Toxin resistance and/or male fertility reversion is correlated with defined transcription changes in the 1.5kb *Ava*I region of *cms*T

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ABSTRACT

Reversion of T type cytoplasmic male sterility (*cms*T) to fertility is correlated with sequence changes in a 1.5kb *Ava*I fragment. This 1.5kb *Ava*I fragment is composed of 5' flanking sequences of ATPase subunit 6, one complete open reading frame (ORF 13) and part of the another (ORF 25). The sequence of the 1.5kb *Ava*I fragment was compared to the sequences of homologous regions in the N(male fertile) and T revertant *V*₃ mitochondrial DNA. Sequences were found to diverge between ORF13 and ORF25 coding regions.

To further characterize the transcription of these rearranged sequences, specific probes for ORF 13, ORF 25 and 5' flanking sequences of ATPase 6 were hybridized to Northern blots of N, *cms*T and the T revertant *V*₃ and *V*₁₈ mRNAs. Each revertant has a single ORF 25 homologous transcript in contrast to the multitranscript pattern in *cms*T. ORF 13 homologous transcripts were not detected in either revertant cytoplasm. The loss of ORF 13 and/or altered ORF 25 transcription in the fertile revertants may be responsible for the male fertility and/or toxin resistance in these plants.

INTRODUCTION

Cytoplasmic male sterility (*cms*) is a maternally inherited type of male sterility in which pollen abortion occurs in the anthers, while development of other plant parts is normal. Since cytoplasmic male sterile plants fail to release functional pollen, they need not be hand emasculated for hybrid crosses.

There are three different male sterile cytoplasm in maize designated *cms*S (U.S.D.A.), *cms*C (Charrua), *cms*T (Texas)^{1,2,3} and one male fertile cytoplasm designated N. Each sterile cytoplasm is distinguished by a unique set of nuclear fertility restorer alleles. *Cms*T cytoplasm is restored to fertility in a sporophytic fashion by two dominant restorer alleles *Rf*₁ and *Rf*₂ located on chromosome 3 and 9 respectively.^{4,5} *S* cytoplasm is restored gametophytically by a single dominant allele *Rf*₃ located on chromosome 2^{6,7}. *C* cytoplasm is restored by *Rf*₄ and like *cms*T exhibits a sporophytic restoration pattern.^{8,3} Fertile *cms*T and *cms*S plants can be obtained through changes in the cytoplasmic gene systems.^{9,10,11,12} In *cms*T, fertile cytoplasmic

revertants have been isolated from *H. maydis* toxin-resistant tissue culture selection schemes.^{9,10,11,13} Each cytoplasm has a unique mitochondrial DNA(mtDNA) organization as illustrated by restriction endonuclease fragment analysis.^{14,15,16,17,18,19} Similarly, the cytoplasmic revertants in both cmsT and cmsS have further mtDNA organizational changes.^{20,11} In contrast, chloroplast DNA (ctDNA) structure from the four major cytoplasms (N, cmsT, cmsS, cmsC) appears invariant except for one HindIII fragment in S cytoplasm.¹⁸

T revertant plants have a very similar restriction pattern to cmsT. There is, however, one rearrangement in an XhoI fragment which is found consistently among a large proportion of T revertant lines investigated. This is seen as a size reduction of a 6.6kb XhoI fragment in cmsT to a 6.2kb fragment in the T revertant pattern.^{11,13} This restriction fragment size change is the result of a recombinational event occurring in an internal region of the 6.6kb XhoI fragment of cmsT. The entire 6.6kb XhoI fragment has been cloned as part of a larger 8.5kb Bam HI fragment. Using this clone (pB8.5T) the region of recombination has been delimited to a 1.5kb AvaI fragment in cmsT.^{21,22} The 1.5kb AvaI region of cmsT mtDNA is part of the chimeric transcription unit designated by Dewey *et al.*²³ as TURF 2H3. TURF 2H3 contains portions of the 5' flanking sequences of ATPase 6, one short open reading frame (ORF 13) and a larger open reading frame (ORF 25).²³ Correlated with fertility reversion in one cmsT revertant (V_3) is the loss of the 1.5kb AvaI fragment and appearance of a 2.1kb AvaI fragment.^{21,22} The 2.1kb AvaI region of V_3 mtDNA has extensive homology to a similar sized region in N as evidenced from Southern hybridization analysis. Two possible mechanisms could explain this. Either the toxin selects for amplification of an N-like minor component of the mitochondrial DNA population in cmsT or the toxin selects a chance recombination in this region reestablishing the progenitor N-like region.

