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Possibility of Developing a Male Sterile Line of Shallot (*Allium cepa* L. Aggregatum Group) with Cytoplasm from *A. galanthum* Kar. et Kir.

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Summary

In an effort to develop a male sterile line of shallot (*Allium cepa* L. Aggregatum group), a continuous backcrossing was carried out using *A. galanthum* Kar. et Kir. as a cytoplasm donor and the shallot as a nucleus donor. Meiosis and fertility in the F_1 hybrids, B_1, B_2, and B_3 progenies were examined.

The F_1 hybrids had low pollen fertility, whereas the B_1 plants were pollen sterile or nearly so; the B_2 and B_3 plants were completely pollen sterile. Pollen sterility observed in the backcross progenies was attributed to nuclear-cytoplasmic incompatibility because the pollen mother cells in most of these plants underwent normal meiosis. Although the seed fertility varied considerably among the progenies at each backcross generation, it could be improved with the advancement of backcrossing. PCR-RFLP analysis of chloroplast DNA proved that all the backcross progenies had the cytoplasm from *A. galanthum*. These results demonstrate that there is a realizable possibility of developing a male sterile line of shallot using this genetic approach.

Key Words: shallot, *Allium cepa*, *Allium galanthum*, cytoplasm, male sterile.

Introduction

Shallot (*Allium cepa* L. Aggregatum group) is an important vegetable crop in Southeast Asia and is also grown in home gardens in Europe and the United States for its bulbs and green leaves (Ochse,1931; Jones and Mann,1963; Hanelt,1990). Shallot is very similar to the common onion (*A. cepa* L. Common onion group) with respect to the inflorescence, karyotype, and meiotic behavior. They differ in that the shallot has a small stature, tillers actively, and forms a cluster of small bulbs (Tashiro et al., 1982). In Southeast Asia, the shallot is more popular and useful than is the common onion because of its high adaptability to tropical and subtropical zones. Although propagated vegetatively, shallot has fertile pollen and seed; therefore, it can be easily crossed with the common onion (Atkin,1953; Tashiro et al.,1982) and *A. fistulosum* L. (Cochran,1942; Tashiro,1984). The shallot germplasm is important as a genetic resource for the improvement of tropical *Allium* crops. Moreover, the establishment of a true seed shallot and the development of F_1 cultivars are expected to have many horticultural advantages, such as high efficiency of propagation and high storage ability and shipping quality of seeds. Furthermore, the seedlings could be made virus free and adaptable to mechanical transplanting. The F_1 cultivars of shallot should have superior agronomic characteristics, such as high uniformity and productivity of bulbs. Male sterile plants are essential to exempt breeders from the difficulty of emasculation and produce large number of the F_1 seeds. In common onion, the first cytoplasmic male sterile (CMS) line was discovered in cv. Italian Red by Jones and Emsweller (1937) and the mode of its inheritance was reported by Jones and Clarke (1943). After that, some CMS lines were identified and characterized (Berninger, 1965; Schweisguth,1973). Using these CMS lines, many superior F_1 seeds of common onion have been released. However, no effective male sterile cytoplasm has been found in shallot yet. The aim of this study is to utilize the cytoplasm of *A. galanthum* Kar. et Kir., a wild species in section *Cepa*, to develop a male sterile line of shallot.

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Materials and Methods

Materials

First, a strain of *A. galanthum* from the IVT (CPRO-DLO) of the Netherlands was crossed with a clone of shallot from Thailand to obtain interspecific F_1 hybrids. *A. galanthum* was used as a seed parent, and shallot as a pollen parent. The F_1 hybrids were continuously backcrossed to shallot; the B_1, B_2, and B_3 progenies were produced. All crossings were made by hand pollination in a screen-covered isolation cage in a greenhouse. The parental plants, F_1 hybrids, and backcross progenies were observed for meiosis and pollen and seed fertility and analyzed for their chloroplast DNA (cpDNA).

