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T. Engelke · T. Tatlioglu

Mitochondrial genome diversity in connection with male sterility in *Allium schoenoprasum* L.

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Abstract Mitochondrial genome diversity in chives (*Allium schoenoprasum* L.) was investigated with respect to different forms of male sterility. Cytoplasmic male-sterile (CMS) and restored genotypes of the known CMS system, compared to plants of the *wi*-, the *st1*- and the *st2*-sterility types and additional fertile plants of different origin were examined by means of RFLP analyses using mitochondrial gene probes. Besides the (S)-cytoplasm of the CMS system four additional cytoplasms were distinguished that differed in the organisation of their mitochondrial genomes. There is consequently a high degree of variability of the mitochondrial genome in chives, especially when compared with the closely related onion. A possible function of the *atp*9 gene in generating the different cytoplasm types of chives is discussed in relation to the origin of known CMS sequences in other plant species. The existence of different cytoplasm types offers the opportunity for further characterisation of the *wi*-, *st1*- and *st2*-sterility systems with respect to cytoplasmic factors which might be causally related to them. Whether these new sterilities are CMS or GMS (genic male sterilities) is of interest to plant breeders in order that restrictions on the genetic basis used in hybrid seed production be avoided.

Key words mtDNA-diversity · atp9 · Male sterility · Chives

Introduction

Mitochondrial genome diversity has received greater attention since it became obvious that recombination events inside the mitochondrial genome are responsible for cytoplasmic male sterility (CMS). CMS has been the most frequently used mechanism in hybrid seed production of different crops since Jones and Clarke (1943) first described the inheritance and the handling of this character in *Allium cepa*. In most cases CMS can be explained by a maternally transmitted factor and an additional factor with Mendelian inheritance. While the Mendelian factor is mostly caused by one nuclear restorer gene, the maternally inherited factor must be traced back to mutations in the mitochondrial genome and not to mutations within the chloroplasts (Belliard et al. 1978; Pelletier et al. 1983; Clark et al. 1985).

The genetic basis of the first characterised male sterility in *Allium schoenoprasum* consists of a sterility-inducing cytoplasm (S) and three independently acting nuclear genes (*X, T, a*) that restore the male fertility in this cytoplasm. The gene *X* leads to a stable restoration, while gene *T* only works at high temperatures and gene *a* causes pollen production only in combination with a tetracycline treatment. Plants carrying the normal cytoplasm (N) are always male-fertile, regardless of the constitution of the three nuclear genes (Tatlioglu 1982, 1987; Tatlioglu and Wricke 1988). Furthermore, three additional male sterilities have been discovered that show a monogenic recessive inheritance in the offspring of sterile plants after pollination with male-fertile plants (Tatlioglu 1994). These three genes, *wi*, *st1* and *st2*, are nonallelic and different from those of the previously described CMS system (Engelke and Tatlioglu 1996). For further investigations concerning cytoplasmic factors which might be causally related to the new sterilities, mitochondrial genome diversity in chives should be examined by means of restriction fragment length polymorphism (RFLP) analysis using mitochondrial gene probes. The results from such investigations would provide the opportunity to transfer the nuclear genes to different cytoplasm types. This in turn would clarify if the genes *wi*, *st1* and *st2* show themselves in these different cytoplasms or if additional cytoplasmic factors are necessary for the occurrence of one of these sterilites.

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T. Engelke (✉), T. Tatlioglu
Abteilung Angewandte Genetik, Universität Hannover,
Herrenhäuser Str. 2, D-30419 Hannover, Germany
e-mail: thomas.engelke@mbox.genetik.uni-hannover.de
Fax: +49-511 762 3608

Materials and methods

Plant materials

Fertile plants as well as plants carrying the (S)-cytoplasm (sterile and restored) were used. With respect to the three new male sterilities (*wi*, *st1* and *st2*) sterile and fertile plants of segregating offspring were chosen. The *wi*-sterile plants were selected from four different F_2 lines descending from female parents of various origins: cv. Wilau, Grolau, Gigantic and plant 111 of the basis materials (Tatlioglu 1994).

