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Plastid Genotyping Reveals the Uniformity of Cytoplasmic Male Sterile-T Maize Cytoplasms[1][OPEN]

Massimo Bosacchi[2], Csanad Gurdon[2], and Pal Maliga*

Waksman Institute of Microbiology, Rutgers, Piscataway, New Jersey 08854–8020

ORCID IDs: 0000-0003-4454-9882 (M.B.); 0000-0002-8063-653X (C.G.); 0000-0002-6202-0029 (P.M.).

Cytoplasmic male-sterile (CMS) lines in maize (*Zea mays*) have been classified by their response to specific restorer genes into three categories: cms-C, cms-S, and cms-T. A mitochondrial genome representing each of the CMS cytotypes has been sequenced, and male sterility in the cms-S and cms-T cytotypes is linked to chimeric mitochondrial genes. To identify markers for plastid genotyping, we sequenced the plastid genomes of three fertile maize lines (B37, B73, and A188) and the B37 cms-C, cms-S, and cms-T cytoplasmic substitution lines. We found that the plastid genomes of B37 and B73 lines are identical. Furthermore, the fertile and CMS plastid genomes are conserved, differing only by zero to three single-nucleotide polymorphisms (SNPs) in coding regions and by eight to 22 SNPs and 10 to 21 short insertions/deletions in noncoding regions. To gain insight into the origin and transmission of the cms-T trait, we identified three SNPs unique to the cms-T plastids and tested the three diagnostic SNPs in 27 cms-T lines, representing the HA, I, Q, RS, and T male-sterile cytoplasms. We report that each of the tested 27 cms-T group accessions have the same three diagnostic plastid SNPs, indicating a single origin and maternal cotransmission of the cms-T mitochondria and plastids to the seed progeny. Our data exclude exceptional pollen transmission of organelles or multiple horizontal gene transfer events as the source of the mitochondrial *urf13-T* (unidentified reading frame encoding 13-kD cms-T protein) gene in the cms-T cytoplasms. Plastid genotyping enables a reassessment of the evolutionary relationships of cytoplasms in cultivated maize.

Cytoplasmic male sterility has been described in many flowering plant species and is linked to mitochondrial genes encoding toxic proteins. Cytoplasmic male-sterile (CMS) proteins are typically encoded by a chimeric mitochondrial gene assembled from rearranged mitochondrial DNA sequences and contain a hydrophobic, membrane-spanning domain (Hanson and Bentolila, 2004; Chase, 2007; Carlsson et al., 2008; Kubo and Newton, 2008; Chen and Liu, 2014). CMS cytoplasms in maize (*Zea mays*) are well characterized. Thirty-eight sources of cytoplasmic male sterility have been examined for fertility restoration in 28 inbred backgrounds and classified by their response to specific restorer genes into the cms-C, cms-S, and cms-T groups. The cms-T group is composed of the earlier identified HA, I, Q, RS, and T cytoplasms, which all respond to the same Restorers of fertility nuclear genes *Rf1* and *Rf2*. Plants with the cms-T cytotype are susceptible to *Bipolaris* (*Helminthosporium*) *maydis* race T, a fungal pathogen that causes southern corn leaf blight (Beckett, 1971; Gracen and Grogan, 1974). A distinct mitochondrial genome representing each of the CMS cytotypes has been sequenced (Allen et al., 2007), with male sterility linked to the *urf13-T* (unidentified reading frame encoding 13-kD cms-T protein) gene in cms-T (Dewey et al., 1987) and the cotranscribed open reading frames (ORFs) *orf355/orf77* in cms-S (Zabala et al., 1997). The 13-kD maize mitochondrial protein encoded by the *urf13-T* gene was shown to confer sensitivity to the T-toxin produced by *B. maydis* in *Escherichia coli*, firmly establishing the linkage between the T-type cytoplasmic male sterility and T-toxin sensitivity (Dewey et al., 1988). A collection of CMS lines is searchable at the Maize Genetics and Genomics Database (MaizeGDB) Web site (Sen et al., 2009), and seed may be obtained upon request. We used this resource to learn whether the independently isolated cms-T cytoplasms in cultivated maize are related. In maize, plastids and mitochondria are transmitted to the seed progeny from the maternal parent (Conde et al., 1979). However, maize yields hybrids with relatively distant wild relatives such as *Zea luxurians*, *Zea diploperennis*, and *Zea perennis* (Allen, 2005), and when doing so, it is possible that the mode of organelle inheritance may change to biparental with an increased frequency of organelle leakage via pollen. In an extreme case of an interspecies hybrid, a shift from maternal to paternal inheritance was

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[2] These authors contributed equally to the article.

* Address correspondence to maliga@waksman.rutgers.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Pal Maliga (maliga@waksman.rutgers.edu).

P.M. designed and supervised the project; C.G. and M.B. assembled and annotated the plastid genomes; M.B. performed the molecular experiments; M.B., C.G., and P.M. analyzed the data; M.B., C.G., and P.M. wrote the article.

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documented (Hansen et al., 2007). Lineages arising outside a strict maternal mode of organelle inheritance in the cms-T cytoplasm can be reconstructed by analyzing plastid types in the cms-T collection. This necessitated a search for plastid markers.

