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Mapping of a chloroplast RFLP marker associated with the CMS cytoplasm of sugar beet (*Beta vulgaris*)

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Abstract The Owen cytoplasm of male-sterile sugar beet is associated with several alterations of mitochondrial DNA and one additional *Hin*dIII site of chloroplast DNA. The region of this *Hin*dIII site has been cloned and sequenced. The site maps in a small reading frame (*orf32*) close to the *ycf7* (*orf31*) gene in the *petG* - *psbE* region of chloroplast DNA. Possible functional implications of the results are discussed. The chloroplast RFLP marker described could be useful for studies on chloroplast-mitochondrial interactions, CMS of sugar beet, and the origin of the Owen cytoplasm.

Key words Chloroplast genes *petG*, *ycf7* (*orf31*), *orf32*, *psbE*, and *psbF* of sugar beet (*Beta vulgaris*) · Chloroplast RFLP · CMS

Introduction

The phenomenon of cytoplasmic male sterility (CMS) has been described for many plant species, and in several well-studied systems the trait appears to be associated with mitochondrial DNA (mtDNA) rearrangements resulting in the expression of novel chimeric genes that are supposed to interfere with pollen development (for review see Hanson 1991; Saumitou-Laprade et al. 1994; Vedel et al. 1994). Differences between chloroplast DNA (ctDNA) of fertile and sterile lines have also been observed in a few cases (Chen et al. 1993), and the involvement of ctDNA in the development of functional pollen was demonstrated in a genetic study with *Oenothera* (Göpel 1970).

In hybrid breeding programs of sugar beet (*Beta vulgaris*) one single CMS cytoplasm is used worldwide – the CMS or S cytoplasm introduced by Owen (1942). Many molecular differences between sterile and fertile sugar beets have been observed in the restriction pattern of mtDNA and in mitochondrial transcripts (Saumitou-Laprade et al. 1993). Alterations of specific mitochondrial genes (Senda et al. 1993; Xue et al. 1994) and inserts specific for the S-type mtDNA (Maggouta et al. 1994) have been identified. An additional *Hin*dIII site has also been described for the ctDNA of sterile sugar beets (Mikami et al. 1985; Fritzsche et al. 1987; Bonavent et al. 1989), and characteristic associations between variant types of mitochondrial and chloroplast DNAs have been found in natural populations of the wild beet, *Beta maritima* (Saumitou-Laprade et al. 1991).

An association between specific mitochondrial and chloroplast DNA variants could be the result of (1) a historical process caused by strict co-transmission and maternal inheritance of both types of organelles or (2) a close functional interaction between mitochondria and chloroplasts. The ctDNA alteration would have no functional meaning in the first instance, however it would reflect an adaptation to the mitochondrial genome and therefore could contribute to CMS if the second assumption is true. To follow up these questions a molecular characterization of the ctDNA alteration is necessary. In this article we report the cloning and sequencing of the polymorphic *Hin*dIII site on the ctDNA from male-sterile sugar beet.

Materials and methods

Plant materials

Beta vulgaris (L) ssp. *vulgaris* (sugar beet) lines 5A3031 (male sterile; S cytoplasm) and 5B3031 (fertile; N or O type cytoplasm) were kindly provided by Kleinwanzlebener Saatzucht AG, Einbeck (Germany).

Isolation of chloroplast DNA

The procedure of Boutin et al. (1987) was followed.

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Cloning of chloroplast DNA

Chloroplast DNA was digested with *Hin*dIII and the 5.5-kb (N), 2.9-kb (S) and 2.6-kb (S) fragments were separated by electrophoresis on agarose gels. The three fragments were isolated by electroelution, ligated into the vector pUC19 and transformed into the *E.coli* strain DH5α as described by Sambrook et al. (1989).

Southern blot analysis

*Hin*dIII-digested ctDNA was separated on 0.8% agarose gels and transferred to Biodyne Transfer Membrane A (PALL Biosupport). The cloned 5.5-kb *Hin*dIII fragment of ctDNA was used as the DNA probe. It was labeled with [α-^{32}P]dATP using the "random primed DNA labelling kit" from Boehringer Mannheim, Germany. Hybridi-zation was carried out at 68 °C as described by Sambrook et al. (1989).

Polymerase chain reaction (PCR)

Synthetic primers used for PCR were: (1) upstream 5′ CAGTTACAA ATAATCCAG 3′; (2) downstream 5′ CACGATATGTGTAGATG 3′ (MWG-Biotech GmbH Germany). The positions of primers are shown in Fig. 2. The reaction was performed with 100 ng of ctDNA and 300 ng of each primer in a Perkin Elmer Cetus thermal reactor programmed as follows: an initial denaturation (94 °C, 5 min), annealing (43 °C, 30 s), and extension (72 °C, 2 min) followed by a final extension step (72 °C, 5 min). The PCR products were extracted with chloroform and gel-purified using the "jetsorb DNA extraction kit" (GENOMED GmbH Germany).