Here we present mtDNA sequencing data and Northern blot analysis of revertant mRNAs illustrating that a recombination 5' to the ORF 25 sequences and 3' to the ORF 13 sequences has occurred in the V_3 revertant. This recombination redefines the ORF 25 transcription unit and eliminates ORF 13 transcription.

MATERIALS AND METHODS

All maize lines (B73N, B73T) except fertile cmsT revertant lines A188N/-Wf9T, were obtained from Pioneer Hi-Bred International.

1. MtDNA and RNA extraction

Mitochondrial DNA from dark grown coleoptiles was isolated by the method of Kemble *et al.*¹⁶ with further purification on a neutral cesium gradients.

Fig 1. Restriction map of the cmsT clone pB8.5T showing the gene map of the 1.5kb *Ava*I fragment. The extent of homology of various cloned probes from other cytoplasms is depicted at the bottom of the Figure. Restriction sites: A, *Ava*I; B, *Bam* HI; E, *Eco* RI; H, *Hind*III; S, *Sal*I; Ss, *Sst*II; X, *Xho*I.

For mRNA extraction, the final mitochondrial pellet was resuspended in 3 ml of 4M guanidinium isothiocyanate mixture and RNA extracted by methods outlined of Maniatis *et al.*²⁴

2. Northern blot hybridizations

Mt RNA samples were electrophoresed on 1.5% formaldehyde agarose gels and blotted as outlined in Thomas.²⁵ Northern blots were prehybridized in a solution containing 5X Denhardt's without BSA (0.1% Ficoll and 0.1% Polyvinylpyrrolidone), 5X SSC, 0.5% SDS and 100ug/ml salmon sperm DNA for 1 hour at 65°C. and hybridized overnight with ³²P nick translated DNA.^{24,26} Filters were washed twice for 15 min. at 65°C with 2X SSC twice in 1X SSC and once in 0.3X SSC.

3. Fragment Isolation and Labeling

DNA fragments were separated by electrophoresis on 0.8% agarose gel in TAE buffer (0.04M Tris-acetate, 0.001M EDTA). Specific fragments were isolated by the electroelution method of Maniatis *et al.*²⁴

4. DNA Cloning and Sequencing

M13 cloning was done by the method of Messing *et al.*²⁷ *Sau* 3A fragments were ligated into the *Bam* HI site of M13mpl8 and M13mpl9. The ligation mixture was transfected in JM 101. Cells are plated on soft agar in presence of IPTG and X-gal. The deletion clones for sequencing were isolated using the method of Dale *et al.*²⁸ The primers RD 22 and RD 29 were obtained from I.B.I

Fig 2. Comparison of 2.1kb HindIII region in *cmsT* with the 3.2kb HindIII regions in *N* and *V₃*. The hatched lines are homologous regions between *N* and *V₃*. The dark lines are homologous regions in *N*, *cmsT* and *V₃*. The region from the right HindIII site to the vertical dotted line has been sequenced in each cytoplasm.

(International Biotechnologies, Inc.). The single stranded M13 DNA containing the appropriate insert was prepared from individual plaques by methods outlined in Messing²⁹ and sequenced by the dideoxy-chain termination method.³⁰

The 2.1kb and 3.2kb HindIII fragments of *cmsT* and *V₃* respectively were sequenced by deletion sequencing methods.²⁸ The 2.1kb HindIII *cmsT* fragment has been previously cloned in its entirety on a pUC8 Bam HI clone designated pB8.5T.²¹ The 3.2kb HindIII fragment of *N* was partially digested with Sau3A, and subcloned into M13 mpl8 and mpl9 vectors.²⁹ Overlapping clones homologous to the 2.1kb HindIII fragment of *cmsT* were selected by plaque hybridization and sequenced by chain termination methods.³⁰

RESULTS

The 1.5kb *Ava*I region of *cmsT* is homologous to a 3.2kb HindIII region in both *N* and the *T* revertant *V₃* mtDNA.

