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Interspecific hybrids between onion (*Allium cepa* L.) with S-cytoplasm and leek (*Allium ampeloprasum* L.)

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Abstract Interspecific hybrids between *Allium cepa* and *A. ampeloprasum* have been generated as a first step for the introduction of S-cytoplasm from onion into leek. Pre-zygotic barriers of crossability were observed after the arrival of pollen tubes at the end of the style when entering the cavity. Nevertheless, micropyle penetration of pollen tubes and the formation of hybrid embryos were also observed. After accomplishing in vitro culture of ovaries and ovules successively, triploid hybrid plants with 24 chromosomes were obtained. Their hybrid nature was confirmed by RAPD analysis, genomic in situ hybridization, and morphological analysis. Southern hybridization with a cytoplasmic probe indicated the transfer of unaltered S-cytoplasm into the hybrid plants.

Key words Interspecific hybrid · Onion · Leek · CMS · GISH

Introduction

Onion and leek belong to cultivated vegetable species of the *Allium* genus. Onion is a diploid (2n = 2x = 16), whereas leek (2n = 4x = 32) is a presumably weak segmental allotetraploid (Khazanehdari et al. 1995). Breeding of the two species proceeds in a quite different way. Modern onion breeding is almost completely based on the production of hybrid seed. This is produced by means of cytoplasmic male sterility (CMS) which is induced by the interaction between S- or T-cytoplasm (Jones and Emsweiler 1936; Berninger 1965) and one or three nuclear genes (Jones and Clarke 1943; Schweisguth 1973).

In leek, no source of CMS is known as yet. Several strategies have been proposed to obtain cytoplasmic male-sterile leek, by the selection of spontaneous and induced mutants or by the combination of the leek nucleus with an alien cytoplasm (Silvertand and van Harten 1992). Kampe (1980) suggested crossing onion and leek to introduce the S-cytoplasm into leek. Regarding the negative results in the generation of the initial interspecific cross reported by Dolezel et al. (1980) this strategy seemed to be unrealizable (Currah 1986). The latter author proposed to identify a sterile cytoplasm in a more closely related species and to use it in leek breeding. From his study of diversity of chloroplast genomes in *Allium*, Havey (1991) concluded that the use of sexual hybridization for gene transfer between *A. ampeloprasum* and *A. cepa* may be difficult. However, Ohsumi et al. (1993) demonstrated successful sexual hybridization between such distantly related species as onion and garlic which taxonomically belongs to the same subgenus *Allium* as leek (Hanelt 1990). They studied pro-gamic barriers by means of fluorescence microscopy and post-gamic ones using differential interference contrast (DIC) microscopy.

The principal aim of our work was to produce an interspecific hybrid between onion with S-cytoplasm and leek that could be used in further experiments for the induction of CMS into leek.

Materials and methods

Plant material

Ten different idiotypes of common onion, nine male-sterile with S-cytoplasm ('Argo' 'Balstora', 'Copra', 'Mambo', 'Summit', ms1, ms2, ms3, ms4) and one male-fertile with N-cytoplasm (Fix1), were

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used as female parents. Inbred lines of four leek varieties ('Pollux', 'Labrador', 'Carentan', 'Bleustar') and one unknown line were used as pollen parents.

The plant material was grown in the greenhouse without temperature control. Except for idiotype Fix1 female onion plants were isolated without emasculation. Fresh leek pollen was applied to receptive stigmata with a paintbrush.

Embryo rescue

Only swollen and greened ovaries were harvested 7–14 days after pollination (dap). After removal of the perianth, selected ovaries were rinsed for 30 s in 70% (v/v) ethanol and surface sterilized in 2% (w/v) sodium hypochlorite solution for 30 min. Then the ovaries were rinsed for 10 min in sterile tap water and transferred to Petri dishes with MS medium. The ovaries were kept for 14 days at 22°C at about 2000 lux with a 16 h day/8 h night. Thereafter, black-coloured ovules were dissected and cultivated in darkness for a further 14 days. Germinating embryos remained 10–12 weeks under illumination until differentiation of roots and green leaves occurred. The plantlets were transferred into soil and kept for 2 weeks at 22/18°C under wet conditions. The plants were potted and cultivated in the greenhouse.

