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CND41, a DNA-binding protein in chloroplast nucleoid, and its function

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Abstract

Plastids, which are organelles unique to plant cells, bear their own genome that is organized into DNA-protein complexes (nucleoids). Regulation of gene expression in the plastid has been extensively investigated because this organelle plays an important role in photosynthesis. Few attempts, however, have been made to characterize the regulation of plastid gene expression at the chromosomal structure, using plastid nucleoids. In this report, we summarize the recent progress in the characterization of DNA-binding proteins in plastids, with special emphasis on CND41, a DNA binding protein, which we recently identified in the chloroplast nucleoids from photomixotrophically cultured tobacco cells. CND41 is a protein of 502 amino acids which consisted of a transit peptide of 120 amino acids and a mature protein of 382 amino acids. The N-terminal of the 'mature' protein has lysine-rich region which is essential for DNA-binding. CND41 also showed significant identities to some aspartyl proteases. Protease activity of purified CND41 has been recently confirmed and characterized. On the other hand, characterization of accumulation of CND41 both in wild type and transgenic tobacco with reduced amount of CND41 suggests that CND41 is a negative regulator in chloroplast gene expression. Further investigation indicated that gene expression of CND41 is cell-specifically and developmentally regulated as well as sugar-induced expression. The reduction of CND41 expression in transgenic tobacco also brought the stunted plant growth due to the reduced cell length in stem. GA3 treatment on apical meristem reversed the dwarf phenotype in the transformants. Effects of CND41 expression on GA biosynthesis will be discussed.

Plastids, which are organelles unique to plant cells, bear their own genome that is organized into DNA-protein complexes (nucleoids) similar to those found in bacteria. Regulation of gene expression in the plastid has been extensively investigated because this organelle plays an important role in photosynthesis. In addition, transcriptional, posttranscriptional, and translational regulatory mechanisms have been shown to control gene expression in plastids [1-4].

Few attempts, however, have been made to characterize the regulation of plastid gene expression at the chromosomal structure, using plastid nucleoids. Differences in the patterns of the plastid nucleoids in chloroplasts and those in chromoplasts, etioplasts, and proplastids suggest that the form of DNA packaging in plastid nucleoids may be linked with transcriptional control of plastid gene expression [5-7]. DNA binding proteins, in addition to RNA binding ones, have been reported in plastid nucleoids [8-11]. In this report, we summarize the recent progress in the characterization of DNA-binding proteins in plastids, with special emphasis on CND41, a DNA binding protein which we recently identified in the chloroplast nucleoids from photomixotrophically cultured tobacco cells, and discuss their functions.

Factors involved in transcriptional machinery

Nucleoids isolated from plastids show transcriptional activity [7, 10, 12], indicating that factors involved in transcription are components of nucleoids. In prokaryotic system, RNA polymerase can bind DNA directly. In fact, some subunits of plastid RNA polymerase have affinity for DNA [13]. Plastids have two types of RNA polymerases; nuclear-encoded RNA polymerase (NEP) and plastid-encoded prokaryotic RNA polymerase (PEP) [14, 15]. While the detail of DNA binding affinity of these RNA polymerases are still not clear yet, recent progress in the isolation of cDNAs of sigma factor (key compo-

