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THEORETICAL AND APPLIED GENETICS

Higher plant mitochondrial DNA expression

1. Variant expression of the plant mitochondrial open reading frame, ORF25, in B37N and B73N maize lines

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Summary. In studying the process of mitochondrial transcription, mutants that show altered gene expression as evidenced from transcript pattern differences are a valuable resource. However, such mutants are difficult to find since changes in mitochondrial gene expression will most likely be lethal. Several laboratories have been investigating cytoplasmic male-sterile mutants in maize and have reported changes in transcription patterns due to nuclear background influences on the complex chimeric gene region TURF-2H3 in T-cms. There have been no reports of altered transcription patterns for N cytoplasm that can be attributed to nuclear background differences. Through a Northern hybridization analysis of ORF25 transcription in a number of N lines, we reported invariant expression of this region. Subsequently, we have discovered a line B37N, which shows the presence of a single ORF25-specific transcript of 3,400 nucleotides, in contrast to the transcript sizes of 3,400, 2,300 and 1,600 displayed by most of the cytoplasms we have examined. Experiments presented in this communication demonstrate that the differences in the B37N, ORF25 transcript pattern map to the 5′ flanking sequences of the reading frame. Using restriction enzyme mapping and Southern hybridization analysis, no detectable differences were found in the transcription unit structure for this reading frame in B37N and B73N, which shows the standard, three-transcript pattern. Analysis of nuclear background influences indicates that the transcript patterns for this open reading frame are dependent on nuclear background. These data are presented in part 2 of this study.

Key words: Plant – Mitochondrial DNA – ORF25 – Transcription – B37N

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Introduction

In contrast to animal and fungal systems, the higher plant mitochondrial genome is much larger and more variable in size. The genome can range from 200 kb in *Brassica* to 2,500 kb in cucurbits (Ward et al. 1981, Palmer and Shields 1984). In general, the principal mitochondrial genome is arranged in a multipartite set of circular molecules, each arising through intra- as well as intermolecular recombination of a master chromosome (Palmer and Shields 1984; Lonsdale et al. 1984; Quetier et al. 1985). As in the case of maize, these recombinations occur at direct and inverted repeat sequences dispersed around the master chromosome (Lonsdale et al. 1984).

Although much larger than its animal or fungal counterparts, the plant mitochondrial genome essentially encodes a similar set of proteins. Most of these are components of the inner mitochondrial membrane and include the *cob* (Dawson et al. 1984), *coxI, coxII, coxIII* (Fox and Leaver 1981; Issac et al. 1985b; Heisel et al. 1987), *atp6, atp8, atp9, atpA* (Braun and Levings 1985; Dewey et al. 1985a, b; Heisel and Brennicke 1985; Issac et al., 1985a). There are also several putative genes which have been identified strictly on the basis of sequence information. These include, the possible complex I genes, *nad1, nad5* (Bland et al. 1986; Stern et al. 1986; Wissinger et al. 1988), and possible ribosomal protein genes *rsp4, rsp13,* and *rsp14* (Bland et al. 1986; Schuster and Brennicke 1987; Wahleithner and Wolstenholme 1988). The genome also encodes the three major ribosomal RNAS, a number of transfer RNAs, and several unassigned reading frames.

Considering the complex multipartite structure of the mitochondrial DNA (mtDNA) in higher plants, it is difficult to imagine that genes are transcribed on large polygenic transcripts, as seen in animal systems. Consistent

with this hypothesis are the observations that several genes have 5′ cappable transcripts initiating at sites upstream of these genes (Mulligan et al. 1988a, b). Several investigators have reported upstream consensus sequences (Isaac et al. 1985b; Young et al. 1986) similar to the yeast nanonucleotide promoter sequence (Christianson and Rabinowitz 1983), however, the actual functional significance of these sites in transcription initiation has not been tested.