Observation of pollen-tube growth and early embryo formation

Pollen-tube growth was detected by aniline-blue staining according to the method of Kho and Baer (1968). Twenty four hours after cross-pollination, the ovaries with styles were excised and treated with 1 N NaOH for 1 h at room temperature, washed with water, and stained with 0.05% (w/v) aniline blue in 0.1 M K_3PO_4. The pistil was dissected gently into its three compartments leaving the style nearly unpartitioned. The pollen tubes were observed by fluorescence microscopy.

Embryo-sac contents was analyzed by the clearing method (Herr 1982). Ovaries were fixed 4–7 dap in formalin/propionic acid/ 50% ethanol (5:5:90, v/v) for 24 h and stored in 70% ethanol. Ovules were excised from ovaries, dehydrated in absolute ethanol, and transferred to clearing fluid (lactic acid/chloral hydrate/phenol/clove oil/xylene/benzyl benzoate, 2:2:2:2:1:1, w/w). After 6 h, the cleared ovules were analyzed by differential interference contrast optics (Nikon Optiphot-2).

Organelle genome characterization

A DIG-labelled 2.5-kb *Eco*RI-fragment of the *cox*II gene from *Zea diploperennis* cloned in pUC9 and a 4.3-kb *Bam*HI fragment of the *cob* gene cloned in pBR322 were used as mitochondrial (mt) DNA probes (A. Brennecke, personal communication). Total plant DNA was isolated from 1 g of leaf tissue using the method of Rogers and Bendich (1985). For restriction-endonuclease digestion and Southern transfer, standard protocols were employed (Sambrook et al. 1989). Hybridization with 100–200 ng of DIG-labelled probe DNA and chemiluminescent detection was done using standard protocols (Düring 1991), except that the hybridization temperature was 37°C.

RAPD analysis

RAPD analysis was performed in 25-µl reactions containing 40 ng of total plant DNA, PCR buffer (50 mMKC1, 10 mM Tris-HCl pH 8.3), 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.1 µM primer, and 0.6 U of Ampli-*Taq* DNA polymerase (Perkin Elmer-Cetus). Fragments of DNA were amplified after a 5-min denaturation step at 95°C for 40 cycles (94°C 45 s, 35°C 45 s, 72°C 1.5 min) in an Autogene II Thermal Cycler (Grant) with the final cycle extended so that the 72°C incubation period was 10 min. Amplification products were analyzed by electrophoresis on 1.5% agarose gels in 1 × TAE buffer. After electrophoresis, gels were stained with ethidium bromide, then de-stained and photographed. Primer OPB 01 (Operon Technologies Inc.) has the sequence 5′-GTTTCGCTCC-3′.

Chromosome preparation

Excised roots of potted plants were pre-treated in α-bromonaphthalene at 4°C for 24 h, fixed in 3: 1 ethanol/acetic acid for 24 h and stored in 70% ethanol. The fixed root meristems were thoroughly washed in water and incubated in an enzyme mixture (2.5% cellulase and 2.5% pectolyase in 75 mM KCI buffer, pH 4.0) for 15 min at 37°C. After enzyme maceration, the root tips were squashed in 2% orcein acetic acid for microscopic observation, or in 45% acetic acid for genomic in situ hybridization.

Genomic in situ hybridization

After removing the cover slips at −80°C and drying for 3–4 h, slides carrying metaphase spreads were treated with RNase (0.1 µg/µl in H_2O for 40 min at 37°C) and Proteinase K (1 ng/µl in 20 mM Tris; 2 mM $CaCl_2$ pH 7.4 for 15 min at 37°C), re-fixed in paraformaldehyde (4% in SSC for 10 min at 20°C), denatured (50% formamide in 2 × SSC for 7 min at 75°C) and quickly cooled in 96% ethanol.

