Theor Appl Genet (1997) 94: 273–278

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Cloning and sequencing of RAPD fragments amplified from mitochondrial DNA of male-sterile and male-fertile cytoplasm of sugar beet (*Beta vulgaris* L.)

Received: 6 July 1996 / Accepted: 19 July 1996

Abstract Mitochondrial DNA fragments of two nearly isogenic lines of sugar beet (*Beta vulgaris* L.) were amplified by RAPD analysis. A number of fragments, most of them unique to either the male-sterile or the male-fertile cytoplasm, were selected for cloning and sequencing. One fragment was present in the PCR fingerprint pattern of both cytoplasms, whereas five of the selected fragments were specifically amplified from only one type of cytoplasm. The mitochondrial origin of all cloned RAPD fragments was confirmed by Southern hybridization. One fragment resulted in a hybridization pattern that suggests its repetitive presence in the mitochondrial genome of sugar beet. Four out of the five cytoplasm-specific RAPD fragments were shown to hybridize specifically to one type of cytoplasm only. One fragment hybridizing with the mtDNA from N-cytoplasm also revealed hybridization signals with both total and nuclear DNAs of N- as well as S-cytoplasm. Sequence alignments of this clone showed strong homologies with a part of the plastidal *ndhC* gene of higher plants, indicating that the male-fertile-specific mtDNA RAPD fragment is derived from chloroplast DNA. Sequence analysis of an amplified sterile-specific fragment revealed the presence of an open reading frame of 288 bp. Northern hybridization showed a transcription signal specific for the male-sterile cytoplasm. No sequence homology of the open reading frame to any known sequences was found. The results reveal an extremely high degree of sequence variability between the mtDNA of the N- and S-cytoplasm of *Beta vulgaris*.

Key words *Beta vulgaris* · Cytoplasmic male sterility · RAPD · Mitochondrial DNA · Chloroplast DNA

Communicated by R. Hagemann

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Introduction

The phenomenon of cytoplasmic male sterility (cms) is a common feature of many higher plants (reviewed in Kaul 1988). The contribution of mitochondrial (mt) DNA to the expression of cms has been shown in a variety of plant species (Braun et al. 1992). Extensive rearrangements in the mitochondrial genomes between cytoplasmic male-sterile and male-fertile cytoplasms are considered as a general source of cms (Braun et al. 1992; Mackenzie et al. 1994). Although no ultimate evidence exists for the direct effect of a mtDNA sequence or a mitochondrially encoded protein on the expression of cms, a number of sterile-specific genes and proteins have been described. The generation of novel sterile-specific open reading frames seems to be a general feature of cms-associated sequences. Differences in the restriction fragment patterns between the mtDNAs of male-sterile and male-fertile cytoplasms were also reported for sugar beet (Powling and Ellis 1983; Mikami et al 1984; Weihe et al. 1991). Recently, we were able to demonstrate differences in the organization of the mitochondrial DNA of male-sterile and male-fertile sugar beet by the method of random amplified polymorphic DNA (RAPD) analysis (Lorenz et al. 1994).

In sugar beet, transcription analysis between male-sterile and male-fertile lines showed variations in the length of the transcription signals of a number of mitochondrial genes (Duchenne et al. 1989), but no evidence for the existence of chimeric genes has been provided thus far. Recently, a cms-associated mtDNA fragment has been identified carrying open reading frames with partial homology to a reverse transcriptase and a truncated single-subunit type RNA polymerase (Weber et al. 1996). However, little is still known about mitochondrial DNA sequences and/or transcripts associated with the expression of cytoplasmic male sterility in sugar beet compared to other plant species. With this objective in mind, we have investigated the mitochondrial DNAs of both cytoplasms by RAPD analysis. Selected polymorphic RAPD fragments from PCR fingerprint patterns of mitochondrial DNA from two nearly isogenic cy-

toplasmic male-sterile and male-fertile lines were cloned and sequenced, and Southern and Northern hybridizations were performed with all cloned fragments.

Materials and methods

Plant material

The two nearly isogenic lines 3011 (cytoplasmic male-sterile) and 3012 (male-fertile) of sugar beet (*Beta vulgaris* L.) were kindly provided by Kleinwanzlebener Saatzucht AG (Einbeck, Germany).

