**Allodiploid nature of *Allium wakegi* Araki
revealed by genomic *in situ* hybridization
and localization of 5S and 18S rDNAs**

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ABSTRACT

Allium wakegi Araki could be originated from cross hybridization between close relatives of a form of *A. cepa* and *A. fistulosum*. Chromosomes of each parental haploid set were identified in the chromosome complement of *A. wakegi* by genomic *in situ* hybridization using probes of the total, parental genomic DNAs of *A. cepa* and *A. fistulosum*, respectively. The results of GISH suggested significant differentiation of genomic DNAs between these closely related species. *Allium cepa* had two 5S rDNA loci at the interstitial regions of the short arms of small chromosome pair (the 7th pair) and *A. fistulosum* had one locus at the interstitial region of the homologous short arms. *Allium wakegi* had two chromosomes carrying one and two 5S rDNA loci which appeared to correspond to those of *A. fistulosum* and *A. cepa*, respectively. Chromosomes carrying 18S rDNA loci originated from those of both *A. cepa* and *A. fistulosum* were also observed in the chromosome complement of *A. wakegi*.

1. INTRODUCTION

Allium wakegi Araki is one of the old cultivated crops in Japan, China, and Southeast Asia. The species is completely sterile and thus vegetatively propagated by bulbing. The karyotype ($2n=16$) of the species is heterozygous and two J-shaped nucleolar chromosomes are quite different in length and morphology from each other (Kurita, 1952, 1953; Yamaura, 1961; Fukushima et al., 1964; Adaniya et al., 1978; Fujieda et al., 1980; Tashiro, 1984c). On the basis of karyotype and behavior of meiotic chromosomes, Kurita (1953) firstly suggested that this species was of allodiploid origin between *A. fistulosum* and unknown species with the large J-shaped chromosome. Pattern of heterochromatin or C-bands which appeared at both terminal ends of all chromosomes of *A. wakegi* supported this hypothesis (Kurita, 1959; Vosa, 1976). An artificial hybrid between a form of *A. cepa* (the aggregatum type, syn. *A. ascalonicum*) and *A. fistulosum* possessed very similar karyotype and meiotic behavior to these of *A. wakegi* (Adaniya et al., 1978; Tashiro, 1980). Tashiro (1984a,b) observed meiotic chromosome pairings in PMCs of triploid hybrids between tetraploid *A. wakegi*

and *A. ascalonicum* or *A. fistulosum*, and also tetraploid hybrids between an artificial amphidiploid (*A. ascalonicum* and *A. fistulosum*) and tetraploid *A. wakegi*. It was cytogenetically demonstrated that *A. wakegi* is the hybrid between *A. ascalonicum* and *A. fistulosum* (Tashiro, 1984c).

In this paper two parental genome sets were identified in the chromosome complement of *A. wakegi* by genomic *in situ* hybridization (GISH called by Schwarzbacher et al. 1989), providing an additional evidence on its allodiploid nature. Localization of 5S and 18S rDNAs supported the conclusion.

2. MATERIALS AND METHODS

Plants

Bulbs of *Allium wakegi* Araki and seeds of *A. cepa* L. cv. 'Sharumu' and *A. fistulosum* L. cv. 'Kujo' were obtained from commercial sources.

DNA extraction

Total genomic DNA was extracted from young leaves or whole seedlings. The samples were ground into powder using a mortar and a pestle with liquid nitrogen and homogenized in the buffer containing 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% cetyltrimethylammonium bromide and 0.5% mercaptoethanol. The homogenate was extracted three times with an equal volume of chloroform-isoamyl alcohol (24 : 1) for 15 min each and the DNA was precipitated with an equal volume of isopropyl alcohol at room temperature. The DNA was treated with DNase-free RNase A (0.2 mg/ml) at 37°C for 1 h followed by extractions with phenol-chloroform and chloroform.

