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Genetic analyses supported by molecular methods provide evidence of a new genic (st1) and a new cytoplasmic (st2) male sterility in *Allium schoenoprasum* L.

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Abstract Spontaneous mutations leading to male sterility have been described for many different crops and are of great importance to hybrid breeding, provided that their inheritance is resolved. This paper describes an efficient method to characterise male sterilities with respect to cytoplasmic factors that might be causally related to them. The differentiation of cytoplasmic (CMS) and genic (GMS) male sterility is achieved by a specific transfer of nuclear male sterility factors to different cytoplasm types which have previously been distinguished by means of RFLP analyses using mitochondrial gene probes. The nuclear sterility factors of *Allium schoenoprasum* used, *st1* and *st2*, showed a monogenic recessive inheritance in their original cytoplasms. While *st1* was expressed in four different cytoplasm types, *st2* did not show itself in a cytoplasm type differing from the original. Therefore, the st1-sterility is a GMS, while a cytoplasmic factor is necessary for the occurrence of st2-sterility. This cytoplasmic factor was verified by a reciprocal cross, and the CMS system was completed by the selection of maintainer genotypes. Neither of these new sterilities were influenced by high temperatures or tetracycline. The benefits of a new CMS system to practical breeding and the advantages and disadvantages of the environmental influences on the expression of male sterility are discussed.

Key words Cytoplasmic male sterility, CMS · Genic male sterility, GMS · Chives · Mitochondrial genome

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Introduction

Cytoplasmic male sterility (CMS) is the most frequently used mechanism in hybrid seed production, as the maternal transmission of specific cytoplasms in combination with Mendelian nuclear genes permits the efficient and complete control of pollination. The seed parent [A-line, (S)*rfrf*] is maintained by pollination with the nearly isogenic B-line, (N)*rfrf*, differing only in the cytoplasm that induces the fertility of this line. If fertility has to be obtained in the hybrid, the dominant *Rf* allele can be brought in from the male parent (C-line), leading to the restoration of the fertility within the (S)-cytoplasm.

Using genic male sterility (GMS), it is not possible to obtain a completely sterile A-line, due to the lack of the maternally inherited factor. GMS is usually inherited in a monogenic recessive way, and the sterility can at best be maintained by pollination with heterozygous plants, resulting in a segregation of sterile and fertile plants in a ratio of 1:1.

In *Allium schoenoprasum* a CMS system has already been described, consisting of a sterility-inducing cytoplasm (S), within which a stable restoration is obtained by the gene *X* (Tatlioglu 1982). A second restorer gene, *T*, only leads to pollen production at high temperatures (24°C/24°C, day/night), while the same genotypes remain male-sterile at lower temperatures (20°C/12°C, day/night) (Tatlioglu 1987). A third gene, *a*, causes pollen production in combination with a tetracycline treatment (Tatlioglu and Wricke 1988). Plants carrying a normal cytoplasm type (N) are always male-fertile, regardless of the constitution of the three nuclear genes. This CMS system permits hybrid breeding in chives, and F_1 cultivars have already been developed in Germany. The selection of maintainer genotypes leading to A-lines with temperature-insensitive expression of the sterility is still a problem in practical breeding, as the dominant *T*-allele can only be recognised and eliminated when the temperature is adjusted precisely during the flowering time.

In addition to the CMS system, a second male sterility has been characterised in chives. This form is exclusive-

ly caused by the *wi* gene and is, consequently, a GMS (Engelke and Tatlioglu 2000a). As described above, GMS can only be used in hybrid breeding by eliminating the fertile plants from a segregating A-line. Although the sterile plants can be vegetatively multiplied, this does not have a real advantage over the use of the CMS system described above.

Yet two different male sterilities were found in an offspring obtained from the self-fertilisation of a maintainer (st1-sterility) and from materials of Danish origin, derived from the Western Regional Plant Introduction Station in Washington (st2-sterility). In earlier studies the original sterile plants had been pollinated with maintainer genotypes of the CMS system as a test for allelism. In the F_1 obtained only fertile plants matured; thus the new sterilities have to be based on genetic mechanisms other than the known CMS system. In F_2 and F_3 progeny produced by self-pollination, the segregation of sterile and fertile plants revealed a monogenic recessive inheritance for both sterilities, and the corresponding genes were named *st1* and *st2* (Tatlioglu 1994; Engelke and Tatlioglu 1996). Tests for allelism showed the nonallelic character of *st1*, *st2* and the gene *wi* (Engelke and Tatlioglu 1996).

This paper will clarify the question of whether an additional factor of a specific cytoplasm is necessary for the occurrence of st1- or st2-sterility. Since such cytoplasmic factors are caused by recombination in the mitochondrial genome, different plants were characterised by means of restriction fragment length polymorphism (RFLP) analyses using mitochondrial gene probes. In addition to the (S)-cytoplasm of the CMS system, four different cytoplasm types were distinguished (Engelke and Tatlioglu 2000b). The genetic analyses of the *st1* and the *st2* gene in different cytoplasm types are described below. In addition, the influence of different temperatures and tetracyline on the new sterilities was examined.

