Biochemical characterization of CND41, a nucleoid DNA binding protein in chloroplast

Shinya Murakami

2000

Biochemical characterization of CND41, a nucleoid DNA binding protein in chloroplast

Shinya Murakami

2000

Contents

Abbreviations

ATP-γ-S	adenosine 5'-*O*-(3-thiotriphosphate)
CABP	2-carboxyarbinitol-1,5-bisphosphate
CBB	Coomassie Brilliant Blue
CND41	41kD chloroplast nucleoid DNA-binding protein
CPBP	2-carboxypentitol-1,5-bisphosphate
EDTA	ethylenediaminetetraacetic acid
EST	expression sequence tag
FITC	fluorescein isothiocyanate
FTC-Hb	FITC-labeled hemoglobin
GA	gibberellic acid
GSH	reduced form of glutathione
GSSG	oxidized form of glutathione
GTP-γ-S	guanosine 5'-*O*-(3-thiotriphosphate)
Hb	hemoglobin
Hepes	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid
Mops	3-(*N*-morpholino) propanesulfonic acid
OEC23	oxygen evolving complex protein of 23 kD in photosystem II
PEG	polyethylene glycol
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene difluoride
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane

Introduction

Plastids, organelles unique to plant cells, bear their own genome. Regulation of gene expression in plastids has been extensively investigated because this organelle has important functions in photosynthesis as well as in nitrogen and sulfur assimilation. Whereas the gene expression of prokaryote, the putative origin of chloroplast, is mainly controlled at the transcriptional level, that of plastids is not only at the transcriptional level but also at the post transcriptional level which includes editing, splicing, and mRNA stability, and at the translational level (Mullet, 1993; Mayfield et al., 1995; Sugita and Sugiura, 1996; Sugiura et al, 1998; Schuster et al., 1999). In the last decade, it has been thought that the amounts of chloroplast gene transcripts were controlled by mRNA stability, and transcriptional control was considered to play only a limited role in the regulation of plastid gene expression (Deng and Gruissem, 1987; Mullet and Klein, 1987). However, recent investigations have revealed the importance of transcriptional control again (Rapp et al., 1992; Sakai et al., 1998).

The investigation of transcriptional regulation in chloroplasts has made great progress in last few years. Two types of RNA polymerases, i. e., plastid-encoded and nuclear-encoded RNA polymerases have been characterized (Allison et al., 1996; Hajdukiewicz et al., 1997), and the genes for σ factors, which are key components of prokaryote type RNA polymerase for the promoter recognition of genes and encoded in nuclear DNA, have been isolated and characterized from several plant species (Isono et al., 1997; Tanaka et al., 1997; Tozawa et al., 1998; Morikawa et al., 1999). A gene for T7-phage type RNA polymerase, a nuclear encoded RNA polymerase in chloroplasts, also has been identified (Hedtke et al., 1997).

Chloroplastic gene expression changes at the transcription level with plastid development (Baumgartner et al., 1993). Characterization of transcriptional activity of the isolated plastid nucleoids indicates that the activity in chloroplast is approximately 25 times higher than that in proplastid (Sakai et al. 1998). Because plastid nucleoids consist of plastid DNA and protein complex (Hansmann et al., 1985; Reiss and Link, 1985, Nemoto et al., 1990, Nakano et al. 1993), proteins in plastid nucleoids are considered to be important for transcriptional regulation of plastid gene expression. Thus, we have isolated a DNA-binding protein of 41 kD in chloroplast nucleoids from photomixotrophically cultured tobacco cells (Nakano et al., 1993, Nakano et al., 1997). The protein (named CND41; chloroplast nucleoid DNA-binding protein of 41 kD) is abundant in non- or low-photosynthetic tobacco cells or tissues, whereas very low in photoautotrophically cultured tobacco cells and leaves. The reverse relationship of CND41 accumulation with chloroplastic gene transcripts and the characterization of antisense CND41 transgenic tobacco with reduced amount of CND41 indicate that CND41 is a possible negative regulator of chloroplast gene transcription.

Because CND41 is non-specific DNA-binding protein, the function of CND41 as a negative regulator of the chloroplast gene expression was first considered to be mediated by this nonspecific binding of CND41 to DNA. However, the presence of active domains of aspartic protease conserved in all the proteases of this class (Fig. 0-1), and about 26 % identities to a typical aspartic protease (mucoropepsin, EC3.4.23.23) over 380 amino acid residues (Fig. 0-2) suggest that CND41 may function as a protease. Aspartic proteases (EC 3.4.23), a class of endopeptidases with acidic pH optima, are present in a wide variety of organisms such as animals, plants, fungi, yeast, some bacteria and viruses, but there was no report in chloroplasts. Whereas many examples of protein degradation in chloroplasts have been documented (Adam, 1996; Andersson and Aro, 1997), knowledge of chloroplast proteases is still limited (Sokolenko et al., 1997; Itzhaki et al., 1998).

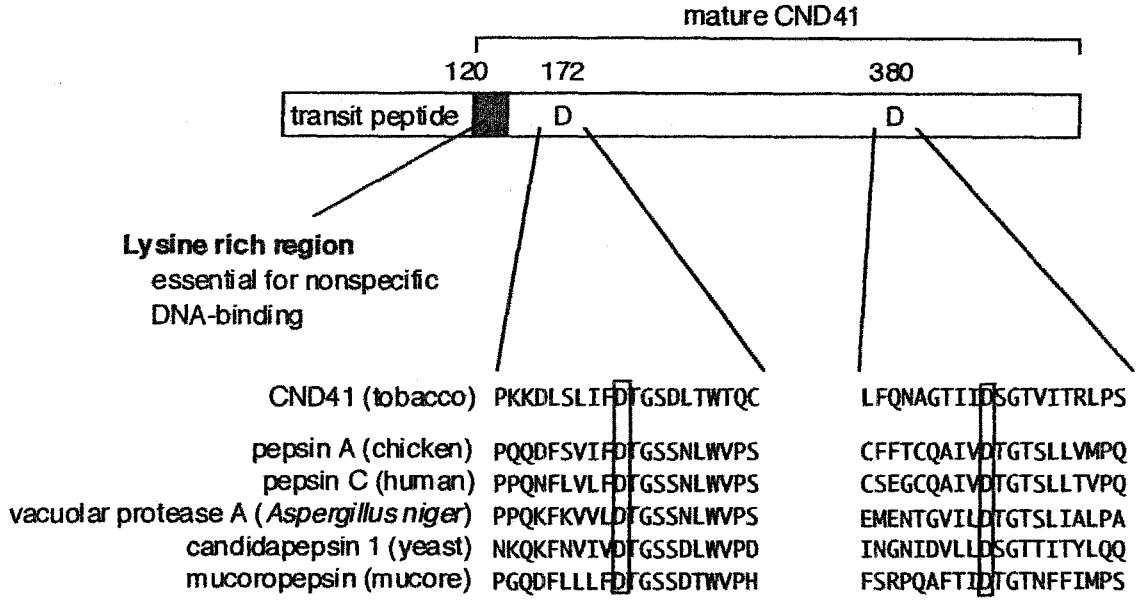


Fig. 0-1. Structure of CND41 and sequence alignment with typical aspartic proteases (pepsin A, PEPA_CHICK; pepsin C, PEPC_HUMAN; vacuolar protease A, CARP_NEUCR; candidapepsin 1, CAR1_CANAL; mucoropepsin; CARP_RHIMI). Active sites of aspartic acid residues were boxed.

If CND41 has proteolytic activity, CND41 can have both protease and DNA-binding activity. Only a few DNA-binding proteins like Lon protease of *E. coli* (Fu et al., 1997 and references therein) are known to have protease activity. Lon contributes to the regulation of several important cellular functions, including radiation resistance, cell division and proteolytic degradation of certain regulatory and abnormal proteins. AEBP1, a mouse carboxypeptidase with site-specific DNA-binding activity, is responsible for transcriptional repression (He et al., 1995). The yeast homologue of mammalian bleomycin hydrolase, GAL6, which has high binding affinity for RNA and single-strand DNA, but lower affinity for double-strand DNA, also shows cysteine protease activity (Xu and Johnston, 1994; Zheng and Johnston, 1998). DNA-binding protein with proteolytic activity would be important regulatory factor both in prokaryotic and eukaryotic genetic systems.

Antisense CND41 transformants with reduced CND41 amount showed pleiotropic

effects on the plant development; dwarfism, retarded senescence and high accumulation of chloroplast transcripts (Nakano et al., 1997, and unpublished data). The characterization of protease activity of CND41 is quite important to understand the multiple functions of CND41. For this purpose, I first purified this protein from cultured tobacco cells and characterized its proteolytic activity with FITC-labeled hemoglobin as the model substrate. Secondly, I examined the protease activity of CND41 at pH 7.5, because the pH in chloroplast stroma where chloroplast nucleoids exist, is weak basic to neutral. Finally, I characterized the *in vivo* function of CND41 protease in plant using antisense CND41 tobacco with reduced amount of CND41, and discussed the multiple functions of CND41 in plant development.

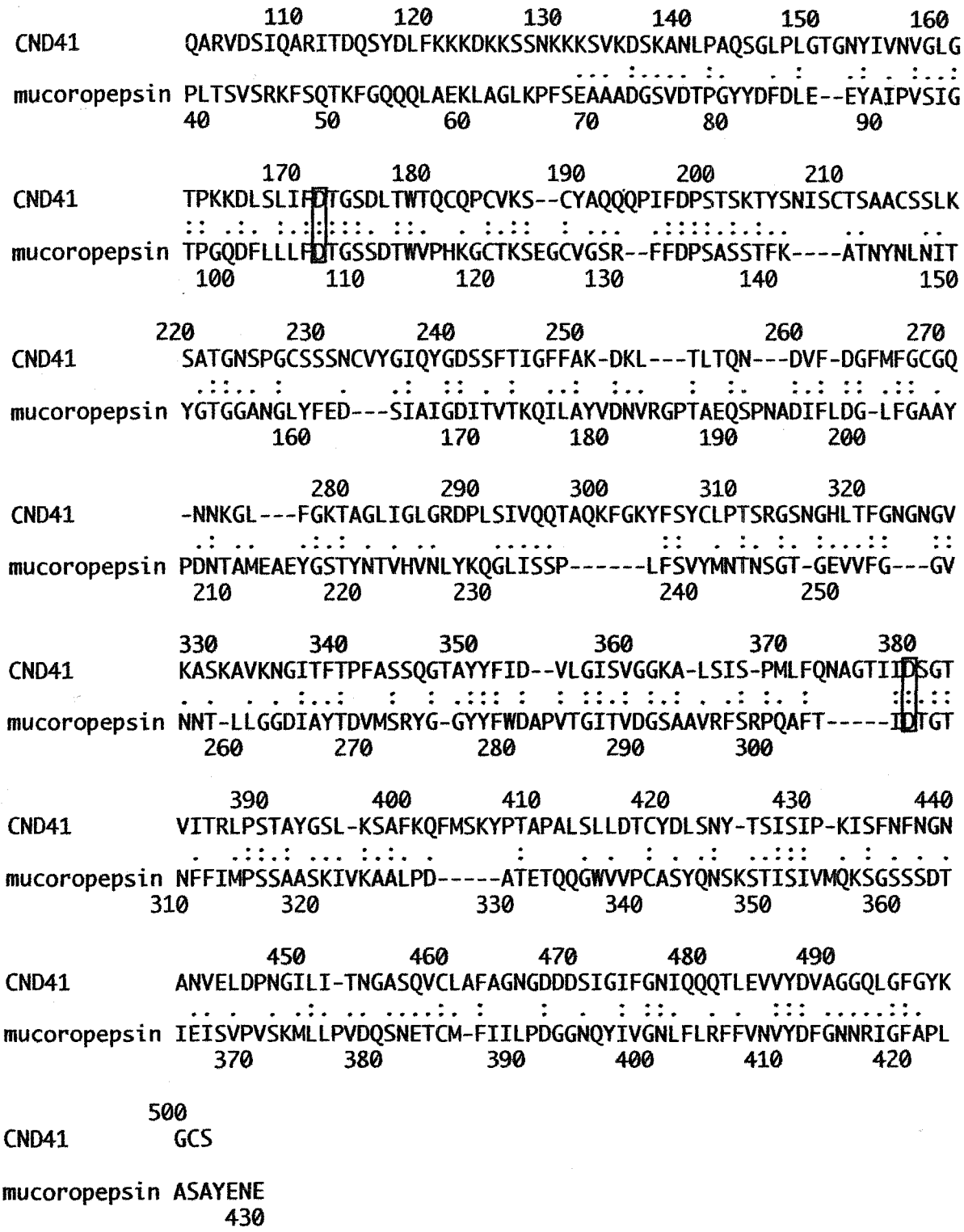


Fig. 0-2. Sequence alignment of CND41 and mucoropepsin precursor (CARP RHIMI). Colons indicate identical residues and dots indicates similar residues. Overall identity in 387 amino acids overlap is 26 %. Active sites of aspartic acids are boxed.

Chapter 1

Purification and proteolytic activity of CND41

As described in the Introduction, the deduced amino acid sequence indicates that CND41 has active domains conserved for aspartic proteases. Because CND41 is proposed to be putative negative regulator of chloroplast gene transcription, I became interested in the bifunctional nature of this novel protein; i. e., possibility that CND41 degrades proteins related to transcription, such as σ factors or RNA polymerases, and decreases the level of chloroplast transcripts. Then, I first attempted to confirm the proteolytic activity of CND41. I purified the CND41 from cultured tobacco cells and assayed its proteolytic activity with FITC-labeled hemoglobin as the substrate. I found that CND41 has strong proteolytic activity at acidic conditions, then precisely determined its nature as a protease. I also examined the proteolytic activity at a weak acidic pH. CND41 degraded chloroplastic proteins, Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) and OEC23 (oxygen evolving complex protein of 23 kD in photosystem II) at sequence specific manner under weak acidic conditions, although CND41 degraded proteins nonspecifically the under acidic condition.

Materials and Methods

Plant materials

Photomixotrophic cultured cells of *Nicotiana tabacum* cv. Samsun NN were maintained under continuous light (about 60 μmol $m^{-2}s^{-1}$) in a modified Linsmaier and Skoog liquid medium containing 3% (w/v) sucrose as described previously (Takeda et al., 1990).

Protein substrates

FITC-labeled hemoglobin was used as a substrate because of its digestibility, purity and solubility at a wide range of pH. The preparation of FITC-labeled hemoglobin (FTC-Hb) was done by the method described by Twining (1984) with some modification. Recombinant tobacco OEC23 5B was prepared as described by Ifuku et al. (1998). Purified spinach Rubisco was gift from Prof. A. Yokota of Nara Institute of Science and Technology.

Examination of extraction condition of CND41

All procedures were done below 4 °C. Cultured cells after 7-10 days of culture were harvested and homogenized in the same volume of 50 mM Na phosphate buffer (pH 7.2) with a blender. The homogenate was filtered through Miracloth (Calbiochem), and centrifuged at 10,000 x *g* for 10 minutes. The precipitate was suspended in 1/10 volume of phosphate buffer alone or phosphate buffer with 10 mM EDTA, 1 % Triton X-100 or NaCl of concentration indicated in Fig. 1-1. Amount of extracted CND41 was determined by immunoblotting or Southwestern blotting following to SDS-PAGE as described by Nakano et al. (1997).

