Theor Appl Genet (1991) 82:723–728

THEORETICAL AND APPLIED GENETICS

Higher plant mitochondrial DNA expression

2. Influence of nuclear background on the transcription of a mitochondrial open reading frame, ORF25

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Received January 25, 1991; Accepted April 30, 1991
Communicated by R. Hagemann

Summary. Mitochondria are semi autonomous organelles, with their own genome and transcription/translation systems. Although the regulation of mitochondrial gene expression is fairly well characterized in the animal system, little is known about these processes in plants. We have been studying the expression of ORF25, a mitochondrial open reading frame, in normal male-fertile maize. In all the N lines that we have examined, the ORF25 transcript pattern is similar, except for that in B37N. We have compared ORF25 transcription patterns between B73N and B37N: B73N has one major transcript of 2,300 nucleotides and two minor transcripts of 3,400 and 1,600 nucleotides, while B37N has a single transcript, 3,400 bases long. The ORF25 reading frame and 5′ flanking regions have been analyzed by restriction mapping and found to be identical in these lines. Interestingly, the F_1 progeny from reciprocal crosses between B73N and B37N have ORF25 transcript patterns identical to B73N. This suggests that the process of mitochondrial transcription is influenced by nuclear factors in normal cytoplasm. This factor(s) appears to be dominant in B73N and the F_1 progeny. S1 nuclease analyses have revealed that identical fragments are protected in B73N and the F_1 hybrids, indicating that the ORF25 transcripts in the F_1 progeny are identical on the 5′ ends to those of the parent B73N. This nuclear regulation may be at the level of initiation of transcription or processing of the mtRNA.

Key words: Plant – Mitochondria – DNA – RNA – ORF25

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Introduction

Higher plants contain two extranuclear genetic systems, the mitochondrial DNA and the chloroplast DNA. Efficient interaction between these two genetic systems and the nucleus is essential for the normal functioning of the plant cell. The mechanisms by which the nucleus regulates mitochondrial and chloroplast gene expression are poorly understood, particularly for the mitochondrial system. Alterations in the way the three genetic systems interact, or in the structure of any of the genomes, can result in changes of the plant phenotype or the expression of a gene.

Mutations leading to molecular or phenotypic change in the organism have been very useful for studying the regulatory mechanisms within a cell. Mitochondrial genes encode for proteins that are required for essential cellular functions, and mutations in these genes are generally lethal to the cell. However, there are a few known mitochondrial mutations, such as cytoplasmic male sterility, which causes pollen abortion (Laughnan and Gabay-Laughnan 1983; Hanson and Conde 1985), and the nonchromosomal stripe 5 (Shumway and Bauman 1967; Newton et al. 1990), which is due to a mutation in the cytochrome oxidase subunit 2 gene.

Nuclear background and chromosomal mutations are also important in studying the regulation of mitochondrial gene expression. Yeast nuclear mutations have been described that block expression of the mitochondrial genes *coxII* and *coxIII* (Fox et al. 1988). In addition to mutational analysis, sexual crosses in plants can generate novel nuclear-cytoplasmic combinations that may alter the processes of transcription or translation. For example, in T-cms plants that are restored to fertility, nuclear restorer alleles change the transcriptional pattern of TURF2H3 (Dewey et al. 1986). By studying the

molecular nature of these changes, key features of the basic processes of mitochondrial gene regulation can be identified.

We have been involved in the study of expression of a mitochondrial open reading frame, ORF25, and the influence of various nuclear backgrounds on its transcription. In all the normal male-fertile lines we have analyzed, ORF25 is expressed and the transcript pattern is identical (Walker et al. 1987), except in B37N (Wang et al. 1991). We have compared the ORF25 transcript pattern between B73N, and B37N. B73N has a major transcript of 2,300 nucleotides and two minor transcripts of 3,400 and 1,600 nucleotides, while B37N has a single transcript of 3,400 nucleotides. Wang et al. (1991) compared the gene structure for ORF25 between B73N and B37N by restriction mapping, and found no major differences in the reading frame or in the 5′ flanking sequences.