Preparation of probes

Parts of 18S rDNA and a full length of 5S rDNA were amplified by polymerase chain reaction (PCR: Saiki et al., 1988). The 18S rDNA primer sets (NS1 and NS2, NS5 and NS8: Dams et al., 1988) and the 5S rDNA primer set (Hizume, 1993) were used. The reaction mixture (50 µl) containing 10 mM Tris-HCl (pH 7.5), 2.5 mM MgCl₂, 50 mM KCl, 200 µM each of dATP, dTTP, dCTP, dGTP, 50 ng genomic DNA, 50 mM each of primer sets and 1 unit of *Taq* DNA polymerase (Perkin Elmer Cetus or Wako Pure Chemical Co.) was subjected to thirty thermal cycles with the first 3 min denaturation at 95°C and the last elongation at 75°C for 5 min. The amplification cycle consisted of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and primer extension at 75°C for 10 sec. Total genomic DNAs and amplified DNA fragments were labeled with biotin-14-dATP by nick translation according to the supplier's instruction (BRL). Probe mixture (400 µl) contained 10% dextran sulfate, 50% formamide, 2×SSC (1×SSC; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 1 µg biotin-labeled DNA. In some experiments, the probe mixture contained the labeled DNA of one species and the

40 fold non-labeled genomic DNA of another species as a blocking DNA.

Chromosome preparation

Root tips were collected and treated with 2 mM 8-hydroxyquinoline at 20°C for 3 h. After fixation in acetic alcohol (1 : 3) the root tips were treated with 2% Cellulase RS (Yakult) and 0.5% Pectolyase Y-23 (Seishin Pharma.) in 0.1 M citrate buffer (pH 4.5) containing 5 mM EDTA at 37°C for 30 min, washed in distilled water and squashed in 45% acetic acid. Some root tips were macerated in 45% acetic acid at 60°C for 5 min before squashing. After removal of coverslips by the dry-ice method the preparations were air-dried overnight.

In situ hybridization

In situ hybridization was performed as previously described (Hizume, 1992) with the following modifications. The chromosomes treated with the cell wall-degrading enzymes were denatured in 70% formamide-2×SSC at 75°C for 3 min, dehydrated through cold ethanol series and air-dried. The probe mixture was applied to chromosomes, and the slide was covered with a coverslip and incubated at 37°C overnight. For the chromosomes prepared by acetic acid maceration, they were simultaneously denatured with the probe DNA by heating the glass slide at 75°C for 10–15 min on a hot plate and then incubated at 37°C overnight. After hybridization and post-hybridization washing, hybridized biotin-labeled probes were cytochemically detected using the avidine-horse radish peroxidase complex (Vector Laboratories) and a diaminobenzidine-H₂O₂ solution. Chromosomes were counterstained with 2% Giemsa (Merck) in 2×SSC for 2 min and then mounted with Eukitt (Kindler).

Total area of all chromosomes of each parental set was measured by an image analyzer (IBAS: Kontron).

3. RESULTS

Identification of parental chromosome sets by GISH

In the chromosome complement of *Allium wakegi* after GISH using the total genomic DNA from *A. cepa* as probe, eight relatively long chromosomes showed heavier signal than the other eight chromosomes (Fig. 1A). Karyotype of the signaled chromosome set was very similar to that of *A. cepa*. On the other hand, eight relatively short chromosomes of *A. wakegi* were more densely signaled than the eight longer chromosomes, when the total genomic DNA from *A. fistulosum* was used as probe (Fig. 1B). Karyotype of the signaled shorter chromosome set was very similar to that of *A. fistulosum*. All the chromosomes belonging to each chromosome set were thus discriminated by GISH. All the chromosomes showed evenly distributed signals over their entire length (Figs 1 and 2). When using the blocking DNA from *A. fistulosum* small satellite of the long subtelocentric

Fig. 1. Genomic *in situ* hybridization of metaphase chromosomes of *Allium wakegi* using the probe of biotin-labeled total genomic DNA of *A. cepa* (A) and *A. fistulosum* (B). Bar = 10 μ m.