Materials and methods

Transfer of the *st1* and the *st2* gene to different cytoplasm types

The transfer of the *st1* and the *st2* gene from their original cytoplasm to different cytoplasm types was achieved using the crossing scheme outlined in Fig. 1. For realisation of the crossing scheme the female parent as the donor of the new cytoplasm type (n) has to be emasculated and pollinated from a plant in the heterozygous condition with respect to the nuclear sterility factor, *St/st*. The offspring consists of plants carrying the new cytoplasm type, half of them in the heterozygous and the other half in the homozygous dominant state. All plants in the F_1 are consequently fertile and can not be distinguished from each other by phenotype. The 50% of the F_2 progenies obtained by selfing of a heterozygous F_1 plant show a fertile : sterile plant segregation ratio of 3:1 when sterility is also formed in the new cytoplasm. In this case the sterility only depends on the nuclear gene and is therefore a genic male sterility (GMS). When the plants of all of the F_2 progenies are fertile, a factor of the original cytoplasm is necessary for the occurrence of the sterility. The latter case would result in a new CMS system.

In order to transfer the *st1* and the *st2* gene into different cytoplasms, we distinguished five cytoplasm types by means of RFLP analyses using mitochondrial gene probes (Engelke and Tatlioglu 2000b). The (S)-cytoplasm of the CMS system was represented by number (4), while the st1-sterility occurred in type (2) and the st2-sterility in type (5). The transfer of the *st1* and the *st2* gene into different cytoplasms was achieved using the following plants as maternal and paternal parents according to the crossing scheme described above.

- Maternal parents:
  - Gr.9 for the transfer to cytoplasm (1)
  - 2035/1 for the transfer to cytoplasm (3)
  - 2023/14 for the transfer to cytoplasm (5).

For a further characterisation of these plants, crosses with a temperature-insensitive CMS genotype (6025/35) and selfings were carried out.

- Paternal parents:
  - 9001/11 and 9002/4 for st1-sterility, (2) *St1 st1*
  - 9046/6 for st2-sterility, (5) *St2 st2*.

Classification of the sterility and statistical analysis

Plants were classified in five groups according to the amount of fertile anthers in at least three umbels (Tatlioglu 1982). Absolutely male-sterile flowers were characterised by class 0, fertile flowers by class 4. Flowers which were not reliably sterile (normal appearing anthers <15%) were grouped in class 1, semi-sterile flowers with up to 60% fertile anthers in class 2 and the remaining flowers with 61–99% fertile anthers in group 3. If the mean of classified flowers is ≤1, the plants were counted as 'sterile' for statistical analyses; plants with a mean >1 were counted as 'fertile'. It should be noticed that semi-sterile flowers are rare and that normally classes 0 and 4 dominate the classification.

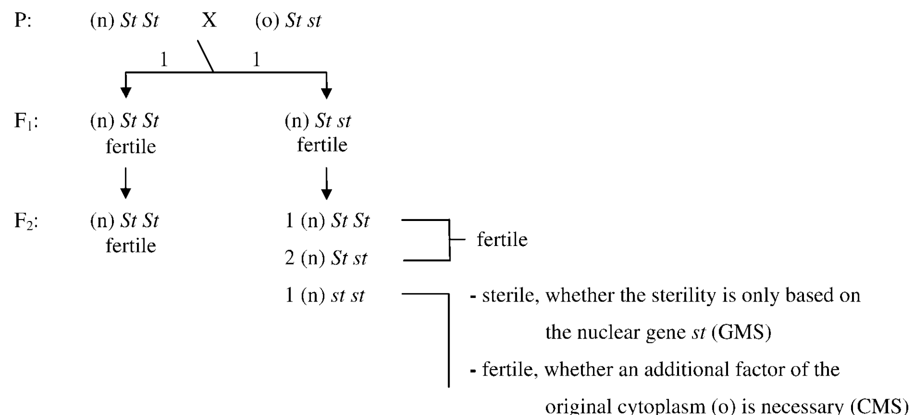

Fig. 1 Crossing scheme showing the transfer of a nuclear male sterility factor *st* from the original cytoplasm (o) to a new cytoplasm type (n)

Statistical comparisons of segregation results with expected ratios were carried out using the χ^2-test (P=5%). For random sample sizes of fewer than 200 the value was corrected according to Yates (1934). To distinguish between segregating and non-segregating lines, the smallest random sample size for the rejection of a 1:1 segregation is 5 and for the rejection of a 3:1-segregation, 11 (at P=5%).

Variation of temperature and tetracycline application

In order to test the influence of temperature and tetracycline application on the expression of st1- and st2-sterility, we allowed sterile and some fertile genotypes from segregating F_2 progenies to multiply vegetatively. The clones were cultivated in greenhouse chambers and the temperatures adjusted to 20°/12°C, 24°/24°C or 30°/24°C (day/night). Tetracycline in a solution of 500 ppm was sprayed twice a week on plants in separate greenhouse-chambers kept at 20°/12°C and 24°/24°C, beginning with the first visible flower bud and continuing for 3 or 4 weeks until the opening of the flowers.