The cloning and characterization of the 1.5kb *Ava*I region of *cmsT* has been previously described.²¹ These studies utilized an 8.5kb Bam HI clone (pB8.5T) of *cmsT* mtDNA which contains the 1.5kb *Ava*I region in its entirety (Fig. 1). The 1.5kb *Ava*I region is contained within the TURF 2B region

		5'							
	N	TGTCATGGAA	TCAAGTCTAT	TTGTTTGAAT	GTTCTTTTTT				
	V3	*****	-----TCG-	-*****	*****				
1500	T	GAC*ATCC*	CTCTC****A	GCC*AA*TA*	TCCTCAA*GA				
	N	T-----	GGAAAAACCA	ACCACAAAAA	AAGTCTCCCT				
	V3	*CGTTTCGTT	*****	*****	*****				
1540	T	CCGATAGCGT	AC**GT****	TGAGGG*CC*	*****				
				ORF13 stop					
	N	TTCTCTT---	-----GGAGC	AGAGCT----	-----TCATC				
	V3	*****TTG	-----*****	*****GAT-	-----TGT**T				
1580	T	*****TTG	GGGTG**G**	G****GAAT	AAATTGT**T				
	N	ATA-AAAGTG	GAGAGTCACA	ATGAGATTTA	GTGGAATGGA				
	V3	T**T*****T	T*****T	*****GAT-	*****GAT-				
1620	T	T**T*****T	T*****T	*****GAT-	*****GAT-				
				ORF25 start					
	N	TATGAAGGGT	ATAAATATGC	TATTTGCTGC	TATTCCATCT				
	V3	*****AAA	*G***A****	*****GAT-	*****GAT-				
1660	T	*****AAA	*G***A****	*****GAT-	*****GAT-				
	N	ATTTGTGCAT	CAAGTCCGAA	GAAGATCTCA	ATCTATAATG				
	V3	*****	*****	*****	*****				
1700	T	*****	*****	*****	*****				
	N	AAGAAATGAT	AGTAGCTCGT	TGTTTTATAG	GCTTCTCAT				
	V3	*****	*****	*****	*****				
1740	T	*****	*****	*****	*****				
	N	ATTAAGTCGG	AAGAGTTTAG	GTAAGACTTT	CAAAGAAACT				
	V3	*****	*****	*****	*****				
1780	T	*****	*****	*****	*****				
	N	CTCGACGGGA	GAATCGAGTC	TATTCAGGAA	TCATTGCAGC				
	V3	*****	*****	*****	*****				
1820	T	*****	*****	*****	*****				
	N	AATTCTTCAA	TCCTAACGAA	GTCATTCTGG	AGGAATCCAA				
	V3	*****G***	*****G***	*****G***	*****G***				
1860	T	*****G***	*****G***	*****G***	*****G***				
	N	TGAACAACAA	CGATTACTTA	ATCTACGGAT	CAGCTTGCGA				
	V3	*****	*****	*****A***	*****A***				
1900	T	*****	*****	*****A***	*****A***				
	N	ATTTCGACGA	CCGTAAAAGT	AGTAGAATCA	TTACCAGCGG				
	V3	*****	*****	*****	*****				
1940	T	*****	*****	*****	*****				
	N	CACGCTGTGC	GCCTAAGTGC	GAAAAGACAG	TGCAAGCTT				
	V3	**-----C**	*****	*****	*****				
1980	T	*****C**	*****	*****	*****				

3'

Fig. 3. Nucleotide sequence of the right side of the 2.1kb HindIII region in cmsT and 3.2kb HindIII regions in N and V₃. Line 1, 2, and 3 represent the N, V₃ and T sequences, respectively. Asterisks signify nucleotides identical to the N nucleotides; dashes represent absence of matching nucleotides. Where bases differ from the N sequence the differing bases are depicted. The putative consensus 5' flanking sequence of ORF25 has been enclosed in a box.