DNA extraction, digestion and blotting

For genomic DNA isolation flowers were collected and frozen in liquid nitrogen. The procedure was performed according to a protocol described by Bentzen et al. (1990). This is an altered CTAB method (Saghai-Maroof et al. 1984) using Serum separation tubes (Becton Dickinson) for separating the organic and inorganic phases during the phenol and chlorophorm extraction that enables the isolation of high yields of DNA (150–450 µg) from only 150 mg of plant material. For a detailed description of the method see Engelke (1999). Fifteen micrograms of DNA was digested using 20 U of the restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Rsa*I (Gibco BRL) according to the manufacturer's instructions. The restriction fragments were separated by flatbed electrophoresis using 0.8% agarose gels in 1 × TAE buffer for 14–16 h (2 V/cm length of the gel). The DNA was cracked by soaking the gel in 0.25 *M* HCl for 10 min and then transferred to Hybond^{TM}-N^{+} nylon membranes (Amersham) in 0.4 *M* NaOH using a vacuum system (Pharmacia: 60 mbar, 45 min). The membranes were rinsed in 2 × SSC and air-dried.

Sources of the probes, hybridisation and autoradiography

Ten heterologous mitochondrial genes were employed as probes: *cox*II (*Eco*RI fragment of 2.4 kb), *cob* (*Pst*I/*Hind*III fragment of 0.68 kb) and *rrn*26 (*Sma*I fragment of 4.8 kb) were derived from *Zea mays*; *cox*III (*Eco*RI/*Pst*I fragment of 1.1 kb), *nad*1 (*Bam*HI fragment of 2.2 kb), *nad*3 (*Eco*RI-fragment of 1.0 kb) and *atp*A (*Pst*I/*Hind*III fragment of 1.9 kb) from *Oenothera berteriana*; as well as *atp*6 (*Bam*HI fragment of 1.66 kb), *atp*9 (*Bam*HI fragment of 1.84 kb) and *rrn*18 (*Apa*I fragment of 1.88 kb) from *Arabidopsis thaliana*. The different plasmids containing the mitochondrial gene fragments were transformed into *Escherichia coli* (XL1-Blue MRF, Stratagene), cloned and recovered (Plasmid Midi Kit, Quiagen) according to the manufacturers' instructions. The mitochondrial genes were separated from the plasmids by digestion of the cloning sites with the corresponding restriction enzymes and a subsequent gel electrophoresis using the UV Band-Elutor E91 from Biometra. The radioactive labelling of the probes was achieved by random priming with the Oligolabelling kit from Gibco using 50 ng of each probe and 1.85 MBq α-[^{32}P]-dCTP (111TBq/mmol, Amersham). Non-incorporated nucleotides were separated out using the Nuctrap Push Columns from Stratagene. Before the labelled probes were added, the membranes were prehybridised in rotating glasstubes (Bachofer) containing 30 ml of 0.25 *M* $NaHPO_4$, 0.25 *M* NaCl, 1 m*M* EDTA, 7% SDS at 65°C. Half of the labelled probes were added to each tube, which contained maximally three membranes. The hybridisation took place overnight at 65°C. The membranes were then washed inside the rotating tubes at the same temperature, twice in 150 ml 2 × SSC/0.5% SDS for 15 min and at least for another 15 min in 1 × SSC/0.1% SDS. Fuji-X-Ray films were exposed to the membranes at –80°C for 2–14 days, depending on the remaining radioactivity. The membranes can be reused several times after being boiled in 0.5% SDS for 15 min.

Results

In adition to the (S)-cytoplasm of the CMS-system we were able to distinguish four other cytoplasms differing in the organisation of their mitochondrial genomes. Examples of the hybridisation patterns originating from the different mitochondrial gene probes are shown in Figs. 1–3.