Extraction of DNA and PCR conditions

Genomic (total cellular), nuclear, mitochondrial (mt) and chloroplast (cp) DNAs were isolated as described previously (Lorenz et al. 1994).

Amplification reactions were performed in volumes of 50 µl containing 15 ng template DNA, 1×*Taq* polymerase buffer (Boehringer Mannheim, FRG), an additional 3 mM of Mg-acetate, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 20 ng of primers and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, FRG), overlaid with 50 µl mineral oil. Amplification was carried out in a BIO-med 60/2 thermocycler (BIO-med, Germany) programmed for 40 cycles of 20 s at 93°C; 1 min at 36°C for the 10-mer primer, 42°C for $(GATA)_4$, 50°C for $(GACA)_4$; a 20 s extension at 72°C and a final cycle of 6 min at 72°C. Amplification products were analyzed by electrophoresis in 1.2% agarose gels run in 1×TAE buffer (Maniatis et al. 1982) and detected by ethidium bromide staining.

Primers $(GATA)_4$ and $(GACA)_4$ were purchased from TIB MOLBIOL (Berlin, Germany) and primer OPB-18 was obtained from Operon Technologies (Alameda, Calif., USA)

Cloning and sequencing

Cloning and sequencing of RAPD fragments was performed as described earlier (Lorenz et al. 1995). Briefly, selected RAPD fragments were excised from agarose gels, re-amplified, and cloned using a TA cloning system (Invitrogen, San Diego, Calif., USA). PCR products were ligated into a pCRII vector and transformed in *E. coli* strain INVαF′. Positive clones were sequenced from both strands with a PRISM dye terminator cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany) using M13 universal and reverse sequencing primers and sequence-deduced custom primers. Sequences were analysed on an automated laser fluorescence sequencer (Applied Biosystems 373A).

Southern and Northern hybridization

Southern hybridizations were performed in 7% SDS, 250 mM sodium phosphate (pH 7.2) at 65°C overnight. Blots were washed in 2×SSC, 1% SDS at 65°C for 30 min; 1×SSC, 0.5% SDS at 65°C for 30 min, and 0.1×SSC, 0.1% SDS at 65°C for 20 min. The blots were exposed to X-ray films for 2–24 h.

Northern hybridizations were performed in 7% SDS, 120 mM sodium phosphate (pH 7.2), 250 mM NaCl and 50% formamide at 42°C overnight. Blots were washed in 2×SSC, 1% SDS at 42°C for 30 min; 1×SSC, 0.5% SDS at 42°C for 30 min; and 0.1×SSC, 0.1% SDS at 42°C for 20 min. The blots were exposed to X-ray films for 2–24 h.

Results and discussion

In order to investigate the contribution of organellar DNA to the PCR fingerprint patterns of total DNA, total, nuclear, as well as the mt and cpDNA of two nearly isogenic male-sterile and male-fertile sugar beet lines were subjected to RAPD analysis (Lorenz et al. 1994). Thereby, considerable differences in the amplification patterns of mtDNAs were detected between the male-sterile and male-fertile lines. Further PCR fingerprint analysis with 18 random decamer primers revealed a high number of polymorphic bands between the mtDNAs of the male-sterile and male-fertile line (data not shown). With primers $(GACA)_4$, $(GATA)_4$ and OPB-18 (sequences see Table 1) highly different fingerprint patterns were obtained from the mtDNAs of male-sterile and male-fertile lines (Fig. 1).