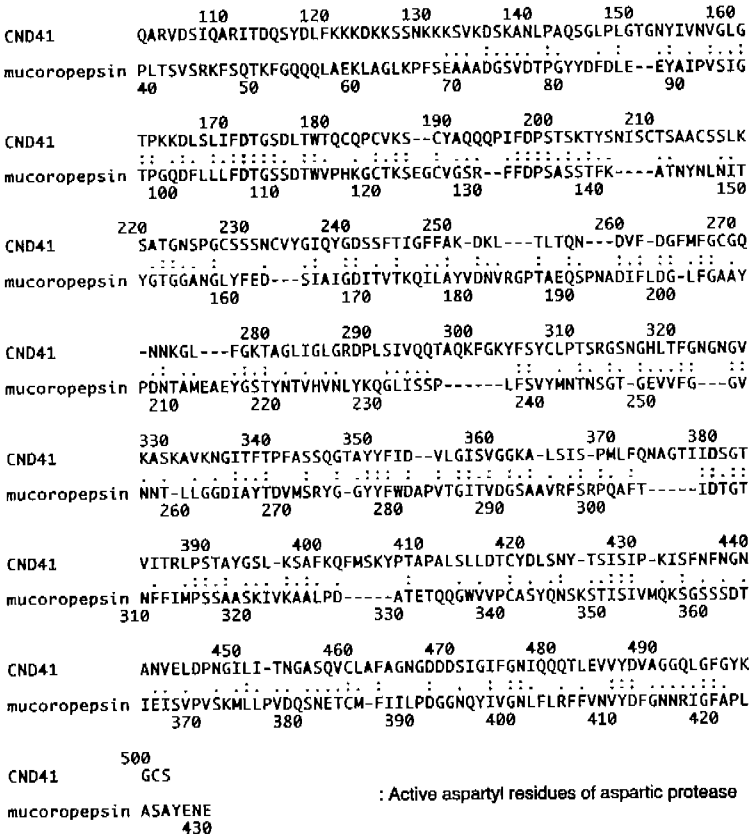

Figure 1. Sequence Alignment of CND41 and Mucopepsin (pepsin; CARP_RHIMI). Protease motif is boxed and active aspartyl residuc is underlined. Colons indicate identical residues and dots indicate homologous residues.

nent of PEP) from several plant species, as well as the isolation of T7-bacteriophage type RNA polymerase facilitate the characterization of regulation of transcription through RNA polymerase [14, 16-18].

Except the subunits of RNA polymerase, several sequence-specific DNA binding proteins, which might be involved in the regulation of transcription, are identified. A 115-kD protein that seems to bind specifically to a region between the large subunit of ribulose bisphosphate carboxylase (*rbcL*) and beta subunit of ATPase (*atpB*) has been detected in pea chloroplasts [19]. The *psbD* operon is transcribed from at least three different promoters. One of the *psbD* promoters is differentially activated when plants are exposed to blue light. This blue light-responsive promoter is highly conserved among plant species and contains multiple AAG sequences, which interacts with a sequence-specific DNA binding factor termed AGF [20]. AGF might be a sigma-factor because light regulated gene expression of sigma-factor is recently reported [16, 21]. Transcription of the spinach plastid *rrn* operon promoter is initiated at three sites: P1, PC, and P2. However, *in vivo*, transcription starts exclusively at PC. Iratni et al. suggest that the sequence-specific DNA-binding factor CDF2 functions as a repressor for transcription initiation of the PEP at P1 and P2 [22].

Factors which might be involved in the DNA replication, recombination and so on.

Proteins in nucleoids may also be involved in the DNA replication, repair, recombination and so on. DNA polymerases [23, 24], DNA topoisomerases [25, 26], and DNA helicase [27] have been purified from the chloroplasts of higher plants. An *Escherichia coli* RecA homolog, essential for DNA repair and recombination, has also been identified in plastids [28]. A DNA binding protein with DNA polymerase 'accessory activity' has also been purified to apparent homogeneity from pea chloroplasts. This 43 kD protein, present abundantly in the pea chloroplast, increases cognate DNA polymerase-primase activity in a dose dependent manner [29]. Plastid nucleoids are known to bind to the envelope membrane in developing chloroplasts. Here, plastid DNA is extensively replicated. cDNA of a DNA-binding protein of 130 kD

(named PEND, 9) in the inner envelope membranes of developing plastids in pea is recently cloned [30]. The PEND protein consists of a basic region plus zipper region and a putative membrane-spanning region. These DNA-binding proteins may play an important role in the replication of pea chloroplast DNA. On the other hand, the 18-kD protein that binds to the chloroplast DNA replicative origin was reported to be an iron-sulfur protein related to a subunit of NADH dehydrogenase [31].

