTION

The polymerase chain reaction (PCR) has facilitated genetic studies in plants and animals (Erlich et al., 1991). This PCR technique has been used in the isolation of particular genes by amplifying random sequences of genomic DNA. These random amplified sequences can in many cases be used as genetic markers that are useful in the molecular analysis of the genome.

C-banding and fluorescence *in situ* hybridization (FISH) are powerful tools for karyotyping and genome identification in plants. Several investigators have described differences in C-banding patterns in *A. cepa* (Joshi and Ranjekar, 1980; Sato, 1981). However, C-banding with giemsa stain identifies only the constitutive heterochromatin on the chromosomes which earlier facilitated to identify the individual chromosomes of the karyotype (Kalkman, 1984). Now, it is possible to locate the DNA sequences on the chromosomes with the *in situ* hybridization (ISH) technique. Fluorescence *in situ* hybridization technique using fluorochromes allows the visualization of multi-gene families (Castilho and Heslop-Harrison, 1995), highly repeated DNA sequences (Irifune et al., 1995) and

even unique sequences on the chromosomes (Ten Hoopen et al., 1996). Recently, chromosome banding and FISH technique have successfully been integrated into a single procedure for the analysis of plant chromosomes (Jiang and Gill, 1993; Mukai et al., 1992).

In the present study, we have combined all the above mentioned techniques, PCR, C-banding and FISH, in order to discover the chromosomal marker in the chromosome complement of *A. cepa*. This study also includes a further characterization of the heterochromatin by C-banding combined with FISH technique with rDNAs and tandemly repeated DNA sequence probes. The aim of this study is to find out a correlation between giemsa stained heterochromatic regions and tandemly repeated DNA sequences of *A. cepa*.

MATERIALS AND METHODS

Plant material and chromosome preparations. *Allium cepa* cv. 'Cheonjudaego' obtained from commercial source were soaked in water, then placed on a moist filter paper in Petri dishes and incubated at 25 °C. After 3–4 days, the root tips about 1 cm long were excised and used for chromosome preparation for C-banding and FISH according to the method of Seo and Kim (1988) and Mukai et al. (1990), respectively.

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PCR analysis and labelling of PCR products.

Genomic DNA was extracted from fresh leaves according to the method of Rogers and Bendich (1988). A set of 20 random decamer oligonucleotides (A01–A20) purchased from Operon Technologies Inc. (Alameda Calif., USA) were used as single primers for the amplification of PCR sequences. The amplification procedure followed was exactly that of Williams et al. (1990). Briefly, the reaction mixture (25 µl) contained 10 ng of template DNA, 200 mM each of primer, 200 mM each of dATP, dGTP, dCTP, and dTTP, and 2.5 U *Ex Taq* Polymerase (Takara Shuzo co. Ltd.) in 1X *Ex Taq* buffer (Takara Shuzo co. Ltd.) with 2 mM magnesium chloride. The PCR reaction was performed in a Perkin Elmer Cetus System 9600 thermal cycler. Amplification involved a preliminary 5 min denaturation at 94 °C followed by 45 cycles of 1 min at 94 °C (denaturation), 1 min at 38 °C (annealing) and 2 min at 72 °C (extension). The final extension was at 72 °C for 5 min followed by slow cooling to room temperature. PCR products were visualized by electrophoresis in 1.2% agarose gels. For the *in situ* hybridization analysis, each PCR band was isolated using a GeneClean II kit (Bio 101, Inc.) and labelled with biotin-16-dUTP (Boehringer Mannheim) by PCR method as described previously using the 10-mer oligonucleotide primer that generated each corresponding PCR product. The FISH experiment followed the procedures described by Mukai et al. (1990).

Cloning and sequencing of the PCR marker.

The chosen PCR marker detected by FISH was excised from the agarose gels. It was purified by a GeneClean II kit (Bio 101, Inc.), cloned into pT7Blue(R) T-vector (Novagen) following the manufacturer's instructions, and introduced into DH5a by transformation (Hanahan, 1983). For the FISH analysis, the same PCR marker was labelled with biotin-16-dUTP (Boehringer Mannheim) by PCR amplification from the corresponding recombinant plasmid (pAc074) using the 10-mer oligonucleotide primer that generated the corresponding PCR product as described previously. Nucleotide sequencing was done on PAGE gel with an ABI Prism 377 sequencer (Perkin-Elmer) as an automated fluorescent sequencing system. CLUSTAL X 1.6 (Thompson et al., 1994), a multiple alignment program, was used to locate homologies of the cloned satellite sequences of *A. cepa*. The BLASTIN program (Altschul et al., 1990) was used to search the homologous region to DNA sequences in database such as GenBank, EMBL, and DDBJ.