Earlier work yielded very few markers that would be useful for plastid genotyping in cultivated maize (Pring and Levings, 1978). Complete plastid genome sequences are convenient sources of plastid DNA (ptDNA) markers. Prior to our study, the only annotated maize ptDNA sequence in GenBank (NC_001666; Maier et al., 1995) was assembled from sequencing clones of two maize hybrids (see "Discussion"). To provide markers specific to the lines used in this study, we sequenced the plastid genomes of three fertile lines: A188 representing cytotype NA and B37 representing cytotype NB (Clifton et al., 2004; Allen et al., 2007). The third fertile line, B73, was chosen because its nuclear genome has been sequenced (Schnable et al., 2009). We also sequenced the plastid genome of cms-C, cms-S, and cms-T lines in the B37 nuclear background (B37C, B37S, and B37T lines), in which the mitochondrial genome sequence has been determined (Clifton et al., 2004; Allen et al., 2007). The maize lines with sequenced plastid and mitochondrial genomes are listed in Table I.

An alignment of the completed plastid genome sequences facilitated the identification of three single-nucleotide polymorphisms (SNPs) that are unique to the cms-T plastid haplotype. We report here that each of the tested 27 cms-T accessions has the same three diagnostic plastid SNPs, indicating a single origin. Our data exclude exceptional transmission of organelles by pollen or independent horizontal transfer of the *urf13-T* gene to fertile mitochondrial genomes during domestication as the source of the *urf13-T* gene in the cms-T cytoplasm.

RESULTS

Plastid Genomes of Fertile Maize Lines

Biotechnological applications require a maize line amenable to plant regeneration. A188 is such a line because it maintains its potential for plant regeneration from cultured cells over an extended period of time. Sustained regeneration potential is linked to a morphology known as type II callus, which is friable and embryogenic (Armstrong and Green, 1985). The Hi-II maize line was developed to combine the sustained regeneration potential of the A188 line with the superior agronomic performance of B73. B73 is an important breeding line with poor tissue culture regeneration potential. The two lines were crossed using A188 as the maternal parent and the segregating progeny selected over several seed generations for tissue culture response. The resulting A and B maize lines are crossed to provide highly regenerable Hi-II immature embryos for transformation (Armstrong et al., 1991). We sequenced total cellular DNA isolated from the A188 line, the Hi-II A and B lines, and B73. The plastid genomes of the Hi-II A and B lines are identical to the A188 line (KF241980), as expected. The B73 plastid genome (KF241981) is somewhat larger (140,447 nucleotides as compared with 140,437 nucleotides; Fig. 1) and differs from A188 by 17 SNPs and 11 insertions/deletions (indels), which are one or two nucleotide differences in length (Fig. 1; Tables II and III). Our B73 ptDNA sequence (KF241981) is eight nucleotides longer than the nonannotated B73 ptDNA sequence in GenBank (AY928077). This discrepancy is due to differences in the length of mononucleotide repeats that were verified in our genomes by direct sequencing of PCR amplicons.

Diversity of Plastid Genomes of CMS Maize Lines

The mitochondrial genomes of the fertile B37 maize (referred to as wild-type or normal NB; Clifton et al., 2004) and the three main CMS types have been sequenced in the B37 nuclear background (Allen et al., 2007). Therefore, we decided to sequence the ptDNA of the fertile B37 (B37N) line and the three CMS cytoplasmic substitution lines. The ptDNA sequences of the B37N (KP966114) and the B73 (KF241981) lines are identical. This is not surprising, since these particular B lines may share a common ancestry. Both lines were developed from different cycles of recurrent selection on Iowa Stiff Stalk Synthetic at Iowa State University. The plastid genomes of the CMS B37 cms-C, cms-S, and cms-T lines differ from their fertile counterparts by 25/18, 21/20, and 17/18 nucleotide substitutions/indels, respectively. Most of the SNPs and all of the indels are in noncoding, intergenic regions. Relative to B37N, four genes carry nucleotide substitutions in their coding regions. Two of these substitutions result in amino acid changes: in the *rpoC2* gene of all lines and in the *infA* reading frames of cms-C and cms-S. The remaining two polymorphisms (in the *rpl36* gene of cms-C and the *psbD* gene of cms-S) are silent. The map positions of all

Table I. *Plastid and mitochondrial genomes of maize*

Line	Cytotype	ptDNA Accession No.	Nucleotides	ptDNA Reference	Mitochondrial DNA Accession No.	Mitochondrial DNA Reference
A188	NA	KF241980	140,437	This study	DQ490952	Allen et al. (2007)
B73	NB	KF241981	140,447	This study		
B37N	NB	KP966114	140,447	This study	AY506529.1	Clifton et al. (2004)
B37C	cms-C	KP966115	140,457	This study	DQ645536	Allen et al. (2007)
B37S	cms-S	KP966116	140,534	This study	DQ490951	Allen et al. (2007)
B37T	cms-T	KP966117	140,479	This study	DQ490953	Allen et al. (2007)

