The Plant Cell, Vol. 5, 963–971, August 1993 © 1993 American Society of Plant Physiologists

Analysis of Leaf Sectors in the NCS6 Mitochondrial Mutant of Maize

Jianying Gu, Donald Miles, and Kathleen J. Newton[1]

Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211

The nonchromosomal stripe (NCS6) mutation of maize is a partial deletion of the mitochondrial cytochrome oxidase subunit 2 (*Cox2*) gene. The *Cox2* deletion and a narrow yellow striping phenotype are inherited together in a maternal fashion. The striped plants are heteroplasmic for mutant and normal *Cox2* genes. Only the mutant *Cox2* gene is detected within the yellow stripes, whereas both normal and mutant forms of the gene are present in the green sectors of the NCS6 plants. In the green leaves of nonstriped relatives, only the normal *Cox2* gene is found. Both the structure and functioning of the chloroplasts in the yellow leaf sectors of NCS6 plants are altered. The pleiotropic effects of the NCS6 mutation suggest that mitochondrial function is required for the development of photosynthetically competent chloroplasts.

INTRODUCTION

Relatively little is known about the importance of specific mitochondrial functions in various types of higher plant cells. Experimental approaches utilizing inhibitors for analyzing the functioning of mitochondria in plant cells have provided valuable information but are limited in scope. Developmental analyses in many organisms have been greatly facilitated by the use of mutants. One would like to use mutations to examine the roles of mitochondria in different cells and tissues over the life span of the plant; however, those affecting vital mitochondrial functions would be expected to be lethal. A way around the problem of lethality is to use defined mutations in mitochondrial DNA that survive heteroplasmically with normal mitochondrial genomes. During development, sectors of abnormal tissue arise due to somatic segregation of the mutant from the normal organelles. These sectors can then be analyzed for defects in cellular metabolism. We have tested the validity of this approach by analyzing defective leaf sectors that arise in plants carrying a defined mitochondrial electron transport mutation.

There are currently a number of nonchromosomal stripe (NCS) mutants in maize that have been reported to carry deletions in specific essential mitochondrial genes (Newton and Coe, 1986; Feiler and Newton, 1987; Newton et al., 1989; Hunt and Newton, 1991). NCS mutant plants grow poorly and are characterized by variable necrotic or pale striping on leaves and aborted kernels on ears. It has been suggested that plants carrying an NCS mutation survive only because they are heteroplasmic for normal and mutant mitochondria and that somatic segregation of the mutant mitochondria results in the sectors of defective growth that are observed on the NCS plants (Newton and Coe, 1986). Indeed, slowly growing plants with more stripes do have correspondingly more of the mutated mitochondrial gene than do sibling plants that are more normal in appearance (Newton and Coe, 1986).

NCS6 plants have a partially deleted mitochondrial cytochrome oxidase subunit 2 (*Cox2*) gene resulting from recombination between 36 bp repeats, one of which is located within the first exon of the *Cox2* gene (Lauer et al., 1990). Both the *Cox2* mRNA and the COX2 protein are specifically reduced, whereas other mitochondrial genes and their RNA products are not affected (Lauer et al., 1990). This mutation is highly deleterious to the development of both vegetative and reproductive tissue, and all the plants analyzed have been heteroplasmic for the mutation. The stunted NCS6 plants exhibit narrow yellow stripes on the leaves and sectors of aborted kernels on the ears. This phenotype is exactly the same as that of NCS5, an independent mutation in *Cox2* (Newton et al., 1990), suggesting that it is the loss of cytochrome oxidase function rather than the particular rearrangement event that gives rise to the abnormalites. The fact that NCS5 and NCS6 plants have yellow leaf stripes is suggestive of chlorophyll and, accordingly, chloroplast defects. Therefore, both maize mitochondrial *Cox2* mutations appear to have pleiotropic effects in leaf cells.

In the current series of studies, we have confirmed the maternal inheritance of the NCS6 leaf sectors by analyzing reciprocal cross data. We have also shown that the yellow stripes on the heteroplasmic plants contain only the mutant mitochondrial *Cox2* gene. In addition, we present ultrastructural and physiological characterizations of the abnormal chloroplasts in mutant leaf sectors. Because the yellow sectors are very narrow, it is challenging to analyze the small number of chloroplasts available. Consequently, emphasis has been placed on utilizing nondestructive, in vivo methods for the analysis of chloroplast function.

[1] To whom correspondence should be addressed.

RESULTS

Maternal Inheritance of NCS6 Striping

Leaves of the NCS6 mutants are characterized by very slow growth and the presence of narrow yellow stripes. This phenotype has always cosegregated with a *Cox2* deletion (Lauer et al., 1990). The maternal inheritance of NCS6 striping was confirmed by reciprocal crosses. Table 1 shows the progeny analysis for one heavily striped (but relatively vigorous) NCS6 plant used as either a female (NCS6 × NOR) or a male (NOR × NCS6) in crosses with the same normal maize line. When the NCS6 plant was used as the male, only a normal ear resulted. A noticeably smaller ear with aborted kernels resulted when the NCS6 plant was used as the female parent in the reciprocal cross. Although more than 90% of the progeny kernels planted from the normal ear germinated and grew into normal, healthy plants, fewer than 60% of the (nonaborted) kernels from the mutant ear germinated or grew beyond the seedling stage. Furthermore, mutant (stunted and yellow-striped) progeny plants were seen only when NCS6 had been the maternal parent. DNA analysis of several of the yellow-striped individuals showed that they were heteroplasmic for the mitochondrial *Cox2* mutation (data not shown). In addition, all of the tested plants resulting from crosses with NCS6 as the male parent had only the normal *Cox2* gene.

