An Abnormal Growth Mutant in Maize Has a Defective Mitochondrial Cytochrome Oxidase Gene

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We describe a new maternally inherited maize mutation, nonchromosomal stripe 5 (NCS5), that adversely affects plant growth and yield. Mutant plants are characterized by reduced height, defective yellow striping on leaves, and aborted kernels on ears. NCS5 striped plants carry both normal and partially deleted versions of the mitochondrial cytochrome oxidase subunit 2 gene and exhibit greatly reduced levels of cox2 transcripts when compared with nonstriped control plants. Other mitochondrial genes and their mRNAs are not affected. Thus, the defective plant phenotype is correlated with a reduction in the number of functional cytochrome oxidase subunit 2 genes. The NCS5 mutant mitochondrial genome appears to have arisen by amplification of a rare homologous recombination product.

INTRODUCTION

Higher plant mitochondrial genomes are much larger and more complex than those of animals and fungi (reviewed by Newton, 1988). Many studies have found variations in mitochondrial genomes to be associated with maternally inherited male sterility (cytoplasmic male sterility; CMS) in higher plants (see Hanson and Conde, 1985; Pring and Lonsdale, 1985). CMS plants fail to shed functional pollen but they are normal in overall growth and morphology. Few studies have addressed maternally inherited growth abnormalities, although they have long been known to occur in maize (Duvick, 1965; Shumway and Bauman, 1967; Coe, 1983). The nonchromosomal stripe (NCS) mutants are characterized by variable leaf striping, poor growth, and decreased yields (Shumway and Bauman, 1967; Coe, 1983; Newton and Coe, 1986).

NCS plants have appeared spontaneously in various maize inbred lines in combination with a number of mitochondrial genotypes (Newton et al., 1989). However, the inbred line WF9 gives rise to NCS plants at a much higher frequency than do other lines studied. The previously described NCS2 and NCS3 mutations arose in Texas male-sterile cytoplasms (cms-T) in the presence of WF9 nuclear genes (Coe, 1983; Newton and Coe, 1986). NCS plants have also been observed in strains with the WF9 nuclear genotype and cms-S mitochondrial genotypes, as well as in a strain of inbred A619 carrying EP cytoplasm, which had been recurrently back-crossed five times by WF9 as the pollen parent (Newton et al., 1989). NCS mutations have arisen not only in male-sterile cytoplasms but also in fertile N and RU cytoplasms, as well as in fertile derivatives of cms-S cytoplasms (Duvick, 1965; Newton et al., 1989). These studies indicate that nuclear genotype plays a role in the generation or selection of these mutations. However, because the aberrant phenotypes are transmitted only through the maternal parent, they result from true cytoplasmic mutations.

In maize, both mitochondria and chloroplasts are transmitted maternally. Mitochondria, rather than chloroplasts, were postulated to be the primary sites of the NCS lesions because mutant plants are characterized by gross reductions in plant vigor, sectors of reduced growth in the leaves, and, in most cases, kernel abortion (Coe, 1983; Newton and Coe, 1986). The mutations appear to be highly detrimental in all cell types. The phenotypes are consistent with heteroplasmy, that is, with affected plants containing a mixture of defective and normal organelles. Somatic segregation of the mutant and nonmutant organelles would account for the observed sectors of defective and normal growth. Previously, it was reported that specific mitochondrial DNA rearrangements are correlated with the expression and inheritance of the aberrant phenotypes for two independent and phenotypically distinct maize mutants, NCS2 and NCS3 (Newton and Coe, 1986; Feller and Newton, 1987); however, the functions altered by these two mutations were not identified.

We now report that a partial deletion of an essential mitochondrial gene, cytochrome oxidase subunit 2, is correlated with the expression of a maternally inherited aberrant plant phenotype.
RESULTS

NCS5 Phenotype and Origin

The phenotype of this new mutant, designated NCS5, is typical of NCS in that plants carrying this mutation exhibit variable stunting and striping. In the case of NCS5, the leaf stripes are yellow, as shown in Figure 1, and sectors of aborted kernels are seen on ears from NCS5 plants. Thus, this mutation can be lethal during kernel development and highly detrimental in other plant tissues.