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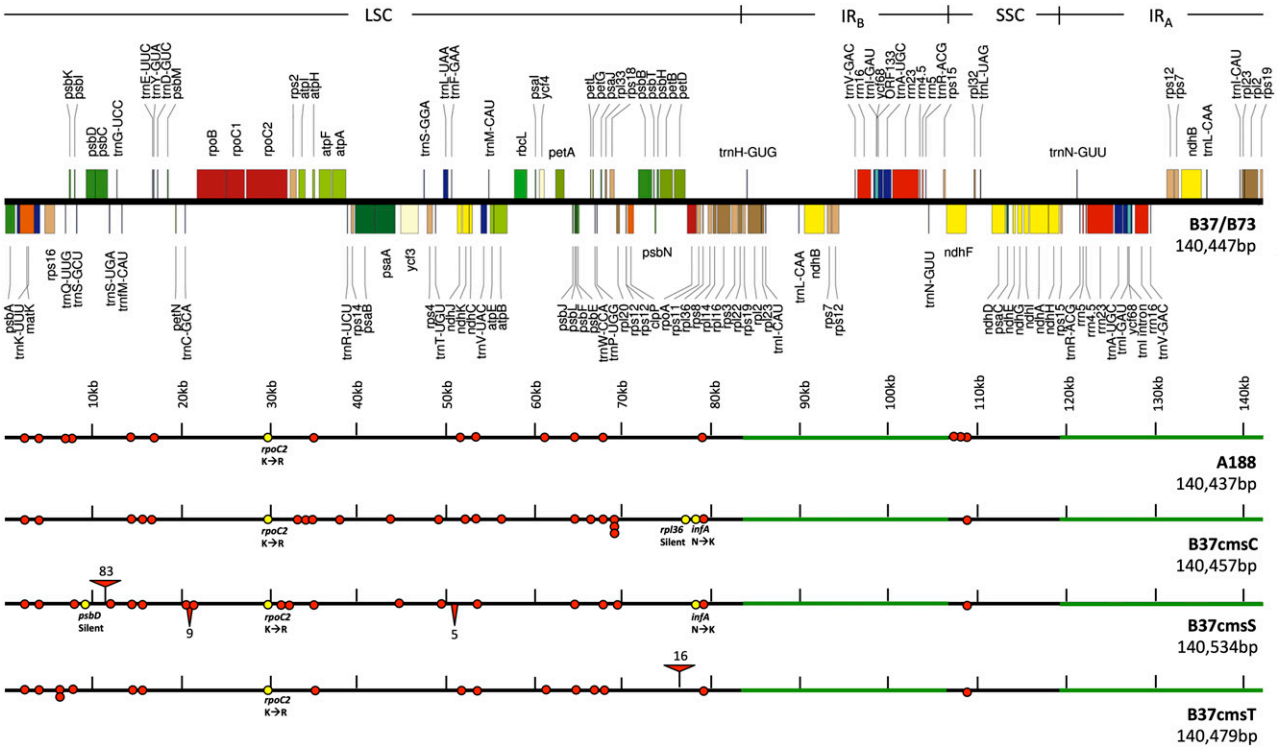

Figure 1. Linear map of maize plastid genomes, with SNPs and select large indels. LSC (large single-copy region), SSC (small single-copy region), and inverted repeats A and B (IR_A and IR_B) are marked on top. In linear maps, unique regions are in black, repeated regions in green, red circles represent intergenic SNPs, and yellow circles represent SNPs in coding regions, with gene name and amino acid change. Insertions are on top, deletions on the bottom. SNPs and indels are represented in relation to the B73 and B37 genomes, which are identical.

SNPs and substantial indels, which may be useful as markers, are shown in Figure 1. A complete listing of ptDNA polymorphisms is in Tables II and III.

The ancestral state of the polymorphic nucleotides could be determined by comparing them with sugarcane (*Saccharum officinarum*; AE009947), the closest maize relative with an available plastid sequence. The ancestors of maize diverged 11.9 million years ago from the lineage leading to sugarcane and sorghum (*Sorghum bicolor*; Swigonová et al., 2004). With one exception, sugarcane ptDNA carries one of two maize alleles for every SNP in maize, the allele we consider the ancestral state. The exception is at nucleotide position 20,313 (Table II), where sugarcane carries a C, while maize lines harbor an A or G. The ancestral state was determined to be A after comparison of this region with publicly available plastid sequences.

We aligned the 1995 maize ptDNA (NC_001666) with the B37 NB ptDNA (KP966115). The two ptDNAs differ by 235 SNPs and 144 indels. This deviation is much more extensive than variation among the newly sequenced genomes, which differ from B37N only by zero to three SNPs in coding regions and by eight to 22 SNPs and 11 to 21 short indels in noncoding regions. We also compared the 1995 sequence (NC_001666) with the ptDNA sequence of sugarcane (AE009947) and sorghum (NC_008602), the two closest relatives of maize with a sequenced chloroplast genome. The overwhelming majority of SNPs in the 1995 sequence (NC_001666) are unique to it, while sorghum, sugarcane, and the six maize plastid genomes reported here carry the same nucleotide. Therefore, we believe that most of the differences in the 1995 sequence are due to sequencing errors inherent to the technology available at the time. With the exception of one locus (Tillich et al., 2001), the sequence has not been updated since the original submission.