Southern hybridization analysis.

Southern hybridization of genomic DNA and recombinant plasmid was done according to Sambrook et al (1989) and Southern (1975). The genomic DNAs digested with *EcoRV*, *BamHI* and *HindIII* were electrophoresed in a 1% agarose gel (1X TAE buffer), then stained with ethidium bromide, after

Table 1. PCR products amplified from genomic DNA in *Allium cepa*

Lane no.	Primer (pM)	Nucleotide sequence (5' to 3')	Products (ca. bp)
1	OPA01 (20)	CAGGCCCTTC	1100, 900, 700, 500, 400, 300
2	OPA01 (40)	CAGGCCCTTC	1100, 1000
3	OPA02 (20)	TGCCGAGCTG	1900, 1500, 1000
4	OPA03 (20)	AGTCAGCCAC	2000, 1400, 1100, 800, 500
5	OPA03 (40)	AGTCAGCCAC	2000
6	OPA04 (20)	AATCGGGCTG	2100, 1800, 800, 600, 390
7	OPA05 (20)	AGGGGTCTTG	1100, 600, 300
8	OPA06 (40)	GGTCCCTGAC	2500, 1300
9	OPA07 (40)	GAAACGGGTG	3100, 800, 500, 400 ¹
10	OPA08 (20)	GTGACGTAGG	1500, 1000, 800, 300
11	OPA09 (40)	GGGTAACGCC	900, 600, 400
12	OPA10 (20)	GTGATCGCAG	300
13	OPA10 (40)	GTGATCGCAG	1000, 800, 600, 300
14	OPA11 (20)	CAATCGCCGT	1400, 800, 500
15	OPA12 (20)	TCGGCGATAG	2500, 1600, 850, 600
16	OPA13 (20)	CAGCACCCAC	4900, 4000, 2300, 1650, 800
17	OPA13 (40)	CAGCACCCAC	2300, 1650, 1000, 800
18	OPA14 (40)	TCTGTGCTGG	700, 400
19	OPA15 (20)	TTCCGAACCC	2500, 2000, 1600, 1000
20	OPA16 (20)	AGCCAGCGAA	3000, 2500, 1800, 1400, 1000
21	OPA16 (40)	AGCCAGCGAA	3000, 2500, 1400, 900
22	OPA17 (20)	GACCGCTTGT	2500, 1500
23	OPA18 (40)	AGGTGACCGT	2500, 1800, 1600
24	OPA19 (20)	CAAACGTCGG	1500, 1000, 800, 500, 300
25	OPA20 (20)	GTTGCGATCC	9000, 4000, 3000, 1800, 1600, 1000, 600

¹ Underlined DNA fragments are FISH-detectable products.

which they were photographed and transferred to nylon membranes (Positively charged, Boehringer Mannheim) by vacuum action. In the case of the probe, the PCR marker amplified from genomic DNA in the clone pAc074 was extracted by double digestion with *Pst*I and *Eco*RI then labelled with 32 P-dCTP (3,000 Ci/mmol; Amersham) by the random primer extension method using a DNA labelling kit (Boehringer Mannheim).

After crosslinking under the ultra-violet light (Stratagene), prehybridization (6X SSC, 2X Denhardt's solution, 100 mg/ml denatured herring sperm DNA, and 0.5% SDS) and hybridization (6X SSC, 100 mg/ml denatured herring sperm DNA, 0.5% SDS and isotope-labeled probe) was carried out at 62 °C.

Probe labelling for FISH. Clone pAc074 was labelled with biotin-16-dUTP by PCR method as described previously. pTa71 was used containing one unit of 18S-5.8S-26S rDNA (8.9 kb) derived from common wheat (*Triticum aestivum* cv. Chinese Spring) (Gerlach and Bedbrook, 1979). The probe was labelled by nick translation method with digoxigenin-11-dUTP (Boehringer Mannheim). The probe pCE605, containing a 352-bp insert for a 120 bp tandem repeated 5S rDNA sequence isolated from *A. cepa*, was labelled with digoxigenin-11-dUTP by PCR according to Do et al. (1999).