RFLP analyses using mitochondrial gene probes

The RFLP analyses using mitochondrial gene probes were performed as previously described (Engelke and Tatlioglu 2000b). The heterologous mitochondrial gene probes were obtained from *Zea mays* (*cox*II and *cob*), *Arabidopsis thaliana* (*atp*6, *atp*9 and *rrn*18) and *Oenothera berteriana* (*nad*3) (for a detailed description see Engelke and Tatlioglu 2000b). The defined hybridisation patterns of the different cytoplasm types were checked in the parental, the F_1, and the F_2 generations, in order to verify the maternal inheritance that is predicted in the crossing scheme.

Results

Characterisation of plants Gr.9, 2035/1 and 2023/14, which carry cytoplasm types (1), (2) and (5), respectively

The plants with the defined cytoplasm types that were used as maternal parents, according to the crossing scheme of Fig. 1, were characterised by selfing and by crossing with a temperature-insensitive CMS genotype (Table 1).

No sterile plants occurred in the progeny obtained by selfing Gr.9. In contrast, progeny obtained by crossing Gr. 9 with the CMS genotype showed a 1:1segregation ratio, i.e. Gr.9 is heterozygous with respect to the *X*/*x*-locus. The sterile plants developed no temperature-sensitivity; therefore, the *T*/*t*-locus is in a homozygous recessive condition. The genotype of Gr.9 is consequently (1)*Xxtt*.

No sterile plants occurred in the progeny obtained by selfing 2035/1. In contrast, progeny that had originated from the crossing with the CMS genotype consisted of sterile plants only, i.e. 2035/1 is able to maintain the CMS and is therefore recessive for the *X*/*x* locus. The sterile plants showed no temperature sensitivity, and the genotype of 2035/1 is consequently (3)*xxtt*.

The progeny obtained by selfing of 2023/14 showed a segregation of 14 sterile : 51 fertile plants (χ^2-value$_{1:3}$=0.25). Half of the sterile plants were temperature-sensitive (χ^2-value$_{1:3}$=3.43). The progeny obtained by crossing 2023/14 with the CMS genotype consisted of 17 sterile and 13 fertile plants (χ^2-value$_{1:1}$=0.3). Four sterile plants were sensitive to high temperatures, while 13 plants were insensitive (χ^2-value$_{1:1}$=3.76). The assumption that the genotype of 2023/14 is (S)*XxTt* could explain the observed segregations and would be in accordance with results obtained from previous studies (Tatlioglu 1982). However, the cytoplasm type of 2023/14 is (5) and the mitochondrial genome organisation is therefore different from the (S)-cytoplasm that was represented by type (4). To provide evidence of the cytoplasmic character, i.e. whether type (5) contains the CMS-inducing sequence as well, we performed an additional crossing of 2023/14 and 2035/1 (maintainer of the CMS, see above). Of the offspring 13 fertile and no sterile plants matured. This is not in agreement with the hypothesis that 2023/14 contains the (S)-cytoplasm and is restored by the gene *X*/*x* in the heterozygous condition as, in this case, crossing with the maintainer would have resulted in a segregation ratio of 1:1. Consequently the sterile plants in the progeny obtained by selfing 2023/14 do not have CMS genotypes, and yet another mechanism must be responsible for the occurrence of these sterile plants, likely one with a recessive inheritance. The corresponding gene will be called *B*/*b* and has to be taken into account, as 2023/14 will be involved in subsequent seg-

Table 1 Segregations resulting from self-pollination of the plants Gr. 9, 2035/1 and 2023/14 and from pollination of a temperature-insensitive CMS genotype (S)*xxtt*

	Self-pollination	Temperature		Pollination of (S)*xxtt* (6025/35)	Temperature	
		20°/12°C	24°/24°C		20°/12°C	24°/24°C
Gr. 9	Sterile : fertile	0:35	–	Sterile : fertile	10:10	6:7
	Temp– : temp.+	–		Temp.– : temp+	6:0	
2035/1	Sterile : fertile	0:6	–	Sterile : fertile	35:0	33:0
	Temp– : temp+	–		Temp– : temp+	33:0	
2023/14	Sterile : fertile	14:51	7:46	Sterile : fertile	17:13	13:16
	Temp– : temp+	7:7		Temp– : temp+	13:4	

temp–, temperature-insensitive (sterile at 20°/12°C and 24°/24°C);
temp+, temperature-sensitive (sterile at 20°/12°C but fertile at 24°/24°C)

Table 2 Segregations resulting from the transfer of the gene *st1* to cytoplasms (1), (3) and (5)