The study of complex cellular processes such as transcription can be facilitated by the analysis of appropriate mutants. One would like to apply this strategy to the study of mitochondrial gene expression in higher plants, however, few mutants in the transcription process are available. During a survey of transcription of the ORF25 reading frame in a number of N lines (Walker et al. 1987), we reported the invariant expression of this reading frame in diverse nuclear backgrounds. Similar results were noted by Kennell et al. (1987), however, they noted that various N cytoplasmic sources can show variation in the expression of this reading frame when there is a recombinant form of the transcription unit present. Subsequently, we have discovered one line B37N, which exhibits a variation in the expression of ORF25 when compared to B73N. However, in contrast to the variants described in Kennell et al. (1987), there do not appear to be any structural differences in the reading frame in these two lines. In this communication, we describe the variant expression of the ORF25 reading frame in the B37N line and discuss the importance of these observations to our understanding of mitochondrial gene expression.

Materials and methods

The maize lines B73N and B37N were obtained from M. Albertsen (Pioneer Hi-Bred International).

mtDNA extraction and labelling

Mitochondrial DNA was isolated (Kemble et al. 1980) with further purification on a neutral CsCl gradient. mtDNA samples were restricted with appropriate enzymes, electrophoresed on 0.8% agarose gels, and blotted (Southern 1975). ^{32}P nick-translated probes were prepared by previously described methods (Maniatis et al. 1982; Rigby et al. 1977). DNA hybridizations were carried out according to Lonsdale et al. 1981.

mtDNA cloning

All mtDNA clones were isolated by shotgun cloning restriction enzyme digested mtDNA from each line into pUC8 and selecting clones with the appropriate inserts by colony hybridization with the ^{32}P-labelled probes (Maniatis et al. 1982).

mtRNA isolation

All mitochondrial RNA samples were isolated as described previously in Abbott and Fauron (1986). All Northern hybridizations were carried out as described in Maniatis et al. (1982).

S1 nuclease analysis

S1 nuclease protection analysis was performed with purified mtRNA samples as described in Maniatis et al. (1982), using the 3.2-kb *Hin*dIII fragment of pH3.2 N as the protector. S1 nuclease products were run on a 0.8% agarose gel with appropriate size markers. The gel was subsequently Southern blotted and hybridized with the 3.2-*Hin*dIII fragment of pH3.2N.

Results

The ORF25 reading frame is an essential feature of the complex mtDNA rearrangement originally described by Dewey et al. (1986) in T-cms mitochondria. In T-cms, ORF25 is cotranscribed with another reading frame, *urf13*, which encodes a 13-kilodalton protein that has subsequently been shown in prokaryotes to confer sensitivity to the toxin produced by *Helminthosporium maydis* race T (Dewey et al. 1988).

Toxin-resistant male-fertile revertants of the T cytoplasm all display recombinations or sequence changes in this chimeric gene region, which alter the transcription unit or the translational reading frames (Abbott and Fauron 1986; Qin et al. 1987; Wise et al. 1987). This implies that this reading frame plays a possible role in male sterility, however, the translation of the ORF25 reading frame has not been established.

In order to characterize further the expression of this reading frame, Northern hybridization analysis was used to evaluate the integrity of the transcription unit for this gene in N cytoplasm with T-, S-, and C-restored nuclear backgrounds. In all cases initally reported, there was no variation in transcript sizes for this reading frame in N cytoplasm (Walker et al. 1987). In contrast, clear changes in the expression of this transcription unit are evident in restored T cytoplasm (Dewey et al. 1986).

In subsequent experiments with various seed sources, we have discovered that the inbred B37N exhibits variant expression of the ORF25 reading frame manifested as a single transcript of 3,400 nucleotides, rather than the major 2,300-nucleotide transcript seen in all other lines examined and exemplified by B73N cytoplasm in Fig. 1. Two hypotheses can be proposed to explain this data. (1) During the generation of the B73N, a recombination or sequence change in the ORF25 transcription unit has occurred altering the appropriate transcription signals. (2) The expression of this reading frame is dependent on the nuclear background associated with the B37N or B73N cytoplasm.