Purification of CND41

All procedures for purification were done below 4 °C. Cells from an 8-day culture (about 1 kg fresh weight) were homogenized in a Polytron (Kinematica) in the same volume of buffer A (50 mM Na phosphate, pH 7.2 and 10 mM 2-mercaptoethanol) containing 10 mM EDTA. The homogenate was filtered through two layers each of gauze and Miracloth (Calbiochem) then centrifuged at 7,000 x *g* for 30 min. The precipitate was resuspended for 1 hour in 1/10 volume of buffer A containing 0.5 M NaCl then centrifuged at 15,000 x *g* for

20 min.

The supernatant diluted with the same volume of buffer A was applied to an SP-Sepharose (Amersham Pharmacia) column equilibrated with buffer A containing 0.25 M NaCl. Proteins were eluted with a 0.25 M-1.2 M NaCl gradient. The CND41 fractions were applied to an Octyl Sepharose CL-4B (Amersham Pharmacia) column, which had been equilibrated with buffer A containing 0.25 M NaCl, then proteins eluted in a gradient of 0-50% ethylene glycol. The eluted CND41 fractions were dialyzed in 50 mM Hepes-NaOH (pH 7.2), 10 mM 2-mercaptoethanol and 0.25 M NaCl then layered on a HiTrap heparin column (Amersham Pharmacia). Proteins were eluted with a gradient of 0.25 M-1.5 M NaCl. The CND41 fractions were concentrated using Centriplus-10 (Millipore) and stored at -70 °C in 25 % (v/v) glycerol until used. The filtrate was similarly stored and used as the mock control in the protease assay. The elution of CND41 was monitored by immunoblotting or by proteolytic activity with FITC-labeled hemoglobin as the substrate.

The concentration of the purified CND41 was measured by the absorbance at 280 nm (Fasman, 1989). Purity was determined by SDS-PAGE with silver staining, immunoblotting and Southwestern blotting as described by Nakano et al. (1997). N-terminal amino acid sequences of the purified proteins were determined with an ABI 476A peptide sequencer (Perkin-Elmer).

Protease activity assay

FTC-Hb (final concentration 0.5 mg/ml) and purified CND41 (20 nM) were mixed on ice in Na citrate buffer at the indicated pH (final concentration 50 mM, final volume 50 μl), and the proteolytic reaction was started by an immediate shift of temperature to 30 °C. After 40 min of incubation, the reaction was stopped by the addition of 120 μl of 5 % (w/v) TCA. After the precipitation of TCA-insoluble protein by centrifugation at 10,000 x *g* for

10 min 4 °C, 120 μl of the supernatant was diluted with 800 μl of 500 mM Tris buffer (pH 8.5), and its fluorescence was measured in a fluorescence spectrophotometer (Excitation: 490 nm, emission: 525 nm, Model F-2000, Hitachi).

Proteolysis of OEC23 and Rubisco

Purified CND41 (20 nM) was mixed with recombinant tobacco OEC23 5B (20 μg/ml) or purified spinach Rubisco (40 μg/ml) on ice in Na citrate buffer at the indicated pH (final concentration 50 mM, final volume 50 μl), and the proteolytic reaction was done as described above. After the precipitation of proteins with 5 % (w/v) TCA, the precipitate was separated by SDS-PAGE with 12.5 % polyacrylamide gel for OEC23 or 7.5 % gel for Rubisco, and stained with CBB. N-terminal amino acid sequences of the digested proteins were determined with an ABI 476A peptide sequencer (Perkin-Elmer) as described by Nakano et al. (1997).

Results

Solubilization of CND41

CND41 first was identified as a DNA-binding protein, but proteolytic activity was not confirmed at the beginning of purification. I therefore purified CND41 using immunological detection with rabbit antiserum prepared against truncated CND41 produced in *Escherichia coli* (Nakano et al, 1997). This specific antiserum detected two proteins of molecular mass about 46 kD and 41 kD in extract of cultured tobacco cells. The 41 kD protein was minor and insoluble but showed DNA-binding activity on Southwestern blotting. The 46 kD protein was major and mainly soluble but did not show DNA-binding activity (Fig. 1-1A). Because CND41 was isolated as DNA-binding protein on Southwestern blotting (Nakano, 1993), I further purified the 41 kD protein. In order to purify it, the extraction

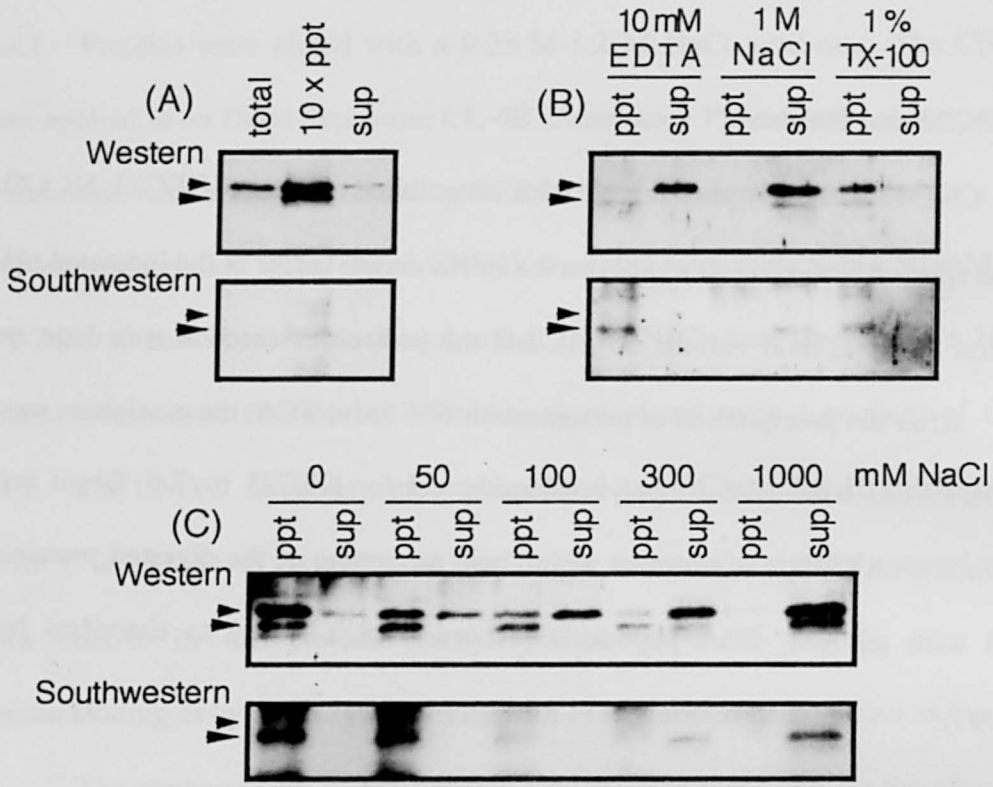


Fig. 1-1. CND41 solubilization condition. (A): Extract of cultured tobacco cells was fractionated by centrifugation at 10,000 x *g* for 10 min. Total extract (total), insoluble fraction which was 10 times concentrated precipitate of total extract (ppt), and soluble fraction (sup) were separated by SDS-PAGE. (B): Solubilization of CND41 from insoluble fraction with 10 mM EDTA (10 mM EDTA), 1 M NaCl (1 M NaCl) and 1 % Triton X-100 (1 % TX-100). (C): Effects of NaCl concentration on solubilization.

In all figures, upper and lower panels, respectively, indicate immunoblotting with anti-CND41 antiserum and Southwestern blotting probed with total chloroplast DNA. The small and large arrowheads indicate 46 kD and 41 kD bands detected by anti-CND41 antiserum, respectively.

condition was examined. Whereas 1 % Triton X-100 did not made CND41 soluble, 10 mM EDTA made the 46 kD protein soluble, and not the 41 kD protein. One M NaCl treatment, however, made both proteins soluble (Fig. 1-1B). Examination of NaCl concentration for the solubilization of CND41 indicated that the 41 kD protein needed higher concentration of NaCl than the 46 kD, and 0.3 M NaCl was sufficient to make 41 kD protein soluble (Fig. 1-1C).

Purification of CND41

Next, I established the purification method of 41 kD-CND41 protein, as summarized in Fig. 1-2. Cultured tobacco cells were homogenized in a buffer containing EDTA to extract the 46 kD protein completely. The precipitate with 41 kD-CND41 was collected and suspended in a high salt buffer containing 0.5 M NaCl to extract the 41 kD protein. The 41 kD-CND41 was purified through successive SP-Sepharose, Octyl-Sepharose and heparin column chromatographies. After the heparin column chromatography, three bands of about 41, 26 and 19 kD were detected in SDS-PAGE analysis with silver staining. All three bands were detected by the anti-CND41 antiserum (Fig. 1-3B). Determination of the N-terminal amino acid sequences indicated that both the 41 and the 26 kD proteins had the N-terminal amino acid sequence of mature CND41 and that the 19 kD band had the internal sequence of CND41 (Fig. 1-3D). I concluded that this fraction contained only CND41 and its degradation products. Because no other contaminant was detected in this fraction after heparin column chromatography, I then used it as highly purified CND41 in order to characterize its biochemical activity.

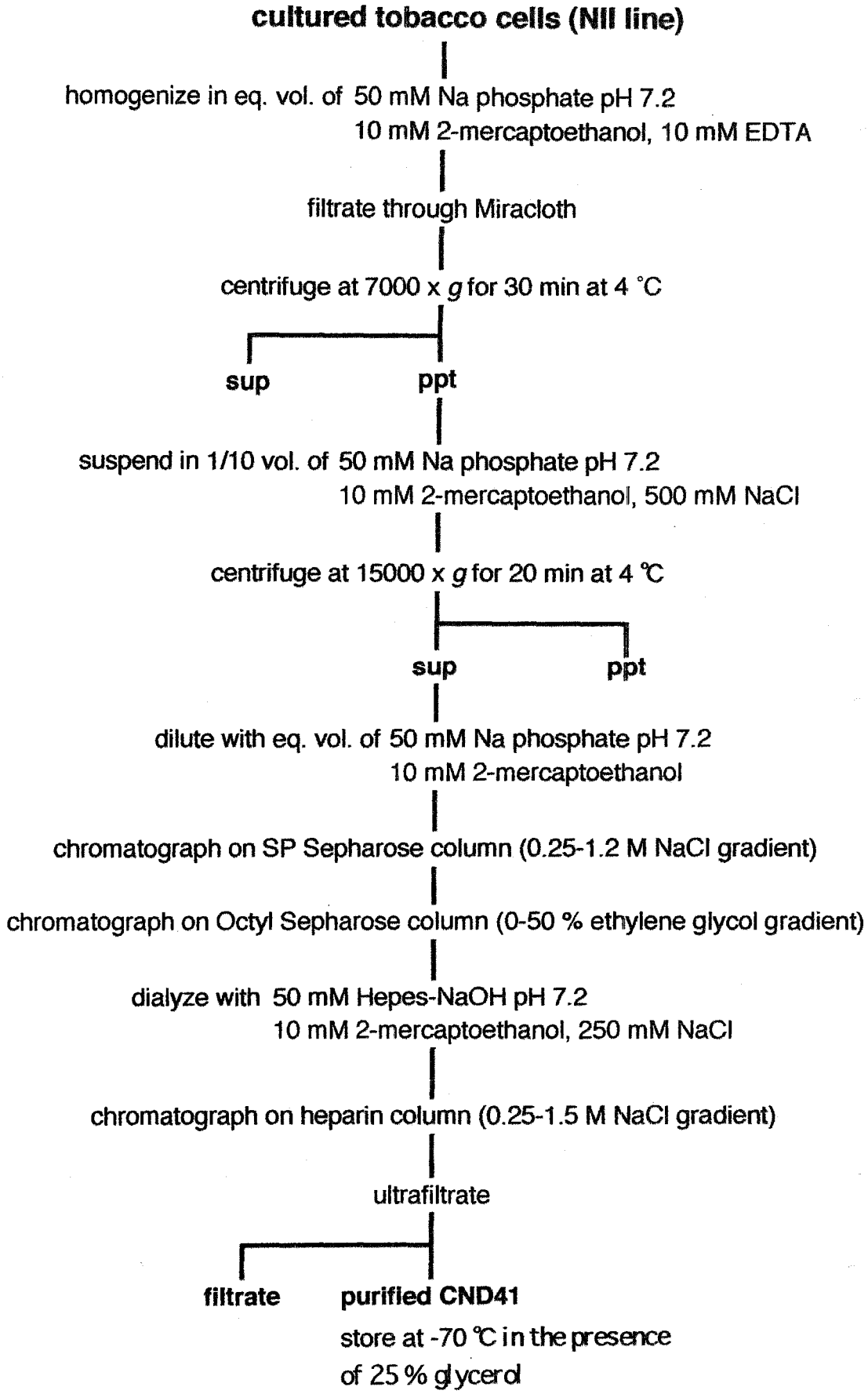
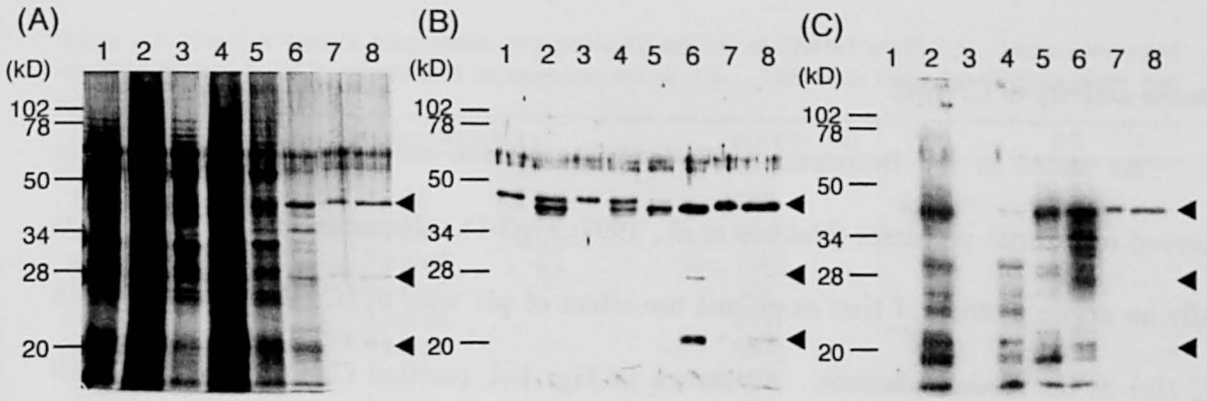


Fig. 1-2. Purification of CND41 from cultured tobacco cells



(D) KKKDKKSXNK: 41 kD and 26 kD protein
XVNGITFTP: 19 kD protein

Fig. 1-3. Purification of CND41 from cultured tobacco cells. Each fraction (10 μl) was analyzed by SDS-PAGE and silver staining (A), immunoblotting using anti-CND41 antibodies (B) or Southwestern blotting with ^{32}P-labeled chloroplast DNAs (C). Lanes are 1, crude homogenate of cultured tobacco cells; 2 and 3, precipitate and supernatant after 7,000 x *g* centrifugation of the crude homogenate; 4 and 5, insoluble and soluble fractions after 500 mM NaCl extraction of the 7,000 x *g* precipitate; 6, 7, and 8, CND41 fractions respectively after SP Sepharose-, Octyl Sepharose- and heparin column chromatography. Proteins of molecular mass of about 41 kD, 26 kD and 19 kD in final preparation were detected by silver staining and indicated by arrowheads. (D) N-terminal sequences of 41 kD, 26 kD and 19 kD proteins.

