PCR-mediated Detection of Heteroplasmy in Maize Mitochondrial Mutants

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The NCS2 (nonchromosomal stripe) mutation arose in maize plants carrying the CMS-T cytoplasm in a WF9 nuclear background. Heteroplasmic plants impaired by this trait are characterized by pale-green leaf striping and sectors of aborted kernels on the ears. Additionally, overall plant growth is reduced and development is delayed.^(1–3)

On the molecular level it was shown that NCS2 mutation results from a rearrangement in mitochondrial DNA (mtDNA). In NCS2 mtDNA, a novel 21-kb *XhoI* restriction fragment could be detected. This fragment shares homology with an 8-kb restriction fragment at the 5' end and a 15-kb *XhoI* restriction fragment at the 3' end. The appearance of the novel fragment is the result of a rearrangement in the mtDNA between sequences present on the 8-kb *XhoI* and the 15-kb *XhoI* restriction fragment.⁽⁴⁾

Because the mutation is lethal during kernel development, surviving NCS2 plants are heteroplasmic, that is, they contain a mix of mutant and normal mitochondria. During plant development, sorting out into mutant and normal sectors occurs through somatic segregation. The occurrence and the amount of the novel 21-kb *XhoI* restriction fragment are correlated with the phenotype and also with the reduction of the normal 8-kb band.

We routinely perform Southern blot analyses on restriction digests of purified mtDNA to detect heteroplasmic plants. This method requires relatively large amounts of material and is rather time consuming. We decided to try a PCR-mediated approach to obtain equivalent data in a short time from small tissue samples. This method uses a set of three primers in the same reaction to distinguish between amplification products representing the normal and the mutant DNA. The use of three PCR primers in a simultaneous reaction has been used previously to confirm the identity of a probe.⁽⁵⁾

Because we wished to examine whether the region of interest is transcribed, we modified this approach. mtRNA was reverse transcribed with random hexamer primers, and the resulting single-stranded cDNA was used in the same PCR protocol.

The methods described in this paper are simple and fast, allowing one to screen multiple samples in a relatively short time.

MATERIALS AND METHODS

Plant Material

The NCS2 mitochondrial mutation arose in maize plants carrying the CMS-T cytoplasm in a WF9 nuclear background.^(1,6) Normal relatives of NCS2 mutants were used as control plants.

Isolation of Total DNA

Fully expanded leaves were selected for heteroplasmic leaf sector analysis. Leaf sectors were excised from NCS2 plants. For comparisons, green tissues from nonmutant relatives were also excised. Excised leaf sectors were quickly frozen and ground in liquid nitrogen, and total DNA was isolated using urea/Sarkosyl lysis and phenol–chloroform extraction.⁽⁷⁾

Isolation of Mitochondria, mtDNA, and mtRNA

Mitochondria, mtDNA, and mtRNA were isolated from unpollinated maize ear shoots as described previously.^(8,9)

First-strand cDNA Synthesis

Approximately 10 µg of mtRNA was incubated in a 50-µl reaction with 13 units of DNase I (Worthington, Freehold, NJ) in 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, for 15 min at 37°C. mtRNA was extracted once with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and once with chloroform/isoamyl alcohol (24 : 1), and ethanol precipitated. After resuspension in DEPC–water, mtRNA concentrations were estimated by measuring the OD₂₆₀. For the reverse transcription reaction, 1 µg of mtRNA was combined with 50 ng of random hexamer primers and incubated at 70°C for 10 min. After chilling on ice, the reaction was brought to 19 µl by adding 5 µl of 5× reaction buffer (250 mM Tris-HCl at pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 µl of 10 mM DTT, and 1 µl of 10 mM dNTP. The reaction mixture was incubated at 42°C for 2 min to adjust the temperature. After adding 200 units of Superscript Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD), the reaction was carried out at 42°C for 1 hr. The single-stranded cDNA was used directly for amplification.