Several other proteins are also believed to have structural roles in packaging chloroplast DNA within the plastid nucleoids. A 17-kD protein that cross-reacts with antiserum prepared against *E. coli* HU was detected in spinach chloroplasts [32]. The *Guillardia theata* chloroplast *hlpA* gene encodes a protein resembling bacterial histone-like protein HU. HlpA protein overexpressed in *E. coli* strongly facilitated cyclization of a short DNA fragment in the presence of T4 DNA ligase, indicating its ability to mediate very tight DNA curvatures [33]. Recently, a 68 kD protein was purified from soybean nucleoid and its DNA compacting activity with inhibition of DNA synthesis *in vitro* was reported [34].

CND41, a DNA-binding protein in chloroplast nucleoids from cultured tobacco cells

Previously, we isolated chloroplast nucleoids from photomixotrophically cultured tobacco cells with actively dividing chloroplasts [10]. Moreover, we identified a DNA binding protein (CND41) in the chloroplast nucleoids that has a subunit molecular mass of 41kD and a basic pI. The cDNA isolated has an open reading frame of 502 amino acids which consisted of a transit peptide of 120 amino acids and a mature protein of 382 amino acids (accession number D26015, 35). No significant homology to other known genes was found in several databases using the FASTA and BLAST programs, when we isolated. Some aspartyl proteases (mucoropepsin; EC 3.4.23.23 , cathepsin ; EC 3.4.23.34, and so on), however, showed about 26% identities to CND41 over 380 amino acid residues (Figure 1). Interestingly, the active sites of these proteases were completely conserved in CND41. Biochemical analysis of purified CND41 confirmed its protease activity (see below).

CND41 has also several structural features, e.g., lysine-rich region in the N-terminal of the 'mature' CND41 (10 of 18 residues). Expression of CND41 in *E. coli* indicated that the lysine-rich region is essential for the DNA binding activity. When different regions of *psbA* and synthesized oligonucleotides (poly dG-dC, poly dA-dT) were reacted at several concentrations to estimate the DNA binding affinity of CND41, CND41 bound all probes with the similar affinity as histone. These results suggest that CND41 would bind chloroplast DNA nonspecifically.

Because chloroplast nucleoids are thought to consist of chloroplast DNA and CND41, the copy number for chloroplast DNA per cell in tobacco cells was determined. Photoautotrophic cultured tobacco NI cells had high chloroplast DNA copies per cells, i.e., 10000 to 26000, whereas the roots had a very low copy number (< 600) [35, 36]. The chloroplast copy in nonselected cells was similar to that in leaves. The lower correlation between the copy number of chloroplast DNA and CND41 accumulation in the cell indicates that CND41 is not a simple structural protein in the chloroplast nucleoid.

CND41 and Chloroplast Transcription

CND41 was abundant in cultured tobacco cells grown on medium containing sucrose, as well as in the stems. The high CND41 accumulation coincided with the low accumulation of the mRNA for the *psbA*, *rbcL*, and the rRNA (16S rRNA) encoded on chloroplast DNA. There was little accumulation of CND41, however, in photoautotrophic cultured cells and leaves of tobacco. The very low accumulation of CND41 in these cells coincided with the large accumulation of *psbA*, *rbcL*, and 16S rDNA transcripts, except in the root where no photosynthetic activity was found to occur.

The characterization of cultured cells of antisense transgenic tobacco (R22 and R28), that had reduced levels of CND41 protein, showed that both R22 and R28 cells had a higher level of expression for *psbA*, *psbD/C,* and *rbcL*, and the 16S and 23S rDNAs compared to the control cells [35]. There was no difference in the chloroplast DNA copy number between the transformants and the control cells. Because these cultured cells were maintained under the same culture condition, the increase in chloroplastic transcript levels would reflect the effect of reduced CND41 level on the chloroplast gene expression. The small increase of chloroplastic transcripts in comparison with the large reduction of CND41 protein in antisense transgenes, however, suggests that CND41 is only one of the rate-limiting steps for the transcriptional regulation of chloroplast in cultured cells. Other factors, e.g., RNA polymerase, its sigma factor and/or post-transcriptional regulation may affect the small accumulation of chloroplast transcripts in cultured cells.

