Theor Appl Genet (1987) 74:531–537

Northern hybridization analysis of mitochondrial gene expression in maize cytoplasm with varied nuclear backgrounds

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Received March 23, 1987; Accepted April 2, 1987
Communicated by R. Hagemann

Summary. Type T cytoplasmic male sterility in *Zea mays* is associated with mitochondrial RNA (mtRNA) coding sequences found on a 1.5 kb AvaI mitochondrial DNA (mtDNA) fragment not found in other cytoplasms (N, C, or S) (Abbott and Fauron 1986). Three probes (pH 3.2N, pH 2.7N, and ORF 13) specific to different parts of the 1.5 AvaI T region were used in a Northern blot analysis of N mtDNAs from lines with diverse nuclear backgrounds (Rf_1, Rf_2 included). The N mtDNA clone pH 3.2N shows homology with the right-hand boundary of the 1.5 AvaI T region and includes a portion of an open reading frame (ORF 25). Southern blots of AvaI and BamHI digestions of N, T, S, and C mtDNA, probed with pH 3.2N demonstrate that sequences in or adjacent to this region are highly active in recombination. The clone pH 2.7N is homologous to an untranscribed region of ATPase 6 and the structural gene; and ORF 13 is a portion of the 1.5 AvaI region derived predominantly from 26S 3′ flanking sequences. pH 3.2N and 1.5 AvaI sequences showed identical hybridization patterns on Northern blots of N, T, Trev and Tres mtRNAs. Transcript sizes of mtRNAs homologous to the pH 3.2N probe in all of these lines were different, however, there was no variation in transcript sizes when pH 2.7N was used as a probe. Northern blots of mtRNA from N cytoplasms with various nuclear restored backgrounds showed no difference in expression when probed with pH 3.2N or pH 2.7N; however, transcripts homologous to an ORF 13 specific probe can be detected in N cytoplasm with a particular nuclear background. This may suggest activity of nuclear restorer alleles in N cytoplasm.

Key words: CMS – Plant – Mitochondria – Genes – Expression

Introduction

Studies on mitochondrial gene function in higher plants are still rather preliminary. This is for several reasons: higher plant mitochondrial genomes are extremely variable in size, easily manipulatable cell culture systems are just becoming available for most plant species, and most importantly appropriate mitochondrial gene mutants are not available.

Cytoplasmically inherited male sterility (cms) is one mutant phenotype in higher plants which has been investigated extensively at the molecular level due to the agronomic importance of the cms plant. Correlative evidence in *Zea mays* suggests that the cms plant has one or more changes in mitochondrial genome (mtDNA) organization which uniquely disrupt normal pollen development generating the male sterile phenotype. Previous data supporting this model are:

1. Comparative agarose gel electrophoresis of restricted and unrestricted mtDNA from all maize cytoplasms (N, T, S, C) shows distinct differences in sequence organization (Pring et al. 1977; Pring and Levings 1978; Thompson et al. 1980; Kemble and Bedbrook 1980; Kemble et al. 1980; Lonsdale et al. 1981).
2. Chloroplast DNA structure as judged by restriction enzyme analysis is invariant among all cytoplasms except for one difference in the S cytoplasm (Pring et al. 1977).
3. T cytoplasm is uniquely sensitive to the fungal toxin produced by *Helminthosporium maydis* (race T) and

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evidence suggests the target of toxin action is the mitochondria (Hooker et al. 1970; Lim and Hooker 1972; Laughnan and Gabay 1973; Arntzen et al. 1973; Gengenbach et al. 1973; Gengenbach et al. 1973).
4. Mitochondrial ultrastructure appears altered in tapetal and pollen cells prior to pollen abortion in cmsT anthers (Warmke and Lee 1977).
5. In vitro translated mitochondrial protein profiles are different for all cytoplasms studied (Forde et al. 1978, 1980; Forde and Lever 1980).
6. Recent evidence suggests unique gene transcipts and gene rearrangements in T cytoplasm may be responsible for the previously delineated differences in mitochondrial protein profiles (Abbott and Fauron 1986; Dewey et al. 1986).

Genetically, the various male sterile cytoplasms are distinguishable on the basis of nuclear inherited, fertility restoration. Each cytoplasm is restored to fertility by unique dominant nuclear restorers (Buchert 1961; Duvick et al. 1961; Beckett 1971). In the case of cmsT there are two restorer alleles designated Rf_1 and Rf_2. S and C cytoplasms are each restored by one major restorer allele designated Rf_3 and Rf_4, respectively (Laughnan and Gabay 1973, 1978; Kheyr-Pour et al. 1981).