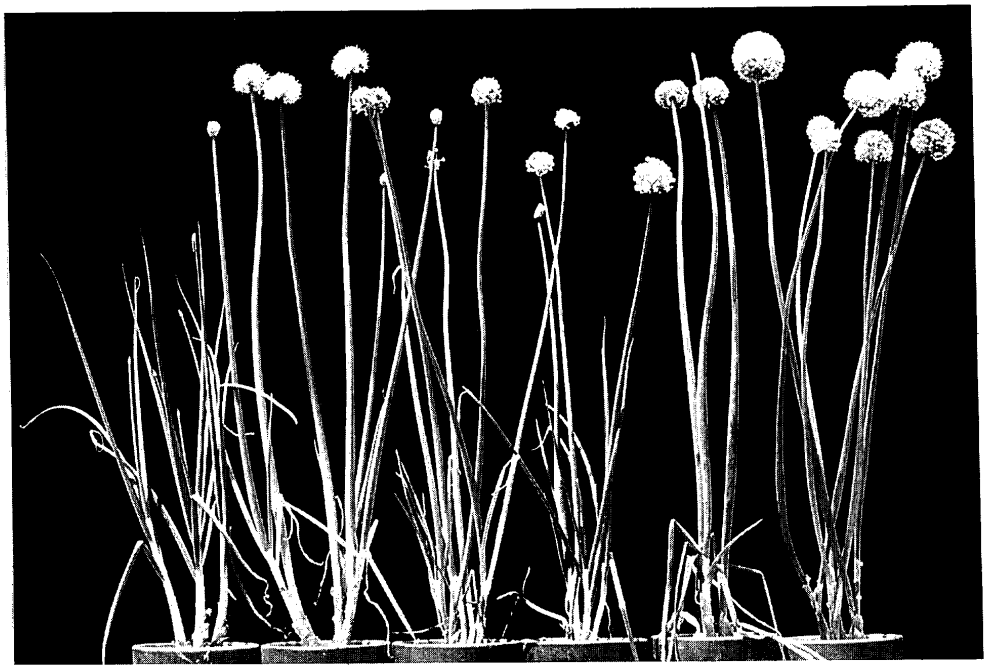

Fig. 1. Plants of *A. galanthum*, F_1, B_1, B_2, and B_3 progenies and shallot (left to right) at flowering stage.

Fig. 2. Basal portion of leaf sheath of *A. galanthum* and bulbs of F_1, B_1, B_2, B_3, and shallot (left to right).

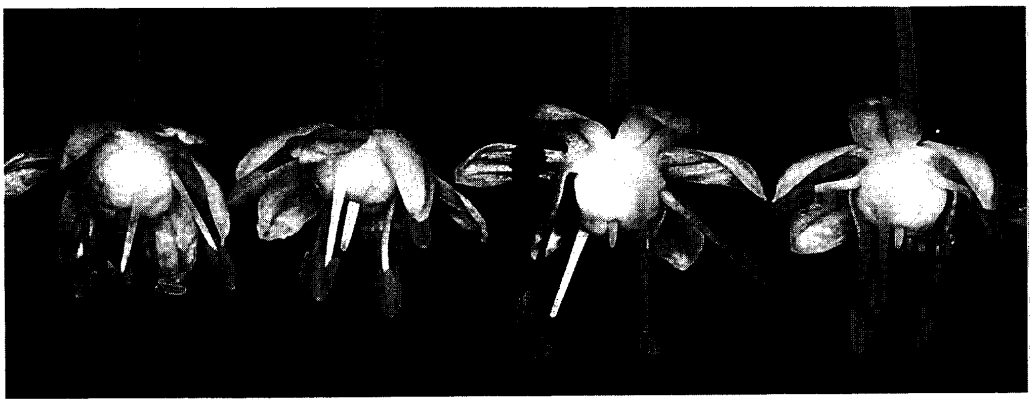

Fig. 3. Florets of shallot (first and second from left) and B_3 plant (third and fourth).