To characterize the polymorphic mtDNA RAPD fragments from the male-sterile and male-fertile sugar beet lines, we have cloned and sequenced selected fragments. Six bands from all three amplification patterns were chosen for a detailed analysis. In particular, a fragment of approximately 800 bp was selected from the fingerprint

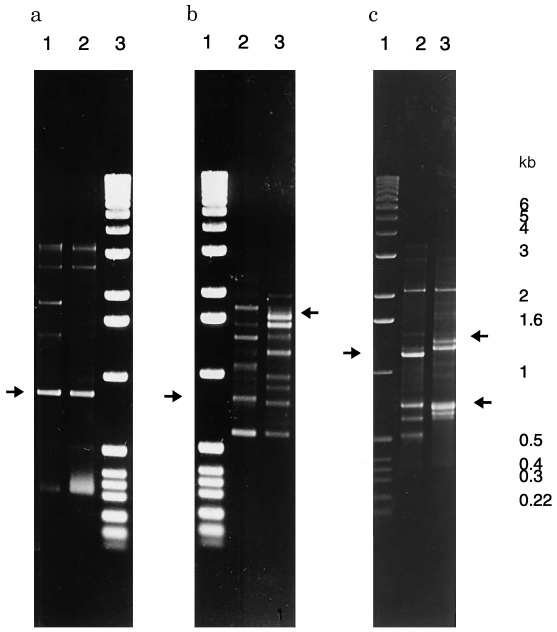

Fig. 1 PCR fingerprint analysis of mitochondrial DNA from nearly isogenic cytoplasmic male-sterile and male-fertile sugar beet lines with primers **a** $(GACA)_4$, **b** $(GATA)_4$ and **c** OPB-18. *Lane 1a* mtDNA from line 3012 (male-fertile, N-cytoplasm); *lane 2a* mtDNA from line 3011 (cytoplasmic male-sterile, S-cytoplasm); *lane 3a* 1-kb marker (Gibco BRL); *lane 1b* 1-kb marker; *lane 2b* N-cytoplasm; *lane 3b* S-cytoplasm; *lane 1c* 1-kb marker; *lane 2c* N-cytoplasm; *lane 3c* S-cytoplasm. Molecular-weight markers are indicated at the right. RAPD fragments selected for cloning and further analysis are marked by *arrows*

Table 1 Nucleotide sequences of primers

Primer	Sequence
$(GACA)_4$	5′-GACAGACAGACAGACA
$(GATA)_4$	5′-GATAGATAGATAGATA
OPB-18	5′-CCACAGCAGT

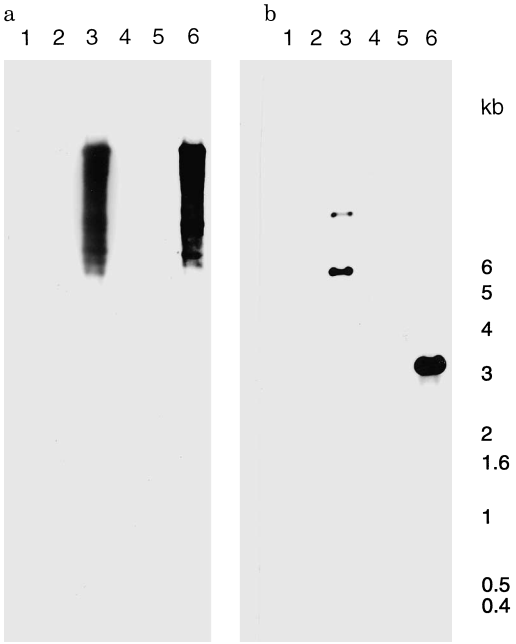

Fig. 2 Southern hybridization of clone **a** GACA S800 with *Eco*RI-digested and **b** GATA S1600 with *Bam*HI-digested total, nuclear, and mtDNA of a male-fertile and a male-sterile sugar beet line. Clone GACA S800 was amplified from both cytoplasms, clone GATA S1600 is unique to the male-sterile amplification pattern. *Lanes 1–3* DNAs from line 3012; *lanes 4–6* DNAs from line 3011; *lanes* (*1, 4*) total DNA; *lanes* (*2, 5*) nuclear DNA; *lanes* (*3, 6*) mtDNA. Molecular-weight markers are indicated at the right; 7 μg of total and nuclear DNA and 3 μg of mtDNA were loaded onto the gel

