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MOLECULAR ANALYSES OF NUCLEAR-CYTOPLASMIC INTERACTIONS AFFECTING PLANT GROWTH AND YIELD

Summary

Mitochondria have a central role in the production of cellular energy. The biogenesis and functioning of mitochondria depends on the expression of both mitochondrial and nuclear genes. One approach to investigating the role of nuclear-mitochondrial cooperation in plant growth and development is to identify combinations of nuclear and mitochondrial genomes that result in altered but sublethal phenotypes. Plants that have certain maize nuclear genotypes in combination with cytoplasmic genomes from more distantly-related teosintes can exhibit "incompatible" phenotypes, such as reduced plant growth and yield and cytoplasmic male sterility, as well as altered mitochondrial gene expression. The characterization of these nuclear-cytoplasmic interactions was the focus of this grant. We were investigating the effects of two maize nuclear genes, *Rcm1* and *Mct*, on mitochondrial function and gene expression. Plants with the teosinte cytoplasm and homozygous for the recessive *rcm* allele are small ("miniature") and slow-growing and the kernels are reduced in size. We mapped this locus to molecular markers on chromosome 7 and attempted to clone this locus by transposon tagging. The effects of the nuclear-cytoplasmic interaction on mitochondrial function and mitochondrial protein profiles were also studied. The product of the *Mct* allele, present in certain maize lines, specifically alters the cytochrome oxidase subunit 2 transcript pattern in the mitochondria from the perennial teosintes. It appears to be a gene-specific transcription factor that can activate transcription from a "cryptic" mitochondrial promoter. It is interesting that the plants carrying perennial teosinte cytoplasm are male sterile in the presence of the dominant *Mct* allele. This suggests that there may be a stage-specific effect of the *Mct* transcription factor that is associated with the expression of cytoplasmic male sterility.

Background

One way to investigate the role of nuclear-mitochondrial cooperation in plant growth and development is to identify combinations of nuclear and mitochondrial genomes that result in altered but sublethal phenotypes. Combinations of nuclear and cytoplasmic genomes that have diverged during evolution can be useful for such investigations.

We have focused on a defined system in the genus *Zea*. Maize is an important crop plant for which many inbred lines have been developed. Wild relatives of maize, the teosintes, have been classified according to their relationships to maize. The cytoplasm from the most distantly related teosintes (*Zea luxurians*, *Zea perennis*, *Zea diploperennis*) have been introduced into different maize inbred lines, using the maize line as the male parent in a program of recurrent backcrossing to the teosinte-derived female line. After several generations of backcrossing, the nuclear genotype is essentially that of the maize inbred, while the maternally inherited cytoplasmic genomes remain those of the original teosinte.

The products of maize nuclear genes interact with the products of teosinte mitochondrial genes to give a variety of interesting morphological and molecular phenotypes. These include effects on plant growth and yield (miniature plant and kernel), cytoplasmic male sterility, altered transcripts for cytochrome oxidase subunit 2 and the production of a novel mitochondrial polypeptide. Allelic variants for one or more nuclear genes have been identified in different maize lines that affect each of these aberrant phenotypes. The products of these nuclear genes

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are probably functional in maize mitochondria and their roles can be revealed by their altered interactions with the teosinte mitochondria. The further study of these phenomena promises to yield insights into nuclear controls of mitochondrial gene expression and the coordinated regulation of nuclear and mitochondrial gene products, processes central to plant growth.

The Effects of the *Mct* locus on *cox2* transcripts

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The nuclear components involved in the production of mitochondrial transcripts are still poorly understood in higher plants. We had shown that a single nuclear gene, *mct* ("Modifier of *cox2* transcripts"), is responsible for a difference in the major stable cytochrome oxidase subunit 2 (*cox2*) transcripts in *Zea perennis* (Zp) and *Zea diploperennis* (Zd) mitochondria. In a W23 (*mct/mct*) nuclear background, these teosinte mitochondria have two abundant transcripts of 1.9 and 1.7 kb, whereas in an A619 (*Mct/Mct*) background, they have three major transcripts of 1.9, 1.5 and 1.3 kb. No effect of nuclear background on *cox2* transcripts was observed for A619 and W23 plants possessing a *Z. luxurians* (Zl) cytoplasm. Maize mitochondria present in the A619 and W23 inbred lines also have identical *cox2* transcript patterns. Immunoblot analysis showed a three-fold reduction of the COXII polypeptide in the Zp-A619 mitochondrial-nuclear combinations compared to Zp-W23 combinations. This suggests that the altered teosinte transcripts may not be translated. The 1.7 and 1.3 kb transcripts are missing untranslated regions 3' to the *cox2* gene; therefore, at least some of the size heterogeneity is due to differential termination or downstream processing. Alleles at the nuclear *Mct* locus are responsible for the observed size differences in the major *cox2* transcripts at their 5' ends. The A619 line carries the dominant allele, which gives rise to the 1.5 kb transcripts. The action of the *Mct* allele is highly specific--working only on *cox2* transcripts (and not on other mitochondrial transcripts such as *cox1* or *cox3*).

