Theor Appl Genet (1986) 72:695–699

Molecular analysis of mitochondrial DNA from rye (*Secale cereale* L.)

P. Tudzynski [1,*], P. Rogmann [1,*] and H. H. Geiger [2]

[1] Lehrstuhl für Allgemeine Botanik, Ruhr-Universität, D-4630 Bochum, Federal Republic of Germany
[2] Institut für Pflanzenzüchtung, Saatgutforschung und Populationsgenetik, Universität Hohenheim, D-7000 Stuttgart 70, Federal Republic of Germany

Received February 20, 1986; Accepted March 26, 1986
Communicated by R. Hagemann

Summary. Molecular characterization of mitochondrial (mt) DNA of rye (*Secale cereale* L.), free of significant amounts of contaminating chloroplast (cp) DNA, was initiated using the open-pollinated cultivar 'Halo' as a source of mtDNA. Based on the compilation of data from restriction patterns, the molecular size of the mtDNA was estimated to be 410 Kb and its buoyant density was determined as 1.705 g/ml. Southern hybridization, using labelled cp genes (P700 and ribulosebiphosphate-carboxylase large subunit), indicated the presence of cpDNA-homologous regions on putative mtDNA fragments. Mt DNAs of inbred lines with fertile and cytoplasmic male sterile (CMS) 'Pampa' cytoplasm were also analysed. Whereas the restriction patterns of mtDNAs of 'Halo' and the fertile line turned out to be identical, 'Pampa' mtDNA showed a unique restriction pattern, indicating (as in most other CMS systems) the involvement of mtDNA rearrangements in the expression of male sterility in rye. All 3 mtDNAs investigated contain regions homologous to the plasmid S1 of the CMS-S cytoplasm of Maize (*Zea mays*), as indicated by hybridization experiments. In 'Pampa' cytoplasm the S-homologous sequence is located within a rearranged region of mtDNA.

Key words: *Secale* – mt DNA – cpDNA – CMS

Introduction

Rye (*Secale cereale* L.) is one of the major crop plants in Central and Eastern Europe. In the late sixties Geiger and Schnell (1970) detected cytoplasmic male sterility in 'Pampa' (P) rye, opening the way for hybrid seed production on a commercial scale. The first hybrid cultivars were meanwhile released (Geiger 1985). To reduce the genetic vulnerability of hybrid rye, various new CMS sources have been developed by Geiger and Morgenstern (1975).

CMS systems in other plants have been studied in detail using genetical and molecular methods (Hanson and Conde 1984). The best analyzed system is that of *Zea mays* (Laughnan and Gabay-Laughnan 1983). In all these systems rearrangements of mitochondrial (mt) DNA seem to be causally related to the CMS phenotype. Molecular methods like restriction analysis of mtDNA have proved to be valuable tools for distinguishing normal from CMS cytoplasms and, more importantly, for classifying different CMS types.

To further improve prospects for hybrid breeding in rye, a molecular analysis of mtDNA was initiated. Since not much information is available of the mtDNA of *Secale* (Vedel et al. 1980), in this paper the mtDNA of a commercial, open-pollinated cultivar is characterized. In addition, data obtained from a homozygous inbred line in CMS and normal cytoplasm is presented.

Materials and methods

Plant material

The commercial winter rye cultivar 'Halo' was obtained from F. von Lochow-Petkus GmbH, Bergen; L201-N and L201-P are the male-fertile (normal) and CMS ('Pampa') versions of inbred line L201 developed by longterm selfing and backcrossing, respectively, in the Hohenheim hybrid rye program (Geiger 1985). Grains were surface-sterilized by incubation with 25% sodiumhypochloride (90 min) and placed on wet sterile Vermiculite in a dark room (for mtDNA isolation) or at permanent light (for cpDNA isolation) at 20 °C.

Plasmids

For hybridization analysis the following clones were used: pBP5 (COI gene of *Neurospora crassa*, Burger et al. 1982),

* Present address: Institut für Mikrobiologie, Universität Düsseldorf, D-4000 Düsseldorf, Federal Republic of Germany

pZmC556 (P700 of *Zea mays,* Fish et al. 1985), pZmC461 (internal fragment of RubLS of *Zea mays*, obtained from U. Kück), pS1 (internal 6.2 Kb fragment of plasmid S1 of *Zea mays* CMS-S, obtained from D. R. Pring).