Figure 2. F2 progeny of transgenic tobacco with antisense CND41 transgene. WT: *Nicotiana tabacum* cv. Samsun NN wild type, R22-1 and R22-2: F2-progeny of R22 antisense CND41 transgenic tobacco, R28-1: F2-progeny of R28 antisense CND41 transgenic tobacco.

Protease activity of CND41

Interesting structural characteristics of CND41 is the aspartyl protease motif (Figure 1). All active site residues are conserved as mentioned above. To examine the protease activity of CND41 and the possibility that CND41 might regulate the chloroplast gene expression through protease activity, we purified CND41 from photomixotrophically cultured tobacco cells [37]. Cultured cells were homogenized with low ionic buffer and CND41 was recovered from insoluble fraction. Then CND41 was solubilized in buffer containing 0.5M NaCl and purified with cation exchange-, hydrophobic interaction- and heparin-column. Purified CND41 showed no protease activity at neutral pH, but high activity was found at acidic pH (i.e. pH 3), with hemoglobin as substrate. Further characterization indicated that protease activity at acidic pH was inhibited by NADPH, nucleoside-triphosphate and ATP/GTP gamma-S analog. Furthermore, a specific substrate can be digested under neutral pH by CND41 (Murakami et al . unpublished data). The characterization of the regulation of proteolysis under physiological condition and of physiological roles of protease activity are on going.

Regulation of gene expression of CND41

CND41 mRNA mainly accumulated in nonphotosynthetic tissues and cells like root, stem and cultured cells. Analysis of accumulation in cultured cells of tobacco indicated nonselected (NII) cells accumulated large amount of CND41 mRNA, while photoautotrophic (NI) cells accumulated little [35]. To investigate the regulation of gene expression of CND41, the expression of CND41 in cultured NII cells was examined. The level of CND41 transcripts in cultured NII cells was found to change during the growth. Low CND41 mRNA level at early log stage started to increase at log to stationary stage, then decreased [38]. Significant change of nucleoid composition during exponential stage to stationary stage is recently reported in *E. coli* [38] and the siliencing of functions by a compaction of the genome DNA is proposed. Induction of CND41 at late growth stage may have similar effect on chloroplast DNA activity. Sucrose in medium also affected the accumulation of CND41 mRNA [35, 38, Chatani *et al.*, unpublished data]. Glucose also induced the gene expression but mannitol did not. These data indicate that CND41 is sugar-inducible gene. The characterization of regulatory mechanism of sugar-induction is on going.

CND41 and plant development

The antisense transformants of F1 and F2 progeny showed trends in dwarfism and highly greened leaves (Figure 2). Observation of cell length of stem indicated that dwarfism is due to the reduced cell length, and not to the reduction of cell division. Antisense transformants also showed reduced number of epidermal hairs on the leaf surface.

To examine the cause of dwarfism, GA3 was fed on apical meristem. GA3 treatment obviously reversed the dwarf phenotype of stem and shorten phenotype of leaf in the transformants. Further analysis of gibberellin contents in transformants indicated the reduction of GA3. While the mechanism of this reduction is not clear, the chloroplast development in apical meristem was better than control plants. Because the apical meristem is the site of GA biosynthesis and plastids are the site of the biosynthesis of GA precursor, reduction of CND41 expression and development of plastid in this region might affect the GA biosynthesis (Nakano et al., unpublished results). Plastids are not only the site of biosynthesis of metabolites, but also the site of plant growth regulators like gibberellin, ABA and so on. It is quite conceiv-

able that modification of chloroplast development may affect the whole plant development.

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