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Gene content and organization of the oat mitochondrial genome

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Abstract The physical organization of the oat mitochondrial genome has been established. The master chromosome, one of the most complex described so far among higher plants, accounts for 596 kb and contains six direct repeats. Reiterated inverted repeats of 12 and 3 kb are also present and imply the possible existence of multiple isomeric forms. Fourteen genes coding for proteins, components of chain respiration and oxidative phosphorylation complexes, and of mitochondrial ribosomes have been detected together with *rrn26*, *rrn18* and *rrn5* genes and a set of 18 tRNA genes (ten genuine and eight cp-like). Some of them are clustered in a conserved form with respect to other monocots. Only the *trnS* (GGA) gene is silent.

Keywords *Avena sativa* L · Mitochondrial genome · Physical map · Gene location

Introduction

The higher-plant mitochondrial genomes are much larger and more complex than those of other eucaryotic organisms (Lonsdale et al. 1984). Analysis of plant mitochondrial DNA (mtDNA) by restriction mapping indicates that these genomes are composed primarly of unique sequences carried on a collection of different-sized circles, that interconvert via recombination between pairs of direct repeats (Falconet et al. 1984; Palmer and Shields 1984; Stern and Palmer 1984, 1986; Palmer and Herbon 1986, 1987). The number of recombination repeats in a master circle defines the complexity of a mitochondrial genome.

The organization of plant mtDNAs is highly variable among plant species (Pring and Lonsdale 1985; Newton 1988) whereas, in contrast to their variability in size, they are rather uniform in base composition (46–51%) (Makaroff and Palmer 1987).Among dicots, several mitochondrial genomes from crucifers have been characterized (Palmer and Herbon 1986). Most of them were found to have a tripartite structure: recombination between a pair of direct repeats results in two smaller circles each with one copy of the repeated sequence. The recombination events apparently occur frequently. A tripartite structure has also been reported for the spinach and sunflower mitochondrial genomes (Stern and Palmer 1986; Siculella and Palmer 1988).

In maize (Lonsdale et al. 1984), wheat (Quetier et al. 1985) and rice (Iwahashi et al. 1992), the genomic organization is much more complex. The 430-kb wheat mitochondrial genome contains a minimum of ten repeats (Quetier et al. 1985). In maize a 570-kb master circle contains six major repeats, five direct and one inverted, which can give rise to multiple circular size classes (Lonsdale et al. 1984). For the rice mtDNA a recombination model consisting of five circular DNA molecules has been postulated, each circle sharing homologous sequences with others. A master circle has been constructed also for the rice mitochondria genome, on the assumption that recombination occurs via these repeated sequences, and its size is 492 kb (Iwahashi et al. 1992).

In this paper we report the physical organization of mtDNA from oat (*Avena sativa* L. cv Argentina) and the mapping of several genes coding for proteins, rRNAs and tRNAs.

Materials and methods

Isolation of the mitochondrial DNA

Oat plants (*A. sativa* L: cv ‘‘Argentina”) were grown in a growth chamber at 25°C. MtDNA was purified from 6-day old etiolated

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shoots, using the DNAase–I procedure (Kolodner and Tewari 1972).

Methods used for restriction endonuclease analysis of mtDNA, agarose-gel electrophoresis, bidirectional transfer from agarose gel to Hybond filters, nick-translation of recombinant plasmid and filter hybridization were as described (Palmer 1986).

Cloning into plasmid pUC19

Clone banks of mtDNA *Pst*I, *Sal*I and *Kpn*I fragments were constructed by ligating *Pst*I, *Sal*I and *Kpn*I digests of mtDNA with appropriate digests of the vector pUC19 and transforming into *Escherichia coli* strain JM105.

Northern-blot analysis

MtRNA was isolated from 6-day old etiolated shoots by the procedure of Stern and Newton (1986). RNA was electrophoresed in 1% agarose gel containing 37% formaldehyde, 20 mM MOPS pH 7.0, 5 mM NaOAc, 1 mM EDTA and blotted to Hybond filters in 20×SSC. Hybridization were performed at 60°C for 12 h in 1 M NaCl, 1% SDS, 5% Dextran sulphate. Filters were washed extensively in 1×SSC, 0.5% SDS at 65°C prior to autoradiography.