In another cross (Table 1; NCS6-nd × NOR), we tested whether a nonstriped plant from another NCS6 family could transmit the striping phenotype. Nonmutant plants have been found occasionally in the same family as NCS6 mutant plants. Presumably, the egg giving rise to such a plant received none of the mutant mitochondria as a result of sorting out of the normal from the mutant mitochondria during development (Newton and Coe, 1986). Indeed, when such plants were tested, they were found to be homoplasmic for the normal *Cox2* gene (Lauer et al., 1990), which is consistent with this hypothesis. The "normal-derivative," or NCS6-nd plants (from the same maternal and paternal lineage as NCS6 but which are not striped), do not transmit the NCS6 striping (Table 1; NCS6-nd × NOR). Thus, there is a direct correlation between expression of the mutant phenotype and the ability to transmit it maternally.

Table 1. Progeny from Reciprocal Crosses between One Heavily Striped NCS6 Plant and a Normal Maize Line (NOR)

Cross	No. Kernels Planted	No. Plants	No. Mutant	No. Normal
NCS6 × NOR	70	39	39	0
NOR × NCS6	90	83	0	83
NCS6-nd × NOR	25	22	0	22

Reciprocal crosses demonstrate the maternal inheritance of the mutation. Plants that have "lost" the mutant phenotype (NCS6-nd) also do not transmit the mutation.

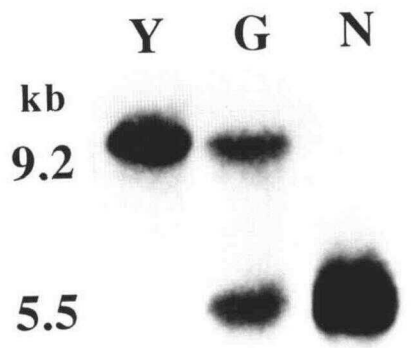

Figure 1. Analysis of Mutant and Normal *Cox2* Genes from NCS6 Leaf Sectors.

Total DNA from the NCS6 leaf stripes and control leaf used for fluorescence studies were digested with XhoI, and the gel blots were probed with a cloned *Cox2* gene. Lane Y, NCS6 yellow sector; lane G, NCS6 green sector; lane N, normal control. The 9.2-kb mutant and 5.5-kb normal *Cox2* XhoI fragments are indicated.

Analysis of Mitochondrial DNA in Leaf Sectors

To test whether the defective leaf stripes are enriched for the mitochondrial mutation, total DNA was extracted from the leaf sectors, and the relative amounts of mutant and normal mitochondrial *Cox2* genes were compared following XhoI digestion and DNA gel blot hybridizations using a cloned *Cox2* gene as the probe. Results of these experiments are shown in Figure 1.

The yellow sectors appeared to be highly enriched for the mitochondrial *Cox2* deletion (Figure 1, lane Y). No normal *Cox2* sequence was detected in the DNA from yellow stripes, even after prolonged exposures of the x-ray film to the blot (data not shown). In DNA from a control plant (a nonmutant relative), the probe hybridized only to the normal 5.5-kb XhoI fragment (Figure 1, lane N). Almost equal amounts of the mutant 9.2-kb and normal 5.5-kb XhoI fragments were detected in a green sector from a striped NCS6 plant (Figure 1, lane G). Although data for only one experiment are shown in Figure 1, these results have been replicated four times with different NCS6 and normal plants.

Chlorophyll Content and Chlorophyll *a/b* Ratios

Because the NCS6 stripes are yellow, a reduction in chlorophyll content was expected. The chlorophyll concentrations in normal green leaves measured between 3.0 and 3.9 mg per gram

fresh weight. In yellow sectors, the chlorophyll was reduced to as low as 28% of normal to as high as 40% of normal. The chlorophyll *a*/*b* ratios of normal green leaves ranged between 2.14 and 2.33. The chlorophyll *a*/*b* ratios were always reduced in the yellow sectors. However, the amount of the reduction was found to be quite variable. Yellow sectors from four different plants had chlorophyll *a*/*b* ratios of 1.21, 1.62, 1.91, and 1.99. This variation in the yellow sectors may depend, to some extent, on the age of the tissue.

Chlorophyll Fluorescence Induction Kinetics

Prior to DNA extractions, fluorescence induction kinetics measurements, which do not destroy the tissues, were conducted on the sectors to examine whether the photosynthetic light reactions were altered in the yellow stripes. A representative set of analyses is displayed in Figure 2. Figure 2, curve N shows a typical fluorescence induction curve for a leaf from a dark-adapted control plant. Immediately upon actinic illumination, an initial level of fluorescence (F_0) is attained. The fluorescence yield then increases more slowly over the following 2 sec to a maximum level (F_m). Fluorescence then decreases as the secondary electron accepters of photosystem II (PSII) are oxidized by photosystem I (PSI). Variable fluorescence (F_v) is $F_m - F_0$ and can be compared using the F_v/F_m ratio to compensate for differences in leaf chlorophyll concentration (Hipkins and Baker, 1986). F_v/F_m is a recognized measure of the quantum efficiency of PSII.