Meiotic observation

Meiosis in each generation was examined to estimate the degree of hybridity of the nucleus. Chromosome pairings at metaphase-I in pollen mother cells (PMCs) from fresh anthers were observed by the smear method

with acetocarmine and the frequency of bivalent chromosomes per PMC was recorded. To observe microsporogenesis, flower buds at different stages were collected and fixed in a mixture of acetic acid and ethyl alcohol (1:3). The microspores in various developmental stages were treated with acetocarmine, smeared as above, and photographed under a light microscope.

Pollen fertility test

Florets were fixed in a mixture of acetic acid and ethyl alcohol (1:3) just before anthesis. Pollen grains were stained with acetocarmine using the smear method and their percentage fertility was evaluated by their morphology and stainability.

Seed fertility test

Seed fertility in both parents, F_1 hybrids, and backcross progenies was estimated from the seed set and the germination rate of seeds when shallot was used as a pollen parent. The seeds were sown on the MS medium without hormone and cultured at 25 ℃ in the dark. Germinated seeds were kept at 25 ℃ under a 12 hr photoperiod; the seedlings were eventually transplanted into pots.

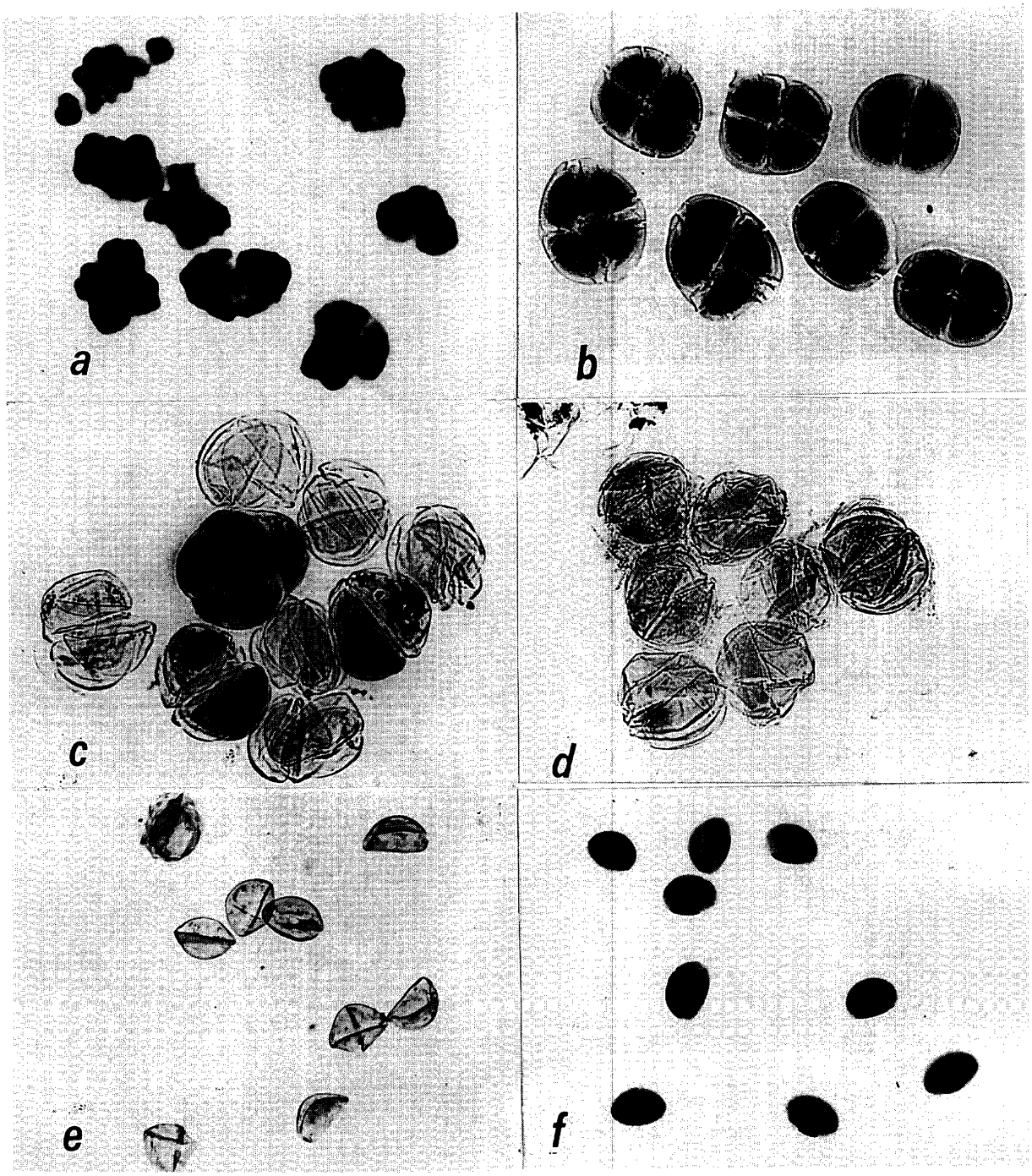

Fig. 4. Chromosome pairing at metaphase-I in a PMC of B_3 plant (a), tetrads of B_3 plant (b), degeneration of protoplasms in microspores of B_3 plant after tetrad stage (c~e), and normal pollen grains of shallot (f).

