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CMS in rye: comparative RFLP and transcript analyses of mitochondria from fertile and male-sterile plants

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Abstract The mitochondrial (mt) genomes of rye (*Secale cereale* L.) lines with "normal" and cytoplasmic male sterility (CMS) inducing "Pampa" cytoplasm were compared by detailed restriction fragment length polymorphism (RFLP) and Northern analyses. RFLP analyses using several heterologous mt genes as probes revealed considerable differences in the overall structure of the two mt genomes. With *cob* and *atpA*, the data indicate intragenic recombination and/or different copy numbers of these genes in the two cytoplasms. In spite of this heterogeneity at DNA level, the transcriptional patterns of nine out of ten mitochondrial genes analysed are unaffected. The exception is in the "Pampa" cytoplasm which contains an additional *cob*-homologous transcript. Since this transcript is strongly reduced in the presence of restorer genes, it might causally be correlated to the CMS phenotype.

Key words CMS-*Secale* · RFLP · Differential transcription · *cob* · *atpA* · *atp9*

Introduction

Cytoplasmic male sterility (CMS), the inability of plants to produce functional pollen grains caused by the interaction of cytoplasmic and nuclear genes, is a widespread phenomenon in the plant kingdom (review: Kaul 1988). In crop plants it is of utmost economic importance because it provides a genetic mechanism of hybrid seed production on a commercial scale (Fehr 1987). Hybrids generally outyield other types of cultivars since they allow the breeder to maximize heterozygosity and thus to fully capitalize on heterosis. In rye (*Secale cereale* L.), a hermaphroditic outbreeder, hybrid breeding became possible by the detection of a CMS-inducing cytoplasm originating from "Pampa" rye (Geiger and Schnell 1970). Although several other CMS sources were detected (Geiger and Morgenstern 1975), "Pampa" is the only CMS cytoplasm being used in commercial hybrids at present. Nuclear genes restoring male fertility in plants with "Pampa" (P) cytoplasm were first detected by Geiger (1972). Restorer genotypes are male fertile in both "normal" and "Pampa" cytoplasm; however, the latter may show a certain reduction in pollen shedding.

In all CMS systems investigated so far, sterility is associated with changes in the mitochondrial genetic system. In some cases, only minor parts of the mitochondrial genome are involved; e.g., in *Helianthus annuus*, differences between sterile and fertile plants are limited to 17 kb of the 300 kb mt genome (Siculella and Palmer 1988). In other systems, a complete rearrangement of mtDNA is observed, e.g., in "Ogura" radish, where at least ten inversions lead to a completely different organization of the mtDNA (Makaroff and Palmer 1988). In the best investigated CMS-system, the T cytoplasm of *Zea mays*, the mtDNA is completely reorganized via recombination at repetitive sequences (Fauron et al. 1989). Apart from structural reorganizations of the mitochondrial genome, differential transcription of mitochondrial genes and the generation of hybrid genes have been described in various CMS systems.

In rye, preliminary analyses have indicated that the overall organization of mtDNA of fertile and CMS plants is different: the mtDNA restriction pattern of fertile plants differs significantly from that of plants carrying the sterile "Pampa" cytoplasm (Tudzynski et al. 1986). In the present paper these differences are examined in detail by RFLP and Northern analyses using heterologous mitochondrial genes as probes.

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Materials and methods

Plant material

The open-pollinated winter rye cultivar "Halo" (kindly provided by F. von Lochow-Petkus GmbH, Bergen) served as a source of "normal" cytoplasm, and the CMS inbred line L301-P (from the Hohenheim hybrid rye program) as a source of "Pampa" cytoplasm. In addition, "normal" and "Pampa" cytoplasms were obtained from the respective versions of restorer inbred line L-287 (L287-N and L287-P, from Hohenheim). Both lines are advanced generations from backcrossings, containing the N and P cytoplasm, respectively, in an isogenic nuclear background. For DNA and RNA preparations, rye seedlings were grown in the dark on vermiculite at room temperature for 6–12 days

Purification of nuclei acids

The mtDNA and mtRNA were purified as described by Tudzynski et al. (1986) and Dohmen and Tudzynski (1994), respectively. Plasmid DNA from *E. coli* was prepared according to Holmes and Quigly (1981).

Standard molecular techniques

Gel electrophoresis was according to Sambrook et al. (1989); DNA: 10 µg/slot, 1% agarose, Tris acetate buffer; RNA: 20 µg/slot, 1.5% agarose, MOPS buffer, 6% formaldehyde. Restriction fragments to be used as probes were eluted from agarose gels using DEAE cellulose (Schleicher and Schuell) following the instructions of the manufacturer. Radioactive labelling of probes was achived by random-primed labelling (Feinberg and Vogelstein 1983). Northern hybridization was performed according to Sambrook et al. (1989); the hybridization temperature was 55 °C for *cob* and 60 °C for *atpA/9*.