Until recently restorer activity was uncharacterized at the molecular level. Dewey et al. (1986) have shown that restored T lines show alterations in transcript sizes encoded in a rearranged portion of the T mitochondrial DNA (designated TURF 2H3). Similiar data from our laboratory has demonstrated that the phenomenon of cytoplasmic fertility reversion is correlated with rearrangements redefining the reading frames in this region (Abbott and Fauron 1986). Data from these studies suggests that T restorers may be processing enzymes.

Alteration of TURF 2H3 encoded mitochondrial transcript sizes in T restored lines raises two questions central to our conception of restorer action. 1. In general, how much influence does nuclear background have on transcript sizes in T as well as other cytoplasms? 2. Do nuclear restorer alleles alter processing of the unique coding construct in T mtDNA or do they affect transcript sizes of homologous N gene regions in N lines with T restorers? The second possibility may not be recognized since there may not be a measurable phenotypic change in N. In the following communication we demonstrate that N mtDNA transcripts homologous to the unique gene construct in T mtDNA described by Abbott and Fauron (1986) and Dewey et al. (1986) are not affected by variation in nuclear background (including the presence of Rf_1, Rf_2). This suggests that nuclear restorer alleles affect only the unique sequence construction in T mtDNA and is consistent with genetic data on the specificity of restorer action in the different cytoplasms.

Materials and methods

mtDNA extraction and labelling

Mitochondrial DNA was isolated by method of Kemble et al. 1980 with further purification on a neutral cesium gradient.

Mitochondrial DNA 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).

All DNA:DNA hybridizations were carried out according to Lonsdale et al. 1981.

DNA:RNA hybridizations were done at 65 °C overnight in 5×SSC, 0.1% polyvinylpyrollidone, 0.5% SDS. They were rinsed 4 times in 1,000 ml of 2×SSC at 65 °C for 15 min each, followed by a final rinse in 0.3×SSC at 65 °C for 15 min. Filters were dried and autoradiographed.

mtRNA extraction and labelling

Mitochondrial pellets for RNA extraction were prepared from 80 grams (fresh wt.) coleoptiles or whole plants (Kemble et al. 1980). The final mitochondrial pellet was resuspended in 3 ml of 4 M guanidium isothiocyanate mixture and RNA extracted (Maniatis et al. 1982). RNA samples were electrophoresed on 1.5% formaldehyde agarose gels and blotted (Maniatis et al. 1982).

Results

The 1.5 kb AvaI fragment of cmsT maize mtDNA is homologous with several uniquely sized transcripts in T mitochondria (Abbott and Fauron 1986). This region contains a unique open reading frame (ORF 13) and a portion of an open reading frame (ORF 25) found in all cytoplasms. ORF 13 and ORF 25 are capable of encoding a 13 kd and 25 kd protein respectively (Dewey et al. 1986).

Hybridization of this 1.5 kb probe onto a Northern blot of various mtRNAs shows that expression of this region is quite different in N, T, and T revertant (Trev) cytoplasms (Abbott and Fauron 1986). Similar experiments on T cytoplasm in restored (Rf_1 Rf_2) backgrounds show further changes in the size of transcripts homologous with this 1.5 kb AvaI region of T mtDNA (Fig. 1A). Similar results on T restored (Tres) cytoplasm have been published recently by Dewey et al. (1986).

In view of the evidence above, several possibilities for restorer action are apparent. Dominant nuclear restorer alleles may only modify transcripts from the unique construct in T cytoplasm defined on the 1.5 kb AvaI fragment or they may affect transcription of homologous gene regions in other cytoplasms as well. The second possibility may not be observable in other cytoplasms since there is no recognizable change in phenotype. We decided to look for restorer activity on the coding sequences defined by the 1.5 kb AvaI region of T, in N lines having T, C, and S restored nuclear

