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BARD

FINAL REPORT
PROJECT NO. IS-2265-93

**Degradation of Abnormal Proteins in Chloroplasts of
Higher Plants**

Z. Adam, E. Pichersky

1997

Date: October 20, 1997

BARD Project Number: IS-2265-93R

Project Title:

Degradation of Abnormal Proteins in Chloroplasts
of Higher Plants

Investigators:
(Principal first)

Zach Adam

Eran Eichersky

Affiliated Institution:

The Hebrew University of Jerusalem

The University of Michigan

Project Start Date: August 1, 1994

Type of Report

1st Annual

2nd Annual

Final X

[Signature]
Signature:

Principal Investigator

[Signature]
Signature:

Authorizing Official, Affiliated Institution

Degradation of Abnormal Proteins in Chloroplasts of Higher Plants

Final report submitted to BARD

by

Zach Adam and Eran Pichersky

October 1997

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Abstract

In this study we attempted to get a better understanding of processes involved in the degradation of abnormal proteins in chloroplasts. To achieve this goal, we used a number of complementary approaches. We first characterized the expression of the two subunits of Clp protease. We demonstrated that both of them were expressed in chloroplasts in a constitutive fashion, but the expression of the regulatory subunit ClpC was enhanced by light. We generated a mutant the luminal protein OEE33 which was targeted to the stroma in *in vitro* experiments. In the wrong compartment it was found unstable, and characterization of its degradation revealed that it was degraded by a soluble, ATP-dependent serine protease, which are also the characteristics of Clp protease. In search of other homologues of bacterial proteases, we found that chloroplasts contain a homologue of the FtsH protease. It is an ATP-dependent metallo-protease, bound to the stromal side of the thylakoid membrane, whose expression is dependent on light. The gene encoding this protease was cloned and characterized. In attempt to generate *Arabidopsis* mutant plants impaired in their capability to degrade abnormal chloroplast proteins, we fused the gene for mistargeted OEE33 to the streptomycin-detoxifying gene. This chimeric gene was introduced into *Arabidopsis* plants, to generate transformed plants. This transformants plants were sensitive to streptomycin due to the rapid turn-over of the chimeric protein. Seeds from these plants were then chemically mutagenised, and seedlings were selected for their capability to grow on streptomycin. The ability of these mutant transformants to grow on streptomycin is presumably due to stabilization of the chimeric protein. These plants will allow us in the future to identify the effected genes, which are likely to be involved in the protein degradation process.

Objectives:

This project was a part of our long term goal to understand the role of protein degradation processes in the biogenesis and maintenance of chloroplasts. Within this framework, the specific goals of the project were the following:

1. Biochemical and molecular characterization of Clp protease in chloroplasts of higher plants.
2. Generation of *Arabidopsis* mutants impaired in their capability to degrade abnormal chloroplast proteins.

Results and Discussion:

When this project was started, the only putative protease in chloroplasts was Clp protease, due to identification of homologous genes to those of bacterial Clp protease, known to degrade abnormal protein in *E. coli*. No information was available at that time whether these genes were expressed or not. We therefore set up to answer this question both at mRNA and protein levels. We first studied the expression of the regulatory subunit ClpP, along with a homologue of another cyanobacterial protease PrcA. The results of this characterization appeared in the article "Immunological detection of proteins similar to bacterial proteases in higher plant chloroplasts", Eur. J. Biochem. 236: 932-936 (see below). We then characterized the expression of the regulatory subunit of Clp protease, ClpC, with respect to the effects of light and temperature on the expression. These results are summarized in "Effects of light and temperature on expression of ClpC, the regulatory subunit of chloroplastic Clp protease, in pea seedlings", Plant Mol. Biol. 31: 673-676.

Using another approach, we mutated the gene encoding the luminal protein OEE33 to direct it to the stroma in *in vitro* import experiments. We found that when mistargeted to the wrong compartment within the chloroplast, this protein has become unstable. We characterized its degradation and shown that the characteristics of this proteolytic process were similar to those of Clp protease. These results are summarized in "Degradation of mistargeted OEE33 in the chloroplast stroma", Plant Mol. Biol. 30: 925-933.

While this study was in progress, the importance of another ATP-dependent bacterial protease, FtsH, became clear. Although not mentioned in the original proposal, we thought that it would be important to test whether this protease has a chloroplastic homologue. We identified it in the chloroplast by immunological means, characterized it and cloned its gene. This work was summarized and appeared in "Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplast of higher plants", J. Biol. Chem. 271: 29329-29334.