The NCS5 mutation appeared in a plant that had the WF9 nuclear genotype and a fertile revertant maize mitochondrial genotype that derived from a cytoplasmic male-sterile strain, cms-ML, a member of the cms-S group (see Methods). The striped NCS plant was discovered within a maternal lineage descended from this cytoplasmic revertant plant. The striped phenotype is also transmitted only maternally.

Identification, Cloning, and Sequencing of Mutant Restriction Fragments

Four separate preparations of mitochondrial DNA from NCS5 striped plants within a single family were compared with six separate mitochondrial DNA preparations from nonstriped control families (see Methods). No clearly correlated differences between the mitochondrial DNAs from NCS5 plants and their fertile nonstriped relatives were detected by ethidium bromide staining of electrophoretically separated restriction digest fragments (data not shown). Hybridization experiments using previously cloned and characterized plant mitochondrial structural genes (including those for subunits of cytochrome oxidase, ATPase, and NADH dehydrogenase; see Methods) as probes on DNA gel blots of the mitochondrial DNA gels revealed that the only consistent striped/nonstriped difference involved the cytochrome oxidase subunit 2 gene (cox2). A 2.4-kb EcoRI fragment containing the whole cox2 gene, as well as 5'- and 3'-flanking regions (pZmE1; Fox and Leaver, 1981) hybridized to a 5.5-kb Xhol fragment in mitochondrial DNAs from both ML sterile and fertile-revertant nonstriped plants. However, Figure 2A shows that, in mitochondrial DNAs from the NCS5 striped, fertile plants, the amount of the 5.5-kb hybridizing fragment was reduced and an additional 8.3-kb Xhol fragment hybridized strongly. The maize cox2 gene consists of two exons separated by a 794-bp intron (Fox and Leaver, 1981). Figure 2B illustrates that a 578-bp HinfI fragment of pZmE1, containing most of the first exon and approximately 300 bp of the intron, did not hybridize to the 8.3-kb Xhol fragment in NCS5 mitochondrial DNA. The first exon and a portion of the intron of the cox2 gene have apparently been deleted from this NCS mutant mitochondrial DNA.

The 5.5-kb Xhol fragment from nonstriped control mitochondrial DNA and the 8.3-kb Xhol fragment from NCS5 mitochondrial DNA were cloned into the pUC8c1 vector (see Methods). Figure 3 shows restriction maps that were constructed of pCLK3, containing the 5.5-kb Xhol fragment, and pCLK2, carrying the 8.3-kb fragment, to determine where the two clones diverged in sequence. Using the 3.1-kb HindIII fragment of pCLK2, which includes the site of divergence, as a hybridization probe onto DNA gel blots, a 4.1-kb Xhol fragment was found to be homologous in both control and mutant mitochondrial DNA (Figure 2C). It should be noted that, in the control DNA, the 4.1-kb fragment hybridized much more strongly with the probe than did the 5.5-kb fragment, suggesting that it might be present in multiple copies. The 4.1-kb Xhol fragment was also cloned, yielding the recombinant plasmid pCLK5.

Detailed restriction mapping of the three clones (Figure 3) suggests that the molecular origin of the NCS5 mutation, i.e., the 8.3-kb Xhol restriction fragment, could have been a recombination event in which the reciprocal product was...
Figure 2. Differences in Mitochondrial DNA between NCS5 and Normal Plants.

DNA gel blots of Xhol-digested mitochondrial DNA from NCS5 plants (5) and nonstriped plants in related lineages (St = sterile; F = fertile) hybridized with 32P-labeled probes.

(A) The probe used was the 2.4-kb EcoRI insert from pZmE1.

(B) The probe used was a 578-kb Hinfl fragment from pZmE1, containing most of exon1 and part of the intron of cox2.