Fig. 2. Chromosome complements in *A. wakegi* hybridized *in situ* using the probe of total genomic DNA of *A. cepa* (A) and *A. fistulosum* (B). Left half of the chromosomes belong to *A. cepa* and the right half to *A. fistulosum*. Dots indicate that signals are from overlapped chromosomes that belong to the corresponding other parental chromosomal set. Bar = 10 μ m.

chromosomes and terminal ends of several other chromosomes were not signaled by the GISH using the probe of *A. cepa* genomic DNA. Fig. 2 shows parental chromosome sets identified by GISH using genomic DNA of each species, arranged in a decreasing order of length. Chromosomes hybridized with *A. cepa* DNA were longer than chromosomes hybridized with *A. fistulosum* DNA when each homologous chromosomes were compared. Total area of the longer chromosomes was about 28% larger than that of the shorter chromosomes. The satellited chromosome similar to that of *A. cepa* was found in the longer chromosomes

and that of *A. fistulosum* in the shorter chromosomes. The shorter satellited chromosomes derived from *A. fistulosum* showed constantly elongated secondary constriction at metaphase in *A. wakegi*.

Localization of 5S rDNA

The 5S rDNA loci were clearly different in number and location between *A. cepa* and *A. fistulosum*: *Allium cepa* had four 5S rDNA loci at the interstitial regions of the short arms of the small metacentric chromosomes (the 7th pair) (Fig. 3A), while *A. fistulosum* had two loci at interstitial region of the short arms of small metacentric chromosomes (the 7th pair) (Fig. 3B).

Allium wakegi had three 5S rDNA loci on two small metacentric chromosomes (Fig. 3C), two were on the short arm of the longer chromosome and the remaining was at the interstitial region of the short arm of the shorter chromosomes. The chromosomes with 5S rDNA locus observed in *A. wakegi* thus appeared to correspond to each one of the chromosomes carrying 5S rDNA loci in *A. cepa* and *A. fistulosum*.

Fig. 3. Localization of 5S rDNA in the chromosomes of *Allium cepa* (A), *A. fistulosum* (B) and *A. wakegi* (C), and that of 18S rDNA in the chromosomes of *A. wakegi* (D). Solid triangles indicate signaled chromosomes of *A. cepa* and open triangles indicate signaled chromosomes of *A. fistulosum*. Bar = 10 μ m.

Localization of 18S rDNA

Allium wakegi had three 18S rDNA loci on three chromosomes in the chromosome complement (Fig. 3D). One long subtelocentric chromosome had a signal at the terminal region of the short arm, while one shorter subtelocentric chromosome had a signal at both the elongated secondary constriction and the satellite. Another short metacentric chromosome had a very small signal at the terminal region of the short arm. The long and short subtelocentric chromosomes with 18S rDNA locus were similar to those of *A. cepa* and *A. fistulosum*, and the short metacentric chromosome carrying the tiny signal to that of *A. cepa* (data not shown, Ricroch et al., 1992). However, the smallest 18S rDNA locus of the shortest chromosome pair of *A. fistulosum* reported by Ricroch et al. (1992) was not detected.

4. DISCUSSION

Each chromosome set of the parental species genomes was identified by GISH (Figs 1 and 2) together with different patterns of 5S and 18S rDNAs loci (Fig. 3). The observations confirmed that *A. wakegi* is an allodiploid between a certain form of *A. cepa* and *A. fistulosum*. These two species can be easily hybridized and interspecific hybrids were frequently obtained by the artificial cross-pollination (see Peffley and Mangum, 1990). Top onion or tree onion (*A. cepa* L. var. *viviparum* = *A. proliferum* (Moench) Schrad.), that is cultivated in home gardens in Europe and North America, was found to be natural hybrids between *A. cepa* and *A. fistulosum* based on karyotype analysis (Bozzini, 1964) and C-band analysis (Fiskesjo, 1975; Vosa, 1976; Schubert et al., 1983). Thus, the top onion and *wakegi* were in fact reported to have same parentage (Hanelt, 1990). However, since they show different forms and characteristics, their parental strain of *A. cepa* might be different from each other. The strain of *A. cepa* that contributed as one of the parents of top onion is believed to be from the Cepa group (Schubert et al., 1983) and that of *wakegi* is from the Aggregatum group (*A. cepa* var. *ascalonicum* Backer = *A. ascalonicum* auct. non Strand) (Tashiro 1984a,b,c). In the present study GISH identified the chromosome set derived from the aggregatum type of *A. cepa* in the chromosome complement of *A. wakegi* using probe DNA extracted from the common onion type of *A. cepa*. This indicated that these two types of *A. cepa* have homologous genomic DNA.

