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REGULATION OF CHLOROPLAST NUMBER AND DNA SYNTHESIS IN HIGHER PLANTS

Final Report

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PROGRESS REPORT

TITLE: Regulation of Chloroplast Number and DNA Synthesis in Higher Plants
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A. OVERALL OBJECTIVE

The long term objective of this research is to understand the process of chloroplast development and its coordination with leaf development in higher plants. This is important because the photosynthetic capacity of plants is directly related to leaf and chloroplast development. This research focuses on obtaining a detailed description of leaf development and the early steps in chloroplast development including activation of plastid DNA synthesis, changes in plastid DNA copy number, activation of chloroplast transcription and increases in plastid number per cell. The grant will also begin analysis of specific biochemical mechanisms by isolation of the plastid DNA polymerase, and identification of genetic mutants which are altered in their accumulation of plastid DNA and plastid number per cell.

B. SUMMARY OF RESULTS

An analysis of pea leaf and chloroplast development in plants grown in darkness, far-red or white light was completed. Quantification of leaf length, DNA, cell number and chlorophyll, cell size, DNA content per nucleus and plastid number per cell and plastid DNA synthesis, DNA copy number, transcription activity, plastid RNA abundance and chlorophyll content led to the following major conclusions.

1. Leaves of dark-grown pea plants grow slowly, have low plastid transcription activity but plastids accumulate approximately 100 genome copies. Cells and plastids in the barley leaf basal meristem and primary leaves of dark-grown pea are at a similar stage of development.
2. Leaf growth in continuous far-red light was a high irradiance response most likely mediated by PHYA.
3. Far-red and white light induce similar increases in leaf growth, cell number per leaf from 4 days post imbibition (dpi) to 8 dpi.
4. Leaf length in white light increases from 12 mm to 24 mm but not in far-red light. Most of the extra increase in leaf size in white light is due to an increase in cell size and air spaces. Plastid number per cell increases during this phase of development in white light but not far-red light consistent with the known relationship between cell size and plastid number.
5. Plastid DNA synthesis activity increases between 5 dpi and 6 dpi in both light conditions. Maximal DNA synthesis activity in far-red illuminated plants is greater than in white light illuminated plants and DNA copy number reaches 600 vs 350. DNA copy number per plastid declines in both treatments in part due to dilution of DNA into new plastids.
6. Plastid transcription activity increases between 4 dpi and 5 dpi and reaches a maximum at 6 dpi in both light treatments although transcription activity is higher in far-red light.

Transcription activity declines after 6 dpi in both treatments which indicates that the decline is not due to photosynthesis.

7. Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in chloroplast development and genes for the photosynthetic apparatus later in development.

8. The stability of plastid RNAs is decreased in white light grown plants after 6 dpi relative to RNAs in far-red illuminated plants. Photosynthetic activity may activate the turnover of plastid RNAs and reduce the capacity of chloroplasts to carry out protein synthesis.

9. *PsbA* mRNA is stable and accumulates in older plastids of dark-grown, far-red and white light illuminated plants.

Results from other projects undertaken as part of this grant are listed below.

10. *Arabidopsis* mutants which vary in the ratio of plastid DNA to nuclear DNA were identified.

11. *Arabidopsis* mutants which have altered plastid number per cell were identified.

12. A lysed cell run-on plastid transcription assay was devised. This assay was used to investigate differential transcription in mesophyll and bundle sheath cells of maize. Only small differences in relative transcription were observed in these plastid populations.

13. Differential accumulation of *rpoB* mRNA relative to *atpB* and *rbcL* was observed when leaf development occurred in the presence of chloramphenicol (to block plastid protein synthesis) or tagetitoxin (inhibits plastid transcription).

14. The plastid DNA polymerase was partially purified.

C. DESCRIPTION OF RESULTS

1. Regulation of Pea Leaf and Chloroplast Development

Rationale:

Our long term goal was to understand the mechanisms which lead to increased plastid number per cell and activation of plastid DNA synthesis and transcription early in chloroplast development. We had previously characterized the sequence of events in barley leaf and chloroplast development and wanted to determine whether the same developmental pathway operated in leaves of dicot plants. This question arises because dicot leaf and chloroplast development is under complex light control not observed in barley. In addition, cell division continues throughout the dicot leaf for much longer than in monocot leaves which restrict cell division to the leaf base early in development. Future analysis of *cis* and *trans*-factors which regulate expression of genes involved in the early stages of chloroplast development will require analysis of transgenic plants. At present, transformation of barley is not routine and therefore, it would be useful to carry out this type of experiment using tobacco or *Arabidopsis*. We decided to carry out the analysis of chloroplast development using pea because of the large number of previous studies on this topic and because pea leaves, unlike *Arabidopsis* leaves, afforded sufficient material to allow *in vitro* plastid transcription assays to be carried out at all stages of leaf development. I thought that if chloroplast development in pea and barley had basic steps in common then we could use barley and pea for biochemical studies and tobacco/*Arabidopsis* for generation of mutants and transgenic plants.

Pea leaf growth and chloroplast development are modulated by phytochrome and leaf expansion occurs when seedlings are exposed to far-red light. Studies in *Arabidopsis* (rev. 1) indicate that several genes are involved in mediating the influence of light on leaf growth. For example, *Arabidopsis* leaves expand and plastid genes are activated in the *det1* and *cop1* mutants (2,3). Expression of plastid genes in dark-grown *det1* resembles that observed in barley (2). Therefore, we were interested in determining the extent of leaf and chloroplast development in pea plants grown in darkness vs those illuminated with far-red and white light.