Technical Tips

Conditions for PCR

Standard conditions for the PCR reactions were 30 cycles of amplification in 50 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, and 1.5 mM dNTP. One hundred nanograms of each primer and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI) were used. The temperature/time scheme used was 1 min at 94°C, 1.5 min at 54°C, and 1.5 min at 72°C. The PCR products were analyzed on 1.5% agarose gels using TBE buffer and were photographed under UV illumination.

Primer Design

The sequences of the primers were based on the DNA sequences for normal and mutant NCS2 (J. Marienfeld and K. Newton, unpubl.). The set of primers (Fig. 1) consists of a common upstream primer (P1), a downstream primer derived from the normal sequence (P2), and a downstream primer derived from the NCS2 sequence (P3). The primer mixture can amplify both the NCS2 mutant and the normal sequence as distinguishable PCR products. The sequences of the oligonucleotides used are PR1, 5'-CCA TAG TTC TTC CGT GCG TC-3', PR2, 5'-TGA TCG TGT CAT GTG CAA CG-3', and PR3, 5'-TTA ATC GGC CCT AAG GGA G-3'.

Southern Analyses

A genomic clone from normal mtDNA covering the region around the rearrangement site (J. Marienfeld and K. Newton, unpubl.) was used in hybridization analysis of DNA from both NCS2 and nonmutant relatives. The fragment

FIGURE 1 Strategy for detection of PCR products in heteroplasmic tissue. Primer 1 (P1) is homologous to both normal and NCS2 mitochondrial DNA, whereas primer 2 (P2) and primer 3 (P3) are specific for normal and rearranged NCS2 mitochondrial DNA, respectively. The expected sizes, deduced from the sequences, are 235 bp for the normal amplification product (P1 to P2) and 132 bp for the NCS2 amplification product (P1 to P3).

was isolated and labeled using the oligo-labeling kit from Pharmacia (Piscataway, NJ) following standard protocols.⁽¹⁰⁾ The conditions for hybridization have been described previously.⁽⁴⁾

RESULTS

We have developed a simple and reliable PCR-mediated method to detect heteroplasmy in different tissues and plants. We have designed a set of three primers that allow amplification of one fragment specific to the normal mitochondrial genome and one fragment specific to the mutant genome in the same reaction. Primer 1, which is common to both normal and mutant DNA, is used in conjunction with two other primers: one that has been derived from the normal 8-kb sequence downstream of the rearrangement site, and another derived from sequence of the rearranged NCS2 mutant gene. By using a set of three primers in the PCR reaction, two differently sized fragments are amplified from the mutant and normal genome (Fig. 1).

Comparison of Southern Data with PCR Results

To test whether the PCR amplification results reflected the amounts of mutant and normal DNA actually present in the samples, we performed both PCR and Southern analysis on the same mtDNA samples.

For Southern analyses, the same mtDNA samples used for PCR were digested with *Xho*I, and Southern blots of the DNA gels were hybridized with a labeled gene probe from normal mtDNA covering the region around the rearrangement site (J. Marienfeld and K. Newton, unpubl.). In mtDNA from the control plants, the probe hybridized only to the normal 8-kb *Xho*I restriction fragment (Fig. 2A, lane 1). In mtDNA isolated from unpollinated ears of NCS2 striped plants, the normal and the mutant fragments were detected in ratios that reflect the severity of the mutation. mtDNA from a heavily striped plant showed mostly the mutant 21-kb *Xho*I restriction fragment and a very light normal 8-kb band (Fig. 2A, lane 2). In comparison, mtDNA samples isolated from moderately affected NCS2 plants contained increased amounts of the normal fragment (Fig. 2A, lane 3).

