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## Chromosomal location of a pollen fertility-restoring gene, *Rf*, for CMS in Japanese bunching onion (*Allium fistulosum* L.) possessing the cytoplasm of *A. galanthum* Kar. et Kir. revealed by genomic in situ hybridization

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**Abstract** In a previous study, we developed cytoplasmic male sterile lines of *Allium fistulosum* possessing the cytoplasm of *A. galanthum*, a wild species, by continuous backcrossing. Furthermore, we reported the presence of a pollen fertility-restoring gene (*Rf*) for cytoplasmic male sterility (CMS) in *A. fistulosum* from segregation of pollen fertility of backcross progenies. In the present study, genomic in situ hybridization (GISH), using genomic DNA of *A. galanthum* as the probe DNA and that of *A. fistulosum* as the blocking DNA, was applied to F<sub>1</sub> hybrids between both species and backcross progenies to determine the chromosomal location of the *Rf* locus. By means of GISH, eight chromosomes from *A. galanthum* were clearly discriminated from those of *A. fistulosum* in the F<sub>1</sub> hybrids, and chromosome substitution process through continuous backcrossing was visualized. Interestingly, the chromosome region from *A. galanthum*, specific to male fertile plants, was detected in one chromosome of BC<sub>4</sub> to BC<sub>7</sub> generations. Based on the karyotype analysis of the male fertile plants, the chromosome was identified as the 5F chromosome. Our results confirm that the *Rf* locus is located on the 5F chromosome of the male fertile plants. This is the first report that identified the chromosomal location of the pollen fertility-restoring gene in *A. fistulosum*.