Objective 1-1: Characterize leaf development and plastid DNA synthesis in peas grown in darkness, far-red and white light. [DuBell, N. Arnold and J.E. Mullet (199) Plant Physiology 109: 95-103 - Appendix 1]

1. *Leaf growth in darkness, far-red and white light was similar from 0 to 4 days post-imbibition (dpi).*

Seeds were germinated and grown in darkness until 2 dpi. At this point a portion of the plants were kept in darkness and others were transferred to continuous far-red light or white light. Leaf size (2 mm), cell number, leaf DNA content and plastid number per cell were elevated to a small extent in plants exposed to far-red and white light relative to dark-grown plants at 4 dpi.

2. *Far-red light stimulates an increase in leaf cell number (but not size) through a high irradiance response from 4 dpi to 8 dpi. Continuous far-red light potentiates leaf growth in white light.*

Between 4 dpi and 8 dpi, leaf length increased in parallel in pea plants exposed to far-red and white light and much faster than in dark-grown plants. Cell size did not change significantly during this period of development indicating that increased leaf size was due to an increase in cell number. The fluence dependence of the increase in leaf length in continuous far-red light was consistent with a high irradiance response. Exposure of plants from 2 dpi to 4 dpi to far-red light potentiated leaf growth if plants were illuminated at 4.5 dpi with white light. However, exposure of far-red illuminated plants to white light at 8 dpi did not allow leaves to expand to their full size.

3. *White light but not far-red light stimulates an increase in cell size and plastid number per cell between 7 dpi and 12 dpi.*

Leaf growth in far-red illuminated plants stopped at 8 dpi when leaves were 12 mm in length. In contrast, leaves of white light illuminated plants increased in length from 12 mm to 24 mm from 7 dpi to 12 dpi primarily due to an increase in cell size and the formation of air spaces in the leaf. Plastid number per cell also increased in white light but not in far-red illuminated plants. This data could indicate that PHYA level (or activity) is reduced after 8 dpi or that this photoreceptor cannot stimulate cell expansion. Cell expansion could be mediated by PHYB, a blue light photoreceptor or the action of another photoreceptor and photosynthesis. The role of PHYB in the last phase of cotyledon expansion mediated by bright red light has been previously documented in *Arabidopsis* (4).

4. *Plastid DNA synthesis is activated by illumination of pea plants with far-red or white light.*

Plastid DNA copy number was 120 in plastids of dark-grown pea plants at 5 dpi. This is similar to levels found in plastids of the barley leaf basal meristem. DNA synthesis rates and

DNA copy number increased in plants illuminated with far-red or white light between 4 dpi and 5 dpi. Maximal DNA synthesis rates were observed at 6 dpi and declined in older plants. Plastid DNA copy number reached 350 in white light grown plants and 600 in far-red illuminated plants. Plastid DNA copy number decreased to 100 per plastid in white light grown plants primarily by dilution of DNA into newly synthesized plastids. Plastid DNA copy number per cell gradually decreased in far-red light to 300 probably through a combination of dilution and DNA degradation. These data show that continuous far-red light, most likely acting through PHYA, is able to fully stimulate plastid DNA synthesis.

Objective 1-2: Light regulated chloroplast gene expression in pea plants grown in darkness, far-red and white light. [DuBell, A.N. and J.E. Mullet (1994) Differential transcription of pea chloroplast genes during light-induced leaf development. Continuous far-red light stimulates chloroplast RNA synthesis. *Plant Physiology* 109:105-112, Appendix 2]

1. *Overall plastid transcription is activated early in chloroplast development in plants illuminated with far-red or white light.*

Plastid transcription activity was low in dark-grown plants at 5 dpi and remained low relative to illuminated plants through 14 dpi. Plants illuminated with white light showed an increase in transcription activity between 4 and 5 dpi and maximal activity at 6 dpi. A similar induction was observed in far-red illuminated plants except that induction started later and the maximum of transcription was 2-fold greater than in white light illuminated plants. Plastid transcription activity decreased in both light conditions from 6 to 9 dpi. A part of this decline could be attributed to dilution of the transcription apparatus into an increasing number of cells and plastids. The peak of transcription activity at 6 dpi corresponded to 20% of full leaf growth and less than 10% of final accumulation of leaf chlorophyll. This suggests that leaves synthesize the plastid transcription apparatus in anticipation of later increases in cell number and chloroplast development. These data also demonstrate that activation of phytochrome is sufficient to fully induce plastid transcription activity during pea leaf development.

2. *Plastid genes encoding the transcription/translation apparatus are differentially transcribed relative to genes encoding the proteins of the photosynthetic apparatus.*

Run-on transcription assays revealed a 2-fold increase in the ratio of transcription of genes encoding the transcription/translation apparatus (i.e., *rpoB*, 16S rRNA, *rpl16*, *rps16*) relative to genes such as *rbcl*, *atpB*, *petD*, *psaB* that encode proteins of the photosynthetic apparatus from 4 dpi to 5 dpi and then a 10-fold decrease in this ratio. This change may facilitate the accumulation of the plastid's decoding apparatus prior to synthesis of the photosynthetic apparatus. A similar broad based change in transcription of these two sets of genes was observed during barley chloroplast development (5). This change in transcription may be due to alterations in the ratio of the two plastid RNA polymerases or the activity of sigma-like factors which modulate plastid transcription (rev. 6).

3. *White light stimulates turnover of many plastid RNAs including the *rpoB* and *rps16* mRNAs. *PsbA* mRNA is stable in mature chloroplasts.*

Plastid transcription in far-red and white light illuminated plants decreased in parallel from 6 dpi to 12 dpi. However, plastid mRNAs and rRNAs declined rapidly in white light but not in far-red light. This result was particularly striking for the *rpoB* and *rps16* mRNAs which remained high in far-red illuminated plants but declined rapidly in white light illuminated plants. This result indicates that white light, perhaps through the action of photosynthesis, caused a destabilization of the plastid's translation apparatus and many mRNAs. One exception to this

general change was the accumulation of the *psbA* mRNA which is needed in mature chloroplasts.