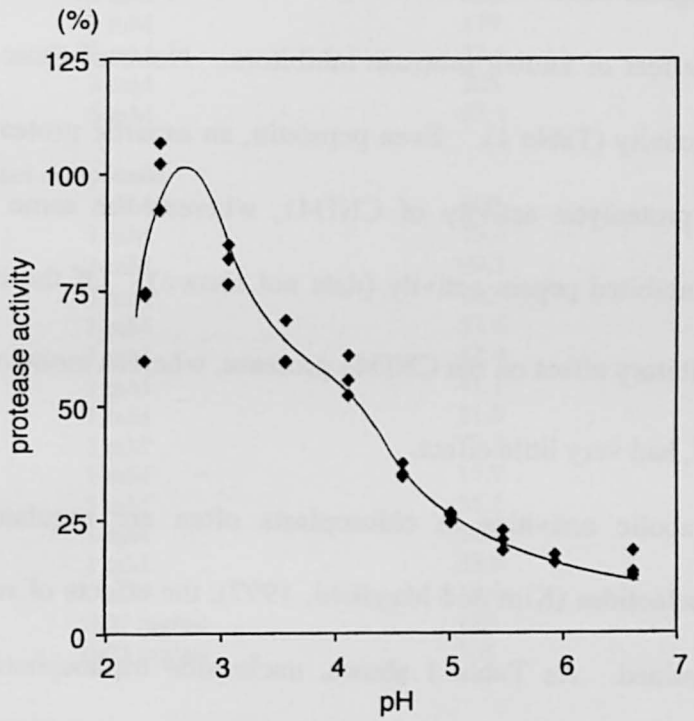


Fig. 1-4. Effect of pH on the proteolytic activity of purified CND41. The activity was determined with FTC-Hb as described in Materials and Methods. The mean value of triple measurements at pH 2.5 was taken as 100%.

Protease activity of CND41

As stated in the Introduction, CND41 has all the residues of the active sites conserved in aspartic proteases (Nakano et al., 1997; Fig0-1). Because aspartic protease is usually an acidic protease, I first examined the effect of pH with FITC-labeled hemoglobin (FTC-Hb) as the model substrate. As shown in Fig. 1-4, purified CND41 had very high activity at acidic pH (pH 2-4); the highest being at pH 2.5 and half maximum was pH 4.0. Although about 10 % activity of the maximum activity was found with FTC-Hb at pH 6.5, immunoblotting of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) showed no degradation of the protein at this pH (data not shown). The maximum activity of CND41 with FTC-Hb at pH 2.5 was about one tenth of pepsin purified from porcine stomach mucosa (Sigma) of 45,000 unit/mg protein on the molecule basis.

Next, I investigated conditions which might affect the proteolytic activity of CND41. First, I examined the effect of known protease inhibitors. None of those examined had a strong effects on the activity (Table 1). Even pepstatin, an aspartic protease inhibitor, only weakly inhibited the proteolytic activity of CND41, whereas the same concentration of pepstatin completely inhibited pepsin activity (data not shown). Of the cations examined, Fe^{3+} had a marked inhibitory effect on the CND41 protease, whereas monovalent and divalent cations, including Fe^{2+}, had very little effect.

Because metabolic activities in chloroplasts often are regulated by the redox potential as well as nucleotides (Kim and Mayfield, 1997), the effects of redox-reagents and nucleotides were examined. As Table 1 shows, nucleoside triphosphates and their non-hydrolysable analogs, e. g. ATP/GTP-γ-S, as well as NADPH strongly inhibited the protease activity of CND41 at an acidic pH. Because NADPH is very unstable at this acidic pH, the

Table 1-1. Effect of various compounds on proteolytic activity of CND41 at pH 2.5. Mean values and standard deviations of 3 independent experiments are shown. The mean value for CND41 alone was taken as 100 %.

compounds	concentration	activity (%)	SD
Protease inhibitors			
PMSF	1 mM	103	4.1
antipain	3 μg/ml	98.4	7.8
chymostatin	3 μg/ml	106	9.6
leupeptin	3 μg/ml	101	6.2
pepstatin	3 μg/ml	81.6	1.1
phosphoramidon	3 μg/ml	85.0	5.2
aprotinin	5 μg/ml	100	1.8
EDTA	1 mM	122	7.2
Ions			
KCl	20 mM	92.6	4.3
NaCl	20 mM	97.2	6.9
$CaCl_2$	2 mM	78.5	4.6
$MgCl_2$	2 mM	95.0	1.8
$MnCl_2$	2 mM	89.8	5.7
$ZnCl_2$	2 mM	93.7	2.7
$FeCl_2$	2 mM	99.8	5.4
$FeCl_2$	2 mM	14.5	2.3
Redox-reagents			
2-mercaptoethanol	5 mM	147	9.2
ditiothreitol	1 mM	129	10
GSH	10 mM	156	2.9
GSSG	5 mM	130	20
NADH	3 mM	82.3	23
NAD	3 mM	139	4.4
NADPH	3 mM	32.1	3.5
NADP	3 mM	105	4.0
Na ascorbate	5 mM	92.1	6.6
Nucleotides and related compounds			
Na phosphate	1 mM	109	5.2
Na pyrophosphate	1 mM	79.4	1.8
AMP	1 mM	99.3	10
ADP	1 mM	102	11
ATP	1 mM	33.8	3.2
GTP	1 mM	17.9	6.1
CTP	1 mM	41.1	5.1
UTP	1 mM	31.6	6.6
dATP	1 mM	10.2	1.0
dGTP	1 mM	15.9	2.7
dCTP	1 mM	55.5	9.1
dTTP	1 mM	28.9	5.3
ATP-γ-S	1 mM	25.8	3.1
GTP-γ-S	1 mM	14.6	0.77
DNA[1]	0.02 mg/ml	119	9.0
RNA[2]	0.02 mg/ml	103	5.0
Detergents			
SDS	0.05 %	0.00	1.6

[1]Salmon sperm DNA (Sigma)
[2]Calf liver RNA (Sigma)

actual inhibitory mechanism of NADPH on protease activity is not clear. SDS, which is known to stimulate protease activity (Otto and Feierabend, 1994; Yamada et al., 1998), also strongly inhibited the activity of CND41. Although CND41 has DNA-binding activity, the protease activity was not effected when salmon sperm DNA or calf liver RNA were added at an acidic pH.

Chloroplast proteins as substrate

I confirmed the proteolytic activity of CND41 at acidic pH, using FTC-Hb as substrate, but Hb is not natural substrate in plants. Therefore, I examined the protease activity of CND41 with chloroplast proteins as substrates, i. e. recombinant tobacco oxygen evolving complex protein OEC23 in photosystem II, and purified spinach Rubisco, whereas no incubation of Rubisco was done at acidic pH because Rubisco was insoluble in the acidic condition. When OEC23 and CND41 were incubated at acidic pH, this protein was completely degraded as Hb (Fig. 1-5). Although no proteolysis was observed at neutral pH as I described above, partial degradation of both OEC23 and Rubisco was observed at weak acidic pH 5.6. Amino acid sequence analysis of degradation products indicated that 6 or 8 amino acids were removed from the N-terminal of OEC23 and Rubisco, respectively (Fig. 1-5B). These results indicate that CND41 has nonspecific proteolytic activity at acidic conditions and sequence specific activity at weak acidic conditions.

Discussion

CND41, a chloroplast-nucleoid DNA-binding protein, was highly purified, and its proteolytic activity confirmed. Like the other aspartic proteases, CND41 has high

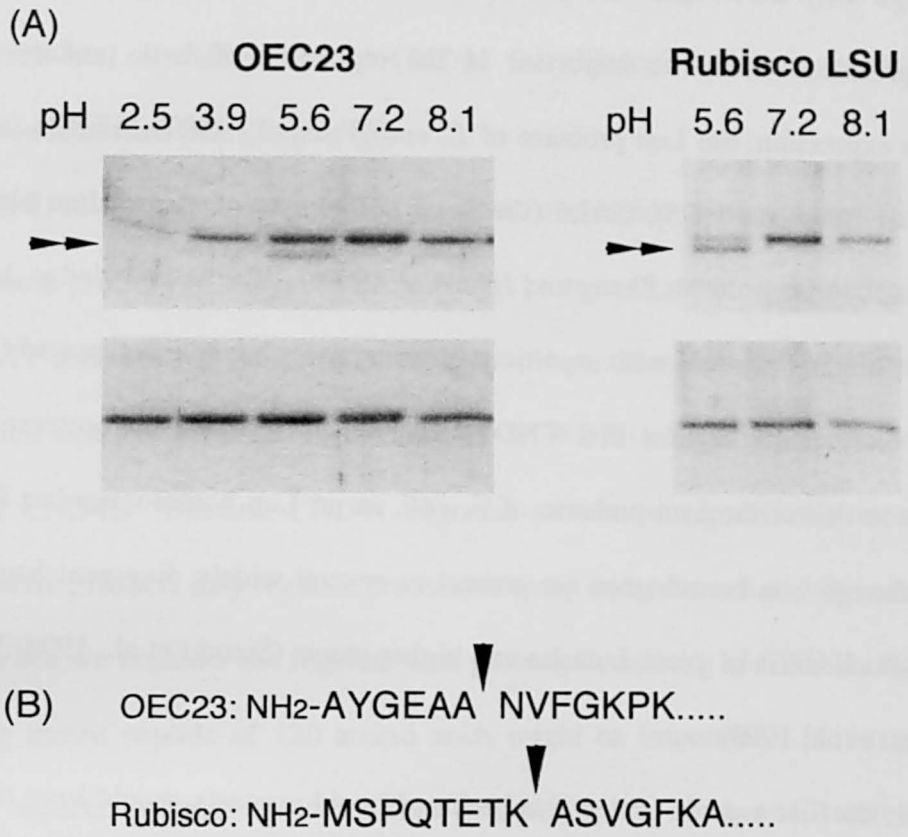


Fig.1-5. Degradation of chloroplast proteins by CND41. (A): Recombinant tobacco OEC23 5B (20 μg/ml) and purified spinach Rubisco (40 μg/ml) were incubated with (upper panel) or without (lower panel) CND41 (20 nM) at 30 °C for 40 min at pH indicated (pH 2.5, 3.9, 5.6 and 7.2, 50 mM Na citrate; pH 8.1, 50 mM Hepes-NaOH) followed by TCA precipitation. The precipitates were analyzed by SDS-PAGE and CBB staining. (B): N-terminal amino acid sequences of OEC23 5B and Rubisco large subunit. Arrowheads indicate the sites of proteolysis by CND41 at pH 5.6, i. e. N-terminal of degraded proteins indicated by double arrowheads in (A).

proteolytic activity under acidic conditions, indicative that as a component of the nucleoid CND41 has both DNA-binding and protease activities.

Although only a few proteases are known to have DNA-binding activity, these DNA-binding proteases would be important in the regulation of both prokaryotic and eukaryotic gene expression: the Lon protease of *E. coli* (Fu et al., 1997), AEBP1 (a mouse carboxypeptidase; He et al., 1995), GAL6 (the yeast homologue of mammalian bleomycin hydrolase; Xu and Johnston, 1994; Zheng and Johnston, 1998). To our knowledge, however, only a few DNA-binding proteins with aspartic protease activity have been reported (Malathi et al., 1994). Our results suggest that CND41 may be a chloroplastic protease with a function similar to that of the Lon protease of *E. coli*, as no Lon homologues has found in chloroplasts, although Lon homologues are present in several widely divergent bacteria, as well as in the mitochondria of yeast, humans and higher plants (Suzuki et al., 1994; Wang et al., 1993; Barakat et al., 1998).

CND41, the first aspartic protease to be found in chloroplasts, would have the broad substrate specificity as the Clp protease (Fig. 1-5 and unpublished data). CND41 might be involved not only in the regulation of gene expression, but also in the biogenesis of the functional apparatus of chloroplasts and in the degradation of denatured proteins. Only a few proteolytic enzymes that are involved in processing for the generation of functional components have been well characterized in chloroplasts (Adam, 1996; Andersson and Aro, 1997). Although CND41 has unusually low optimal pH and such a low pH would not occur in chloroplasts under normal physiological conditions, certain environmental stresses, e. g. freeze-induced dehydration stress, are known to lower the cytosolic pH due to disturbance of the tonoplast membranes (Murai and Yoshida, 1998).

I also found the proteolytic activity at weak acidic conditions, even though the proteolysis was sequence specific in contrast to the nonspecific proteolysis at acidic pH; i. e.

partial degradation of N-terminals of OEC23 and Rubisco. The amino acid sequence removed from Rubisco was the same as that by trypsin (Gutteridge et al., 1986), although no further digestion was observed by CND41. These partial digestions of chloroplast proteins may indicate the regulatory role of CND41 for chloroplast functions, although further investigation is needed (see Chapter 2).

CND41 does not need ATP or other nucleoside triphosphates for proteolysis, whereas some proteolytic enzymes, such as Clp protease, Lon protease and proteasome do. Instead, ATP and other nucleoside-triphosphates (ribonucleosides or deoxyribonucleosides as well as such non-hydrolysable nucleosides as ATP/GTP-γ-S) strongly inhibit the protease activity of CND41. Because CND41 degrades proteins nonspecifically at a low pH, the photosynthetic products may regulate protease activity under normal conditions.

When we consider the regulation of proteolytic activity of CND41, the existence of rather long transit peptide of 120 amino acids might be important. It is reported that all eukaryotic aspartic proteases are translated with propeptide in N- or C-terminal, which has multiple functions such as an intermolecular chaperon, signal for distinct targeting or inhibition of proteolytic activity (Koelsch et al., 1994). Aspartic proteases commonly have an active site cleft that divides two β-barrel domains which contain catalytic aspartic acids. The N-terminal propeptide alone (James and Sielecki, 1986), or propeptide and N-terminal region of mature enzyme cooperatively block the active site in cleft to inactivate proteases (Kervinen et al., 1999). Similarly N-terminal 120 amino acids of the precursor CND41 protein may inactivate the protease activity of CND41, whereas any similarity between the N-terminal propeptide of precursor CND41 protein and propeptides of other aspartic proteases.

Whereas the protease activity of mature CND41 was confirmed, the function of the 46 kD protein detected by anti-CND41 antiserum is not clear. Purification and determination of the N-terminal and internal sequences of the 46 kD protein showed that this

protein was different from CND41 (data not shown). Antisense transgenic tobacco plants with reduced amount of CND41, however, showed concomitant decrease of the 46 kD protein. This result suggested that the 46 kD protein would be structurally related protein to CND41, although the sequence of the 46 kD protein was different from CND41 and no DNA-binding and protease activities were detected. Further characterization of this protein is needed.

