CONF-9203214--1

DE93 004513

情報交換セミナー報告書

REPORT OF THE INFORMATION EXCHANGE SEMINAR ON MOLECULAR REGULATION OF GENES AND ENZYMES IN PHOTOSYNTHESIS JAPAN-US COOPERATIVE PHOTOCONVERSION AND PHOTOSYNTHESIS RESEARCH PROGRAM

MEITETSU INUYAMA HOTEL, INUYAMA, JAPAN

MASTER

MARCH 15-19, 1992.

FG05-89ER14002

CONF-9205214--1

TITLE: Manipulating the Chloroplast Genome of Chlamydomonas: Present Realities and Future Prospects.

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Biolistic technology has now been developed for in vitro modification and stable reintroduction of chloroplast genes in Chlamydomonas reinhardtii (1-4) and Nicotiana tabacum (5-6) by homologous recombination. This permits, for the first time, structure : function analyses of plastid encoded proteins involved in photosynthesis (7,8), characterization of sequences which regulate expression of plastid genes at the transcriptional and translational levels (9-14), targeted disruption of chloroplast genes (15-18) and molecular analysis of processes involved in chloroplast recombination (19-21). Our laboratory has ongoing projects in each of these areas.

We are interested in the role played by the multifunctional D1 reaction center polypeptide of PS II encoded by the chloroplast psbA gene in a plant's photosynthetic response to high light. Excessive light is hypothesized to unbalance the D1 cycle of synthesis, maturation, light-dependent damage and, degradation (22), causing the rate of photodamage to exceed the rate of D1 replacement. To test this hypothesis, we have introduced site-specific psbA mutations and mutations in the 16S rRNA (19) conferring resistance to spectinomycin (spr) and streptomycin (sr) into the chloroplast genome of wild type Chiamydomonas. The psbA mutations alter function of the D1 protein (23), while the 16S rRNA mutations perturb chloroplast protein synthesis. When the 16S sr spr transformants are grown at high light (600 umol/m^2/s) they show both a 60% reduction in the initial rate of D1 synthesis (determined by ^{35}S incorporation) and enhanced photodamage, with up to 50% reductions in the maximum rate and quantum efficiency of photosynthesis, and 40% lower phototrophic growth rates compared to wild type. The same transformants grown at low light (90 umol/m^2/s) have essentially normal

photosynthetic performance. These results are consistent with synthesis being the limiting step of the D1 cycle in sr spr cells grown under high light. Since transformants carrying only the 16S spr mutation do not show this light sensitive photosynthetic phenotype, we believe the reduced chloroplast protein synthesis in the sr spr transformants results from the the 16S sr mutation which is known to affect chloroplast ribosome assembly (24). A Ser_{264} -> Ala mutation we have introduced into the quinone/herbicide binding site of D1 also causes a similar reduction in photosynthetic performance at high, but not low light. While such mutations are likely to affect photosythetic electron transfer directly (23), we cannot yet rule out the possibility that they may alter the propensity of D1 for photodamage or its rate of degradation. We have also begun to examine the importance of D1 maturation and modification in relation to its function and stability under high light and have obtained photosynthetically competent psbA transformants with altered C-terminal D1 processing (Ala_{345} -> STOP) and a blocked putative N-terminal phosphorylation site (Thr_2 -> Ala,Ser).

We are also characterizing the molecular basis for the differences in translational regulation of chloroplast genes encoding photosynthetic and ribosomal proteins. Under conditions of reduced chloroplast protein synthesis, ribosomal proteins are preferentially translated compared to photosynthetic proteins (25). Gel retardation and UV cross linking assays have been carried out using in vitro synthesized RNAs corresponding to short sequences of the 5' untranslated region (UTR) from both the rps12 and atpB genes and DNA/RNA binding protein fractions enriched by heparin-actigel chromatography. Proteins of 56-60 kDA and 47 kDa were observed to bind 5' UTR sequences from mRNAs of both genes. In addition, a protein of 36.5 kDa bound specifically to the 5' UTR of the rps12 mRNA and a second protein of 92 kDa bound specifically to the 5' UTR of atpB mRNA. Deletion and site directed mutagenesis studies are now in progress to localize more precisely the UTR sequences necessary and sufficient for binding of these proteins. We are attempting to obtain transformants homoplasmic for chimeric rps12 and atpB genes in which the 5' and 3' UTR sequences are exchanged or mutated to verify in vivo the regulatory roles of these protein-binding sequences deduced in vitro. We are especially interested in the translational

