FINAL REPORT
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Genetic Analysis of Chloroplast Translation, A. Barkan, PI

This project concerned two nuclear genes in maize, *crp1* and *atp1*, whose function is required for the translation of one or several chloroplast mRNAs. Mutations in these genes cause a reduction in the number of ribosomes bound to the regulated mRNAs, implying a defect in translation initiation or early elongation. The proposed experiments were designed to elucidate how their gene products activate translation.

The specific aims of the proposal were to:

- 1. Identify biochemical activities associated with purified native and recombinant Crp1p.**
- 2. Further optimize the maize chloroplast *in vitro* translation system.**
- 3. Explore the role of the other components of the Crp1p complex.**
- 4. Clone the *atp1* gene and initiate functional studies of its gene product.**

Modification to original aims: Because CRP1 biochemistry proved to be intractable because of its insolubility after expression in *E. coli*, we modified the original aims to include an exploration of the functions of other members of the PPR family. The hope was that other members of the family would be more amenable to biochemical analysis than CRP1. Thus, we used reverse genetic approaches to determine the *in vivo* roles of two other chloroplast-localized PPR proteins.

Summary of Findings

CRP1 is the founding member of a very large gene family in plants containing a novel, repeated motif. We found that CRP1 contains 14 tandem copies of a degenerate 35 amino acid motif that resembles, but is distinct from the tetratricopeptide (TPR) motif. TPRs mediate protein-protein interactions. We performed extensive bioinformatic analysis of the CRP1 motif, finding that it is predicted to fold into paired hydrophilic helices that are anticipated to pack differently than the helical hairpins in TPRs. These insights indicated that the CRP1 motif is distinct from the TPR motif. We also found the motif to be present in tandem arrays in hundreds of predicted proteins in rice and Arabidopsis. Before we were able to publish these findings, a group in Europe published a description of the CRP1 motif, including an analysis of its representation in the Arabidopsis genome, and dubbed it a “PPR motif”.

That the PPR family is so large and so unexplored makes our studies of CRP1 broadly relevant. The majority of PPR proteins are predicted to be targeted to mitochondria and/or chloroplasts. Given the role of CRP1 in chloroplast gene expression, it seems likely that other members of the family function in gene expression in mitochondria and chloroplasts. We anticipated that the PPR motif mediates intermolecular interactions (to protein and/or RNA), as does its well-characterized cousin, the TPR motif. However, no substrates of any PPR protein have been

identified. We had previously shown that CRP1 is present in a multiprotein complex in the chloroplast. Thus, identifying its partners in this complex will not only elucidate the biochemical role of CRP1 in chloroplast translation, but will also lead to a general understanding of how PPRs bind specific substrates.

2. Progress toward identification of CRP1s molecular partners.

We ran up against one roadblock after another in our attempts to identify proteins that interact with CRP1. Although CRP1 is readily over-expressed in *E. coli*, exploration of a wide variety of expression vectors, host strains, and induction conditions failed to yield soluble recombinant CRP1. Therefore, we have been unable to use recombinant CRP1 to test its ability to bind candidate substrates, such as chloroplast RNAs and ribosomes.

We constructed a yeast 2-hybrid cDNA library from maize seedling leaf mRNA and performed a 2-hybrid screen using a portion of CRP1 as “bait”. However, the CRP1 screen came up empty, despite the fact that we screened ~ 1 million cDNAs.

We attempted to purify the CRP1 complex from chloroplast stroma by standard chromatographic techniques (heparin affinity, ion exchange, and gel filtration chromatography). However, the complex did not elute as a single peak from either heparin or ion exchange columns, suggesting that it is heterogeneous. We therefore tried an immuno-affinity approach. Affinity purified anti-CRP1 antibodies were covalently attached to Protein A Sepharose beads and used to precipitate CRP1 complex from stroma. Although we could immunoprecipitate a reasonable amount of CRP1, we were not able to get the complex(es) sufficiently pure for proteomics analysis.

We sought a null-mutation in the CRP1 ortholog in *Arabidopsis*, because we think its phenotype might clarify some aspects of CRP1 function and because we could then use the mutant as a host for introduction of mutant forms of the gene, in structure/function studies. We worked with the Wisconsin T-DNA insertion group through two rounds of screening. The first screen yielded a T-DNA in the 3'UTR of the presumed *crp1* ortholog, that did not disrupt the expression of the gene. The second round yielded a T-DNA insertion in an exon, that should disrupt expression. However, we were unable to recover homozygous mutants for phenotypic analysis, suggesting that the mutation is embryonic lethal.

We showed that CRP1 is tightly associated *in vivo* with the 5'-untranslated regions of the two chloroplast mRNAs whose translation it activates (Schmitz-Linneweber et al, *in press*), showing for the first time that a PPR protein is associated with specific RNAs *in vivo*.

3. Genetic and Biochemical Analysis of two new "PPR" proteins.

We sought mutants in other members of the PPR/CRP1 family, to gain insight into the general functions of PPR proteins, and to identify family members that are more amenable than CRP1 to biochemical analysis. We used the genetic resources generated in the lab to construct our own reverse genetic resource that is tailored for studies of chloroplast biogenesis. In brief, we prepared DNA samples from each of the ~2000 *Mu*-induced non-photosynthetic maize mutants in our collection and organized them into pools. These pools can be screened by PCR to identify mutants with *Mu* insertions in genes of known sequence but unknown function. From this collection, we identified loss-of-function alleles of two new PPR genes (PPR2 and PPR4), both predicted to encode chloroplast-localized proteins. We showed that *ppr2* mutants are defective in the accumulation of plastid ribosomes (Williams and Barkan, 2003) and that PPR4

mutants are defective in the trans-splicing of the chloroplast rps12 mRNA (in preparation). We showed further that both proteins are found in large complexes in the chloroplast stroma, and that PPR4 is bound in vivo specifically to the intron whose splicing it facilitates (in preparation). Taken together, these results suggest that most of the hundreds of predicted chloroplast-localized PPR proteins influence post-transcriptional steps in chloroplast gene expression through interaction with specific RNAs.

4. Progress toward Cloning the *atp1* gene. We conducted an exhaustive search for a *Mu*-insertion that was genetically-linked to *atp1*, but came up empty-handed. We concluded, disappointingly, that the reference *atp1* allele is NOT tagged by *Mu*. We recently recovered a new mutant *atp1* allele and hope to try a novel micro-array based strategy to clone the gene.

5. Publications.

Barkan A and Goldschmidt-Clermont M. (2000) Participation of nuclear genes in chloroplast gene expression. *Biochimie* 82: 559-572.

Williams PM and **Barkan A.** (2003) A chloroplast-localized PPR protein required for plastid ribosome accumulation. *Plant J.*, 36:675-686.

Stern DB, Hanson MR, and **Barkan A.** (2004) Genetics and genomics of chloroplast biogenesis: maize as a model system. *Trends Plant Sci.* 2004 Jun;9(6):293-301.

Schmitz-Linneweber C, Williams-Carrier R, and **Barkan A** (2005) RNA immunoprecipitation and microarray analysis shows a chloroplast PPR protein to be associated with the 5'- region of mRNAs whose translation it activates. *Plant Cell*, in press.

Schmitz-Linneweber C, Williams-Voelker P, and **Barkan A.** A PPR protein required for the trans-splicing of the maize chloroplast rps12 mRNA. Manuscript in preparation.