Southern hybridization was as described by Johnson et al. (1984; hybridization temperature: 60 °C).

Probes

Heterologous mitochondrial genes used as probes were: *cob*, *coxI*, *coxII*, *rrn18* and *rrn26* from *Z. mays* (obtained from D. Lonsdale); *atpA* (1.9-kb *PstI/Hin*dIII fragment), *atp9* (1.4-kb *BamHI/Hin*dIII fragment), *coxIII*, *nad3* and *nad5* (5′ part with exon 1 and intron 1) from *Oenothera* (obtained from A. Brennicke).

Results

RFLP analyses

The observed differences in restriction patterns of mtDNAs from fertile and CMS rye plants (Tudzynski et al. 1986) were analysed in detail using several heterologous mtDNA genes as probes (see Materials and methods): *coxI*, *coxII*, *cob*, *rrn26*, and *rrn18* from *Z. mays* and *coxIII*, *atp9*, and *atpA* from *Oenothera berteriana*. MtDNA of fertile and male-sterile rye was digested with various restriction enzymes, transferred to nylon membranes and hybridized to the various probes. The results are summarized in Table 1. As expected from the significant difference in restriction pattern observed in ethidium bromide-stained agarose gels, the sizes of hybridizing fragments differed considerably, their number being, in most cases, constant. Two gene probes gave differences in the number of major hybidizing bands which could indicate differences within the coding region. Using *cob* as a probe with *Sal*I-digested DNA, two strong signals appeared in "Normal" mtDNA and only one in "Pampa" mtDNA (Fig. 1 B).

A similar observation was made with *atpA*: in this case *Sal*I-digested "Pampa" mtDNA contains an additional

Table 1 RFLP analysis of mtDNA from fertile ("N=Halo") and male-sterile ("P=Pampa") rye. The sizes of the major restriction fragments hybridizing to the mitochondrial gene probes are presented; they are the means of at least three determinations. Significant size differences (RFLPs) are underlined. Description of probes: see Materials and methods. Values in brackets indicate faint hybridization; stars indicate double fragments

Probe	Restriction enzyme	Fragment sizes (kb)	
		"N"	"P"
coxI	*Bam*HI	6.4	6.4
	*Hin*dIII	4.4	4.3
	*Pst*I	6.3	8.8
	*Xba*I	5.9	5.1
coxIII	*Bam*HI	7.0	7.0
	*Pst*I	9.2	12.8
	*Xba*I	8.6	10.7
	*Sal*I	10.7	10.7
cob	*Bam*HI	5.4	14.0
		3.6	3.6
	*Hin*dIII	4.2	13.5
	*Pst*I	17.0	23.7
	*Xba*I	9.0	10.0
		5.9	5.9
	*Sal*I	15.0	21.2
		6.6	
rrn26	*Bam*HI	10.5	11.4
	*Hin*dIII	3.0	3.4
		2.0	2.0
	*Pst*I	7.7	7.4
	*Xba*I	14.5	19.2
rrn18	*Bam*HI	3.3	3.2
	*Pst*I	(25.0)	(23.0)
		14.0	13.4
	*Xba*I	10.1	10.1
		6.0	6.0
	*Sal*I	7.0	10.5
atpA	*Bam*HI	5.6	5.6*
		2.8	2.8*
	*Pst*I	3.4	3.4*
	*Xba*I	5.6	5.6*
	*Sal*I	18.5	18.5
		16.0	16.0*
			11.0
atp9	*Bam*HI	2.8	2.8*
		0.9	0.9
	*Pst*I	3.4	3.4*
		19.0	16.5
	*Xba*I	5.6	5.6*
		9.8	8.4
	*Sal*I	16.0	16.0*
		18.7	18.7