Overall, the process of chloroplast development in pea and barley are similar even though the rate of development is regulated by light in pea but not barley. Both plants show an early increase in plastid DNA synthesis activity and copy number. Plastid DNA copy number then declines in plastids due to dilution and/or degradation. Plastid transcription is activated early in chloroplast development and then declines rapidly as chloroplasts mature. Activation of plastid transcription can occur in darkness (barley), or in far-red or white light (pea). Differential transcription of plastid genes encoding the transcription/translation apparatus relative to genes encoding the photosynthetic apparatus is observed in both plants. A light-induced decrease in stability of many plastid mRNAs occurs as chloroplasts mature and in both pea and barley, *psbA* mRNA is stable in mature chloroplasts (7).

Objective 1-3: Isolate the plastid DNA polymerase which increases in activity early in chloroplast development.

The aim of this objective was to isolate the plastid DNA polymerase in order to obtain protein sequence information and antibodies which in turn would allow isolation of the corresponding genes. We have followed the purification procedure described by McKnown and Tewari (9) and Heinhorst et al. (8) with some success. In brief, plastids are isolated from pea plants on Percoll gradients. The intact plastids are lysed in 2% Triton X-100. The mixture of membranes and soluble phase is treated with ammonium sulfate and the DNA polymerase activity is precipitated between 30% and 70%. The precipitate is dialyzed and loaded on a DEAE column and the DNA polymerase is eluted with 150 mM KPO₄. The DNA polymerase fractions are concentrated, loaded on a glycerol gradient and centrifuged. Fractions from the glycerol gradient are loaded on a P-11 column and eluted with a salt gradient. At this point the DNA polymerase has been purified approximately 3000-fold with 10% recovery. One additional purification step will be tested to see if we are approaching homogeneity (heparin sepharose or Mono-S). Once a nearly homogeneous preparation was obtained, we proceeded to antibody preparation and partial sequence analysis. The antibodies were tested for reactivity against the purified DNA polymerase preparation and subsequently used to identify clones expressing the corresponding proteins.

Objective 1-4: Screen mutagenized *Arabidopsis* for mutants varying in plastid number per mesophyll cell and DNA content.[Appendices 3,4]

Dr. Daryll Morishige joined this project in August, 1993 and set up screens for the mutants described above. He decided to focus first on screens for plastid DNA content because these were technically the most difficult. Our general approach was to isolate total DNA from one half of a primary leaf of a two week old seedling and to amplify the DNA with primers for a nuclear gene (plastocyanin) and a plastid gene (*atpE*). The amplified DNA was then blotted onto a nylon membrane and probed with radiolabeled DNA probes for the nuclear and plastid target genes. The ratio of hybridization to the two gene probes provides a measure of the ratio of plastid DNA to nuclear DNA. The method needed to be easy and reproducible enough to allow at least 3,000 mutants to be screened.

Preliminary results with assay development were encouraging. Using purified *Arabidopsis* DNA, target DNA could be amplified reproducibly and the relative ratio of nuclear to plastid DNA determined. The target genes, plastocyanin and *atpE* were chosen such that the amplified DNAs were similar in size (469 bp, 663bp). The ratio of plastid to nuclear gene revealed by the PCR assay is less than what we would predict from other plants. However, a

reproducible ratio is all that is required for screening and we will determine the actual copy numbers for these genes in wild type and mutant plants by quantitative hybridization at a later point in this analysis. Extraction of DNA proved to be very time consuming until the alkali release method of Klimyuk et al (1993) was adopted. Using this method, 600 samples can be screened per week. This is well within the numbers needed to successfully screen for the desired mutants. At present, Dr. Morishige has screened over 5,000 mutagenized *Arabidopsis* plants. He has found 56 mutants which have plastid/nuclear gene ratios 2-fold (or more) greater or less than wild type. These mutants will be rescreened and further characterized. Dr. Morishige has also identified a mutant with an increased plastid number per cell (2-fold) and two mutants with giant chloroplasts.

2. Analysis of plastid RNA polymerases and understanding the initial activation of transcription during chloroplast development.

Rationale:

Analysis of plastid transcription was not originally part of the proposed research. However, several significant observations led us include these studies as part of this grant. First, analysis of plastid transcription during pea chloroplast development revealed that light activation of transcription was rapid, occurred in parallel with activation of DNA synthesis and was an early event in chloroplast development. The pea study also provided evidence for differential transcription of genes encoding the transcription/translation apparatus relative to genes encoding proteins of the photosynthetic apparatus. This suggested that activation of plastid transcription during chloroplast development might be controlled by increased synthesis of an RNA polymerase or regulatory factor which stimulates synthesis of the plastid-encoded RNA polymerase. Evidence for two plastid RNA polymerases was strengthened by observation of transcription in *Epifagus* which lacks *rpoB*, differential RNA and transcription in the barley mutant *albostrans* which lacks plastid ribosomes and purification of a plastid RNA polymerase which lacks the plastid-encoded β -subunit (rev. 6). This latter RNA polymerase is apparently encoded by nuclear genes.

Objective 2-1: Test the influence of chloramphenicol and tagetitoxin on RNA accumulation during barley chloroplast development.