To test the first hypothesis, it was necessary to know on which side, 5′ or 3′, of the reading frame the transcripts differed between these cytoplasms. For this reason, a comparative analysis of the ORF25 transcript structure in B73N and B37N was performed using Northern hybridization and S1 nuclease digestion tech-

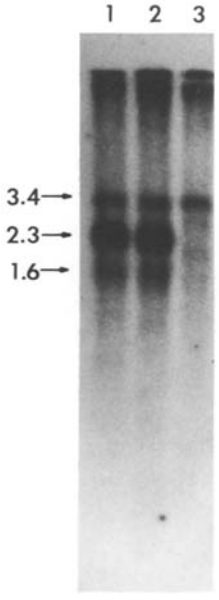

Fig. 1. Northern hybridization analysis of mtRNA samples from: lane *1*: B73N root tissue; lane *2*: B73N coleoptile tissue; lane *3*: B37N coleoptile tissue. The probe was the 3.2-kb *Hin*dIII fragment of pH3.2N (a pUC8 clone of B73N mtDNA). Note that B37N mtRNA lacks the 2.3 and 1.6 kb transcripts

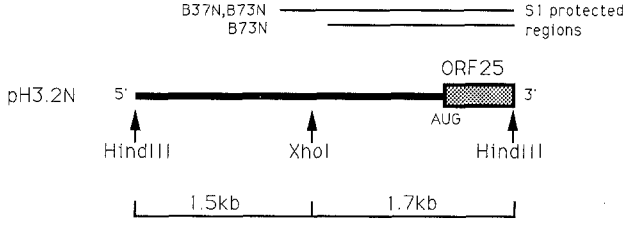

Fig. 2. A restriction map of the 3.2-kb *Hin*dIII fragment of pH3.2N showing the reading frame position and the transcript homologies in this region

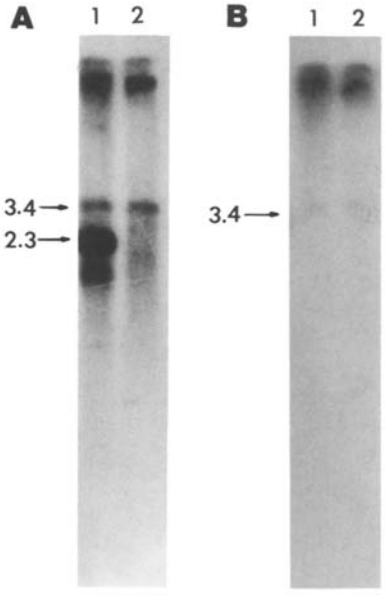

Fig. 3. A and B. Northern blot hybridization of mtRNA from: lane *1*: B73N coleoptile tissue; lane *2*: B37N coleoptile tissue. The probe was the 1.7-kb *Xho*I fragment of pH3.2N. **B** Identical blot to that of panel **A**, however the hybridization probe in this case was the 1.5-kb *Xho*I fragment of pH3.2N. Note that the 1.7-kb *Xho*I fragment contains homology to all transcripts, however the 1.5-kb *Xho*I fragment is homologous only to the 3.4-kb transcript

niques. Data from these analysis should locate putative mtDNA structural differences, if they exist.

Northern hybridization analysis of mtRNA from these lines was done using probes specific for portions of the 5′ adjacent sequences and the ORF25 reading frame. The probes were derived from clone pH3.2N (Walker et al. 1987), which contains a 3.2-kb *Hin*dIII fragment of B73N mtDNA carrying the 5′ half of ORF25 and 2,700 nucleotides of 5′ flanking sequence (Fig. 2). These probes detected a precursor transcript 3,400 nucleotides in length (Fig. 3), with a putative processing or alternate initiation site within 1,200 nucleotides of the 5′ end of the ORF25 reading frame. This is deduced from the fact that a 1.7-kb *Xho*I fragment of pH3.2N hybridizes with the 3,400 and 2,300 nucleotide transcripts in B73N, but the remaining 1.5 kb, 5′ to the *Xho*I site, has homology only to the 3,400 base transcript. The single 3,400 base transcript in B37N appears to be identical in size to the putative precursor transcript in B73N.