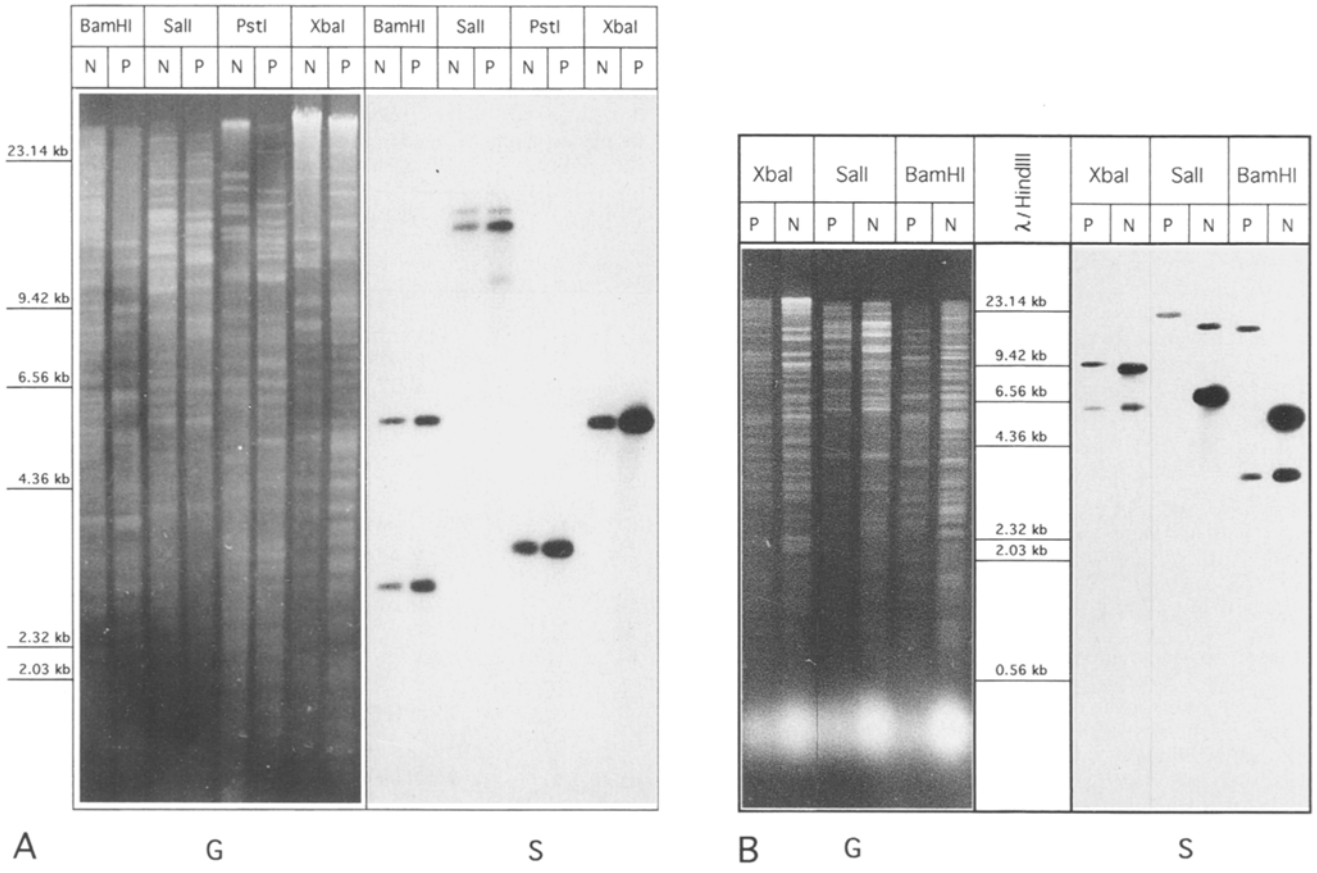

Fig. 1 A, B RFLP analyses of mtDNA of male-fertile (*N*) and male-sterile (*P*) rye. *G*, ethidium bromide-stained agarose gel; *S*, Southern blot using *atpA* (**A**) and *cob* (**B**) as probes, respectively. *Hin*dIII-digested lambda-DNA was used as a molecular weight marker

11-kb hybridizing fragment (Fig. 1 A). In the case of *atpA*, detailed analyses have shown that "Pampa" mtDNA contains an additional gene copy: by combined DNA sequence and Southern analyses (Hessberg and Tudzynski, in preparation) it could be shown that the additional 11-kb-*Sal*I fragment (Table 1) contains the 5′ part of the gene (the *Sal*I site lies within the gene) which is also found in the 18.5-kb fragment present in both mtDNAs. The 16-kb fragment containing the 3′ part of the gene is present in more than one copy in the "Pampa" cytoplasm (Fig 1 A) which therefore seems to be identical in both copies. A detailed analysis showed that "Normal" and "Pampa" mtDNA differ also in copy number of the *atp9* gene (data not shown). The *atp9* gene is closely linked to the *atpA* gene, and is also present on the 16-kb *Sal*I fragment (once in "Normal", twice in "Pampa" mtDNA).

