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A PCR-based marker system monitoring CMS-(S), CMS-(T) and (N)-cytoplasm in the onion (*Allium cepa* L.)

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Abstract The chimerical mitochondrial CMS_1-specific sequence in chives (*Allium schoenoprasum*) was used to develop a PCR-marker that distinguishes both male-sterility inducing cytoplasms, CMS-(S) and CMS-(T), from the normal cytoplasm in onion (*Allium cepa*). In combination with a previously described marker for CMS-(S), which anchors in the upstream region of the mitochondrial gene *cob*, all of the three known cytoplasms in the onion are distinguishable. The PCR-marker system was tested in 361 onion plants, which were selected from F_1-hybrids and different open-pollinated varieties. The latter are mainly landraces from Turkey, in which all three cytoplasm types were detected.

Keywords Cytoplasmic male sterility · Mitochondria

Introduction

In onion, (*Allium cepa* L.), two types of cytoplasmic male sterility (CMS) are used in hybrid breeding. The CMS-(S) was discovered in the onion cultivar 'Italian Red' and is conditioned by the sterility inducing cytoplasm (S) and the single nuclear restorer gene *Ms*/*ms* in its recessive condition (Jones and Emsweller 1936; Jones and Clarke 1943). The CMS-(T) was discovered in the onion cultivar 'Jaune paille des Vertus' (Berninger 1965) and is influenced from three independently segregating restorer loci. Fertility is restored by a dominant allele at one locus, *A*/*a*, or at both of two complementary loci, *B*/*b* and *C*/*c* (Schweisguth 1973).

Onion is a biennial plant and 4 to 8 years are required to determine a cytoplasm type by test-crossings. The characterization of polymorphisms in the mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) by use of RFLP (restriction fragment length polymorphism) analysis is a significantly faster method than test crossing to distinguish the different cytoplasm types. Restriction patterns of mitochondrial DNA have been permitted to distinguish between (S)-cytoplasm, on the one hand, and (N)- and (T)-cytoplasm, on the other hand (De Courcel et al. 1989; Holford et al. 1991a; Satoh et al. 1993; Havey 1995, 2000). The latter two types could not be properly differentiated and are therefore assigned to the (M) cytoplasmic group. According to the authors, these findings indicate that the (S)-cytoplasm might be of alloplasmic origin, while the (T)-cytoplasm has to be considered as an autoplasmic mutation of the (N)-type. Havey (1993) corroborated this theory by RFLP investigations of the cpDNA. All these RFLP-analyses either need restricted DNA of purified mitochondria or chloroplasts, or the use of suitable probes in Southern hybridization. RFLP analyses are therefore still time-consuming, especially due to the large amounts of highly purified DNA that are required but that may not be available from small plants. Compared to this, the Polymerase chain reaction (PCR) would allow a quick and confident identification of the cytoplasm of individual plants.

The first published PCR-marker for cytoplasm types in onion amplifies the intergenic spacer (IGS) between *trn*T and *trn*L in the cpDNA. Havey (1995) demonstrated that there is a 100-bp insertion in the IGS in (N)-cytoplasm, resulting in a bigger amplicon of corresponding size. Alcalá et al. (1999) reported DNA sequence variation within this IGS and described a second plastome variant within the (N)-cytoplasm. Further variation within the (M) cytoplasmic group was found by our own investigations (Terefe et al. 2002). Though the overall organisation of the plastid genome is conserved, minor rearrangements consisting of small insertion/deletion mutants are common and occur principally in non-coding intergenic spacer regions and introns. The IGS-region between *trn*T and *trn*L shows a cluster of these small-length mutations. The tendency of insertion/deletion mutants to cluster in "hot spot" regions is already known, especially in AT-rich

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regions (Kung et al. 1982; Downie and Palmer 1992; Ogihara et al. 1992). The IGS-sequence contains many direct and inverted repeats with AT contents up to 100% that may contribute to the instability of this segment. However, variation within the (S)-cytoplasm has not yet been found, and the amplicon in (S)-cytoplasm is the smallest and can be used as the diagnostic fragment for this cytoplasm type.

A second PCR-marker anchors in the upstream region to the mitochondrial gene *cob* (Sato 1998). According to the author, the (S)-cytoplasm contains an insertion in this region, which is homologous to the chloroplast *orf*1708 of *Nicotiana tabacum*, and which can be used to anchor a (S)-specific primer. Sato suggested a second primer which should be (N)-specific, and a common antisense primer. In this paper we report on our experiences with this marker in (S)-, (N)- and also in (T)-cytoplasm.

