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Genetic variation of petaloid male-sterile cytoplasm of carrots revealed by sequence-tagged sites (STSs)

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Abstract The mitochondrial DNA of various carrot lines was characterized by random amplified polymorphic DNA (RAPD) analysis, and six sequence-tagged sites (STSs) led to identification of the petaloid type of cytoplasmic male sterility (CMS). Using six STS primer combinations, we were able to classify five CMS lines into two groups and eight fertile carrots into six groups. Both the STS1 and the STS4 primer combinations differentiated CMS cytoplasms from the fertile cytoplasms, and the STS2 primer combination revealed two different types of CMS cytoplasms – of Wisconsin Wild and Cornell origins. Cybrid carrot lines with petaloid flowers which had been obtained by asymmetric cell fusion could also be separated from fertile cybrids by the STS1 primer combination. The STS1 fragment contained a homologous sequence with the *orfB* gene. DNA gel blot analysis indicated that homologous regions to the STS1 fragment existed in fertile types as well as the CMS types, although the restriction fragment size patterns differed. These observations demonstrate that rearrangements involving this region occurred in the mitochondrial genome. The STS4 fragment had a more complicated gene structure, including retrotransposon-like sequences and small segments of chloroplast genome.

Key words *Daucus carota* spp. *sativus* · RAPD · Cytoplasmic male sterility (CMS) · Asymmetric cell fusion

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Introduction

In cytoplasmic male sterile (CMS) lines, developmental gene expression of flower formation is not correctly regulated, and abnormal flower organs have been observed (Laser and Lersten 1972; Hanson 1991). The CMS phenotype, a maternally inherited trait, is usually correlated with rearrangement of the mitochondrial genome. However, many CMS restorer genes are encoded by the nuclear genome, indicating that morphological expression of the CMS phenotype is also affected by a concerted gene expression from the nuclear genome (Hanson 1991).

In carrot [*Daucus carota* spp. *sativus* (Hoffm.) Arcang.] two distinct CMS phenotypes, the petaloid and brown anther types, have been observed. The phenotype of brown anther CMS is characterized by brownish anthers, in which pollen growth ceases prior to maturation of this phenotype. In contrast, the petaloid CMS types have petals or petal-like structures instead of anther organs, which also do not form functional pollen. At least two different types of petaloid CMS, the Cornell and the Wisconsin Wild types, have been identified in carrots (Morelock et al. 1996), and their cytoplasms were thought to have originated from wild carrots. The petal of the Wisconsin Wild type generally exhibits a distinct white color compared with that of the Cornell petaloids. Some differences in the restriction fragment length polymorphism (RFLP) patterns of mitochondrial DNA (mtDNA) have been noted between these two types (Bowes and Wolyn 1998). The molecular mechanism of the CMS phenotypes in carrots has remained unclear because key molecules coded by both the nuclear and mitochondrial genomes still remain unidentified (Peterson and Simon 1986; Börner et al. 1995).

We reported earlier that the mitochondrial genome of carrots could be classified by random amplified polymorphic DNA (RAPD) (Nakajima et al.1997). By means of RAPD analysis, highly reproducible fragment patterns were obtained with a small amount of mtDNA and the dendrogram of the mitochondrial genome showed four

differentiated groups of carrots. In the investigation presented here, we selected six RAPD fragments amplified from the mtDNA in both CMS and fetile carrot varieties and then converted them into sequence-tagged sites (STSs) for a more convenient detection of the CMS genotype. Using these STSs, we attempted to distinguish CMS and fertile cytoplasms. Cybrid plants were produced by cell fusion of a CMS variety and a fertile cytoplasm, and these were examined for mtDNA diversity by STS analysis. We also present here structural analysis of the STS fragments from CMS lines and the characterization of rearrangements of the CMS mitochondrial genome.

Materials and methods

Plant materials and extraction of mtDNA

Thirteen varieties of carrot [*Daucus carota* ssp. *sativus* (Hoffm.) Arcang.] were used for this study, including five CMS lines (493S, 2566A, 9304A, MS-1 and MS-2), three inbred fertile lines (493N, 2566B and 9304B; Simon et al. 1987, 1990) and five fertile cultivars ('Nagafutori-kintoki', 'Kokubun-senkou-oonaga', 'Koizumi-risou-gosun', 'Imperator' and 'Scarlet Nantes') (Table 1). 493S, 2566A and 9304A are petaloid cytosterile counterparts of 493N, 2566B and 9304B, respectively. MS-1 and MS-2 also have petaloid type flowers and are derived from the progenies of F_1 hybrid cultivars (Nakajima et al. 1997).