In this paper, we report the modulation of the ORF25 transcription by varying the nuclear background. S1 nuclease-protected fragments in the parents and the F_1 progeny were mapped on pH3.2N, a clone containing the 5′ flanking sequence of the ORF25 reading frame (Walker et al. 1987). This experiment indicates that the 5′ ends of the transcripts in the F_1 hybrids are similar to those in B73N, and the difference between the 2,300 and 3,400 nucleotide transcripts lies in the 5′ flanking sequences of ORF25. Densitometric analysis of the Northern hybridizations reveals that the ratios of these transcripts differ in the F_1 hybrids and B73N. This indicates that the nuclear regulators may behave in a dosage-dependent fashion.

Materials and methods

Seeds for the two normal male-fertile lines B73N and B37N were provided by Pioneer Hi-Bred International. Reciprocal crosses between B73N and B37N and subsequent backcrosses were set up in the fields at Clemson University.

Mitochondrial DNA isolation, cloning, and analysis

Mitochondrial DNAs were isolated from etiolated coleoptiles, according to the procedure of Kemble et al. (1980), and further purified on a neutral CsCl gradient. DNA samples were digested with restriction enzymes and analyzed on 0.8% agarose gels. DNA gels were blotted according to the procedure of Southern (1975). *Bam*HI and *Hin*dIII genomic libraries for the two maize lines had been previously constructed in pUC8, and recombinants were grown on X-gal and ampicillin plates. Further screening for the appropriate clones was done using ^{32}P labelled probes (Maniatis et al. 1982).

Mitochondrial RNA isolation and analysis

All mitochondrial RNA samples were isolated from etiolated coleoptiles, as previously described by Abbott and Fauron (1986). The RNA samples were rinsed with 2 *M* LiCl, followed by a 75% ethanol wash, before analysis. RNA samples were denatured and analyzed on 1.5% agarose formaldehyde gels. Gels were directly blotted on nylon membranes and hybridized as described by Maniatis et al. (1982).

DNA labelling

DNA probes used for Southern and Northern hybridizations were prepared by nick-translation according to previously described procedures (Rigby et al. 1977; Maniatis et al. 1982).

S1 nuclease protection experiments

Mitochondrial RNAs from B73N, B37N, and F_1 progeny were hybridized with the 3.2-kb *Hin*dIII fragment and digested with S1 nuclease (Maniatis et al. 1982). The protected fragments were separated on a 1.0% agarose gel, and then blotted and hybridized with the 3.2-kb *Hin*dIII fragment from pH3.2N.

Densitometric analysis

Autoradiograms identical to the ones in Figs. 4 and 11 were used for densitometric analysis. The amount of individual transcripts is represented by the area under the respective peaks. The quantitative variation is represented as the ratios of the 2,300 to the 3,400 nucleotide transcripts. The numbers used for obtaining the ratios shown in Figs. 5 and 12 are average values from three separate readings.

Results

The open reading frame, ORF25, was originally described in T-cms cytoplasm (Dewey et al. 1986) and is part of a chimeric gene region, TURF2H3. ORF25 is cotranscribed with *urf13*, also a part of TURF2H3, using the *atp6* promoter. ORF25 could encode a 24,657-dalton protein, but no translation product has been identified so far and its role in cytoplasmic male fertility not established. This region of the mtDNA is transcribed in all the normal male-fertile cytoplasms that we have studied.

Mitochondrial RNA from two normal male-fertile cytoplasms, B73N and B37N, were analyzed by electrophoresis and Northern hybridization. In B73N, the 5′ end of ORF25 and flanking sequences are located on a 3.2-kb *Hin*dIII fragment (Fig. 1), and this fragment was used as a probe to study ORF25 transcript patterns. B73N has three transcripts homologous to the 3.2-kb fragment, a major transcript of 2,300 and two minor transcripts of 3,400 and 1,600 nucleotides. In contrast, B37N has a single transcript of 3,400 nucleotides (Fig. 2). When only the 5′ end of the 3.2-kb fragment is used as a probe (1.5-kb *Hin*dIII-*Xho*I fragment), only the 3,400