To determine the degree of similarity of the 3,400 base transcripts in both lines, an S1 nuclease protection analysis was done using mtRNA of both lines and the 3.2-kb *Hin*dIII fragment of pH3.2N as the protector. S1 nuclease products were electrophoresed on a 1% agarose gel, Southern blotted, and hybridized with a ^{32}P-labelled 3.2-kb *Hin*dIII fragment (Fig. 4). In both lines there is a fragment of 2,200 bases protected. In the B73 mtRNA there is also a fragment of 1,100 bases representing the 5′ protected region of the 2,300 base transcript. From this analysis, the 5′ end of the 2,300 base transcript of B73N and the 5′ end of the 3,400 base transcript seen in both cytoplasms are positioned 1,100 and 2,200 nucleotides, respectively, from the internal *Hin*dIII site in ORF25. The difference in the number of bases between the 3,400 and the 2,300 base transcript is equal to the difference in bases for the protected fragments in the S1 analysis. This suggests that structural differences in the ORF25 transcription units, if they exist in these two lines, must be in those sequences that flank the gene on the 5′ side.

To examine the organization of the 5′ flanking regions for the ORF25 reading frame, a Southern hybridization analysis was done on *Ava*I restriction-digest-

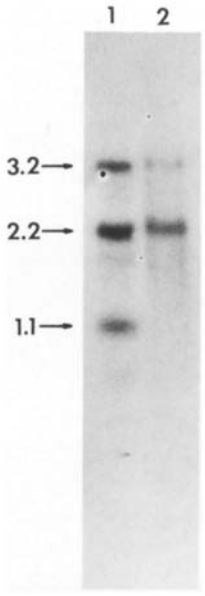

Fig. 4. Southern blot hybridization of an S1 nuclease protection analysis of those transcripts homologous to the 3.2-kb *Hin*dIII fragment of pH3.2N in mtRNA samples from: lane *1*: B73N coleoptiles; lane *2*: B37N coleoptiles. The S1 products were run on a 0.8% agarose gel, blotted, and hybridized with the 3.2 kb *Hin*dIII fragment of pH3.2N

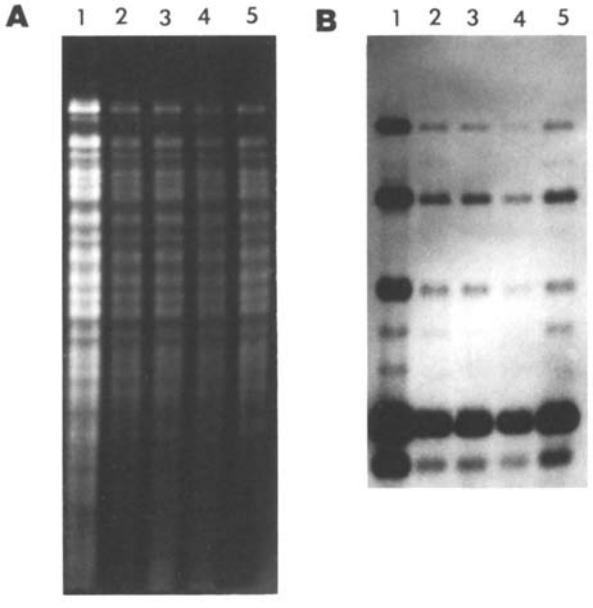

Fig. 5. A and B. 0.8% agarose gel of *Ava*I restriction-digested mtDNAs from: lane *1*: B37N root tissue; lane *2*: B37N coleoptile tissue; lane *3*: a different isolate of B37N root tissue; lane *4*: B37N coleoptile tissue from isolate used in lane *3*; lane *5*: B73N coleoptile tissue. **B** Southern blot hybridization of the gel in panel **A** using the 3.2-kb *Hin*dIII fragment of pH3.2N as a probe. Note that there are no detectable fragment differences in this region, regardless of the source of DNA