Genotyping cms-T Plastid Genomes

CMS lines were collected independently from multiple sources and subsequently classified by their response to restorers of male fertility. Five different groups of cms-T isolates were derived from different maize varieties: HA (Hasting's Yellow variety), Q (uncertain, perhaps Mo988), T (Texas sterile cytoplasm or cms-T, derived from Golden June variety), I (derived from a line carrying the *iojap* gene), and RS (unknown; Beckett, 1971; Gracen and Grogan, 1974). Male sterility in the cms-T lines is conferred by *urf13-T*, a chimeric mitochondrial gene that is toxic to plant cells and whose effect is masked at the posttranscriptional level by certain fertility restorer genes (Dewey et al., 1987). Cytotype-specific ptDNA markers,

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Table II. *Nucleotide substitutions in ptDNA to distinguish plastid types*

Homologs in sugarcane (AE009947) are shown for comparison. Numbering refers to the location in the B37 plastid genome (KP966114). SNPs that differ from B37 are shown in boldface; maize polymorphisms that differ from the ancestral state in sugarcane are marked by asterisks. Amino acids encoded by the codons that contain genic polymorphisms are shown in B37 and the polymorphic line (B37N AA and SNP AA). Regions are as follows: LSC and SSC.

Substitution	Location	B73/B37	B37 cms-C	B37 cms-S	B37 cms-T	A188	Sugarcane	B37N AA	SNP AA	Region
1,338	Intergenic	*T*	**G**	**G**	**G**	**G**	**G**			LSC
3,353	Intergenic	*A*	**G**	**G**	**G**	**G**	**G**			
6,422	Intergenic	C	C	C	***G***	C	C			
6,423	Intergenic	A	A	A	***G***	A	A			
7,423	Intergenic	C	C	C	***A***	***A***	C			
7,527	Intergenic	G	G	G	G	***T***	G			
8,695	Intergenic	C	C	***A***	C	C	C			
9,633	**psbD**	C	C	***T***	C	C	C	F	F	
12,449	Intergenic	C	C	***A***	C	C	C			
14,856	Intergenic	*A*	**G**	**G**	**G**	**G**	**G**			
16,048	Intergenic	*C*	**T**	**T**	**T**	*C*	**T**			
16,107	Intergenic	T	***G***	T	T	T	T			
16,293	Intergenic	A	A	A	A	***C***	A			
20,313	Intergenic	A	A	***G***	A	A	C			
20,656	Intergenic	T	T	***G***	T	T	T			
29,991	**rpoC2**	*A*	**G**	**G**	**G**	**G**	**G**			
31,586	Intergenic	T	T	***G***	T	T	T			
31,652	Intergenic	T	T	***G***	T	T	T			
33,790	Intergenic	G	***A***	G	G	G	G			
34,115	Intergenic	*A*	**C**	*A*	*A*	*A*	**C**			
35,732	Intergenic	*A*	**T**	**T**	**T**	**T**	**T**	**K**	**R**	
38,167	Intergenic	*A*	**T**	*A*	*A*	*A*	**T**			
44,891	Intergenic	A	***C***	A	A	A	A			
45,973	Intergenic	T	T	***G***	T	T	T			
49,356	Intergenic	T	***A***	T	T	T	**T**			
49,532	Intergenic	T	T	***G***	T	T	T			
52,307	Intergenic	T	T	T	***C***	***C***	T			
53,045	Intergenic	G	***A***	G	G	G	G			
53,751	Intergenic	*A*	**T**	**T**	**T**	**T**	**T**			
56,123	Intergenic	G	***A***	G	G	G	G			
61,312	Intergenic	A	A	A	***G***	***G***	A			
64,854	Intergenic	*T*	**A**	**A**	**A**	**A**	**A**			
66,701	Intergenic	T	***C***	T	T	T	T			
66,839	Intergenic	T	T	T	***C***	T	T			
66,848	Intergenic	*T*	**G**	**G**	**G**	**G**	**G**			
69,228	Intergenic	C	***A***	C	C	C	C			
69,229	Intergenic	T	***A***	T	T	T	T			
69,230	Intergenic	T	***G***	T	T	T	T			
69,522	Intergenic	G	G	***T***	G	G	G			
77,919	**rpl36**	G	***A***	G	G	G	G	Y	Y	
78,382	**infA**	G	***T***	***T***	G	G	G	**N**	**K**	
78,983	Intergenic	*T*	**A**	**A**	**A**	**A**	**A**			
108,040	Intergenic	A	A	A	A	***C***	A			SSC
108,639	Intergenic	C	C	C	C	***T***	T			
108,873	Intergenic	*T*	**A**	**A**	**A**	**A**	**A**			

based on our sequence information, allowed us to test whether all T cytotype lines carry the same ptDNA. We acquired 27 seed accessions representing the five different groups of origin and tested them for the three cms-T-specific ptDNA polymorphisms (at positions 6,422, 6,423, and 66,839) and three polymorphisms shared only with the A188 ptDNA (at positions 7,423, 52,307, and 61,312; Table II). All 27 seed accessions listed in Supplemental Table S1 carried the six ptDNA polymorphisms characteristic of cms-T plastids.

DISCUSSION

Sequencing Maize Plastid Genomes Using Total Cellular DNA

Plastid genome assembly from total cellular DNA sequence reads has become the predominant method to obtain complete chloroplast genomes (Nock et al., 2011). The feasibility of this approach is based on the relatively high number of plastid genome copies compared with the mitochondrial and nuclear genomes per

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Table III. *Insertions and deletions in ptDNA to distinguish plastid types*

Cognate regions in sugarcane (AE009947) are shown for comparison. Numbering refers to the location in the B37 plastid genome (KP966114). Insertions and deletions that differ from B37 are in boldface. Regions are as follows: LSC, IR_A, and IR_B.