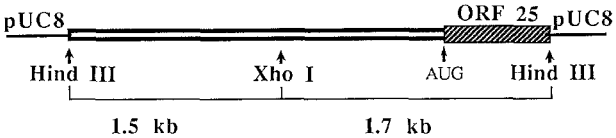


Fig. 1. A restriction map of the 3.2-kb *Hin*dIII fragment from pH3.2N showing the location of the ORF25 reading frame. The 3.2-kb *Hin*dIII fragment has an internal *Xho*I restriction site

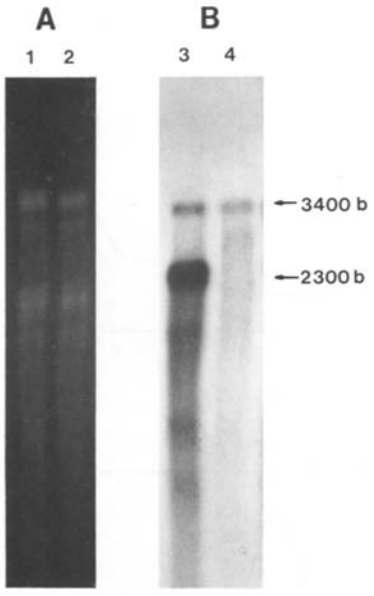


Fig. 2. **A** mtRNA separated on a 1.5% formaldehyde agarose gel. **B** Northern blot using the 3.2-kb *Hin*dIII fragment as a probe. *Lane 1*: B73N; lane *2*: B37N

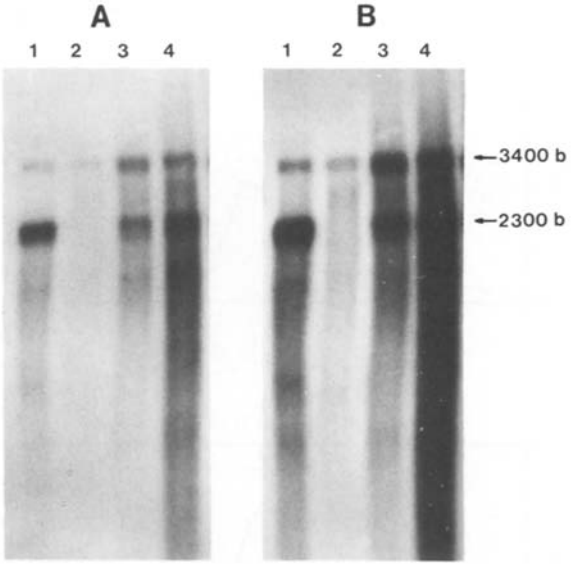


Fig. 3. **A** Hybridization of the 3.2-kb *Hin*dIII fragment from the clone pH3.2N to coleoptile mtRNA samples from: lane *1*: B73N; lane *2*: B37N; lane *3*: F_1 progeny from the cross female B73N × male B37N; and lane *4*: F_1 progeny from the cross female B37N × male B73N. **B** Darker exposure of the same blot shown in **A**, to clearly demonstrate that B37N has a single transcript of 3,400 nucleotides

