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PROGRESS REPORT**DE-FG03-89ER13993****Signal Transduction Pathways that Regulate *CAB* Gene Expression**

Summary: The specific strategies and long-term goals of this proposal remain intact relative to the original proposal submitted in 1991 and activated in 1992. We have completed the initial genetic and phenotypic characterization of several classes of new mutants that affect *CAB* gene expression. The *doc* mutants (for *dark overexpression* of *cab*) are characterized by elevated levels of *CAB* gene expression in the dark; however, unlike the previously isolated *de-etiolated* mutants (also isolated in my lab), the *doc* mutants still appear etiolated. The *doc* alleles define 3 loci, each of which maps to a separate chromosome. The details of the mutant isolation scheme and the genetic and phenotypic description of these new mutants is detailed further below and in a manuscript that has been submitted for publication. I have included a preprint of the manuscript with this Progress Report (Li, Altschmied, and Chory, 1993, submitted). The second class of mutants, the *gun* mutants (for genomes *uncoupled*) show accumulation of *CAB* mRNA in the absence of chloroplast gene expression and development. Thus, the normally tightly coordinated expression between the chloroplast and nuclear genes that encode chloroplast-destined proteins has been uncoupled. This work was described in a manuscript that recently appeared in *Cell* (Susek et al., 1993) and is included with this Report. Further, we have utilized DOE funds to partially support our work on phytochrome mutants. During this funding period, we have shown that the Arabidopsis *HY3* locus encodes the type B phytochrome apoprotein gene and we have characterized the phenotypes of null *hy3* alleles to ascertain a role for this phytochrome in Arabidopsis development (Nagatani et al., 1991; Reed et al., 1993). Simultaneously, we have isolated and characterized a number of alleles of the phytochrome A gene (Nagatani et al., 1993). Finally, three invited reviews and a commentary in the genetics of photomorphogenesis were published (Reed et al., 1992; Pepper et al., 1993; Li et al., 1993; and Chory, 1993). These seven reprints are also included with this project update.

During the past year, we have also identified a number of new mutants that down-regulate *CAB* gene expression in the light. Phenotypic characterization of these new mutants is in progress. A detailed report describing these results is presented below.

During the rest of this funding period and in year 3 of the award, we will concentrate on fine-mapping the *doc* and *gun* mutants. Using this information, we will begin the molecular cloning of one *doc* and one *gun* gene within the next six month period. We will continue biochemical analyses of these mutants to further define the molecular lesions.

Screening Strategy for Isolation of Mutations that Affect the Expression of the Arabidopsis *CAB3* Promoter.

(1). **Strategy.** One of our long-term goals is to saturate the photoreceptor pathways with mutations so that we can reconstruct the number and order of events associated with chloroplast biogenesis. One difficulty in saturating the pathway with mutations is that all the possible phenotypes will be difficult or impossible to predict. Therefore, we have pursued a parallel molecular genetic approach in which we identify mutants by aberrant gene expression patterns, rather than by phenotype. We chose the nuclear *CAB3* gene of Arabidopsis as an indicator of light-regulated, developmental gene expression. The *CAB* promoter-marker gene chimeras needed for these studies have been introduced into wild-type plants at a single site in each transgenic line. The transcriptional chimeras (see Fig) contain a fully regulated *CAB3* promoter sequence fused to either of two selectable marker genes: (a) the *HPH* (hygromycin phosphotransferase) gene, which confers hygromycin resistance allowing for positive selection strategies; or (b) the Arabidopsis *ADH* (alcohol dehydrogenase) gene which allows either positive or negative selection. Both constructs show low basal expression from the *CAB* promoter and

carry a screenable marker *GUS* gene (*E. coli* B-glucuronidase) under control of a second *CAB* promoter. Our strategy is to mutagenize and select for plants which aberrantly express the marker transgenes from the *CAB3* promoter under a variety of conditions. Following selection, we also screen for *GUS* activity, which is under a second *CAB3* promoter contained in the construct. This step is important so that true signal transduction mutants can be distinguished from *cis*-acting promoter mutations.