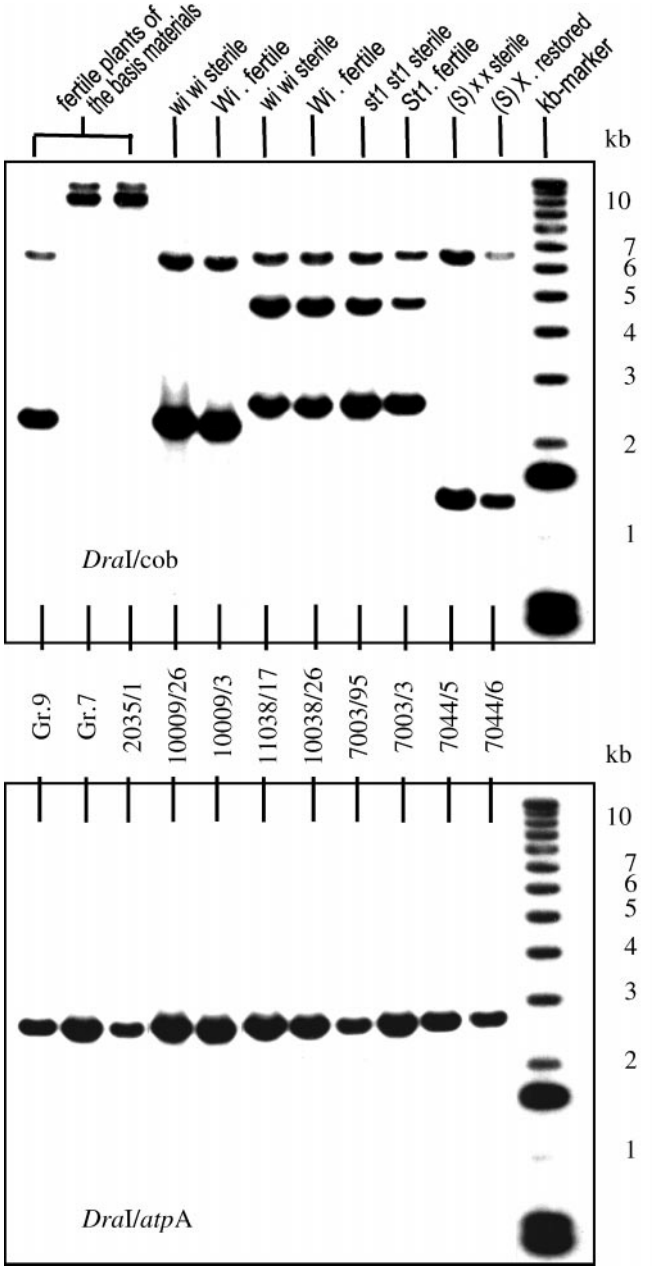

Fig. 1 Autoradiogram of total chive DNA, *Dra*I-digested and probed with the mitochondrial genes *cob* and *atp*A, respectively. Fertile plants of the basis materials and lines segregating with respect to *wi*- or *st1*-sterility are compared to sterile and restored CMS genotypes. Each lane represents DNA from a single plant