PCR amplification

The PCR amplifications were carried out in the presence of 2.5 units of *Taq* polymerase (Perkin Elmer) using as a template 20 ng of mtDNAs or 10 ng of plasmid DNA, in the conditions suggested by the enzyme supplier. The products of PCR were resolved on a 1% agarose gel.

Sequencing analysis

The dideoxy chain-termination method of (Sanger et al. 1977) was used to obtain the sequence. Sequencing reactions were primed by the specific synthetic oligonucleotides (MWG Biotech) or with universal sequencing primers (Stratagene).

Results

Construction of the oat mitochondrial DNA physical map

Oat mtDNA digested with a variety of different restriction endonucleases demonstrates a complex restriction profile with both unimolar and submolar restriction fragments. Some fragments are present in roughly equal levels relative to each other and at about half the level of unimolar fragments, as judged by their reduced fluorescence (data not shown). Size estimation based on these profiles indicates a genome having a complexity of about 500 kb.

In order to establish a restriction map of the oat mitochondrial genome, plasmid DNA clones were selected from mtDNA libraries generated with *Pst*I, *Sal*I and *Kpn*I. Small-scale plasmid DNA preparations were analyzed by restriction endonuclease digestion and gel electrophoresis to establish a set of unique clones for each enzyme. These clones were labelled with ^{32}P and hybridized to Southern blots containing single and double digests of oat mtDNA generated by six restriction enzymes. The results of hybridizations with all the plasmid clones led to construction of a complete physical restriction map. Its entire sequence complexity can be accounted for by a circular DNA molecule (master circle) of 596 kb (Fig. 1). Without considering small repeated sequences under 500 bp, seven major sequence reiterations have been identified on the master chromosome by filter hybridization. An example of the hybridization of a repeat-containing *Pst*I fragment is reported in Fig. 2: a cloned *Pst*I fragment of 10.6 kb (p324 clone) is shown to cross-hybridize with *Pst*I fragments of 25 kb, 23 kb and 15 kb. The four repeat-containing *Pst*I fragments are substoichiometric as judged by their reduced fluorescence relative to other *Pst*I fragments in ethidium bro-

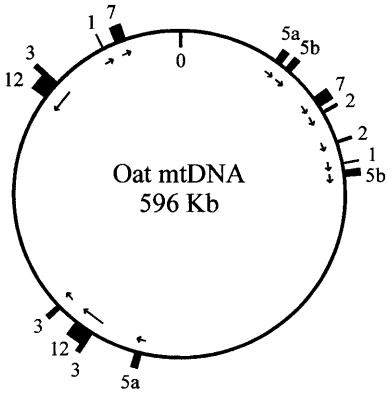

Fig. 1 The master chromosome of the oat mitochondrial genome with the location of repeats, represented by *closed boxes* outside the circle. *Numbers* close to the boxes indicate their size in kb. The zero point corresponds to the first *Pst*I site downstream from the *trnE* gene (see also Fig. 4)

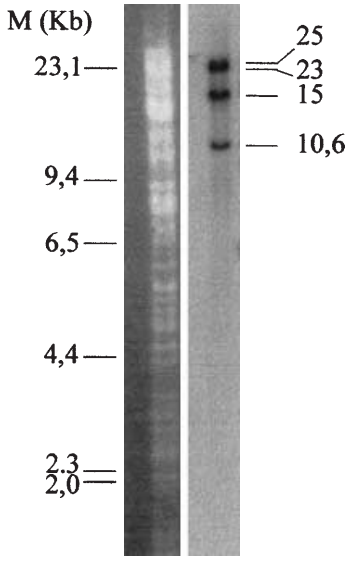

Fig. 2 Hybridization analysis of the 7-kb recombination repeat. Purified oat mtDNA was digested with *Pst*I, electrophoresed in 0.7% agarose gel (left panel), transferred to a Hybond filter and hybridized with nick-translated p324 (*right panel*). This clone has a *Pst*I insert of 10.6-kb containing the 7-kb recombination repeat (see also Fig. 4)