Table 2 Designation of cloned mitochondrial RAPD fragments

Name	EMBL accession	Amplified from (type of cytoplasm)
CACA S800	Z70028	N- and S-cytoplasm
GATA S1600	Z70029	S-cytoplasm
GATA N800	Z70025	N-cytoplasm
P18 S1200	Z70027	S-cytoplasm
P18 N1100	Z69797	N-cytoplasm
P18 S750	Z70026	S-cytoplasm

pattern generated by $(GACA)_4$ (indicated by an arrow in Fig. 1a). This fragment was present in the mtDNA patterns of both cytoplasms. From the fingerprint pattern amplified by $(GATA)_4$ a fertile-specific fragment of about 800 bp and a male-sterile-specific fragment of 1600 bp were selected (indicated by arrows in Fig. 1b). Two bands of about 750 bp and 1200 bp, polymorphic for the male-sterile cytoplasm, and one fragment of about 1100 bp, specific for the male-fertile cytoplasm, were chosen from the fingerprint pattern of the mtDNA generated by primer OPB-18 (marked by arrows in Fig. 1c). All fragments were excised from the gel, re-amplified and cloned. Positive clones were designated as outlined in Table 2.

Positive clones were hybridized to Southern blots of total, nuclear and mtDNA from male-sterile and male-fertile lines of sugar beet in order to demonstrate the mitochondrial origin of the cloned RAPD fragments, to determine their copy number in the mitochondrial genome, and to confirm their association with the male-sterile or male-fertile cytoplasm, respectively. Figure 2a shows the results of hybridization of clone GACA S800 with *Eco*RI-digested DNAs of both cytoplasms. This clone hybridized only to the mtDNAs and, after longer exposure, also to total DNAs of both cytoplasms, indicating its mitochondrial origin (lanes 3 and 6 in Fig. 2a). The type of hybridization signal suggests that this clone is present as a repetitive sequence in the mitochondrial genome of both male-sterile and male-fertile sugar beet. Apart from repetitive sequences involved in recombination events, no highly repetitive sequences have yet been reported in mitochondrial genomes. Sequencing of the clone did not reveal any obvious repetitive structure and, as in similar investigations (Weising et al. 1995; Lorenz et al. 1995), no accumulation of the simple tandem repeat $(GACA)_4$, which served as primer in the PCR fingerprint analysis, was found. In the light of the observed differences between the N- and S-cytoplasm of sugar beet, the identical hybridization pattern between the mtDNAs of the male-sterile and the male-fertile sugar beet line is remarkable. The clone GACA S800 seems not to be affected by recombination, although the sequence hybridizes to fragments of relatively high molecular weight.

Clone GATA S1600 was hybridized to *Bam*HI-digested total, nuclear, and the mtDNAs of both cytoplasms (Fig. 2b). Also, in this case, hybridization exclusively with the mtDNAs (lanes 3 and 6 in Fig. 2b) confirmed its mitochondrial origin. The fragment was specifically amplified from the mtDNA of the male-sterile cytoplasm, but it hybridized to a restriction fragment showing length polymorphism between both cytoplasms. Differences in the hybridization pattern between the male-sterile and the male-fertile cytoplasm of sugar beet have been reported for a number of genes (Duchenne et al. 1989; Weihe et al. 1991).

In contrast to the fragments GACA S800 and GATA S1600, giving rise to signals with the mtDNAs of both cytoplasms, clones GATA N800, P18 S1200, P18 S750 and P18 N1100 contained cytoplasm-specific sequences. Clone GATA N800, isolated from the fingerprint pattern of the N-cytoplasm, hybridized only with the mtDNA of the male-fertile line (Fig. 3a). Surprisingly, an additional hybridization signal at about 1.1 kb was detected in nuclear DNA and, after longer exposure, also in total DNA of both cytoplasms (marked by an arrow in Fig. 3a). There are two plausible explanations for this phenomenon. Part of the sequence could have been transferred to the nucleus, or else the fragment contains sequences from chloroplast (cp) DNA. To investigate this possibility, we performed further hybridizations with chloroplast DNA. In Fig. 3b, a Southern blot of *Hin*dIII-digested DNAs with clone GATA N800 confirmed its specific hybridization with the mtDNA of the male-fertile cytoplasm (lane 2), but significant hybridization signals were also obtained with the cpDNA of both cytoplasms (Fig. 3b, lanes 7 and 8). Signals of the same size in nuclear DNAs are most probably due to a contamination of nuclear DNAs with cpDNA.