described by Dewey *et al.*²³ In V_3 mtDNA a recombination has occurred in this 1.5kb *Ava*I region approximately 500bp 5' of the right *Hind*III site centered in ORF25.²² This recombination reestablishes a 3.2kb *Hind*III fragment in V_3 which is very similar in sequence structure to a 3.2kb *Hind*III fragment in N mtDNA (Fig. 2). To position precisely the recombination point in the revertant V_3 mtDNA and compare the homologous 3.2kb *Hind*III N mtDNA region, clones of these fragments were sequenced. For this purpose, the 3.2kb *Hind*III fragment of V_3 was subcloned from a cosmid (VH3.3-1) and a 3.2kb *Hind*III cloned fragment of N mtDNA (pH3.2N) was selected from a pUC8 *Hind*III library. The extent of homology of these fragments to the *Ava*I 1.5kb region of *cmsT* was determined by restriction mapping and Southern hybridization (Fig. 2).

Sequence comparison of the 1.5kb *Ava*I T homologous regions in N and the T revertant V_3 mtDNAs

In order to be consistent with the published sequence of TURF 2H3 we have chosen the same nucleotide numberings as Dewey *et al.*²³ Figure 3 shows the sequences of the homologous regions in N, *cmsT* and V_3 aligned by the right end *Hind*III site of the 2.1kb *cmsT* region designated nucleotide 2018. The first line depicts the sequence of N, the second line the sequence of the T revertant V_3 and the last line the sequence of *cmsT*.

Comparing N and T sequences over the region defined by the ORF 25 start codon (nucleotide 1640) to the *Hind*III site ending at nucleotide 2018 reveals a 97.5% sequence homology, with differences occurring at nucleotides 1667, 1668, 1669, 1671, 1675, 1866, 1899, 1926, and 1987. These differences have been described previously by Stamper, S. (personal communication). The N and T sequences between the ORF 25 start codon and the point of complete sequence divergence (nucleotide 1568) are 63.3% matched with differences occurring at nucleotides 1587-1594, 1597, 1600, 1606-1616, 1619, 1620, 1623, 1629, 1630. 5' to nucleotides 1568 the N and T sequences totally diverge (24.6% match). It is interesting to note that ORF 25 coding sequences are much more highly conserved than the immediate 5' flanking sequence; although the differences are most pronounced in one region defined by base 1587-1616. The high degree of sequence variation in this region suggests that this may be the site of recombination.

Comparing the N and fertile revertant V_3 sequences 5' to nucleotide 1568 (Fig. 3), the V_3 sequence is similar to the N sequence with a 72.4% nucleotide match. The nucleotide differences occur at position 1516, 1517, and 1518. In this same region V_3 has an additional 9 nucleotides not present in N (nucleotide 1541-1549) and N has 6 nucleotides not present in V_3 (nucleotide

Fig. 4. Northern blot hybridizations of N (lane 1), cmsT (lane 2) and V_3 (lane 3) mRNAs with A. ORF 25 specific probe; C. ORF 13 specific probe; B and D are the longer exposures of A and C. Transcript sizes are in nucleotides.

1510-1513, 1519, 1520). 3' to base 1568 up to the ORF25 start codon V_3 has a 63.3% nucleotide match to N in contrast to a 81.6% nucleotide match to T. From the ORF25 start codon to the HindIII site at nucleotide 2018 the V_3 and N sequences are 98.1% nucleotide matched while the V_3 and T sequences are 99.4% nucleotide matched. In this region, the V_3 sequence is more similar to the T than the N sequence as judged by the nature of the nucleotide differences, particularly those in the region of nucleotides 1667-1675. As found in the 5' flanking sequences of the Cytochrome Oxidase II genes of various plant species, the consensus sequence GGAGCAGAG³¹ has been located between nucleotide 1595-1604 in N and V_3 . The T sequence in this region has two different nucleotide substitutions at position 1597 and 1600.