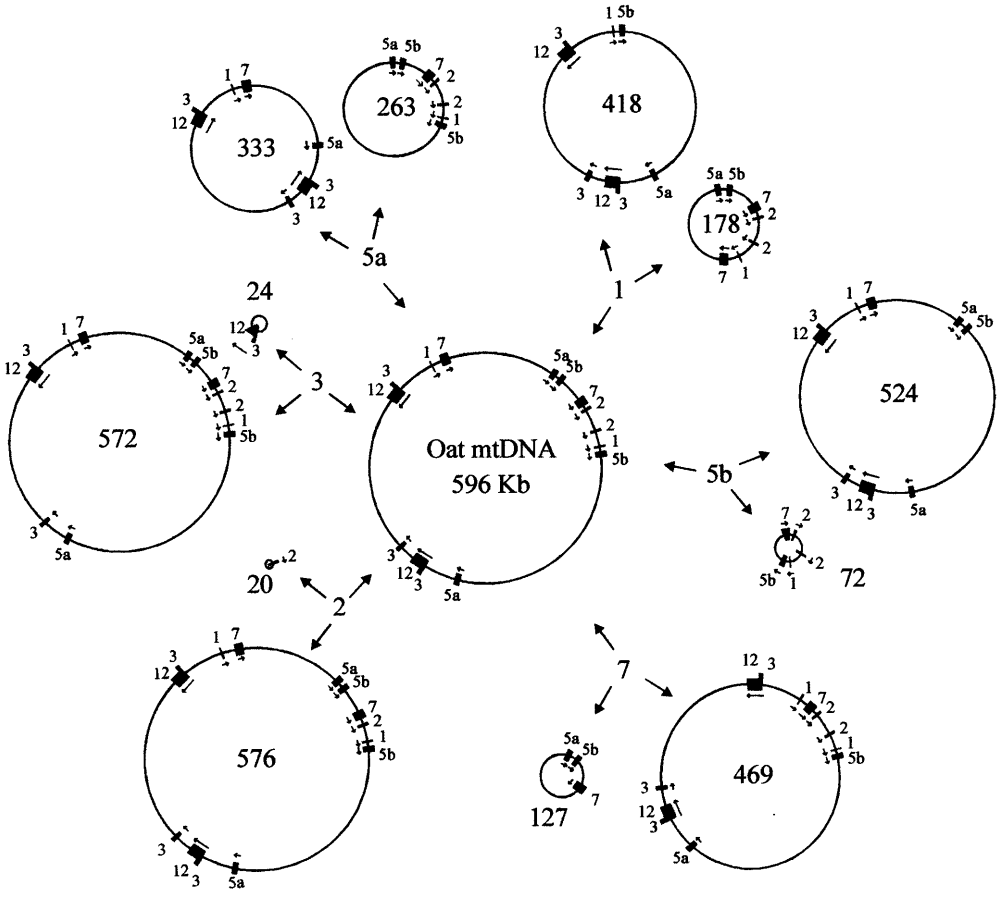

Fig. 3 The hypothetical multipartite structure of oat mtDNA. The isomeric form I in the center of the figure represents the master chromosome as mapped and described in Fig. 4. It can give rise to the isomeric form II by recombination between the 12-kb inverted repeats. Each isomeric form contains six sets of repeats, shown as *closed boxes* outside the circles and flanked by a *number* indicating their length in kb. The *number* inside each circle indicates its size in kb

mide-stained gels (Fig. 2 left panel). Among these submolar *Pst*I fragments, only those of 25 kb and 10 kb are accounted for by the restriction map shown in Fig. 4. The 23-kb and 15-kb *Pst*I fragments, however, can be generated by a single postulated recombination event between the reiterated sequences, embedded within the 25-kb and 10-kb fragments.

The identified repeats having estimated lengths of 1, 2, 5, 7, 12 kb have a reiteration number of two. The 12-kpb pair has inverted orientation and contains a 3-kb repeat, one copy of which is also present elsewhere on the genome in direct orientation. The size of each repeat has been estimated by restriction mapping and, therefore, it is not a precise value.