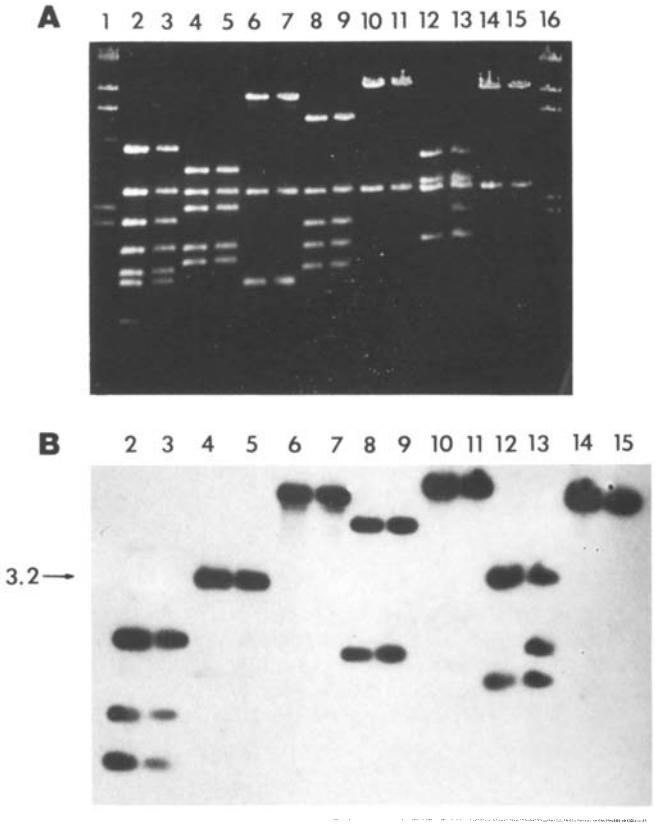

Fig. 6. A and B. A 0.8% agarose gel of restriction enzyme digestions of the two *Bam*HI clones, pB10N(B73N) and pB10NR(B37N). Lanes *1* and *16*: *Hin*dIII fragments of lambda phage DNA; lanes *2, 4, 6, 8, 10, 12,* and *14*: pB10N; lanes *3, 5, 7, 9, 11, 13,* and *15*: pB10NR; lanes *2* and *3*: *Ava*I and *Bam*HI; lanes *4* and *5*: *Hin*dIII and *Bam*HI; lanes *6* and *7*: *Pst*I and *Bam*HI; lanes *8* and *9*: *Xho*I and *Bam*HI; lanes *10* and *11*: *Bam*HI; lanes *12* and *13*: *Eco*RI and *Bam*HI; lanes *14* and *15*: *Sal*I and *Bam*HI. **B** A Southern blot hybridization of the gel depicted in panel **A.** The probe was the 3.2-kb *Hin*dIII insert of pH3.2N. The position of the 3.2-kb *Hin*dIII fragment in each of these clones is denoted by the *arrow*

ed mtDNA from B37N and B73N, using the 3.2-kb *Hin*dIII fragment of pH3.2N as a probe (Fig. 5). From the lack of detectable restriction fragment differences between these lines, it is evident that no major rearrangements in this area have occurred.

For more extensive restriction analysis of this region in the two lines, *Bam*HI clones carrying the ORF25 gene were obtained from pUC8 shotgun libraries, using the insert of pH3.2N as a probe. In both lines the ORF25 sequences are carried on a *Bam*HI fragment of approximately 10 kb. Clones carrying this fragment were obtained for each line (denoted pB10N for B73N and pB10NR for B37N), and these were used in a comparative restriction enzyme analysis using seven different restriction enzymes (Fig. 6). To examine those specific fragments carrying the 5′ flanking sequences of the ORF25 reading frame in each case, a Southern blot of the gel in

Fig. 7. A 1.2% agarose gel of *Sau*3A-digested inserts of the M13 subcloned 3.2-kb *Hin*dIII fragments from the *Bam*HI clones pB10N(B73N) and pB10NR(B37N). Note that no differences in fragment pattern are detectable

Fig. 6A was hybridized with ^{32}P-labelled insert of pH3.2N (Fig. 6B). In all but one case the fragment patterns were identical. In the exceptional case, lane 13, the 2-kb unique *Eco*RI/*Bam*HI fragment was found to be a partial digestion product and did not represent an actual difference. No major mtDNA organizational differences appear to exist between B37N and B73N for the region surrounding and including the ORF25 reading frame. This analysis does not rule out the possible existence of small sequence differences in the 5′ flanking regions. To examine this possibility, the 3.2-kb *Hin*dIII fragment from each *Bam*HI clone was subcloned in M13 and the inserts were isolated, digested with *Sau*3A, and the products were compared by agarose gel electrophoresis. As before, there appear to be no detectable differences in the 5′ flanking sequences of the ORF25 region in these two lines (Fig. 7).