Sequencing of DNA

The 5.5-kb insert of clone 5.5 was digested with exonuclease III (Boehringer Mannheim) to obtain a deletion library (conditions as suggested by the supplier). Overlapping subclones and PCR products were sequenced using the "T7 sequencing kit" (Pharmacia). DNA sequence analysis was performed with the GCG software package of the University of Düsseldorf.

Results and discussion

Chloroplast DNA prepared from sterile (S) and fertile (N) sugar beet was digested with *Hin*dIII and electrophoresed. Preparations from fertile sugar beet are characterized by a 5.5-kb *Hin*dIII fragment that is replaced by two *Hin*dIII fragments of 2.9 and 2.6 kb in digests of ctDNA from sterile plants (Fig. 1). The three *Hin*dIII fragments were cloned into the pUC19 vector, and the respective clones were designated clone 5.5, clone 2.9 and clone 2.6. A 2074-bp sequence was determined and compared between N and S ctDNA. The two sequences were observed to be identical with the exception of four alterations at positions 519 (T to C), 795 (deletion of one T), 935 (T to A) and 1117/1118 (AA to TT) of Fig. 2. The new C in the ctDNA type S at position 519 creates the new *Hin*dIII site observed by restriction analysis. This sequence difference was verified by PCR amplification using uncloned ctDNA from S and N plants and two primers selected from the DNA sequence of Fig. 2. The PCR products were directly sequenced, and the T to C change is shown in Fig. 3. The three additional changes described will not be discussed here because they have no obvious influence on the restriction pattern and the putative reading frames.

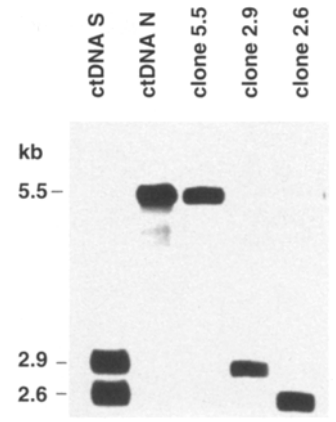

Fig. 1 Southern blot analysis of *Hin*dIII digests of ctDNA from male-sterile (*S*) and male-fertile (*N*; O-type) *B. vulgaris* lines. The three *Hin*dIII fragments of ctDNA, 5.5, 2.9 and 2.6 kb in size, were cloned into the pUC19 vector. *Hin*dIII digests of these three clones are included in the analysis. Hybridization was performed with the radiolabeled 5.5-kb *Hin*dIII fragment of ctDNA (line N) isolated from clone 5.5

The region studied contains the conserved genes *petG*, *ycf7* (*orf31*), *psbE* and *psbF* of ctDNA (Fig. 4). Similarities between the four *B. vulgaris* genes and the respective chloroplast genes of *Cuscuta reflexa*, *Nicotiana tabacum*, *Oryza sativa*, *Zea mays*, *Pinus thunbergii* and *Marchantia polymorpha* have been calculated and are shown in Table 1. The chloroplast-derived *petG* sequence of *Beta* mtDNA has recently been published by Kubo et al. (1995) and is identical to our N and S sequences. The *Beta* ctDNA region sequenced contains also four small open reading frames as indicated in Fig. 4. The polymorphic *Hin*dIII site

Table 1 Amino acid similarities between plastid-encoded genes from *Beta vulgaris* (this study) and corresponding genes from other sources

Gene Organism	*petG*	*ycf7*	*orf32*	*psbE*	*psbF*
C.r.	91.9	87.1	83.3		
N.t.	100.0	90.3	58.2	98.8	92.3
O.s.	100.0	87.1	25.0	98.8	94.9
Z.m.	100.0	87.1	28.1	98.8	94.9
P.t.	83.8	58.6	–	92.8	97.4
M.p.	86.5	64.5	–	89.0	92.3

[a] C.r., *Cuscuta reflexa* (Haberhausen et al. 1992); N.t., *Nicotiana tabacum* (Shinozaki et al. 1986); O.s., *Oryza sativa* (Hiratsuka et al. 1989); Z.m., *Zea mays* (Haley and Bogorad 1990); P.t., *Pinus thunbergii* (Wakasugi et al. 1994); M.p., *Marchantia polymorpha* (Ohyama et al. 1986).
An *orf32* homologous sequence is not found in *Pinus* and *Marchantia* ctDNA