### Introduction

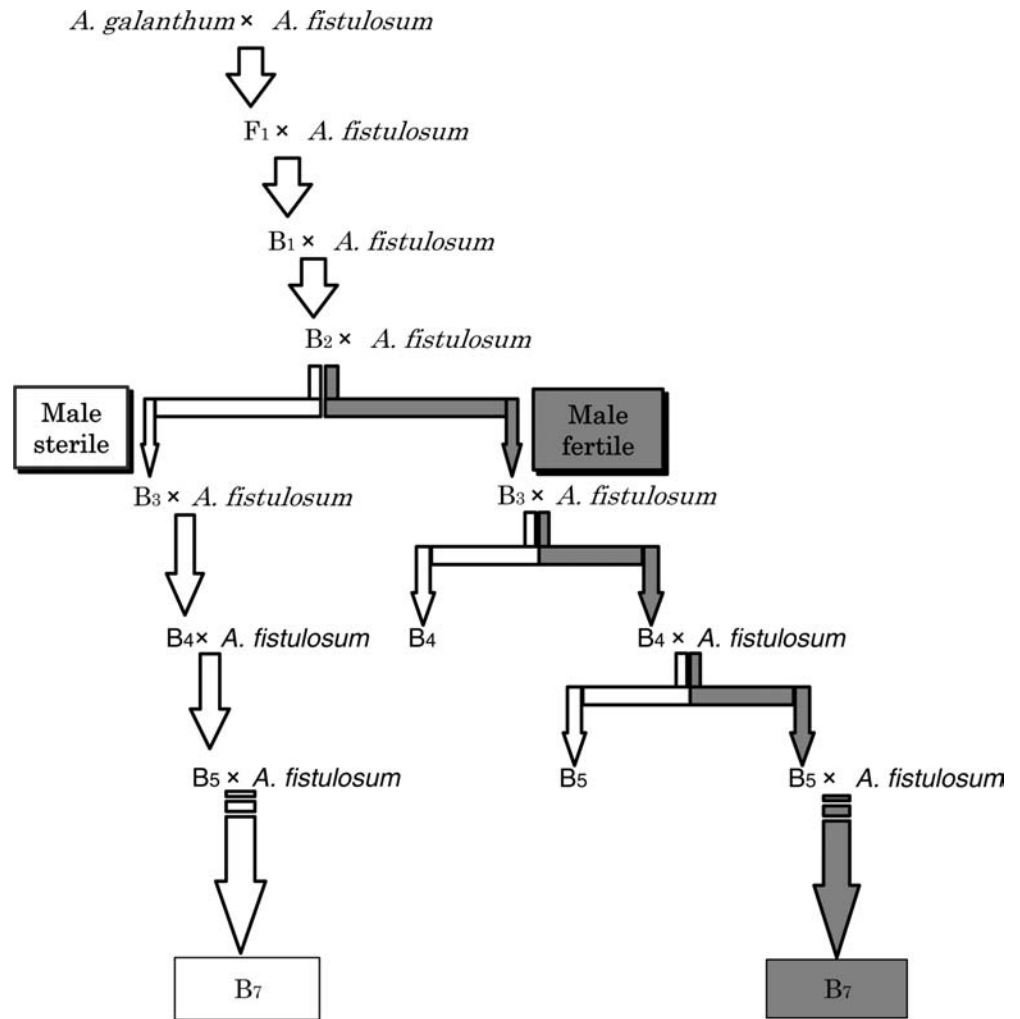
Japanese bunching onion (*Allium fistulosum* L.), which belongs to section *Cepa* of genus *Allium*, is an important vegetable crop in east and southeast Asian countries, especially Japan, China, and Korea (Kumazawa and Katsumata 1965; Inden and Asahira 1990). This species is also one of useful genetic resources for onion (*A. cepa* L. Common onion group), because it has some beneficial characters, e.g., resistance to *Fusarium* basal rot, onion leaf blight, and pink root diseases (Emsweller and Jones 1935; van der Meer and van Bennekom 1978; Entwistle et al. 1990). *A. fistulosum* can be distinguished into two types: one type grown for green leaves, and the other type grown for blanched pseudostems. In Japan, *A. fistulosum* has been consumed long before onion was introduced. At present, many cultivars and strains adapted to different regions exist, and the year-round cultivation is common. Releases of F<sub>1</sub> hybrids to a market have been advanced in *A. fistulosum*; few reports concerning the male sterile line, essential for production of F<sub>1</sub> hybrid seeds, have been published. Spontaneous male sterile plants were found in *A. fistulosum* populations by Nishimura and Shibano (1972), and this male sterility was elucidated to be cytoplasmic male sterility (CMS) by Moue and Uehara (1985). However, the CMS line has not been used for practical F<sub>1</sub> seed production because of disease susceptibility of the CMS line (T. Moue, personal communication). In a previous study, we successfully developed alloplasmic male sterile lines of *A. fistulosum* possessing the cytoplasm of *A. galanthum* Kar. et Kir., a wild species of section *Cepa*, by the continuous backcrossing in which *A. galanthum* was used as a cytoplasm donor and *A. fistulosum* cultivar 'Kujyo' as the recurrent parent (Yamashita et al. 1999a; Yamashita and Tashiro 2004). Backcross progenies were selected in two directions of male sterility and high pollen fertility from the BC<sub>2</sub> generation: all plants of BC<sub>3</sub>, BC<sub>4</sub>, and BC<sub>5</sub> generations selected to the direction

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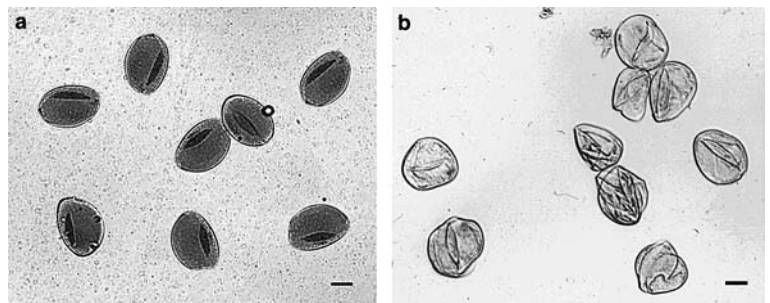
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**Fig. 1** Segregation of pollen fertility in backcross progenies between *Allium galanthum* and *A. fistulosum*