Discussion

Expression of the complex chimeric TURF 2H3 region of T-cms varies with nuclear background. There is no evidence to suggest that nuclear background can influence expression of the individual sequence components of this region in N cytoplasm. Variation in the expression of the ORF25 region has been noted in several N cytoplasms, however, in these cases the variation was due to changes in the transcription unit structure (Kennel et al. 1987). In this communiction, a comparative analysis of ORF25 expression in the two lines B37N and B73N is presented. Expression of this reading frame differs in these two lines, as illustrated by Northern hybridization analysis of mtRNA samples using probes specific for the reading frame and 5′ flanking sequences. In B37N mtRNA there is a single transcript of 3,400 nucleotides, in contrast to three transcripts of 3,400, 2,300, and 1,600 nucleotides found in B73N. S1 nuclease analysis of the 5′ half of the ORF25 homologous transcripts reveals that in both lines a fragment of 2,200 bases is protected and in B73N a unique fragment of 1,100 bases is protected. The difference in the size of the protected fragements in B73N is equal to the difference in size of the 3,400 and 2,300 base transcripts, suggesting that these transcripts have different 5′ ends but that the 3′ ends are similar. This latter needs verification.

We have compared the structural organization of the 5′ flanking regions of ORF25 in these two lines using restriction enzyme digestions and Southern hybridizations. In no case could we detect any major differences in the mtDNA structure in the 5′ flanking sequences of ORF25 in these lines. If differences exist, then they must involve a few bases.

It is possible that the differences in nuclear genetics could be responsible for this mitochondrial transcriptional variation. Nuclear background has not been demonstrated to affect transcription of this mtDNA region in N cytoplasm, however, in T cytoplasm the presence of restorer genes does alter transcript patterns for the TURF 2H3 region of which ORF25 is a component. To examine possible influences of nuclear backgrounds on ORF25 transcription in B37N and B73N, reciprocal crosses have been done and transcription analysis of the resultant progeny are presented part 2 of this study.

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References

Abbott AG, Fauron CMR (1986) Structural alterations in a transcribed region of the T-type cytoplasmic male-sterile maize mitochondrial genome. Curr Genet 10:777–783

Bland MM, Levings III CS, Matzinger DF (1986) The tobacco mitochondrial ATPase subunit 9 gene is closely linked to an open-reading frame for a ribosomal protein. Mol Gen Genet 204:8–16

Braun CJ, Levings III CS (1985) Nucleotide sequence of the F_1-ATPase a-subunit gene from maize mitochondria. Plant Physiol 79:571–577

Christianson TW, Rabinowitz M (1983) Identification of multiple transcriptional initiation sites on the yeast mitochondrial

genome by in vitro capping with guanyltransferase. J Biol Chem 258:14025–14033

Dawson AJ, Jones VP, Leaver CJ (1984) The apocytochrome b gene in maize mitochondria does not contain introns and is preceded by a potential ribosome-binding site. EMBO J 3:2107–2113

Dewey RE, Levings III CS, Timothy DH (1985a) Nucleotide sequence of ATPase subunit 6 gene of maize mitochondria. Plant Physiol 79:914–919

Dewey RE, Schuster AM, Levings III CS, Timothy DH (1985b) Nucleotide sequence of F_0-ATPase proteolipid (subunit 9) gene of maize mitochondria. Proc Natl Acad Sci USA 82:1015–1019

Dewey RE, Siedow JN, Timothy DJ, Levings III CS (1988) A 13-kilodalton maize mitochondrial protein in *E. coli* confers sensitivity to *Bipolaris maydis* toxin. Science 239:293–295

Fox TD, Leaver CJ (1981) The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. Cell 26:315–323

Heisel R, Brennicke A (1985) Overlapping reading frames in *Oenothera* mitochondria. FEBS Lett 193:164–168

Heisel R, Schobel W, Schuster W, Brennicke A (1987) The cytochrome oxidase subunit I and subunit III genes in *Oenothera* mitochondria are transcribed from identical promotor sequences. EMBO J 6:29–34