Indels	Location	B73/B37	B37 cms-C	B37 cms-S	B37 cms-T	A188	Sugarcane	Region
3,543–3,553	Intergenic	$(A)_{11}$	**$(A)_{10}$**	**$(A)_{12}$**	$(A)_{11}$	$(A)_{11}$	**$(A)_9$**	LSC
3,755–3,765	Intergenic	$(A)_{11}$	**$(A)_{13}$**	**$(A)_{13}$**	**$(A)_{14}$**	**$(A)_9$**	**$(A)_{15}$**	
4,097–4,107	Intergenic	$(A)_{11}$	$(A)_{11}$	$(A)_{11}$	**$(A)_{10}$**	$(A)_{11}$	**$(A)_{10}$**	
7,424–7,434	Intergenic	$(A)_{11}$	**$(A)_{17}$**	**$(A)_{15}$**	**$(A)_{16}$**	**$(A)_9$**	$(A)_{11}$	
8,366–8,376	Intergenic	$(A)_{11}$	**$(A)_{12}$**	$(A)_{11}$	$(A)_{11}$	$(A)_{11}$	**$AT(A)_7$**	
8,711–8,720	Intergenic	$(T)_{11}$	$(T)_{11}$	$(T)_{11}$	**$(T)_{12}$**	$(T)_{11}$	$(T)_{11}$	
11,851–11,852	Intergenic			**83-bp Insertion**			**83-bp Insertion**	
12,962–12,973	Intergenic	$(T)_{12}$	**$(T)_{17}$**	**$(T)_{17}$**	**$(T)_{16}$**	**$(T)_{11}$**	**$(T)_{11}$**	
12,987–12,995	Intergenic	$(A)_9$	**$(A)_{10}$**	$(A)_9$	$(A)_9$	$(A)_9$	**$(A)_5T(A)_8$**	
16,052–16,065	Intergenic	$(T)_{14}$	**$(T)_{13}$**	$(T)_{14}$	$(T)_{14}$	$(T)_{14}$	$(T)_{14}$	
16,145–16,153	Intergenic	$(A)_9$	$(A)_9$	$(A)_9$	**$(A)_{10}$**	$(A)_9$	$(A)_9$	
17,177–17,188	Intergenic	$(G)_{12}$	**$(G)_{13}$**	**$(G)_{13}$**	$(G)_{12}$	$(G)_{12}$	**$A(G)_8$**	
20,571–20,579	Intergenic			**9-bp Deletion**			**53-bp Deletion**	
20,815–20,825	Intergenic	$(A)_{11}$	**$(A)_9$**	**$(A)_9$**	**$(A)_9$**	**$(A)_9$**	**$(A)_8$**	
31,819–31,824	Intergenic	$(A)_6$	$(A)_6$	**$(A)_5$**	$(A)_6$	$(A)_6$	$(A)_6$	
35,298–35,305	Intergenic	$(T)_8$	**$(T)_9$**	**(T)9**	**$(T)_9$**	**$(T)_9$**	**$(T)_{10}$**	
36,534–36,551	Intergenic	$(A)_{13}(T)_2$	**$(A)_{12}(T)_2$**	$(A)_{13}(T)_2$	**$(A)_{13}(T)_3$**	$(A)_{13}(T)_2$	**$(A)_9(T)_2$**	
38,107–38,108	Intergenic	AT	AT	AT	**ATT**	AT	AT	
38,168–38,175	Intergenic	$(T)_8$	**$(T)_9$**	$(T)_8$	$(T)_8$	$(T)_8$	**$(T)_{10}$**	
43,710–43,721	Intergenic	$(T)_{12}$	**$(T)_{11}$**	**$(T)_{11}$**	**$(T)_{11}$**	$(T)_{12}$	**$(T)_9$**	
48,257–48,267	Intergenic	$(A)_{11}$	$(A)_{11}$	**$(A)_{10}$**	**$(A)_{10}$**	**$(A)_{10}$**	**$(A)_9$**	
51,065–51,069	Intergenic			**5-bp Deletion**			**5-bp Deletion**	
52,733–52,747	Intergenic	$(T)_{15}$	**$(T)_{16}$**	$(T)_{15}$	$(T)_{15}$	$(T)_{15}$	**$(T)_7$**	
58,311–58,320	Intergenic	$(A)_{10}$	**$(A)_9$**	**$(A)_9$**	**$(A)_9$**	**$(A)_9$**	**$(A)_6G(A)_2$**	
59,489–59,499	Intergenic	$(T)_{11}$	$(T)_{11}$	$(T)_{11}$	**$(T)_{10}$**	**$(T)_{10}$**	$(T)_{11}$	
65,014–65,029	Intergenic	$(T)_{16}$	**$(T)_{12}$**	**$(T)_{17}$**	**$(T)_{19}$**	$(T)_{16}$	**$(T)_3C(T)_6$**	
65,809–65,817	Intergenic	$(T)_9$	$(T)_9$	**$(T)_{10}$**	$(T)_9$	$(T)_9$	**$(T)_{10}$**	
65,936–65,946	Intergenic	$(A)_{11}$	**$(A)_{12}$**	**$(A)_{12}$**	**$(A)_{13}$**	$(A)_{11}$	**$(A)_9$**	
67,503–67,513	Intergenic	$(T)_{11}$	$(T)_{11}$	**$(T)_{14}$**	$(T)_{11}$	**$(T)_{12}$**	**$(T)_{12}$**	
73,336–73,345	Intergenic	$(A)_{10}$	$(A)_{10}$	**$(A)_{12}$**	$(A)_{10}$	$(A)_{10}$	**$(A)_{11}$**	
77,833–77,834	Intergenic				**16-bp Insertion**		**Ambiguous**	
81,829–81,838	Intergenic	$(T)_{10}$	**$(T)_{11}$**	**$(T)_{11}$**	**$(T)_{11}$**	$(T)_{10}$	**$(T)_8$**	
88,310–88,317	Intergenic	$(A)_8$	$(A)_8$	$(A)_8$	$(A)_8$	**$(A)_7$**	$(A)_8$	IR_B
134,506–134,513	Intergenic	$(T)_8$	$(T)_8$	$(T)_8$	$(T)_8$	**$(T)_7$**	$(T)_8$	IR_A