Isolation of mitochondrial DNA
(modified after Leaver, personal communication)

Etiolated shoots (about 100 g) were harvested after 10–12 days, ground in a mortar with 2 volumes of buffer A (0.4 M mannitol, 1% bovine serum albumin (BSA), 0.001 M EGTA, 0.01 M cysteine, 0.025 M MOPS). The homogenate was filtered through cheesecloth and centrifuged for 5 min at 1,000 g. Mitochondria were pelleted from the supernatant by centrifugation at 15,000 g for 15 min. Differential centrifugation was repeated in buffer B (like A, but with 0.005 M MOPS, without cysteine). The crude mitochondrial pellet was resuspended in buffer C (0.4 M mannitol, 0.01 M $MgCl_2$, 0.05 M Tris-HCl, pH 8.0) and incubated with DNASE I (75 mg/kg (fresh weight)) for 30 min at 4 °C. DNAse-activity was stopped by the addition of 3 volumes buffer D (0.1 M EGTA, 0.4 M mannitol, 0.05 M Tris, pH 7.2) and by centrifugation (15 min 14,000 g). The mitochondrial pellet was resuspended in buffer B.

Mitochondria were purified by centrifugation through a discontinous sucrose gradient (0.6, 0.9, 1.2, 1.45, 2.0 M sucrose in 0.01 M Tricine, 0.001 EGTA, 1% BSA, pH 7.2; rotor SW 28 25,000 rpm, 60 min). Mitochondria collected from this gradient were diluted in buffer D (0.4 M mannitol, 0.001 M EGTA, 0.01 M tricine, pH 7.2) pelleted (13,000 g, 15 min), resuspended in lysis buffer (0.1 M EDTA, 0.1 M TrisHCl, pH 8.0) and incubated in the presence of sarcosyl (0.4%) and proteinase K (0.1 mg/ml) for 30 min at 60 °C. MtDNA was purified by centrifugation through a CsCl-ethidiumbromide-gradient (TV 865, 45,000 rpm, 15 °C, 21 h). Ethidiumbromide was extracted by isopropanol, mtDNA was dialysed and concentrated, if necessary, by ethanol precipitation.

Isolation of chloroplast DNA

The method of Kolodner and Tewari (1975) was used with the following modifications: seedlings were kept dark 24 h before harvest. DNAse-concentration was 50 mg/kg fresh weight; DNase-activity was stopped by 250 mM EDTA-solution and centrifugation through a sucrose cushion (0.5 M sucrose, 0.05 M Tris-HCl, 0.02 M EDTA, pH 8.0; 6,000 × g 20 min). Chloroplasts were lysed by incubation with lysis buffer (0.05 M Tris HCl, 0.02 M EDTA, 2% sarcosyl, 0.2 µg/ml proteinase K, pH 8.0) for 30 min at 37 °C.

After phenol extraction and ethanol precipitation incubation with RNase, sarcosyl and proteinase K was repeated. After a second phenol extraction and dialysis cpDNA was pelleted by ethanol.

Molecular methods

Restriction analyses, agarose gel electrophoresis, Southern blotting, DNA-DNA-hybridization and the determination of buoyant density were all performed as described previously (Tudzynski et al. 1983).

For estimation of molecular size of mtDNA agarose gel electrophoresis was modified as follows: 0.7% agarose, a total run of 44 h (photographs were taken at 24, 36 and 44 h); as molecular weight markers fragments of lambda DNA were used (SalI and HindIII).