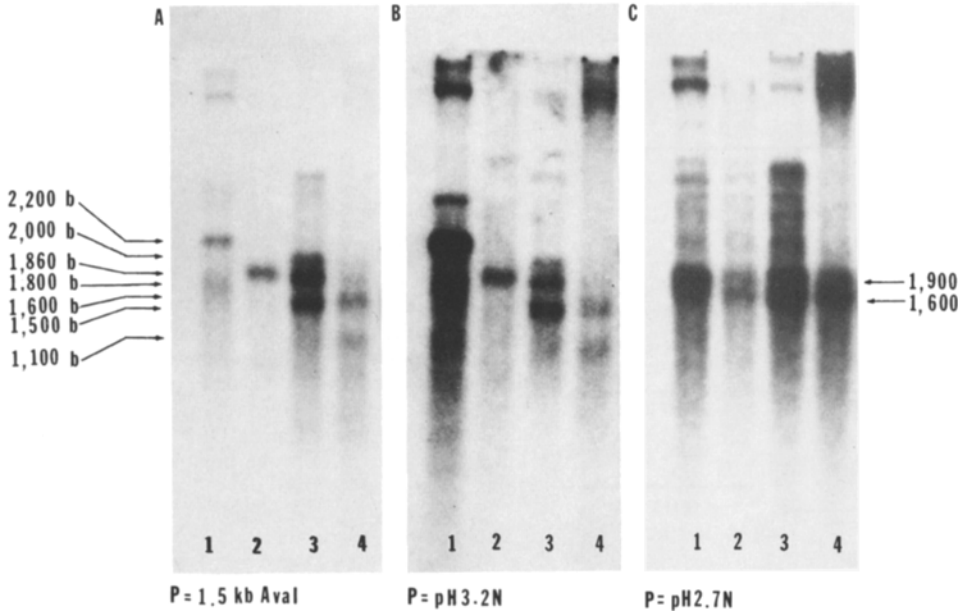

Fig. 1 A–C. Northern blot hybridizations of *lane 1,* N; *lane 2,* T revertant; *lane 3,* T; and *lane 4,* T restored ($Rf_1 Rf_2$) mtRNA. **A** The probe utilized was the 1.5 kb AvaI fragment from pB8.5T; **B** The probe utilized was pH3.2N; **C** The probe utilized was pH2.7N

backgrounds. Since the 1.5 region in T is complex in its coding capacity, we chose to obtain N mtDNA clones carrying the individual gene constituents homologous to the 1.5 region of T for this analysis. We also utilized an ORF 13 specific fragment obtained from a clone which carried a portion of the 1.5 region.

The 1.5 kb AvaI region in cmsT has major homology to two HindIII fragments in N cytoplasm each carrying a coding sequence

In order to obtain clones of the gene regions in N cytoplasm homologous to the 1.5 AvaI T region, a pUC8 HindIII clone bank of N mtDNA was hybridized with the cloned 1.5 AvaI fragment of cmsT. A number of clones were identified of which two were chosen for further study. They are denoted pH3.2N and pH2.7N with inserts of 3.2 and 2.7 kb, respectively. Restriction mapping and Southern hybridization of these N clones onto the AvaI 1.5 region of T have revealed the extent of their sequence homology to this T region (Fig. 2, blots not shown). The 2.7 clone shares sequences with the left hand boundary of the 1.5 kb AvaI fragment and the 3.2 clone shares homology with the right hand boundary. Sequencing data of the 3.2 clone and the T region reveal that ORF 25 sequences are shared in common (Abbott, unpublished results), and that the 2.7 clone homology is an untranscribed upstream region of ATPase 6 (Dewey et al. 1986).

pH 3.2 N hybridized to all the novel transcripts homologous to the 1.5 T region

In order to examine the RNA coding potential of these N clones, Northern blots of N, Trev, T, and Tres mtDNA were hybridized with the pH 3.2 N and pH 2.7 N probes (Fig. 1 B, C). The pH 2.7 N probe hybridizes with two major transcripts of 1900 b and 1600 b and a number of minor transcripts. There are no differences in expression of this gene region among the cytoplasms studied. Recent sequencing data by Dewey et al. (1986) and Southern analysis of the 6.6 kb XhoI region with pH 2.7 N as a probe have revealed that this N HindIII fragment carries the ATPase 6 gene (Abbott, unpublished results).