C-banding and FISH. The method of Seo and Kim (1988) was used for C-banding. Chromosome measurements were on 10 C-banded onion chromosome. Some preparations were used for sequential C-banding and FISH. FISH was carried out following C-banding. The C-banded slides were incubated in xylene for 1 h, washed in 95% ethanol for 20 min, placed in 45% acetic acid for 20 min, and oven dried at 60 °C for about 5 h before *in situ* hybridization (Xu et al., 1994). Clone pAc074- and 18S-5.8S-26S rDNA-specific probe were first hybridized to chromosomes of *A. cepa* followed by washing and reprobing of 5S rDNA-specific probe. Five-hundred ng/ml of biotin-labelled pAc074 clone and 500 ng/ml of digoxigenin-labelled 18S-5.8S-26S rDNA were used as probes at first. FISH was made following procedures described by Mukai et al. (1990). Preparations were counterstained with 1 mg/ml DAPI (4', 6-diamino-2-phenylindole) and mounted in 5 ml of Vectashield (Vector Laboratories). The slides were then destained according to Xu et al. (1994). Reprobing with the 500 ng/ml of digoxigenin-labelled 5S rDNA was performed to the same metaphase cell of *A. cepa*. For detection of hybridization signals, we used Carl Zeiss epifluorescent microscope equipped with the filter sets No. 15 (Rhodamine), 09 (FITC), and 02 (DAPI). Photographs were taken on Kodak super clear 400 color film. Double exposures were made with a triple band pass filter, using separate excitation filters for each fluorochrome.

RESULTS

PCR amplification and selection of the PCR marker.

To obtain the PCR products that can be detected with FISH technique, 20 kinds of 10-mer primers (OPA kit) were applied to a total genomic DNA of *A. cepa*. Ninety-one kinds of different reproducible DNA bands were obtained (Table 1). To determine which of these PCR products should be used as a potential genetic marker in the FISH, all PCR bands showing reproducibility were isolated from agarose gel, labelled with biotin-16-dUTP by PCR amplification, and detected by FISH. As a result, a PCR fragment from OPA07 primer was detected in the terminal region of all chromosomes except for the short arm of chromosome 6. The fragment was cloned and designated as pAc074.

Identification and sequence analysis of the clone pAc074.

The selected PCR marker, pAc074, was used as a probe for Southern hybridization of genomic DNA to de-

Fig. 1. Southern hybridization of *A. cepa* genomic DNA. (2.0 mg) digested with *Eco*RV (lane 1), *Bam*HI (lane 2) and *Hind*III (lane 3) and probed with *Pst*I/*Eco*RI fragment of pAc074. Ladder-like pattern was detected in the three digestions.

Fig. 2. Multiple alignment of nucleotide sequences of the clone pAc074 and the satellite DNA sequences (ACSAT1, ACSAT2 and ACSAT3) of *A. cepa*. Satellite sequences of ACSAT1, ACSAT2 and ACSAT3 have been published by Barnes et al. (1985). Primer sequences used, 5'-GAAACGGGTG-3', in both terminals are deleted in this alignment. The nucleotide sequences of the clone pAc074 is registered in the EMBL/GenBank/DBJ Nucleotide Sequence Data Base under accession No. AF227152. The hyphens indicate the gaps assumed. Sequence homology decreases from black to white.

termine its level of sequence repetition. In the case of *EcoRV*, *Bam*HI and *Hind*III digestion, pAc074 detected bands showed ladder-like pattern (Fig. 1). It indicated that the pAc074 contained tandemly repeated DNA sequences. We compared the pAc074 nucleotide sequence (314 bp) with the satellite DNA sequences (ACSAT1, ACSAT2, and ACSAT3) of *A. cepa* (Barnes et al., 1985; Fig. 2). The pAc074 sequence had 81%, 81% and 78% similarity with the satellite sequences, respectively.