Analysis of chloroplast DNA

Total DNA was extracted from fresh leaves, using the procedure described by Hong et al. (1997). The oligonucleotide primers, homologous to the region containing the ribulose-1,5-bisphosphate carboxylase gene (*rbc*L) and ORF106, were used for polymerase chain reaction (PCR), according to Arnold et al. (1991). The reaction mixture (50 μl) contained 10mM Tris-HCl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.1mM each dATP, dTTP, dCTP and dGTP, 36pmol of each primer, 128ng of total DNA, and 1.25 units of *Taq* DNA polymerase (TAKARA). DNA amplification was carried out, using the PROGRAM TEMP CONTROL SYSTEM PC-800 (ASTEC). The thermal cycler was programmed for 1min at 92 ℃, followed by 35 cycles of 1 min at 92 ℃, 1min at 55 ℃, and 4min at 70 ℃, with a final 7 min at 70 ℃. The PCR product was digested with restriction enzyme *Alu*I and electrophoresed on 1.8% agarose gel, containing ethidium bromide in 1 × TAE buffer with a size marker. The restriction fragment length polymorphism (RFLP) pattern of PCR product was made visible on a UV transilluminator.

Results and Discussion

The morphological characters and flowering time of

Table 1. Chromosome pairing at meiotic metaphase-I in PMCs, pollen fertility, and seed fertility in *Allium galanthum*, shallot, F_1, B_1, B_2, and B_3 progenies.