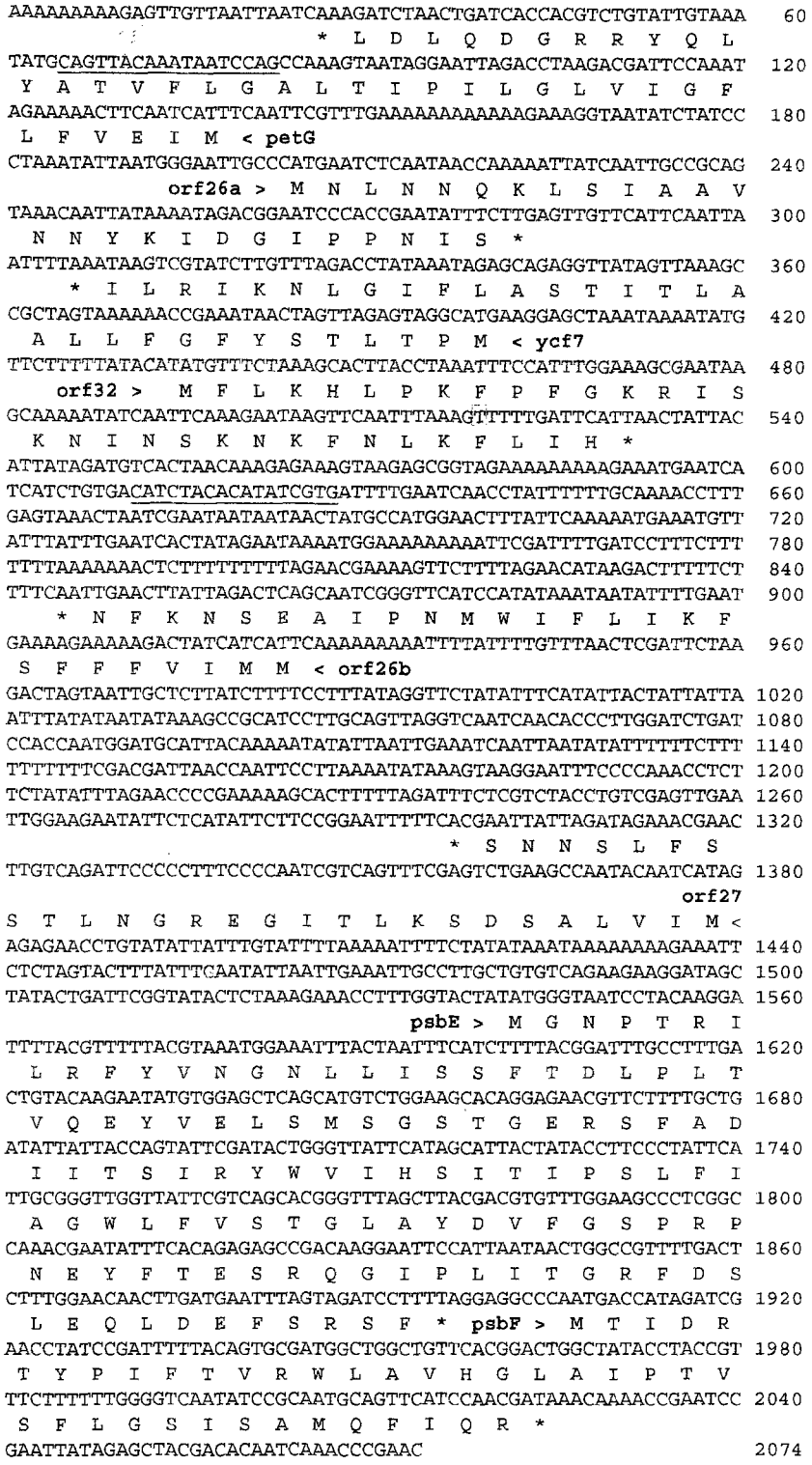

Fig. 2 Nucleotide sequence and derived amino acid sequences of the N ctDNA region containing the conserved *petG*, *ycf7* (*orf31*), *psbE* and *psbF* genes. Sequences of the two primers used for PCR amplification are *underlined*. The T to C transition creating a new *Hin*dIII restriction site is located at nucleotide 519 (*boxed*)

of ctDNA type S maps in *orf32*, one of the four small less conserved or not conserved orfs of Fig. 4. The T to C change creates a phe to leu substitution at the C-terminus of the deduced *orf32* protein. Does *orf32* embody a specific function and code for a protein? The presence of a similar orf adjacent to *ycf7* in four additional angiosperms and a conserved motive MKHLPKF (Fig. 5) point to a specific functional role of this ctDNA region either as a cis- or trans-acting genetic element. Further studies are necessary to determine the function of *orf32* and to test whether the polymorphic *Hin*dIII site can be used as a marker for chloroplast-mitochondrial interactions, cytoplasmic male sterility, and the origin of the Owen CMS cytoplasm of sugar beet.