Chapter 2

CND41 degrades denatured proteins at physiological pH

Although the proteolytic activity of CND41 was confirmed as shown in Chapter 1, its optimum at acidic pH suggests that CND41 is not functional as a protease at physiological pH in chloroplasts where CND41 locates. However, it is well known that protein modification of enzyme and/or existence of cofactors as well as the modification of substrates may regulate the protease activity. Indeed, the activities of some ATP-dependent proteases are regulated by ATP level in prokaryotes and eukaryotes (Gottesman and Maurizi, 1992) and ATP-dependent proteolysis is also reported in chloroplast. The activity involved in the degradation of the major light-harvesting chlorophyll *a/b* protein of photosystem II (LHCII) was strictly dependent on ATP (Lindahl et al., 1995). FtsH, ATP dependent zinc-stimulated protease, would be involved in the degradation of the unassembled Reiske FeS protein (Ostersetzer and Adam, 1997). The proteolysis of active-oxygen modified Rubisco with chloroplast stroma fraction was also ATP-dependent (Desimone et al., 1998). On the other hand, it is also known that unassembled subunits are degraded rapidly; e. g. reduced amount of Rubisco large subunits induced the degradation of excess small subunits in *Clamydomonas* (Schmidt and Mishkind, 1983) as well as Riske FeS protein described above. The proteins without cofactors are less stable too; e. g. light-harvesting chlorophyll a/b binding protein (CAB) failed to accumulate when chlorophyll supply was limited (Apel and Kloppstech, 1980). Targeting to wrong component also induces the degradation; artificially mistargeted OEC33 and OEC23, components of lumenal oxygen-evolving complex, were degraded rapidly (Halperin and Adam,1996; Roffey and Theg, 1996). It is also reported that active-

oxygen modified Rubisco increased the susceptibility to proteolysis (Peñarrubia and Moreno, 1990).

In this Chapter, I used purified Rubisco as the substrate for CND41 protease. Rubisco is the most important CO_2-fixing enzyme in photosynthesis and the most abundant protein, which concentration in the chloroplast stroma is as high as 4 mM of protomer (Pyke and Leach, 1987) and composed about 50 % of total soluble protein of higher plants. Thus, Rubisco is the major organic nitrogen resource in higher plants and its specific degradation in aged leaves and subsequent translocation as amino acids to newly developing organs are important in plant development. The actual mechanisms of degradation of Rubisco in leaves, however, are not well known yet, although the gene expression, the synthesis, assembly and regulation of enzyme activity have been studied extensively.

Materials and Methods

Proteins and chemicals used

Purified Rubisco, aldolase and 2-carboxypentitol-1,5-bisphosphate (CPBP) were gift from Prof. A. Yokota of Nara Institute of Science and Technology. The CPBP is mixture of epimer (2-carboxyarabinititol-1,5-bisphosphate (CABP) and 2-carboxyribitol-1,5-bisphosphate). Recombinant tobacco OEC23s and maize ferredoxin were prepared as described by Ifuku et al. (1998) and by Walker (1987), respectively. Salmon sperm DNA purchased from Sigma was used after extraction with phenol/chloroform.

Denaturation of protein substrates and proteolysis with CND41

Purified Rubisco insolubilized in 20 % PEG-4000 and 20 mM $MgCl_2$ was precipitated by centrifugation at 10,000 x *g* for 15 min at 4 °C, then denatured as following. Rubisco precipitate (1 mg/ml) was acid-treated in 50 mM Na citrate buffer at pH 4-6 on ice

for 30 min, or Rubisco precipitate (40 μg/ml) was boiled in 50 mM Hepes-KOH buffer (pH 7.5) for 10 min, or incubated in 50 mM Hepes-KOH buffer (pH 7.5) containing 0.2 % SDS, 0.2 % TritonX-100 or 2 M urea on ice for 30 min. The proteolysis was started by mixing denatured Rubisco (40 μg/ml) and purified CND41 (20 nM) in 50 mM Hepes-KOH and sift of temperature to 30 °C. After 40 min incubation at 30 °C, reaction was stopped with the addition of equal volume of the double concentration of SDS-PAGE loading buffer (50 mM Tris-Cl (pH 6.8), 2 % SDS, 10 % 2-mercaptoethanol, 0.1 % bromophenol blue and 10 % glycerol) and boiling for 10 min, or with the addition of one tenth volume of 100 % (w/v) TCA. The precipitate by TCA was dissolved in SDS-PAGE loading buffer and boiled for 10 min. Digested proteins were separated by SDS-PAGE and detected by CBB staining, or by immunoblotting using anti-Rubisco antibodies after transfer to PVDF membranes. Other chloroplastic proteins were similarly denatured and degraded with CND41. The reactions were stopped with the addition of an equal volume of the double concentration of SDS-PAGE loading buffer and boiling or with 1/10 volume of 100 % (w/v) TCA. The degradation products were analyzed by SDS-PAGE and CBB staining.

Activation of Rubisco and its degradation with CND41

Rubisco was successively activated as follows and used for the degradation with CND41. Purified Rubisco (56.3 μg/ml; 0.79 μM protomer) was incubated in a 70.4 mM Hepes-KOH buffer with/without 14.1 mM $NaHCO_3$ and $MgCl_2$ at 25 °C for 15 min, followed by the incubation with 6.85 μM CPBP, about 10 times the mole of Rubisco protomer, at 25 °C for 30 min. Activated Rubisco was denatured in 2 M urea on ice for 30 min. The proteolytic reaction was started by the addition of CND41 (20 nM) and incubated at 30 °C for 40 min. The reaction was stopped by the addition of an equal volume of the double concentration of SDS-PAGE loading buffer and boiling. Degradation products were

analyzed by SDS-PAGE and with CBB staining.

Results

Activation of protease activity of CND41 at pH 7.5

To examine proteolytic activity of CND41 at physiological pH (neutral to weak basic), I measured the activity of CND41 in 50 mM Hepes-KOH buffer at pH 7.5 using Rubisco as the substrate. As described in the previous Chapter, no degradation of Rubisco was observed at this pH range (Fig. 2-1 lane 1). Then, effects of several chemicals, which may activate proteolytic activity of CND41 at the physiological pH, were examined. Because metabolic activities in chloroplasts often are regulated by the redox potential as well

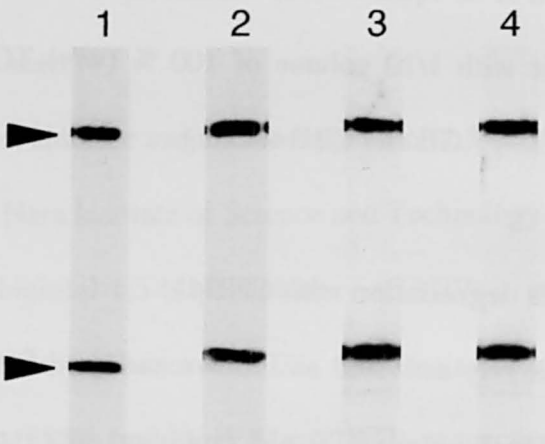


Fig. 2-1. Addition of ATP, NADP or NADPH did not change the protease activity of CND41 at physiological pH. Purified CND41 (20 nM) and purified Rubisco (40 μg/ml) were incubated without chemicals (lane 1), or with 2 mM ATP (lane 2), 2 mM NADP (lane 3) or 2 mM NADPH (lane 4) in 50 mM Hepes-KOH (pH 7.5). The reaction was stopped by the addition of equal volume of double concentration of SDS-PAGE loading buffer and boiled. Reaction product was analyzed by SDS-PAGE and immunoblotting with anti-Rubisco antibodies. Arrowheads indicate large and small subunits of Rubisco.

as nucleotides (Kim and Mayfield, 1997; see Chapter 1), the effect of ATP, NADP and NADPH were determined. However none of them showed any activation (Fig. 2-1). Other chemicals such as 1 mM DTT, 5 mM 2-mercptoethanol, 20 mM ascorbate, 5 mM GSH and 2.5 mM GSSG, 5 mM H_2O_2 and 1 mM EDTA also showed no effects on activity of CND41 (data not shown).

Denaturation of Rubisco and its degradation by CND41

Whereas I could not find chemicals to activate protease the activity of CND41 at a physiological pH, I noticed that some treatments enhanced the proteolysis by CND41. More careful examination indicated that denaturation of Rubisco increased the proteolysis by CND41 at the physiological pH. Thus, I denatured Rubisco by the following treatments and measured the degradation by CND41. All denaturation treatments (acidic pretreatment in citrate buffer (pH 4.5), boiling for 10 min, 0.2 % SDS, and 2 M urea treatment) except 0.2 % TritonX-100 enhanced the proteolytic degradation of Rubisco by CND41 (Fig. 2-2). When the acidic treatment conditions were examined (Fig. 2-3), the pretreatments at pH lower than 5.0 were found to be effective to enhance the proteolysis by CND41, and the pretreatment of pH 6.1 had no effect. The pretreatment at pH 4.0 obviously showed less protein signal, indicating that further degradation occurred at this pH. The concentration of urea in denaturation treatment was also important for the degradation (Fig 2-4). As shown above, 2 M urea was effective to induce the degradation of Rubisco large subunit by CND41, and higher concentration of urea induced the degradation of both large and small subunits of Rubisco by CND41 more strongly.

Activation states of Rubisco and degradation

Because Rubisco is one of the most important enzymes in Calvin cycle, random

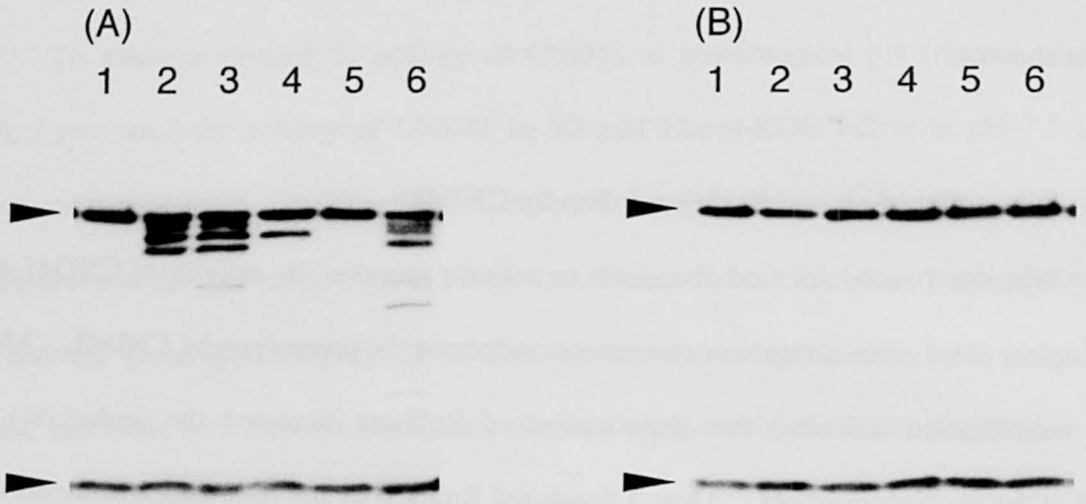


Fig. 2-2. Denaturation of Rubisco enhanced the proteolysis by CND41 at physiological pH. Purified spinach Rubisco was denatured in 50 mM sodium citrate buffer (pH 4.5; lane 2) or under following conditions in 50 mM Hepes-KOH (pH 7.5); boiling for 10 min (lane 3), incubation with 0.2% SDS (lane 4), 0.2 % Triton X-100 (lane 5) or 2 M urea (lane 6) for 30 min on ice. Non-denatured (lane 1) or denatured Rubisco (40 μg/ml) was incubated with (A) or without (B) purified CND41 (20 nM) at 30 °C for 40 min. Reaction was stopped by the addition of double concentration of SDS-PAGE loading buffer and boiled. Degradation products were analyzed by SDS-PAGE and immunoblotting using anti-Rubisco antibodies. Arrowheads indicate non-degraded Rubisco large and small subunits.

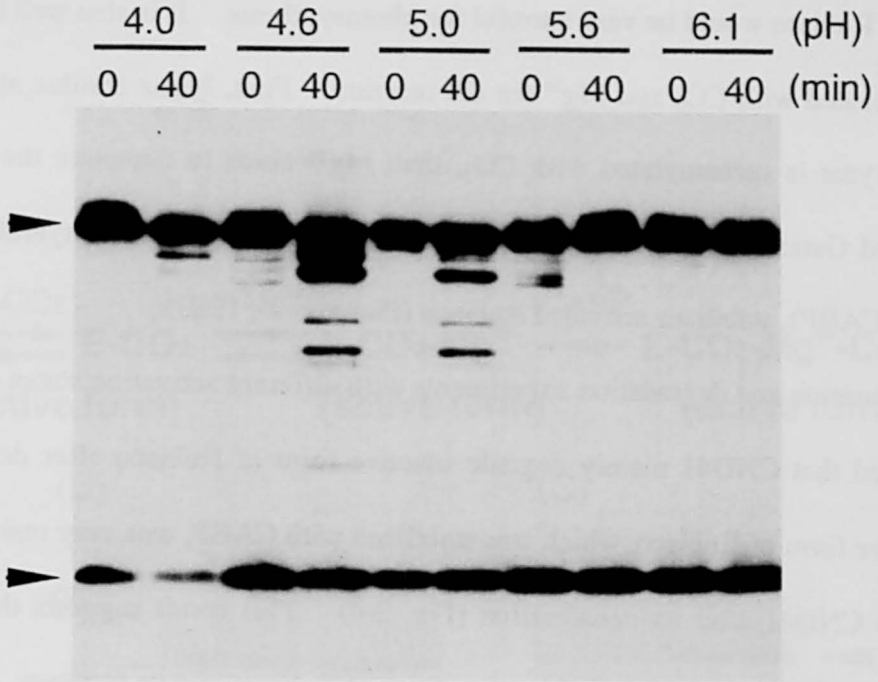


Fig. 2-3. Acid-pretreatment enhanced Rubisco degradation by CND41 at neutral pH. Purified spinach Rubisco (1 mg/ml) was kept in 50 mM sodium citrate buffer at indicated pH on ice for 30 min. Degradation of pretreated Rubisco (40 μg/ml) was measured in the presence of CND41 (20 nM) in 50 mM Hepes-KOH (pH 7.5) at 30 °C for indicated time. Reactions were stopped by the addition of double concentration of SDS-PAGE loading buffer and boiled. Each reaction product was analyzed by SDS-PAGE and immunoblotting with anti-Rubisco antibodies. Arrowheads indicate Rubisco large and small subunits of Rubisco.