cell. Estimates of plastid genomes per leaf cell range from 700 to 1,400 copies (Golczyk et al., 2014) and from 3,000 to 4,000 copies (Ma and Li, 2015) in maize. The number of maize mitochondrial DNA copies is 30 to 110 in young leaf cells (Ma and Li, 2015). ptDNA fragments are also present in the other genetic compartments: seven to 10 genome equivalents in the maize nuclear genome (Roark et al., 2010; Yoshida et al., 2014) and 17 to 29 kb of the 140-kb ptDNA in the mitochondrial genome (Allen et al., 2007). ptDNA in the heterologous compartments accumulates mutations over time. The ptDNA sequence is a majority consensus of thousands of sequence reads; thus, low-coverage sequences derived from the ptDNA copies integrated in the nuclear and mitochondrial genomes are excluded from the majority consensus. The relatively modest Illumina MiSeq V3 platform we use today generates 25 million 2- × 300-nucleotide-long reads, generating approximately 15 GB of useful data that are sufficient to assemble multiple plastid genomes in a single run. De novo assembly of paired-end sequence reads yielded complete plastid genomes with ambiguity only about the length of mononucleotide runs. These ambiguities were eliminated by Sanger sequencing of PCR amplicons. Additionally, we confirmed each polymorphism in the assembled plastid genomes by Sanger sequencing, as we believe that error-free chloroplast sequences are necessary for phylogenetic comparisons.

The 1995 maize plastid genome sequence (NC_001666) was obtained using dideoxy chain-termination sequencing of ptDNA libraries. These sequence data were compared with the rice (*Oryza sativa*) and wheat (*Triticum aestivum*) chloroplast genomes in a 2002 evolutionary study, which identified five genes with a higher gene divergence in maize than in wheat or rice (Matsuoka et al., 2002). Four of the maize genes (*ndhK*, *psbD*, *rrn16*, and *rrn23*) harbor 35 SNPs in the 1995 maize sequence NC_001666 that are absent in the six maize ptDNA sequences we report here. We conclude that these polymorphisms are most likely sequencing errors; therefore, the rate of evolution of the four maize genes may not be as rapid as assumed (Matsuoka et al., 2002).

Plastid Genotyping Markers

The plastid genotyping markers reported here are useful for the quick classification of plastid types.

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Identical plastid genomes in the B37 and B73 lines reflect the fact that these inbreds, both developed at Iowa State University, share a common lineage. However, not all B lines necessarily have identical cytoplasms. The B designation reflects the location of the breeding program and has no biological significance. The NA (A188; a line developed at the University of Minnesota) and NB (B37 and B73) fertile lines differ in the structure of their mitochondrial genomes and have a total of 17 distinguishing SNPs between their plastid genomes. The plastid genomes of cms-C, cms-S, and cms-T lines differ from the B37/B73 fertile lines by 25/18, 21/20, and 17/18 nucleotide substitutions/indels, respectively. The number of plastid polymorphic sites in the maize cultivars reported here is somewhat lower than between *indica* and *japonica* rice (72 SNPs and 27 indels; Tang et al., 2004) or between upland and lowland switchgrass (*Panicum virgatum*; 116 SNPs and 46 indels; Young et al., 2011).