Isolation of mtDNA was done as previously described (Nakajima et al. 1997). Protoplasts were prepared from suspension culutres, and mitochondrial fractions were collected by centrifugation. After DNase treatment, mitochondrial membrane components were digested with Proteinase K, and the mtDNA was purified by phenol-chloroform extraction.

RAPD analysis and the cloning procedure of polymorphic bands

RAPD analysis of mtDNA was carried out using 59 primers consisting of FPT11–20 (Yamamoto et al. 1994); OBP02–10, OPE01–20 and OBJ01–20 (Operon Technologies, Calif.). Amplification was done in 20-µl volumes consisting of 10 m*M* TRIS-HCl (pH 8.30), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.01% gelatin, 0.2 m*M* each of dATP, dCTP, dGTP, dTTP, 1 µ*M* RAPD primers, 20 ng mtDNA and 2.5 U Taq polymerase (Takara Shuzo). Polymerase chain reaction (PCR) amplification was for 40 cycles of 94°C for 1 min, 40°C for 2 min, 72°C for 3 min, for denaturing, annealing and primer extension, respectively. Five PCR fragments from CMS plants and one PCR fragment from fertile plants were selected and cloned into the pCRII vector (Invitrogen) for DNA sequence analysis.

STS analysis

Sequencing of the cloned RAPD fragments was done according to the manufacturer's (Applied Biosystems) protocol. The nucleotide sequences of both ends (approximately 200 bp) of the DNA fragments were determined using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and DNA sequencer Model 370A (Applied Biosystems). STS primers were designed based on the nucleotide sequence of each fragment.

Total DNA was isolated from 2-week-old leaves by the CTAB extraction method described by Yamamoto et al. (1994). The prepared DNA (100 ng) served for PCR amplification under conditions of 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C in 20-µl reaction mixtures consisting of 10 m*M* TRIS-HCl (pH 8.30), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin, 0.2 m*M* each of dATP, dCTP, dGTP and dTTP, 1 µ*M* each primer, 1 U Taq polymerase (Takara Shuzo). Amplified products were separated on 1.5% agarose gels.

Cell fusion

MS-2 and 'Kikuyou-gosun' were used for asymmetric cell fusion. MS-2, with petaloid stamens, is a CMS line and 'Kikuyou-gosun' is a stable fertile cultivar which has a mitochondrial genome organization similar to that of 'Scarlet Nantes' (Nakajima et al. 1997). Protoplasts of the two varieties were prepared as described above (Nakajima et al. 1997). Protoplasts of 'Kikuyou-gosun' were resuspended in fusion medium (0.1 m*M* $CaCl_2$, 0.5 *M* mannitol, pH 5.7) and irradiated with 85 krad X-ray to prevent cell division. Protoplasts of MS-2 were treated with 15 m*M* iodoacetamide in fusion medium at 4°C for 20 min to inactivate cell growth. A 0.4-ml aliquot of each protoplast (1×10^7/ml) was mixed and exposed to an alternating current of 1.0 MHz at 100 V/cm in the electric field for 10 s, then exposed to 1 or 2 square direct current pulse of 50 ms duration at 1.0 kV/cm by the Somatic Hybridizer (SSH-1, Shimazu). The fused protoplasts were cultured in MS (Murashige and Skoog 1962) medium (0.3 *M* sorbitol, 2% sucrose and 0.1 mg/l 2,4-dichlorophenoxyacetic acid) at 25°C and shaken at 50 rpm for 1 month.

Colonies 0.5 mm in diameter were transferred into the liquid MS medium without sorbitol for 2 weeks. Somatic embryos formed in liquid MS medium with 2% sucrose after a 1-month incubation were then plotted onto solid MS medium with 3% sucrose and 0.8% agar. Plantlets derived from cybrid cells were transplanted into potting soil, and the leaves were harvested for PCR analysis. Testing for STS fragments was performed by PCR as described above. Cybrid plants were grown to maturity in a greenhouse and the flower organs observed.