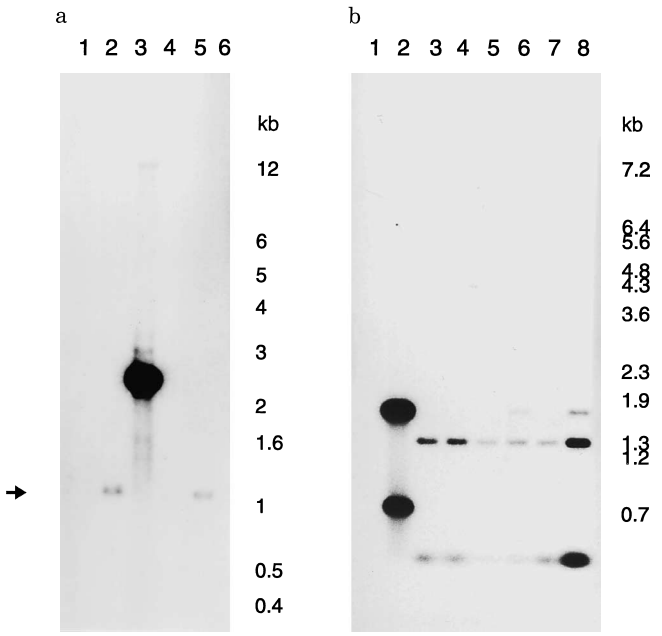

Fig. 3 Southern hybridization of clone GATA N800 with **a** *Eco*RI- and **b** *Hin*dIII-digested DNAs of a male-sterile and male-fertile sugar beet line. **a** *Lanes 1–3* DNAs of the male-fertile sugar beet line 3012; *lanes 4–6* DNAs of the male-sterile line 3011; *lanes* (*1, 4*) total DNA; *lanes* (*2, 5*) nuclear DNA; *lanes* (*3, 6*) mtDNA; the *arrow* indicates the additional hybridization signal at 1.1 kb in the total and nuclear DNA of both cytoplasms. **b** Hybridization of clone GATA N800 with cpDNA from both cytoplasms. (**1**) mtDNA-S; (**2**) mtDNA-N; (**3**) nuclear DNA-S; (**4**) nuclear DNA-N; (**5**) total DNA-S; (**6**) total DNA-N; (**7**) cpDNA-S; (**8**) cpDNA-N. In every case 3 µg of chloroplast DNA was loaded onto the gel