Hybridization and detection steps were according to Jacobsen et al. (1995) with the following modifications. The hybridization mix contained 10 ng/µl of *A. cepa* DNA labelled with DIG-11-dUTP and 0.2 µg/µl of *A. ampeloprasum* competition DNA that was sheared by autoclaving for 5 min, generating fragments of about 150 bp (data not shown). For post-hybridization washes slides were incubated 2× for 10 min in 50% formamide, 1 × SSC at 37°C and 3× for 5 min in 0.5 × SSC at 50°C. We performed detection with anti-DIG-FITC from sheep and amplified the signal with anti-FITC from mouse and anti-mouse-FITC from sheep (all from Boehringer-Mannheim). Vectashield (Vector) was used as an antifade.

Results

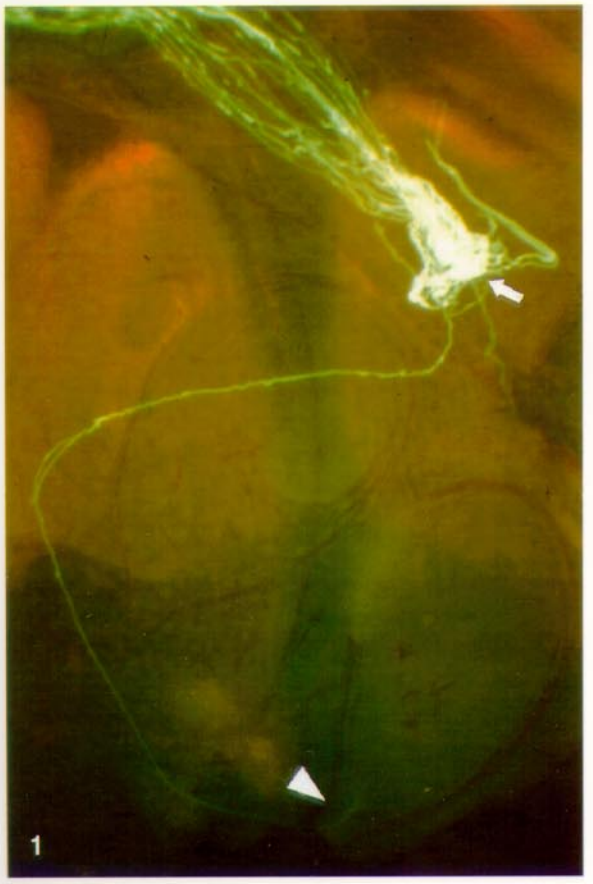
Pollen tube growth and fertilization

Germination of leek pollen on onion stigma and further growth throughout the style appeared undisturbed under optimized crossing conditions. A major part of the pollen tubes reached the end of the styles but there

Fig. 1 Growth of leek pollen tubes in an onion ovary. At the bottom of the style most pollen tubes cease growing (*arrow*). A single pollen tube penetrates the micropyle (*arrowhead*) of an ovule. (× 40)

Fig. 2 Early embryo development after onion × leek hybridization. A few-celled hybrid embryo 7 days after pollination (*arrowhead*) is visible. (× 200)

Fig. 3A, B Somatic metaphase cell of an interspecific onion × leek hybrid after genomic in situ hybridization. **A** PI-stained chromosomes. **B** Orange-red PI fluorescence of 16 *A. ampeloprasum* chromosomes and yellow-green fluorescence of eight *A. cepa* chromosomes hybridized to DIG-labelled DNA of *A. cepa*. (both × 1200)