Table 1 Carrot varieties used (*NT* not tested)

Variety name	Fertility	Cytoplasm origin (CMS origin)	Cytoplasmic group[a] by RAPD
493S	Sterile	USA (Cornell)	NT
2566A	Sterile	USA (Wisconsin Wild)	NT
9304A	Sterile	USA (Cornell)	NT
MS-1	Sterile	USA	I
MS-2	Sterile	USA	I
493N	Fertile	USA	NT
2566B	Fertile	USA	NT
9304B	Fertile	USA	NT
Kokubun-senkou-oonaga	Fertile	Japan	II
Nagafutori-kintoki	Fertile	Japan	II
Imperator	Fertile	USA	III
Koizumi-risou-gosun	Fertile	Japan	III
Scarlet Nantes	Fertile	USA	IV

[a] Cytoplasmic groups were classified by Nakajima et al. (1997)

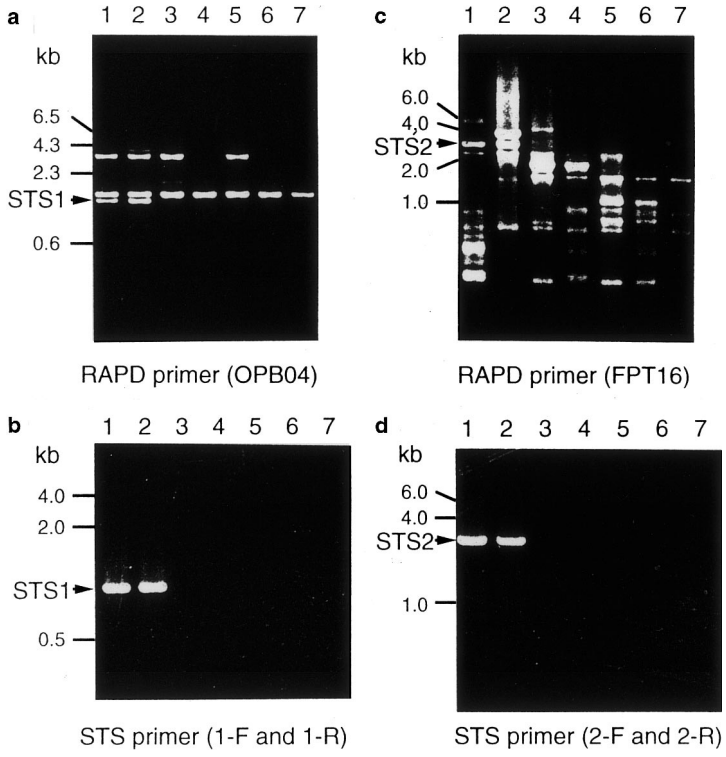

Fig. 1a–d Mitochondrial RAPD patterns and PCR products obtained with the designed STS primers. PCR products obtained with: **a** OPB04 RAPD primer, **b** STS1 primer combinations (1-F and 1-R), **c** FPT16 RAPD primer, **d** STS2 primer combinations (2-F and 2-R). *Lanes 1–7* Amplified fragments as follows: *1* MS-1, *2* MS-2, *3* 'Kokubun-senkou-oonaga', *4* 'Nagafutori-kintoki', *5* 'Imperator', *6* 'Koizumi-risou-gosun', *7* 'Scarlet Nantes'

Table 2 Characterization of polymorphic RAPD fragments and the designed STS primers

RAPD primers	Obtained RAPD fragment (kb)	Determined STS primers (no.)		Amplified bands
GGACTGGAGT (OPB04)	1.1	ACATATAGGATACACCGGTGC	(1-F)	STS1
		TGAGTCGAGCCTACTCGATT	(1-R)	
AGATCGGCAT (FPT16)	2.4	CAACAACTGCTTTCTTTCACCTG	(2-F)	STS2
		TGGGCCAATTGGGACTCTCTT	(2-R)	
CTCCATGGGG (OPJ05)	1.8	TCGACCTGTGAACAAGGTAA	(3-F)	STS3
		CCTTATCATCAGTTGCAGGG	(3-R)	
AAGCGGCCTC (OPJ20)	1.0	AGAGGAATGGGAACGAAACA	(4-F)	STS4
		ATATATGCCCCACAAGCTAA	(4-R)	
AAGACCCCTC (OPE06)	1.1	CTTACCGTAATGCGAATCTC	(5-R)	STS5
		TTTTCAAGGAAGTGAGTCTC	(5-R)	
CCGAACACGG (OPJ04)	2.4	CATCTATCGCCAACTGAGTC	(6-F)	STS6
		ATTGAAATAGGAGCCGGTTC	(6-R)	

Southern blot analysis

Mitochondrial DNA (0.3–0.6 µg) was digested with *Bam*HI (Takara Shuzo), and DNA fragments were separated on 0.8% agarose gels run at 40 V for 16 h. Southern blot analysis was done as previously described (Nakajima et al. 1997). The STS1 fragment was used as the probe.