Northern blot analysis of ORF 13 and ORF 25 transcripts in N, cmsT and T revertant (V_3)

To further characterize how the rearranged coding sequences in the fertile revertant mtDNA alter the transcription unit defined by transcripts homologous to the 1.5kb AvaI region of cmsT, two specific probes were hybridized to a Northern blot containing N, cmsT and T revertant V_3 mtRNA. The first probe is an ORF 13 specific probe, isolated from a deletion clone of the 1.5kb AvaI region of pB8.5T that contains all the ORF 13 sequences and 300 nucleotides of the 5' ATPase 6 flanking sequences. The second probe is an ORF 25 specific clone (pH3.2N). Sequencing experiments, described above, show that the 1.5kb AvaI homologous region in pH3.2N is located at the right side of the 3.2kb HindIII region. The homology of these probes to the 1.5kb AvaI fragment is shown in Figure 1.

Figure 4A shows the hybridization pattern for the ORF25 specific probe on a Northern blot of N, cmsT and T revertant V_3 mtRNAs. This clone hybridizes to a 2900 nucleotide transcript in V_3 , a 2100 and 1600 nucleotide transcripts in N, and several transcripts in cmsT ranging from 4000 to 1500 nucleotides (Fig. 4A). A long exposure of this blot is included (Fig. 4B).

Hybridization of the ORF13 specific probe is presented in Figure 4C. As shown previously by Dewey *et al.*²³, ORF 13 is absent in N and cotranscribed with ORF 25 in cmsT. Our data demonstrates the absence of ORF 13 transcription in the fertile revertant V_3 . In the revertant V_3 , rearrangement of the 1.5kb region of cmsT has redefined the transcription unit for ORF 25 and eliminated transcription of ORF 13.

Since the ORF 13 probe contains some 5' flanking sequence of the ATPase 6 gene (Fig. 1), any detectable hybridization signal might be due to this sequence. To eliminate this possibility a Northern blot of N, cmsT and T

Fig. 5. Southern blot hybridization of the 1.5kb *Ava*I fragment of pB8.5T to *Ava*I digestions of a V_3 cosmid DNA (lane 1), cmsT mtDNA (lane 2), N mtDNA (lane 3) and V_{18} mtDNA (lane 4)

revertant mtRNA was probed with a pUC 8 N mtDNA clone (pH2.7N) which includes the ATPase 6 gene and its 5' flanking region.³² The expression of the ATPase 6 gene is the same in N, cmsT and the T revertant mitochondria and the minor hybridization signal to the ORF13 probe in the revertant and N mtRNA samples is caused by the 300bp of 5' flanking sequence of ATPase 6 (data not included).

During fertility reversion, rearrangements occur in the transcription units defined on the chimeric 1.5kb *Ava*I fragment of cmsT. Rearrangements in this chimeric gene region can affect the transcription of one or both reading frames; therefore two hypotheses explaining cytoplasmic reversion are evident. One hypothesis would suggest that toxin resistance and/or male fertility requires a defined rearrangement creating a specific ORF 25 transcription unit. Alternatively, another hypothesis would suggest that toxin resistance and/or male fertility arises by loss of ORF 13 transcription and not altered transcription of ORF 25.

In order to examine these hypotheses, we chose to investigate other revertants for the molecular structure and expression of the sequences homologous to the 1.5kb *Ava*I region of cmsT.

Southern blot and Northern blot analysis of ORF 13 and ORF 25 regions in V_{18}

One T revertant examined was a sister plant V_{18} regenerated from the same

Fig. 6. Northern blot hybridization of V_{18} (lane 1), cmsT (lane 2) and N (lane 3) mtRNAs with A. ORF 25 specific probe; B. ORF 13 specific probe. Transcript sizes are in nucleotides.

callus that gave the V_3 plant. A Southern blot of *Ava*I digested N, cmsT, V_{18} mtDNA and a V_3 cosmid clone hybridized with the 1.5kb *Ava*I fragment of cmsT is shown in (Fig. 5). In N, V_3 and V_{18} mtDNA, 1.5kb *Ava*I homologous sequences are present on a 2.1kb fragment in N and V_3 and two fragments of 1.2kb and 1.25kb in V_{18} . This suggests the mtDNA rearrangements are not identical in V_3 and V_{18} .