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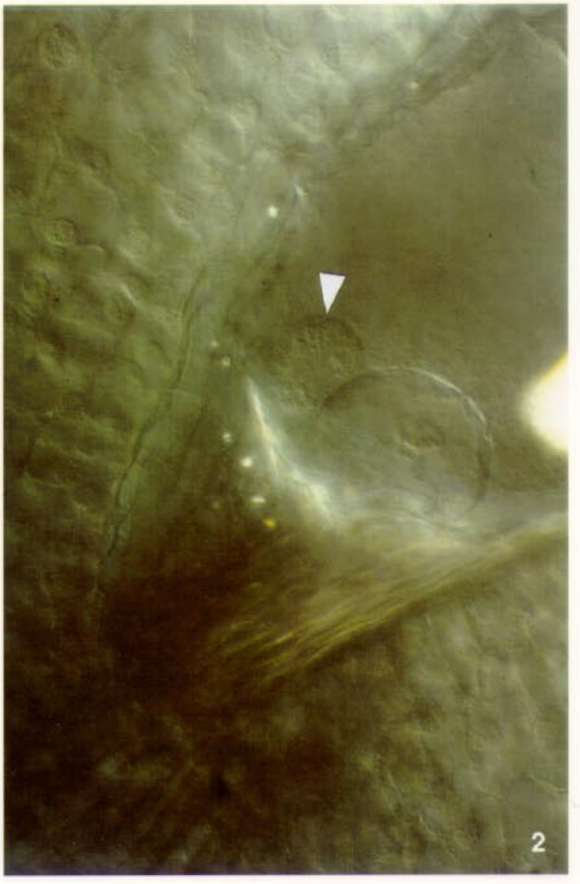

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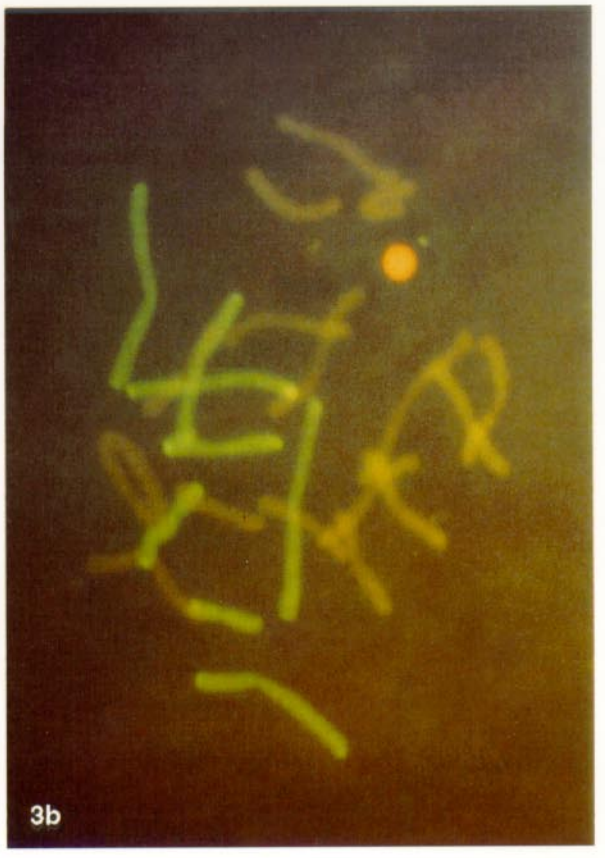
3a



3b

they stopped growing and did not elongate into the cavity or penetrate the micropyle. In only very few pistils was a single pollen tube seen to grow into the micropyle (Fig. 1). Tips of pollen tubes must have reached the egg apparatus so that fertilization could occur because formation of hybrid embryos in embryo sacs was observed (Fig. 2).

Formation of hybrid embryos and plants

From the 1081 ovaries harvested, 449 black-coloured ovules were selected, which corresponds to a percentage of 6.9% taking into account that each ovary contains six ovules (Table 1). Only 23 (5.1%) of the ovules contained germinating embryos growing with a white-tipped root pole out of the micropylar opening. Two ovules contained more than one embryo. Only one ovule, which gave rise to plant 105/1, had a normally developed embryo and endosperm.