The fluorescence induction kinetics of a green sector from an NCS6 plant (Figure 2, curve G) shows a pattern similar to that of the normal leaf, except that it had a slightly lower F_0, whereas the F_m was 80% of the control leaf. As shown in Table 2, the F_v/F_m was 0.62 for the NCS6 green sector compared to 0.67 for the normal leaf. The fluorescence induction curve for a dark-adapted NCS6 yellow sector had a different pattern (Figure 2, curve Y). The F_0 and F_m values for the yellow sector were both reduced. Moreover, after F_m was reached, the curve did not decline in fluorescence, as is normally observed in plant leaves. The F_v/F_m was 0.61 (Table 2), suggesting that the functioning of PSII reaction centers and secondary electron acceptors is near normal. The slow decline in fluorescence after F_m suggests that electron transport subsequent to PSII is partially blocked or delayed.

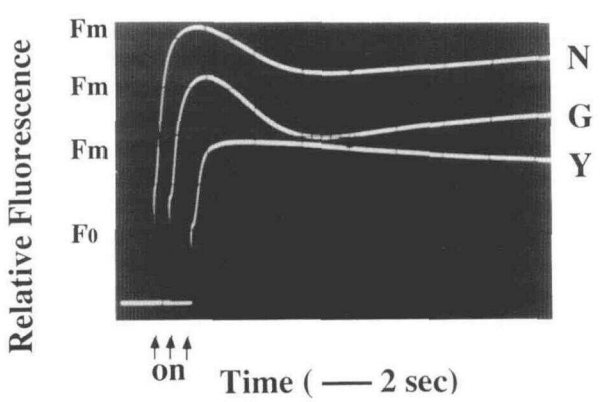

Figure 2. Chlorophyll Fluorescence Induction Kinetics of NCS6 Leaf Sectors.

Y, NCS6 yellow sector; G, NCS6 green sector; N, normal control. The fluorescence actinic light is on at the position of the arrow for each of the superimposed curves.

Table 2. Comparison of Fluorescence Induction Kinetics Parameters for NCS6 Leaf Sectors and Normal Leaf Tissue

Leaf Tissue	F_0	F_m	F_v	F_v/F_m
Normal	15	46	31	0.67
NCS6 Green	14	37	23	0.62
NCS6 Yellow	10	26	16	0.61

The shapes of the fluorescence induction curves for each type of leaf sector were reproducible from leaf to leaf and from plant to plant. Because the F_v/F_m ratios are not greatly different between the NCS6 leaf sectors and normal control leaves, the function of PSII appears to be little altered in NCS6 leaf sectors. The reduced values of F_0 and F_m in the yellow sectors are probably due to the reduced chlorophyll content in these sectors compared with normal green leaves. However, the unusual shape of the fluorescence trace cannot be explained by the decrease in chlorophyll content. The slow decline in fluorescence following F_m would suggest some limitation in electron transport occurring after PSII.

Chlorophyll Fluorescence Emission Spectra

To further characterize the photosynthetic apparatus using nondestructive methods in the very narrow mutant stripes, chlorophyll fluorescence emission spectra were conducted. Segments of NCS6 yellow and green leaf sectors and normal control leaves were excised immediately before measurement. Figure 3 shows a representative set of fluorescence emission of both NCS6 and normal control leaf tissues. A typical normal leaf (Figure 3, curve N) has a maximum fluorescence emission at 680 to 685 nm, in which the majority of the emission is from chlorophylls associated with PSII, and another emission peak at 740 nm, which is more closely associated with PSI chlorophyll–protein complex (Hipkins and Baker, 1986). The green sectors from NCS6 leaves (Figure 3, curve G) show the same pattern except that the level of the emission is reduced compared with the control leaf tissues. However, although the NCS6 yellow leaf sectors (Figure 3, curve Y) have the maximum emission at ~680 to 685 nm, there is only a small shoulder at 740 nm. There is some reduction of the 685 band, which probably reflects the reduced

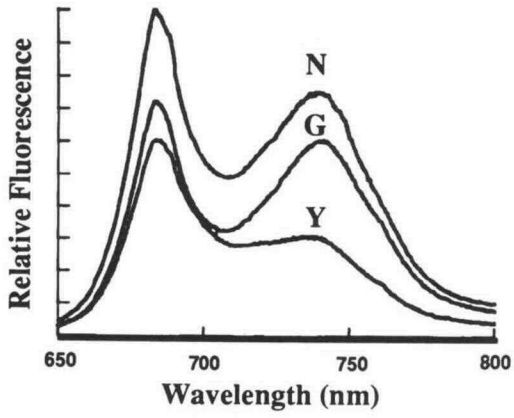

Figure 3. Chlorophyll Fluorescence Emission Spectra of Leaf Sectors.

Y, NCS6 yellow sector; G, NCS6 green sector; N, normal control.

concentration of chlorophyll. However, the loss of the 740 band in the yellow sectors suggests an additional reduction of the PSI chlorophyll–protein complex.

Thylakoid Membrane Proteins

Although most NCS6 yellow stripes are very narrow and yield only a little leaf tissue for thylakoid membrane preparations, enough material was collected for an extraction by pooling stripes from an individual. A small portion of each thylakoid preparation was tested for chlorophyll concentration and chlorophyll *a*/*b* ratios. Samples were equalized for loading on gels on the basis of chlorophyll concentration (15 μg/lane). Figure 4 shows the electrophoretic separation of thylakoid membrane proteins isolated from both NCS6 leaf sectors and normal control leaf tissue by lithium dodecyl sulfate (LDS)-PAGE. The chlorophyll *a*/*b* ratio of each sample is given in the figure legend. Because the samples were prepared and separated by electrophoresis under nondenaturing conditions, the positions of chlorophyll–protein "green" bands could be noted. After the electrophoretic separation, the gel was stained with Coomassie Brilliant Blue. A few differences in the polypeptide patterns can be seen. The major quantitative difference in thylakoid membranes isolated from NCS6 yellow stripes (Figure 4, lanes 4 and 5) is a reduction in an ~110-kD chlorophyll protein. This blue-green band has low visual fluorescence under UV light and all the distinctive characteristics of the chlorophyll–protein I complex of PSI (CPI; Metz and Miles, 1982), including dissociation when the thylakoids are heated prior to electrophoresis (data not shown). This complex has also been called PS 110 (Green, 1988) and the 110-kD Core Complex I (Bredenkamp and Baker, 1988). In contrast, thylakoid membranes isolated from NCS6 green leaf sectors (Figure 4, lanes 2 and 3) and a normal leaf (lane 1) show no significant differences in the levels of this chlorophyll–protein complex.