Plant material		Chromosome pairing	Pollen fertility (Mean ± S.E.) (%)	Seed set[z] (%)	Germination rate (%)
Allium galanthum		8.0 II	85.0 ± 7.2	6.1	27.3
Shallot		8.0 II	89.4 ± 2.0	21.1	87.0
F_1	104[y]	6.1 II +3.9 I	5.0 ± 1.6	0.04	100
	106	5.5 II +4.9 I	8.8 ± 2.1	0	—
B_1	57[x]	7.6 II +0.8 I	0	1.2	80.0
	58	6.5 II +3.1 I	0	0	—
	1	7.8 II +0.3 I	6.6 ± 3.9	2.0	77.8
B_2	1[w]	7.9 II +0.2 I	0	1.6	50.0
	2	7.8 II +0.3 I	0	4.3	100
	3	7.8 II +0.4 I	0	10.0	97.5
	4	7.8 II +0.4 I	0	1.7	81.8
	6	8.0 II	0	1.0	50.0
	7	7.7 II +0.6 I	0	6.5	82.1
	10	7.8 II +0.4 I	0	7.7	95.1
B_3	11	7.9 II +0.1 I	0	1.4	85.0
	12	7.9 II +0.3 I	0	0.8	14.3
	13	6.1 II +3.9 I	0	0.1	—
	14	3.9 II +8.2 I	0	0.3	66.7
	15	7.7 II +0.6 I	0	1.4	87.5
	16	6.0 II +4.1 I	0	10.2	88.2
	18	7.9 II +0.2 I	0	9.3	84.0
	19	8.0 II	0	3.5	86.0
	20	7.9 II +0.2 I	0	3.0	75.0
	21	7.9 II +0.1 I	0	4.4	86.3
	22	6.9 II +2.3 I	0	3.5	81.8
	23	7.9 II +0.3 I	0	2.4	77.8
	24	7.6 II +0.7 I	0	5.0	90.2
	26	7.0 II +2.0 I	0	6.0	86.7
	27	7.9 II +0.3 I	0	3.9	83.9
	28	7.9 II +0.3 I	0	0.2	100
	29	5.3 II +5.4 I	0	2.9	56.6

[z] Seed set= $\frac{\text{Number of seeds produced}}{\text{Number of flowers pollinated} \times \text{Number of ovules per flower(6)}} \times 100$

[y] Seed parent of B_1.

[x] Seed parent of B_2.

[w] Seed parent of B_3.

the F_1 hybrids were intermediate between *A. galanthum* and shallot. The more backcrossing was repeated, the more the backcross progenies resembled the shallot (Fig. 1). Shallot formed bulbs, but *A. galanthum* did not. All the F_1 hybrids and backcross progenies formed bulbs; B_2 and B_3 plants had almost the same bulb morphology as the shallot (Fig. 2). Most of the backcross progenies showed some abnormalities in anther shape and color, i.e. their anthers were more slender than those of shallot (Fig.3). Moreover, their anthers were whitish yellow, whereas those of the shallot were dark green. Tashiro (1984) produced reciprocal F_1 hybrids between *A. fistulosum* and shallot; he observed hypoplasia of anthers only in progenies between *A. fistulosum* (seed parent) and shallot (pollen parent). He interpreted this phenomenon as a genetic disharmony between the cytoplasm from *A. fistulosum* and the nuclear genome from shallot. Ulloa-G et al. (1995) attempted to introduce the cytoplasm of *A. fistulosum* into the common onion as a source of CMS; they reported the presence of morphological abnormalities, including lack of anther, short petal, small pistil, and shortened filament in B_1 and B_2 progenies. They concluded that the flower abnormality was evidence of a strong incompatibility between the cytoplasm of *A. fistulosum* and the nucleus of the common onion. The abnormalities of anthers observed in our study may, likewise, be evidence of such genetic disharmony or incompatibility between the cytoplasm and nuclear genome from different species.

Both parents formed eight bivalent chromosomes per PMC (Table 1). The frequency of bivalent chromosomes was lower in the F_1 hybrids than in the parents. Compared with the F_1 hybrids, B_1 and B_2 progenies had increased bivalent chromosomes, which indicates that nucleus substitution to shallot nucleus progressed gradually with frequency of backcrossing. Although most plants in the B_3 generation had improved chromosome pairings, the frequency of bivalent chromosomes varied from 3.9 to 8.0. Desynapsis occurred in plants that showed low frequencies of bivalent chromosomes. These plants may have some genetic factor which induces desynapsis.

Pollen fertility in F_1 hybrids decreased drastically in comparison with both parents (Table 1). Of the three B_1 plants, one possessed low pollen fertility, whereas the other two were completely pollen sterile. All plants in B_2 and B_3 generations were completely pollen sterile independent of the frequency of bivalent chromosomes per PMC. A possible cause for low pollen fertility in the F_1 hybrids is attributed to the hybridity of nuclei. However, it is difficult to relate pollen sterility observed in the backcross progenies to the hybridity of nuclei because these plants exhibited high frequency of bivalent chromosomes (Fig. 4a). Therefore, we attribute the pollen sterility in the backcross progenies to an incompatibility between nucleus and cytoplasm.