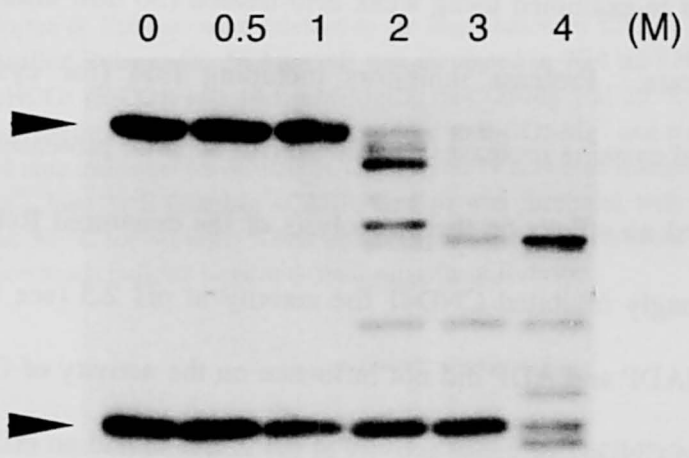


Fig. 2-4. Urea treatment enhanced Rubisco degradation by CND41 at neutral pH. Purified spinach Rubisco (40 μg/ml) was incubated in 50 mM Hepes-KOH (pH 7.5) containing indicated concentration of urea on ice for 30 min. Rubisco degradation was measured in the presence of CND41 (20 nM) at 30 °C for 40 min. Each reaction product was analyzed by SDS-PAGE and immunoblotting using anti-Rubisco antibodies. Arrowheads indicate large and small subunits of Rubisco.

degradation of Rubisco would be very harmful for photosynthesis. It is also well known that Rubisco is activated with CO_2 and Mg^{2+} for the reaction. First, lysine residue at the active site of the enzyme is carbamylated with CO_2, then Mg^{2+} binds to complete the activation (Gutteridge and Gatenby, 1995). Transition intermediate analog, 2-carboxyarabinitol-1,5-bisphosphate (CABP), stabilizes activated Rubisco (Pierce et al, 1980).

Denaturation and degradation experiments with different activation states of Rubisco clearly indicated that CND41 mainly degrade inactive form of Rubisco after denaturation. In fact the active form of Rubisco, which was stabilized with CABP, was very resistant to the degradation by CND41 after its denaturation (Fig. 2-4). This result suggests that CND41 would be involved in the degradation of inactive form of Rubisco and its turnover.

Effects of chemicals on the degradation of denatured Rubisco by CND41

Now, the degradation of denatured Rubisco by CND41 became evident at a physiological pH. Thus, effects of several chemicals on the proteolytic activity of CND41 at a physiological pH was re-examined using weak acid-treated (50 mM citrate buffer pH 4.5) Rubisco as the substrate. Protease inhibitors including E64 (for cysteine proteases), leupeptin (for serine and cysteine proteases), pepstatin (for aspartic proteases) and EDTA (for metal proteases) showed no effects on the proteolysis of the denatured Rubisco. Although NADPH and ATP strongly inhibited CND41 the activity at pH 2.5 (see Chapter 1), these chemicals as well as NADP and ADP did not influence on the activity of CND41 at pH 7.5. SDS, strong inhibitor of CND41 protease activity at pH 2.5, also had no effect on the activity at pH 7.5. On the other hand, DNA showed inhibition of proteolysis of Rubisco by CND41 at pH 7.5, whereas DNA had a little effect at pH 2.5.

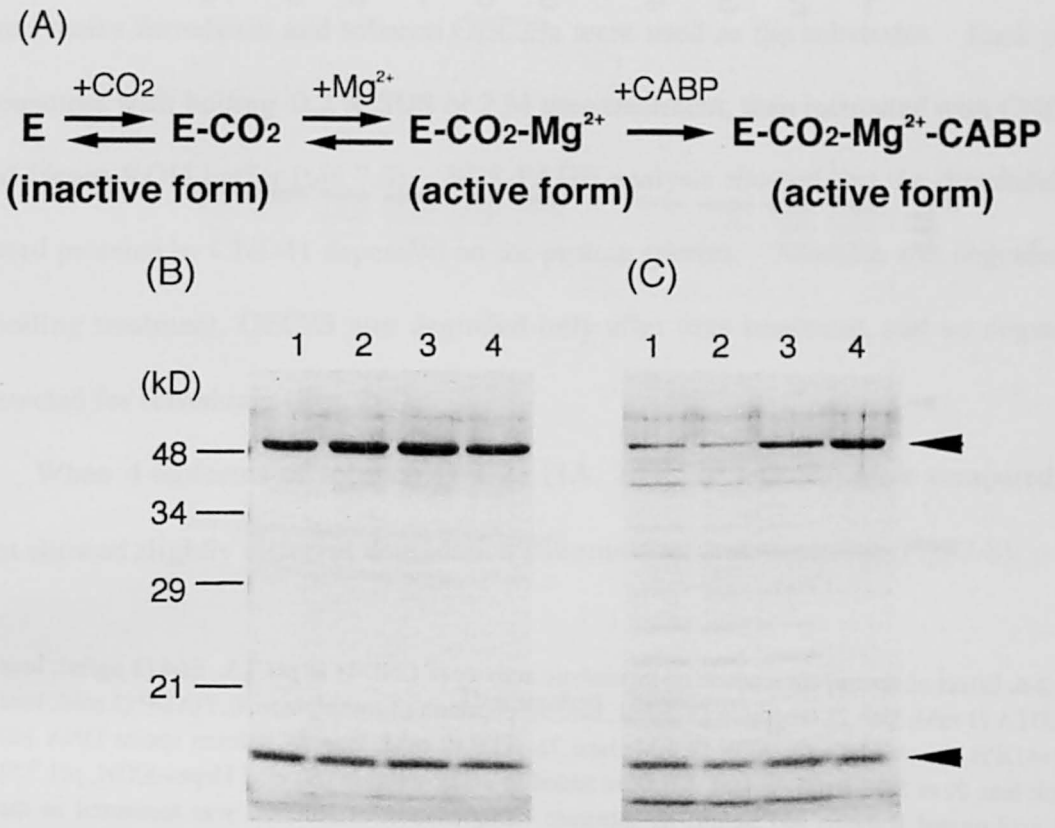


Fig. 2-5. Active forms of Rubisco were tolerant to the degradation by CND41. Model of Rubisco activation (A). Purified Rubisco (E; 56.3 μg/ml) was incubated in 70.4 mM Hepes-KOH (pH 7.5) with 14.1 mM $NaHCO_3$ (E-CO_2) and 14.1 mM $MgCl_2$ (E-CO_2-Mg^{2+}) at 25 °C for 15 min. CPBP (6.85 μM, 2-carboxypentitol-1,5-bisphosphate) was added to E-CO_2-Mg^{2+} and incubated at 25 °C for 30 min. After 2 M urea treatment for 30 min on ice, a series of activated Rubisco (E, lane 1; E-CO_2, lane 2; E-CO_2-Mg^{2+}, lane 3; E-CO_2-Mg^{2+}-CABP, lane 4) was incubated with (B) or without (C) CND41 (20 nM) at 30 °C for 40 min. These reaction products were analyzed by SDS-PAGE and CBB staining. Arrowheads indicate large and small subunits of Rubisco.

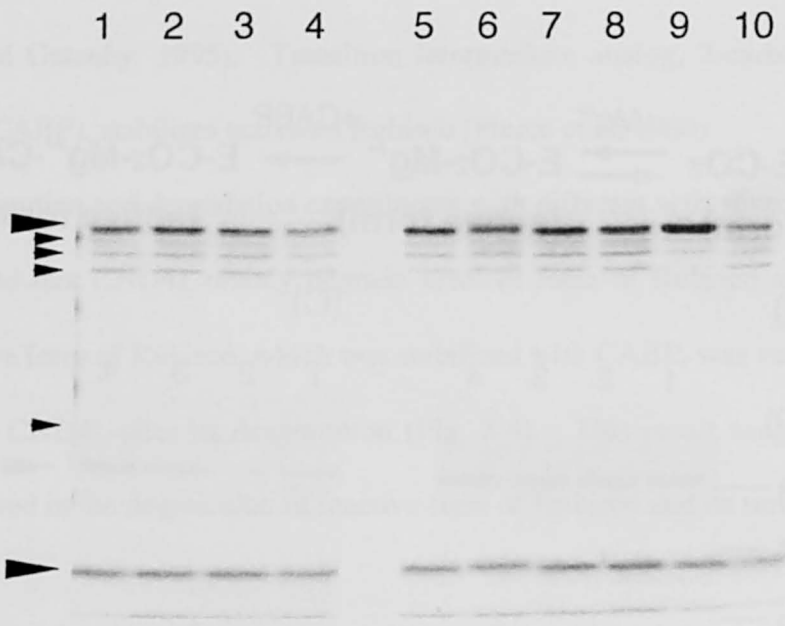


Fig. 2-6. Effect of several compounds on proteolytic activity of CND41 at pH 7.5. E64 (3 μg/ml; lane 1), EDTA (1 mM; lane 2), leupeptin (3 μg/ml; lane 3), pepstatin (3 μg/ml; lane 4), NADP (3 mM; lane 5), NADPH (3 mM; lane 6), ADP (2 mM; lane 7), ATP (2 mM; lane 8), salmon sperm DNA (40 μg/ml; lane 9) or SDS (0.05 %; lane 10) were added in assay solution (50 mM Hepes-KOH, pH 7.5) with acid-treated Rubisco (40 μg/ml) as substrate. Degradation of Rubisco was measured in the presence of CND41 (20 nM) at 30 °C for 40 min. Each reaction product was analyzed by SDS-PAGE and CBB staining. Large arrowheads indicate native large and small subunits of Rubisco. Small arrowheads indicate degradation products.

Other substrates of CND41 protease at pH 7.5

Degradation of other chloroplastic proteins was examined to know whether the proteolysis by CND41 at physiological pH is specific for denatured Rubisco. Spinach aldolase, maize ferredoxin and tobacco OEC23s were used as the substrates. Each protein was denatured with boiling, 0.2 % SDS or 2 M urea treatment, then incubated with CND41 in 50 mM Hepes-KOH buffer (pH 7.5). SDS-PAGE analysis showed that the digestability of denatured proteins by CND41 depended on the protein species. Aldolase was degraded only after boiling treatment, OEC23 was degraded only after urea treatment, and no degradation was detected for ferredoxin (Fig. 2-7).

When 4 isoforms of tobacco OEC23 (1A, 2AF, 3F and 5B) were compared, each isoform showed slightly different degradation patterns after urea treatment (Fig. 2-8).

Discussion

Proteolytic activity of CND41 at a physiological pH was determined with denatured Rubisco. Rubisco is the most abundant protein that reaches to 4 mM of the protomer (Pyke and Leech, 1987), which concentration is more than 7,000 fold higher than that used in my experiment. Because the activity generally depends on the substrate concentration, observed activity would be important even *in vivo* system, although only denatured Rubisco is degraded.

Because Rubisco is an important protein in photosynthesis, its degradation is very harmful for plant. The result that Rubisco was degraded only when its inactive form was denatured, indicates that active Rubisco under normal physiological condition is not the substrate of CND41 and photosynthesis runs safely. On the other hand, it is known that senescence or stress condition induce the degradation of Rubisco drastically (Makino et al.

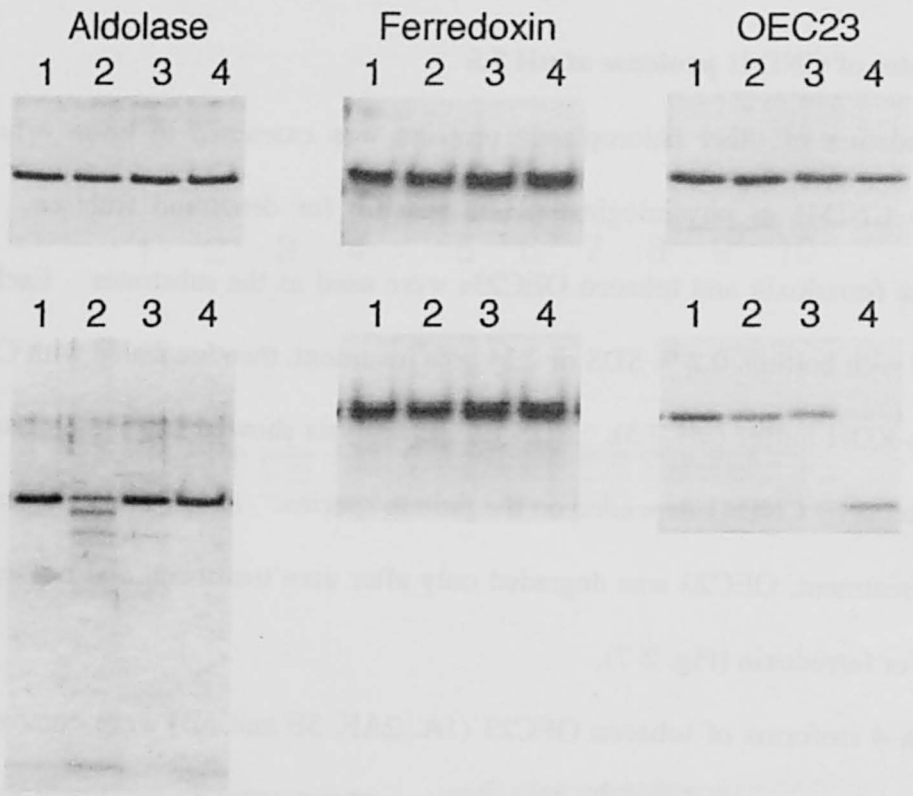


Fig. 2-7. Degradation of chloroplast proteins by CND41. Purified spinach aldolase, maize ferredoxin and recombinant tobacco OEC23 5B (40 μg/ml) were denatured under following conditions in 50 mM Hepes-KOH (pH 7.5); boiling for 10 min (1), incubation in 0.2% SDS (2) or in 2 M urea (3) for 30 min on ice. These proteins were incubated with (lower panel) or without (upper panel) 20 nM CND41 at 30 °C for 40 min, and analyzed by SDS-PAGE and CBB staining.

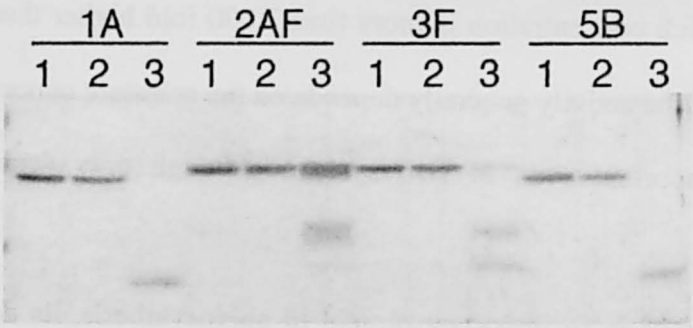


Fig. 2-8. Degradation of OEC23 isomers by CND41. Recombinant tobacco OEC23 1A, 2AF, 3F and 5B (40 μg/ml) were denatured under following conditions in 50 mM Hepes-KOH (pH 7.5); boiling for 10 min (2), or incubation in 2 M urea (3) for 30 min on ice. These proteins were incubated with (lane 2 and 3) or without (lane 1) 20 nM CND41 at 30 °C for 40 min, analyzed by SDS-PAGE and CBB staining.

1984; Ferreira and Teixeira, 1992; García-Ferris and Moreno, 1994). Thus, it is highly possible that the denatured Rubisco is produced under such stress condition and induces the proteolysis by CND41. Active oxygen species generated in the chloroplast is one of the factors to destabilize Rubisco (Desimone et al., 1996; Ishida et al., 1997), whereas active oxygen-treated Rubisco, i. e. treated with 5 mM H_2O_2, $^{\cdot}OH$ (generated by 5 mM H_2O_2, 10 mM $FeSO_4$, 1 mM DTT and 20 mM Ascorbate) or $^{\cdot}O_2^-$ (0.1 mM xanthine and 2.5 mU/ml xanthine oxygenase (Wako)), was not good substrate of CND41 protease (data not shown).