The data described above led us to propose that the initial activation of chloroplast transcription during chloroplast development might be controlled by synthesis of the nuclear-encoded RNA polymerase which in turn drives the synthesis of the plastid-encoded RNA polymerase (6). As a first test of this hypothesis, barley plants were grown in the presence of chloramphenicol or tagetitoxin and changes in plastid mRNA analyzed. Both of these treatments would be expected to prevent build-up of the chloroplast-encoded RNA polymerase. The data showed that *rpoB* mRNA levels increase in the presence of the inhibitors whereas *rbcL* and *atpB* mRNAs do not. If the nuclear-encoded RNA polymerase differentially transcribes *rpoB* relative to *rbcL* and *atpB*, then this result is consistent with a role for the nuclear-encoded RNA polymerase in the activation of plastid transcription early in chloroplast development.

Objective 2-2: Develop a run-on transcription assay for plastid genes which does not require the isolation of plastids. [Appendix 5]

The plastid run-on transcription assay has been a very useful tool to analyze relative rates of plastid gene transcription (6). However, up to the present time, its use has been limited to plastid populations which can be purified. Therefore, transcription in plastids located in shoot apical meristems have been difficult to assay because the proplastids in this tissue can not be easily isolated. Furthermore, analysis of differential transcription in chloroplasts of bundle sheath cells cannot be done until after protoplasting which raises the question whether these

assays reflect the *in vivo* state. To circumvent this limitation, we have developed a run-on transcription assay which does not require the isolation of intact plastids. We noted that plastid DNA and the elongating RNA polymerase remains associated with plastid membranes when plastids or cells are ruptured. Plastid membranes released from lysed plastids can be easily separated from nuclei on Percoll gradients. If run-on transcription assays are performed using these membranes in the presence of heparin, then we obtain results similar to those obtained with the standard run-on transcription assay. This assay was used to investigate the contribution of differential transcription to gene expression in mesophyll and bundle sheath cells of maize. The differences in RNA abundance in these two cells types could not be explained by variation in rates of transcription. These results are consistent with results obtained in *Amaranthus* (11) and do not support the proposed differential methylation and plastid gene transcription model presented by Ngerprasirtsir (12).

References Citing Support from this DOE Grant

1. Mullet, J.E. (1993) Dynamic regulation of chloroplast transcription. *Plant Physiology* 103: 309-313.
2. DuBell, N. Arnold and J.E. Mullet (1994) Continuous far-red light activates plastid DNA synthesis in pea leaves but not cell enlargement or an increase in plastid number per cell. *Plant Physiology* 109:95-103.
3. DuBell, A.N. and J.E. Mullet (1994) Differential transcription of pea chloroplast genes during light-induced leaf development. Continuous far-red light activates chloroplast transcription. *Plant Physiology* 109:105-112.
4. LaFlamme, D. and J.E. Mullet (1994) A plastid run-on transcription assay for cell lysates. *Plant Mol. Biol. Reporter* (submitted) - Appendix 5

LITERATURE CITED

1. Deng, X.-W. (1994) Fresh view of light signal transduction in plants. *Cell* 76:423-426.
2. Chory, J., Peto, C., Feinbaum, R., Pratt, L. and Ausubel, F. (1989) *Arabidopsis thaliana* mutant that develops as a light-grown plant in the absence of light. *Cell* 58:991-999.
3. Deng, X.-W. and Quail, P.H. (1992) Genetic and phenotypic characterization of *cop1* mutants of *Arabidopsis thaliana*. *The Plant Journal* 2:83-95.
4. Neff, M.M. and Van Volkenburgh, E. (1994) Light-stimulated cotyledon expansion in *Arabidopsis* seedlings. The role of phytochrome B. *Plant Physiol.* 104:1027-1032.
5. Baumgartner, B.J., Rapp, J.C. and Mullet, J.E. (1993) Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley (*Hordeum vulgare*) chloroplast development. Evidence for selective stabilization of *psbA* mRNA. *Plant Physiol.* 101:781-791.
6. Mullet, J.E. (1993) Dynamic regulation of chloroplast transcription. *Plant Physiol.* 103:309-313.
7. Kim, M., Christopher, D.A. and Mullet, J.E. (1993) Direct evidence for selective modulation of *psbA*, *rpoA*, *rbcL* and 16S RNA stability during barley chloroplast development. *Plant Mol. Biol.* 22:447-463.
8. Heinhorst, S., Cannon, G.C. and Weissbach, A. (1990) Chloroplast and mitochondrial DNA polymerases from cultured soybean cells. *Plant Physiol.* 92:939-945.
9. McKnown, R.L. and Tewari, K.K. (1984) Purification and properties of a pea chloroplast DNA polymerase. *Proc. Natl. Acad. Sci. USA* 81:2354-2358.
10. Pyke, K.A. and Leech, R.M. (1994) A genetic analysis of chloroplast division and expansion in *Arabidopsis thaliana*. *Plant Physiol.* 104:201-207.
11. Boinski, J.J., Wang, J.-L., Xu, P., Hotchkiss, T. and Berry, J.O. (1993) Post-transcriptional control of cell type-specific gene expression in bundle sheath and mesophyll chloroplasts of *Amaranthus hypochondriacus*. *Plant Mol. Biol.* 22:397-401.
12. Ngernprasirtsir, J., Chollet, R., Kobayashi, H., Sugiyama, T. and Akazawa, T. (1989) DNA methylation and the differential expression of C4 photosynthesis genes in mesophyll and bundle sheath cells of greening maize leaves. *J. Biol. Chem.* 264:8241-8248.

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APPENDIX 3

SCREENING FOR PLASTID DNA COPY NUMBER MUTANTS

Determination of Relative Plastid/Nuclear DNA ratios

The polymerase chain reaction has been used to determine relative gene copy number levels in various organisms (1-3). In this study specific oligonucleotide primers for nuclear and chloroplast genes are used for limited PCR amplification of crude DNA extracts from leaf tissue. Amplification of a single copy nuclear gene is used as an internal control to compensate for any variations in tissue preparation (see below) prior to PCR. The relative ratio of PCR products derived from plastid and nuclear DNA was determined to identify *Arabidopsis* seedlings with altered levels of plastid DNA.