Microsporogenesis occurred in all the pollen sterile backcross progenies and resulted in a large number of normal tetrads (Fig. 4b). However, the protoplasm gradually degenerated after the tetrad stage (Fig.4c), finally resulting in empty tetrads or a few empty pollen grains in the anthers (Fig. 4d,e). The empty pollen grains were almost the same size as normal pollen grains of shallot (Fig. 4e,f). Thus, we conclude that the cytoplasm from *A. galanthum* intensely influenced the gene expression of the pollen fertility of shallot at microsporogenesis just after the tetrad stage. Anatomical studies of microsporogenesis in male sterile onions reported by Tatebe (1952), Chauhan (1979), Chauhan and Kinoshita (1980), and Holford et al. (1991) attributed the male

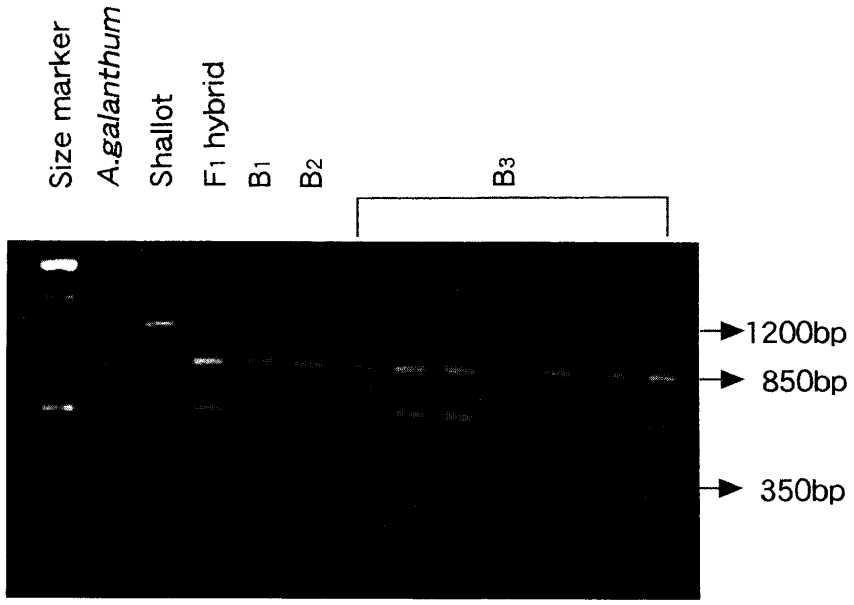

Fig. 5. PCR-RFLP patterns of *rbc*L-ORF106 region of cpDNA in *A. galanthum*, shallot, F_1, and backcross progenies. Amplified DNA fragments were digested with *Alu* I

sterility to abnormal tapetal behavior. Hence, further study is necessary to clarify the mechanism of pollen sterility in our backcross progenies.

F_1 and B_1 plants had seed sets less than 6.1 % in *A. galanthum* (Table 1), whereas seed sets in B_2 and B_3 generation varied from 1.0 to 10.0% and from 0.1 to 10.2 %, respectively. Seed set improved with frequency of backcrossing. Most of the seeds yielded from the backcross progenies were viable, which reveals that the cytoplasm from *A. galanthum* gently governs the expression of gene(s) concerning the seed fertility of shallot. Ulloa-G et al. (1995) reported that most of B_2 progenies between *A. fistulosum* and common onion were completely sterile with respect to both pollen formation and seed set. Therefore, the influence of nuclear-cytoplasmic incompatibility on pollen and seed fertility seems to vary with the sources and combinations of nuclei and cytoplasms within *Allium*.