Results

Construction of STS primers

The RAPD analysis was first carried out using 59 primers for mtDNA of both CMS and fertile lines to select the sequence-tagged sites (STSs). When the primer OPB04 was used for this analysis, PCR products of 3.0, 1.5 and 1.1 kb were observed for two CMS lines, 'MS-1', 'MS-2' and five fertile lines. Both the 3.0- and 1.5-kb bands were present in sterile and in fertile carrots. However, the 1.1-kb product was only evident in the CMS lines, MS-1 and MS-2 (Fig. 1a). Similarly, a 2.4-kb PCR product was amplified only from the CMS plant with FPT16 (Fig. 1c). Both the 1.1-kb and 2.4-kb PCR products were designated as the STS1 and STS2 fragments, respectively. To design STS primers for CMS lines, we determined the nucleotide sequences of about 200 bp of both 5′ and 3′ ends of the STS1 and STS2 fragments. On the basis of the nucleotide sequences determined, the STS primer combinations 1-F and 1-R, and 2-F and 2-R were synthesized. As expected, the synthesized STS primer combination 1-F and 1-R amplified the 1.1-kb fragment (Fig. 1b) and 2-F and 2-R amplified the 2.4-kb

Table 3 Genetic variability of mtDNA identified using STS primers

Variety name	STS primers[a]						Cytoplasmic group
	1-F,R	2-F,R	3-F,R	4-F,R	5-F,R	6-F,R	
493S	+	–	+A	+B	+B	+	S I
2566A	+	+	+A	+B	+B	+	S II
9304A	+	–	+A	+B	+B	+	S III
MS-1	+	+	+A	+B	+B	+	S II
MS-2	+	+	+A	+B	+B	+	S II
Kokubun-senkou-oonaga	–	–	+B	+A	+A	–	F I
Nagafutori-kintoki	–	–	+B	+A	–	–	F II
Imperator	–	–	+A	+A	+A	+	F III
Koizumi-risou-gosun	–	–	+A	+A	+A	+	F III
Scarlet Nantes	–	–	+B	+A	–	+	F IV
493N	–	–	+B	+A	+B	+	F V
2566B	–	–	–	+A	–	–	F VI
9304B	–	–	+B	+A	+B	+	F V

[a] +, +A and +B indicate the presence of amplified bands. When two different bands were amplified, the two bands were designated as +A and +B, respectively. Sizes of +A bands by 3-F&R, 4-F&R and 5-F&R primers are 1.8, 1.9 and 1.3, respectively. The sizes of +B bands by 3-F&R, 4-F&R and 5-F&R primers are 1.6, 1.1 and 1.1 kb, respectively. –, The absence of amplified bands

fragment (Fig. 1d). Furthermore, the OPJ05, OPJ20, OPE06 and OPJ04 RAPD primers produced a unique DNA band pattern of 1.8, 1.0, 1.1 and 2.4 kb in length, respectively (Table 2). Four STS primer combinations were similarly synthesized according to the determined nucleotide sequences of the amplified DNA. A total six combinations of STS primers were produced to individually analyse these diagnostic DNA fragments.