Materials and Methods

Tissue is prepared according to the method of Klimyuk et al. (4) with modifications. A two-week old primary leaf is gently crushed and incubated in 40 μ l of 0.25M NaOH at room temperature for 5 minutes. Samples are placed in a boiling water bath for 30 seconds and then placed on ice. The solution is neutralized with 40 μ l 0.25M HCl and 20 μ l of 0.5M Tris pH 8.0/0.25% Nonidet P-40. Samples are incubated in a boiling water bath for 90 seconds and placed on ice. The solution is removed and the tissue is washed with dH₂O. To each tube 25 μ l PCR reaction mixture [1x Taq polymerase buffer (Promega; 50mM KCl, 10mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 2.50mM MgCl₂, 12.5 μ M each dNTP, 0.50 μ M each *atpE* primer, 0.25 μ M each plastocyanin primer, 0.25 units Taq DNA polymerase) and 20 μ l light mineral oil are added. Primers were designed from published nucleotide sequences of *Arabidopsis* plastocyanin (5; PC, nucleotides 1-471) and the *atpE-trnM-trnV* gene cluster (6; *atpE*, nucleotides 189-852). Each primer was designed as a 20-21mer with a calculated T_m of 60-61°C. Amplification under optimized conditions yields products of 471 and 663 bp for PC and *atpE* primers, respectively. Multiplex PCR is carried out for 18 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes for 48 individual samples using a Perkin-Elmer DNA thermal cycler. The conditions used for the PCR reactions in this study were empirically derived to produce the greatest efficiency in amplification with a minimal amount of starting material (primers, etc.).

After 18 rounds of amplification, the reactions are diluted with 250 μ l of 2x SSC. For quantitation of PCR products, 100 μ l aliquots of the diluted PCR reactions are vacuum blotted to duplicate GeneScreen Plus (DuPont) filters. Pairs of filters are hybridized separately with randomly primed *atpE* or plastocyanin probes. Up to 5x10⁶ cpm/ml of radiolabeled probe are used for each hybridization. Relative amounts of PCR products are determined by quantitation of the radioactive signal derived from the hybridized labeled probe using a Betascope 603 Blot Analyzer (Betagen). Ratios of *atpE*/PC signal are calculated for single sets of 48 reactions to negate any variability in amplification efficiency between single runs. Seedlings producing an *atpE*/PC ratio less or greater than two standard deviations of the mean are tested again to confirm the initial results. Plants producing a positive result after the second round of screening are transplanted to individual pots and allowed to produce seed.

The relative amounts of PC and *atpE* products with increasing rounds of PCR was studied. Tissue was prepared for PCR and amplified for an increasing number of cycles. Amplification of both nuclear and plastid DNA is logarithmic for both *atpE* and PC up to 20 cycles of PCR. Beyond 20 cycles the ratio between *atpE* and PC decreases, reflecting a reduced rate in amplification of the *atpE* product with a continued logarithmic accumulation of PC product. The decrease in rate of *atpE* accumulation is probably due to a reduced amount of primer available to the amplification reaction.

The PCR conditions used for screening have a wide response range. From about 1000 to 1×10^8 molecules of both *atpE* and PC were amplified for 18 cycles. In this example, accumulation of PCR products is logarithmic up to 3.2×10^6 molecules of each starting DNA. Amplification of the two products is independent of each other.

Utilizing this protocol it is possible for one person to screen 600 seedlings per week. About 4,000 M2 seedlings derived from X-ray treated seeds have been screened with 35 seedlings apparently containing greatly aberrant levels of plastid DNA. An additional 1,500 M2 seedlings derived from EMS treated seeds have been screened by this method. Of these, 21 seedlings have been identified with altered plastid DNA levels. Each of the putative mutants have been tested at least twice by PCR. Seeds are now being bulked for further analysis of M3 seedling populations. Currently, this PCR screening approach is being applied to the available T-DNA tagged lines with the goal of obtaining additional plastid DNA copy number mutants.

References

1. Neubauer, A., Neubauer, B., Liu, E. (1990) *Nuc. Acids Res.* 18: 993-998.
2. Volkenandt, M., Dicker, A.P., Banerjee, D., Fanin, R., Schweitzer, B., Horikoshi, T., Danenberg, K., Bertino, J.R. (1992) *Proc. Soc. Exp. Biol. Med.* 200: 1-6.
3. Wester, L., Somers D.E., Clack, T., Sharrock, R.A. (1994) *Plant J.* 5: 261-272.
4. Klimyuk, V.I., Carroll, B.J., Thomas, C.M., Jones, J.D.G. 1993. *Plant J.* 3: 493-494.
5. Vorst, O., Oosterhoff-Teertstra, R., Vankan, P., Smeekens, S., Weisbeek, P. (1988) *Gene* 65: 59-69.
6. Chen, H.-C., Wintz, H., Weil, J.-H., Pillay, D.T.N. (1988) *Nuc. Acids Res.* 16: 10372.

APPENDIX 4

ANALYSIS OF PLASTID NUMBER/CELL

Preparation of leaf material for the light microscope

Arabidopsis seeds were planted on Metromix and grown under a 16hr/8hr light/dark cycle in a growth chamber. Primary leaves from two week old seedlings were fixed in 10 mM sodium phosphate, pH 7.4, 3.5% glutaraldehyde in the dark for 1.5 hours. Tissue was washed with dH₂O and incubated in 100mM EDTA, pH 9.0, for 2 hours at 60°C. Samples were observed under the microscope using Nomarski optics.