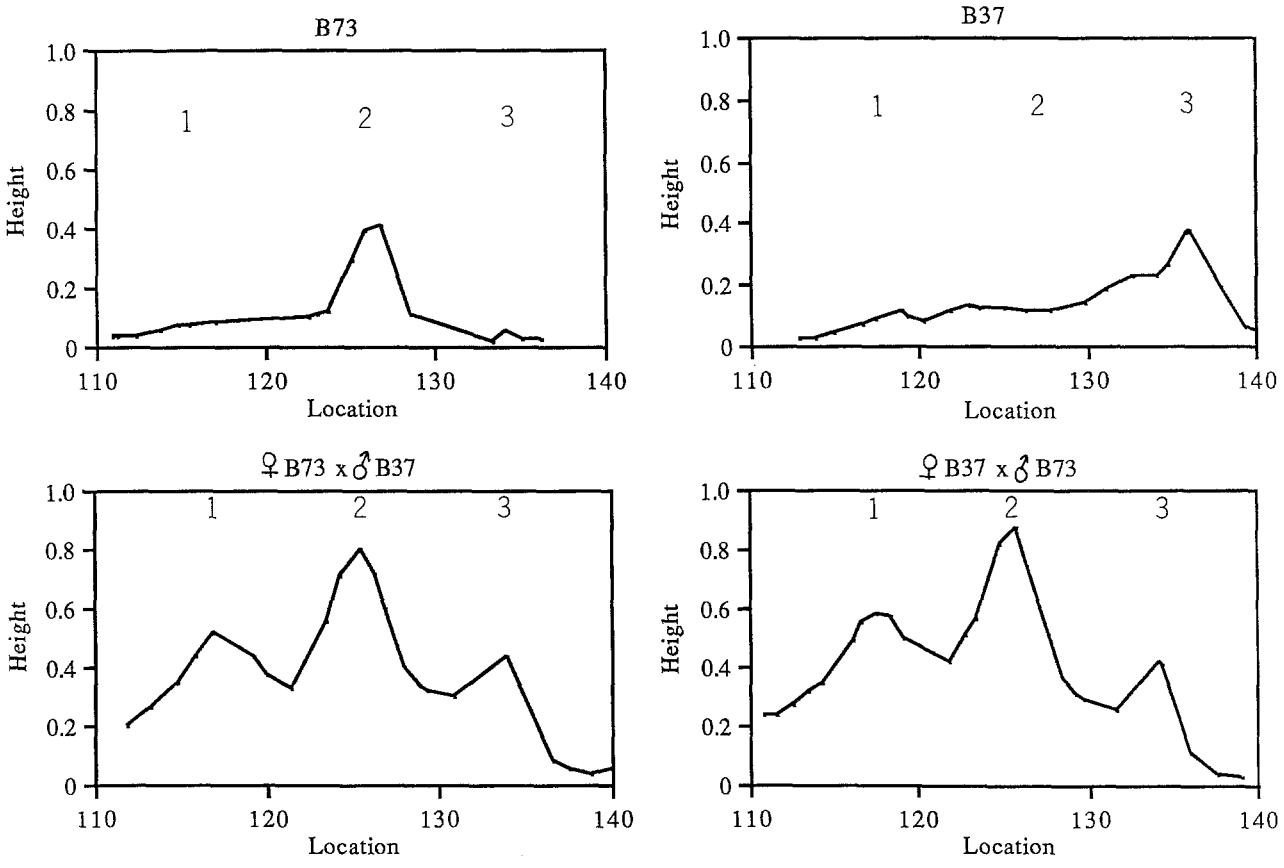
nucleotide transcript is identified (Wang et al. 1991). This indicates that the 3,400 and the 2,300 nucleotide transcripts differ in their 5′ ends. Thus, the 2,300 nucleotide transcript could be a processed form of the larger transcript or the two transcripts could be generated from separate initiation sites.

In order to test the possibility of nuclear influence on transcription of ORF25, reciprocal crosses between B73N and B37N were done and the F_1 progeny were analyzed for ORF25 transcript patterns. A Northern blot with mtRNA samples for the parents and the F_1 progeny was hybridized with the 3.2-kb *Hin*dIII fragment isolated from pH3.2N (Fig. 3). ORF25 homologous transcripts are detected in all the RNA samples. The F_1 progeny from the two crosses have a transcript pattern similar to that in B73N, with a major transcript of 2,300 and two minor transcripts of 3,400 and 1,600 nucleotides, in contrast to that of B37N. This indicates that the transcript pattern is independent of cytoplasmic origin, since the F_1 hybrids have either B73N cytoplasm or B37N cytoplasm.

The transcript patterns are similar in the F_1 hybrid and the B73N parent. However, individual transcript concentrations vary. To compare the relative amounts of ORF25 transcripts between the F1 progeny and B73N, an autoradiogram similar to that in Fig. 3 was scanned by densitometric analyses and the ratios of the 2,300/3,400 nucleotide transcripts were compared. These results are shown in Fig. 4. The ratios are similar between the F_1 progeny from the two crosses, but differ from the value for the B73N parent. This result indicates that the nuclear influence on ORF25 may be dosage dependent or codominant. Interestingly, the 1,600 base transcript, a very minor component in the B73N pattern, is a major transcript in the hybrid patterns. At this point, we have focused our attentions only on the major component RNAs of the B73N and B37N transcript patterns.