The pH 3.2 N probe showed Northern hybridization identical to the 1.5 kb AvaI fragment, suggesting that the coding region present on the 3.2 kb HindIII fragment of N is involved in the recombination creating the 1.5 kb region in T. This data also demonstrates that the 3.2 kb HindIII region of N mitochondrial DNA does not carry any other transcribed sequences but those homologous to a single major transcript in N at the 2,200 base size. Sequencing of this clone reveals that it has a 5′ portion of the ORF 25 sequences as well as the flanking sequences (Abbott, unpublished data). Southern blot analysis of AvaI digestions of cmsT and N mtDNA using pH 3.2 N as a probe demonstrate that this N region is involved in recombination creating the

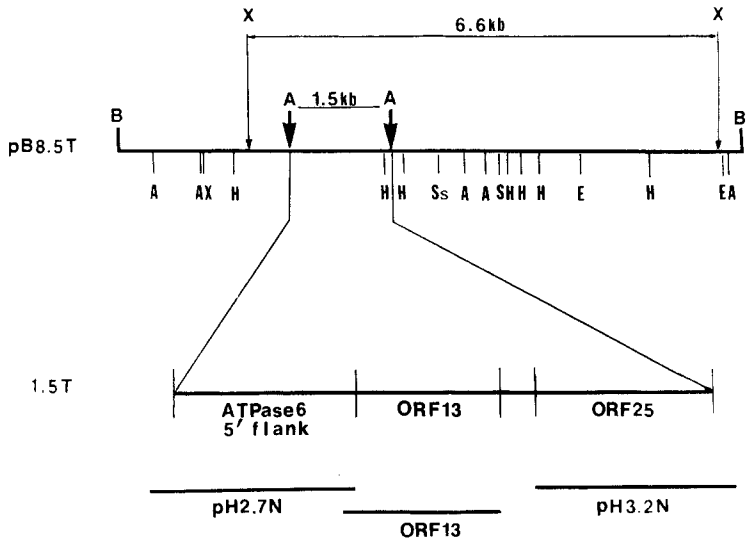

Fig. 2. Restriction map of the cmsT 8.5 kb BamHI fragment. *Small arrows* denote the 6.6 kb XhoI fragment and *large arrows* denote the 1.5 kb AvaI fragment. Expanded area shows ATPase 6 5′ flanking sequences, and reading frames ORF 13 and ORF 25. Lines depict extent of probe homologies to these regions

TURF 2H3 region in cmsT (Fig. 3). In N the 3.2 homologous sequences are found on a 2.1 kb AvaI fragment and in T they are found on a 1.7 kb AvaI fragment with limited homology to the 1.5 AvaI region (Fig. 3). Interestingly, the BamHI restriction profiles of all maize cytoplasms show rearrangements in sequences surrounding this region. This suggests that this area of the genome is active in intermolecular or intramolecular recombination. Since the sequences on pH 3.2 N have homology to all mitochondrial transcripts identified by the 1.5 AvaI probe of T, we decided to examine transcripts from this coding region for restorer modifications in N cytoplasms with nuclear restorers for T, C, and S cytoplasm. Transcripts homologous to pH 2.7 N were also examined for any size variation in N in order to estimate the overall influence of varied nuclear background on mitochondrial transcript size.

Major transcripts homologous to pH 3.2 N and pH 2.7 N show no size variation in N cytoplasm with nuclear restored backgrounds

To examine the possibility of nuclear restorer action on gene transcripts in N cytoplasm from the 3.2 and 2.7 regions, which may not result in an observable phenotypic alteration, several N lines were studied by Northern blot analysis using the 1.5 AvaI T fragment, pH 3.2 N, and pH 2.7 N as probes (Figs. 4A, B and 5B). The N lines examined included two inbreds (B73, OH51), a hybrid (Pioneer Brand 3377), plus inbreds with various combinations of nuclear restorer alleles as follows: one restorer (rf_1Rf_2) for T (A632); restorers ($Rf_1Rf_2rf_3Rf_4$) for T and C (F5DD1); and restorers ($rf_1Rf_2Rf_3Rf_4$) for C and S (CE1).

If transcript size in N cytoplasm varies with nuclear background, then dominant nuclear restorer alleles are