Results

Initial determinations of cell size and chloroplast number in young *Arabidopsis* seedlings.

	<u>Average Cell Size*</u>	<u>Average # Chloroplasts/Cell*</u>
14-day old	774 (+/-136) μm^2	24 (+/-5)
17-day old	1287 (+/-299) μm^2	34 (+/-7)
24-day old	1387 (+/-363) μm^2	31 (+/-8)

*-data determined from 30 cells for each time point

A direct relationship between cell size (area) and total number of chloroplasts per cell is observed. Cell size reaches a maximum between 17 and 24 days under the growth conditions used in this study. Since cell size (and chloroplast number) does not increase appreciably after 17 days, 17 day old tissue was used in the screen for chloroplast number mutants.

We screened for mutants with deviations in chloroplast number per cell (i.e. during the initial screening process, mutants were screened visually for changes in number, size and shape). Cell area and chloroplast number per cell were determined for any plants that appear worthy of further characterization. Approximately 900 *Arabidopsis* seedlings derived from X-ray treated M2 lines (Columbia background) and 1000 T-DNA tagged seedlings have been screened to date.

One seedling from the X-ray deletion lines has approximately twice the number of chloroplasts per cell. The chloroplasts of this seedling also appear much smaller. In all other respects this seedling has a normal phenotype.

A T-DNA tagged seedling has also been identified that contains a greatly reduced number of chloroplasts per cell. In primary leaves only one large chloroplast is apparent in mesophyll cells. In some of these cells smaller plastids are also visible. In younger leaves (second and third leaves of the rosette) up to five chloroplasts per cell can be observed. This plant appears very pale green compared to its phenotypically wild type siblings. The pigmentation of the individual chloroplasts of the mutant seedling appears normal when compared to wild type, indicating that the pale green phenotype is due to the reduced number of chloroplasts per cell and not to reduced pigment accumulation. In addition the plant is much smaller and seed production is greatly reduced. We are currently in the process of determining if a T-DNA insert co-segregates with this mutant phenotype.

APPENDIX 5

A PLASTID RUN-ON TRANSCRIPTION ASSAY FOR CELL LYSATES OR FROZEN TISSUES

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Abstract: The plastid transcription assay has been extended to allow relative rates of plastid gene transcription to be measured in whole cell lysates or in previously frozen tissues. The method is based on the observation that plastid DNA and actively transcribing RNA polymerase is associated with plastid membranes. Therefore, plastid membranes isolated from whole cell lysates or frozen tissue can be used for plastid run-on transcription assays. This extends the utility of the assay to tissues where it is difficult to isolate intact plastids. The method was used to analyze plastid transcription in mechanically isolated and frozen bundle sheath cells of maize and the results were compared to transcription in mesophyll cells. This analysis revealed only small differences in the relative rate of *rbcL* and *psbA* transcription in these cell types.

Introduction

Information on the regulation of plastid gene transcription has been obtained using transcription run-on assays (Mullet and Klein, 1987; Deng et al, 1987; Rapp et al., 1992). This technique requires the isolation of intact plastids which are lysed in reaction mixtures containing radiolabeled UTP. RNA polymerases which had initiated transcription of plastid genes *in vivo* are allowed to transcribe for 5 min followed by isolation and analysis of radiolabeled RNAs. Addition of heparin to reaction mixtures limits initiation of transcription during the assay by DNA-free RNA polymerase and heparin blocks RNA turnover (Klein and Mullet, 1990). The plastid transcription run-on assay has been used to detect differential transcription of *psbD-psbC* in response to blue light (Sexton et al., 1990) and revealed that *rpoB* and several other plastid genes are differentially transcribed early in barley chloroplast development (Baumgartner et al., 1993).

In the past, plastid run-on transcription assays have required the isolation of intact plastids immediately prior to performing the assay. In most situations this is a relatively straight-forward task requiring approximately 1 h. However, there are times when isolation of intact plastids is difficult or not convenient. For example, it is difficult to isolate proplastids from meristematic tissues because the density of proplastids makes them difficult to separate from mitochondria on Percoll gradients. Similarly, intact amyloplasts are often difficult to isolate because starch grains lyse the plastids during centrifugation. Furthermore, researchers analyzing plastid transcription in mesophyll and bundle sheath cells of maize have primarily relied upon production of protoplasts from the two cell types prior to analysis of plastid transcription (i.e., Boinski et al., 1993). The procedure for protoplast production normally involves incubation of leaf material for several hours. Therefore, any conclusions regarding relative plastid transcription rates in plastids isolated from protoplasts must assume that the techniques used to prepare protoplasts do not alter *in vivo* patterns of plastid gene transcription.

In order to extend the plastid run-on transcription assay to the situations described above, we have developed a modified run-on transcription assay which requires the isolation of plastid membranes rather than intact plastids. Plastid DNA and actively transcribing RNA polymerases are to a large extent found associated with plastid membranes. Plastid membranes containing bound DNA/RNA polymerase can be separated from nuclei on Percoll gradients and used in standard transcription run-on assays. In addition, we find that membranes can be isolated from frozen tissue which extends the convenience and utility of this assay.

Protocols

Isolation of intact barley and maize mesophyll cell plastids.

* Maize seedlings (FR20A X FR140, Illinois Foundation Seeds) were grown at 25 C for 11 d (16 h light (500 μ E/m²); 8 h dark). Leaf tissue was harvested from the most developed regions of leaves, generally greater than 8 cm above the leaf base. The tissue was transferred to

grinding medium and ground with a Polytron (Brinkman Instruments) using short pulses. Alternatively, leaf tissue was harvested from 7 d old barley seedlings grown in continuous light.

- * Ground leaf material was filtered through a layer of miracloth and a 50 μ M polyester filter and centrifuged at 4,000g for 5 min.