Carbon Dioxide Fixation

To determine whether chloroplasts fix carbon dioxide in the yellow stripes of NCS6 leaves, the ability of leaves to take up radiolabeled carbon dioxide was tested. Leaves of both NCS6 and normal control plants were exposed to $^{14}CO_2$ for 1 min and were then excised from the plant and quickly frozen for autoradiography.

Figure 5 shows autoradiographs of both NCS6 (Figure 5A) and normal control leaf segments (Figure 5B). The exposed (dark) areas of the film correspond to tissue that had fixed the $^{14}CO_2$, and the unexposed areas on the film that appear as clear areas represent the leaf tissue that did not fix $^{14}CO_2$. All of the normal control leaves were strongly and evenly labeled, suggesting that the carbon was being fixed efficiently during

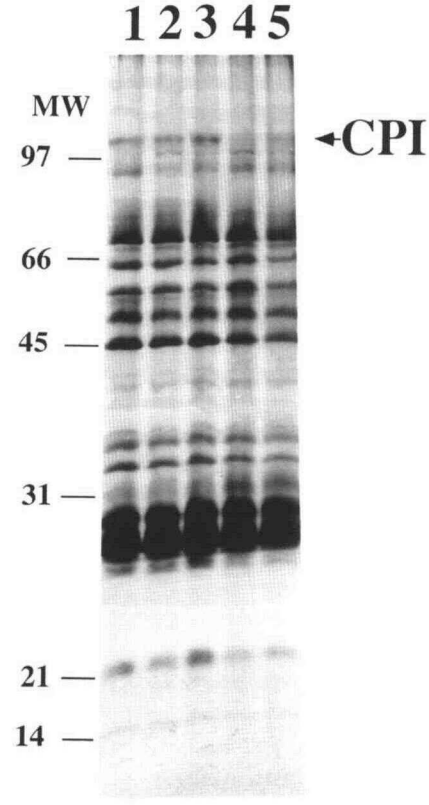

Figure 4. PAGE Analysis of Thylakoid Proteins from NCS6 Leaf Sectors.

The thylakoid preparations loaded in each lane were standardized by chlorophyll concentration and solubilized; the proteins were separated by LDS-PAGE on a linear gradient gel of 10 to 15% polyacrylamide at 4°C. Lane 1, normal control (chlorophyll *a*/*b* = 2.14); lane 2, green sector from NCS6 plant 1 (chlorophyll *a*/*b* = 2.27); lane 3, green sector from NCS6 plant 2 (chlorophyll *a*/*b* = 2.23); lane 4, yellow sector from NCS6 plant 1 (chlorophyll *a*/*b* = 1.91); lane 5, yellow sector from NCS6 plant 2 (chlorophyll *a*/*b* = 1.99). Positions of the molecular weight markers are given in kilodaltons to the left of the figure. The 110-kD CPI band is indicated.

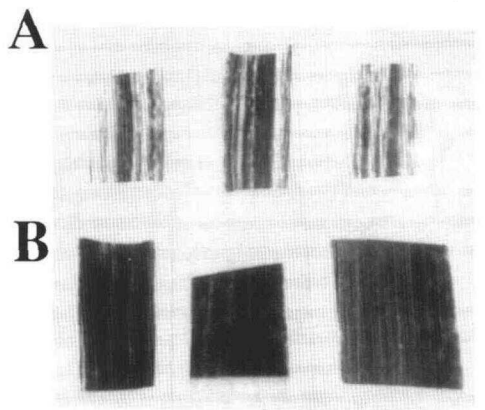

Figure 5. Autoradiographs of NCS6 and Normal Control Leaves Exposed to $^{14}CO_2$.

(A) Autoradiograph of labeled NCS6 leaf pieces.
(B) Autoradiograph of labeled control leaf pieces.

the brief exposure. In contrast, leaves from NCS6 plants showed variable labeling, ranging from no detectable labeling of the yellow sectors to heavy labeling of fully green sectors. Because stomata appear to be present and functional within the yellow sectors (data not shown), the observed dramatic reductions of labeling within the yellow sectors are probably due to the reduction in CO_2 fixation, rather than to a defect in leaf gas exchange.

Ultrastructural Analyses of NCS6 Yellow Sectors

Electron microscopy was used to determine whether the structure of the chloroplasts in NCS6 yellow sectors was altered. Young leaf tissues from field-grown NCS6 and normal control plants were simultaneously harvested and processed for electron microscopic analysis. Figure 6A illustrates a section of normal control leaf tissue showing chloroplasts in both bundle sheath and mesophyll cells. The bundle sheath chloroplasts (upper cell) had accumulated multiple starch granules (despite having been collected in the early morning of a cloudy day), and the mesophyll chloroplasts (lower cell) contained the expected grana stacking. In contrast, a section of an NCS6 yellow stripe shows abnormal chloroplasts (Figure 6B). Chloroplasts from the yellow sector have reduced amounts of thylakoid membranes and less grana stacking, and none of the chloroplasts accumulates starch granules. In addition, there appear to be fewer chloroplasts within smaller cells (data not shown).