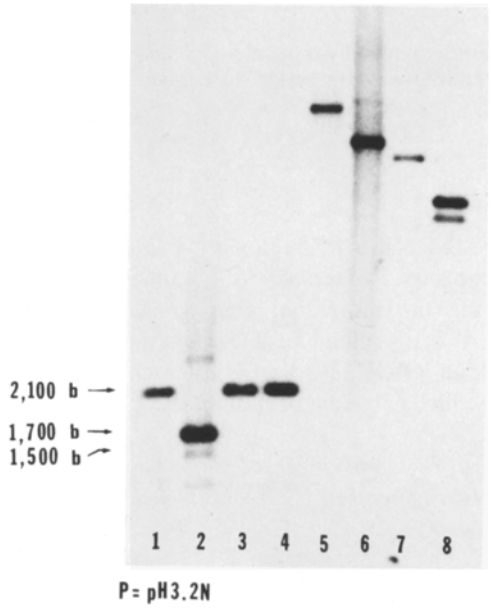

Fig. 3. Southern blot hybridization of AvaI (*lanes 1–4*) and BamHI (*lanes 5–8*) digested mtDNA from *lanes 1 and 5*, B73N; *lanes 2 and 6*, B73T; *lanes 3 and 7*, B73C; and *lanes 4 and 8*, B73S, probed with the 3.2 region of N mtDNA. *Arrows* denote important restriction fragment differences

ubiquitous in their action. If this is not the case, activity of nuclear restorers for the gene regions examined would appear to affect only transcripts from the unique 1.5 kb construct in T cytoplasm.

The pH 2.7 N probe showed identical Northern hybridization in all N lines (Fig. 5B). Since pH 2.7 N showed no differences in expression in N, Trev, T, and Tres, no differences were expected among the N lines.

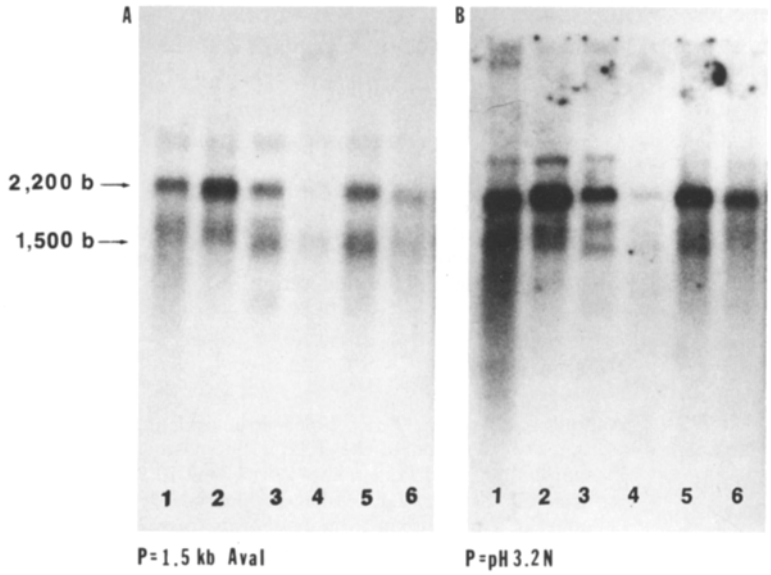

Fig. 4A, B. Northern blot hybridizations of mtRNA from various N lines *lane 1,* B73N; *lane 2,* 3377 (a hybrid); *lane 3,* A632 (Rf_1, Rf_2); *lane 4,* OH51; *lane 5,* F5DDI (Rf_1, Rf_2, Rf_4); *lane 6,* CE1 (Rf_3, Rf_4). **A** Probed with 1.5 kb AvaI; **B** Probed with pH3.2N