- * Pelleted material was resuspended in grinding buffer and layered on a discontinuous PBF Percoll gradient consisting of 40% and 85% PBF Percoll. The gradient was centrifuged at 4,000g for 15 min. Intact plastids were located at the 40%/85% Percoll interface. Plastids were collected, diluted with 5 volumes of grinding medium and recovered by centrifugation. The plastids were resuspended in grinding buffer and counted using a hemacytometer.

Isolation of maize bundle sheath cell plastid membranes.

- * The material retained on the miracloth following the initial Polytron treatment was resuspended in grinding buffer. This material was ground and filtered several times until the filtrate was colorless.

- * Mechanically isolated bundle sheath strands were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle and subsequently resuspended in grinding buffer.

- * The sample was filtered through miracloth and a 50 μ M polyester filter and centrifuged for 5 min at 4,000g. The membrane pellet was resuspended in grinding buffer and layered onto 40% Percoll in a 30 mL Corex tube. The Corex tube was centrifuged for 10 min and the material at the interface was collected, diluted with 5 volumes of grinding buffer and the membranes were recovered by centrifugation.

Run-on plastid transcription assays (following Rapp et al, 1992)

- * Reaction mixtures containing 50 mM HEPES-KOH (pH 8.0), 10 mM $MgCl_2$, 25 mM potassium acetate, 10 mM dithiothreitol, 0.5 mM CTP, GTP, ATP and 250 μ Ci [α - ^{32}P]UTP (specific activity 3,000 Ci/mmol) were set up and warmed to 23 C.

- * Transcription was initiated by adding 2×10^7 plastids in a volume of less than 20 μ L to the reaction mix to bring the total volume to 200 μ L. When plastid membranes were analyzed, membrane material corresponding to 2×10^7 plastids was added to the reaction mixture based on chlorophyll content (Jagendorf, 1982).

- * Transcription reactions were carried out for 5 min after which the reactions were extracted once with an equal volume of TE-saturated phenol and once with an equal volume of chloroform:isoamyl alcohol (24:1).

- * Incorporation of total [^{32}P]UMP into RNA was determined by spotting aliquots on DE-81 filter paper (Hallick et al., 1976). Filters were washed twice for 5 min each with 5% sodium phosphate dibasic, once for 5 min with water, once for 5 min with 100% ethanol and rinsed once with ether to facilitate drying.

- * Analysis of specific gene transcription was carried out as previously described (Rapp et al, 1992). Radiolabeled transcripts from assays were hybridized to dot blots containing 1 pmol/dot of *in vitro* synthesized antisense RNA for each of the eight plastid genes being studied. Hybridizations were carried out with equal counts of probe RNA. Antisense RNAs were applied to the filters in duplicate. Following hybridization and washing of filters, the amount of radioactivity hybridized was determined by excising the dots followed by scintillation counting. This data was corrected for hybridization efficiency (Rapp et al, 1992) and probe size and expressed as fmols of UMP incorporated per kb of DNA per 2×10^7 plastids per 5 mins.

Solutions Required

Grinding Medium

50 mM HEPES-KOH, pH 8.0

0.33 M Sorbitol

2 mM EDTA

PBF Percoll (per 27.5 mL, 2 gradients)

0.8 g PEG (mol wt 3,500)

0.27 g bovine serum albumin

0.27 g ficoll

Percoll to 27.5 mL

Percoll Gradients

50 mM HEPES-KOH, pH 8.0

1.25 mM EDTA

6% Sorbitol

40% and 80% PBF Percoll (v/v)

Results and Discussion

Effect of freezing and thylakoid membrane isolation on transcription of barley plastid genes. In many applications it would be desirable to freeze tissue prior to analysis of plastid gene transcription. To test this possibility, barley leaves were isolated and frozen in liquid nitrogen. The frozen leaves were ground and subsequently thawed on ice in grinding medium. The resulting cell lysate was loaded on Percoll gradients, centrifuged and the thylakoid membrane fraction at the 40% Percoll interface isolated. In parallel, intact barley plastids from the same set of plants were isolated on Percoll gradients. Normal plastid run-on assays were set up containing 2×10^7 intact plastids or an equivalent amount of plastid membranes from frozen leaf tissue based on chlorophyll content. Analysis of total counts incorporated into RNA in each reaction indicated that on a chlorophyll basis, transcription in membranes from frozen tissue was reduced 20% compared to intact plastids which had not been frozen. The relative rates of plastid gene transcription in the two fractions were compared by hybridizing run-on transcripts to gene specific probes. The results of this assay are shown in Table 1. Transcription rates of the 5 plastid genes assayed varied 6-fold when intact plastids from non-frozen tissue was assayed. A similar range of transcription rates were observed when plastid membranes from frozen tissue was the source of DNA/RNA polymerase. These results indicate that this method should allow differences in plastid transcription to be analyzed using plastid membranes isolated from frozen tissues.

Transcription rates in mesophyll and bundle sheath chloroplasts. Previous studies have indicated that transcription of *rbcL* is enhanced in maize bundle sheath cell chloroplasts relative to other plastid genes (Ngernprasirtsir et al., 1989). This reported difference in transcription was correlated with differential methylation of *rbcL* in mesophyll cell chloroplasts. In contrast, in *Amaranthus hypochondriacus*, *rbcL* transcription in mesophyll and bundle sheath cells was similar and differential expression in the two cell types was ascribed to regulation at a post-transcriptional level (Boinski et al., 1993).