**Fig. 2** Fertile (a) and sterile (b) pollen grains of BC<sub>5</sub> plants. Bar = 10 μm



of male sterility showed male sterility (Figs. 1, 2), whereas, in the BC<sub>3</sub>, BC<sub>4</sub>, and BC<sub>5</sub> plants selected to the direction of high pollen fertility, male fertile and male sterile plants segregated at the ratio of 1:1. From these results, the presence of a single dominant pollen fertility-restoring gene (*Rf*), which originated from the nuclear genome of *A. galanthum*, was confirmed (Yamashita et al. 1999a). The gene *Rf* is essential to control systematically male fertility of *A. fistulosum* possessing the *A. galanthum* cytoplasm in the breeding of F<sub>1</sub> cultivars. Then, we reported that an isozyme gene

locus *Phosphoglucosomerase-1* (*Pgi-1*), a randomly amplified polymorphic DNA (RAPD) fragment in size of 700 bp (OPE03700), and a sequence-characterized amplified region fragment in size of 700 bp (OPJ15700) could be used as markers linked to the *Rf* locus (Yamashita et al. 1999b, 2002). Chromosomal locations of various isozyme gene loci (Shigyo et al. 1994, 1995a, b, 1996) and RAPDs (Shigyo et al. 1997) of shallot (*A. cepa* L. *Aggregatum* group) were determined using alien monosomic addition lines of *A. fistulosum* with extra chromosomes from shallot. Amplified fragment

length polymorphism (AFLP) linkage groups of onion were assigned to the chromosomes of *A. cepa* via the monosomic addition lines (van Heusden et al. 2000). Although production of monosomic addition lines of *A. fistulosum* with wild species chromosomes and genetic analyses of them are now in progress (Tashiro et al. 2000), chromosomal location of the markers linked to the *Rf* locus has not been identified yet. Genomic in situ hybridization (GISH) has been evidenced to be a powerful tool for discriminating different genomes or direct signal detection of chromosome regions introgressed from other species in various *Alliums* (Keller et al. 1996; Buiteveld et al. 1998; Khrustaleva and Kik 1998, 2000; Puizina et al. 1999; Shigyo et al. 1998, 2003; Yamashita et al. 2000a, 2001; Hou and Peffley, 2000; Peterka et al. 2002). In this study, we determined chromosomal location of the *Rf* locus by comparing GISH profiles of the male fertile and male sterile plants of advanced backcross generations.

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## Materials and methods

### Plant material

Two plants of F<sub>1</sub> hybrids between *A. galanthum* (seed parent) and *A. fistulosum* (pollen parent), two plants each from BC<sub>1</sub> to BC<sub>4</sub>, seven plants of BC<sub>5</sub>, four plants of BC<sub>6</sub>, and 12 plants of BC<sub>7</sub> generations were analyzed by GISH. The pollen fertility of the materials had been investigated beforehand by the smear method with acetocarmine: their percentage fertility was calculated from the morphology and stainability of 500 pollen grains per floret in three florets per plant.

### GISH experiment

Root tips of material plants were collected and treated with 0.05% colchicine at 20°C for 2.5 h, after which they were fixed in a mixture of acetic acid and ethyl alcohol (1:3). The procedure of GISH was according to Yamashita et al. (2000a). Total genomic DNA of *A. galanthum* was extracted from fresh leaves by using the cetyltrimethylammonium bromide method, modified by Yamashita et al. (2000b), labeled with biotin-16-dUTP, using the Nick Translation Kit (Roche Diagnostics) and used as the probe DNA, whereas excess of unlabeled genomic DNA of *A. fistulosum* was used as the blocking DNA. One microgram probe DNA and 200 µg blocking DNA were contained in 100 µl hybridization mixture (Yamashita et al. 2000a). After hybridization, probe hybridization signals were amplified with fluorescein avidin DN (Vector Laboratories, Burlingame, Calif., USA) and counterstained with propidium iodide. The GISH image was observed on the fluorescence microscope DMRXA (Leica, Germany) by using the software QFISH (Leica).