Transfer of the *st1* gene from cytoplasm (2)		Segregation in the separate lines:		Sum of		
		Sterile plants	Fertile plants	The lines	Sterile plants	Fertile plants
To cytoplasm (1)						
P: Gr. 9×9002/4						
F_1: 13012		0	29			
F_2: Non-segregating:	15021	(1)[a]	19			
	15023	0	20			
	15024	0	15			
	15026	0	32			
	15027	(1)[a]	17	5	(2)[a]	103
Segregating:	15020	11	34			
	15022	3	5			
	15025	1	4	3	15	43
To cytoplasm (3)						
P: 2035/1×9001/11						
F_1: 14002		0	33			
F_2: Non-segregating:	15081	0	16			
	15082	0	25			
	15083	0	31			
	15084	(1)[a]	16			
	15087	0	15			
	15088	0	21			
	15089	(1)[a]	20	7	(2)[a]	144
Segregating:	15075	3	19			
	15076	2	7			
	15078	10	21			
	15079	5	13			
	15080	8	22			
	15085	4	27	6	32	109
To cytoplasm (5)						
P: 2023/14×9001/11						
F_1: 13013		0	25			
F_2: Non-segregating:	15031	0	12			
	15033	0	26	2	0	38
Segregating:	15028	3	27			
	15029	2	10			
	15030	4	16			
	15032	(19)[a]	(13)[a]			
	15034	7	13			
	15035	5	19			
	15036	(6)[a]	(7)[a]	6	26	113
	15037	5	28	(2)[a]	(25)[a]	(20)[a]

[a] Plants which can not be traced back to the monogenic recessive inheritance of the *st1* gene (for explanations see main text)

regation studies. Interestingly, some of the b-sterile plants become fertile at 24°/24°C. The b-sterility is therefore affected by high temperatures. Segregation indicates a ratio of 7:9, suggesting a complementary gene action of two genes. The segregation of progeny derived from crossing with the CMS genotype can also be explained by this assumption (for details, see Engelke 1999), but further investigations have to prove this hypothesis. The temperature effect is of no significance for the analyses of st1- and st2-sterility, since the segregation studies concerning the transfer of the *st1* and *st2* gene to different cytoplasm types were carried out at 20°/12°C.

Genetic analysis of the *st1* gene within cytoplasms (1), (3) and (5)

The transfer of the *st1* gene to cytoplasm types (1) and (3) resulted in a F_1 generation consisting of fertile plants only and in segregating and non-segregating F_2-offspring in a ratio of 1:1, as would be expected for a GMS (Table 2).

The monogenic recessive inheritance of st1-sterility was confirmed by the occurrence of sterile and fertile plants in the expected ratio of 1:3 (15:43, χ^2-value$_{1:3}$=0.00; 32:109, χ^2-value$_{1:3}$=0.29). The lines 15021, 15027, 15084, and 15089 can be classified as non-segregating offspring with respect to the *st1* gene, although 1

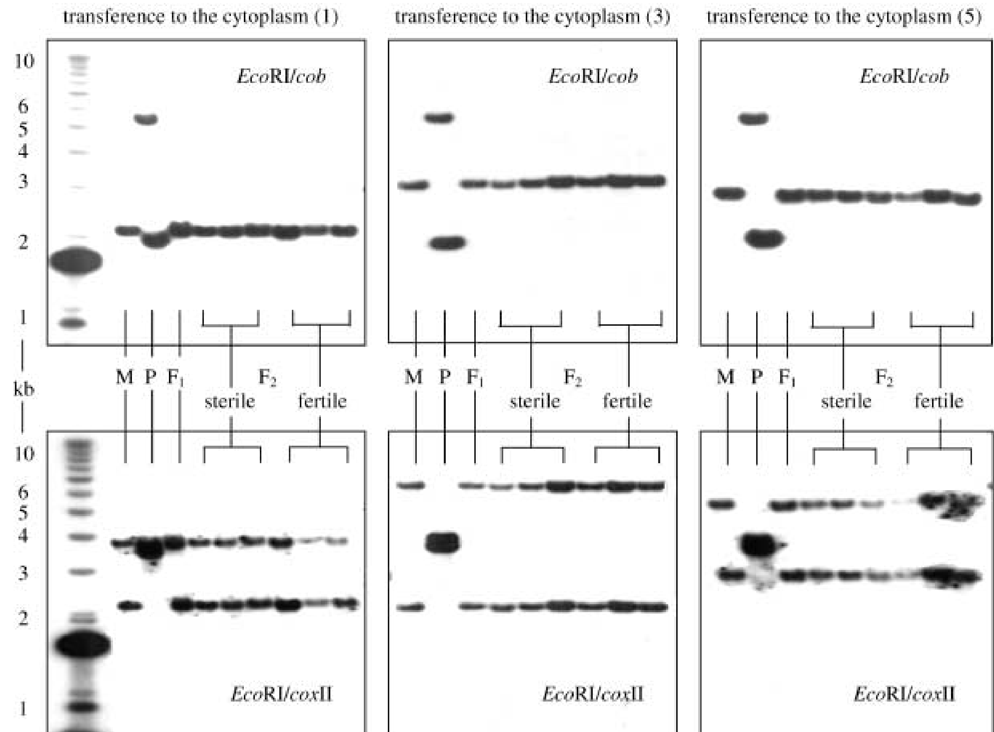

Fig. 2 Hybridisation patterns (*Eco*RI digestion and probed with *cob* and *cox*II) from the maternal (M) and paternal (P) parent and the first (F_1) and second (F_2) filial generation, obtained from the transfer of the gene *st1* from cytoplasm type (2) to cytoplasm types (1), (3) and (5)

sterile plant occurred in each of these lines, and the $\chi^2_{1:3}$-values are not significant if calculated by means of the correction described by Yates (see Materials and methods). The phenotype of the sterile plants in these lines varied from the typical st1-phenotype and the observational data indicated an inheritance pattern of duplicated genes, resulting in a ratio of 1:15. An additional crossing of Gr.9 and 2035/1 did in fact result in segregation ratios which confirm this hypothesis (Engelke 1999, the genes were named *M/m* and *N/n*).