The filter hybridizations, the substoichiometric levels of several repeat-containing *Pst*I fragments, and their physical relationship are consistent with the occurrence of intragenomic recombinations. The results of intramolecular recombinations between direct repeats fractionate the master chromosome into a population of smaller subgenomic circular DNA molecules.

Recombination between the inverted repeats interconverts the master circle, described in Fig. 3, into several distinct isomeric circular molecules in which the location and orientation of the *trnP, rrn18, rrn5, cox I, nad I* and *rps3* genes can differ markedly. Some of the possible arrangements of these genes can be related to their expression (see also Discussion). Recombination events through any one of the direct repeats of each isomeric form give rise to subgenomic circles, whose sizes are dependent upon the location of the repeat within the master chromosome. Although it has not yet been possible by electrophoresis or electron microscopy to visualize any of these different-sized circles, the population of oat mitochondrial subgenomic circular DNA molecules can be predicted from the mapping study according to the scheme described in Fig. 3. Subgenomic circles generated from the master chromosome vary in size from 20 kb to 596 kb. Recombination between the two copies of the 7-kb repeat, for example, generates two large subcircles of 127 kb and 469 kb respectively. Furthermore, because some of the these subgenomic molecules still possess direct repeats, they in turn may mediate recombination to generate more subcircles. Finally, intermolecular recombination between various subcircles may take place to give rise to an essentially infinite variety of other circular DNA molecules.

Protein gene location

Several plant mitochondrial gene probes previously characterized from sunflower (Siculella and Palmer 1988) and *Arabidopsis* (Unseld et at. 1997) were hybridized to the same filters used for restriction mapping, and their hybridization profiles made possible to clarify some am-

Table 1 Origin of probes used for gene localization and mapping of oat mitochondrial genes. Note: sources of probes were as follows: clones of sunflower mitochondrial genome correspond to fragments indicated in Siculella et al. (1988) and Ceci et al. (1996) for protein and ribosomal RNA genes and for tRNA genes respectively. When amplification products were used as probes, they were obtained using the templates indicated in the second column and suitable primers deduced from conserved regions of monocot genes (for oat or maize amplified products) and of dicot genes for *Arabidopsis* (Unseld et al. 1997) and sunflower (Ceci et al. 1996) amplified products. The accession numbers of the EMBL Nucleotide Sequence database are indicated for genes fully sequenced in their coding region