Taken together, these data show that differences in restriction patterns of mtDNAs of fertile and sterile rye do not just reflect a reshuffling of genes due to recombination between non-coding intergenic sequences, but that variations in the copy numbers of mitochondrial genes, and sequence variations within or around the coding regions, are probably involved. It is unclear whether these differences are correlated with the expression of the CMS phenotype; however, restoration to fertility of "Pampa" cytoplasm by the appropriate nuclear background was not reflected in changes of the mtDNA organization (hybridization patterns remained "Pampa" specific; data not shown). Since a comparison of translation products (by in-organello protein synthesis) revealed no difference between "Normal" and "Pampa" mitochondria (Smit, unpublished data), we started a detailed Northern analysis to look for CMS-specific differences in transcription patterns.

Transcript analyses

Using ten heterologous mitochondrial genes as probes (see Materials and methods), Northern analyses were performed with mtRNA of male-fertile and CMS plants. With most of the probes no differences in transcript pattern were observed, apart from variations in the relative intensity of single transcripts. Since such variations were also observed between different RNA preparations of "Normal" and "Pampa" cytoplasm itself, they were not considered to be significant. Also with *atpA* and *atp9* no qualitative differences in transcription patterns were observed (Fig. 2); obviously the presence of additional gene copies of these genes in "Pampa" mtDNA does not lead to additional/altered transcripts. As documented in Fig. 2, the *atpA* and *atp9* genes were co-transcribed: the two large transcripts detected by the *atpA* probe (2.7 and 2.5 kb), also bound to the *atp9* probe; the latter probe hybridized to several other bands (1.4, 1.0, 0.8, 0.7, 0.5, 0.3-kb) leading to a rather

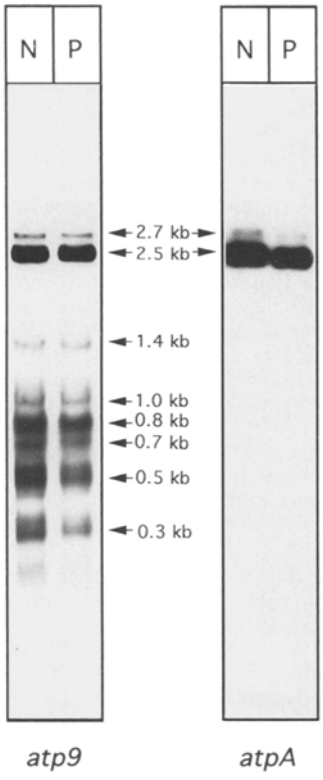

Fig. 2 Northern analysis of mtRNA from male-fertile ("*N*") and male-sterile ("*P*") rye using *atp9* and *atpA* as probes. Sizes of the major transcripts are indicated

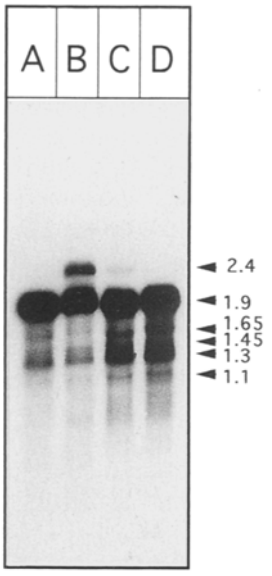

Fig. 3 Northern analysis of mtRNA from rye using *cob* as a probe. *A*, fertile ("*N*"), *B*, male-sterile (L301-P); and *C*, *D*, restorer lines L287-P, L287-N of rye. Sizes of major transcripts are indicated

complex transcription pattern, which might be due to partial homology to other transcripts or splice products.

However, using *cob* as a probe, in addition to five transcripts present in both lines (1.9, 1.65, 1.45, 1.3, 1.1 kb), a transcript of about 2.4-kb could be detected in "Pampa" mtRNA (Fig. 3). This qualitative difference was confirmed using several independent RNA preparations. To investigate the relationship of this difference to the CMS phenotype, RNA from lines L287-N and L287-P (carrying "Normal" and "Pampa" cytoplasm, respectively, in a nuclear restorer background) was used as a control. As can be seen from Fig. 3, the additional *cob*-homologous transcript of "Pampa" is not present in the restorer line L287-N (lane D), whereas in the line L287-P the transcript is present but significantly reduced (lane C). This indicates that this transcript is under nuclear control and is correlated with the expression of the CMS phenotype.