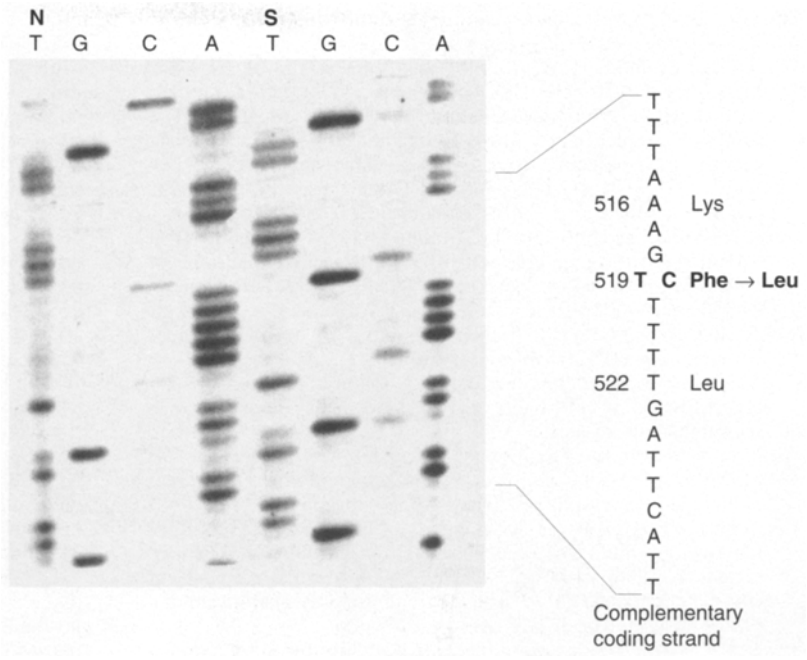

Fig. 3 Identification of the *Hin*dIII polymorphic site by direct sequencing of PCR-amplified ctDNA from *B. vulgaris* lines *S* and *N*. For primers and nucleotide numbering, see Fig. 2. Nucleotide sequences of the N and S non-coding strands are shown. The corresponding coding sequences are AAG*TTTTTG* (Lys *Phe* Leu) for *orf32* from N and AAG*CTTTTG* (Lys *Leu* Leu) for *orf32* from S ctDNA. The amino acid change occurs at position 29 of *orf32*

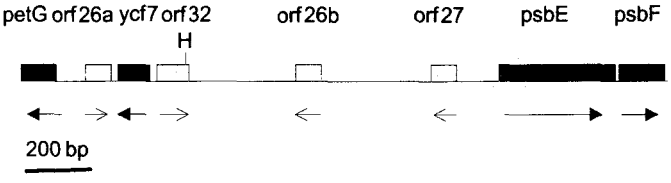

Fig. 4 Map of the ctDNA region sequenced. *Black boxes* and *arrows* denote coding regions and orientations of conserved genes. Four putative orfs are indicated by *dotted boxes*. Location of the *Hin*dIII polymorphic site is indicated (H)

Fig. 5 Sequence alignment between chloroplast *orf32* of *B. vulgaris* line N and homologous regions from four other plants. A conserved motive is *boxed*. *B.v. Beta vulgaris, C.r. Cuscuta reflexa, N.t. Nicotiana tabacum, O.s. Oryza sativa, Z.m. Zea mays*. For references see Table 1

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                10                                                  60
B.v.  .......... ...MFLKHLP KFPFGKRISK NINSKNKFNL KFLIH..... .......... ..
C.r.  .......MFF IRHMFLKHLP KFLFF..... .......... .......... .......... ..
N.t.  ....MKYVLF I.HMFKKHLP KFQIFQNKIG G..........  .......... .......... ..
O.s.  MKGLNSIFFT TLHMLVKHLP K..LWKIHNS SVILDNTKKQ DRVRYRSVK. .ENGFIRWGM SP
Z.m.  MKGFNSIFFT TLHMLVKHLP KFKLWKIHNS SVILDNTKKQ DRVRYRSVKK RENRFIRRNM SP
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Note added in proof

Sequences determined throughout this work have been given the following accession numbers in the EMBL Data Library: X87636 for the ctDNA region of the *Beta vulgaris* N cytoplasm and X87637 for the respective region of the S cytoplasm.

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