Issac PG, Brennicke A, Dunbar SM, Leaver CJ (1985a) The mitochondrial genome of fertile maize (*Zea mays*) contains two copies of the gene encoding the a-subunit of the F_1-ATPase. Curr Genet 10:321–328

Issac PG, Jones VP, Leaver CJ (1985b) The maize cytochrome c oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male-sterile plants. EMBO J 4:1617–1623

Kemble RJ, Gunn RE, Flavell RB (1980) Classification of normal and male-sterile cytoplasms in maize. II. Electrophoretic analysis of DNA species in mitochondria. Genetics 95:451–458

Kennell JC, Wise RP, Pring DR (1987) Influence of nuclear background on transcription of a maize mitochondrial region associated with Texas male sterile cytoplasm. Mol Gen Genet 210:399–406

Lonsdale DM, Thompson RD, Hodge TP (1981) The integrated forms of the S1 and S2 DNA elements of maize male-sterile mitochondrial DNA are flanked by a large repeated sequence. Nucleic Acids Res 9:3657–3669

Lonsdale DM, Hodge TP, Fauron CM-R (1984) The physical map and organization of the mitochondrial genome from fertile cytoplasm of maize. Nucleic Acids Res 12:9249–9261

Maniatis T, Fritsch EF, Sambrook J (1982) In: Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY

Mulligan RM, Maloney AP, Walbot V (1988a) RNA processing and multiple transcription initiation sites result in transcript size heterogeneity in maize mitochondria. Mol Gen Genet 211:373–380

Mulligan RM, Lau GT, Walbot V (1988b) Numerous transcription initiation sites exist for the maize mitochondrial genes for subunit 9 of the ATP synthase and subunit 3 of cytochrome oxidase. Proc Natl Acad Sci USA 85:7998–8002

Palmer JD, Shields CR (1984) Tripartite structure of the *Brassica campestris* mitochondrial genome. Nature 307:437–440

Quin J, Fauron CM-R, Brettell RIS, Milhous M, Abbott AG (1987) Toxin resistance and/or male fertility reversion is correlated with defined transcription changes in the 1.5-kb *Aval* region of cms T. Nucleic Acids Res 15:6091–6103

Quetier F, Lejeune B, Delorme S, Falconet D (1985) Molecular organization and expression of the mitochondrial genome of higher plants. In: Douce R, Day DA (eds) Encyclopedia of plant physiology. Springer, Berlin, pp 25–36

Rigby PWJ, Diekmann M, Rodes C, Berg P (1977) Labelling DNA to high specific activity by nick translation with DNA polymerase I. J Mol Biol 113:237–251

Schuster W, Brennicke A (1987) Plastid, nuclear and reverse transcriptase sequences in the mitochondrial genome of *Oenothera*: is genetic information transferred between organelles via RNA? EMBO J 6:2857–2863

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Stern DB, Bang AG, Thompson WF (1986) The watermelon mitochondrial URF-1 gene: evidence for a complex structure. Curr Genet 10:857–869

Wahleithner JA, Wolstenholme DR (1988) Ribosomal protein S14 in borad bean mitochondrial DNA. Nucleic Acids Res 16:6897–6913

Walker NH, Qin J, Abbott AG (1987) Northern hybridization analysis of mitochondrial gene expression in maize cytoplasm with varied nuclear backgrounds. Theor Appl Genet 74:531–537

Ward BL, Anderson RS, Wolstenholme DR (1981) The mitochondrial genome is large and variable in a family of plants (Cucurbitaceae). Cell 25:793–803

Wissinger B, Heisel R, Schuster W, Brennicke A (1988) The NADH-dehydrogenase subunit 5 gene in *Oenothera* mitochondria contains two introns and is co-transcribed with the 5S rRNA gene. Mol Gen Genet 212:56–65

Wise RP, Pring DR, Gengenbach BG (1987) Mutation to fertility and toxin insensitivity in Texas (T) cytoplasm maize is associated with a frameshift in a mitochondrial open-reading frame. Proc. Natl Acad Sci USA 84:2858–2862

Young EG, Hanson MR, Dierks PM (1986) Sequence and transcription of the Petunia gene for the ATP synthase proteolipid subunit. Nucleic Acids Res 14:7995–8006