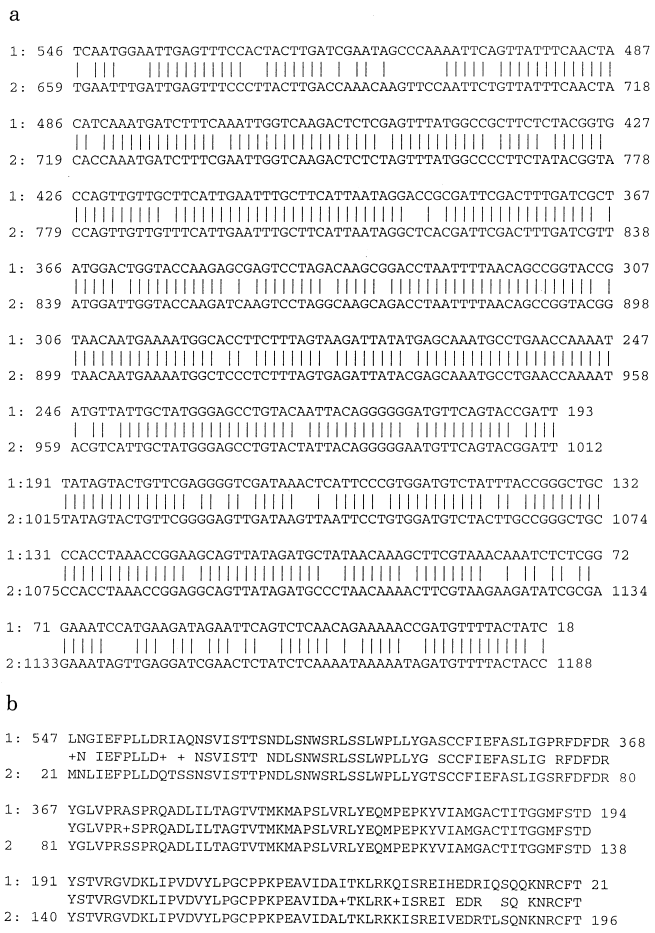

Fig. 4 a, b Alignment of clone GATA N800 (*1*) with part of the *ndhC* gene from chloroplasts of wheat (*T. aestivum*) (*2*) (SWISSPROT accession number: P26304). Both nucleotide (**a**) and amino-acid alignments (**b**) are shown. The translation of nucleotide to amino-acid sequence is in reading frame +3. At position 192 the open reading frame of clone GATA N800 contains a frame shift

Although the results of Southern hybridization suggested that clone GATA N800 shares homology with cpDNA sequences, confirmation of this had to be obtained by sequence analysis. Sequence alignments revealed a high homology of clone GATA N800 to a number of plastidal *ndhC* genes from higher plants (Sugiura 1992). The homology begins immediately downstream from the primer sequence and covers about 70% of the coding region of the *ndhC* gene (Fig. 4). This high conservation of homology at the amimo-acid, and even the nucleotide, level is an indication of a recent evolutionary transfer event. The transposition of DNA sequences from chloroplasts to mitochondria has been reported for many plant species (e.g. Stern and Palmer 1984, 1986; Schuster and Brennicke 1988). In rice, about 6% of the mitochondrial genome is of plastidal origin (Nakazono and Hirai 1993). Whether the transposition of cpDNA sequences to the mitochondrial genome of the male-fertile cytoplasm has an evolutionary advantage for the trait of male fertility is not known. In maize, however, 9 out of 40 kb of the mtDNA which are unique to the normal N-cytoplasm (compared to the male-sterile T cytoplasm) consist of cpDNA sequences (Fauron and Havlik 1989).

The results of Southern hybridizations with the cloned PCR fingerprint fragments amplified by primer OPB-18 (Table 1) are shown in Fig. 5. With clones P18 S1200 and P18 S750, both of which were derived from fingerprint patterns of the male-sterile cytoplasm, specific hybridization signals were obtained exclusively with mtDNAs of the S-cytoplasm (Fig. 5a, b). Clone P18 N1100 hybridized exclusively with mtDNA of the N-cytoplasm (Fig. 5c). Thus, four (together with clone GATA N800) out of five fragments in the PCR fingerprint patterns polymorphic for one cytoplasm, hybridized specifically with either the male-sterile or the male-fertile cytoplasm in the Southern analysis. These surprising findings suggest that sugar-beet mitochondria contain a considerable portion of DNA sequences, unique to the male-sterile and male-fertile cytoplasm, respectively. This is in agreement with the markedly different restriction fragment patterns of the male-sterile and male-fertile cytoplasms of sugar beet (Powling and Ellis 1983; Mikami et al 1984; Weihe et al. 1991). In maize, investigation of the structure and sequence organization of the mitochondrial genome between the male-fertile N-cytoplasm and the male-sterile T type also revealed