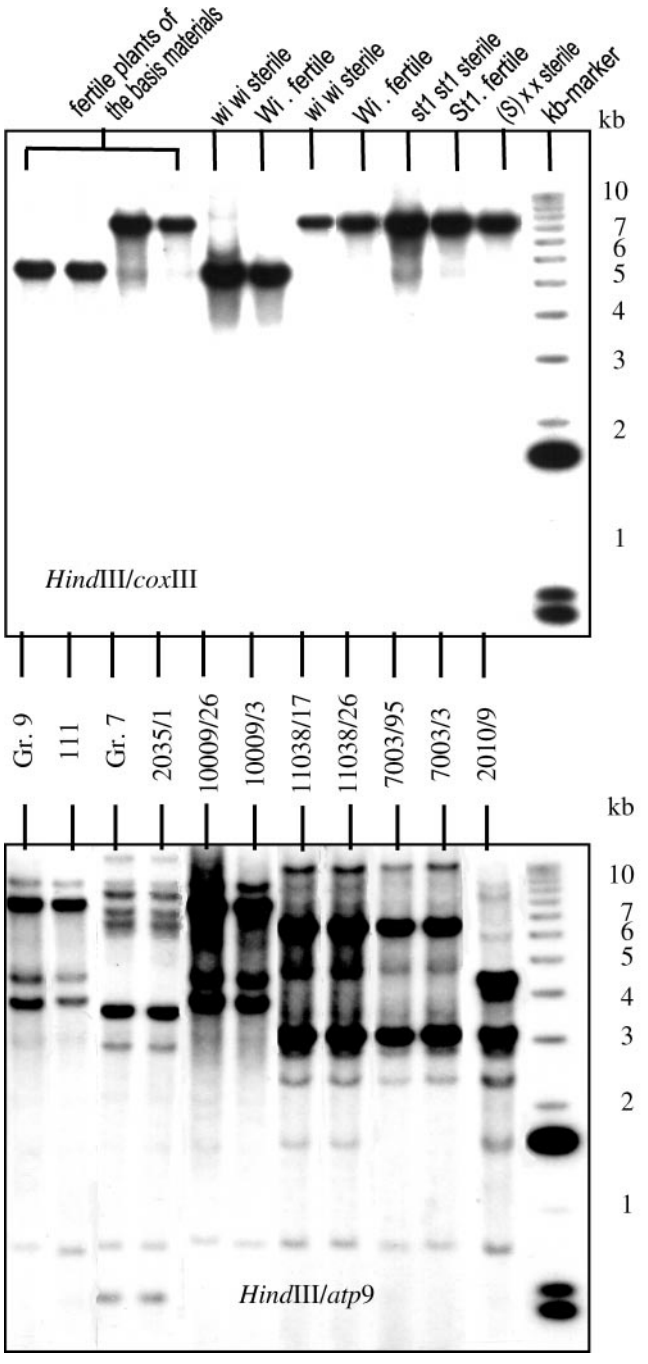

Fig. 2 Autoradiogram of total chive DNA, *Hind*III-digested and probed with the mitochondrial genes *cox*III and *atp*9, respectively. Fertile plants of the basis materials and lines segregating with respect to *wi*- or *st1*-sterility are compared to a CMS genotype. Each lane represents DNA from a single plant