Gene	Species of probes	Hybridization with oat mtDNA fragments (kb)		Accession numbers
		*Sal*I	*Pst*I	
rrn26	Sunflower clone	10	10.6	
		30	21.7	
rrn18	Sunflower clone	25	6.0	
		13.5	15.2	
rrn5	Sunflower clone	25	6.0	
		13.5	15.2	
cob	Sunflower clone	7.2	18.0	
coxI	Sunflower clone	10	4.4	
		7	5.2	
coxII	Sunflower clone	3.4	4.8	
coxIII	Sunflower clone	6.3	7.0	
atpA	Sunflower clone	2.2	4.5	
atp6	Sunflower clone	30.0	11.0	
atp9	Sunflower clone	30.0	7.8	
nad1	Sunflower clone	19.8	11.0	
nad3	mtDNA *Avena* amplified product	12.5	7.8	
nad5 Exon A+B	mtDNA *Arabidopsis* amplified product	30.0	7.8	
rps12	mtDNA *Avena* amplified product	12.5	7.8	
rpl5	mtDNA *Arabidopsis* amplified product	6.3	7.0	
rpl16	mtDNA *Arabidopsis* amplified product	7.2	18.0	
rps3	mtDNA *Arabidopsis* amplified product	19.8	11.0	
Genuine				
trn D (GTC) (Asp)	mtDNA *Avena* amplified product	11.7	12.4	AJ012130
trn E (TTC) (Glu)	mtDNA sunflower amplified product	17.0	25.7	AJ011897
trn I (CAT) (Ile)	mtDNA sunflower amplified product	11.7	12.4	AJ011898
trn K (TTT) (Lys)	mtDNA *Avena* amplified product	12.5	14.7	AJ011899
trn Mi (CAT) (fMet)	mtDNA sunflower amplified product	4.5	1.0	AJ011900
trn P (TGG) (Pro)	mtDNA sunflower amplified product	10.0	31.0	AJ011901
		11.0	7.0	
		13.5	15.2	
trn Q (TTG) (Gln)	mtDNA sunflower amplified product	12.5	14.7	AJ011902
trn S (GCT) (Ser)	mtDNA sunflower amplified product	12.5	7.8	AJ011903
trn S (TGA) (Ser)	mtDNA *Avena* amplified product	2.8	31.0	AJ011904
trn Y (GTA) (Tyr)	mtDNA sunflower amplified product	30.0	4.1	AJ011905
Cp-like				
*trn C** (GCA) (Cys)	mtDNA *Avena* amplified product	13.5	15.2	AJ012131
*trn F** (GAA) (Phe)	mtDNA *Avena* amplified product	11.3	8.2	AJ012132
*trn H** (GTG) (His)	mtDNA sunflower amplified product	0.9	25.7	AJ012137
*trn M** (CAT) (Met)	mtDNA sunflower amplified product	20.0	1.4	AJ012133
*trn N** (GTT) (Asn)	mtDNA sunflower amplified product	11.7	12.4	AJ012134
*trn P** (TGG) (Pro)	mtDNA *Avena* amplified product	27.1	14.7	AJ012136
*trn S** (GGA) (Ser)	mtDNA *Avena* amplified product	11.3	8.2	AJ012135
trn W *(CCA) (Trp)	mtDNA *Avena* amplified product	27.1	14.7	AJ012136

biguities in the physical map and to locate corresponding genes on the map. A description of clones and a list of hybridizing fragments is given in Table 1. In Fig. 4 the restriction map of the master chromosome, linearized at a specific *Pst*I site, is reported. Figure 4 also shows the entire sequence complexity together with the location of repeated sequences and genes coding for proteins, 26S, 18S and 5S ribosomal RNAs, and for tRNAs. With the exception of the genes *coxI*, *rrn26*, *rrn18*, *rrn5* and *trnP** being coincident with the inverted 12 kb, and the direct 7-kb and 3-kb repeats respectively, all genes detected are present as single copies.

Most of the localized genes are widely scattered throughout the genome and are probably unlinked transcriptionally, as in other plant mtDNAs (Dawson et al. 1986; Stern and Palmer 1986; Makaroff and Palmer 1987). A sole exception to this pattern is the close linkage of the *rrn18* and *rrn5* genes, which are also adjacent