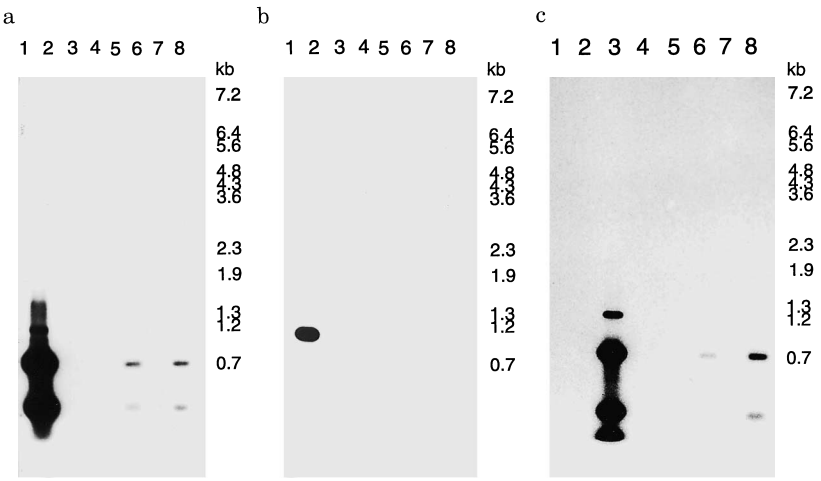

Fig. 5 a–c Southern hybridization of clones previously amplified by primer OPB-18 (see Table 1) with *Hin*dIII-digested DNAs of a male-sterile and male-fertile sugar beet line. **a** clone P18 S1200; **b** clone P18 S750; (**c**) clone P18 N1100. Lanes: (*1*) mtDNA-S; (*2*) mtDNA-N; (*3*) nuclear DNA-S; (*4*) nuclear DNA-N; (*5*) total DNA-S; (*6*) total DNA-N; (*7*) cpDNA-S; (*8*) cpDNA-N

Table 3 General features of cloned mitochondrial RAPD fragments

Fragment	bp	A/T	ORFs	Comments
GACA S800	885	54%	No	Amplified from S- and N-cytoplasm; repetitive hybridization pattern, no recombination between S and N
GATA S1600	1688	58%	No	RFLP in Southern analysis
GATA N800	844	60%	Yes	Specific to N-cytoplasm; homology to plastidal *ndhC* genes
P18 S1200	1355	58%	ORF (288 bp)	Specific to S-cytoplasm; specific transcription signal in male-sterile cytoplasm
P18 N1100	1212	55%	No	Specific to N-cytoplasm
P18 S750	710	57%	No	Specific to S-cytoplasm

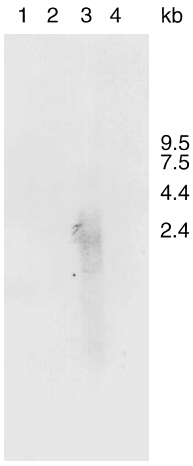

Fig. 6 Northern hybridization of clone P18 S1200 with total and mtRNA of sugar beet. Lanes (*1*) total RNA-S; (*2*) total RNA-N; (*3*) mtRNA-S; (*4*) mtRNA-N. In each case 25 µg of total and 15 µg of mitochondrial RNA were loaded onto the gel

a high portion of unique sequences in both cytoplasms. About 40 kb are unique to the male-fertile cytoplasm and the maize cms T type possesses unique DNA sequences of about 30 kb (Fauron and Havlik 1989).

No repetitive structures of the cloned fragments were found by sequence data analysis, and they do not contain any obviously repetitive sequences. Furthermore, with exception of GATA N800 and P18 1200, sequence data analysis revealed no open reading frames in these clones. Some general features of all cloned mitochondrial RAPD fragments are outlined in Table 3.

In order to investigate the transcription of the cloned fingerprint fragments, we hybridized them to Northern blots of total and mtDNA. For clone GATA N800, which shows strong homology to chloroplast *ndhC* genes, the plastidal, but no mitochondrial, transcripts were identified (data not shown). The results of Northern analysis for clone P18 S1200, unique to the male-sterile cytoplasm in Southern hybridization, is shown in Fig. 6. Whereas in total RNAs no signals were detectable, there is a hybridization signal in the mtRNA of the male-sterile cytoplasm (Fig. 6, lane 3). Sequence analysis of this clone revealed the presence of an open reading frame of 288 bp starting downstream from the primer sequence and terminated by a stop codon within the fragment. Sequence alignments with the DNA and protein databases did not reveal homologies with any known sequences. Transcription of male-sterile specific open reading frames in sugar beet mitochondria has been reported recently (Weber et al. 1996) and was also

shown for a number of other higher plants (Boeshore et al. 1985; Dewey et al. 1986; Pruitt and Hanson 1991; Moneger et al. 1994). Whether the male-sterile-specific transcript of the open reading frame detected on RAPD fragment P18 S1200 has any function and/or importance for the expression of male sterility in sugar beet requires further investigation. The extremely high degree of difference observed between the N- and S-cytoplasm of sugar beet suggests that the two cytoplasms were derived from two different species. Therefore, DNA analysis alone probably will not lead to the identification of the DNA sequences causally involved in male sterility.

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