The results of the PCR amplifications

FIGURE 2 Heteroplasmy analysis of maize mtDNA. (A) Southern analysis of mtDNA. Approximately 2 µg of mtDNA per lane was loaded. (B) PCR products of the same mtDNA as in A. Approximately 4 ng of mtDNA was used in each PCR reaction. (C) PCR analysis of total DNA from leaf tissue. Approximately 10 ng of total leaf DNA was used in each PCR reaction. (A,B, lanes 1) mtDNA from a normal plant. (A,B, lanes 2) mtDNA from a very affected plant. (A,B, lanes 3) mtDNA from a moderately affected NCS2 plant. (C, lane 1) Total DNA from a NCS2 leaf; (lane 2) total DNA from a normal control leaf. Normal (N, 235 bp) and mutant (M, 132 bp) bands in both Southern analysis and PCR are indicated.

were consistent with those from Southern analyses. The relative ratios of mutant and normal bands from the Southern analyses correlated well with the PCR amplification data (Fig. 2A,B). As in the Southern analysis, only the normal band was detected from the normal control sample (Fig. 2B, lane 1). In the heteroplasmic samples, the ratios of normal and mutant bands reflected the severity of the defective phenotype (Fig. 2B, lanes 2,3).

Leaf Sector Analysis

For the analysis of leaf sectors, samples were taken from striped mutant plants or from normal relatives. Total DNA was extracted, and the mitochondrial genomes were analyzed by PCR amplification of the rearrangement region. Typical results are shown in Figure 2C. A heteroplasmic NCS2 plant showed both normal and mutant mitochondrial gene amplification (Fig. 2C, lane 1). The control leaf showed no detectable mutant sequence by PCR amplification (Fig. 2C, lane 2).

Reverse Transcription-PCR

To determine whether the normal and/or the NCS2 gene were actually transcribed, we reverse-transcribed mtRNA from both mutant and normal mito-

chondria using random hexamer primers. Using the single-stranded cDNA (sscDNA) as template for PCR amplifications, we were able to detect a fragment of 235 bp representing the transcript of the normal mitochondria (Fig. 3, lane A). When sscDNA from mitochondria of NCS2 striped plants was used as a template, a fragment of 132 bp was also amplified (Fig. 3, lanes B,C).

Differences in the amount of amplified fragment could be correlated with the severity of the mutation in the plants investigated: the more severely affected plants had greater amounts of the 132-bp fragment representing the mutant transcript. For the 235-bp fragment representing the normal gene transcript, we observed a decrease in band intensity with increasing severity of the mutation (Fig. 3, lanes B,C).

DISCUSSION

The PCR-mediated approach described in this paper is a simple and reliable method to screen for heteroplasmy. It facilitates screening of large numbers of plants and different tissues because it can be performed on total DNA that is isolated easily from small amounts of tissue. The PCR method yields comparable results with those obtained by standard mtDNA gel blot analyses. These results were highly reproducible. When we used the same DNA samples in different amplification reactions, we obtained simi-

lar band intensities of the PCR products as judged by visual inspection of the gels (data not shown).

To determine whether the normal and the mutant NCS2 genes were actually transcribed, we modified the PCR-mediated approach such that sscDNA generated from normal and NCS2 mtRNA templates was used in the PCR reactions. The results presented in this paper show that the mutant NCS2 gene, as well as the normal gene, is transcribed. Furthermore, the band intensities yielded from different samples reflected the severity of the mutant trait.

The method described here has the potential to be used for quantitative PCR^(11,12) to determine copy numbers and levels of expression, respectively. However, our main focus was the detection of heteroplasmy in different tissues from small samples. The simple PCR-mediated approach described here allows one to assay the relative amounts of mutant and normal alleles in heteroplasmic plants. Furthermore, the reverse transcription-PCR (RT-PCR) based method facilitates the detection of transcript levels in different plants. This simple and reliable technique should be useful in the analysis of other heteroplasmic traits, especially in tissues where the impairment of organellar function does not result in an observable phenotype.

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FIGURE 3 PCR amplification following reverse transcription of normal and NCS2 mitochondrial RNA. (Lane A) A 235-bp product representing the normal transcript is amplified. (Lane B) Amplification of sscDNA generated from mtRNA of a very affected plant. The 132-bp fragment representing the NCS2 transcript is prominent; the normal 235-bp fragment is barely detectable. (Lane C) Amplification of sscDNA generated from mtRNA of a moderately affected plant. Both the normal 235-bp fragment and the mutant 132-bp fragment representing the NCS2 transcript are present.

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