Figure 1: pOCA107 allows selection for hygromycin resistance in the dark with subsequent screening for *GUS* activity. *uidA* stands for the gene for the B-glucuronidase enzyme from *E. coli*. Many colorimetric and fluorometric substrates are available which make *GUS* screening feasible on small amounts of tissue. In pOCA108, either a positive or negative selection can be performed for the presence or absence of the alcohol dehydrogenase (ADH) gene. As for pOCA107, screening for *GUS* activity subsequent to selection should help delineate promoter mutations from trans-acting mutations. In both cases, the selection for the transformation event is by selection for kanamycin resistance, which is expressed from a highly and constitutively expressed plant promoter from cauliflower mosaic virus (35S).

We first transformed wild-type or *adh*⁻ *Arabidopsis* with either the pOCA107 or pOCA108 constructions and obtained several independent transgenic lines that were (a) diploid and (b) showed proper light/dark and tissue-specific expression of the *cab3* promoter. 50,000 seeds from each transgenic line were mutagenized with EMS, the plants were selfed, and the M2 seeds collected in pools of 200 families. We have been screening for trans-acting regulatory mutations that affect the expression of the two *CAB* promoters in the pOCA107 and pOCA108 lines. The rationale for the screens follows.

The expression of *CAB* genes is tightly regulated. These genes are expressed only in the light in green plants, with little or no detectable levels in etiolated seedlings. In addition, *CAB* genes are expressed in a tissue-specific manner, transcripts being most abundant in leaves and lower or undetectable in other organs. Finally, the developmental stage of the chloroplast itself appears to regulate the expression of *CAB* genes. For example, in photooxidative mutants of maize or in a variety of plants where chloroplast development is arrested with an inhibitor, *CAB* genes are not transcribed. Thus, *CAB* gene expression is regulated by light, intrinsic developmental signals, and must also be sensitive to signals originating from the chloroplast itself. We have looked for and obtained mutants where the *CAB3* promoter is aberrantly expressed in 3 situations. Specifically, we searched for mutants where the *CAB* promoter was expressed either: (1) in the dark at abnormally high levels; (2) in the presence of inhibitors that eliminate chloroplast function; or (3) in the light at abnormally low levels. The results from each screen are described separately.

(2). Trans-acting Mutations that Affect the Etiolated Expression Levels of *CAB3*. As mentioned in the Summary, we have designed a screen in which the expression levels of *CAB* are affected in the absence of morphological changes in the etiolated seedling. We used the pOCA107 transgenic line to isolate mutants that over-expressed the *CAB* genes in the dark. Eight mutants were isolated which showed aberrant expression of both *CAB3* promoters, and were designated *doc*, for dark over-expression of *CAB*. Genetic and phenotypic analyses indicate that most of the mutations are recessive

and define at least three loci (*doc1*, 2, 3) that fall into two phenotypic classes. Mutants of the first class have both elevated *CAB* and *RBCS* transcript levels in the dark, while mutants of the second class specifically repress the expression of *CAB* genes in the dark but do not affect the expression of *RBCS* genes. The phenotypes of *doc* mutants suggest that morphological changes can be genetically separated from changes in *CAB* gene expression. Moreover, the regulation of *CAB* gene expression can be further separated from the regulation of *RBCS* gene expression. Double mutant studies of *det1-doc1* and *det3-doc2* suggest that the *DOC* gene products are downstream from *DET1* and on a different branch from *DET3* in the light-regulated signal transduction pathway. Mutations in *doc1*, 2, or 3 also impair plant growth under short day conditions, suggesting these loci may play a role in down-regulating *CAB* gene expression during the night. A preprint describing these results in greater details is included with this Progress Report (Li, Altschmied, and Chory, 1993, submitted). Our current studies are focussed on completing the characterization of the short-day phenotypes, performing *in vitro* biochemical studies to study DNA-binding activities in nuclear extracts from the mutants, and fine mapping *doc1* so that cloning studies can be initiated.