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## Results

Pollen fertility, number of chromosomes, and chromosome constitution of material plants are shown in Table 1. Both species of *A. galanthum* and *A. fistulosum* have 16 chromosomes (2n). All the plants of F<sub>1</sub> hybrids and backcross progenies analyzed had also 16 chromosomes (2n), and chromosome deletion or fragment chromosome was not observed. In GISH profiles of the F<sub>1</sub> hybrids, eight chromosomes with yellowish green fluorescence were clearly discriminated from the other eight chromosomes with orange fluorescence (Fig. 3a). The former were identified as *A. galanthum* chromosomes and the latter as *A. fistulosum* chromosomes. In both BC<sub>1</sub> plants, three and five recombinant chromosomes were observed (Fig. 3b; Table 1). With the progress of the backcrossing, chromosome regions of *A. galanthum* per cell drastically decreased (Fig. 3b–e; Table 1). The chromosomes of a male fertile BC<sub>3</sub> plant consisted of 14 chromosomes of *A. fistulosum* and two recombinant chromosomes, whereas that of a male sterile BC<sub>3</sub> plant consisted of 16 chromosomes of *A. fistulosum*. After the BC<sub>4</sub> generation, interestingly, remarkable difference of the amount of *A. galanthum* chromosome region was detected between male fertile and male sterile plants; all of 14 male fertile plants from BC<sub>4</sub> to BC<sub>7</sub> generations possessed one chromosome with the *A. galanthum* chromosome region on its one arm. In contrast, all 11 male sterile plants from BC<sub>4</sub> to BC<sub>7</sub> generations did not possess the chromosome (Fig. 4a, b; Table 1). Except for the chromosome, all the male fertile plants had no recombinant chromosomes. Karyotype analysis was conducted in the male fertile plants of BC<sub>7</sub> generation on the basis of relative chromosome length and position of a centromere according to the nomenclature of Kalkman (1984). Chromosomes 1, 3, 5, and 7 are metacentric, chromosomes 2, 4 and 8 are submetacentric, and the chromosome 6 is subtelocentric in both species. The chromosome with the *A. galanthum* chromosome region was identified as the chromosome 5F of the male fertile plants (Fig. 4c).

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## Discussion

In this study, chromosome substitution process between *A. galanthum* and *A. fistulosum* through the continuous backcrossing was visualized by means of GISH. The chromosomes of F<sub>1</sub> hybrids consisted of each eight chromosomes from the parental species. In a previous study, we reported high frequency of bivalent chromosomes in meiotic metaphase I of pollen mother cells (7.9) in the F<sub>1</sub> hybrid (Yamashita et al. 1999a). Theoretically, eight recombinant chromosomes from the F<sub>1</sub> hybrid should be transmitted to BC<sub>1</sub> plants. However, in both BC<sub>1</sub> plants, the number of recombinant chromosomes was five or three, respectively. The present results illustrate that even if homoeologous chromosomes formed

**Table 1** Pollen fertility and chromosome constitution of F<sub>1</sub> hybrids and backcross generations between *Allium galanthum* and *A. fistulosum*

