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Final Technical Report:**Project DE-FG03-88ER13900, Structure and Expression of Nuclear Genes Encoding Rubisco Activase, Raymond E. Zielinski, PI****Project Summary**

Rubisco activase (*Rca*) is a soluble chloroplast protein that catalyzes the activation of rubisco, the enzyme that initiates the photosynthetic carbon reduction cycle, to catalytic competency. *Rca* in barley consists of three polypeptides, one of 46- and two of 42-kDa, but the quaternary structure of the protein is not known. We have isolated and completely sequenced 8.8 kb of barley genomic DNA containing two, tandemly oriented activase genes (*RcaA* and *RcaB*) and three different cDNAs encoding the 42- and 46-kDa *Rca* polypeptide isoforms. Genomic Southern blot assays indicate that these sequences represent the entire *Rca* gene family in barley. Pre-mRNAs transcribed from the *RcaA* gene are alternatively spliced to give mRNAs encoding both 46- (*RcaA1*) and 42-kDa (*RcaA2*) *Rca* isoforms. The *RcaB* gene encodes a single polypeptide of 42 kDa. Primer extension and northern blot assays indicate that *RcaB* mRNA is expressed at a level that is 10- to 100-fold lower than *RcaA* mRNA. It is not yet known whether there are any significant biochemical differences in the rubisco activating properties of the polypeptides encoded by the *RcaA* gene compared with the isoform encoded by the *RcaB* gene. Analyses at the mRNA and protein level showed that *Rca* gene expression is coordinated by that of the rubisco subunits during barley leaf development. The major issues to be addressed in future work include: (1) identifying the cis-acting DNA sequences that regulate *Rca* gene expression; (2) quantifying the relative contributions of the two *Rca* structural genes to the ultimate production of *Rca* polypeptides; and (3) establishing the nature of rubisco activation catalyzed by the different *Rca* polypeptides.

Summary of Activities for the Period January through December 1991

1. Barley rubisco activase gene structure and expression. The results of our earliest work was described in previous progress reports and are summarized in the form of published journal articles in Appendices I and II. In the final year of the project, we initially completed our characterizations of rubisco activase gene expression by comparing the amounts of specific *Rca* mRNAs and polypeptides that were accumulated during the course of barley leaf cell development, and in response to different regimes of illumination. These studies were published in the *Journal of Biological Chemistry* (Appendix III) and are summarized here.

RcaA and *RcaB* mRNAs are differentially expressed during barley leaf development, and during the course of a diurnal cycle. By using gene-specific probes, we found that *RcaA1*, *RcaA2* and *RcaB* mRNAs accumulate differentially through the course of leaf cell differentiation such that the total amount of *Rca* mRNA parallels that of the mRNAs encoding the rubisco subunits. We infer from these results that there must be subtle differences in the abilities of the different activase isoform polypeptides to activate rubisco. However, because of degradation problems during the extraction of activase protein from barley leaves, and because we have no way to specifically identify the *RcaB* polypeptide, it has not been possible to accurately measure the accumulation of individual *Rca* proteins during leaf cell maturation. Run-on transcription assays

using isolated barley leaf nuclei showed that the differences in *RcaA* and *RcaB* gene expression can be attributed largely to differences in transcription rates. A point of particular curiosity in this work is the fact that *RcaB* mRNA is expressed maximally during times when one would expect rubisco to be deactivated; that is, *RcaB* mRNA (and presumably *RcaB* polypeptides) accumulates maximally in the basal region of barley leaves, and during the dark periods of diurnal cycles. One interpretation of these studies is that *RcaB* *inactivates* rubisco at appropriate times when carbon reduction activity is inappropriate, but it maintains rubisco in a form that can be rapidly activated when it is physiologically appropriate. Our aim in the future is to test this possibility *in vitro* by expressing the barley *Rca* polypeptides in *E. coli* and testing their abilities to activate purified rubisco.

Because we have defined the basic differences in *RcaA* and *RcaB* mRNA accumulation in the barley system and have cloned the relevant genomic and cDNA sequences, and because we are currently constructing monoclonal antibody tools to detect specific barley rubisco activase isoforms (described in section 3 below), we are in a position to examine in detail the mechanisms regulating differential expression of the barley *Rca* genes.