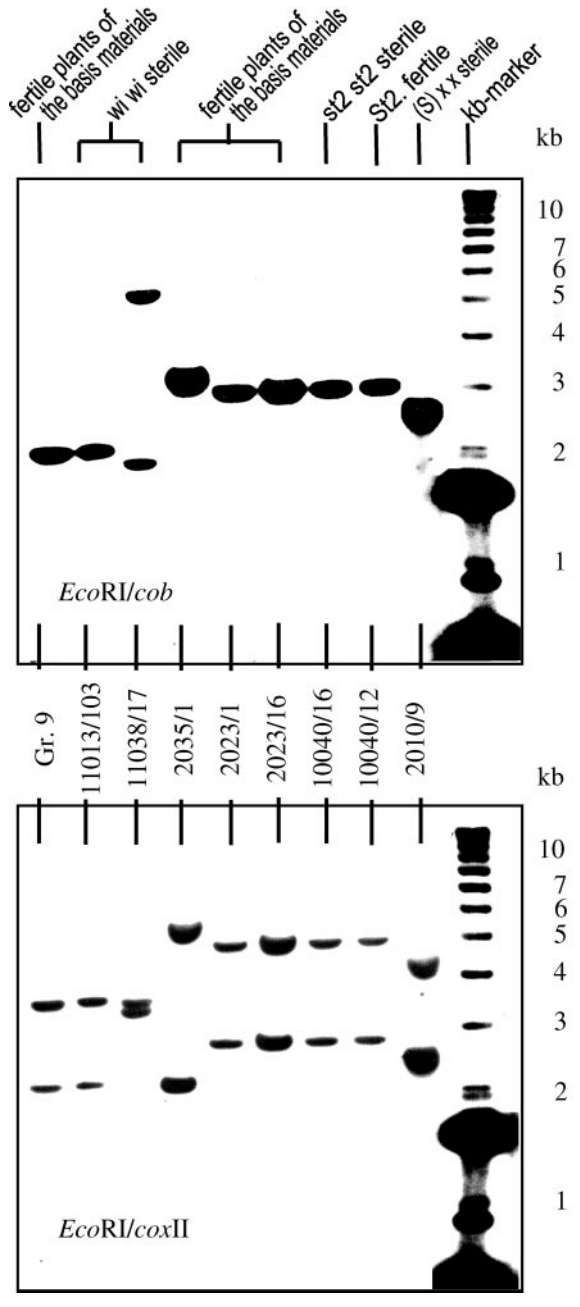

Fig. 3 Autoradiogram of total chive DNA, *Eco*RI-digested and probed with the mitochondrial genes *cob* and *cox*II, respectively. Fertile plants of the basis materials, two *wi*-sterile plants from different lines, a *st2*-sterile and a fertile plant from a single segregating line are compared to a CMS genotype. Each lane represents DNA from a single plant

While *atp*A detected no differences in *Dra* I-restricted DNA, the probe *cob* was able to distinguish between five cytoplasm types, of which four are given in Fig. 1. On the left side there are three lanes with the hybridisation patterns obtained from fertile plants of the basis materials. While Gr. 9 and Gr. 7, two plants selected from cv Grolau, differ from each other, plant 2035/1 corresponds to Gr. 7. The plants from line 10009 show the same hybridisation pattern as Gr. 9 but differ from the plants of line 11038. Both lines, 10009 and 11038, segregate in fertile and *wi*-sterile plants, i.e. the *wi*-sterility shows itself in two different cytoplasm types. In one of these cytoplasms the *st1*-sterility also occurred, as shown for line 7003. In the outer lanes, next to the molecular weight marker, the hybridisation pattern of plants carrying the (S)-cytoplasm is shown. This pattern is different from those of all the other types.

Table 1 Differentiation[a] of the hybridisation patterns from fertile plants of the basis materials in comparison with sterile and restored CMS genotypes

	W1115/47	Gr. 9	111	Gr. 7	2035/1	2010/9 (S)*xx*	7044/5 (S)*xx*	7044/6 (S)*X.*	2023/1	2023/16
*Dra*I-digested:										
*cox*II	–	1	–	3	3	4	4	4	5	5
*cox*III	1	1	1	–	–	–	–	–	–	–
*nad*3	1	1	1	3	3	4	4	4	5	5
*atp*A	–	1	–	1	1	1	1	1	–	1
*atp*6	–	1	–	3	3	4	4	4	5	5
*atp*9	1	1	1	3	3	4	4	4	–	5
cob	–	1	–	3	3	4	4	4	5	5
*rrn*18	–	1	–	3	3	1	1	1	–	1
*Eco*RI-digested:										
coxII	–	1	–	–	3	4	–	–	5	5
coxIII	1	1	1	2	2	2	2	2	–	2
*nad*3	1	1	1	3	3	4	4	4	5	5
*atp*A	–	1	–	3	3	4	4	4	–	5
*atp*6	1	1	1	3	3	4	4	4	5	5
*atp*9	1	1	1	3	3	4	4	4	–	5
cob	–	1	–	3	3	4	4	4	–	5
*rrn*18	–	1	–	3	3	4	4	4	–	4
*Hind*III-digested:										
coxII	–	1	–	2	2	2	2	2	–	–
coxIII	1	1	1	–	–	–	–	–	–	–
*nad*3	1	1	1	3	3	4	4	4	–	5
*atp*A	–	1	–	3	3	4	4	4	–	5
*atp*6	1	1	1	3	3	4	4	4	–	5
*atp*9	1	1	1	3	3	4	4	4	–	5
cob	–	1	–	1	1	1	1	1	–	1
*rrn*18	–	1	–	3	3	1	1	1	–	1