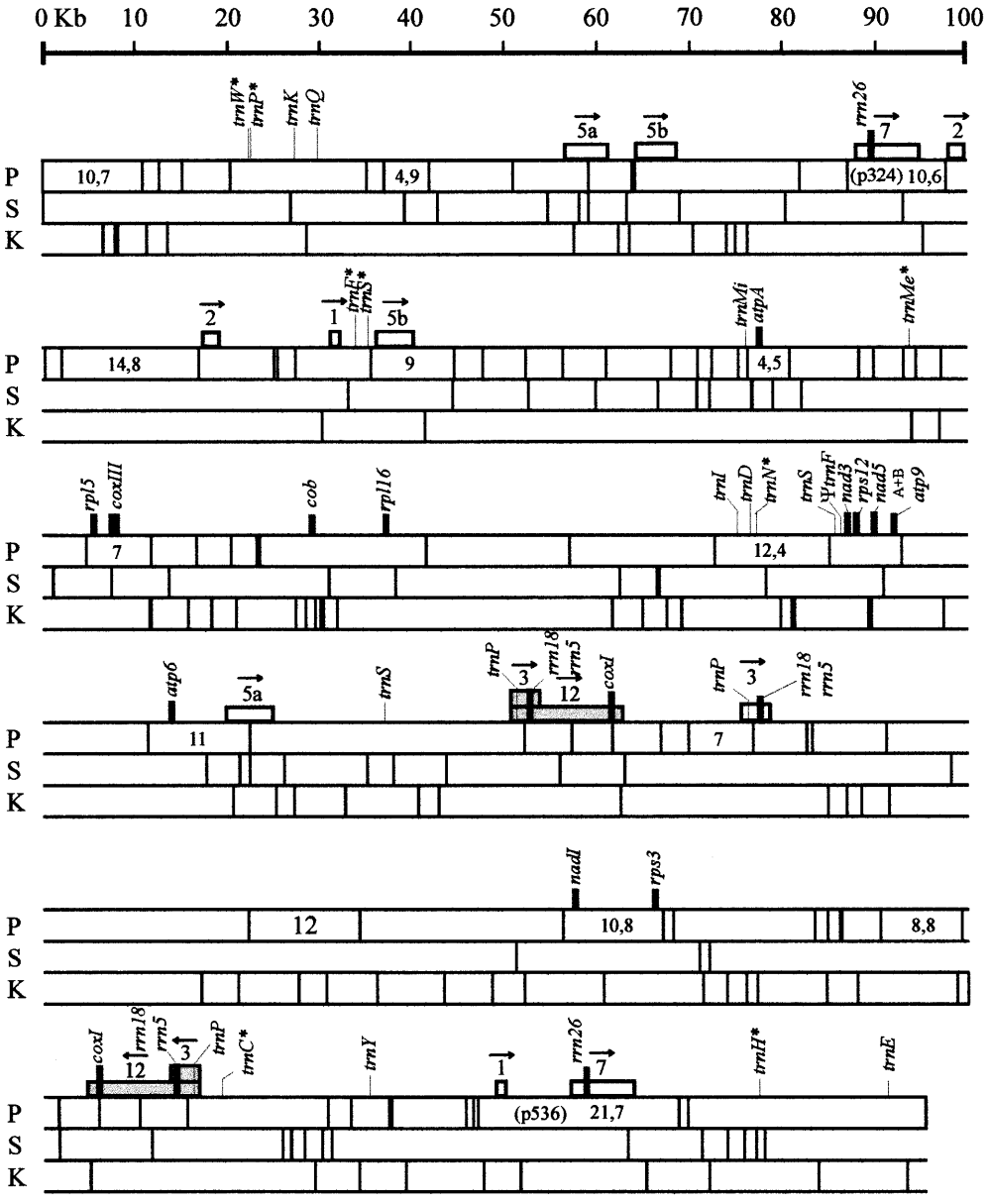

Fig. 4 Restriction map of the oat master chromosome, showing the entire sequence complexity. The circular map linearized at the first *Pst*I site downstream from the *trnE* gene is shown. Fragment sizes are in kb. Direct repeats are represented by *open boxes* with their size in kb. *Sal*I (*S*) and *Kpn*I (*K*) sites are also reported. Inverted repeats are indicated by *dashed boxes*. The *arrows* indicate repeat orientation. The rRNA and protein-coding genes are represented by *closed boxes*. tRNA genes, indicated by *asterisks*, are considered to be of chloroplast origin (cp-like genes)

in other plant mitochondrial genomes (Bonen and Gray 1980; Stern et al. 1982).

Detection and expression of tRNA genes

Table 1 and Fig. 4 describe the tRNA genes identified on the oat mitochondrial genome. Ten belong to the genuine (or native) class, eight to those of defined chloroplast origin (cp-like genes).

Their expression and relative extent can be deduced from the Northern-blot experiments described in Fig. 5. Taking into account that all the experiments were carried out using similar amounts of total mitochondrial RNAs, and probes prepared with similar procedures, the extent of expression can be considered directly comparable among each other (see also legend of Fig. 5). Contamination by tRNA of chloroplast origin could be ruled out because a labelled probe obtained as amplification product of the sunflower *trnV** gene (Ceci et al. 1996) strongly hybridyzed with total RNA extracted from oat chloroplasts. No hybridization signals could be detected whether the same probe was hybridized with the preparations of total mitochondrial RNA described in Fig. 5 (data not shown).The results of the experiments described in Fig. 5 demonstrate that the only silent gene is the (cp-like) *trnS** (GGA). Furthermore the gene *trnP** is transcribed to a lower extent in comparison with the *trnW** gene which is located in tandem on the genome (see Fig. 4). Finally most of the cp-like tRNA genes show a transcription pattern more complex than those of all genuine genes. These points will be considered in the Discussion that follows.