Embryo formation was observed after crosses with each of the five leek idiotypes and with four of the ten onion idiotypes (Table 1). This rules out a strong idiotypic dependence of crossability between the two species. Not all embryos differentiated into plants. Altogether eight plants were obtained after embryo culture, which corresponds to a growth rate of about 20%. Flowering occurred after vernalization at temperatures between 10 to 15°C for 6–8 weeks.

Hybridity

Plant 105/1 was presumably a selfing plant possessing 16 chromosomes. All the remaining seven plants obtained were interspecific hybrids. Their somatic chromosome number was $3x = 24$ as would be expected from the parents, onion having 16 and leek having 32 chromosomes (Fig. 4).

Three chromosomes with satellites and two chromosomes with intercalary pseudosatellites could be identified in the mitotic metaphase of the hybrid, which are expected from the parental karyotypes. Onion possesses two chromosomes with satellites (Kalkman 1984). Leek has four satellited chromosomes and four chromosomes with intercalary pseudosatellites (Murin 1964).

The chromosomes of the parental genomes could be unambiguously distinguished by genomic in situ hybridization using an onion DNA probe (Fig. 3). Eight chromosomes of the hybrid plant 123/1 hybridized with onion DNA and 16 chromosomes did not.

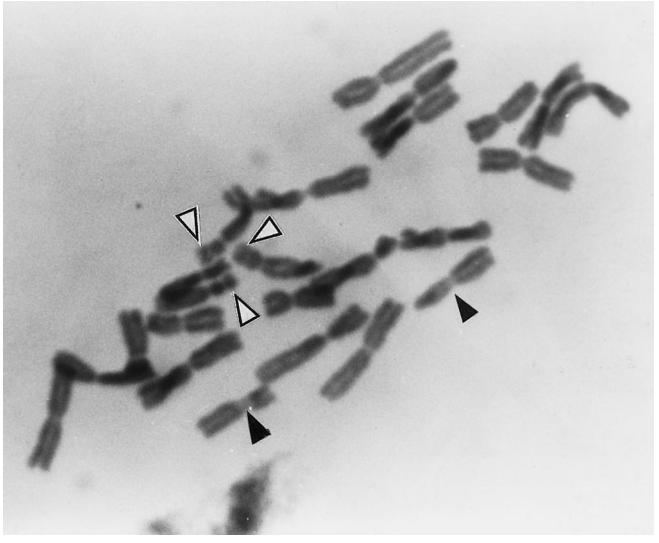


Fig. 4 Chromosomes of the interspecific hybrid plant 123/1 in mitotic metaphase ($2n = 3x = 24$). Three chromosomes have satellites (*hollow arrowheads*) and two chromosomes have pseudosatellites (*filled arrowheads*). (× 1000)

Table 1 Production of hybrid plants after crosses with ten onion idiotypes as females and five leek idiotypes as males

Cross combination		Number of harvested ovaries	Number of prepared ovules	Number of germinated embryos	Number and code of developed plants
A. cepa idiotype	*A. ampeloprasum* idiotype				
'Argo'	'Pollux'	44	116	0	0
'Balstora'	'Pollux'	21	10	0	0
'Copra'	'Pollux'	40	60	0	0
'Mambo'	'Pollux'	21	0	0	0
'Summit'	'Pollux'	103	14	4	1 (99/1)
Fix1	'Pollux'	18	0	0	0
ms1	'Pollux'	40	57	1	1 (105/1)
ms1	'Labrador'	138	59	6	2 (114/1), (114/2)
ms1	'Carentan'	29	30	3	1 (123/1)
ms1	'Bleustar'	54	46	6	2 (124/1), (124/4)
ms2	'Pollux'	126	46	1	1 (84/1)
ms3	'Pollux'	25	1	0	0
ms4	'Pollux'	238	9	1	0
ms4	unknown	184	1	1	0
Total		1081	449	23	8

In Fig. 5 RAPD-profiles of the four onion × leek hybrid plants, having the same onion parent ms1, are compared with parental profiles. The hybrid plants displayed DNA fragments of both parents. Because of the heterozgosity of the parents not all parental fragments occurred in the hybrid profiles.