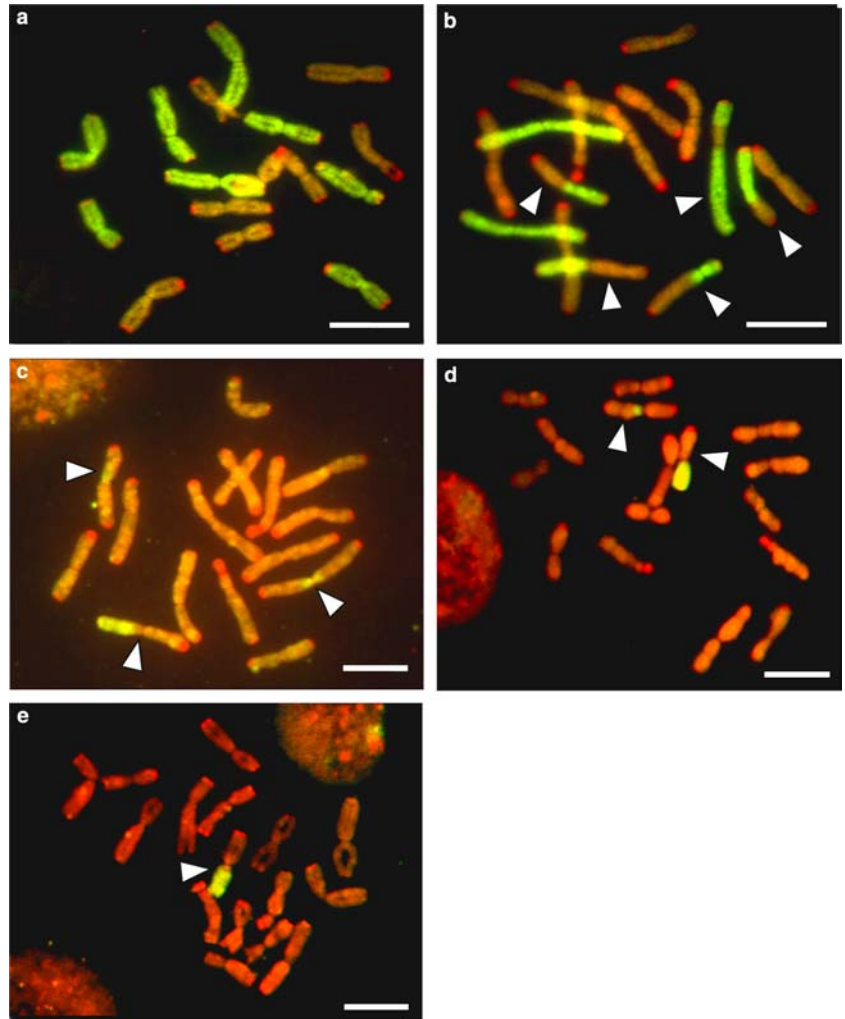
Generation	Line number <sup>a</sup>	Pollen fertility (%)	Number of chromosomes	Number of chromosomes from <i>A. galanthum</i>	Number of chromosomes from <i>A. fistulosum</i>	Number of recombinant chromosomes
F <sub>1</sub>	1 <sup>z</sup>	65.1	16	8	8	0
	2	60.2	16	8	8	0
BC <sub>1</sub>	1-1 <sup>y</sup>	74.2	16	2	11	3
	1-2	10.2	16	2	9	5
BC <sub>2</sub>	1-1-1	0	16	0	14	2
	1-1-2 <sup>x</sup>	97.5	16	0	13	3
BC <sub>3</sub>	1-1-2-1 <sup>w</sup>	95.2	16	0	14	2
	1-1-2-2	0	16	0	16	0
BC <sub>4</sub>	1-1-2-1-1 <sup>v</sup>	97.3	16	0	15	1
	1-1-2-1-2	0	16	0	16	0
BC <sub>5</sub>	1-1-2-1-1-1 <sup>u</sup>	95.3	16	0	15	1
	1-1-2-1-1-2 <sup>t</sup>	97.5	16	0	15	1
	1-1-2-1-1-3	97.5	16	0	15	1
	1-1-2-1-1-4	0	16	0	16	0
	1-1-2-1-1-5	0	16	0	16	0
BC <sub>6</sub>	1-1-2-1-1-6	0	16	0	16	0
	1-1-2-1-1-7	0	16	0	16	0
	1-1-2-1-1-1-1	94.7	16	0	15	1
	1-1-2-1-1-1-2 <sup>s</sup>	96.3	16	0	15	1
	1-1-2-1-1-2-1	0	16	0	16	0
	1-1-2-1-1-2-2 <sup>r</sup>	91.4	16	0	15	1
	1-1-2-1-1-2-1	90.9	16	0	15	1
BC <sub>7</sub>	1-1-2-1-1-2-2	0	16	0	16	0
	1-1-2-1-1-2-3	96.9	16	0	15	1
	1-1-2-1-1-2-4	0	16	0	16	0
	1-1-2-1-1-2-5	0	16	0	16	0
	1-1-2-1-1-2-6	95.9	16	0	15	1
	1-1-2-1-1-2-7	96.7	16	0	15	1
	1-1-2-1-1-2-2-1	97.1	16	0	15	1
	1-1-2-1-1-2-2-2	0	16	0	16	0
	1-1-2-1-1-2-2-3	90.1	16	0	15	1
	1-1-2-1-1-2-2-4	91.2	16	0	15	1
	1-1-2-1-1-2-2-5	0	16	0	16	0

<sup>a</sup>Superscripted r, s, t, u, v, w, x, y, z: Seed parents for next generations