(C) The probe used was a 3.1-kb Hindlll fragment from pCLK2 (8.3-kb Xhol insert).

lost. We have never detected the expected 1.3-kb Xhol reciprocal product, despite using higher percentage gels and all three clones as hybridization probes (not shown). It appears to be absent from the mutant genome. The relative locations of the two "parental" Xhol restriction fragments (5.5 kb and 4.1 kb) in the control mitochondrial genome are unknown. However, they do not appear to be adjacent to each other; when used as hybridization probes in DNA gel blot analyses, the 5.5-kb and 4.1-kb Xhol fragments identify different Hindlll and Pstl restriction fragments (not shown).

The 5.5-kb and 4.1-kb Xhol restriction fragments apparently lack long regions of homology with one another, as assayed by stringent hybridization; however, the possibility of a very short region of homology at the site of the rearrangement was not eliminated by this analysis. To ascertain whether a very short sequence of homology was present at the presumptive recombination site, portions of each of the three clones were subcloned into the Bluescript SK- and KS- vectors (see Methods), and single-stranded DNA preparations were sequenced by the dideoxy method. The sequences surrounding the junction were compared and all three clones were shown to have two short regions of homology: 6 nucleotides at the junction itself and 5 nucleotides 3 bp (in the 4.1-kb Xhol fragment) or 5 bp (in the 8.3-kb and 5.5-kb Xhol fragments) distal to the junction, as illustrated in Figure 4A. This supports the hypothesis that the NCS5-associated rearrangement arose by a homologous recombination event. Because of the limited homology, this event is expected to occur at extremely low frequencies. One hundred twenty nucleotides of sequence distal to the BamHI site in the intron of our cox2 gene were compared with those of the published cox2 intron sequence from maize N mitochondrial DNA (Fox and Leaver, 1981). Only two minor differences were found, as illustrated in Figure 4B.

Analysis of cox2 mRNA in NCS5 Plants

Mitochondrial RNA was isolated from NCS5 striped plants and from nonstriped controls. Figure 5 presents an RNA gel blot analysis that shows that transcripts corresponding to the cox2 gene are detected at very reduced levels in the plants carrying the partially deleted cox2 gene. This reduction appears to be specific because transcripts for cytochrome oxidase subunits I and III, as well as for ATPase subunit 9 and mitochondrial ribosomal RNA (18.5S rRNA), are present at relatively normal levels (Figure 5).

This finding of a quantitative reduction, rather than of aberrant transcripts, suggests that the partially deleted cox2 gene is not transcriptionally active. The presence of low levels of normal cox2 transcripts in the striped plants is expected, as they would be products of the intact cox2 genes in nonmutant mitochondria, which are isolated from the heteroplasmic NCS5 plants together with the mutant mitochondria.

DISCUSSION

Our evidence suggests that the NCS5 mutation is a rearrangement in mitochondrial DNA resulting in a mitochon-
The mitochondrial genome that lacks a portion of the cytochrome oxidase subunit 2 gene, including the first exon and the 5' flanking region. The appearance of aborted kernels on ears of NCS5 plants suggests that the mutation can be lethal during kernel development. Thus, plants carrying the mutant genomes should also carry some normal mitochondrial genomes. According to this hypothesis, mutant and normal mitochondria segregate during development, giving rise to the observed defective and normal sectors. The hypothesis is supported by the observation that mitochondrial DNA from nonstriped control plants has only the normal cox2 gene, whereas that from striped NCS5 plants has both normal and defective cox2 genes. The levels of transcripts homologous to cox2 are specifically reduced in striped plants, presumably because of the loss of promoter sequences from the mutant genomes. There are no reductions in other mitochondrial RNAs, including those for subunits 1 and 3 of cytochrome oxidase.

The cms-S fertile revertant and NCS-type abnormal plants arise in WF9 nuclear backgrounds at similar frequencies (1% to 2%; Laughnan, Gabay-Laughnan, and Carlson, 1981; Newton and Coe, 1986). In the pedigree of the NCS5 mutant, both these phenotypic changes occurred. Rearrangements in the mitochondrial genome are associated with reversion of an S male-sterile cytoplasm to fertility. Multiple rearrangements have been observed in mitochondrial DNAs following reversion of cms-S to fertility (Small et al., 1988) in the absence of other phenotypic effects. A more specific rearrangement resulted in an NCS phenotype. Together, these changes converted a non-striped cms-S sterile plant to a fertile, NCS plant. The mechanism by which nuclear genes present in the WF9 line lead to rearrangements of mitochondrial genomes is unknown, but such an effect is not without precedent; a strong influence of the M825 nuclear genotype on the frequency of cytoplasmic reversion to fertility of cms-S has been reported (Laughnan et al., 1981).