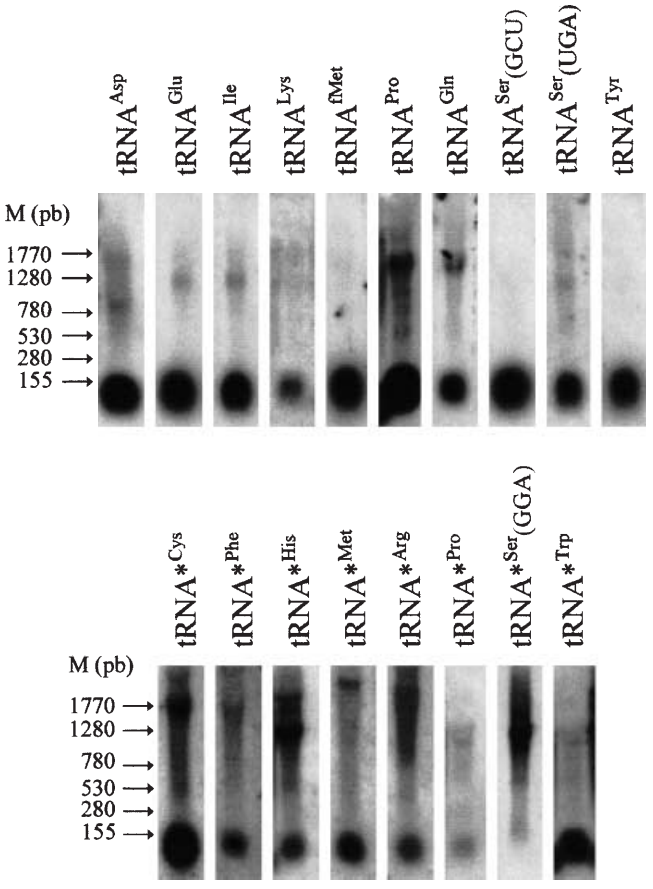

Fig. 5 Northern-blot analysis of tRNA transcripts in oat mitochondria. Total mitochondrial RNA was electrophoresed and transferred as reported in the Materials and methods section. Probes were according to the list in Table 1. Labelling was performed using the random priming procedure. Specific activity of probes ranged from 10^8 to 10^9 cpm/µg of DNA. Three different RNA preparations were used. The first was employed for experiments with probes corresponding to *trnK*, *trnQ* and *trnY*, the second with probes corresponding to *trnF**, *trnH**, *trnMe** and *trnN**, and the third with the remaining probes. The amount of loaded RNA ranged between 12 and 15 µg

Discussion

Large, complex mitochondrial genomes are a striking feature of higher plants (Ward et al. 1981; Lonsdale et al. 1984; Sederoff 1984; Lonsdale 1994). Even for oat mtDNA the total genetic information can be arranged into a large, single, circular molecule that is referred to as the master chromosome having a size of 596 kb. Because of its dimension, comparable to those of other monocots such as corn (Lonsdale et al. 1984; Fauron et al. 1989; Fauron and Casper 1994), wheat (Quetier et al. 1985) and rice (Iwahashi et al. 1992), the oat mitochondrial genome is postulated to have a highly complex multipartite organization. The 596-kb master chromosome and six subgenomes could interconvert by reciprocal recombination within six pair of direct repeats, respectively of 1, 2, 3, 5a, 5b and 7 kb. Recombination between the 3- and 12-kb inverted repeats interconverts the master circle into several isomeric subcircular DNA molecules. In each of them, *trnP*, *rrn18*, *rrn5*, *cox I*, *nad I* and *rps3* can have different location and orientations.