The transfer of the *st1* gene to cytoplasm type (5) led to a segregation, that of the *st1* and *b* genes (see above). The F_1 generation consisted only of fertile plants. As a result of this, *st1* and *b* can be considered to be nonallelic. One quarter of the F_1 plants were dominant for both genes, a fourth heterozygous for the *st1* gene, a fourth heterozygous for the *b* gene and a fourth heterozygous for both loci. The expected ratio of non-segregating and segregating offspring in the F_2-generation is therefore 1:3 and corresponds to the observed data (Table 2). Among the segregating offspring, two thirds were expected to show sterile and fertile plants in a ratio of 1:3 and the remaining plants were expected to show a segregation of 7:9, as a result of the segregation of both genes. Line 15032 gives evidence for the latter segregation because the $\chi^2_{1:3}$-value=12.76 is significant, while the $\chi^2_{7:9}$-value=2.57 is not significant. The observational data in line 15036 (6 sterile : 7 fertile plants) are likewise rather adapted to a 7:9 ratio, although the test against the 1:3-segregation is not significant (χ^2-value=2.08). The remaining 6 F_2 progenies showed a 1:3 segregation, as expected for the segregation of one of either gene.

RFLP analyses using mitochondrial gene probes were carried out in order to confirm the formation of st1-sterility in the different cytoplasm types, i.e. that the transfer of the *st1* gene from the original cytoplasm type (2) to cytoplasm types (1), (3) and (5) was really achieved as predicted by the crossing scheme. For the transfer to cytoplasm (1) the combinations *Eco*RI/ *atp*6, *cob*, *cox*II, *nad*3, *rrn*18 and *Dra*I/*atp*6, *cob*, *cox*II, *nad*3, *rrn*18 were examined; for the transfer to cytoplasm type (3) the combinations *Eco*RI/ *atp*6, *cob*, *atp*9, *cox*II, *rrn*18 and *Dra*I/*atp*6, *cob*, *cox*II; and for the transfer to cytoplasm type (5) the combinations *Eco*RI/*atp*6, *cob*, *cox*II, *nad*3, *rrn*18 and *Dra*I/ *atp*6, *cob*, *cox*II, *nad*3, *rrn*18 (Engelke 1999). The hybridisation patterns of the F_1 and F_2 generations were identical with those of the maternal parents in each cross (an example is shown in Fig. 2). The inheritance of the mitochondria in *Allium schoenoprasum* is therefore maternal, as assumed in the crossing scheme.

The genetic analysis and the RFLP analyses provided evidence that the *st1* gene creates sterility in all four cytoplasms. Consequently, the sterility depends only on this nuclear gene and an additional factor of a specific cytoplasm can be excluded. Thus, we have proved st1-sterility to be a genic male sterility (GMS).

Genetic analysis of the *st2* gene within cytoplasm (1)

The segregation of sterile and fertile plants in the F_1 and F_2 generations, resulting from the transfer of the *st2* gene to cytoplasm type (1), is presented in Table 3.

Table 3 Segregations resulting from the transfer of gene *st2* to cytoplasm (1)

Transfer of the *st2* gene from cytoplasm (5)		Segregation in the separate lines:		Sum of		
		Sterile plants	Fertile plants	The lines	Sterile plants	Fertile plants
To cytoplasm (1)						
P: Gr. 9×9046/6						
F_1: 13022, 13023		0	45			
F_2: Non-segregating:	15038	0	11			
	15039	0	20			
	15040	0	17			
	15041	0	35			
	15043	0	29			
	15044	0	31			
	15045	0	15			
	15047	0	17			
	15048	(1)[a]	29			
	15049	(1)[a]	25			
	15050	0	32			
	15051	0	18			
	15052	0	16			
	15053	0	14			
	15054	0	24			
	15055	0	11	16	(2)[a]	389
Segregating:		–	–	0	–	–

[a] Plants which can not be traced back to monogenic recessive inheritance of the *st2* gene (for explanations see main text)

Table 4 Verification of the cytoplasmic factor of st2 sterility in a reciprocal cross

Genotypes forming the cross:	(5)*St2st2* 9046/6	×	(1)*st2st2* 13022/1
F_1:		16002	
Segregation sterile : fertile		11:7	
Crossing in the opposite direction:	(1)*st2st2* 13022/1	×	(5)*St2st2* 9046/6
F_1:		16001	
Segregation sterile : fertile		0:28	