Whereas *in vitro* results indicate that CND41 degrades denatured Rubisco, I have no direct evidence to indicate the involvement of this novel protease in *in vivo* degradation of Rubisco. So far, some proteases are reported to degrade Rubisco in chloroplasts. The homologues of *E. coli* ClpA/P proteases in stroma (Nakabayashi et al., 1999) are considered to be substrate nonspecific protease. ATP-dependent protease(s) in stroma is also reported to be involved in the proteolysis of active-oxygen modified Rubisco (Desimone et al., 1998). In addition, another zinc protease EP1 in stroma is reported to degrade Rubisco (Bushnell et al., 1993). However, no one identified the protease(s) which degrade Rubisco *in vivo*. Experiments to evaluate the *in vivo* function of CND41 as the protease for Rubisco degradation is really important to understand the senescence and/or stress response in plants.

Identification and isolation of CND41 homologue from *Arabidopsis* would be useful to characterize the function of CND41, because reverse genetical methods are more available in *Arabidopsis*, such as T-DNA tagging. In fact many sequences of *Arabidopsis* were assigned as putative chloroplast nucleoid DNA binding protein due to the overall similarity to CND41 (30-40 % identity; accession number AL132959-21, AC006193-13, AC000132-13 or ACC006580-31). Although they have considerable similarity to the aspartic protease region of CND41, none has the lysine rich region of CND41 which is essential for DNA binding. Because CND41 seems to be expressed in rather limited developmental stage and cells in

plant as shown by Northern analysis and also because CND41 genome DNA contains a large intron in a transit peptide region, (Nakano et al, 1997 and unpublished data), it might be difficult to assign the CND41 in *Arabidopsis*. An EST from tomato ovary (AI487587) with 77 % identity in 236 amino acid overlap also lacks the lysine rich region.

The inhibition of proteolytic activity of CND41 by DNA suggests the interaction of DNA-binding and proteolytic activity. The inhibition of protease activity with 40 μg/ml DNA for 20 nM CND41 indicates that the presence of a fragment of *ca.* 3 kb DNA is sufficient to block the protease activity of CND41 molecule. Because chloroplast DNA has 120-150 kb length, a copy of chloroplast DNA can inhibit protease activity of 50 CND41 molecules at least. This CND41/DNA ratio corresponds to the value estimated in green leaves, whereas the ratio would be more than 10 times higher in nonphotosynthetic cultured cells (Shoji et al., unpublished data). Under such condition, i. e. in cultured cells, CND41 might be more active as a protease. It is also known that the copy number of chloroplast DNA changes considerably during chloroplast development; e. g. while the number of chloroplast remains constant throughout the growth of the coleoptile of rice, chloroplast nucleoids disappear before the degeneration of chloroplast (Sodmergen et al., 1989). This reduction of DNA copy during senescence might be the trigger for the activation of protease function of CND41. The DNA sequence for the inhibition of protease activity of CND41 would be non-specific, because salmon sperm DNA was effective. However, more careful re-examination of DNA sequence specificity might be needed, because large DNA fragments were used for DNA-binding experiments.

Although the mechanism of inhibition of protease activity by DNA is not clear, three-dimensional structure analysis shows that typical aspartic proteases contain one substrate-binding cleft and two lobes and the propeptides of some aspartic proteases mask the cleft to prevent the binding of substrate to active sites (James and Sielecki, 1986; Yang et al.,

1997; Kervinen et al., 1999). The lysine rich region, especially with DNA might mask the cleft in the same way, although it is not clear whether the removal of lysine rich region is necessary for the protease activity.

The substrate specificity of protease activity of CND41 is another remaining question to be answered. Denaturation of several different proteins and proteolysis showed that some proteins after denaturation are good substrate for CND41. CND41 degraded boiled aldolase or urea-treated OEC23, but did not ferredoxin with any treatment tested. In addition, degradation patterns of 4 isomers of tobacco OEC23 were different. Rubisco small subunit was degraded after 4 M urea treatment, whereas 2 M urea treatment was not effective. These results suggest that CND41 has different tendency to degrade proteins which are denatured under certain conditions. Because proteins involved in transcriptional machinery such as RNA polymerase, σ factor or another transcriptional factors are generally labile, it is quite reasonable that CND41 degrades these proteins and might reduce transcriptional activity, whereas further examination is needed.

Chapter 3

Characterization of *in vivo* function of CND41 protease during senescence

In the previous Chapter, I reported the *in vitro* proteolytic activity of CND41 using denatured Rubisco as the substrate at a physiological pH. These results show that CND41 degrades inactive form of Rubisco, indicating that CND41 would be involved in Rubisco degradation during senescence. However, *in vivo* activity of CND41 as a protease is not clear yet. Because of the importance of Rubisco in photosynthesis and its localization in chloroplasts, I characterized the *in vivo* function of CND41 on Rubisco degradation during senescence.

Rubisco, the essential carboxylation enzyme in photosynthesis, is also important reservoir of organic nitrogen. During foliar senescence due to flowering and depletion of nitrogen etc., the degradation products of Rubisco are redistributed from aged leaves to reproductive organs or younger leaves (Makino et al., 1984). Although several groups reported that proteolytic activity in chloroplasts which degrades Rubisco *in vitro* (Bushnell et al., 1993; Ishida et al., 1997; Desimone et al., 1998), the actual component *in vivo* is not clarified yet.

Fortunately, we established transgenic tobacco with reduced amount of CND41 by antisense DNA technique (Nakano et al., 1997). These antisense tobacco plants would be useful to determine *in vivo* function of CND41 as a protease, namely for Rubisco degradation during senescence. First, I examined the expression of CND41 during the leaf development. Then, I examined the effect of nitrogen-depletion on the Rubisco degradation and redistribution of organic nitrogen both in control and in CND41 antisense tobacco. This

nitrogen-depletion treatment induced senescence of lower leaves in control tobacco but not in antisense tobacco. Further analysis of soluble proteins confirmed that the involvement of CND41 protease in the degradation of denatured Rubisco during senescence.

Materials and Methods

Plant materials

Transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN; line R22, R28) plants with reduced amount of CND41, were used in this experiment, whereas transgenic tobacco transformed with modified pBI121 vector in which β-glucuronidase gene was removed was used as control (X6). The seeds of T1 generation were obtained by self-pollination.

Culture of T1 plants

The seeds of T1 generation (R22-1, R22-2 and R28-1) were sown in the soil and grown in the 12L/12D light condition (200 μmol $m^{-2}sec^{-1}$) at 26 °C.

RNA gel blot analysis

Total RNAs were prepared according to the method described by Nagy et al. (1988). The RNAs were electrophoresed on a formaldehyde-denaturing-agarose gel in 1 x Mops buffer (20 mM Mops-KOH, pH 7.0, 5 mM Na acetate and 1 mM EDTA) and blotted onto a charged nylon membrane Hybond N+ (Amersham Pharmacia), according to standard protocols (Sambrook et al., 1989). Full-length fragments of the CND41 cDNA (Nakano et al., 1997) and the *Vicia faba* nuclear rRNA clone (VER1; Yakura and Tanifuji, 1983) were labeled with ^{32}P and used as probes.

Nitrogen-depletion treatment

T0 generation of antisense or control plantlets, which were developed from axillary bud *in vitro*, were transplanted to a quarter strength of Linsmaier and Skoog medium without sucrose and hydroponically cultured under the 16L/8D light condition (40 μmol $m^{-2}s^{-1}$) at constant temperature (28 °C) for 12 day. Nitrogen-depletion treatment was done in hydroponic culture as described above with nitrogen-free a quarter strength of Linsmaier and Skoog medium containing 4.70 mM KCl instead of 5.15 mM NH_4NO_3 and 4.70 mM KNO_3 for 14 days.

Analysis of protein degradation

Leaves of each plant were harvested, numbered from lower to higher position, frozen by liquid nitrogen and stored at -70 °C. The frozen leaves were powdered in liquid nitrogen with mortar and extracted in 3 times volume of extraction buffer (100 mM Tris-Cl pH 8.0, 10 mM EDTA, 10 mM 2-mercaptoethanol) by sonication. The homogenates were centrifuged at 10,000 x *g* for 10 min and its supernatants were used for protein assay according to the method of Bradford using Bio-Rad Protein Assay (Bio-Rad). The soluble proteins extracted from 0.5 mg of fresh weight leaves were analyzed by SDS-PAGE with 14 % acrylamide gel and stained with CBB.

Results

Phenotype of antisense transformants

Previously, we have established several lines of antisense CND41 transformants with reduced amount of CND41 (Nakano et al., 1997). The line R22 accumulated the least amount of CND41 and R28 did the second least. These transformants showed remarkable dwarf phenotype. Simultaneously, they showed retarded senescence of lower leaves at

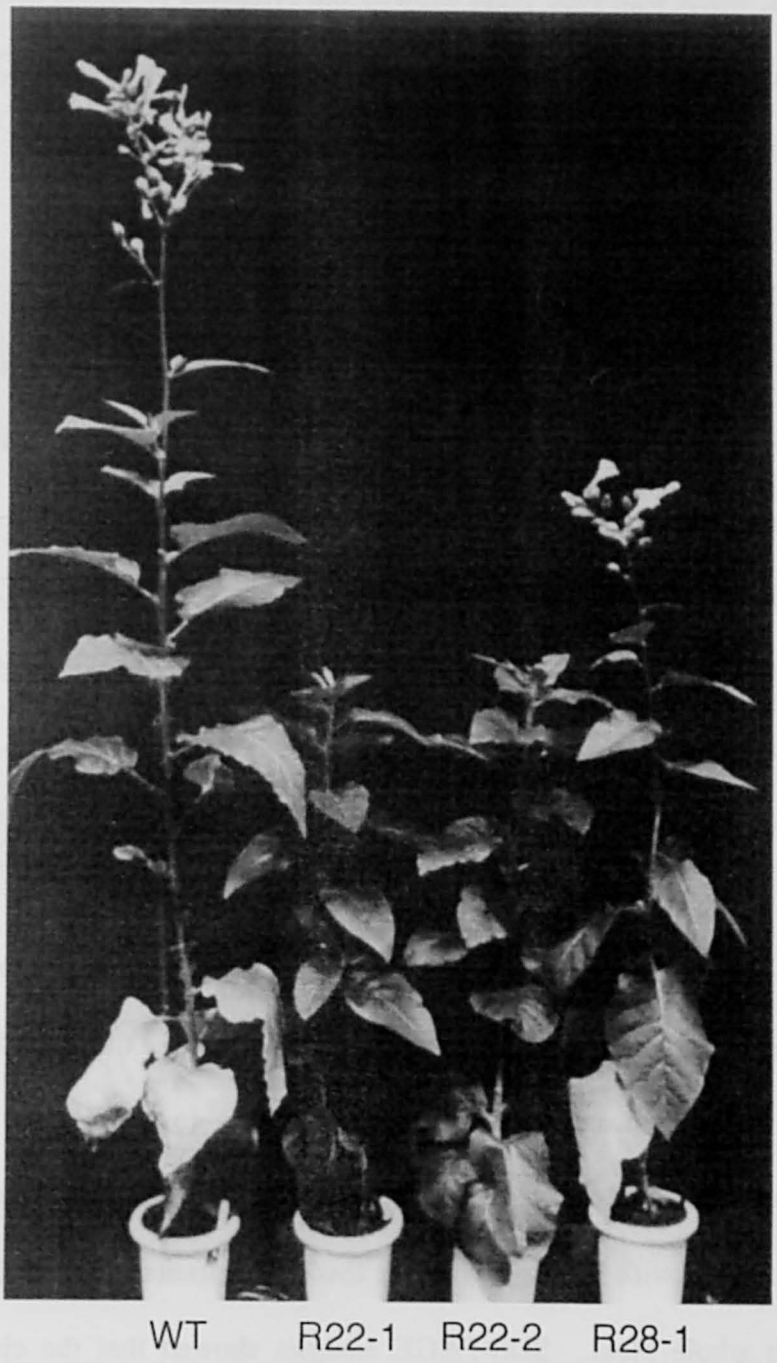


Fig. 3-1. Antisense transformants showed retarded senescence of lower leaves.
WT: *N. tabacum* cv. Samsun NN. R22-1, R22-2 and R28-1: T1 generation of R22 and R28 antisense CND41 transformants.

flowering period, when wild-type plant showed the symptom of senescence of lower leaves (Fig. 3-1). This retarded senescence provided indirect support that CND41 might be involved in the *in vivo* degradation of Rubisco during senescence.

Expression of CND41

Before we characterized the *in vivo* function of CND41 using antisense CND41 tobacco, we examined the developmental expression of CND41 in leaves, because the expression in young mature leaves was reported to be very low. As Fig. 3-2 shows, CND41 mRNA accumulated abundantly in root, stem and low in young leaf. However, the transcript level in lower leaves was as high as stem or root (Fig. 3-2). High CND41 mRNA level in lower leaves suggested that CND41 can function in these tissues. Thus, I further examine the effect of reduction of CND41 amount on leaf senescence.

Soluble protein and Rubisco contents in antisense CND41 tobacco plants

Above data suggested that CND41 might be involved in the degradation of Rubisco. To confirm this idea, I first measured the contents of soluble proteins and Rubisco, both in control and antisense CND41 plants. Fig. 3-3 clearly shows that soluble protein contents in control plant decreased with leaf age, whereas those in antisense tobacco (R22) were rather constant through whole plant. SDS-PAGE analysis showed that the change of Rubisco content coincided with soluble protein contents both in control and CND41 antisense plants (Fig. 3-4), supporting my idea that CND41 might be involved in the Rubisco degradation.