The modified plastid run-on transcription assay which can utilize frozen tissue provided a new method to examine the question of differential *rbcL* transcription in maize mesophyll and bundle sheath cells. Mesophyll cells were isolated by mechanical grinding followed by separation on Percoll gradients. Bundle sheath strands were subsequently isolated, frozen, ground in liquid nitrogen and plastid membranes isolated. RNA isolated from the mesophyll cell chloroplasts and bundle sheath strands were separated on RNA gels and probed with several plastid gene probes. Consistent with previous studies (cf., Sheen and Bogorad, 1985, 1988; Westhoff et al., 1991) *psaA* RNA abundance is similar in plastids of the two cell types whereas *rbcL* mRNA is relatively more abundant in bundle sheath cells and *psbA* is more abundant in mesophyll cell plastids.

The relative rates of transcription of 8 plastid genes in the two cell types were analyzed and the data, normalized to *psaA* is shown in Table 2. The ratio of transcription of most of the genes analyzed was similar in the two cell types. The one exception to this observation was the 16S rRNA which was transcribed at rates 2.5-fold greater in mesophyll cells than in bundle sheath cells. These data indicate that differential transcription of plastid genes can explain only a small part of the observed differences in plastid gene expression observed in the two cell types of maize. This conclusion is consistent with observations made in *Amaranthus* and differ from previous observations made in maize (Ngernprasirtsir et al., 1989).

References

- Boinski, J.J., J.-L. Wang, P. Xu, T. Hotchkiss and J.O. Berry. (1993) Post-Transcriptional control of cell type-specific gene expression in bundle sheath and mesophyll chloroplasts of *Amaranthus hypochondriacus*. *Plant Mol. Biol.* 22:397-410
- Deng, X.-W., Stern, D.B., Tonkyn, J.C., and W. Gruissem (1987) Plastid run-on transcription. *J. Biol. Chem.* 262: 9641-9648.
- Hallick, R.B., C. Lipper, O. C. Richards, and W.J. Rutter. (1976) Isolation of a transcriptionally active chromosome from chloroplasts of *Euglena gracilis*. *Biochemistry* 15:3039-3045.
- Jagendorf, A.T. (1982) In: *Methods in Chloroplast Molecular Biology* (eds. M. Edelman, R.B. Hallick and N.-H. Chua), pp. 881-897, Elsevier Biomedical Press, Amsterdam.
- Klein, R.R., and J.E. Mullet (1990) Light-induced transcription of chloroplast genes. *J. Biol. Chem.* 265: 1895-1902.
- Mullet, J.E. and R.R. Klein (1987) Transcription and RNA stability are important determinants of higher plant chloroplast RNA levels. *EMBO J.* 6:1571-1579.
- Ngernprasirtsir, J., Chollet, R., Kobayashi, H., Sugiyama, T., and T. Akazawa (1989) DNA methylation and the differential expression of C4 photosynthesis genes in mesophyll and bundle sheath cells of greening maize leaves. *J. Biol. Chem.* 264: 8241-8248.
- Rapp, J.C., B.J. Baumgartner and J. Mullet. (1992) Quantitative analysis of transcription and RNA levels of 15 barley chloroplast genes. *J. Biol. Chem.* 167:21404-21411.
- Sheen, J.Y. and L. Bogorad. (1985) Differential expression of the ribulose biphosphate carboxylase large subunit in bundle sheath and mesophyll cells of developing maize leaves is influenced by light. *Plant Physiol.* 79:1072-1076.
- Sheen, J.Y. and L. Bogorad. (1988) Differential expression in bundle sheath and mesophyll cells of maize of genes for Photosystem II components encoded by the plastid genome. *Plant Physiol.* 86: 1020-1026.
- Sexton, T.B., Christopher, D.A., and J.E. Mullet (1990) Light-induced switch in barley *psbD-psbC* promoter utilization: a novel mechanism regulating chloroplast gene expression. *EMBO J.* 9: 4485-4494.
- Westhoff, P., Offermann-Steinhard, K.O., Hofer, M., Eskins, K., Oswald, A., and M. Streubel. (1991) Differential accumulation of plastid transcripts encoding photosystem II components in the mesophyll and bundle-sheath cells of monocotyledonous NADP-malic enzyme-type C4 plants. *Planta* 184: 377-388.

Table 1. Relative Transcription Rates of Genes From Frozen Tissue or Intact Barley Etioplasts¹

Gene	Intact Plastids Transcription Rate	Frozen Plastids Transcription Rate	Ratio of Transcription Rates (Frozen/Intact)
<i>trnK</i>	1.60	1.56	1.02
<i>trnfM/G</i>	2.01	1.68	1.20
<i>psbA</i>	2.39	2.83	0.84
16S rRNA	4.94	6.47	0.76
<i>rbcL</i>	1.00	1.00	1.00

¹ The standard deviation of the assays was less than 10%.

Table 2. Relative Transcription Rates of Plastid Genes From Maize Mesophyll and Bundle Sheath Cells

Gene	Mesophyll Transcription Rate	Bundle Sheath Transcription Rate	Ratio of Transcription Rates (BS/M)
16S rRNA	7.7	4.1	0.5
<i>trnK</i>	2.8	2.4	0.9
<i>trnfM/G</i>	2.8	2.6	0.9
<i>rbcL</i>	1.9	2.5	1.3
<i>psbA</i>	9.6	9.4	1.0
<i>atpB</i>	0.86	1.0	1.2
<i>ndhA</i>	0.48	0.42	0.9
<i>psaA</i>	1.0	1.0	1.0

Figure Legend

Figure 1. Autoradiogram of an RNA blot of total RNA from bundle sheath strands and RNA from chloroplasts isolated from mesophyll cells of maize. RNA loading was adjusted to allow approximately equal signals from *psaA*. Sections of the blot were hybridized with single stranded gene specific probes from *psaA*, *rbcL*, *atpB*, 16S rRNA and *psbA*.

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