The parental species of *A. wakegi*, i.e., *A. cepa* and *A. fistulosum*, are very closely related to each other and classified in the same subgenus *Rhizirideum* (G. Don ex Kock) Wendelbo, section *Cepa* (Mill.) Prokh. (Hanelt, 1990). Amount of genomic DNA per nucleus of *A. cepa*, however, was reported to be 30% more than that of *A. fistulosum* (Jones and Rees, 1968). This agrees well with the estimation obtained in the present study based on the total chromosomal area of each

parental chromosome set. In the artificial hybrids between the two species eight heteromorphic bivalents were most frequently observed with a few multivalents in some PMCs (Emsweller and Jones, 1935; Levan, 1936; Maeda, 1937; Jones and Rees, 1968; Cochran, 1950). Jones and Rees (1968) observed unpaired free ends and loops on pachytene chromosomes in PMC of the hybrids and suggested that the observed difference in DNA amount between *A. cepa* and *A. fistulosum* might be due to segmental duplication or deletion. Peffley (1986) also reported that genomes of the two species had high homology with a few structural differences on the basis of meiotic pairing in the hybrid. Among the clones of *A. wakegi* number of bivalents varied from 0 to 8 (Tashiro, 1980). Genomic discrimination by GISH is not directly related to meiotic pairing in this combination of the species. Genomic differentiation revealed by GISH occurred at whole area of the chromosome arms in each species but not segmentally.

Organization of genomic DNA in *A. cepa* and *A. fistulosum* is relatively well understood (Koravanov and Iordanskii, 1973; Stack and Comings, 1979; Cremonini et al., 1981; Durante et al., 1985). *Cot* analysis of their genomic DNA showed that the highly repetitive, moderately repetitive and unique sequences occupy 29%, 3% and 68% of the genome in *A. cepa* and 8%, 35% and 57% in *A. fistulosum*, respectively (Koravanov and Iordanskii, 1973). Analysis of reassociation of the heterologous DNA from the two species showed that highly repetitive sequences were homologous but moderately repetitive and unique sequences were with low homology. The tandemly repeated sequence (375 bp) of *A. cepa*, which occupies 4% of the total genomic DNA and is localized at the terminal C-banded region, was cloned from the heavy satellite DNA and sequenced (Barnes et al., 1985). The tandemly repeated sequence (380 bp) was also cloned and sequenced from genomic DNA of *A. fistulosum*, which was highly homologous (about 80%) to the 375 bp sequence of *A. cepa* and hybridized at the terminal C-band regions of all chromosomes of *A. fistulosum* (Irifune, K., personal communication). Because of the high homology of these repetitive sequences between *A. fistulosum* and *A. cepa* they were expected to cross-hybridize even under the high stringency washing conditions. However, although half the number of *A. wakegi* chromosomes showed hybridization signals in the experiment using the genomic DNA of one species as a probe, the terminal C-band regions of the other set of chromosomes did not simultaneously show signals even in the absence of blocking DNA. The results suggested that the tandemly repeated sequence families are not involved in the hybridization under the present GISH condition. Durante et al. (1985) reported the fast annealing fraction (fraction I) that hybridized in both segmental and homogeneous patterns depending on chromosomes and indicated that the fraction I contains at least two classes of repetitive sequences. Stack and Comings (1979) reported the existence of two middle repeated (fraction I and II) and single copy (fraction III) sequences in *A. cepa*, and showed that the RNA complementary to the fraction I hybridized *in situ* throughout the length of

chromosomes. Sequences detected by GISH in the present study may thus correspond to the fraction I reported by Stack and Comings (1979) and/or a part of the fraction I of Durante et al. (1985).

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