[a] An identical number denotes an indistinguishable hybridisation pattern, while different numbers indicate polymorphic signals. –, Combination of the restriction enzyme and probe was not tested

In Fig. 2 a comparison of the hybridisation patterns from *cox*III and *atp*9 is shown. The *cox*III probe only detected one fragment, and not all different cytoplasm types were distinguishable, as is the case with the (S)-cytoplasm and the cytoplasm in lines 11038 and 7003. The *atp*9 probe detected more fragments than all the other probes with a varying intensity of the hybridisation signals.

All five cytoplasm types are represented in Fig. 3. In the last cytoplasm type the *st2*-sterility occurred (line 10040). This type is also present in 2023, a fertile flowering line of the basis materials. All combinations of the restriction enzymes *Dra*I, *Eco*RI and *Hind*III and the mitochondrial gene probes tested for the different single plants are given in Table 1 and 2.

On the basis of these results all investigated single plants could be assigned to the five cytoplasm types (Table 3). The (S)-cytoplasm is described with number (4), while the *wi*-sterility is present in types (1) and (2), the *st1*-sterilty in type (2) and the *st2*-sterility in type (5).

Discussion

The Southern hybridisation experiments described here using mitochondrial gene probes revealed that there are at least five cytoplasms differing in the organisation of the mitochondrial genome in *Allium schoenoprasum*. Previous studies have aimed to differentiating plants carrying the (S)-cytoplasm from those with (N)-cytoplasm (Mannschedel 1989; Potz and Tatlioglu 1993). However, in these previous studies the hybridisation patterns had not always been reproducible (Potz 1992). This might be explained by the fact that in these former studies no single plants were investigated; instead, the plants were pooled according to their genetic behaviour either as sterile and restored plants or as those carrying the (N)-cytoplasm. The mixture of plant materials within the (N)-group was reconstructable, as the vegetatively preserved plants Gr. 7 and Gr. 9 (both from cv. Grolau) were assigned to cytoplasm-type (N) in the former studies, while the present investigation revealed differences concerning the hybridisation patterns of both plants. Besides this, plants of the line 2023 were falsely classified as such with (S)-cytoplasm, restored by the gene *X* in heterozygous condition. This was deduced from the segregation of sterile and fertile plants in a ratio of 1:3 in the offspring produced by self-fertilisation, and in a ratio of 1:1 in the offspring obtained by crossing with a male-sterile plant (Tatlioglu 1982). However, the hybridisation patterns of plants from line 2023 differ from those of plants carrying the (S)-cytoplasm, as shown in this paper

Table 2 Differentiation[a] of the hybridisation patterns from offsprings segregating with respect to *wi*-, *st1*- or *st2*-sterility