Based on these findings, I analyzed the change of protein contents, especially of Rubisco, during the senescence induced by nitrogen deficiency. Both control and antisense CND41 plants were hydroponically cultured with or without inorganic nitrogen in medium. As Fig. 3-5 shows, nitrogen-depletion induced the clear symptom of senescence in control

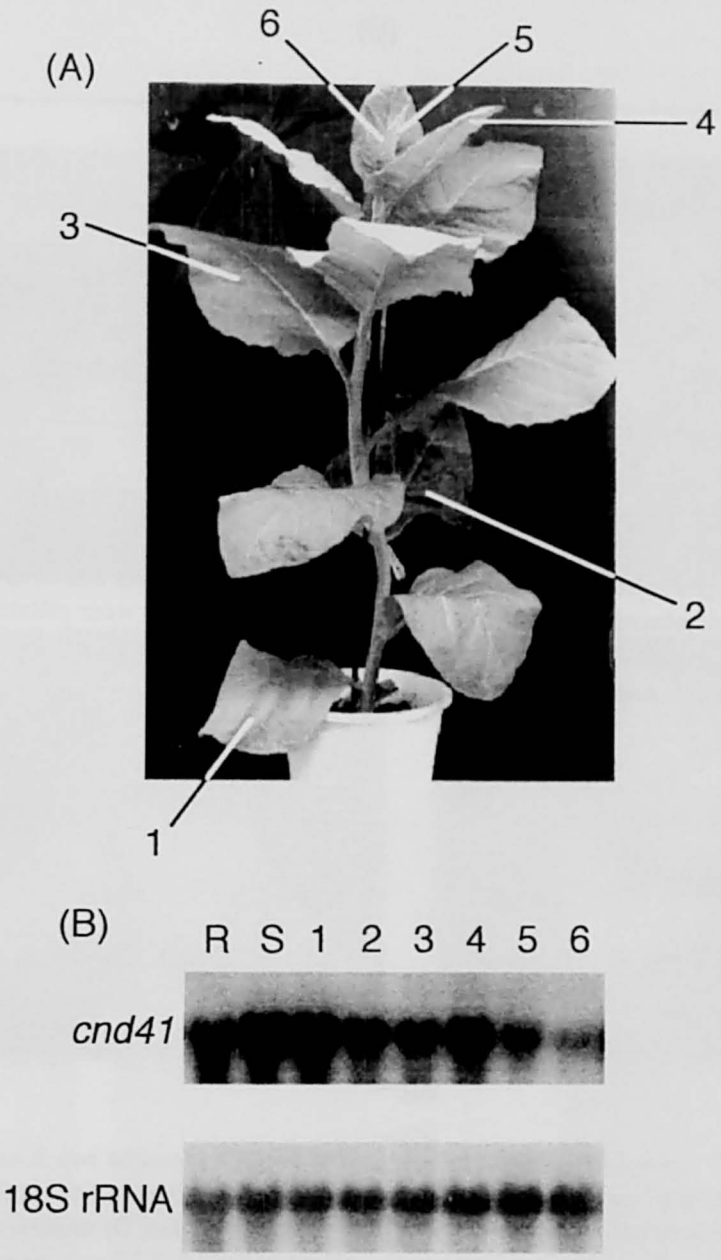


Fig. 3-2. Accumulation of CND41 mRNA in *N. tabacum* plant. (A): Plant material for RNA extraction. (B): RNA gel blot analysis. Total RNAs (10 μg each) were extracted from root (R), stem (S) and leaves as numbered as shown in (A) were hybridized with ^{32}P labeled CND41 cDNA and 18S rRNA clone.

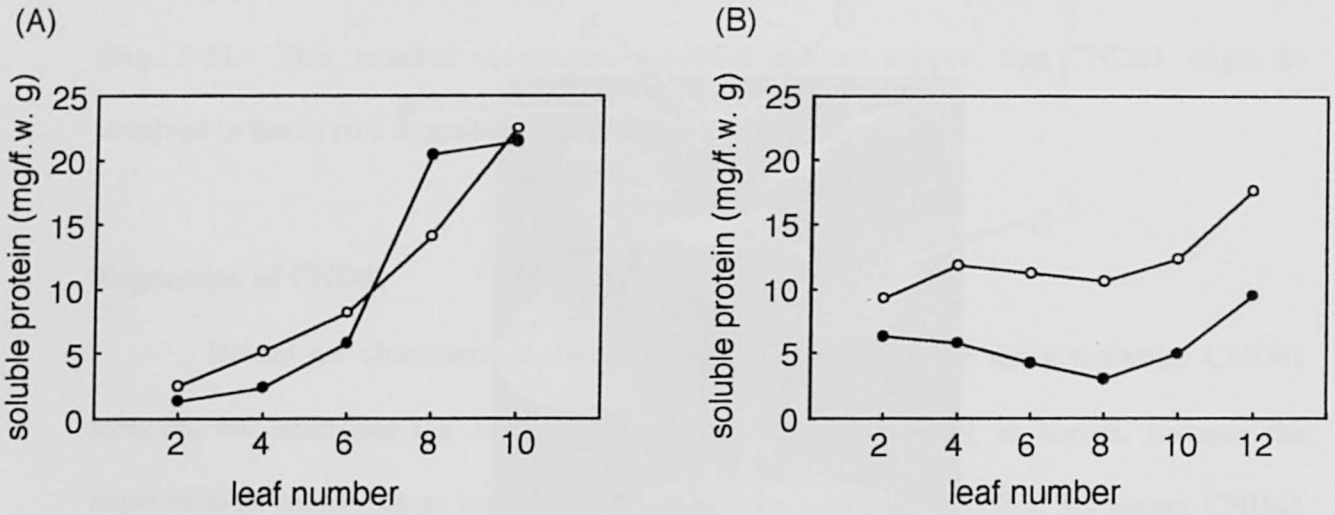


Fig. 3-3. Effect of nitrogen-depletion on the soluble protein contents in control and CND41 antisense tobacco. Leaves were numbered from bottom to top. These tobacco plants were cultured in normal (open-circle) or nitrogen-depleted (closed circle) medium. (A), Soluble protein in control tobacco (X6). (B), Soluble protein in antisense CND41 transformants (R22).

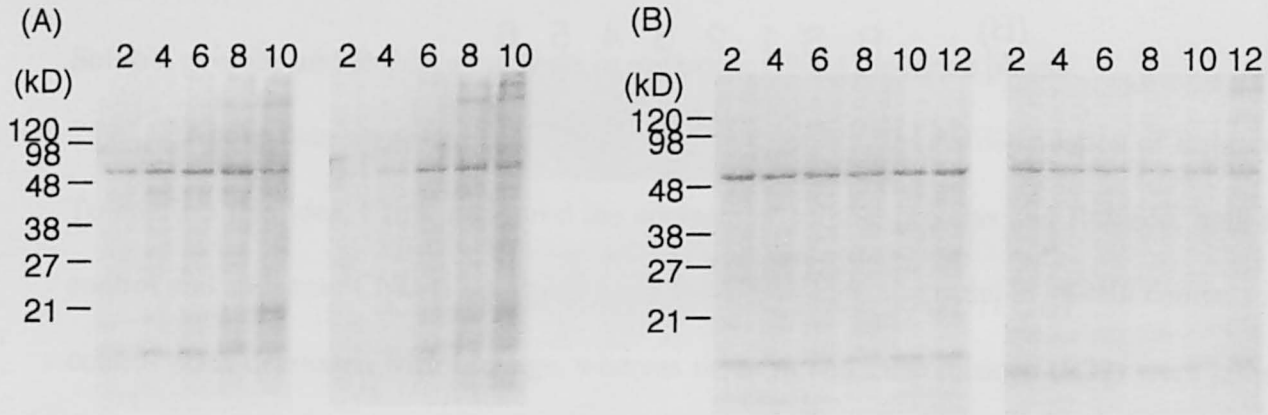


Fig. 3-4. SDS-PAGE analysis of soluble proteins of control and antisense CND41 tobacco under nitrogen depletion condition. (A), control tobacco plants (X6) grown in normal (left) and nitrogen-depleted medium (right). (B), antisense CND41 transformants (R22) grown in normal (left) and nitrogen-depleted medium (right). Soluble proteins extracted from 0.5 mg leaves were analyzed. Leaves were numbered as indicated in Fig. 3-3.

Fig. 3-5. Control and antisense CND41 tobacco under nitrogen-depletion. Control tobacco plants (X6; A and C) and antisense CND41 transformants (R22; B and D) were hydroponically cultured with (A and B) or without (C and D) nitrogen. R28 antisense tobacco also showed similar response to nitrogen-treatment.

plants; yellow leaves at lower parts whereas top parts were green. On the other hand antisense CND41 tobacco showed much less symptom of senescence, i. e. green leaves at lower positions and rather pale green leaves at top parts. This result provided additional evidence that CND41 would be involved in senescence.

Measurement of protein contents in leaves provided direct evidence that CND41 was involved in the degradation of protein, especially Rubisco, in senescent leaves. As Fig. 3-3 shows control plant had very low protein contents in lower leaves, and kept high contents in upper leaves after nitrogen-depletion treatment (Fig. 3-3 A). However, antisense CND41 transformant showed rather constant concentration of proteins through whole plant, whereas the level of contents was reduced after nitrogen-depletion treatment (Fig.3-3 B). SDS-PAGE confirmed that the remarkable decrease of protein in lower leaves in control plant under nitrogen-depletion was due to the degradation of Rubisco. No such decrease of Rubisco in antisense CND41 transformant suggested that CND41 was main protease involved in protein degradation , especially of Rubisco, under nitrogen-depleted condition (Fig. 3-4).

Discussion

In this Chapter, I investigated *in vivo* function of CND41 as a protease using antisense CND41 tobacco. High protein contents in lower leaves of antisense CND41 tobacco under the nitrogen-depletion in comparison with very low protein contents in control plants indicated the involvement of CND41 in protein degradation during senescence. The reduction of protein contents in upper leaves with retained Rubisco in lower leaves of antisense CND41 tobacco under the nitrogen-depleted condition suggests that controlled degradation of Rubisco by CND41 in chloroplasts is needed for the transport of amino acids to replenish the developing organs. On the other hand, this overall reduction of protein

contents suggested that other proteolytic system, e. g. autophagocytosis, might be activated in nitrogen-depleted antisense CND41 tobacco to replenish inorganic nitrogen. High expression of CND41 in lower leaves well explains the function of CND41 as the protease in senescence.

Whereas the involvement of CND41 in protein degradation in senescence is evident, it is not clear how CND41 is activated in senescence. In previous Chapter, I indicated that denaturation of Rubisco is necessary for the proteolysis by CND41 at physiological pH. It is quite possible that nitrogen-depletion may induce the denaturation of Rubisco, through generation of active oxygen and/or other unknown processes. In fact, in antisense tobacco plants, I could detect many degraded protein bands which were detected by anti-Rubisco antibodies, whereas these degradation products were not seen in control plants under either normal or nitrogen-depleted condition. Accumulation of these degradation products of Rubisco in antisense tobacco, especially under the nitrogen-depleted condition, suggests that senescence induces the denaturation of Rubisco and CND41 degrades these degradation products further. How chloroplast DNA is involved in the regulation of the proteolytic activity of CND41 in senescence is open question, whereas chloroplast DNA degradation may occur simultaneously in senescence.

Although I reported here the possible *in vivo* function of CND41 as the protease for the Rubisco degradation in senescence, CND41 has several other functions; i. e. higher accumulation of chloroplast gene transcripts, dwarfism and developed chloroplast in apical meristematic cells were observed in antisense CND41 plants (Nakano et al. unpublished data). Some functions, such as negative regulation of chloroplast gene expression, might be mediated by the protease activity of CND41, as discussed in Chapter 2. Further characterization of the mechanism of other pleiotropic functions of CND41 would be very useful to understand not only the development of chloroplast but also of whole plant.

Summary

Chapter 1

To confirm the proteolytic activity of CND41 that has active domains conserved for aspartic proteases, it was purified from cultured tobacco cells and assayed its activity with FITC-labeled hemoglobin as the substrate. Purified CND41 showed high proteolytic activity under acidic condition, indicative that CND41 has both DNA-binding and protease activities as a component of nucleoid. Nucleoside triphosphates and their non-hydrolysable analog, e. g. ATP/GTP-γ-S, as well as NADPH strongly inhibited the protease activity of CND41 at an acidic pH, whereas protease inhibitors examined, including pepstatin, did not inhibit strongly. CND41 also showed proteolytic activity at weak acidic conditions, even though the sequence specific in contrast to nonspecific at pH 2.5.

Chapter 2

To characterize the proteolytic activity of CND41 at physiological pH in stroma where CND41 exists, several reaction conditions were examined. Finally, denatured Rubisco was found to be a good substrate for the CND41 protease at pH 7.5. When Rubisco was activated with CO_2 and Mg^{2+}, then stabilized with CABP, this Rubisco was tolerant to the proteolysis by CND41 after denaturation, indicating that active form of Rubisco under normal physiological condition is not the substrate. Inhibition of CND41-induced proteolysis with DNA suggests that the proteolytic activity of CND41 is regulated by the DNA binding. Denaturation of several chloroplast proteins and their proteolytic degradation showed that some denatured proteins are good substrates for CND41 at physiological pH.

Chapter 3

Analysis of proteins of control and antisense CND41 tobacco under normal and nitrogen-depleted condition clearly showed that CND41 is one of the main proteases involved in degradation of Rubisco *in vivo* during senescence, especially induced by nitrogen-depletion. Detection of degradation intermediates of Rubisco in antisense CND41 tobacco under nitrogen-depleted condition suggests that senescence induces the denaturation of Rubisco and CND41 degrades these degradation products further. Although the actual mechanism is not clear, this dual activities for DNA binding and protease would provide the multiple functions for CND41 both in chloroplast and whole pant development.

References

Adam, Z. (1996). Protein stability and degradation in chloroplasts. Plant Mol. Biol. 32, 773-783.

Allison, L.A., Simon L.D., and Maliga P. (1996). Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants. EMBO J. 15, 2802-2809.

Andersson, B., and Aro, E.M. (1997). Proteolytic activities and proteases of plant chloroplasts. Physiol. Plant. 100, 780-793.

Apel, K., and Kloppstech, K. (1980). The effect of light on the biosynthesis of the light-harvesting chlorophyll *a/b* protein: Evidence for the requirement of chlorophyll a for the stabilization of the apoprotein. Planta 150, 426-430.

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. Plant Physiol. 101, 781-791.

Barakat, S., Pearce, D.A., Sherman, F. and Rapp, W.D. (1998). Maize contains a Lon protease gene that can partially complement a yeast *pim1*-deletion mutant. Plant Mol. Biol. 37, 141-154.

Bushnell, T.P., Bushnell, D., and Jagendorf, A.T. (1993). A purified zinc protease of pea chloroplasts, EP1, degrades the subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant Physiol. 103, 585-591.

Deng, X.W., and Gruissem, W. (1987). Control of plastid gene expression during development: The limited role of transcriptional regulation. Cell 49, 379-388.

Desimone, M., Henke, A., and Wagner, E. (1996). Oxidative stress induces partial

degradation of the large subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase in isolated chloroplasts of barley. Plant Physiol. 111, 789-796.

Desimone, M., Wagner, E., and Johanningmeier, U. (1998). Degradation of active-oxygen-modified ribulose-1,5-bicphosphate carboxylase/oxygenase by chloroplastic proteases requires ATP-hydrolysis. Planta 205, 459-466.

Fasman, G.D. (1989). In Practical Handbook of Biochemistry and Molecular Biology, (Boca Raton, France: CRC Press).

Ferreira, R.M.B., and Teixeira, A.R.N. (1992). Sulfur starvation in *Lemna* leads to degradation of ribulose-1,5-bisphosphate carboxylase without plant death. Planta 193, 208-215.

Fu, G.K., Smith, M.J., and Markovitz, D.M. (1997). Bacterial protease Lon is a site-specific DNA-binding protein. J. Biol. Chem. 272, 534-538.

García-Ferris, C., and Moreno, J. (1994). Oxidative modification and breakdown of ribulose-1,5-bisphosphate carboxylase/oxygenase induced in *Euglena gracilis* by nitrogen starvation. Planta 193, 208-215.

Gottesman, S., and Maurizi, M. R. (1992) Regulation by proteolysis: energy-dependent proteases and their targets. Microbiol. Rev. 56, 592-621.