There are several other *Ava*I fragments homologous to the 1.5kb *Ava*I fragment in cmsT (Fig. 5). These are 1kb and 3.7kb in size and have identical structure in N, cmsT and V_3 mtDNAs. These have been identified previously and are due to the complex chimeric structure of the 1.5kb *Ava*I probe.²² A Northern blot hybridization analysis of V_{18} mtRNA (Fig. 6A, 6B) was done using ORF 13 and ORF 25 specific probes identical to those used for the V_3 Northern hybridization analysis described above. In V_{18} , ORF 25 homologous sequences are present on a 1860 nucleotide transcript (Fig. 6A). This transcript size contrasts to those ORF 25 homologous transcripts in N and V_3 , suggesting that a defined ORF 25 transcription unit is not necessary for fertility reversion

and/or toxin resistance. As seen in the case of V_3 , ORF 13 transcripts were not detected in V_{18} mtRNA. It is interesting to note that all fertile revertants we've examined have no detectable ORF 13 transcripts. This evidence supports but does not prove that ORF 13 transcription and translation may be responsible for male sterility in cmsT.

DISCUSSION

Reversion of T type cytoplasmic male sterility (cmsT) is correlated with sequence changes in 6.6kb XhoI fragment. This region carries mtRNA coding sequences on a 1.5Kb AvaI fragment located at the left side of 6.6kb XhoI region.²¹ This 1.5kb AvaI fragment is composed of the 5' flanking sequences of ATPase 6 gene, one complete open reading frame (ORF 13) and part of another (ORF 25) as described previously by Dewey *et al.*²³

Southern blot hybridization analysis of N, cmsT and T revertant mtDNAs (V_3 , V_{18}) indicates that the 1.5kb AvaI homologous sequences are present on a 2.1kb AvaI fragment in both N and V_3 ²² and two smaller fragments 1.2kb and 1.25kb in V_{18} . These new fragments appear to arise by sequence rearrangements in 1.5 AvaI cmsT region.

The molecular structure of sequences homologous to 1.5kb AvaI fragment of cmsT in N and V_3 have been examined by sequence analysis. These data confirm cosmid mapping experiments which suggest that during fertility reversion rearrangements occur in a region located between ORF 13 and ORF 25.²² The complete change in sequence between T versus N and V_3 is located a few base pairs after the ORF 13 stop codon at nucleotide 1568. 5' to this point, V_3 mtDNA is very similar to the N mtDNA sequence while in T the ORF13 sequence is found. 3' to this base up to the ORF 25 start codon, there is much less homology among the three genomes, indicating this region may be active in recombination.

The 5' flank of ORF25 may be crucial for translation of this gene. The presence of a consensus sequence in this region identical to a previously described sequence in the COII genes of other plant species³¹ is supportive of this hypothesis; however, it must be noted that this sequence was not found in the 5' flank of the maize COII gene.³¹ Interestingly recombination in this region during fertility reversion reestablishes this consensus sequence in V_3 .

A Northern blot hybridization analysis of N, cmsT and V_3 mtRNA using two probes each specific for a portion of the 1.5kb AvaI coding region (ORF 13, ORF 25) was done. As evidenced from this study, the ORF 25 region is transcribed differently in N, cmsT and V_3 mtRNA. The homologous transcript

sizes in bases are 2200 and 1600 in N, 4000, 1900, 1800 and 1500 in cmsT, and 2900 in V_3 mRNA. ORF 13 homologous transcripts are present in cmsT mRNA, but are not detectable in N or V_3 .

From the data above, fertility reversion and/or toxin resistance is correlated with loss of ORF 13 transcription and the presence of a uniquely sized ORF 25 transcript. Other fertile T revertants as well as T restored lines have changes in ORF 13 transcription patterns furthering supporting the hypothesis that ORF 13 transcription and translation are responsible for cmsT male sterility.^{33,23}

At this juncture further work needs to be done to assess the relative importance of ORF25 transcription pattern changes and how these may effect translation of this gene.

Further studies will focus on determining the molecular nature of the sequence rearrangement in V_{18} . This will provide informative comparisons with V_3 , N and cmsT homologous coding regions.

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