(3). **Mutations that Affect Signalling Between the Nucleus and Chloroplast.** The herbicide norflurazon is known to block carotenoid accumulation, which results in photobleaching and the inhibition of chloroplast development in bright light. In a variety of plants that have been treated with norflurazon, the *CAB* genes are not expressed. This has led to the hypothesis that a signal originating from the chloroplast is necessary for optimal transcription or mRNA accumulation for nuclear genes encoding proteins destined for the chloroplast. Nothing is known about the nature of the signal, or the signal transduction pathway(s) involved in coupling gene expression in the chloroplast and nucleus during photosynthesis. We used the mutagenized pOCA107 transgenic line to select for mutant plants in which the *CAB3* promoter is expressed at high levels in the presence of norflurazon. Ten mutant alleles, defining at least 3 loci, were recovered. The mutants were called *gun* for genomes uncoupled, since mutations in any one of the three *GUN* loci resulted in plants in which the nuclear *CAB* and *RBCS* genes were expressed in the absence of chloroplast gene expression and development. We have examined *gun1* mutants in the greatest detail. The *gun1* mutants develop normally in the dark but are defective in the escape from the etiolated state. Thus, *gun1* mutations interrupt a signal transduction pathway from the chloroplast to the nucleus, but they also impede the transition from etioplast to chloroplast. These studies were published in *Cell*, and a reprint is included here. We are focusing our current biochemical and molecular studies on the *GUN1* locus.

(4). **Use of pOCA108 to Select for Mutations Involved in the Down-Regulation of *CAB3* Transcription.** We have recently established the conditions for screening for down-regulation of *CAB3* promoter expression using transgenic R002 lines that contain pOCA108. R002 is an *adh* null mutant isolated as an allyl alcohol resistant line in the mutagenized Bensheim background. In the pOCA108 line, ADH and GUS activities are expressed in a light-regulated and tissue-specific pattern that corresponds to wild-type expression of the *CAB3* promoter. To perform screens for down-regulation of the *CAB3* promoter, seeds are germinated and grown in liquid for 5 days. At that time, the growth medium is exchanged for medium containing 3.0 mM allyl alcohol and the seedlings are treated for 1 h. After removal of allyl alcohol, the seedlings are allowed to recover on synthetic medium. Control pOCA108 plants are bleached and killed within 48 h of the allyl alcohol treatment. In contrast, R002 seedlings lacking the pOCA108 construct are completely resistant to the identical allyl alcohol treatments. Fifty thousand mutagenized seeds were screened for allyl alcohol resistance using this method in order to identify mutants with low levels of ADH activity from the *CAB3* promoter. We are currently analyzing 11 mutants that also showed low levels of GUS activity. Seven of these 11 mutants have less than 15% of the wild-type levels of *CAB3* expression in the light and are potential candidates for *trans*-acting regulatory mutations involved in the down-regulation of *CAB3* transcription.

In summary, we have isolated mutants that have aberrant *CAB* promoter expression under any of several conditions, and have demonstrated for the first time in plants the feasibility of a strategy that should be applicable to a variety of regulated promoters. We are currently analyzing these new mutants at the genetic, molecular, and biochemical levels. Our future studies will focus on cloning some of these genes by the strategies outlined in my original proposal.

Education and Human Resources.

This research project has partially funded a postdoctoral research associate, Ronald Susek, and has funded the supply funds for two additional postdoctoral fellows, Lothar Altschmied (supported by the Human Frontier Research Program) and Hsou-min Li (supported by the Noble Foundation). In addition, an excellent undergraduate student from UCSD, Kevin Culligan, has helped with the mapping of the *doc* loci and the characterization of the *CAB* underexpressing mutant lines. Ron, Hsou-min, Kevin, and Lothar have done all the work described in this progress report. Funding by the DOE has provided Drs. Susek, Li, and Altschmied with valuable practical experience in plant molecular genetics, as well as exposed them to current research in other areas of plant biology at The Salk Institute and University of California at San Diego.

Manuscripts Enclosed With This Report.

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