2. Polymerase Chain Reaction (PCR) Assays of Barley *RcaA1* and *RcaA2* mRNAs. The differential expression of *Rca* mRNA in barley involves not only differences in transcription from two separate genes (*RcaA* and *RcaB*), but also differential accumulation of alternatively spliced mRNAs (*RcaA1* and *RcaA2*) produced from a single pre-mRNA. One shortcoming of our original experiments, however, was that we had to indirectly estimate the levels of *RcaA1* mRNA because it contains no sequences that are not also encoded in *RcaA2* mRNA. A second shortcoming was that poly(A⁺) RNA had to be used to obtain reasonable hybridization signals, particularly in the case of *RcaB* mRNA. To circumvent these problems, we devised a PCR assay that permits us to visualize both *RcaA* mRNA species directly using small amounts (0.1 to 1 µg) of total RNA (R. Zielinski, unpublished experiments). More recently, we found that we can qualitatively observe differences in the accumulation patterns of *RcaA1* and *RcaA2* mRNAs in the acropetal leaf development gradient of seven day-old barley leaves that directly confirm our earlier slot blot hybridization experiments (Rundle and Zielinski, 1991, Appendix III). We are currently examining different approaches to quantify these PCR assays, so that we can fully appreciate the role alternative mRNA splicing plays in the control of *Rca* gene expression.

3. Antibody Reagents for Barley Rubisco Activase Isoforms Produced from cDNA Clones Expressed in *E. coli*. One of the major limitations in our studies of rubisco activase expression is that we have not been able to determine accurately whether a given increment of mRNA expression results in a corresponding increment of protein accumulation. This shortcoming is a consequence of two factors: (1) the 46-kDa rubisco activase polypeptide produced from the *RcaA1* mRNA is very susceptible to proteolysis during extraction and purification, and is degraded to a 42-kDa form that is indistinguishable from the 42-kDa activase polypeptide produced from the *RcaA2* mRNA; and, (2) the much less abundantly expressed *RcaB* mRNA also encodes a 42-kDa form of rubisco activase that cannot be distinguished from the 42-kDa form produced from *RcaA2* mRNA. To overcome these problems, we initiated experiments designed to generate monoclonal antibodies specific for the different forms of rubisco activase. Barley *RcaA1* and *RcaB* cDNAs were modified by PCR amplification to delete their transit peptide

sequences, except for two amino acids (NH_3^+ -Met-Ala- COO^-) upstream from the putative cleavage site (determined by analogy to the spinach polypeptides, which have been examined directly). The sequences encoding the amino-terminal Met residues were altered in this PCR experiment to introduce *NdeI* restriction enzyme sites in both clones. The cDNAs were then subcloned into *NdeI*-*EcoRI*-cleaved pRK172, which is a T7 RNA polymerase-based expression plasmid derived from the pET series plasmids of Studier and co-workers. In this system when the recombinant plasmids are introduced into *E. coli* BL21(DE3) lysogens, production of T7 RNA polymerase is under *lac* control. Addition of IPTG to the growing culture induces T7 RNA polymerase, which then transcribes the cDNA of interest cloned in pRK172. Thus far, we have succeeded in purifying 5-10 mg of each of the three rubisco activase isoforms [46-kDa (*RcaA1*), 42-kDa (*RcaA2*), and 42-kDa (*RcaB*)], without significant degradation, from 1-liter cultures of transformed, induced bacteria using only minor modifications of published purification protocols for rubisco activase from leaves. The 46-kDa isoform was used to immunize mice in December 1990, and we are now characterizing the specificity of several hybridoma cell lines. The 42-kDa *RcaB* isoform was recently used for immunizations. We anticipate to begin making fusions for monoclonal antibody-producing cell lines based on the *RcaB* polypeptide isoform shortly. In both cases, antibody-producing cell lines will be examined for their specificity by western blotting, using purified barley rubisco activase polypeptides produced in *E. coli*.

Our rationale in conducting these experiments is that there are sufficient differences between the various barley activase polypeptides that we will be able to produce three different monoclonal antibodies: two that distinguish between polypeptides encoded by *RcaA* and *RcaB*; and one that recognizes the 46-kDa activase polypeptide produced by *RcaA1* mRNA, but not the 42-kDa activase isoforms produced by *RcaA2* or *RcaB* mRNAs. These antigenic differences occur by virtue of the fact that: (1) the polypeptides derived from *RcaA* and *RcaB* differ over the span of 15 amino acid residues at the mature amino termini of the proteins; and (2) the 46-kDa activase polypeptide contains a 37-amino acid, carboxy-terminal sequence that is not found in the 42-kDa activase isoforms (ref. 39; Appendix I). A method for monitoring accumulation of the different polypeptides will, in turn, permit us to determine whether post-transcriptional controls operate to regulate rubisco activase expression.

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