	10009/		11013/		11061/		11038/		7003/		10040/	
	26 *wiwi*	3 *Wi.*	103 *wiwi*	64 *Wi.*	20 *wiwi*	98 *Wi.*	17 *wiwi*	26 *Wi.*	95 *st1st1*	3 *St1.*	16 *st2st2*	12 *St2.*
*Dra*I-digested:												
*cox*II	1	1	–	–	–	–	2	2	2	2	5	5
*cox*III	1	1	1	1	1	1	2	2	–	–	–	–
*nad*1	1	1	1	1	1	1	2	2	–	–	–	–
*nad*3	1	1	1	1	1	1	2	2	2	2	5	5
*atp*A	1	1	1	1	1	1	1	1	1	1	–	–
*atp*6	1	1	–	–	–	–	2	2	2	2	–	–
*atp*9	1	1	1	1	1	1	2	2	2	2	–	–
cob	1	1	1	1	1	1	2	2	2	2	5	5
*rrn*18	1	1	–	–	–	–	2	2	2	2	–	–
*Eco*RI-digested:												
*cox*II	1	1	1	1	1	1	2	2	2	2	–	–
*cox*III	1	1	1	1	1	1	2	2	–	–	2	2
*nad*3	1	1	1	1	1	1	2	2	2	2	5	5
*atp*A	1	1	1	1	1	1	2	2	2	2	–	–
*atp*6	1	1	1	1	1	1	2	2	2	2	5	5
*atp*9	1	1	1	1	1	1	2	2	2	2	–	–
cob	1	1	1	1	1	1	2	2	2	2	5	5
*rrn*18	1	1	–	–	–	–	2	2	2	2	–	–
*rrn*26	1	1	1	1	1	1	1	1	–	–	–	–
*Hind*III-digested												
*cox*II	1	1	1	1	1	1	2	2	2	2	–	–
*cox*III	1	1	1	1	1	1	2	2	–	–	–	–
*nad*1	1	1	1	1	1	1	2	2	–	–	–	–
*nad*3	1	1	1	1	1	1	2	2	2	2	–	–
*atp*A	1	1	1	1	1	1	2	2	2	2	–	–
*atp*6	1	1	1	1	1	1	2	2	2	2	–	–
*atp*9	1	1	1	1	1	1	2	2	2	2	–	–
cob	1	1	1	1	1	1	1	1	1	1	–	–
*rrn*18	1	1	–	–	–	–	2	2	2	2	–	–
*rrn*26	1	1	1	1	1	1	1	1	–	–	–	–

[a] An identical number denotes an indistinguishable hybridisation pattern, while different numbers indicate polymorphic signals, also in corresponding to the numbers given in Table 1. –, Combination of the restriction enzyme and probe was not tested

Table 3 Classification of the investigated single plants into five cytoplasm types

Cytoplasm (1)	Cytoplasm (2)	Cytoplasm (3)	Cytoplasm (4)	Cytoplasm (5)
Fertile plants from the basis materials and cytoplasmic male-sterile and restored plants:				
W1115/47		Gr. 7	2010/9 (S)*xx*	2023/1
Gr. 9		2035/1	7044/5 (S)*xx*	2023/16
111			7044/6 (S)*X.*	
wi-, *st1*- and *st2*-sterile and fertile plants from segregating lines:				
10009/26 *wiwi*	11038/17 *wiwi*			10040/16 *st2st2*
10009/3 *Wi.*	11038/26 *Wi.*			10040/12 *St2.*
11013/103 *wiwi*	7003/95 *st1st1*			
11013/64 *Wi.*	7003/3 *St1.*			
11061/20 *wiwi*				
11061/98 *Wi.*				

(Fig. 3). Following pollination with maintainer plants only fertile plants developed, as recent investigations have shown (Engelke 1999). Therefore, the genetic constitution (S)*Xx* does not apply to the plants from line 2023, otherwise the offspring of the cross with the maintainer (N)*xx* would have shown a segregation of sterile and fertile plants in a ratio of 1:1. For that reason the sterile plants among the offspring obtained after self-fertilisation have to be traced back to another gene, denoted as gene *b* (Engelke 1999).

Considering the relatively small number of plants investigated, the finding of five cytoplasms with different mitochondrial genome organisations implies a high degree of variability with respect to this characteristic in

Allium schoenoprasum. Compared to this, the diversity of the mitochondrial genome seems to be small in the closely related *Allium cepa*, although two CMS systems have been genetically characterised in this species (Jones and Clarke 1943; Berninger 1965; Schweisguth 1973). Studies on the mitochondrial and the chloroplast genome have enabled the clear differentiation of the (S)- and (N)-cytoplasm in the first system, while the sterility-inducing cytoplasm (T) in the second system has not yet been distinguished from the (N)-cytoplasm. Both latter types are therefore brought together in one group (M), despite their different genetic behaviour (De Courcel et al. 1989; Holford et al. 1991; Havey 1993).