Normal leaf mitochondria have a characteristic appearance with extensive inner membranes and large intermembrane volumes (Figure 6C). These have not been seen within the cells from the yellow leaf stripes. The most normal looking mitochondria within mutant sectors appear to have reduced cristae and relatively little intermembrane space (Figure 6D), although they do not appear to be much reduced in size.

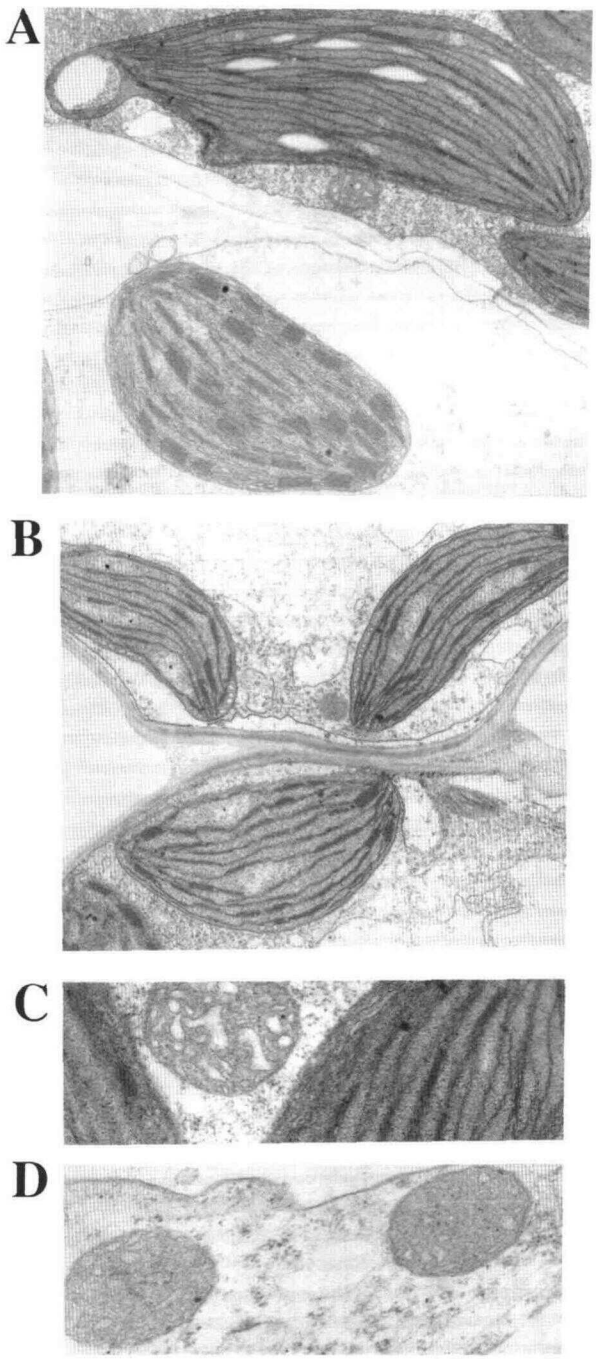

Figure 6. Electron Micrographs of Leaf Tissue.

(A) Chloroplasts in a normal control plant (×10,000). The upper cell is a bundle sheath cell and the lower one is a mesophyll cell.
(B) Chloroplasts in an NCS6 yellow sector (×10,000).
(C) A mitochondrion in a normal control plant (×30,000).
(D) Mitochondria in an NCS6 yellow sector (×30,000).

DISCUSSION

Reciprocal cross data confirm that NCS6 sectoring is maternally inherited and that NCS6 striped plants only arise from plants with visible sectors. NCS6 striped plants had previously been shown to be heteroplasmic for a mitochondrial *Cox2* mutation (Lauer et al., 1990), which is consistent with the inheritance pattern of the mutation. The yellow leaf stripes on NCS6 plants have now been shown by DNA gel blot analysis to contain detectable amounts of only the mutant *Cox2* genes. This provides a direct test for homoplasmy of a mitochondrial mutation in an NCS pale stripe.

We had previously postulated that the pale leaf sectors in NCS mutants are homoplasmic for the mitochondrial mutation due to segregation of the defective mitochondria from the normal organelles during somatic divisions from the initially mixed (heteroplasmic) egg cells (Newton and Coe, 1986; Newton et al., 1989). We have assumed that the mitochondria contain either mutant or normal genomes. The data presented here demonstrate that the defective NCS6 leaf sectors are indeed highly enriched for the mutant mitochondria. However, our results do not rule out the possibility that there may be a few normal mitochondria in a yellow leaf cell. They could be present at a very low level, below the detection limits of the DNA hybridization procedure used. In this case, the mutant phenotype could be explained by a "threshhold" effect: the few normally functioning mitochondria would not be enough to rescue the cell.

The data from the present study and from previous work (Lauer et al., 1990) strongly support the hypothesis that the genetic basis of the NCS6 mutation is a mitochondrial DNA rearrangement that interrupts the *Cox2* gene and leads to a partial deletion of that gene. Thus, we propose that the chlorophyll and chloroplast abnormalities we report are secondary, pleiotropic effects of the NCS6 mitochondrial electron transport lesion.