Our proposed plan to generate *Arabidopsis* mutants impaired in protein degradation in chloroplasts was based on a previous report that demonstrated that a specific single amino acid substitution in the small subunit of Rubisco (SSU) destabilized this protein. However, when we repeated and elaborated these experiments, we discovered that that mutant was a slowly-assembled rather unstable mutant. These results appeared in "A mutation in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase that reduces the rate of its incorporation into holoenzyme", Photosynthesis Res. 43: 143-147. Since this mutant protein was stable, we had to design an alternative approach for generating an unstable chloroplast protein. This goal was achieved by mistargeting a luminal protein, OEE33, to the chloroplast stroma. Indeed, this mutant was unstable, and apart from being characterized *in vitro*, as mentioned above, could be used for generation of transgenic mutant *Arabidopsis* plants impaired in protein degradation in their chloroplasts. Mutant OEE33 was first fused to the

aadA gene, capable of detoxifying streptomycin. We found that the fusion protein rendered *E. coli* insensitivity to streptomycin, and that the fusion protein, when imported into isolated chloroplasts, was as unstable as the mutant OEE33. These preliminary results assured us that we could continue with our experimental scheme. The chimeric gene was introduced into *Arabidopsis* via *Agrobacterium* transformation by infiltration. Transgenic plants were screened for sensitive to streptomycin, as an indication for the rapid turn-over of the chimeric construct. The selected plants were further assayed for the presence of the introduced gene by PCR and for its expression at the mRNA level by RT-PCR. Seeds from transgenic plants that passed all these tests were subjected to chemical mutagenesis by EMS, and seedlings were selected for their ability to grow now in the presence of streptomycin. At this stage, we have transgenic mutant plants, presumably impaired in their capability to degrade proteins in the chloroplast stroma. These plants are now being genetically and phenotypically characterized, and are expected to allow us a further insight into the process of protein degradation in chloroplasts.

The work done under this project, together with other projects going on in our lab, greatly contributed to our understanding of protein degradation processes in chloroplast. The current status of our knowledge in this important field was recently summarized in the review article "Protein stability and degradation in chloroplasts", *Plant Mol. Biol.* 32: 773-783.

Cooperation

Characterization of expression of Clp protease at the mRNA level was initiated in Adam's lab. Simultaneously, Pichersky's lab concentrated on over-expression of ClpC, which turned out

to be a complicated task. The successful expression allowed us the generation of ClpC-specific antibodies.

The introduction of the OEE33 construct into the project, both for the *in vitro* work and for the analysis of the transformant *Arabidopsis*, required a high-quality antibody against this protein. Pichersky's lab over-expressed this protein, purified it, and generated antibodies, which are very valuable in the analysis of the transformants, carried out in Adam's lab.

When the chloroplastic homologue of FtsH was found in Adam's lab, it became apparent that further progress in the study of this protease requires the isolation of its cDNA. Therefore, while Adam's group concentrated on the characterization of the protein, Pichersky's group cloned the cDNA and sequenced the gene. The cloned gene was then expressed in Adam's lab and tested for import into chloroplasts.

Overall, the close collaboration between the labs, taking advantage on specific expertise in each one of them, enabled us the progress described above.

Research achievements

1. We characterized the expression of the two subunits of Clp protease, for which genes were known, but no information was available whether they were expressed at all.
2. We characterized the degradation of a mistargeted protein in the chloroplast, and found that it was degraded with characteristics similar to those of Clp protease. This correlation provides the first, and so far the only suggestion for a role for Clp protease.
3. We identified and characterized a new chloroplast protease, the membrane-bound homologue of bacterial FtsH.

4. We generated *Arabidopsis* transgenic mutant plants in which protein degradation in the chloroplast stroma is impaired.

These achievements add a fair amount of new information on the identity and characteristics of chloroplast proteases, in particular on those involved in degradation of abnormal proteins, and provide important tools for continuing the research on this important subject.

List of publications

1. Adam Z. (1995). A mutation in the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase that reduces the rate of its incorporation into holoenzyme. *Photosynthesis Res.* 43: 143-147.
2. Halperin T. & Adam Z. (1996). Degradation of mistargeted OEE33 in the chloroplast stroma. *Plant Mol. Biol.* 30: 925-933
3. Ostersetzer O., Tabak S., Yarden O., Shapira R. & Adam Z. (1996). Immunological detection of proteins similar to bacterial proteases in higher plant chloroplasts. *Eur. J. Biochem.* 236: 932-936
4. Ostersetzer O. & Adam Z. (1996). Effects of light and temperature on expression of ClpC, the regulatory subunit of chloroplastic Clp protease, in pea seedlings. *Plant Mol. Biol.* 31: 673-676
5. Lindahl M., Tabak S., Cseke L., Pichersky E., Andersson B. & Adam Z. (1996). Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants. *J. Biol. Chem.* 271: 29329-29334
6. Adam Z. (1996). Protein stability and degradation in chloroplasts - a mini-review. *Plant Mol. Biol.* 32: 773-783