regulation of photosynthetic and ribosomal proteins encoded within the same chloroplast operon, (rps12-atpI-petJ and rps7-atpE) in contrast to individually transcribed genes such as atpB and rbcL.

Two different approaches for targeted disruption of chloroplast genes of Chlamydomonas have been published within the past year, both of which depend upon the fact that chloroplast transformation normally occurs by homologous gene replacement. We have used a cotransformation method (4,17) to introduce a selectable antibiotic resistance marker in the chloroplast 16S rRNA gene on one plasmid and a copy of a disrupted or mutated photosynthetic gene (atpB or rbcL) on a second plasmid (15). About 50% of the transformants selected under conditions permissive for survival of cells with photosynthetic defects initially carry copies of both the unselected, disrupted rbcL or atpB genes and the resident wild type genes. Nonphotosynthetic isolates homoplasmic for the disrupted rbcL or atpB genes are readily obtained as single cell clones of the initially heteroplasmic transformants. Transformants with both insertion and deletion null mutations of these monocistronic genes have been isolated. Whether the gene disruption techniques can also be applied to disruption of genes in polycistronic operons remains to be established. An alternative approach involving insertion of a heterologous selectable marker has been pioneered by Goldschmidt-Clermont (16). In this case a selectable disruption cartridge was created by flanking the bacterial aadA coding sequence for spectinomycin and streptomycin resistance with upstream and downstream regulatory sequences from atpA and rbcL respectively and adding polylinkers at each end. Homoplasmic nonphotosynthetic transformants for two different chloroplast genes have been isolated after several rounds of spectinomycin selection, while spectinomycin resistant transformants for an ORF of unknown function remained heteroplasmic for the disruption. Heteroplasmicity in the latter case may result because an essential chloroplast gene has been targeted for disruption.

The homologous integration of donor fragments during chloroplast transformation provides an unusual opportunity to study the processes of chloroplast recombination. We have shown that the integration events during

chloroplast transformation are biased to occur at the ends of the donor fragment, near the vector : insert junction (19). A recombination hot spot located near the 3' end of the psbA gene that we originally identified by genetic crosses also functions in chloroplast transformation (20). Sequence analysis of this 0.7 kb region revealed four pairs of 18-37 bp direct repeats and no ORF. Deletion of the repeats in the donor fragment has shown that they are probably not responsible for the recombinogenic activity. Comparison of the hotspot sequences between two interfertile strains suggests that the activity is either localized in a conserved AT rich region of 135 bp immediately 3' to the end of the psbA gene or within the last 260 bp of the psbA coding region containing a conserved topoisomerase binding motif. Deletion analysis is now in progress to localize the recombination hotspot further and gel shift and UV crosslinking assays are being used to define regions that bind recombination specific proteins. We are also attempting to isolate hyper-recombination mutants of Chlamydomonas using strains carrying specific chloroplast constructs that require elevated levels of chloroplast recombination for survival under selective conditions. Our long term goal is to identify and characterize the spectrum of proteins mediating the distinct recombination/repair processes operating in the chloroplasts of both Chlamydomonas and higher plants.

Acknowledgement: Supported by NIH Grant GM-19427, DOE Grant DE-FG05-89ER/4005, NIH predoctoral traineeship GM-07754 to PH and NIH Postdoctoral traineeships GM-14046 to CH and GM-12934 to SN.

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