Yellow sectors have only ~30% of the normal concentration of chlorophyll and both the structure and function of the chloroplasts are abnormal. Electron microscopic analysis of the chloroplasts shows that they are smaller and have a less well-developed membrane system. The observation that the total mass of thylakoid membrane is reduced in the mutant sectors (although we have not quantified the extent of the reduction) is consistent with a reduction in total chlorophyll. In addition, analysis of the thylakoid membrane proteins suggests that there is a generally proportionate reduction in the proteins; that is, with the exception of CPI, most thylakoid proteins appear to be present at similar levels in normal leaves and NCS6 yellow stripes when they are normalized on the basis of chlorophyll concentration.

Because the NCS6 plants are generally very defective and the yellow stripes tend to be small, it is difficult to isolate large enough amounts of thylakoid membranes for traditional oxygen evolution studies of photosynthesis. For this reason, we chose to use primarily nondestructive, fluorescent measurements of chloroplast function. The data from fluorescence induction kinetics and from fluorescence emission spectra suggest a limitation, but not a block, in electron transport after PSII in the NCS6 sectors. The limitation is probably not at the cytochrome b_6/f membrane complex. If it were, we would expect a much more rapid rise in the fluorescence kinetics and a reduced F_v, as is observed for the maize b_6/f mutants (Metz et al., 1983). The fluorescence kinetics profile in the NCS6 yellow sectors more closely resembles the shape of the PSI *hcf* (high chlorophyll fluorescence) mutants described by Cook and Miles (1990), although the level of fluorescence in the NCS6 stripes is not high, as would be expected in an *hcf* mutant.

The fluorescence emission spectrum of the NCS6 mutant sector does suggest that, in addition to a general reduction of all fluorescence forms, there is a greater loss of the 740-nm band. This is expected if there is a nonuniform loss of CPI in the chloroplasts. If the reduction in chlorophyll were due to loss of the light harvesting complex, as in the well-documented barley *chlorina f2* mutant (Burke et al., 1979), a shift in the 740-nm band to the 720- to 725-nm region would be expected. The decrease in the chlorophyll *a*/*b* ratio as well as the reduction, rather than a shift of the 740-nm fluorescence band, argues for an effect on the PSI/CPI thylakoid complex in the yellow sectors. Support for this effect is provided by gel analyses of thylakoid proteins from the mainly yellow sectors. Although most of the proteins are decreased proportionally to the decrease in chlorophyll concentration, there is a significant additional reduction in the ~110-kD chlorophyll–protein complex of PSI. This CPI band, associated predominantly with chlorophyll *a*, reflects the amount of stably assembled PSI complex (Cook and Miles, 1990), and the reduction in this band does not necessarily mean that any specific polypeptide components of PSI are missing from the yellow sectors. The assembled CPI complex becomes evident at a late stage during the development of the chloroplasts (Bredenkamp and Baker, 1988). Thus, we suggest that the effect of the mitochondrial mutation is a late arrest of chloroplast biogenesis.

The effect on photosynthetic electron transport of the NCS6 mutation in yellow stripes cannot account for the apparently complete loss of carbon reduction. If the sectors retain 30% of the chlorophyll with limited function, we would expect to detect some level of CO_2 fixation. In addition to the lack of detectable carbon fixation, chloroplasts in the yellow sectors accumulate no starch granules. These observations implicate the mitochondrion in the development or activity of photosynthetic carbon metabolism.

Chloroplast abnormalities appear to be associated with several different mitochondrial mutations in higher plants. We have previously reported that NCS2 pale green leaf sectors have functionally and structurally altered chloroplasts, although the primary defect is in the mitochondrial DNA (Roussell et al., 1991). A mitochondrial DNA rearrangement also appears to

be correlated with the maternally inherited variegation of plants carrying the *chm* ("chloroplast mutator") gene in Arabidopsis (Martínez-Zapater et al., 1992).

Biochemical experiments have demonstrated an association between mitochondrial oxidative phosphorylation and photosynthetic function. Low levels of oligomycin, which do not interfere with photosynthesis in isolated chloroplasts, have been shown to partially inhibit photosynthetic oxygen evolution in intact protoplasts and leaves of barley (Krömer and Heldt, 1991). The partial inhibition of photosynthesis is apparently due to the specific inhibition of mitochondrial ATPase by oligomycin and corresponding effects on cellular ATP levels and reducing equivalents (Krömer and Heldt, 1991).

It is still not clear exactly how mitochondria affect chloroplast biogenesis and function. One possibility to explain our results is that the lack of a specific metabolite or energetic compound (such as ATP) supplied by the mitochondria during leaf development leads to the arrest of chloroplast biogenesis in NCS6 yellow sectors. There is some evidence that chloroplasts can import ATP during early leaf development. Exogenous ATP stimulated photosynthetic oxygen evolution in isolated chloroplasts from young pea shoots, but not from older pea leaves (Robinson and Wiskich, 1977). Our results demonstrate that a lack of oxidative phosphorylation is associated with abnormal chloroplast morphology. Conversely, relatively normal chloroplast morphology can be seen for some of the maize *hcf* mutants despite complete blocks in photosynthesis, provided cellular energy is provided from the aerobic metabolism of starch (Miles, 1982). Growth of such *hcf* plants continues for ~14 days until starch reserves are exhausted.

The possible roles of mitochondrial respiration in chloroplast biogenesis have long been a subject of speculation in the literature (reviewed by Wellburn, 1984). However, such roles have been difficult to ascertain using only physiological tests, inhibitor studies, and electron microscopy. The addition of a genetic approach has proved invaluable in many studies of developmental phenomena. In this study, we have analyzed the effects of a defined mitochondrial mutation in leaf sectors of heteroplasmic plants, in which mutant tissues are defective but still viable. Our results suggest that mitochondrial oxidative phosphorylation may be required not only for maintenance of full photosynthetic function, but also for the development of photosynthetically competent chloroplasts. Additional analyses of chloroplast development in normal and mutant leaves will be necessary to determine more precisely how and when chloroplast biogenesis is interrupted or altered in the NCS6 mutant tissue.