DNA fingerprint produced by STS primers

As observed in the MS-1 and MS-2 CMS lines of Fig. 1b, the STS1 primer combination (1-F and 1-R) produced the 1.1-kb CMS-related PCR fragments in other CMS lines such as 493S, 2566A and 9304A (Table 3). In contrast, no amplified DNA bands were obtained in any fertile lines. Therefore, the STS1 primer combination is a specific and convenient primer set by which to detect carrot CMS genotype. In the case of STS2 primers, the 2.4-kb PCR fragment was observed in MS-1, MS-2, and 2560A, and this DNA amplification was not found in other CMS lines such as 493S and 9304A. 493S and 9304A are thought to belong to the Cornell type of petaloid CMS and the remaining CMS lines to the Wisconsin Wild type. This STS2 primer combination (2-F and 2-R) can thus be used to differentiate the CMS genotype of the Wisconsin Wild type carrots from the Cornell type. The STS3 (3-F and 3-R) and STS5 (5-F and 5-R) primer combinations exhibited complicated amplification patterns. When two different bands were amplified by the same primer combination, the band with the higher molecular weight (MW) was named A and that with the other lower MW as B (Table 3). With the STS3 primer combination, two different types of amplified bands, the 1.8-kb and 1.6-kb bands, were seen in almost all of the fertile lines, but no band was obtained in the case of the 2566B fertile line, and this fertile line may have some specific disorder on its mitochondrial genome. Interestingly, the 1.8-kb band was found among all CMS lines and even some fertile lines, ‘Imperator’ and ‘Koizumi-risou-gosun’, but the 1.6-kb band was found in only some fertile lines – ‘Kokubun-senkou-oonaga’, ‘Nagafutori-kintoki’, ‘Scarlet Nantes’, 493N and 9304B. A similar pattern was also observed for the STS5 primer combinations. The STS4 primer combination (4-F and 4-R) also can be utilized as CMS-specific primers. All CMS lines produced the 1.1-kb band, but all fertile lines had the 1.9-kb band. The STS6 primer combination (6-F and 6-R) was initially selected as the fertile specific primer for the variety ‘Scarlet Nantes’. However, this amplified band was also found in CMS and fertile lines. Consequently, five CMS lines were classified into two groups and eight fertile carrot varieties into six groups using the synthesized STS primers (Table 3).

Determination of the cytoplasm genotypes of the cybrids generated by asymmetric cell fusion

Asymmetric cell fusion was carried out between MS-2, a petaloid-type CMS line, and ‘Kikuyou-gosun’, a normal fertile cultivar. Both the STS1 and STS2 primer combinations produced the CMS-specific DNA fragments in MS-2 (as described above) but not in ‘Kikuyou-gosun’. Approximately 100 plantlets were regenerated and the flowers of 9 cybrid plants examined. The flower morphology of the cybrid lines was carefully examined and compared to that of flowers from fertile and CMS parents at several stages of development. The cybrid plants were classified into three types (fertile, brown anther-type sterile and petaloid-type sterile), and their cytoplasm genotypes were tested by the STS1 primer combination (Fig. 2). The cybrid lines 2 and 5, which set petaloid-type flowers, had the same 1.1-kb amplified band as petaloid parent, MS-2.

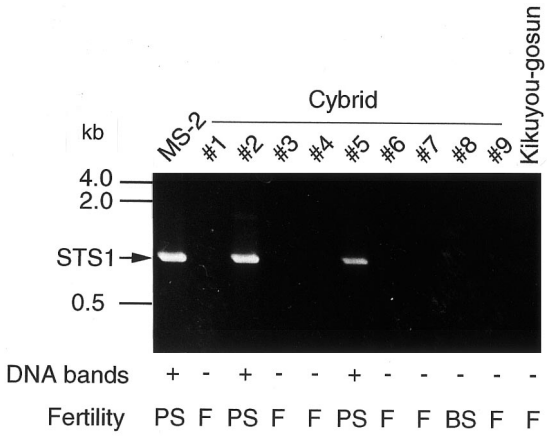

Fig. 2 PCR products of cybrids amplified with 1-F and 1-R primer combintions. *F*, *PS*, *BS* indicate fertile, petaloid sterile type and brown anther sterile type, respectively

On the contrary, the STS1 primer combination did not amplify DNA from cybrids 1, 3, 4, 6, 7 and 9. Flower morphology for all these cybrid lines is of the fertile type. Thus, a clear correlation was observed between the 1.1-kb STS-amplified band and the petaloid CMS phenotype. Interestingly, cybrid 8 with the brown anther type did not produce a positive amplified band. A similar correlation between the petaloid phenotype and the CMS-specific bands was also observed when the primer combinations were used to analyse other cybrid lines (data not shown). These results strongly suggest that the STS1 primer combination is a useful and reliable approach by which to identify the petaloid type CMS.

Southern blot analysis of mtDNA of CMS lines

The mtDNA of carrot varieties was digested with *Bam*HI and the DNA gel blots were hybridized using the 1.1-kb product of the STS1 fragment as a probe. There were no *Bam*HI restriction sites within the STS1 fragment. The STS1 DNA fragment hybridized to the mtDNA of all cultivars, but the band patterns of the CMS lines differed from those of the fertile lines (Fig. 3). In both MS-1 and MS-2, the hybridized bands had relatively strong signals.