Discussion

The RFLP analyses comparing mtDNA from male-fertile and CMS ("Pampa") rye plants showed that their mtDNAs differ considerably in their structure, probably due to extensive rearrangements in the mtDNA of the sterility inducing cytoplasm. Comparable differences between mtDNA of fertile and CMS-inducing cytoplasms have been observed in several higher plants, e.g., *Z. mays, Helianthus, Petunia* (reviews: Hanson 1991; Braun et al. 1992 Hanson and Folkerts 1992;). Most of the RFLPs observed here could be due to recombination or mutations in intergenic regions, leading to differently sized restriction fragments carrying the respective mitochondrial genes. In the case of *cob* (the probe used is a short internal sequence of the *Z. mays* gene) the existing data indicate a modification which leads to the loss of the *Sal*I site in "Pampa" mtDNA. The sum of the two *Sal*I fragments of "Normal" corresponds well to the size of the single *Sal*I fragment of "Pampa" (21.6/21.2 kb).

The polymorphic *atpA* region has been analyzed in detail. "Pampa" mtDNA contains two copies of *atpA*, differing in the region upstream of the gene. Since the *atp9* gene is closely linked to *atpA*, an additional copy is also present in "Pampa" mtDNA. Whether this different organization of "Normal" mtDNA is in any way correlated to the expression of the CMS phenotype, cannot be proven; in the presence of nuclear restorer genes suppressing the sterility inducing effect of "Pampa" cytoplasm, the organization of "Pampa" mtDNA is not affected.

CMS-associated differences in copy number of the *atpA* gene have been observed in other systems: in wheat the *atpA/atp9* region is almost identical to that of rye (Begu et al. 1989,;Schulte et al. 1989); a fertile *Triticum aestivum* line contains only one copy of this region, whereas a sterile alloplasmic line has two copies, like "Pampa". In *Z. mays* the *atpA* region is involved in CMS-associated changes; mtDNA of the normal (N) cytoplasm (in B 37 nuclear background) contains two copies, whereas the sterile (T, S and C) cytoplasms contain one copy of *atpA* (Isaac et al. 1985). In these two systems, as in rye, this different structural organization is not reflected in a different transcription pattern of the *atpA* and/or *atp9* genes.

For most of those mitochondrial genes examined, the numbers and sizes of transcripts were not different between the "Normal" and "Pampa" cytoplasms. In rye as in most of the other CMS systems analysed most of the genes are not affected by the enormous structural reorganization of the mitochondrial genome. An interesting exception is the occurrence of an additional *cob* transcript in "Pampa" cytoplasm. Since this transcript is significantly repressed by the nuclear restorer genotype of L287 (Fig 3), it obviously is CMS-associated.

There are only a few comparable examples of restorer gene-controlled CMS-specific transcripts in other plants: e.g., in *Raphanus sativus* transcription patterns of fertile and sterile ("Ogura") plants with a specific nuclear background differ with respect to *atpA*, atp6 and *coxI*. Under the control of nuclear restorer genes, only the *atpA* transcript pattern was "restored" to that of the fertile cytoplasm (Makaroff and Palmer 1988). In T cytoplasm of *Z. mays*, the gene product of the *T-urf13*-gene, a hybrid gene which is most probably causally correlated to the CMS phenotype, is strictly reduced by the restorer gene Rf1 (Dewey et al. 1987).

Several explanations are possible for the presence of an additional *cob* transcript in "Pampa" cytoplasm. The rearrangement of mtDNA may have created a new transcription start signal upstream of the *cob* gene, leading to an additional longer transcript; or in "Pampa" mtDNA a hybrid gene, like *T-urf13* in *Z. mays*, is formed which contains part of the *cob* coding region (this is rather unlikely, since it should have been detected in the RFLP analyses); or maturation of *cob* transcripts in "Pampa" is not effective, therefore a substantial amount of the precursor is present.

The observed CMS-specific transcription of the *cob* gene in "Pampa" mtDNA is the first report of a CMS-correlated character in rye. To investigate the function of this gene in the expression of the CMS phenotype, a detailed structural analysis of the *cob* region is necessary, and an expression analysis including anther tissue (the expression of the *pcf* gene of *Petunia* is increased five-fold in anther tissue, Young and Hanson 1987). Also for those genes which showed no differences in transcript pattern in the present study, re-examination with anther tissues could be important, since pollen-specific expression of genes has been demonstrated (*ATP B* of *Nicotiana*, De Paepe et al. 1993).

Since all commercial rye hybrids carry "Pampa" cytoplasm and the acreage covered by these hybrids is rapidly expanding, the understanding of the underlying molecular mechanism(s) is of growing importance. A first practical consequence from the observed molecular differences could be the development of a PCR-based test system for the differentiation of "Normal" and "Pampa" cytoplasms in breeding programs.

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