There are substantial differences between our maize plastid genome sequences and the only one previously available in GenBank (NC_001666). Relative to our B37/B73 sequence, NC_001666 has 235 SNPs, of which 73 are in coding regions. These would cause 37 amino acid changes in 15 genes. In addition, the two ptDNAs differ by 144 indels. However, we believe that a significant fraction of the differences are due to sequencing errors in the original study. The first maize plastid genome sequence was reported relatively early, in a pioneering article on plastid RNA editing (Maier et al., 1995). The somewhat older tobacco (*Nicotiana tabacum*) ptDNA sequence, published in 1986 (Shinozaki et al., 1986), has been updated twice by the original research group. In the process, the plastid genome of cv Bright Yellow 4 has grown in size, first to 155,939 bp from the original 155,844 bp, and more recently to the current 155,943 bp. The maize ptDNA sequence had only one minor update since it was originally published (Tillich et al., 2001). Although genotype information was not given in the original publication, we were able to reconstruct which maize lines were used to obtain the original deposited sequence. We know from Fritzsche (1988; cited in Maier et al., 1995) that the source of plastid DNA was Inrakorn or INRA 258, a four-way hybrid (F115-W33 × F7-EP1; MaizeGDB; Chaubet et al., 1989). The plastid ribosomal RNA region in GenBank NC_001666 was obtained by sequencing clone pZmc134 obtained from the Bogorad laboratory (Edwards and Kössel, 1981). This fact was pointed out in a document that accompanied the maize ptDNA library (H. Kössel, personal communication). The pZmc134 plasmid was obtained by cloning ptDNA fragments from the maize hybrid WFGTMS × BS7 (Bedbrook et al., 1977). Thus, GenBank NC_001666 is a compilation of sequences obtained from two different maize lines, neither of which is used in research or grown commercially today. The sequencing errors do not affect the conclusions of the original publication, which is the first genome-wide study of plastid organellar editing and is a classic in the field.

Phylogenetic Tree of Plastid Genomes in Maize

Plastid and mitochondrial DNA sequence variation in species and subspecies of the genus *Zea* has been studied to obtain information about the progenitors of cultivated maize (Timothy et al., 1979; Doebley et al., 1987a, 1987b; for review, see Doebley, 2004). Without the benefit of full sequence information, the RFLP analysis used at that time could distinguish only the cms-S plastids from normal (fertile) cms-C and cms-T ptDNAs (Pring and Levings, 1978). Phylogenetic relationships based on the mitochondrial genome sequences describe NA and NB as being the most closely related mitochondrial genomes, followed by cms-C, cms-S, and cms-T. On the basis of their nucleotide divergence, cms-S and cms-T were suggested to be the earliest diverged cytotypes. cms-S was considered to be a relatively ancient cytoplasm, most likely derived from maize ssp. *mexicana* (Allen et al., 2007; Darracq et al., 2010).

Our ptDNA sequence data allow us to reevaluate the taxonomic position of the cms-T cytotype. We built phylogenetic trees from the chloroplast sequences of the same CMS and fertile lines to find whether chloroplast data independently validate the mitochondrial phylogeny. The sugarcane plastid genome (*Saccharum* ssp.; AE009947) was used as an outgroup, because chloroplast sequences from wild *Zea* spp. lines are lacking. The ancestor of sorghum and sugarcane split from the progenitors of maize 11.9 million years ago (Swigonová et al., 2004), and sugarcane and sorghum ancestors split 8 to 9 million years ago (Jannoo et al., 2007). Thus, sorghum and sugarcane are sister clades equidistant from maize. Phylogenetic trees were built from the single-copy regions of ptDNAs, as there was only one polymorphism in the inverted repeats (Table III). Using all data (Fig. 2) resulted in the same tree as the tree based on SNPs only (data not shown). As the maize plastid genomes harbor few intraspecies differences, branch lengths are very short and bootstrap support is low for some branches. However, we can conclude that cms-T is closest to the fertile NA and NB plastid genomes, while the cms-S and cms-C ptDNAs

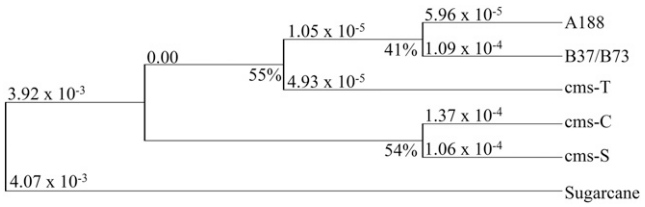

Figure 2. Phylogenetic relationship of maize plastid genomes. A bootstrap consensus tree of maize plastid genomes and sugarcane as an outgroup is shown. Branch distances are shown above and bootstrap support below the branches. The tree was obtained using concatenated single-copy regions. Note that the distances are short because the maize plastid genomes are conserved and bootstrap support is low for the branches.

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are more ancient. The cms-S plastid type shares an 83-nucleotide insertion and a five-nucleotide deletion with sugarcane relative to B37 (Table III). Its placement as part of a basal branch on the maize phylogenetic tree is in accord with the phylogenetic placement of the cms-S mitochondrial DNA, the origins of which predate the domesticated maize (Allen et al., 2007; Darracq et al., 2010).

Single Origin of cms-T Cytoplasm in Maize

Plastids and mitochondria in flowering plants may be inherited maternally, paternally, or biparentally (Mogensen, 1996; Nagata, 2010; Hagemann, 2013). In maize, both organelles are assumed to follow a maternal mode of inheritance. Data supporting the maternal inheritance of maize plastids were obtained in reciprocal crosses of maize and *Z. perennis* interspecies hybrids by testing RFLPs in organellar DNAs (Conde et al., 1979). Evidence supporting the maternal inheritance of mitochondria came from studies of nonchromosomal stripe mutations and cytoplasmic male sterility encoded in mitochondrial genes (Kubo and Newton, 2008). Our objective was to test whether exceptional transmission of organelles by pollen, or independent horizontal transfer of the *urf13-T* gene to fertile mitochondrial genomes, could have contributed to the evolution of cms-T cytoplasm during domestication.