Furthermore we have developed a new PCR marker, which was derived from a marker sequence for CMS_1 in chives (*Allium schoenoprasum* L.). The CMS-(S) in onion and the CMS_1 in chives bear similarities with regard to tapetum behaviour and to the moment and the form of microspore degeneration (reviewed in Holford et al. 1991b; Ruge et al. 1993; Engelke et al. 2002). Besides this, there are reports about an influence from high temperatures on CMS_1 in chives (Tatlioglu 1987), and also in CMS-(S) in onion in some genetic backgrounds (reviewed in van der Meer and van Bennekom 1969). However, the phenotypes of CMS-(S) and CMS-(T) in onion are very similar; thus, it seems possible that some of these reports deal unwittingly with (T)-cytoplasm. As mentioned above, the (S)-cytoplasm of *A. cepa* is believed to be of alloplasmic origin (Holford et al. 1991a; Havey 1993), whereas both alleles of the restorer gene are common in all onion populations and therefore the mutation had to be realised a long time ago in the evolutionary pathway (Little et al. 1944; Davis 1957). From these facts it can be deduced that the CMS-(S) in onion and the CMS_1 in chives might have the same molecular origin. Recently we have developed a PCR-marker for the CMS_1-inducing cytoplasm in chives, which is exclusively amplified in the sterility inducing cytoplasm of CMS_1, but neither in CMS_2 nor in the three known normal cytoplasm types (Engelke and Tatlioglu 2000a, 2002). The sequence of this marker is of chimerical nature, consisting of *atp*9-homologous regions on its end, which are interrupted from an insertion that is composed of one *atp*6-homologous part and one part of unknown origin. The chimerical marker sequence forms a putative open reading frame (*orf*A501), from which CMS_1 might originate (Engelke and Tatlioglu 2002). Therefore we have derived primers from the marker sequence in chives, which span nearly the complete *orf*A501, and tested the amplification in the (S)-, (T)- and (N)-cytoplasm of the onion. The indication of the cytoplasm types is compared to the PCR-marker described from Sato (1998). We applied both markers to 361 onion plants representing 15 open-pollinated and 14 hybrid onion cultivars.

Materials and methods

Plant materials

Five F_1-hybrids from Germany, one from Japan, seven from the Netherlands and one from Turkey were sown in 2000 from commercially available seeds (see Table 2). For molecular analyses, three single plants were randomly selected from each F_1-hybrid. 15 open pollinated varieties were obtained from different regions in Turkey and one from Germany (see Table 2). The number of investigated plants varied between 3 and 40 per open-pollinated variety.

Isolation of DNA, primer-design and PCR-amplifications

Highly purified total DNA was isolated as previously described (Engelke and Tatlioglu 2000b). When the PCR-protocols have been optimised, an alternative DNA-isolation protocol, originally described by Edwards et al. (1991), was used, which is much less-time and cost intensive and needs very less plant material.

Since the primers for the 762-bp marker fragment which is exclusively amplified in (S_1) cytoplasm in chives (Engelke and Tatlioglu 2002) do not amplify a sequence in the onion, new primers were derived which span nearly the complete *orf*A501, that is part of the 762-bp fragment in chives. These primers are 5'ATGGCTCGCCTTGAAAGAGAGC3' and 5'CCAAGCATTTGGCGCTGAC3', and the corresponding annealing temperature is 60 °C.

Primers which anchor in the upstream region to the mitochondrial gene *cob* were used as suggested from Sato (1998). Namely, (S)- specific: 5'-GTCCAGTTCCTATAGAACCTATCACT-3', (N)-specific: 5'-TCTAGATGTCGCATCAGTGGAATCC-3' and a common primer: 5'-CTTTTCTATGGTGACAACTCCTCTT-3' with an annealing temperature of 53 °C.

PCR was performed using 50 ng of total DNA in a final volume of 20 μl, containing 0.25 μM of each primer, 150 μM of each dNTP, and 0.25 units of Dynazyme in reaction buffer recommended by the supplier (Biometra). The reaction mixture was incubated in a thermocycler (Biometra) for 2 min at 94 °C, followed by 40 cycles: 30 s at 94 °C, 1 min for annealing, 2 min at 72 °C and a final extension for 5 min at 72 °C. The annealing temperatures (see above) were calculated by the given T_M-values of the different primers and optimised, using the temperature gradient of the thermocycler. PCR-products were separated by flatbed electrophoresis using 1.5% agarose gels in 1 × TAE buffer.