The nuclear *Mct* locus encodes a mitochondrial gene-specific transcription factor

Potentially, differences in mRNAs could occur at various steps: initiation, termination, post-transcriptional processing or mRNA stability. Our initial hypothesis was that the difference conditioned by the *Mct* nuclear gene was an RNA processing event and that the product of the *Mct* locus is an RNA-binding protein.

For this reason, just prior to the start of the grant, I went on research leave to learn the necessary biochemical approaches to RNA analysis, particularly *in vitro* RNA processing and UV-crosslinking, in David Stern's laboratory (Boyce-Thompson Institute, Cornell). However, none of these types of experiments showed any of the expected differences, although cross-linking proteins with *in vitro* transcribed *cox2* RNA did identify a potential ribosome binding site. These negative results indicated that the action of the *Mct* locus was on transcript initiation, rather than processing.

In order to directly test these possibilities, we modified the methods of M. Mulligan to compare the results using *in vitro* capping of unprocessed 5' ends with results using processed transcripts (labeled at their 5' hydroxyl group with T4 polynucleotide kinase). The radiolabeled, capped mtRNAs (and kinased mtRNAs) from Zp-A619 were hybridized to Southern blots of subclones from the 5' regions of the *cox2* genes. This experiment told us approximately where the initiation sites are located in the DNA. To identify the actual transcript initiation sites, RNase protection analysis of heteroduplexes between the capped RNAs and either sense or antisense *in vitro* transcribed RNAs were performed.

The subclones that were nearest the *Mct*-controlled initiation site (e.g. RH0.6 and HRV0.4) were sequenced. Using the sequence information, we then used standard primer extension experiments to map and sequence this 5' terminus. It was found that the region surrounding the *Mct*-initiation site does not resemble the previously-described putative maize mitochondrial promoters. In addition, when the region was tested in the Rapp and Stern *in vitro* transcription system, this "optional" promoter did not work. The *in vitro* transcription system does not work with every type of maize mitochondrial promoter (including those for

ribosomal RNAs). Thus, if the *Mct* locus does encode a transcription factor that can function as part of the promoter complex, it is lost or inactivated in the preparation of the extract for *in vitro* transcription.

Several known transcription factors are known to directly bind to the DNA. If the *Mct* locus encodes a transcription factor, we might expect it to be able to demonstrate this binding with gel shift and DNA footprinting assays. Figure 4 shows that indeed labeled fragments containing the "optional promoter" are gel-shifted by various extracts. This suggests that proteins can bind to this region. However, there were only slight differences in binding that we could attribute to the presence of different *Mct* alleles.

As a result of these experiments, we suggested that the MCT protein does not bind directly to the promoter, but that its role is to activate a transcription complex that can form at that position.

Most of the data discussed in this section was included in a publication:
Newton, K.J., B. Winberg, K. Yamato, S. Lupold, D. Stern. 1995. Evidence for a novel mitochondrial promoter preceding the *cox2* gene of perennial teosintes. *EMBO J.*, 14:585- 593.

Genetic and Mapping Studies of *Rcm1*

Cloning and analyzing the *Rcm* gene may be the best way to understand its function. Because there is a visible phenotype of plants carrying Zp mitochondria and the *rcm* allele, we attempted to clone this locus by transposon tagging using *Spm* and *Mutator*. However, none of several putatively-tagged kernels showed the characteristic miniature plant phenotype. It is possible that a knockout mutation is lethal to the plant. However, it is also possible that we were unlucky despite large scale screens.

Using a mapping F2 population and the set of cDNA clones available in the University of Missouri maize mapping center, the *Rcm 1* gene locus was mapped between cDNA markers on the short arm of chromosome 7. In the future, it may be possible to use this knowledge to identify putative *Rcm1* clones within this genic region, although map-based cloning is not yet a reality in maize.

Analysis of mitochondrial function in Zp mitochondria differing in the *Rcm1* allele

Plants with the teosinte cytoplasm and homozygous for the recessive *rcm1* allele are small and slow-growing and the kernels are reduced in size. We have used sibling plants, which differ only in the region of the nuclear genome surrounding the *Rcm1* locus, in an attempt to identify specific altered mitochondrial functions. We have measured rates of dark respiration in isolated mitochondria (using an oxygen electrode to measure oxygen consumption), comparing mitochondria isolated from ear shoots from small (*rcm/rcm*) and large (*Rcm/rcm*) plants. Unpollinated maize ear shoots are excellent sources of mitochondria and these mitochondria have high levels of respiration. We expected that analyses of oxygen consumption in the presence of specific inhibitors would give a preliminary indication of which respiratory complexes might be deleteriously affected in the *rcm/rcm* homozygotes. However, no significant differences were found. Therefore, we cannot say which complexes might be specifically affected in these plants. In future studies, we decided to concentrate on protein analyses to identify the molecular changes in teosinte mitochondria that occur in the presence of the recessive allele of the *rcm1* nuclear gene.