In order to determine the 5′ ends of the transcripts in the F_1 hybrids, an identical blot as shown in Fig. 3 was hybridized to the 1.5-kb *Hin*dIII-*Xho*I fragment. This region is homologous only to the 3,400 nucleotide transcript (Fig. 5), which indicates that the 3,400 and the 2,300 nucleotide transcripts differ in their 5′ ends in the F_1 hybrids and in B73N.

A more precise mapping of these transcripts was accomplished by S1 nuclease protection experiments. These experiments were done using the 3.2-kb *Hin*dIII fragment. Identical fragments are protected between B73N and F_1 progeny from the two crosses (Fig. 6). The protected fragments in these three mtRNA samples are 2,200 and 1,100 bases long. As described previously, B37N has a single S1 nuclease-protected fragment, 2,200 bases in size (Wang et al. 1991). The two predominant S1 products in B73N and the F_1 progeny from the two reciprocal crosses differ by 1,100 bases. The two large transcripts in B73N and F_1 progeny that hybridize to ORF25 are 3,400 and 2,300 nucleotides in size, which is also a difference of 1,100 bases. The DNA used for the S1 nuclease experiments was the 3.2-kb *Hin*dIII fragment, which carries 5′ flanking sequences and a portion of the transcription



	B73N	B37N	♀B73 x ♂B37	♀B37 x ♂B73
Ratio of $\frac{2300}{3400}$ nucleotide transcripts	22.1	N.A.	2.9	3.4

Fig. 4. Scanning densitometric analyses of a Northern blot similar to the one shown in Fig. 3 Numbers 1, 2, 3 denote peaks of the 1,600, 2,300, and 3,400 base transcripts, respectively. The relative proportions of the 2,300 and 3,400 base transcripts are tabulated as the ratio of peak 2 area/peak 3 area

unit for ORF25 (Fig. 1). This confirms our previous data, which positioned the region of transcript differences to the 5′ flanking sequences of ORF25.

This analysis indicates that the 5′ ends of the ORF25 homologous transcripts in the hybrids are most likely the same as those in B73N, and could be the result of an identical transcription initiation or processing event (Fig. 7).

Discussion

We have studied the modulation of a mitochondrial open reading frame, ORF25, by the different nuclear backgrounds of B73N and B37N. The normal male-fertile line, B37N, has a variant ORF25 transcript pattern compared to the pattern we observed in other male-fertile lines, including B73N. Kennell et al. (1987) reported variations in ORF25 transcription patterns among different N cytoplasms. These differences were attributed to recombinant forms of the transcription unit.

The major transcript in B73N is a 2,300-nucleotide transcript with two minor transcripts of 3,400 and 1,600 nucleotides, in contrast to B37N, which has a single transcript of 3,400 nucleotides. The difference between the 3,400 and 2,300 nucleotide transcripts is in the 5′ flanking region of the gene and does not appear to be due to major structural changes.

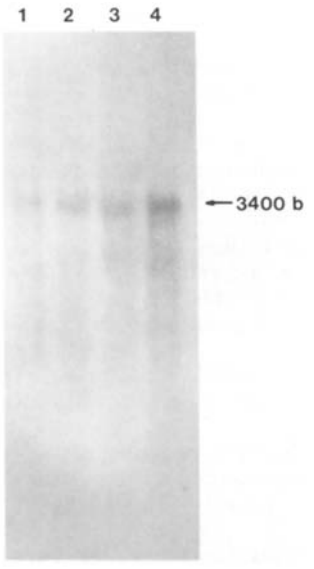


Fig. 5. Northern hybridization with the 1.5-kb *Xho*I-*Hin*dIII fragment. Lane *1*: B73N; lane *2*: B37N; lane *3*: F_1 progeny from the cross female B73N × male B37N; and lane *4*: F_1 progeny from the cross female B37N × male B73N