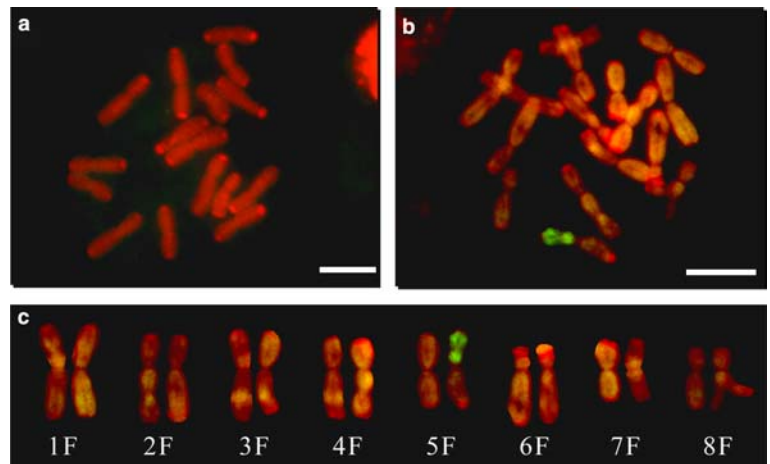
bivalent chromosomes at high frequency in meiotic metaphase I of the interspecific  $F_1$  hybrid, recombination, detectable by GISH, does not always occur. In the continuous backcrossing, the  $BC_1$  plant having such

chromosome constitution was spontaneously selected and used as a seed parent for  $BC_2$  generation (Yamashita et al. 1999a), which would consequently advance the chromosome substitution. Previously, we

**Fig. 3** Genomic in situ hybridization (GISH) profiles of somatic chromosomes in  $F_1$  (a),  $BC_1$  (b),  $BC_2$  (c),  $BC_3$  (d), and  $BC_4$  (e) plants between *A. galanthum* and *A. fistulosum*. Bar = 10  $\mu$ m. Arrowheads show recombinant chromosomes. For more details, see the text



**Fig. 4** GISH profiles of somatic chromosomes in male sterile (a) and male fertile (b) plants of  $BC_7$  generation between *A. galanthum* and *A. fistulosum*, and karyotype of the male fertile plant (c). Bar = 10  $\mu$ m. For more details, see the text



reported that GISH was a useful tool for efficient cytoplasmic substitution between *A. galanthum* and *A. cepa*, provided plants in which the nucleus substitution had advanced earlier in each backcross generation were selected (Yamashita et al. 2000a). The degree of chromosome substitution varied between plants in each BC<sub>1</sub> and BC<sub>2</sub> generations regardless of pollen fertility, though the number of plants analyzed was limited. Therefore, GISH technique is effective for efficient cytoplasmic substitution between *A. galanthum* and *A. fistulosum*, too.

The chromosome on which the *Rf* locus is located was successfully determined to be the 5F chromosome of the male fertile plants by GISH. This is the first report that determined chromosomal location of the pollen fertility-restoring gene locus in *A. fistulosum*. The crossing-over genetically occurs between homoeologous chromosomes from the parental species in meiotic metaphase I of egg mother cells in the F<sub>1</sub> hybrids. Therefore, it is inferred that the gene *Rf* originates from the 5G chromosome of *A. galanthum*. Shigyo et al. (1995b) reported that an isozyme gene locus *Pgi-1* was located on the chromosome 5A of *A. cepa* by analyzing alien monosomic addition lines of *A. fistulosum* with extra chromosomes from *A. cepa*. We previously confirmed that the gene locus *Pgi-1* of *A. galanthum* was linked to the *Rf* locus (Yamashita et al. 1999b). It is presumed that the locus *Pgi-1* of *A. galanthum* is also located on the chromosome 5G of *A. galanthum*.

Large amount of blocking DNA was required in contrast to probe DNA (1:200) for discriminating *A. galanthum* chromosomes from *A. fistulosum* ones in this study. Less than this ratio, it was difficult to discriminate clearly chromosomes of both species due to cross-hybridization of probe DNA to *A. fistulosum* chromosomes. The ratios of probe DNA to blocking DNA needed for discriminating chromosomes of *A. galanthum* from *A. cepa*, and those of *A. cepa* from *A. fistulosum* were 1 to 20 and 1 to 10, respectively (Yamashita et al. 2000; Shigyo et al. 1998). The probe-blocking DNA ratios required in such species combinations would correspond to the degree of their genome homology.