Type of cytoplasm

Southern hybridization with the mt-DNA probe *cox*II revealed no differences between the hybridization pattern of the hybrids and the S-cytoplasm of the female parent ms1 (Fig. 6). Hybridization patterns of another mt-DNA probe (*cob*) gave the same result (data not shown).

Morphology

The hybrid plants developed flat, keeled leaves (Fig. 7) and a round, solid scape. Flowers are globular in shape and never open widely as they do in *A. cepa* (Figs. 8, 9). Individual hybrids differ in their ability to form lateral bulbs. Hybrid 84/1 developed clumps of green tops whereas in hybrid 99/1 no lateral buds were formed.

Discussion

The production of hybrid varieties could allow a more effective and rapid breeding in a tetraploid, biennial crop reacting sensitively to inbreeding. Hybrid leek is

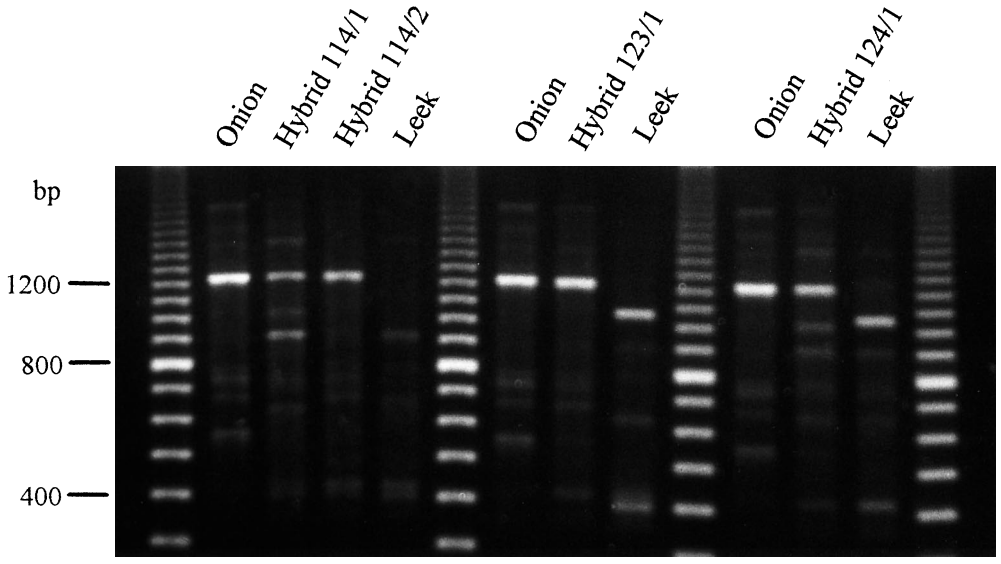


Fig. 5 RAPD-profiles of four interspecific hybrids 114/1, 114/2, 123/1 and 124/1 and their corresponding onion and leek parents. The size marker is a 100-bp molecular-weight standard of Pharmacia