Table 5 Maintainer selection for the new CMS system

Crossing type:		F_1	Segregation	
(5)*st2st2*×	(1)*st2st2*		Sterile	fertile
10040/45	15038/3	16017	9	0
10040/45	15038/11	16018	10	0
10040/45	15040/1	16019	8	0
10040/45	15045/10	16021	7	0
10040/45	15049/4	16026	12	0
10040/45	15049/13	16027	17	0
10040/45	15050/3	16028	26	0
10040/38	15049/15	16033	29	0

In each of lines 15048 and 15049 1 sterile plant occurred, but the phenotype of these plants was different from the typical st2-phenotype and the $\chi^2_{1:3}$-test is significant (χ^2-values=5.13 and 6.4). Thus, the sterility of the plants was not caused by the *st2* gene. Observed data show a ratio of 1:15 and the parents of the cross indicate the segregation of the duplicated genes *m* and *n* (see above). The transfer of the *st2* gene to cytoplasm type (1) resulted in 16 non-segregating F_2-lines, as $P_{1:3}$ is less than 5% in each line, and therefore a segregation can be excluded ($P_{1:3}$ = 4.2% for the line with 11 individuals, and $P_{1:3}$ is even lower in the remaining lines, depending on the sample sizes). Consequently, there is no division in segregating and non-segregating F_2-lines, as it would be expected for a GMS ($P_{1:1}$=0.00%). The *st2* gene does not create male sterility in cytoplasm (1), hence a factor of the original cytoplasm (5) must be necessary for the occurrence of the sterility. This cytoplasmic factor was verified by a reciprocal cross (Table 4). A segregating offspring emerged, when the maternal parent carried cytoplasm (5), while a non-segregating offspring resulted from crossing in the opposite direction.

Interestingly, 13022/1 has to be in homozygous recessive condition with respect to the *st2* gene, otherwise the segregation would not result in a ratio of 1:1. Plant 13022/1 was selected from the F_1 generation of the original crossing between Gr.9 and 9046/6. Consequently, Gr. 9, as the maternal parent of the original crossing, is not homozygous dominant with respect to the nuclear male sterility factor, as assumed in the crossing scheme (Fig.1).

The new CMS system can also be confirmed by the selection of the maintainer genotypes which are predicted to be present in the F_2 generation obtained from the crossing of Gr.9 with 9046/6. Eighteen test-crossings of F_2 plants with sterile plants resulted in 8 non-segregating sterile lines (Table 5). Therefore, the F_2 generation included more maintainer-genotypes than the one-eight frequency predicted by the crossing scheme (Fig. 1). As mentioned above, Gr. 9, as the maternal parent of the original cross, was not homozygous dominant but heterozygous for the nuclear male sterility factor. There-

Table 6 Classification of the sterility/fertility of genotypes belonging to the new CMS system and the st1 GMS, respectively, in different temperature and tetracycline (+tetra.) treatments. The first number gives the number of classified umbels, the second number the classification value (0=sterile, 4=fertile)