Although we do not know the exact role of the nuclear genotype in promoting the recovery of rearranged mitochondrial genotypes, our restriction mapping and sequence analyses suggest that the NCS5 mutant resulted from amplification of a homologous recombination product.

Figure 4. DNA Sequences Surrounding the Junction of the NCS5 Mutation.

(A) Six nucleotides of sequence (bold, underlined) are shared among the 4.1-kb, 5.5-kb, and 8.3-kb Xhol restriction fragments at the rearrangement site. Near the rearrangement junction, an additional 5 nucleotides of homology (underlined) are found.

(B) One hundred twenty nucleotides of sequence within the intron of the cox2 gene carried on the 5.5-kb Xhol restriction fragment, including the BamHI site and the junction repeat (boldface). Two differences from the Fox and Leaver (1981) cox2 intron are found in this region. In their sequence, the underlined G is a C and an additional C is present at the site indicated with a caret (\textasciicircum).
Such a mechanism has been proposed to account for mitochondrial DNA rearrangements associated with CMS (Lonsdale et al., 1988). Because the duplicated segment at the site of the NCS5 rearrangement is so short (6 nucleotides), the frequency of homologous recombination might be expected to be extremely low and the recovery of such a mutation rare. However, a short, 13-bp direct repeat in human mitochondrial DNA has recently been shown to be a hotspot for recombination and deletion (Schon et al., 1989). Only one recombination product is represented in the NCS5 mutant genome and it is missing part of the cox2 gene. This loss is correlated with the reduced expression of cox2 and with lethality (during kernel development). Thus, although the mutant genome can be amplified, it cannot exist alone in the plant. In NCS5 striped plants, the mutant genomes survive only in a heteroplasmic state, together with normal mitochondrial genomes.

Different organs are differentially affected by the NCS5 mutation: developing kernels abort, but leaf tissue survives, albeit poorly. The functioning of chloroplasts apparently partially rescues cox2-deficient leaf cells, perhaps by supplying an essential metabolite normally provided by mitochondria. Differential survival of tissues is also observed for human mitochondrial defects and is postulated to be due to differences in the relative importance of mitochondrial function in different cell types (Wallace, 1989). Plant mitochondria contain not only the cyanide-sensitive cytochrome oxidase, but also a cyanide-resistant alternative oxidase (Douce, 1985). Because the NCS5 mutant leaf sectors are expected to lack a normal cytochrome oxidase, they could be used to study the functioning of the alternative oxidase pathway.

**METHODS**

**Plant Materials**

Maize kernels containing the ML cytoplasm, a member of the cytoplasmic male-sterile S group, and WF9 nuclear genes were provided by J. Beckett (1971). Plants of this genotype were maintained by crosses with pollen from the WF9 inbred line. The nonstriped ML sterile control plants trace to this Beckett accession. Cytoplasmic (maternally inherited) revertants to fertility occur within this lineage at a rate of approximately 2% (Laughnan et al., 1981). Such a revertant appeared in the 1981 Hawaii nursery and was crossed with WF9 inbred line pollen. The resulting kernels were planted in the 1982 summer nursery in Illinois. Two maternal lineages were established from this 1982 planting by recurrent crossing with WF9 as the male parent. A single ear of a revertant nonstriped plant from one of these lineages is the source of the striped NCS5 mutant plants used in this study. The other lineage is the source of the nonstriped revertant (fertile) WF9 control plants.