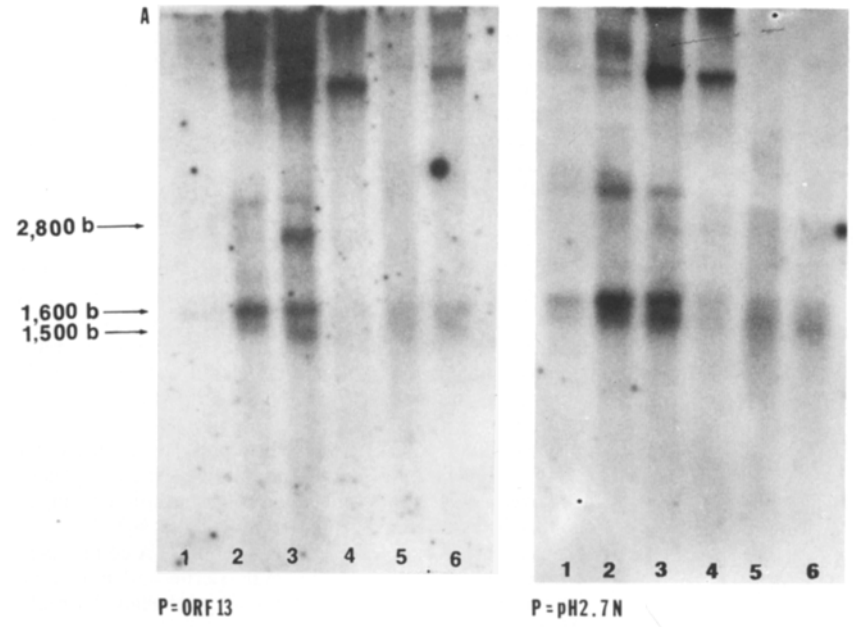

Fig. 5A, B. Northern blot hybridizations of mtRNA from *lane 1,* B73N; *lane 2,* 3377 (a hybrid); *lane 3,* A632 (Rf_1, Rf_2); *lane 4,* OH51; *lane 5,* F5DDI (Rf_1, Rf_2, Rf_4); *lane 6,* CE1 (Rf_3, Rf_4). **A** Probed with ORF 13; **B** Probed with pH2.7N

This suggests that nuclear background may not alter transcription in a general fashion.

The 1.5 T AvaI and pH 3.2 N probes show identical hybridization patterns by Northern blot analysis of N lines (Fig. 4A, B). There are no differences in major homologies. This confirms that expression of this region in N cytoplasm is not affected by the presence of nuclear restorer alleles and that activity of these alleles appears to be specific for transcripts of the unique 1.5 kb construct in T cytoplasm. Long exposure of the Northern blots probed with the pH 3.2 N and the 1.5 AvaI region of T reveals minor homologies which appear to differ in size among the lines examined. We are currently investigating the origin of these transcripts.

MtRNA transcripts homologous to ORF 13 can be detected in N cytoplasm with a particular nuclear background

Experiments presented above demonstrate little variation in expression of N mitochondrial genes in different nuclear backgrounds. Surprisingly, we were able to detect some variation in expression of mtDNA when an ORF 13 specific probe was used on Northern blots

of the various N lines (Fig. 5 A). Since the probe also contained 300 bp of ATPase 6.5′ flanking sequences, an identical blot was hybridized with the ATPase sequences as a control (Fig. 5 b). Comparing these autoradiograms two novel transcripts at 2,800 b and 1,500 b sizes are present uniquely in the A632 line. We are currently investigating the molecular nature of these transcripts. This is the first evidence for the transcription of ORF 13 homologous sequences in N cytoplasm.

Discussion

Studies of nuclear restoration phenomenon at the molecular level have been hampered by the lack of physiological data describing mitochondrial functions during pollen development. The suggestion that the nuclear restorer alleles Rf_1, Rf_2 are potentially involved in mitochondrial transcription and/or processing events leads to experiments designed to question the specific actions of these restorer proteins and how these functions relate to restoration of male fertility. Experiments presented in this communication address two important questions involving restorer gene function:

1. Does broad variation in nuclear background influence mitochondrial transcription in a general fashion?
2. Are T restorer alleles able to change the transcription pattern of N cytoplasm for those gene sequences previously shown to be altered in T vs. T restored cytoplasm?

Data from these experiments demonstrates that nuclear background does not affect the mature transcript sizes of ATPase 6 and ORF 25 in the cytoplasms we've examined. This contrasts markedly with our data and data presented by Dewey et al. (1986) for the ORF 25 sequences in T. Dewey et al. (1986) have shown that in (Rf_1Rf_2) T lines, the variation in transcript sizes in Turf 2H3 are most likely due to changes in the ORF 13 portion of the transcript from this region. Taken together these data suggest that the unique transcription unit in T cytoplasm defined by the ORF 13 and ORF 25 reading frames is uniquely susceptible to restorer modulation. Under this hypothesis it is not surprising that ORF 25 transcripts in N cytoplasm show no change in transcript size when T restorer alleles are present.

ORF 13 RNA sequences appear to be involved in the novel mitochondrial RNA processing present in T restored cytoplasm and these sequences are derived from the flanking and coding sequences of the 26S mitochondrial ribosomal gene. This suggests a possible role for T restorer alleles as constituents of the ribosomal RNA processing system. For this reason, we examined the various N lines for the presence of transcripts which are homologous to the ORF 13 sequences. No such transcripts had been detected by Dewey et al. (1986). Interestingly, we find transcripts homologous to these sequences in one N line (A632). We are currently examining the molecular nature of this novel transcript.

Acknowledgement. We thank Dr. Mark Albertson of Pioneer Hi-Bred International for graciously providing the seeds and for critically reviewing the manuscript.

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