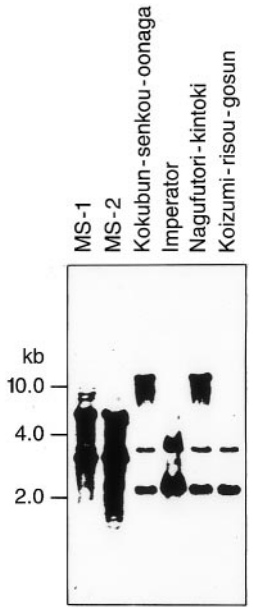

Fig. 3 Southern blot analysis of six carrot varieties. The mtDNA was digested with *Bam*HI, and the DNA fragments obtained were hybridized with the 1.1-kb RAPD fragment of the STS1 fragment as a probe

The STS1-related DNA was also found in fertile plants, although the amplified band obtained with the STS1 primer combination was not observed in fertile lines (Fig. 1a). The mitochondrial genome organization of the surrounding STS1 fragment is thought to be different in both CMS and fetile lines.

Homology search analysis for STS fragments

The nucleotide sequences of all STS fragments were compared with registered genes in the GenBank database. Four STS fragments (STS1, STS3, STS4 and STS6) had homologous sequences with registered genes (Table 4). The STS1 fragment obtained by PCR, which was observed only in sterile lines, contained a portion of the *orfB* gene. The *orfB* gene of sunflower and *Oenothera* codes a similar mitochondrial open reading frame, about 480 bp in length (Hiesel et al. 1987; Spassova et al. 1994). The STS1 fragment covered the amino-terminal region of this gene. The STS1 fragment also involved in the inverted repeat sequence which is analo-

Table 4 Homologysearch analysis of the isolated DNA fragment

Cloned STS name[a]	Identified gene	Origin	Identity (%)
STS1	Mitochondria *orfB*	*H. annuus*	198/208 (95%)
	Mitochondria *orfB*	*O. berteriana*	190/208 (91%)
STS3A, -B	Mitochondria NADH dehydrogenase subunit 5	*O. berteriana*	311/326 (95%)
	Mitochondria NADH dehydrogenase subunit 5	*T. aestivum*	233/247 (94%)
STS4A	Chroloplast DNA	*N. tabacum*	122/123 (99%)
	Chroplast NADH dehydrogenase subunit B (intron)	*H. vulgar*	118/123 (95%)
STS6	Mitochondria *orf250*	*O. berteriana*	414/450 (92%)
	Mitochondria *orf256*	*A. thaliana*	399/419 (95%)

[a] The DNA sequences of STS3A, STS3B and STS4A were submitted as full-length and have been assigned GenBank Accession Nos. AF112970, AF112971 and AF112969, respectively

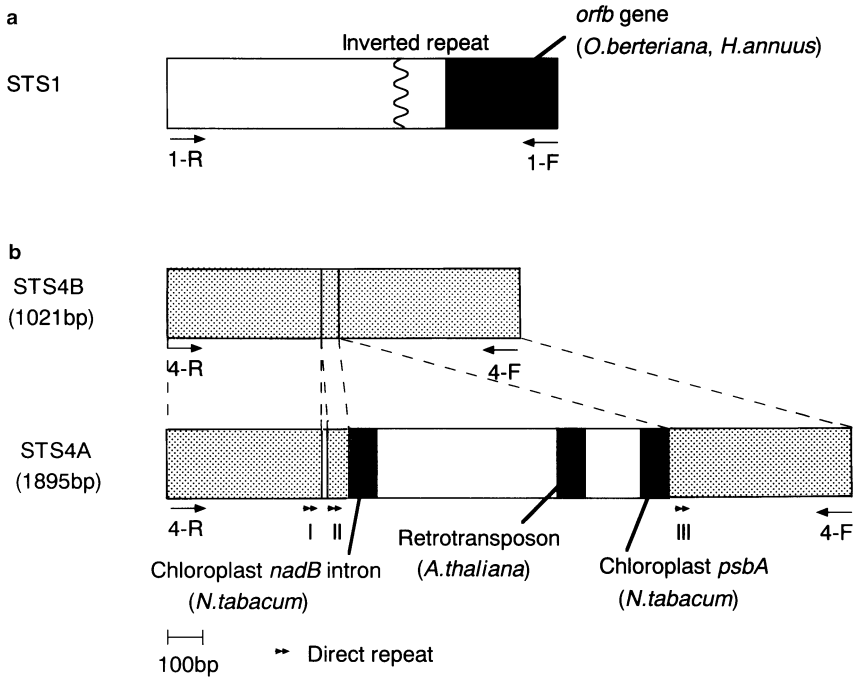

Fig. 4a, b Schematic representations of DNA structure of the STS1 and STS4A and -4B fragments. *Short arrows* show direct repeat sequences. 1-F, 1-R, 4-F and 4-R indicate the STS primers used. **a** DNA structure of the STS1 fragment, **b** DNA structures of the STS4A and -4B fragments

gous to that of sunflower mtDNA (Köheler et al. 1991) (Fig. 4a).