METHODS

Plant Materials

The genotype of the nonchromosomal stripe (NCS6) cytochrome oxidase (*Cox2*) deletion has been reported in detail by Lauer et al. (1990). The mutation arose spontaneously in a fertile revertant strain of a cytoplasmic male sterile type S mitochondrial genotype in the WF9 nuclear background. The normal plants used as controls were close relatives of the mutants. The 1- to 2-month-old plants used in this study were grown in Columbia, MO, in the greenhouse during the winter months or in the field during the summer.

Total DNA Extraction and DNA Gel Blot Analysis

Following the chlorophyll fluorescence measurements, the same leaf sectors were frozen and ground in liquid nitrogen. Total DNA from both NCS6 and nonmutant relatives was isolated using urea/sarkosyl lysis and phenol/chloroform extractions (Cone, 1989). DNA was digested with XhoI, separated by electrophoresis on 0.7% agarose gels, blotted onto nylon membranes, and hybridized under stringent conditions with radiolabeled probe following standard techniques (Sambrook et al., 1989). The 5.5-kb XhoI fragment (from pCLK3) carrying the normal *Cox2* gene was used as the probe (Newton et al., 1990).

Determination of Chlorophyll Content and Chlorophyll *a*/*b* Ratios

Leaf pieces were excised, measured, and weighed. Chlorophyll was extracted with 80% acetone, and absorption at 663 and 645 nm was measured in a spectrophotometer (Lambda 6; Perkin-Elmer). Formulas given in Arnon (1949) were used to calculate the amounts of chlorophylls.

Chlorophyll Fluorescence Measurements

The chlorophyll fluorescence induction kinetics experiments were conducted as described previously (Miles, 1980, 1982). Fully expanded leaves were selected for the measurements. From very affected NCS6 plants, narrow sectors that appeared either yellow or green were excised. For comparison, green tissue from nonmutant normal relatives was also excised. The excised leaf sectors were dark adapted for 2 min. The upper leaf surface was then exposed to actinic light (through a 0.2- × 2-cm slit) at 100 μmol m^{-2} sec^{-1} with an illumination peak of 658 nm in an MF-1 chlorophyll fluorometer (constructed locally by D. Miles). Fluorescence emission was measured 45° to the actinic light. The fluorescence signal was monitored with a storage oscilloscope (model 5103N; Tektronix Inc., Beaverton, OR) and recorded by photography. After the measurements, the leaf sectors were retained for total DNA isolations. For fluorescence emission spectra (Hipkins and Baker, 1986), leaf sectors were excised from fully expanded leaves and the upper leaf surface was exposed (through a 0.2- × 2-cm slit) to actinic light at 100 μmol m^{-2} sec^{-1} with an illumination peak of 470 nm at 23°C using a Fluorolog (model F-112; SPEX Industries, Edison, NJ). Fluorescent emission angle was measured 22.5° to the actinic beam and 12.25° to the upper leaf surface to reduce reabsorption of the fluorescence. This geometry improves the ability to collect fluorescence in the 740-nm region (D. Miles, unpublished data).

Thylakoid Membrane Isolation and LDS-PAGE

Yellow and green leaf sectors were excised from NCS6 plants, and control sectors were excised from green leaves of nonmutant related

plants. Approximately 0.5 g fresh weight was used for each preparation, and this amount of the yellow samples could be obtained only by pooling from several leaves of an individual plant. Thylakoid membranes were isolated by the method of Metz and Miles (1982) and were washed three times. The chlorophyll content of the thylakoid protein samples was measured (Arnon, 1949), and chlorophyll *a*/*b* ratios were calculated. Protein samples for lithium dodecyl sulfate (LDS)-PAGE were standardized on the basis of chlorophyll (15 μg of chlorophyll per lane). Prior to electrophoresis, LDS and dithiothreitol were added to the samples to yield final concentrations of 2% (w/v) and 30 mM, respectively. Linear gradient gels of 10 to 15% (w/v) polyacryamide were used for 4°C electrophoresis, as described by Delepelaire and Chua (1979). Gels were stained with Coomassie Brilliant Blue R250, destained, and photographed.

CO_2 Fixation

Leaves attached to 6-week-old greenhouse-grown plants were placed in a sealed illuminated cuvette (providing 850 μmol photons m^{-2} sec^{-1} PPFD at the leaf surface), preequilibrated (with 37.0 Pa CO_2), and exposed to 15 μCi $^{14}CO_2$ for 1 min at room temperature, as previously described (Roussell et al., 1991). Labeled leaf sectors were rapidly excised and quickly frozen between two aluminum plates prechilled in liquid nitrogen. X-ray film (Kodak RP) was placed directly on the frozen leaf sectors, and the film was developed after 14 days exposure at −80°C.

Electron Microscopy

Leaf tissue samples were collected, fixed, and embedded in polybed 812 resin, as previously described (Thompson et al., 1983; Roussell et al., 1991). Two-micron plastic sections were stained with alkaline toluidine blue and examined by light microscopy to locate areas of interest. Ultrathin sections cut with a diamond knife were placed on uncoated copper grids and poststained with uranyl acetate and lead citrate (Roussell et al., 1991). The stained sections were examined and photographed on an electron microscope (Hitachi H-600).