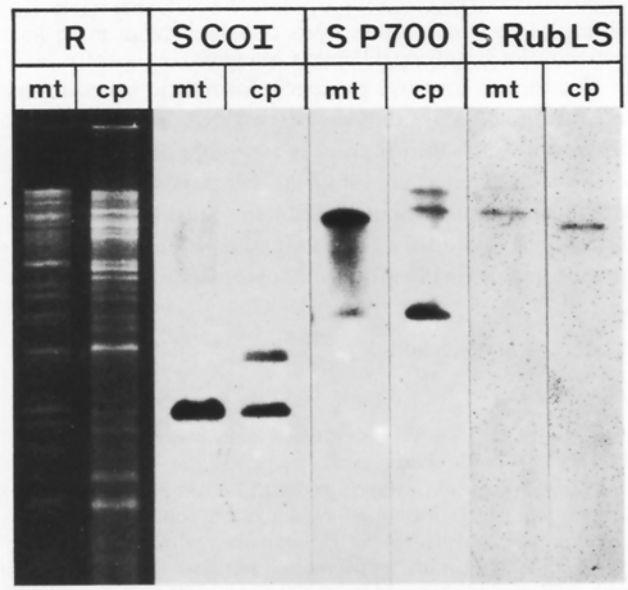

Fig. 1. XhoI- restriction pattern (R) and corresponding Southern blots (S) of mt and cpDNA of rye (cultivar 'Halo'). Hybridization probes: 32P dATP labelled plasmids pBP5 (Cytochrome-C-oxidase subunit I of *Posospora anserina*), pZmC556 (P700 of *Zea mays*) and pZmC461 (Ribulosebisphosphate-carboxylase, large subunit of *Zea mays*). Hybridization conditions: 30% formamide, 37 °C

Results

1 Characterization of mtDNA

Although various mtDNA isolation protocols were tested, including several modes of growing plants, the method described above turned out to be the best with respect to reproducibility, purity and yield of DNA. In about 30 independent preparations the same restriction pattern was observed (see Fig. 1). This complex restriction pattern is typical for mtDNA of higher plants: many bands of varying intensities. There are two possible explanations for this phenomenon: either the existence of one large "masterchromosome", with the different intensities representing multiple equally sized fragments, or the presence of several mt "chromosomes", occurring in non-stöchiometric amounts (like in *Zea mays* or *Brassica,* Lonsdale 1984). For a rough estimate of molecular size, the first alternative was chosen as a working hypothesis, i.e. bands with high intensity were taken as double or triple fragments. Using a special mode of agarose gel electrophoresis (see "Materials and methods") sizes of restriction fragments were determined for 3 enzymes: in Table 1 data for BamHI digestion are presented. The other enzymes used gave comparable results: SalI (31 fragments, 396 Kb) and XbaI (43 fragments, 410 Kb). Taken together these data point to a molecular size of about 410 Kb, which fits well with the published data obtained from other cereals (see below).

Table 1. Estimation of molecular size of rye ('Halo') mtDNA by restriction analysis (BamHI; size of fragments in Kb)

Fragment no.	Size (Kb)	Relative intensity	Fragment no.	Size (Kb)	Relative intensity
1	43.10		21	5.40	2×
2	35.00		22	5.32	2×
3	28.13		23	4.99	2×
4	21.67		24	4.81	
5	16.15		25	4.62	
6	14.60		26	4.13	
7	13.72		27	3.79	2×
8	12.28	2×	28	3.65	
9	11.00		29	3.50	
10	10.36	2×	30	3.35	2×
11	9.27		31	3.27	
12	8.53		32	3.15	
13	8.16		33	2.95	
14	7.38		34	2.75	2×
15	6.96	2×	35	2.62	
16	6.79	2×	36	2.40	2×
17	6.33		37	2.30	
18	6.13		38	2.05	
19	5.84	2×	39	2.00	
20	5.58	2×			
Total				412.65 Kb	

As a further molecular parameter buoyant density of mtDNA was determined using an analytical ultracentrifuge. An average value of 1.705 g/ml for mtDNA was obtained, compared to 1.702 g/ml for bulk (nuclear) DNA. Comparable values have been reported for maize and other higher plants. These data show that as far as complexity of restriction pattern, estimated molecular size, and buoyant density are concerned, the mtDNA of rye is comparable to that of other cereals and most higher plants.

2 Comparison of mitochondrial and chloroplast DNA

For a further detailed analysis it was considered essential to know the degree of purity of the mtDNA preparations, especially with respect to contamination with chloroplast (cp) DNA. For this purpose cpDNA was purified and compared with mtDNA preparations. As expected, cpDNA preparations showed a restriction pattern distinct from that of mtDNA (Fig. 1), with a limited number of restriction fragments. The pattern obtained is similar to that reported by Vedel et al. (1980).