The diversity of the mitochondrial genome is attributed to recombination events, especially between repeated sequences (Lonsdale 1984; Small et al. 1989; André et al. 1992). In this context, it is interesting to note that the mitochondrial gene probe *atp*9 detected more fragments than all the other probes used. This might show that the *atp*9 region is a hot spot for recombination events in *Allium schoenoprasum*. Indeed, this seems to be also true for other plant species. Investigations on intergeneric and intrageneric *Brassica* cybrids show that recombination events associated with the *atp*9 gene are much more frequent than those in association with all other genes (Walters and Earle 1993; Landgren and Glimelius 1994). Specific CMS sequences have been described in a number of plant species. Although these sequences differ in the various species and also in different CMS systems of the same species, it is remarkable that in many cases coding or flanking regions of the *atp*9 gene are involved in the causally related recombination events. This applies to the *pcf* gene in Petunia (Young and Hanson 1987), the *atp*6C sequence in the C (Charrua)-cytoplasm of maize (Dewey et al. 1991), the *orf*H522 in the cytoplasm from *Helianthus petiolaris* (Köhler et al. 1991), the Owen-cytoplasm of sugar beet (Xue et al. 1994), the *orf*263 in the cytoplasm from *Brassica tournefortii* (Landgren et al. 1996), the *orf*107 in sorghum-IS1112 C (Tang et al. 1996), the *orf*-C9 in *Lolium perenne* (Kiang and Kavanagh 1996) and the R-sequence in S-(USDA) maize (Zabala et al. 1997).

The question remains whether in some plant species the recombination events occur frequently, while they seem to be rare in others. Besides the reason for the occurrence of the recombination events, the mechanism of stoichiometric maintenance of the resulting mitochondrial DNA-structures is also not yet understood. That these DNA structures, sometimes called “mitochondrial chromosomes”, are maintained in particular proportions, is illustrated by the hybridisation patterns of *atp*9 (Fig. 2). The varying intensity of the hybridisation signals within one lane might be caused by different quantities of distinct mitochondrial DNA structures. These varying signal intensities within one lane constantly occur in plants with the same cytoplasm type. Consequently, the proportion of mitochondrial DNA structures within the same cytoplasm type seems to be preserved. In this context it is interesting to know that the restorer gene *Fr* in the very special CMS system of *Phaseolus vulgaris* is capable of eliminating the mitochondrial circle carrying the *pvs* sequence, or rather to reduce this circle to a substoichiometric level (Mackenzie and Chase 1990; He et al. 1995; Janska et al. 1998). Such an influence of a restorer gene on the organisation of the mitochondrial genome has not been observed in any other CMS system. In the S-(USDA) CMS system of *Zea mays* the nuclear background has an influence on the existence of linear episomes (Escote-Carlson et al. 1990). These episomes are able to recombine with homologous regions in the CMS-specific R-sequence, leading to fertile revertants (Schardl et al. 1984; Escote-Carlson et al. 1988; Zabala et al. 1997). Consequently, the nuclear background has an indirect influence on the stability of this CMS system. Apart from the CMS, an influence of the nuclear background on the organisation of the mitochondrial genome has also been described for other higher plants (e.g. *Brassica*: Erickson and Kemble 1990, 1993; *Nicotiana*: Håkansson and Glimelius 1991).

In addition to the ideas concerning the origin of the mitochondrial genome diversity discussed above, the results of the present paper may also be of benefit to plant breeding. The variability of the mitochondrial genome provides the opportunity to solve the question of whether the genes *wi*, *st1* and *st2* show themselves in different cytoplasm types or whether additional cytoplasmic factors are necessary for the occurrence of these sterilities. The latter case would result in a new CMS system, otherwise the sterilities would only depend on the genes *wi*, *st1* and *st2* and therefore be genic male sterilities (GMS). The *wi*-sterility seems to be a GMS as this form shows itself in at least two different cytoplasm types [cytoplasm (1) and (2), see Table 2 and 3]. Results concerning the behaviour of all three genes in the various cytoplasm types will soon be presented.

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