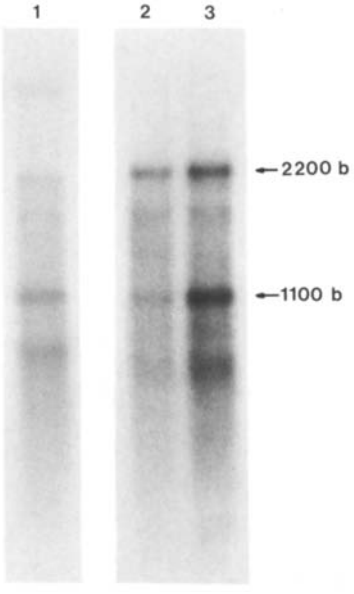


Fig. 6. S1 nuclease products were blotted on nylon membranes and probed with the 3.2-kb *Hin*dIII fragment from the clone pH3.2N. Coleoptile mtRNA from, lane 1: B73N; lane *2*: F_1 progeny from the cross female B73N × male B37N; and lane *3*: F_1 progeny from the cross female B37N × B73N, was hybridized with the 3.2-kb *Hin*dIII fragment and treated with S1 nuclease

In order to identify the cause of transcript pattern differences in these lines, reciprocal crosses between B73N and the inbred B37N were done. mtRNAs from the F_1 hybrids were analyzed by Northern hybridizations using a cloned ORF25 probe. F_1 progeny from both the crosses have a similar transcript pattern to that of the

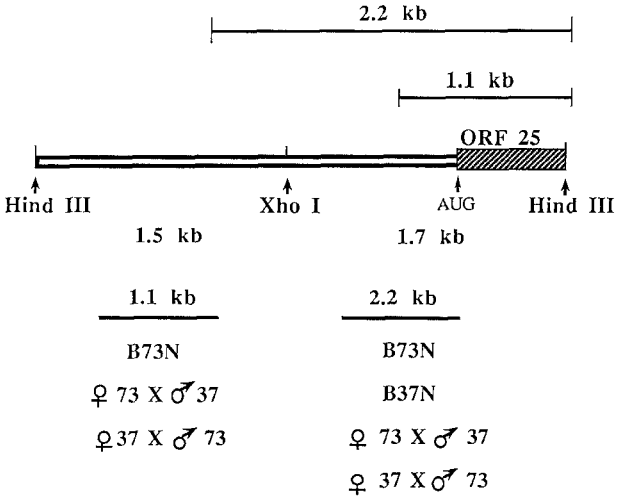


Fig. 7. A restriction map of the 3.2-kb *Hin*dIII region of the clone pH3.2N. S1 nuclease-protected fragments are shown with respect to the region of homology to the 3.2-kb fragment

B73N parent. The F_1 progeny had either the B73N or B37N as the female parent, which argues that it is not the source of the cytoplasm, but the nuclear background that determines the ORF25 transcript pattern.

Although the ORF25 transcript patterns are similar for the F_1 hybrids and B73N, the quantities of individual transcript are not the same. In order to compare the quantitative ratios of ORF25 transcripts, a densitometric analyses was done on Northern blots. The data from these experiments indicate that the ratio of the 2,300 to the 3,400 nucleotide transcripts is similar between the F_1 hybrids, but different from that in B73N. This indicates that a dosage-dependent relationship may exist between the nuclear and cytoplasmic components of this system.

B73N and the F_1 progeny have similar S1 nuclease-protected fragments. This indicates that the 5′ ends of the transcripts are similar in the F_1 hybrids and the parent B73N. We have not examined the 3′ ends of these transcripts, but the differences in the transcripts are completely accountable by the differences noted in the 5′ end.

In order to determine whether the nuclear influence on ORF25 transcription involves a single or multiple genes, plants from the F_1 hybrids were pollinated with B37N. The F_2 progeny plants are being analyzed by Northern hybridization for ORF25 transcription using total RNA extracts. Preliminary data suggests that there may be segregation of transcript patterns in these progeny.

Acknowledgements. This work was done by Dipika Gupta, in partial fulfillment for her Ph.D. degree in Plant Physiology. We would like to thank Pioneer Hi-Bred International for supporting this project and, in particular, Dr. M. Albertsen for providing the seed material. We would also like to thank Linda Eldredge for the field work.

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