**Mitochondrial DNA Isolation and Analysis**

Mitochondrial DNAs were isolated from unfertilized ear shoots of NCS5 striped plants and from nonstriped control plants and were analyzed by digestion with restriction endonucleases and agarose gel (0.8%) electrophoresis as previously described (Newton and Coe, 1986). Duplicate nitrocellulose or nylon (Magna 66; MSI) blots were obtained from each gel and they were hybridized with radiolabeled probes as previously described (Feller and Newton, 1987).

**Mitochondrial RNA Isolation and Analysis**

Mitochondrial RNAs were isolated from unfertilized ear shoots using aurintricarboxylic acid to inhibit RNases, denatured, and electrophoresed on 1.2% agarose/6% formaldehyde gels as previously described (Stern and Newton, 1986). Gels were blotted onto uncharged nylon membranes (Magna 66; MSI), UV-irradiated, and hybridized with radiolabeled probes. Following exposures to x-ray films, the RNA blots were stripped of probes by washing in 80% formamide, 0.1 x SSC, and 0.1% SDS for 1 hr to 2 hr. After autoradiography to confirm removal of the previous probes, the RNA blots were reused in hybridizations with additional probes.

**Mitochondrial Gene Probes**

Previously cloned and characterized plant mitochondrial genes were used in hybridization analyses of mitochondrial DNA and mitochondrial RNA from NCS5 and nonstriped controls. The structural genes tested were cytochrome c oxidase subunits I, coxI (Isaac, Jones, and Leaver, 1985); II, cox2 (Fox and Leaver, 1981); III, cox3 (Heise et al., 1987); ATP synthase subunits 6, atp6 (Dewey, Levings, and Timothy, 1985a); 9, atp9 (Dewey et al., 1985b); atpA (Braun and Levings, 1985); NADH dehydrogenase subunit l, nad1 (Stern, Bang, and Thompson, 1989), and cytochrome b, cob (Dawson, Jones, and Leaver, 1984). The plasmid DNAs were digested with appropriate restriction enzymes and the mitochondrial DNA inserts were excised from agarose gels. The DNAs were labeled with α-32P-dCTP using the oligolabeling kit supplied by Pharmacia LKB Biotechnology Inc.

**Cloning of Restriction Fragments from NCS5**

Mitochondrial DNAs from NCS5 and control plants were digested with XhoI, fractionated on 10% to 40% linear sucrose gradients, and cloned into a plasmid vector following standard procedures (Maniatis, Fritsch, and Sambrook, 1982). The vector used was pUC8c1, a derivative of pUC8 from G. Crouse, in which an XhoI linker has been introduced into the Xmal site of the polylinker (cited in Stern and Newton, 1985). XL1 Blue host cells were used for the transformations and plasmid DNAs were isolated from bacteria that hybridized with the labeled 2.4-kb EcoRI insert (containing cox2) from pZmEl. The recombinant plasmids pCLK3, containing the 5.5-kb XhoI fragment from control mitochondrial DNA, and pOLK2, carrying the 8.3-kb XhoI fragment from NCS5, were restriction mapped using the enzymes HindIII, EcoRI, BamHI, BgII, and PvuII. Following the same procedure, but using
restriction fragments from pCLK2 as hybridization probes, the
4.1-kb XhoI fragment was cloned (pCLK5) and restriction mapped.

Subcloning and Sequence Determination

Restriction fragments found to span the junction from each of
the three original clones were subcloned into Bluescript SK+ and KS+
vectors (Stratagene Cloning Systems). The fragments used for
subcloning were a 1.0-kb BamHI/HindIII fragment from pCLK3
(5.5-kb XhoI insert), a 0.63-kb PstI/XhoI fragment from pCLK5
(4.1-kb XhoI insert), and a 1.23-kb HindIII/PstI fragment from
pCLK2 (8.3-kb XhoI insert). Single-stranded DNAs were recovered
and sequenced by the dideoxy chain termination method (Sanger,
Nicklen, and Coulson, 1977) using Sequenase (US Biochemicals)
and T7 (Pharmacia) kits. M13 universal and reverse primers were
used to obtain the initial sequences, and additional sequence
information resulted from the use of specifically synthesized oli-
gonucleotide (15-mer) primers.

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