Because of the high number and varying intensity of mtDNA restriction fragments, the presence or absence of cpDNA fragments in these preparations could not be detected visually. Therefore, we decided to use DNA-DNA hybridization as a tool for the detection of cross-contamination, with cloned organellar genes as probes: cytochrome-c-oxidase subunit I (COI, from *Neurospora crassa*), which should be specific for mtDNA, and two chloroplast genes (P700 and the Ribulosebisphosphate-carboxylase large subunit, both from *Zea mays*).

The results of such hybridization experiments are presented in Fig. 1. As expected, the mtDNA preparation shows homology to the labelled COI gene; the cpDNA also shows a weaker band in the same position. This indicates some contamination of cpDNA with mtDNA. The degree of this contamination was varying: in some cpDNA preparations there was no homology to the COI probe at all. However, the cp genes used as probes hybridized with comparable intensity to both mt and cpDNA preparations. Since the hybridizing fragments had different sizes in both DNA types (with 3 enzymes tested), impurities could not account for this cross-reaction. Thus, there is no significant cross-contamination of mtDNA preparations with cpDNA.

3 Analysis of CMS-lines

After this general characterization of the mtDNA of an open-pollinated rye cultivar, the analysis of a homozygous inbred line (L201) was initiated, with the aim of distinguishing between different cytoplasmic types. MtDNA was prepared from the N and P version of this line. As may be seen from Fig. 2, the two cytoplasms differ considerably with respect to restriction pattern, i.e. they are easily distinguishable by this method despite the high number of restriction fragments. Interestingly, restriction patterns of 'Halo' and L201-N are indistinguishable. L201-N is derived from 'Petkus' rye, and obviously mtDNA has undergone no major detectable change during the various breeding steps.

As a probe for the detection of rearrangements of mtDNA sequences in P cytoplasm, a cloned fragment of the S1 plasmid of *Zea mays* was used. This plasmid is associated with the CMS S system in maize (Pring et al. 1977), it occurs in free and integrated form in high-molecular-weight mtDNA and it is involved in gross rearrangements of mtDNA in S cytoplasm. In Fig. 2 Southern hybridization data obtained with a labelled S1 probe are presented. Fragments with significant homology to the S1 plasmid occur in all 3 mt preparations. In L201-N and 'Halo' equal-sized fragments are involved, whereas in L201-P a differently sized fragment hybridizes. Apparently, sequences homologous to the S1 plasmid are contained within the rearranged part of P mtDNA.

Discussion

Physical properties of mtDNA of rye obtained in this study are comparable to those reported for other