Results

Amplification of the upstream region to the mitochondrial gene *cob*

The PCR-marker which anchors in the upstream region to the mitochondrial gene *cob* (Sato 1998) will be referred to as the 5' *cob*-marker below. According to Sato (1998) the (S)-specific primer should amplify a 414-bp fragment in the (S)-cytoplasm, and the (N)-specific primer a 180-bp fragment in the (N)-cytoplasm. However, in our investigations it was clearly visible that the (S)-marker fragment is also amplified in the (N)-cytoplasm and vice versa, if only one of these primers is used together with the common antisense primer (Fig. 1). Quantitative differences in the amplification of the 414-bp fragment became obvious from the comparison of the amplicon in both cytoplasms when only the (S)-specific primer is used. The lower amplification in (N)-cytoplasm gives a hint that the

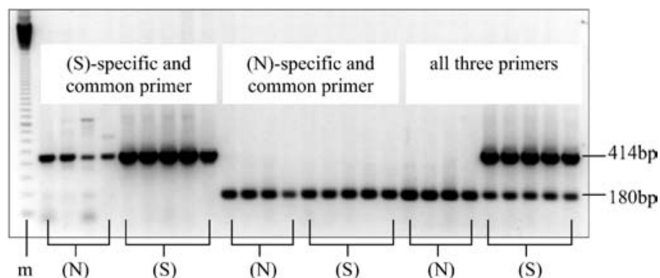

Fig. 1 Amplification of the upstream region of the mitochondrial gene *cob* (5' *cob*-marker) in single plants representing (N)- and (S)-cytoplasm, using the primers according to Sato (1998) in different combinations

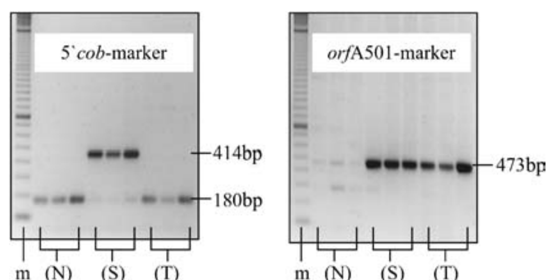

Fig. 2 Amplification of the 5' *cob*-marker (Sato 1998) and of the *orf*A501-marker in (N)-, (S)- and (T)-cytoplasm, in three single plants each

template DNA is present in this cytoplasm type as sub-stoichiometric DNA (sublimon). When both primers, the (S)- and the (N)-specific, are used together with the common primer in one and the same PCR-reaction, the 414-bp fragment is not amplified in (N)-cytoplasm (Fig. 1). The amplification seems to be inferior to the amplification of the 180-bp fragment, as a consequence of the stoichiometric differences of the template-DNA. In (S)-cytoplasm both fragments are amplified, thus both cytoplasm types can be distinguished.

Sato (1998) disregarded the (T)-cytoplasm. The F_1-hybrid ‘Hygro’, which is produced by using the (T)-system according to the breeding company and previous investigations from Havey (2000), was integrated in our experiments as a source of this cytoplasm type. The 5' *cob*-marker does not distinguish this cytoplasm type from the (N)-type, and in both cytoplasm types the 180-bp fragment is amplified (Fig. 2).

Amplification of the mitochondrial *orf*A501 homologous region

The 473 bp representing the *orf*A501 homologous region is clearly amplified in (S)- and (T)-cytoplasm, while there is only a weak amplification in the (N)-cytoplasm, indicating that the template DNA is present as a sublimon in the latter cytoplasm type (Fig. 2).

Table 1 Differentiation of (N)-, (T)- and (S)-cytoplasm by combining the 5' *cob*- and the *orf*A501-marker. +, amplification; –, no amplification

Cytoplasm	5' *cob*		*orf*A501 473 bp
	180 bp	414 bp	
(N)	+	–	–
(T)	+	–	+
(S)	(+)	+	+

The combination of both primer systems allows the differentiation of all three cytoplasm types. The (S)-cytoplasm is characterized by the amplification of the 414-bp fragment of the 5' *cob*-marker.While the (N)-cytoplasm is characterized by the 180-bp fragment of the 5' *cob*-marker and the failure of an amplification product of the *orf*A501-marker; the (T) cytoplasm is characterized by the same result concerning the 5' *cob*-marker and the amplification of the 473-bp fragment of the *orf*A501-marker (Table 1). In other words, both sterility inducing cytoplasms can be distinguished from the (N)-cytoplasm by using the *orf*A501-marker, and separated from each other by using the 5' *cob*-marker.