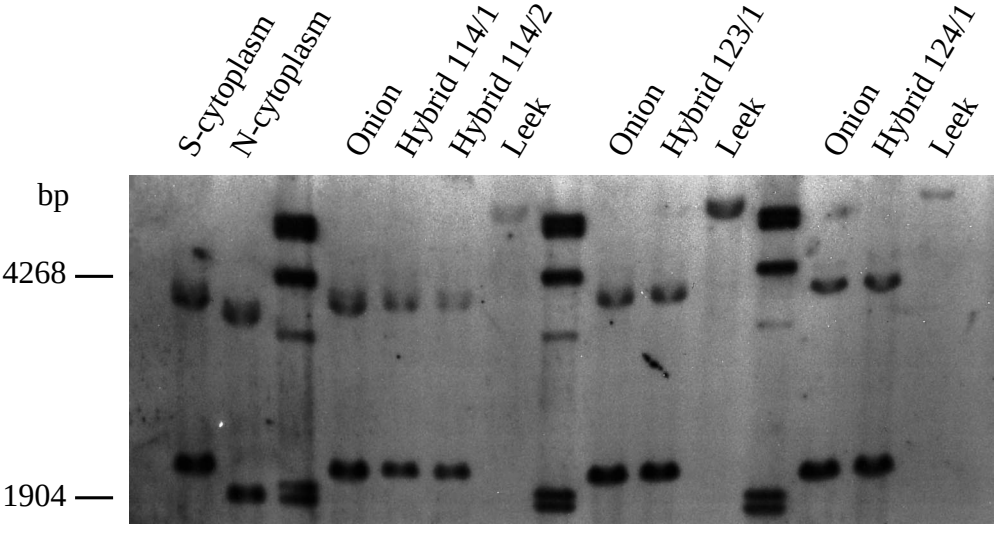


Fig. 6 Southern-blot hybridization of *Eco*RI-digested total DNA from *A. cepa* with S-cytoplasm and with N-cytoplasm, interspecific hybrids 114/1, 114/2, 123/1 and 124/1, and their corresponding onion and leek parents using a *coxII* probe. The size marker is the DIG-labelled molecular-weight standard III of Boehringer Mannheim

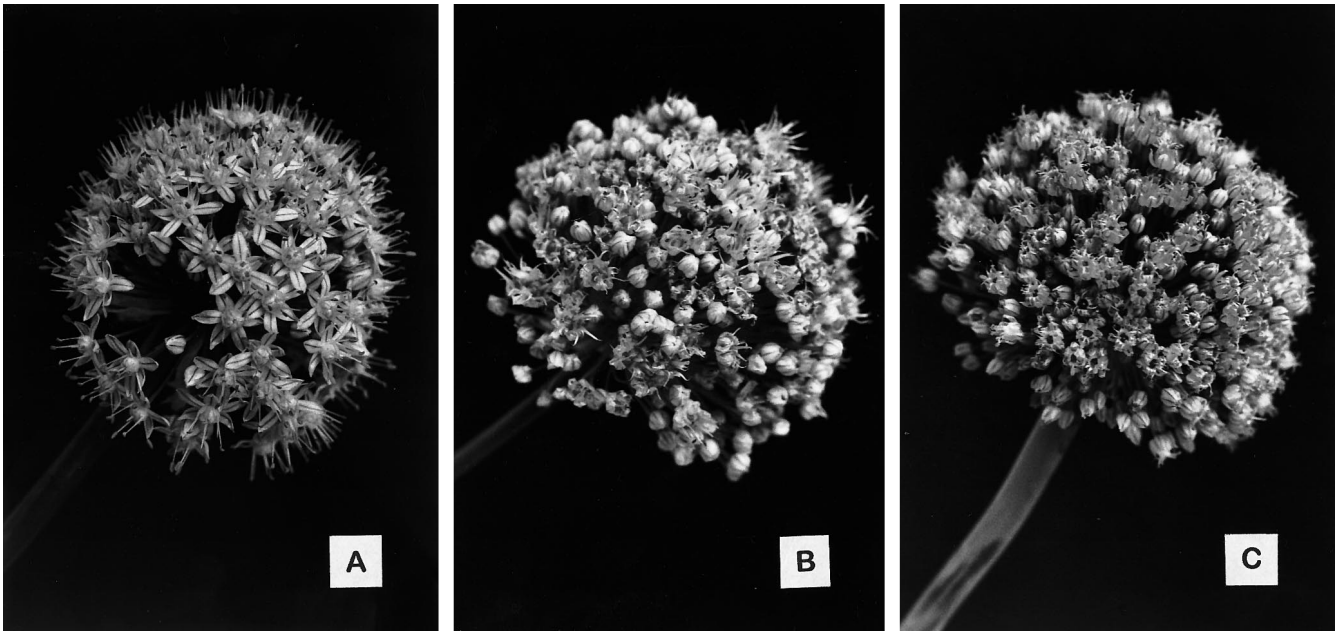
Fig. 7 Interspecific hybrid of *Allium cepa* and *A. ampeloprasum*. *A. cepa* (left), hybrid plant (center), and *A. ampeloprasum* (right) are shown