Analysis of maize mitochondrial porin genes:

Attempts to identify a difference in mitochondrial proteins associated with the teosinte-cytoplasm-associated miniature led a graduate student, Carlyn Keith, to the intriguing initial conclusion that porin levels were reduced substantially in *rcm/rcm* small plants. Porins are channel-forming proteins found in the outer membrane of the mitochondria and they control the

flow of small metabolites, including ATP across this membrane. The reduction in porin levels appeared to be even more interesting when it was determined that a major porin locus was also found to map to chromosome 7S in the region of *Rcm1*. However, so far we have been unable to confirm co-segregation of *Rcm1* with reduced mitochondrial porin levels.

Nonetheless, Carlyn began a study of porin genes, obtaining 6 putative porin ESTs from Pioneer HiBred. We have now fully sequenced four of them that appear to be similar to other mitochondrial porins. They fall into two classes. Two of them are full length and have sequence similarities to other mitochondrial porins of the "VDAC1" type. Two others appear to be very related and of the VDAC2 class. Thus, porins are a small multigene family in maize and should be useful in evolutionary studies.

Linear plasmids in a teosinte mitochondria

An undergraduate in the lab, Kelly Grace, found that one type--the Mazoti strain of *Zea luxurians* carries 7.5 kb and 5.4 kb linear mitochondrial plasmids. These Mazoti plasmids were shown to be related to the S plasmids of cytoplasmic male sterile maize and to the R plasmids of a South American strain of corn. Comparisons of partial sequences from the M1 plasmids to the published S1 plasmid and to the "integrated" R1 sequences confirmed this close relationship. Indeed, the data suggested that either the M1 plasmid is the R1 plasmid or that they are very closely related. Although the R and M plasmids are very similar, the mitochondrial genome of Mazoti is otherwise not characteristic of the South American strains of maize that carry R plasmids.

The origin of R-like plasmids in the mitochondria derived from Mazoti's isolate of *Zea luxurians* has not yet been determined. The source and the crossing history of Mazoti's teosinte are difficult to trace. In maize, no case of paternal transmission of mitochondrial plasmids has been reported. However, a rare paternal transmission could explain the unique presence of R-type plasmids in cytolines derived from the Mazoti strain of *Z. luxurians*. However attempts to demonstrate rare paternal events were not successful. Another possibility is that all *Z. luxurians* strains originally contained the M (R) plasmids. Because these plasmids do not appear to be necessary for the survival of the plant, they could have a tendency to be lost over time.

Alternatively, the plasmids may have been lost during the backcrossing programs which yielded the cytolines used in this study. The loss of S plasmids occurs at a high rate when CMS-S cytoplasm is present in the nuclear background of the M825 line of maize.

Much of the work on the M1 plasmid is published in:

Grace, K.S., J.O. Allen and K.J. Newton 1994. R-type plasmids in the mitochondria from a single source of *Zea luxurians* teosinte. *Current Genetics*, 25:258-264.

In order to determine how related the R and M plasmids are, the R1 plasmid was sequenced into its terminal repeats by undergraduate David Riddle. Although the two plasmids, M1 and R1 are closely related there are significant differences that can be used for "diagnostic" purposes. With graduate student Jeff Thornsberry, David showed that each type of plasmid is characteristic and there is little if any heterogeneity for each. Currently, a manuscript is in preparation on the sequencing and comparison of R1 plasmids.

More recently, postdoctoral associate Eugene Kuzmin has been analyzing the structure of the main mitochondrial genome of Mazoti cytoplasm. It was shown that ZI-Mazoti mitochondria contain longer plasmid-like linear subgenomes in addition to M plasmids. The sizes of these subgenomes range from 10 kb to more than 20 kb (pulse-field gel analysis of Mazoti mtDNA is now in progress). Hybridization and partial sequencing of the linear subgenomes suggests that they have been formed through the integration of M plasmids into the main mitochondrial "chromosomal" DNA and subsequent deletions. Interestingly, the copy number of M plasmids and of the larger linear subgenomes is dependent on the particular maize nuclear background.

It was also shown that there is low-level continuous recombination between M plasmids and a "chromosomal" mtDNA target sequence that shares 73 bp of identity with the terminal inverted repeats (TIRs) of M plasmids. In CMS-S maize mitochondria similar recombination was

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shown to occur between S plasmids and a TIR-homologous sequence in the "chromosomal" mtDNA. Preliminary comparisons of the extent of integration of S and M plasmids into their respective cognate "chromosomal" mtDNAs suggest that M plasmids recombine several times less efficiently. This difference could be explained by the shorter length of the TIR sequence (73 bp vs. 187 bp) that is used as a recombination target sequence in the ZI Mazoti mitochondrial "chromosome".

Comparison of the M plasmid-homologous sequences in different cytolines of *Zea luxurians* suggests that Mazoti could have a progenitor mitochondrial genome type to other *Zea luxurians*, including G36, Honduras and Guatemala, that have subsequently lost free M plasmids, but retained plasmid-homologous sequences integrated into the main mitochondrial genome.