The cytoplasm of *A. galanthum* was demonstrated to induce CMS in shallot (Yamashita and Tashiro 1999) and onion (Havey 1999), as well as *A. fistulosum* (Yamashita et al. 1999a). In the three CMS-cultivated species with *A. galanthum* cytoplasm, the pollen fertility-restoring gene has been found out only in CMS *A. fistulosum*. Alleles to restore male fertility for onion possessing S cytoplasm showed no male fertility restoration for the CMS onion with *A. galanthum* cytoplasm (Havey 1999), indicating that the gene *Rf* regulates the fertility restoration system different from that the *Ms* of onion regulates. We previously observed microsporogenesis of the CMS *A. fistulosum* (Yamashita et al. 1999a): after pollen grain mitosis, binucleate pollen grains were produced. Then, protoplasm of the pollen grains drastically degenerated, finally resulting in empty pollen grains in anthers. The cytoplasm of *A. galanthum* appears to influence the

microsporogenesis at the last stage in *A. fistulosum*. We will embark to verify gene expression of the *Rf* in microsporogenesis of the male fertile *A. fistulosum* possessing the *A. galanthum* cytoplasm.

King et al. (1998) constructed low-density genetic maps of onion consisting of morphological, RAPD, and restriction fragment length polymorphism (RFLP) markers. The *Ms* locus was affiliated to the linkage group B. A most closely related RFLP marker positioned at 0.9 cM from *Ms* locus (Gokce et al. 2002). Most recently, Shigyo et al. (2005) reported that the RFLP linkage group including the *Ms* locus was located on the chromosome 2A of *A. cepa*. The comparison of genetic backgrounds of the genes, *Rf* and *Ms*, such as their functions and constructions will lead to inclusive evaluation of the genes concerning pollen fertility in genus *Allium*. In *A. fistulosum*, the development of molecular markers as AFLP, simple sequence repeat, and cleaved amplified polymorphic sequence, and construction of linkage maps have been just started (Ohara et al. 2003; Song et al. 2004; Tsukazaki et al. 2004). After BC<sub>4</sub> generations, the chromosome region from *A. galanthum* possessing the *Rf* locus was preserved on the 5F chromosome of the male fertile plants. Emsweller and Jones (1935) reported that homologous chromosomes formed plural interstitial chiasmata at diplotene, even if such chromosomes formed localized chiasmata at metaphase I in pollen mother cells of *A. fistulosum*. In contrast, our GISH result represents that the recombination hardly occurred in the chromosome region of 5F of the male fertile plants. The high stability of the region will make the construction of linkage maps of the *Rf* locus difficult, though development of the number of molecular markers linked to the *Rf* locus would be possible. For constructing the linkage maps, it would be necessary to make recombination take place in the chromosome region. Past GISH analyses have visualized various introgression modes of chromosome regions from other species in genus *Allium*: the chromosome regions of *A. galanthum* distributed sparsely in interstitial and terminal regions of chromosomes in BC<sub>1</sub> progeny, but only in terminal regions in BC<sub>3</sub> between *A. galanthum* and shallot (Yamashita and Tashiro 1999). The recombination points distributed randomly in the first and second generations of the bridge cross for introducing *A. fistulosum* genome into *A. cepa* using *A. roylei* as a bridge species (Khrustaleva and Kik 2000). van Heusden et al. (2000) constructed linkage maps of AFLP markers of *A. cepa* and *A. roylei* by analyzing their F<sub>2</sub> populations. By the sib crossing between the male sterile and fertile *A. fistulosum* using *A. roylei* as a bridge species, inducing of recombination might be possible in the *A. galanthum* chromosome region of the 5F chromosome. In those cases, it is premised that *A. roylei* genome does not interfere the gene expression of the *Rf*. Recently, detection of short DNA sequences as RAPD-generated PCR products and single-copy tDNA sequences became possible by fluorescence in situ hybridization or in situ PCR (Benabdelmouna et al.

1999; Khrustaleva and Kik 2001; Mukai and Appels 1996). Hereafter, we intend to determine chromosomal localization of the *Rf* locus by applying such techniques to somatic chromosomes of the male fertile *A. fistulosum*.

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