Plant-number	CMS, (5)*st2st2* and (5)*St2*.[a]				
	20°/12° (control)	20°/12° (+tetra.)	24°/24°	24°/24° (+tetra.)	30°/24°
10031/ 5	4×4	6×4	1×4	–	–
8	7×0	8×0	4×0	8×0	–
11	4×0	4×0	6×0	4×0	5×0
14	6×0	2×0	7×0	7×0	5×0
20	10×0	3×0	3×0	4×0	5×0
25	8×0, 1×4	9×0, 2×4	9×0, 2×4	4×0, 3×4	5×0, 3×4
27	10×4	7×4	3×4	6×4	7×4
10032/ 2	3×0	6×0	10×0	3×0	7×0
10	4×0	3×0	4×0	–	–
12	5×0	4×0	3×0	3×0	–
10033/ 2	8×0	3×0	10×0	6×0	5×0
5	4×0	5×0	10×0	3×0	4×0
10	4×0	3×0	5×0	6×0	3×0
12	6×0	4×0	4×0	6×0	2×0
14	5×0	10×0	6×0	5×0–1	7×3–4
10037/ 4	7×0	3×0	7×0	6×0	10×0
8	4×0	1×0	9×0	10×0	6×0
10	4×4	3×4	–	–	–
11	12×0	5×0	10×0	15×0	8×0
10038/ 1	5×4	4×4	–	–	1×4
7	1×0	5×0	9×0	7×0	5×0
9	4×0	4×0	7×0	5×0	6×0
10040/ 3	2×4	–	1×4	2×4	–
5	7×4	1×4	1×4	6×4	5×4
8	3×0	3×0	3×0	2×0	2×0
10	1×4	1×4	3×4	–	–
11	1×4	1×4	2×4	1×4	–
14	4×4	7×4	–	–	–
19	1×4	10×4	2×4	1×4	–
20	2×0	5×0	4×0	2×0	–
34	2×0	–	–	–	–
36	2×0	1×0	1×0	3×0	–
38	2×0	1×0	1×0	1×0	–
41	2×0	4×0	1×0	1×0	3×0
45	4×0	1×0	1×0	4×0	1×0
51	5×0	4×0–1	2×1, 1×3	3×0, 1×3	–
53	5×0	4×0	1×0	1×0	1×0
55	2×0	–	–	–	1×0
56	1×0	2×0	–	1×0	1×0
60	3×0	2×0	5×0	–	3×0
61	2×0	–	–	–	–
63	3×0	2×0	–	–	2×0
64	1×0	2×0	2×0	1×0	–
66	2×0	1×0	–	–	3×0–1
67	4×0	1×0	1×0	1×0	6×0
68	4×0	5×0	1×0	5×0	4×0
10041/ 14	2×4	5×4	–	1×4	–
20	17×0	18×0	9×0	6×0	4×0
22	2×0	–	–	–	–
23	5×4	4×4	–	–	–
27	3×4	4×4	5×4	–	–
10041/ 33	2×0	1×0	3×0	–	2×0
34	5×0	2×0	–	–	–
47	6×0	–	1×0	1×4	–
54	2×0	1×0	–	–	–
56	10×0	1×0	6×0	6×0	5×0
10042/ 5	5×4	6×4	2×4	3×4	–
9	7×4	3×4	5×4	7×4	4×4
19	3×0	4×2	7×4	–	4×4
20	6×4	1×4	4×4	1×4	–
25	3×4	4×4	–	–	–
26	8×0	5×0	12×0	5×0	11×0
31	8×0	11×0	7×0	5×0	2×0
32	3×0	3×0	6×0	2×0	4×0
41	6×0	10×0	12×0	10×0	5×0
50	3×0	2×0	5×0	–	–
	GMS, (2)*st1st1* and (2)*St1*.[a]				
10046/ 3	7×0	2×0	3×0	5×0	–
4	1×0	1×0	2×0	8×0	–
9	3×0	1×0	6×0	3×0	–
11	5×0	3×0	–	–	–
15	2×0	6×0	5×0	3×0	2×0
16	2×0	–	–	–	–
19	1×0	8×0	5×0	4×0	–
10047/ 20	4×0	3×0	2×0	–	5×0
30	2×0	3×0	3×0	3×0	5×0
37	2×0	2×0	–	–	–
10048/ 1	4×0	2×0	1×0	2×0	–
3	2×4	3×4	–	–	–
10	2×4	9×4	6×4	4×4	10×4
30	5×0	5×0	4×0	7×0	–
10053/ 1	3×4	1×4	3×4	–	–
4	2×0	1×0	–	3×0	1×0
11	1×4	7×4	3×4	4×4	6×4
13	1×0	1×0	2×0	–	–
14	3×4	2×4	–	3×4	1×4
16	1×4	1×4	–	–	1×4
18	1×0	2×0	2×0	3×0	1×0
23	4×0	–	2×0	1×0	2×0
24	2×0	1×0	1×0	–	–
25	3×0	–	2×0	–	–
28	1×0	2×0	2×0	–	–
32	1×0	1×0	2×0	–	–
33	2×0	1×0	2×0	–	–
39	2×0	1×0	3×0	–	1×0
40	2×4	3×4	1×4	3×4	1×4

[a] The dot (.) indicates the unknown allele (dominant or recessive)

fore, the expected frequency for the maintainer-genotypes in the F_2 generation must be increased from one-eighth to three-eights. In fact, the number of 8 maintainers out of 18 test-crossings comes close to this theoretical value.

Influence of high temperatures and tetracycline treatment on st1- and st2-sterility

Clones of genotypes selected from segregating offspring were cultivated in greenhouse-chambers and subjected to different temperature and tetracycline regimes. Table 6 shows the classification of the flowers occurring under the different treatments.

Neither the GMS genotypes nor the CMS genotypes were influenced by high temperatures or tetracycline.

Likewise, the treatments had no effect on the few fertile plants which were selected from the same segregating lines and integrated as a control. Only 6 out of 42 CMS genotypes (10031/25; 10033/14; 10040/51 and 10040/66; 10041/47; 10042/19) varied for the degree of sterility. These variations were also observed under one and the same treatment and can not be traced back to the effect of a special treatment. There probably was a mixing of plant materials or pollution by seeds during the vegetative preservation and propagation of these genotypes.

Discussion

The present paper describes an efficient method to characterise male sterility with respect to cytoplasmic factors, which might be causally related to them. Whether new sterilities are CMS (cytoplasmic male sterilities) or GMS (genic male sterilities) is of interest to plant breeders, as CMS permits the efficient and complete control of pollination in a hybrid breeding programme. In comparison with CMS, a completely sterile A-line can not be obtained using GMS due to the lack of maternally inherited factor. These maternally inherited factors of CMS systems result from recombinations within the mitochondrial genome. Mitochondrial genome diversity is therefore a precondition for the formation of a CMS system and can be examined by means of RFLP analyses using mitochondrial gene probes. For *Allium schoenoprasum*, this has recently been described (Engelke and Tatlioglu 2000b). The subject of the present paper was the transfer of the nuclear sterility factors *st1* and *st2* to previously distinguished cytoplasms. The segregation analysis of the F_2 generation provided evidence that st1-sterility shows itself in four different cytoplasms. Consequently, the sterility only depends on the nuclear gene *st1*, i.e. an additional factor of a specific cytoplasm can be excluded. Thus, st1-sterility is a genic male sterility (GMS). In contrast, transfer of the *st2* gene to a new cytoplasm type resulted only in non-segregating F_2 lines, so that the *st2* gene does not create sterility in this cytoplasm. A factor of the original cytoplasm (type 5) is therefore necessary for the occurrence of the sterility, and this was also verified by a reciprocal cross. The new CMS system was completed by the selection of maintainer-genotypes.