Evaluation of F_1-hybrids and open-pollinated varieties by using the combined marker system

Three hundred and sixty one onion plants from 15 open-pollinated and 14 hybrid onion cultivars were evaluated with the 5' *cob*- and the *orf*A501-marker, in order to demonstrate the applicability of cytoplasm determination. The F_1-hybrids were represented by three single plants each. The (S)-cytoplasm was found in the F_1-hybrid ‘Suluova’ from Turkey, in ‘Takmark’ from Japan, in ‘Berta’ from Germany and in 5 F_1-hybrids from the Netherlands (Table 2). The varieties ‘Stamford’ and ‘Hygro’ (Netherlands) possess the (T)-cytoplasm. The German varieties ‘Hermes’ and ‘Laura’ showed a mixture of plants with (T)- and (N)-cytoplasm, the variety ‘Frieda’ demonstrated plants with (S)- and (N)-cytoplasm, and all three investigated plants of the variety ‘Elsa’ had (N)-cytoplasm (Table 2). These findings contradict the F_1-hybrid character of these latter varieties.

In five of the open-pollinated varieties a mixture of plants with (S)- and (N)-cytoplasm, and in seven only (N)-cytoplasm was found (Table 2). In three of the open-pollinated varieties (‘KocabasI’, ‘Akca soganI’ and ‘Kayseri’) plants with (N)- and (T)-cytoplasm were found (Table 2). However, additional cytoplasm types per variety might occur, if the number of investigated plants would be enhanced.

Discussion

The investigations of the 5' *cob*-marker (Sato 1998) clearly demonstrated that the (S)-marker sequence is also

Table 2 The distribution of cytoplasm types in F_1-hybrids and open-pollinated varieties, determined by comparing the 5' *cob*- and the *orf*A501-marker

Variety	Origin	Number of plants	473 bp (*orf*501)	414 bp (5'-*cob*)	Cytoplasm
Suluova F1	cp. Agromar, Turkey	3	+	+	(S)
Takmark F1	cp. Takii, Japan	3	+	+	(S)
Briso F1	cp. Nunhems, Netherlands	3	+	+	(S)
Cimarron F1	cp. Nunhems, Netherlands	3	+	+	(S)
Rio Bravo F1	cp. Nunhems, Netherlands	3	+	+	(S)
Rojo F1	cp. Nunhems, Netherlands	3	+	+	(S)
Vaquero F1	cp. Nunhems, Netherlands	3	+	+	(S)
Berta F1	cp. Saatzucht Aschersleben, Germany	3	+	+	(S)
Hygro F1	cp. Nunhems, Netherlands	3	+	–	(T)
Stamford F1	cp. Novartis, Netherlands	3	+	–	(T)
Hermes F1	cp. Saatzucht, Quedlinburg, Germany	2	+	–	(T)
		1	–	–	(N)
Laura F1	cp. Saatzucht Aschersleben, Germany	1	+	–	(T)
		2	–	–	(N)
Frieda F1	cp. Saatzucht Aschersleben, Germany	2	+	+	(S)
		1	–	–	(N)
Elsa F1	cp. Saatzucht Aschersleben, Germany	3	–	–	(N)
Stuttg. Riesen	cp. Juliwa, Germany	40	–	–	(N)
Texas 502	cp. Agromar, Turkey	3	–	–	(N)
Valenciana	cp. Agromar, Turkey	4	+	+	(S)
		26	–	–	(N)
Akca sogan-I	Landrace from Turkey	1	+	–	(T)
		29	–	–	(N)
Akca sogan-II	Landrace from Turkey	7	–	–	(N)
Akgün	Landrace from Turkey	1	+	+	(S)
		29	–	–	(N)
Bozgüney	Landrace from Turkey	17	+	+	(S)
		13	–	–	(N)
Corum Tipi	Landrace from Turkey	14	–	–	(N)
Csardas	Landrace from Turkey	30	–	–	(N)
Imrali Sogani	Landrace from Turkey	2	–	–	(N)
Kantartopu	Landrace from Turkey	2	+	+	(S)
		28	–	–	(N)
Kayseri	Landrace from Turkey	8	+	–	(T)
		22	–	–	(N)
Kocabas-I	Landrace from Turkey	2	+	–	(T)
		5	–	–	(N)
Kocabas-II	Landrace from Turkey	6	–	–	(N)
Yalova	Landrace from Turkey	19	+	+	(S)
		11	–	–	(N)

present in the (N)-cytoplasm and vice versa, at least as a sublimon. However, when we used both primers, the (S)- and the (N)-specific, together with the common primer in one and the same PCR-reaction, (S)- and (N)-cytoplasm can be clearly distinguished. The (T)-cytoplasm, which was not studied by Sato (1998), showed the same amplification as the (N)-cytoplasm did. Compared to the cpIGS-marker which shows a cluster of insertion/deletion mutants and an emerging length variation within the (M) cytoplasmic group (see Introduction), we prefer the 5' *cob*-marker for indication of the (S)-cytoplasm, since the explanation was easier in our experiments.