The complex multipartite structure resulting from recombination between homologous repeated sequences explains the existence of submolar and multimolar restriction fragments which are a distinguishing feature of plant mitochondrial restriction profiles. The mapping analysis illustrates how the number, location and orientation of recombinogenic reiterated sequences determine the organizational complexity of the oat mitochondrial genome, dictate its fluidity and have the potential to influence its function. In particular it can be speculated that the presence of overlapping inverted repeats (3 and 12 kb) having a third counterpart (3 kb) within them, generates different locations and orientations of genes included in this region (see above) which can be relevant for the regulation of their expression. Although DNA recombination in higher-plant mitochondria can be involved in so many aspects of gene location and expression, nothing is known about the mechanism of recombination, its induction or its regulation. In particular it is intriguing to imagine how the master circle could be distributed faithfully to the dividing cells.

Finally, the monocots examined thus far, corn (Fauron et al. 1989; Fauron and Casper 1994; Lonsdale et al. 1984), wheat (Quetier et al. 1985), rice (Iwahashi et al. 1992) and oat, mark multiple pairs of reiterated sequences, whereas spinach (Stern and Palmer 1986), sunflower (Siculella and Palmer 1988), radish and several other species of *Brassica* (Palmer and Herbon 1986; 1987; Makaroff and Palmer 1988), possess only a single major repeat. We note that all the above observations are based on a limited number of samples. Thus, it can not be ruled out that a very large dicot genome may have a more complex structure than the smallest monocot genome mapped so far.

Most of the genes mapped in this study are widely scattered throughout the genome their order being markedly different from that detected in maize (Dawson et al. 1986), rice (Iwahashi et al. 1992), spinach (Stern and Newton 1986), turnip (Makaroff and Palmer 1987) and sunflower (Siculella and Palmer 1988). Therefore the order of genes in plant mtDNAs is likely to have little effect on their function and thus varies widely from species to species. All the genes are present only once per genome, except for *coxI* and *rrn26* which, being coincident with the 12-kb inverted repeats and the 7-kb direct repeats, are present in two copies per genome. Finally, the *rrn18*, *rrn5* and *trnP** genes are present on the 3-kb repeats and therefore are present in three copies.

To what extent circular DNA molecules exist in vivo within plant mitochondria is still controversial (Bendich 1993), although the circular map is a reliable and valuable tool for studies concerning higher-plant mitochondrial genomes. Northern-blot experiments performed in order to study tRNA gene expression gave rise to some speculations. The transcription of the gene tandem *trnP*-trnW** is apparently different in the ‘‘graminacea”

in comparison to what has been described in maize (Leon et al. 1989) where both genes are transcribed but only the mature $tRNA^{Trp}$ is accumulated. By contrast the experiments described in Fig. 5 clearly show that significant amounts of $tRNA^{Pro}$ molecules as products of *trnP** gene transcription are present in the oat mitochondria. This observation suggests, in our opinion, that the transcription of this gene in oat mitochondria is at an intermediate evolving situation in which, despite the existence of an active genuine *trnP* gene, the accumulation of the $tRNA^{Pro}$ is not yet completely switched off. Obviously an alternative hypotesis can be considered; that is, in oat the *trnP** gene transcription has only recently (in evolutionary times) been switched on. The second alternative is of course difficult to support considering the high levels of genuine $tRNA^{Pro}$ molecules existing in the oat mitochondria. These considerations suggest accurate experiments are needed to study the tandem *trnP*-trnW** gene transcription (that is, to study the detection of initiation transcription sites, promoters, consensus sequences etc.) and these are actually in progress in our laboratory.

The second aspect of tRNA gene expression is concerned with the complexity of patterns related to all the cp-like genes (Fig. 5). This observation suggests that each of them has been inserted into transcription units already active in the mitochondria for the expression of genes of different function(s). According to the results described in Fig. 5 these seem to be part of transcripts containing other information which ‘‘in organello” must be processed to the mature molecules.

The transcription pattern of genuine tRNA genes should, by contrast, be much simpler, as it is with the exception only of the *trnP* gene, because the transcription would start from promoters specific for each gene.

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