As known, male sterility can be influenced by temperature. The st1- and st2-sterility were therefore tested under different temperature conditions [20°/12°C, 24°/24°C and 30°/24°C (day/night)]. Both sterilities showed a stable expression of the phenotype, therefore temperature was of no significance. Likewise, there was no influence of tetracycline on these sterilities.

An influence of environmental factors on male sterilities has been described for different plant species. For the previously described CMS system of chives, it was discovered that the reaction to high temperatures and tetracycline treatments is causally related to certain genotypes (see Introduction). The selection of temperature-insensitive genotypes, leading to A-lines with a stable expression of the sterility, enables the use of this CMS system in hybrid breeding of chives. However, such a selection is time- and cost-intensive. In other plant species, like in the CMS S-system in onion (*Allium cepa*, van der Meer and van Bennekom 1969) or in the polima and napus CMS systems in rape (*Brassica napus*, Fan and Stefansson 1986), the genetics concerning the sensitivity to high temperatures is not solved. Consequently, the development of A-lines with a stable expression of these sterilities is a problem in practical breeding.

On the other hand, the influence of environmental factors might be of benefit when these factors can be predicted and therefore controlled. Effects of photoperiodism might be used in the breeding of wheat (cytoplasm from *Aegilops crassa*). During short days, pollen production within the A-line permits self-fertilisation, while long days prevent pollen production and enable hybrid fertilisation (Murai 1997). It is therefore not necessary to have a nearly isogenic B-line for the maintenance of the hybrid seed parent (A-line).

Concerning the use of GMS systems the induction or repression of pollen production within the same genotype by controlling the environmental factors is of particular interest, as this is the only way to obtain a completely sterile A-line. In rice there is a GMS, sensitive to photoperiodism, which is used in hybrid breeding as described above for the CMS in wheat (Yuan et al. 1993; Zhang et al. 1994). The use of temperature effects for the maintenance of an A-line is discussed for an additional GMS in rice (reviewed in Subudhi et al. 1997) as well as for a GMS in broccoli (*Brassica oleracea* var. *italica*, Dickson 1970).

However, the prediction and control of environmental factors, especially of temperature, is not always possible and does not, therefore, give a real advantage over a reliably working CMS system. The characterisation of a new CMS system in chives is therefore of direct benefit to hybrid breeding. In order to broaden the genetic basis, both available systems should be used, as it is not yet clear if one of the sterility-inducing cytoplasms transmits undesirable characters. These could include a vulnerability to pathogens, as described for the fungal pathogens *Bipolaris maydis* and *Phyllosticta maydis* in the CMS T-system in maize (Ullstrup 1972; Levings and Siedow 1992), for *Peronosclerospa sorghi* in maize (Borges 1987) and for *Erysiphe graminis* in wheat (Voluevich and Buloichik 1992). There are also cytoplasmic effects influencing the vulnerability to insects (e.g. to *Schizaphis graminum* in sorghum, Dixon et al. 1990) and to abiotic factors (e.g. of the cytochrome-c-oxidase to carbon monoxide in sorghum, Munjal et al. 1988). Besides affecting the vulnerability to pathogens, cytoplasmic effects have been described for the suitability for *in vitro*-techniques (maize: Chernysheva and Shamina 1991, rice: Yan et al. 1996, wheat: Ekiz and Konzak 1991). Furthermore, the nuclear genes which are involved in a CMS system could cause pleiotropic effects or might be in close linkage to other undesirable genes, like the restorer-gene and a

gene coding high amounts of glucosinolate in rape (Delourme et al. 1998; Renard et al. 1998). These examples illustrate the necessity to avoid restrictions on the genetic basis used in hybrid seed production. In order to examine cytoplasmic effects on important agronomical traits in chives, we need to develop nearly isogenic lines with both sterility-inducing cytoplasms. Genotypes which are able to maintain both CMS forms have been selected and the back-crossings are already in progress. The direct comparison between the nearly isogenic lines will reveal characters that are inherited by the cytoplasms. A comparison between test hybrids, produced by crossings with the same paternal parents (C-lines), will provide evidence of whether there are interactions between the cytoplasms and different nuclear backgrounds. Such results could supply information on the involvement of the cytoplasm in the "heterosis phenomenon."

The results presented here provide evidence of a second CMS system and a second GMS system, in addition to the CMS and GMS already existing in *Allium schoenoprasum*. Thus, the existence of two CMS and two GMS systems in the same plant species offers important advantages for further investigations of the molecular and functional basis of male sterility in plants.

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