ACKNOWLEDGMENTS

We are grateful to Steve Pallardy for his assistance in the carbon fixation experiments and to Darrell Kinden and Preston Stogsdill for help with the electron microscopic techniques. This work was supported by National Science Foundation Grants No. DCB8858257 and No. DMB9019249 to K.J.N. and U.S. Department of Agriculture Grant No. 90-37280-5459 to C.D.M.

Received May 6, 1993; accepted June 22, 1993.

REFERENCES

Arnon, D. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. **24,** 1–15.

Bredenkamp, G.J., and Baker, N.R. (1988). The changing contribution of LHCI to photosystem I activity during chloroplast biogenesis in wheat. Biochim. Biophys. Acta **934,** 14–21.

Burke, J.J., Steinback, K.E., and Arntzen, C.J. (1979). Analysis of the light-harvesting pigment–protein complex of wild type and a chlorophyll-b–less mutant of barley. Plant Physiol. **163,** 237–243.

Cone, K. (1989). Yet another rapid plant DNA prep. Maize Genet. Coop. Newslet. **63,** 68–69.

Cook, W. B., and Miles, D. (1990). Anomalous electron transport activity in a Photosystem I–deficient maize mutant. Photosyn. Res. **24,** 81–88.

Delepelaire, P., and Chua, N.-H. (1979). Lithium dodecyl sulfate/polyacrylamide gel electrophoresis of thylakoid membranes at 4°C: Characterizations of two additional chlorophyll *a*–protein complexes. Proc. Natl. Acad. Sci. USA **76,** 111–115.

Feiler, H.S., and Newton, K.J. (1987). Altered mitochondrial gene expression in the nonchromosomal stripe 2 mutant of maize. EMBO J. **6,** 1535–1539.

Green, B.R. (1988). The chlorophyll–protein complexes of higher plant photosynthetic membranes or just what green band is that? Photosyn. Res. **15,** 3–32.

Hipkins, M.F., and Baker, N.R. (1986). Spectroscopy. In Photosynthesis Energy Transduction. A Practical Approach, M.F. Hipkins, and N.R. Baker, eds (Oxford: IRL Press), pp. 51–101.

Hunt, M.D., and Newton, K.J. (1991). The NCS3 mutation: Genetic evidence for the expression of ribosomal protein genes in *Zea mays* mitochondria. EMBO J. **10,** 1045–1052.

Krömer, S., and Heldt, H.W. (1991). On the role of mitochondrial oxidative phosphorylation in photosynthesis metabolism as studied by the effect of oligomycin on photosynthesis in protoplasts and leaves of barley (*Hordeum vulgare*). Plant Physiol. **95,** 1270–1276.

Lauer, M., Knudsen, C., Newton, K.J., Gabay-Laughnan, S., and Laughnan, J.R. (1990). A partially deleted mitochondrial cytochrome oxidase gene in the NCS6 abnormal growth mutant of maize. New Biologist **2,** 179–186.

Martínez-Zapater, J.M., Gil, P., Capel, J., and Somerville, C.R. (1992). Mutations at the Arabidopsis *CHM* locus promote rearrangements of the mitochondrial genome. Plant Cell **4,** 889–899.

Metz, J.G., and Miles, D. (1982). Use of a nuclear mutant of maize to identify components of photosystem II. Biochim. Biophys. Acta **681,** 95–102.

Metz, J.G., Miles, D., and Rutherford, A.W. (1983). Characterization of nuclear mutants of maize which lack the cytochrome f/b-563 complex. Plant Physiol. **73,** 452–459.

Miles, D. (1980). Mutants of higher plants: Maize. In Photosynthesis, Part C, Methods in Enzymology, Vol. 69, A. San Pietro, ed (New York: Academic Press), pp. 3–22.

Miles, D. (1982). The use of mutations to probe photosynthesis in higher plants. In Methods in Chloroplast Molecular Biology, M. Edelman, R.B. Hallick, and N.-H. Chua, eds (New York: Elsevier), pp. 75–107.

Newton, K.J., and Coe, E.H. (1986). Mitochondrial DNA changes in abnormal growth mutants of maize. Proc. Natl. Acad. Sci. USA **83,** 7363–7366.

Newton, K.J., Coe, E.H., Gabay-Laughnan, S., and Laughnan, J.R. (1989). Abnormal growth phenotypes and mitochondrial mutants in maize. Maydica **34,** 291–296.

Newton, K.J., Knudsen, C., Gabay-Laughnan, S., and Laughnan, J.R. (1990). An abnormal growth mutant in maize has a defective mitochondrial cytochrome oxidase gene. Plant Cell **2,** 107–113.

Robinson, S.P., and Wiskich, J.T. (1977). Pyrophosphate inhibition of carbon dioxide fixation in isolated pea chloroplasts by uptake in exchange for endogenous adenine nucleotides. Plant Physiol. **59,** 422–427.

Roussell, D.L., Thompson, D.L., Pallardy, S.G., Miles, D., and Newton, K.J. (1991). Chloroplast structure and function is altered in the NCS2 maize mitochondrial mutant. Plant Physiol. **96,** 232–238.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Thompson, D.L., Walbot, V., and Coe, E.H. (1983). Plastid development in iojap- and chloroplast mutator-affected maize plants. Am. J. Bot. **70,** 940–950.

Wellburn, A. R. (1984). Ultrastructural, respiratory and metabolic changes associated with chloroplast development. In Chloroplast Biogenesis, N.R. Baker, and J. Barber, eds (Amsterdam: Elsevier Science Publishers), pp. 253–303.