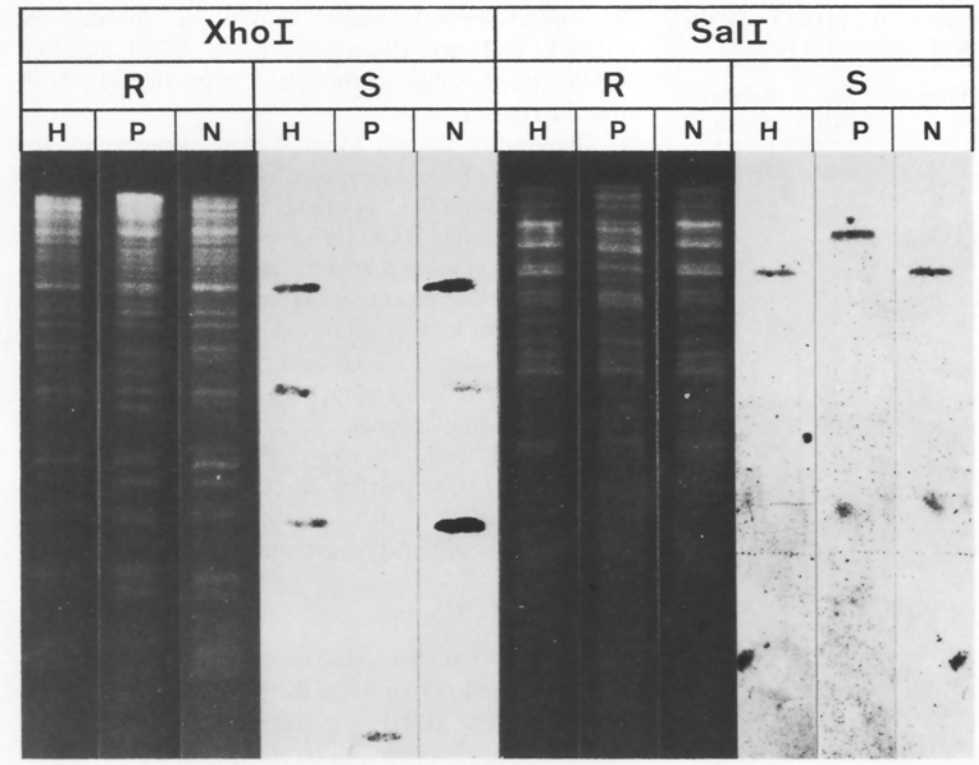

Fig. 2. Restriction analysis (R) and corresponding Southern blots (S) of mtDNA of 3 rye samples: 'Halo', L201-N and L201-P. Hybridization probe: plasmid pS1 (6.2 Kb internal fragment of S1 from CMS-S), 30% formamide, 37 °C

cereals. Rye mtDNA has an estimated size of 410 Kb (wheat: 210 Kb, maize: 570 Kb), a buoyant density of 1.705 g/ml (maize: 1.706 g/ml), and a complex restriction pattern. Interestingly, a test designed to detect cross-contamination of mtDNA with cpDNA revealed instead the presence of cp-homologous sequences in mtDNA preparations. The different sizes of hybridizing fragments ruled out contamination of mtDNA as the basis for hybridization. The presence of cpDNA sequences within the mt genome has been reported for several other higher plants (see Timmis and Scott 1984) where the two cp genes used as probes in this study, Rub LS (*Zea mays,* Lonsdale et al. 1983) and P700 (*Spinacea oleracea,* Whisson and Scott 1985), were also used. Obviously, mtDNA of rye is comparable in this regard to other systems. It should be noted, however, that only by sequence analysis of corresponding fragments can exchange of DNA between organelles be proved unequivocally. This has not been the aim of this paper; as this study is intended to develop tools for a detailed analysis of the CMS-system of rye. The first results obtained with P cytoplasm showed that in rye (like in almost all other CMS systems analysed to date) rearrangements of mtDNA are present in the CMS cytoplasm.

In *Zea mays* the great risk of using a single CMS cytoplasm in hybrid breeding was demonstrated in 1970, when a plant pathogen, *Helminthosporium maydis,* was able to develop epidemically in US corn because commercially grown corn mostly contained the T-cytoplasm which turned out to be highly susceptible to a certain race of the pathogen (Laughnan and Gabay-Laughnan 1983). Therefore, cytoplasmic diversity is an important prerequisite for reducing the genetic vulnerability of hybrid cultivars produced by means of CMS. Molecular methods like restriction analysis and Southern hybridization may prove to be helpful in defining different CMS types in rye as well.

The impact of host-pathogen interaction is also under investigation in this system. *Claviceps purpurea,* a common parasite of rye (and most other grasses), has been intensively studied in the last years. Interestingly, this fungus contains mt plasmids comparable to the S plasmids of maize (Tudzynski et al. 1983; Tudzynski and Düvell 1985). Preliminary hybridization data indicate that mtDNA of rye contains regions of homology to these fungal plasmids, which points to some kind of coevolution or genetic interaction of host and pathogen at the level of mtDNA.

Acknowledgements. We wish to thank Prof. Dr. Dr. h.c. K. Esser for his interest and support, the technical staff of our laboratories for assistance, D. Lenke and Ch. König for typing the manuscript, H. Rathke for the art work and Prof. P. A.

Lemke (Auburn) for critically reading the manuscript. Plasmids used for heterologous hybridization were the kind gifts of D. R. Pring (Gainesville), G. Burger (München) and U. Kück (Bochum). The work was supported by a research grant of the DFG.

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