One mechanism that could yield new combinations of plastids and mitochondria is the exceptional pollen transmission of plastids, a well-documented process in species with a maternal mode of plastid inheritance. Examples include the monocot species *Setaria italica* (Wang et al., 2004) and the dicots tobacco (Avni and Edelman, 1991; Ruf et al., 2007; Svab and Maliga, 2007), petunia (*Petunia hybrida*; Derepas and Dulieu, 1992), and Arabidopsis (*Arabidopsis thaliana*; Azhagiri and Maliga, 2007). Another mechanism that could yield new combinations of plastids and CMS mitochondria is the horizontal gene transfer of mitochondrial DNA, an evolutionary mechanism described in multiple species (Richardson and Palmer, 2007; Bock, 2010).

We tested six cms-T plastid markers to determine whether the different cms-T lines have the same ptDNA. Three of the markers are unique to cms-T plastids and three are shared only with NA plastids. We found that, based on the six markers, all 27 cms-T accessions have the same plastid type. This indicates that the cms-T cytotype originated from a single event. Furthermore, it suggests that the cms-T plastids and mitochondria have always been cotransmitted, with both organelles following a strict maternal mode of inheritance. These findings exclude exceptional pollen transmission of plastids, or horizontal transfer of the *urf13-T* gene, as mechanisms contributing to the evolution of cms-T cytoplasm during domestication.

MATERIALS AND METHODS

Plant Lines

Seeds of maize (*Zea mays*) lines B73, A188, and Hi-II A and B were obtained from Hugo Dooner (Rutgers University). Kathleen Newton (University of Missouri) provided seeds of B37N, B37C, B37S, and B37T. We searched the MaizeGDB (Lawrence et al., 2004; Sen et al., 2009) to identify available cms-T accessions, which were subsequently ordered from the Maize Genetics Cooperation Stock Center. Plants were grown in soil in the greenhouse from seed.

Sequencing and Assembly of Plastid Genomes

Total cellular DNA was isolated from leaves using the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). B73, A188, and A line and B line DNA were sequenced on the SOLiD 5500XL platform using 75-nucleotide reads. The reads were mapped to the B73 ptDNA sequence (AY928077), with one inverted repeat removed, using the Burrows-Wheeler Alignment Tool reference-guided assembly program, version 0.7.1 (Li and Durbin, 2009). De novo contigs were assembled from quality-filtered (90% of bases having a quality cutoff value of 20) and unfiltered reads using the Velvet de novo assembly program, version 1.1, at hash length 67 with otherwise default settings (Zerbino and Birney, 2008). B37N, B37C, B37S, and B37T lines were sequenced on the Illumina MiSeq platform using 300-nucleotide paired-end reads and 600-nucleotide insert paired-end libraries made from total cellular DNA. De novo contigs were assembled using the ABySS program, version 1.3.7, at default settings (Simpson et al., 2009). Sanger sequencing of PCR amplicons was used to eliminate ambiguities and to confirm each ptDNA polymorphic site.

Fully assembled plastid genome sequences were annotated using DOGMA, a software tool developed specifically for organellar genomes (Wyman et al., 2004). DOGMA output (features table) was manually adjusted in Microsoft Excel to resolve intron/exon borders and include stop codons in protein-coding genes. Gene annotation was verified by comparison with the Inrakorn ptDNA (NC_001666). Each fully assembled plastid genome sequence was deposited as a FASTA file, along with a corrected DOGMA features table into Sequin (National Center for Biotechnology Information), which compiled sequence and annotation information into the correct format for submission to GenBank. Maps were prepared using OrganellarGenomeDRAW (Lohse et al., 2013).

PCR Genotyping of cms-T ptDNA

Seed of 27 cms-T accessions was provided by the Maize Genetics COOP Stock Center (University of Illinois). Total cellular DNA was isolated from leaves of 10-d-old seedlings using the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). PCR primers (Supplemental Table S2) were designed to amplify each of the six SNPs used as markers. PCR products ranged from 335 to 465 bp in length. The amplicons were treated with ExoSAP-IT (Affymetrix) and Sanger sequenced. Sequence reads were aligned using SeqMan Pro (DNASTAR) software. We checked for cms-T polymorphisms at positions 6,422, 6,423, 7,423, 52,307, 61,312, and 66,839.

Phylogenetic Analysis

The concatenated large and small single-copy regions of the five maize chloroplast sequences, and the single-copy region of sugarcane (*Saccharum officinarum* hybrid 'SP-80-3280'; National Center for Biotechnology Information locus AE009947), were aligned by MUSCLE (Edgar, 2004), then the alignment was manually checked and adjusted when necessary. Maximum likelihood trees were assembled from the alignment in MEGA6 (Tamura et al., 2013) using the two-parameter model of Kimura (1980), allowing some sites to be evolutionarily invariable, with 1,000× bootstrap support (Felsenstein, 1985). Initial trees for the heuristic search were obtained by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. Trees were assembled with or without the sugarcane outgroup: using gaps (96,131 positions used with or without sugarcane) and discarding gaps (94,406 positions with sugarcane and 94,880 positions without).

The ptDNA sequences have been deposited in GenBank with the following accession numbers: A188, KF241980; B73, KF241981; B37N, KP966114; B37C, KP966115; B37S, KP966116; and B37T, KP966117.

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Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. ptDNA genotyping in cms-T accessions.

Supplemental Table S2. Primers for PCR amplification of markers in Tables II and III.

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