The STS4A fragment had identical 5′ and 3′ regions to the STS4B fragment but contained an additional fragment of 0.9 kb in the middle; this additional part consisted of small segments of the mitochondrial genome, the chloroplast genome and the retrotransposon-like sequence (Fig. 4b). These fragments, ranging from 70 to 170 bp, seemed to be non-functional in themselves. The chloroplast gene segment coded for a portion of the chloroplast NADH dehydrogenase subunit B. Interestingly, direct repeat structures were also found outside of the additional sequence.

The sequences of the STS3A and STS3B were also determined. The common region of two STSs shared a portion of the mitochondrial NADH dehydrogenase subunit 5 gene, but the origin of the extra segment of the STS3A region was unclear. The STS6 fragment contained a homologous region of the mitochondrial *orf250* gene, described for *Arabidopsis* and *Oenothera*.

Discussion

In the present work, two different CMS cytoplasms, the Cornell cytoplasm (493S, 9304A; Peterson and Simon 1986) and the Wisconsin Wild cytoplasm (2566A; Morelock et al. 1996) were used. The STS2 primer combination could differentiate these two CMS types. MS-1 and MS-2 were classified into the group of the Wisconsin Wild cytoplasm (Table 3). On the contrary, the five CMS types, which were also classified into the above two groups, were differentiated from the fertile lines by STS1 and STS4. Thus, the fertile lines and two different types of CMS lines could be easily differentiated using the three primer sets of STS1, -2 and -4.

Fertility conversion using asymmetric cell fusion has been observed for various plants (Tanno-Suenaga et al. 1988; Akagi et al. 1989). As observed in the CMS lines, the mitochondrial genotype of the cybrid lines obtained could be easily predicted using PCR and the STS1 primer.

The 1.1-kb PCR fragment amplified by the STS1 primer combination was not present in the fertile lines but was present in both the CMS lines and the petaloid sterile cybrids. The nucleotide sequence of the STS1 fragment included a portion of the *orfB* gene, and DNA gel blot analysis indicated that reorganization of the mitochondrial genome is associated with *orfB* sequences. The STS1 fragment also included a unique AT-rich sequence which is highly homologous to the 261-bp inverted repeat sequence found in the 5′-flanking region of the *orfH522* gene of sunflower CMS cytoplasm (Köheler et al. 1991). In the case of the Baso CMS cytoplasm of the sunflower, inversion of the mitochondrial genome and insertion of the unknown sequence produced a new orf, the *orfH522* gene, whose first 57 bp is identical to the 5′ region of the *orfB* gene (Monéger et al. 1994). This genome rearrangement may be the result of recombinational events within the inverted repeat. Similarly, genome rearrangements in the vicinity of the *orfB* gene occur in the CMS carrot lines. Interestingly, mitochondrial genome rearrangements surrounding the *orfB* gene have been noted in other CMS lines, such as rapeseed (Handa et al. 1995) and sugar beet (Kubo and Mikami 1996).

The STS4 primer combination amplified the STS4B (1.1 kb) fragment for the fertile lines and the STS4A (1.9 kb) fragment for the CMS lines. The STS4A fragment additionally contained 0.8 kb of an unique insertion sequence in addition to the entire nucleotide sequence of the STS4B. This insertion fragment, which invovles the

mitochondrial genome, the chloroplast genome and the retrotransposon-like sequences, was present in the middle of the STS4A fragment. Three direct repeat sequences were formed at both flanking regions of the insertion. Rearrangement of the mitochondrial genome by insertion of the chloroplast DNA and retrotransposon has been noted in other plants (Unseld et al. 1997; Wolstenholme and Fauron 1995); however, most of these insertions were short gene segments and appeared to be non-functional. To determine the function of these insertions in flower organ development, we can generate transgenic plants carrying the modified mitochondrial genome.

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