PCR-RFLP patterns of cpDNA revealed species specific bands of 850bp and 350bp in *A. galanthum* and that of 1200bp in shallot (Fig. 5). F_1 hybrids and all the backcross progenies had the same pattern as *A. galanthum*, which demonstrates that F_1 hybrids and the backcross progenies inherited the cytoplasm from it.

Some wild species in *Allium* have been regarded as genetic resources to improve cultivated species, and they have been crossed with the common onion and *A. fistulosum* to introduce their useful characters (Saini and Davis, 1967, 1969; McCollum, 1974, 1982; van der Meer and de Vries,1990; Kofoet et al.,1990; de Vries et al.,1992a, 1992b; van Raamsdonk et al.,1992). There have been no previous reports on hybridization between *A. galanthum* and shallot, although researchers have crossed *A. galanthum* with the common onion and investigated the fertility of their F_1 hybrids (Saini and Davis, 1967, 1969; McCollum, 1971; van Raamsdonk et al., 1992). According to their studies, the F_1 hybrids obtained from crossing between *A. galanthum* (seed parent) and common onion (pollen parent) had low pollen fertility. Seed fertility of these F_1 hybrids was generally low, although the detailed data were not reported. McCollum (1971) described a hybrid sterility barrier based on seed sterility in the F_1 hybrids between the common onion and *A. galanthum* or *A. pskemense* B. Fedtsch. He concluded that this sterility barrier should not prevent the use of these species in onion breeding, but it would cause difficulties in early generations of crosses. Some isolation barriers between *Allium* species were also mentioned by van Raamsdonk et al. (1992), based on the cross incompatibility and sterility of F_1 hybrids. In our study, pollen and seed fertility was very low in the F_1 hybrids between *A. galanthum* and shallot; therefore, it seems that a severe isolation barrier existed in the F_1 hybrids. However, once the B_1 generation was obtained, B_2 and B_3 generations were easily obtained. Most fertility studies on interspecific crossings in *Allium* mentioned above finished with the F_1 or B_1 generations. Our results suggest that it is worthwhile to backcross continuously with the different species in *Allium* even if the F_1 hybrids or early backcross generations have low seed fertility.

Our data indicate that the cytoplasm from *A. galanthum* significantly influences only the pollen fertility of shallot and that there is a possibility of developing a male sterile line of shallot with the cytoplasm from *A. galanthum*. Additional backcrosses are in progress. Our study also suggests that the cytoplasms of other wild species may be useful genetic resources for the breeding in *Allium*. Currently, the cytoplasms of other wild species, *A. oschaninii* O. Fedtsch., *A. vavilovii* M. Pop. et Vved., and *A. altaicum* Pall., are being explored.

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Allium galanthum Kar. et Kir. の細胞質を有するシャロット雄性不稔系統の育成の可能性

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摘　要

*Cepa*節野生種*A. galanthum* Kar. et Kir. の細胞質を利用したシャロット雄性不稔系統の育成の可能性を検討するため，これら2種間で連続戻交雑 (*A. galanthum* が細胞質提供親) を行い，F_1，B_1，B_2 および B_3 について減数分裂，花粉稔性および種子稔性の調査を行った．F_1 ではわずかに稔性花粉が観察されたが，B_1 世代では花粉不稔を示す個体が出現し，B_2 および B_3 ではすべての個体が花粉不稔となった．戻交雑後代で花粉不稔となった個体の多くは正常な減数分裂を行ったため，この花粉不稔はシャロットの核と*A. galanthum* の細胞質の不和合によって引き起こされると考えられた．種子稔性は各世代の個体間で分離したが，戻交雑が進むにつれて回復した．葉緑体DNAのPCR-RFLP分析の結果，すべての戻交雑後代が*A. galanthum* 由来の細胞質を有していた．本研究の結果は，*A. galanthum* の細胞質を利用したシャロット雄性不稔系統の育成が可能であることを示している．