Chromosomal location of the clone pAc074 studied by sequential C-banding and FISH. Sequential C-banding and FISH analyses with the clone pAc074, 18S-5.8S-26S and 5S rDNAs were carried out in the metaphase chromosomes to investigate the distribution and relationship between the terminal repeat sequence and the occurrence of C-band heterochromatin in the *A. cepa* chromosomes (Fig. 3 and 4). Large bands of C-heterochromatin were present in the terminal regions of all 16 chromosomes and two satellites, while small bands of C-heterochromatin were present in the interstitial positions of short arm of chromosome 7 (Fig. 3a and Fig. 4a). The pAc074 clone had practically hybridized with the terminal heterochromatic regions in both arms of all 16 chromosomes except for the short arm of chromosome 6

(Fig. 3b). The pAc074 locations corresponded to most of the major C-banded regions of *A. cepa* except the short arm of chromosome 6 (Fig. 4b) and interstitial C-bands on short arm of chromosome 7. Instead 5S rDNA sequences corresponded to these interstitial bands. The digoxigenin-labelled 18S-5.8S-26S rDNA sites were detected on the short arm of chromosome 6 where pAc074 hybridization signals were absent and on short arm of chromosome 8 along with the hybridization signals of pAc074 (Fig. 3c and Fig. 4b). Moreover, hybridization signals of pAc074 occur in the terminal region of chromosome 8 together with 18S-5.8S-26S rDNA. Interestingly, pAc074 loci are present on the interstitial position of long arm of chromosome 7. The 5S rDNA sites are accordant with the C-heterochromatin region (Fig. 3d and Fig. 4b).

DISCUSSION

This study has demonstrated that the PCR technique using random 10-mer primers can be successfully applied to onion to select useful chromosome markers. This technique can provide genetic markers that are useful in the molecular analysis of the genome. We selected the tandemly repetitive chromosome marker, pAc074, in onion. The presence of tandem repeated, satellite DNA sequences in onion telomeric heterochromatin were first

Fig. 3. Sequential analysis of the C-banding (a) and FISH (b, c and d) on metaphase chromosomes of *A. cepa*. Biotin-labelled probe (clone pAc074-specific probe) detected with avidin-FITC conjugate (green) and digoxigenin-labelled probes (5S and 18S-5.8S-26S rDNA probes) detected with antidigoxigenin-rhodamine conjugate (red). a C-banding patterns. All 16 chromosomes are indicated by numbers. b Subsequent FISH on the same metaphase cell with the biotin-labelled clone pAc074-specific probe. Clone pAc074-specific signals at all chromosome ends except the short arm of chromosome 6 (thick arrows). Note the presence of a specific interstitial site for the clone pAc074 in the long arm of chromosome 7 (thin arrows). c The digoxigenin-labelled 18S-5.8S-26S rDNA sites are detected on the short arm of chromosomes 6 and 8. d Reprobing with the digoxigenin-labelled 5S rDNA reveals major FISH sites in the short arm of chromosome 7. Bar = 10 μ m.

reported by Barnes et al. (1985) using radioactive *in situ* hybridization (ISH). By means of same technique, Schubert and Wobus (1985) performed ISH with a 125 I-rDNA clone from *Vicia faba* against *A. cepa* and three strains of top onion.

Studies on repetitive sequences in the bulb onion ge-

nome have mainly focused on a 375-bp satellite sequence (Barnes et al., 1985), and the rDNA repeats (Pich et al., 1996a and b). So far, only a few tandemly repeated DNA sequences including one satellite DNA in *A. cepa* have been reported (Pich et al., 1996b; Pich and Schubert, 1998). A 375-bp satellite sequence has been shown using

Fig. 4. Idiogram of *A. cepa* chromosomes showing the physical location of C-banding pattern (a) and FISH markers (b). The corresponding chromosome complements after FISH with the clone pAc074-specific sequences, 18S-5.8S-26S rDNA, and 5S rDNA (see legends). The chromosomes are in order of descending length.