Fig. 9 Flower morphology of parents and hybrid. Intermediate flower characteristics of an interspecific hybrid (center) in comparison to *A. cepa* (left) and *A. ampeloprasum* (right)

superior to open-pollinated commercial cultivars in relation to uniformity, yield, and quality (Kampe 1980; Smith and Crowther 1995). A difficulty arising in the realization of hybrid breeding in leek is the lack of a cytoplasmic male sterility system. Transfer of CMS from other *Allium* species by somatic or sexual hybridization could solve this problem. The latter would be preferred because alien CMS-plasm will not mix with leek cytoplasm, as in protoplast fusion, which would probably diminish the desired male-sterile effect.

Using in vitro culture of selected ovaries and subsequently of ovules, we have shown that onion and leek can be successfully hybridized though these two species are not closely related. Onion (*Allium cepa*) belongs to the section *Cepa* of the subgenus *Rhizirideum* of the genus *Allium* whereas leek (*A. ampeloprasum*) is a member of the section *Allium* of the subgenus *Allium* (Hanelt 1990). This is the third report of hybrid plant production between the two subgenera, following that of hybridization of onion with garlic by Ohsumi et al. (1993) and of *A. cepa* with *A. sphaerocephalon* (Keller et al. 1996). Obviously, wide taxonomic distances between *Allium* species do not hinder fertilization between them. On the contrary, our preliminary attempts to cross *A. sphaerocephalon* with leek, which belong to the same subgenus, indicated strong barriers early after pollina-

Fig. 8A–C Inflorescences of parents and an interspecific hybrid. An umbel is shown for *A. cepa* (**A**), the hybrid plant (**B**) and *A. ampeloprasum* (**C**)



tion (data not shown). There is no close correlation between the level of relationship and crossability in the genus *Allium* (Van Raamsdonk et al. 1992).

The triploid constitution of hybrid plants was confirmed by cytological analysis. Genomic in situ hybridization with onion DNA allowed the identification of onion and leek chromosomes. The ultimate goal of this project is to combine leek genomes with the S-cytoplasm of onion. Therefore, this technique should be a valuable tool for subsequent backcrosses to leek in order to identify whole onion chromosomes, or parts of them, in backcross plants, similar to work with somatic hybrids between potato and tomato (Jacobsen et al. 1995). Our investigations in onion, leek, and the hybrids demonstrate the maternal inheritance of mt-DNA. Studies of cp-DNA transmission after intraspecific crosses of onion demonstrated exclusively maternal inheritance (Corriveau and Coleman 1988). It is not known if paternal transmission of cytoplasmic components can occur in leek. Therefore, it is necessary to control the type of cytoplasm in the intended repeated backcrosses.

The events which result in the abortion of the microspores in male-sterile onion containing S-cytoplasm are preceded by the abnormal development of the tapetum (Tatebe 1952; Holford et al. 1991). Pollen viability of onion × leek hybrid plants was almost zero. However, this can not only be the effect of S-cytoplasm. Rather, it could be due to irregular meiotic chromosome pairing of parental genomes in the triploid hybrid.

Until now, no embryo formation has been observed using hybrids in backcross pollinations with leek. Further investigations are required to determine if crossing barriers or disturbed development of the female gametophyte are the responsible factors. In the latter case the use of chromosome-doubled interspecific hybrids could be of value.

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