The new *orf*A501-marker, which was developed from a CMS_1-specific sequence in chives, is amplified in (S)- and (T)- cytoplasm of the onion, but not in (N)-cytoplasm. The combination of the 5' *cob*-marker with the *orf*A501-marker allows us to distinguish between all three cytoplasm types in individual plants a few weeks after sowing. This might be helpful to reduce the efforts in a breeding programme, when new sterile lines and the corresponding maintainer lines will be developed. If one of the sterility inducing cytoplasms is detected by the PCR-markers, the selected plant can never be used as a maintainer for this CMS system; however, the plant might be sterile or in selfed progenies sterile plants may segregate and can be used as the starting point for a sterile line. The selection of sterile and corresponding maintainer plants from one and the same provenance might reduce the number of backcrosses for reaching homozygous male-sterile lines with corresponding maintainers, which are needed in a successful hybrid breeding programme.

The identification of the cytoplasm type might also help to classify the genetic nature of sterile plants, which spontaneously occur in open-pollinated varieties. Genetic analyses of such plants revealed that the factors *ms* and (S) may occur in highly different frequencies in onion varieties of different countries (reviewed in van der Meer

and van Bennekom 1971). Evidence on the occurrence of CMS-(S) in onions from Turkey was presented from Davis (1958) and Havey (1997) among introductions to Parma, Idaho, for the first time, and is confirmed by our investigations. Little et al. (1944) suggested that the common occurrence of the recessive *ms* allele in most onion populations and shallot indicates that the mutation from *Ms* to *ms* must have occurred early in the evolution of onion or has occurred many times. Data concerning the CMS-(T) system are rarely available until now, since this system is not in common use in breeding for such a long time as the CMS-(S), and until now it was not possible to distinguish the (T)-cytoplasm from the (N)-cytoplasm by use of molecular methods. The represented PCR-marker might enhance our knowledge of the origin of this system. As our investigations have shown, the (T)-cytoplasm is present in Turkish landraces and German varieties; thus it is not exclusively present in the French variety 'Jaune paille des Vertus' (Berninger 1965), in which it was discovered.

In addition to insights of the area of distribution of the CMS-systems and the advantages for the selection in practical breeding, the marker system might help to check on the F_1-hybrid character of varieties. The results of our investigations using the marker system demonstrate that ten of the F_1-hybrids investigated are in accordance with the information of the breeding companies. However, the segregation of the markers and the finding of plants with (N)-cytoplasm in the remaining four F_1-varieties 'Hermes', 'Laura', 'Frieda' and 'Elsa' clearly contradicts the F_1-hybrid character. Even if there would be further unknown cytoplasm types in the onion, which cannot be distinguished by the markers, one would not expect segregation of a mitochondrial marker in a F_1-hybrid, which was harvested from one pure-breeding homozygous inbred line. Thus our findings give a strong hint, that these latter varieties were not pure F_1-hybrids.

The mitochondrial sequences that cause male sterility in (S)- or (T)-cytoplasm of the onion are still unknown. It is interesting to note that the same *orf*A501-related sequence was amplified in CMS_1 in chives, in CMS-(S) and in CMS-(T) in the onion, but neither in the remaining four cytoplasm types of chives nor in the (N)-cytoplasm of the onion. The question arises, is this sequence just an indirect marker, or is it the cause of CMS. As mentioned in the introduction of this paper, the possibility clearly exists that the CMS-(S)-system in onion and the CMS_1 in chives might have the same molecular origin. The finding of this sequence in (T)-cytoplasm was unexpected, since untill now the (T)-cytoplasm was not properly distinguished from (N)-cytoplasm by the use of molecular methods, and thus alterations between both cytoplasms seem to be limited to a small part of the mitochondrial genome (see Introduction). In order to obtain more information about the significance of the *orf*A501-sequence we will investigate the transcription in sterile and restored plants of the CMS-(S)- and CMS-(T)-system of onions and the CMS_1-system of chives in further studies.

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