FISH to be located in the terminal position on metaphase chromosomes by Barnes et al. (1985). Beginning with the work of Barnes et al. (1985), ISH analysis with biotinylated repetitive DNA probes has been used for the physical mapping of defined sequences in onion and its relatives (Pich and Schubert, 1998; Pich et al., 1996a). The nucleotide sequences of the clone pAc074 is partially homologous to the satellite DNA sequences (Barns et al., 1985), ACSAT1, ACSAT2, and ACSAT3, of *A. cepa* with 81%, 81% and 78% similarity, respectively (Fig. 2). *In situ* hybridization with the pAc074 probe reported here also exhibited strong hybridization signals at telomeric regions of all chromosomes except the short arm of chromosome 6 of *A. cepa* (Fig. 3). The FISH patterns and sequences of the pAc074 clone are similar to that described earlier (Barns et al., 1985; Pich et al., 1996b; Pich and Schubert, 1998), suggesting that pAc074 and the satellite sequences reported before belong to the same family of repeats. The pAc074-type repeated sequence appears to be a family of a highly repetitive sequence that makes up parts of the genome in onion. Pich et al. (1996b) suggested that this type of repeated sequences also form telomeres in other related species that lack at their heterochromatic chromosome termini. The TTTAGGG telomeric sequences were lost and replaced by highly repetitive satellite and rDNA during evolution of *A. cepa*

(Pich et al., 1996b).

In *A. fistulosum*, small bands or dots of C-heterochromatin were present in the interstitial region in some of chromosomes, but there were no hybridization signals of highly repeated DNA sequences in these regions (Irifune et al., 1995). In our results, pAc074 sequence did not correspond to C-heterochromatin on the interstitial region of the long arm of chromosome 7. This result indicates that the pAc074 repeated DNA sequences in the terminal region of the chromosomes is not associated with minor heterochromatin blocks in the nucleus of *A. cepa*. Thus, the pAc074 loci of the interstitial region in the long arm of chromosome 7 are likely to be useful for identification of the long arm of chromosome 7.

Irifune et al. (1995) reported that the repeated DNA sequence of *A. fistulosum* is closely associated with the major C-heterochromatin in the terminal regions of all 16 chromosomes at mitotic metaphase. In our FISH result of pAc074, telomeric region sequence of *A. cepa* is also closely related to the C-heterochromatin. We observed that the signals of repetitive sequences of pAc074 corresponded with the C-banding pattern at telomeric region of all the chromosomes except chromosome 6, confirming earlier reports (Barnes et al., 1985; Pich and Schubert, 1998). The pAc074 loci were associated with 5S and 18S-5.8S-26S rDNA sites. When the pAc074 locations were

compared with the 18S-5.8S-26S rDNA sites, the NOR regions of the chromosome 8 corresponded to pAc074 loci, but the NOR regions of chromosome 6 did not (Fig. 4). In chromosome 8, either rDNA or satellite sequences may yield distal signals after bi-color FISH at chromosome termini where both occur together. The simplest explanation is that both sequences in fact may occupy outer positions and substitute each other. DNA of the terminal region of chromosome 6 did not carry the telomeric region sequence. Our data confirm the previous inference that the short arm of chromosome 6 with the NOR in terminal position is flanked distally by a small heterochromatic satellite (Schubert, 1984), which seems to be composed of condensed rDNA sequences (Ricroch et al., 1992).

The correlation between constitutive heterochromatin and ribosomal DNAs has been reported in *A. fistulosum* (Irifune et al., 1995; Seo et al., 1999), *A. cepa* (Pich et al., 1996b), and *A. wakegi* (Lee and Seo, 1997). In these studies, the rDNA locations corresponded to most of the major C-banded regions. When the 18S-5.8S-26S rDNA sites were compared with the C-banding pattern, NOR sites corresponded to C-bands (Fig. 4). Our data confirmed the various earlier results, also obtained by *in situ* hybridization using 18S-5.8S-26S rDNA probe (Schubert and Wobus, 1985; Ricroch et al., 1992; Pich et al., 1996b). Similarly, the FISH analysis also showed that the chromosomal region containing 5S rDNA corresponded to the C-heterochromatin region (Fig. 3d and Fig. 4b). In onion, although 5S rDNA loci have been assigned to chromosome 7 (Lee et al., 1999), there are few reports which show the coexistence of 5S rDNA and C-bands in *A. cepa*. Mukai et al. (1990) reported that the chromosomal region containing 5S rDNA does not correspond to C-heterochromatin in wheat. However, Dvorak et al. (1989) speculated that C-bands may be the sites of 5S rDNA loci and our data support this hypothesis. This is the first evidence on the relationships between the 5S rDNA loci and C-bands in *A. cepa*.

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