Gutteridge, S., Millard, B.N., and Parry, M.A.J. (1986). Inactivation of ribulose-bisphosphate carboxylase by limited proteolysis: Loss of the catalytic activity without disruption of bisphosphate binding or carbamylation. FEBS Lett. 196, 263-268.

Gutteridge, S., and Gatenby, A.A. (1995). Rubisco synthesis, assembly mechanism, and regulation. Plant Cell, 7, 809-829.

Hajdukiewicz, P.T.J., Allison, L.A., and Maliga P. (1997). The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in

plastids. EMBO J. 16, 4041-4048.

Halperin, T., and Adam, Z. (1996). Degradation of mistargeted OEE33 in the chloroplast stroma. Plant Mol. Biol. 30, 925-933

Hansmann, P., Falk, H., Ronai, K., and Sitte, P. (1985). Structure, combination, and distribution of plastid nucleoids in *Narcissus pseudonarcissus*. Planta 164, 459-472.

He, G.-P., Muise, A., Li, A.W., and Ro H.-S. (1995). A eukaryotic transcriptional repressor with carboxypeptidase activity. Nature 378, 92-96.

Hedtke, B., Boerner, T., and Weihe, A. (1997). Mitochondrial and chloroplast phage-type RNA polymerases in *Arabidopsis*. Science 227, 809-811.

Ifuku, K., Shiga, M., Kanda, Y., and Sato, F. (1998). Reconstitution of photosynthetic oxygen evolving activity with heterologous OEC23 protein expressed in *E. coli*. In Photosynthesis: Mechanisms and Effects, Vol. II, G. Grab, ed (Dordrecht, The Netherlands: Kluwer Academic publishers), pp. 1439-1442.

Ishida, H., Nishimori, Y., Sugisawa, M., Makino, A., and Mae, T. (1997). The large subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase is fragmented into 37-kDa and 16-kDa polypeptides by active oxygen in the lysates of chloroplasts from primary leaves of wheat. Plant Cell Physiol. 38, 471-479.

Isono, K., Shimizu, M., Yoshimoto, K., Niwa, Y., Satoh, K., Yokota, A., and Kobayashi, H. (1997). Leaf-specifically expressed genes for polypeptides destined for chloroplasts with domains of σ70 factors of bacterial RNA polymerases in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 94, 14948-14953.

Itzhaki, H., Naveh, L., Lindahl, M., Cook, M., and Adam, Z. (1998). Identification and characterization of DegP, a serine protease associated with the lumenal side of the thylakoid membrane. J. Biol. Chem. 273, 7094-7098.

James, M.N.G., and Sielecki, A.R. (1986). Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 Å resolution. Nature 319, 33-38

Kervinen, J., Tobin, G.J., Costa, J., Waugh, D.S., Wlodawer A., and Zdanov, A. (1999). Crystal structure of plant aspartic proteinase prophytepsin: inactivation and vacuolar targeting. EMBO J. 18, 3947-3955

Kim, J., and Mayfield, S.P. (1997). Protein disulfide isomerase as a regulator of chloroplast translational activation. Science 278,1954-1957.

Koelsch, G., Mares, M., Metcalf, P., and Fusek, M. (1994). Multiple functions of pro-parts of aspartic proteinase zymogens. FEBS Lett. 343 6-10.

Lindahl, M., Yang, D.H., and Andersson, B. (1995). Regulatory proteolysis of the major light-harvesting chlorophyll *a/b* protein of photosystem II by a light-induced membrane-associated enzymic system. Eur. J. Biochem. 231, 503-509.

Makino, A., Mae, T., and Ohira, K. (1984). Relation between nitrogen and ribulose-1.5-bisphosphate carboxylase in rice leaves from emergence through senescence. Plant Cell Physiol. 25, 429-437.

Malathi, K., Ganesan, K., and Datta, A. (1994). Identification of a putative transcription factor in *Candida albicans* that can complement the mating defect of *Saccharomyces cerevisiae ste12* mutants. J. Biol. Chem. 269, 22945-22951.

Mayfield, S.P., Yohn, C.B., Cohen, A., and Danon, A. (1995). Regulation of chloroplast gene expression. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 147-166.

Morikawa, K., Ito, S., Tsunoyama, Y., Nakahira, Y., Shiina T., and Toyoshima, Y. (1999). Circadian-regulated expression of a nuclear-encoded plastid σ factor gene (*sigA*). in wheat seedlings. FEBS Lett. 451, 275-278.

Mullet, J.E., and Klein, R.R. (1987). Transcription and RNA stability are important determinants of higher plant chloroplast RNA levels. EMBO J. 6, 1571-1579.

Mullet, J.E. (1993). Dynamic regulation of chloroplast gene transcription. Plant Physiol. 103, 309-313.

Murai, M., and Yoshida, S. (1998). Vacuolar membrane lesions induced by a freeze-thaw cycle in protoplasts isolated from deacclimated tubers of Jerusalem artichoke (*Helianthus tuberosus* L.). Plant Cell Physiol. 39, 87-96

Nagy, F., Kay, S.A., and Chua, N.-H. (1988). Analysis of gene expression in transgenic plants. In Plant molecular biology manual, S.B.Gelvin, R.A. Schilperoort, and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), B4: pp. 1-29.

Nakabayashi, K., Ito, M., Kiyosue, T., Shinozaki, K., and Watanabe, A. (1999). Identification of *clp* genes expressed in senescing *Arabidopsis* leaves. Plant Cell Physiol. 40, 504-514.

Nakano, T., Sato, F., and Yamada, Y. (1993). Analysis of nucleoid-proteins in tobacco chloroplasts. Plant Cell Physiol. 34, 873-880.

Nakano, T., Murakami, S., Shoji, T., Yoshida, S., Yamada, Y., Sato, F. (1997). A novel protein with DNA binding activity from tobacco chloroplast nucleoids. Plant Cell 9, 1673-1682.

Nemoto, Y., Kawano, S., Kondoh, K., Nagata, T., and Kuroiwa, T. (1990). Studies on plastid-nuclei (nucleoids) in *Nicotiana tabacum* L. III. Isolation of chloroplast -nuclei from mesophyll protoplasts and identification of chloroplast DNA-binding proteins. Plant Cell Physiol. 31, 767-776.

Ostersetzer, O., and Adam, Z. (1997). Light-stimulated degradation of an unassembled Rieske FeS protein by a thylakoid-bound protease: the possible role of the FtsH protease. Plant Cell 9, 957-965.

Otto, S., and Feierabend, J. (1994). Assay and comparative characterization of the proteolytic degradation of isolated small subunit and holoenzyme of ribulose-1,5-bisphosphate carboxylase/oxygenase in chloroplasts from rye leaves. J. Plant Physiol. 144, 26-33.

Peñarrubia, L., and Moreno, J. (1990). Increased susceptibility of ribulose-1,5-bisphosphate carboxylase/oxygenase to proteolytic degradation caused by oxidative treatments. Arch. Biochem. Biophys. 281, 319-323.

Pierce, J., Tolbert N.E., and Baker R. (1980). Interaction of ribulosebisphosphate carboxylase/oxygenase with transition analogues. Biochemistry 19, 934-942.

Pyke, K.A., and Leech, M. (1987). Cellular levels of ribulose 1,5 bisphospate carboxylase and chloroplast compartment size in wheat mesophyll cells. J. Exp. Bot. 38, 1949-1956.

Rapp, J.C., Baumgartner, B.J., and Mullet, J.E. (1992). Quantitative analysis of transcription and RNA levels of 15 barley chloroplast genes: Transcription rate and mRNA levels vary over 300-fold; predicted mRNA vary 30-fold. J. Biol. Chem. 267, 21404-21411

Reiss, T., and Link, G. (1985). Characterization of transcriptionally active DNA-protein complexes from chloroplasts and etioplasts of mustard (*Sinapis alba* L.). Eur. J. Biochem. 148, 207-212

Roffey, R.A., and Theg, S.M. (1996). Analysis of the import of carboxyl-terminal truncations of the 23-kilodalton subunit of the oxygen-evolving complex suggests that its structure is an important determinant for thylakoid transport. Plant Physiol. 111, 1329-1338.

Sakai, A., Suzuki, T., Miyazawa, Y., Kawano, S., Nagata, T., and Kuroiwa, T. (1998). Comparative analysis of plastid gene expression in tobacco chloroplasts and proplastids: Relationship between transcription and transcript accumulation. Plant Cell Physiol. 39, 581-589

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor Laboratory, NY: Cold Spring Harbor Laboratory Press).

Schmidt, G.W., and Mishkind, M.L. (1983). Rapid degradation of unassembled ribulose 1,5-bisphosphate carboxylase (EC. 4. 1. 1. 39) small subunit in chloroplasts. Proc. Natl. Acad. Sci. USA 80, 2623-2636.

Schuster, G., Lisitsky, I., and Klaff, P. (1999). Polyadenylation and degradation of mRNA in the chloroplast. Plant Physiol. 120, 937-944.

Sodmergen, S.K., Kawano, S., Tano, S., and Kuroiwa, T. (1989). Preferential digestion of chloroplast nuclei (nucleoids) during senescence of the coleoptile of *Oryza sativa.* Protoplasma 152, 65-68.

Sokolenko, A., Altschmied, L., and Herramnn, R.G. (1997). Sodium dodecyl sulfate-stable proteases in chloroplasts. Plant Physiol. 115, 827-832

Sugita M., and Sugiura M. (1996). Regulation of gene expression in chloroplasts of higher plants. Plant Mol. Biol. 32, 315-326.

Sugiura, M., Hirose, T., and Sugita M. (1998). Evolution and mechanism of translation in chloroplasts. Annu. Rev. Genet. 32, 437-459.

Suzuki, C.K., Suda, K., Wang, N., and Schatz, G. (1994). Requirement for the yeast gene LON in intramitochondrial proteolysis and maintenance of respiration. Science 264, 273-276.

Takeda, S., Sato, F., Ida, F., and Yamada, Y. (1990). Characterization of polypeptides that accumulate in cultured *Nicotiana tabacum* cells. Plant Cell Physiol. 31,215-221.

Tanaka, K., Tozawa, Y., Mochizuki, N., Shinozaki, K., Nagatani, A., Wakasa, K., and Takahashi, H. (1997). Characterization of three cDNA species encoding plastid RNA polymerase sigma factors in *Arabidopsis thaliana*: evidence for the sigma factor

heterogeneity in higher plant plastid. FEBS Lett. 413, 309-313

Tozawa, Y., Tanaka, K., Takahasi, H., and Wakasa, K. (1998). Nuclear encoding of a plastid σ factor in rice and its tissue- and light-dependent expression. Nucleic Acids Res. 26, 415-419.

Twining, S.S. (1984). Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. 143, 30-34.

Wang, N., Gottesman, S., Willingham, M.C., Gottesman, M.M., and Maurizi M.R. (1993). A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease. Proc. Natul. Acad. Sci. USA 90, 11247-11251.

Walker, D. (1987). The use of the oxygen electrode and fluorescence probes simple measurements of photosynthesis. (Sheffield, UK: Oxygraphics Limited).

Xu, H.K. and Johnston S.A. (1994). Yeast bleomycin hydrolase is a DNA-binding cysteine protease: Identification, purification, biochemical characterization. J. Biol. Chem. 269, 21177-21183.

Yakura, K., and Tanifuji, S. (1983). Molecular cloning and restriction analysis of *Eco* RI-fragment of *Vicia faba* rDNA. Plant Cell Physiol. 24, 1327-1330.

Yamada, T., Ohta, H., Masuda, T., Ikeda, M., Tomita, N., Ozawa, A., Shioi, Y., and Takamiya, K. (1998). Purification of novel type of SDS-dependent protease in maize using a monoclonal antibody. Plant Cell Physiol. 39, 106-114.

Yang, J., Teplyakov, A., and Quail, J.W. (1997). Crystal structure of the aspartic proteinase from *Rhizomucor miehei* as 2.15 Å resolution. J. Mol. Biol. 268, 449-459.

Zheng, W., and Johnston S.A. (1998). The nucleic acid binding activity of bleomycin hydrolase is involved in bleomycin detoxification. Mol. Cell. Biol. 18, 3580-3585.

List of Publications

Nakano, T., Murakami, S., Shoji, T., Yamada Y., and Sato, F. (1995). CND41; a novel chloroplast-nucleoid DNA-binding protein. In "Photosynthesis: from Light to Biosphere", Vol. III, P. Mathis ed (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 575-578.

Sato, F., Nakano, T., Murakami, S., Shoji, T., and Yamada, Y. (1996). A novel protein with DNA-binding activity from tobacco chloroplast nucleoids: A possible negative regulator in chloroplast gene expression. In "Proceedings of US-JAPAN Workshop on Advances in the Molecular Biology of Photosynthesis", November 21-25, 1996, Grand Canyon National Park, USA, pp. 78-81.

Nakano, T., Murakami, S., Shoji, T., Yoshida, S., Yamada, Y., and Sato, F. (1997). A novel protein with DNA binding activity from tobacco chloroplast nucleoids. The Plant Cell, 9, 1673-1682.

*Murakami, S., Nakano, T., and Sato, F. (1998). Purification of CND41; chloroplast nucleoid DNA-binding protein with proteolytic activity. In "Photosynthesis: Mechanisms and Effects", Vol. IV, G. Garab, ed (Dordrecht, The Netherlands : Kluwer Academic Publishers), pp. 3035-3038 .

*Sato, F., Murakami, S., Chatani, H., and Nakano, T. (1999). CND41, a DNA-binding protein in chloroplast nucleoid, and its function. In "Proceedings of the 1999 Korea-Japan Joint Symposium on Plant Biotechnology", July 15-16, 1999, Seoul, Korea, 13, pp. 51-56

*Murakami, S., Nakano, T., and Sato, F. (2000). Protease activity of CND41, a chloroplast nucleoid DNA-binding protein, isolated from cultured tobacco cells. FEBS Lett.. In press

*Murakami, S., Kato, Y., Kondo, Y., Chatani, H., Nakano, T., Yokota, A., and Sato, F. CND41 degrades denatured Rubisco in senescence. In preparation

* indicates the muscripts which are presented in this thesis

Acknowledgments

I would like to express my deepest gratitude to Professor Fumihiko Sato, Kyoto University, for his continuous guidance, and warm supports throughout this study.

I express my sincere thanks to Emeritus Professor Yasuyuki Yamada, Kyoto University, for his kind encouragement.

I also like to express my appreciation to Associate Professor Kazufumi Yazaki and Dr. Tsuyoshi Endo, Kyoto University, for their kind encouragement and valuable advice.

I am indebted to Dr. Takeshi Nakano, The Institute of Physical and Chemical Research, for his continuous support and useful discussions.

I wish to thank Professor Akiho Yokota, Nara Institute of Science and Technology, for his valuable advice and generous gift of purified proteins and a chemical.

I wish to thank Associate Professor Hiroshi Ueno, Kyoto University, for N-terminal amino acid sequence analysis of several proteins.

I am grateful to Mr. Hiroshi Chatani, Mr. Yoshihiko Kondo and Mr. Yusuke Kato, Kyoto University, for their collaboration, and Mr. Kentaro Ifuku, Kyoto University, for his gift of the purified OEC23s.

I also thank all the members of the laboratory of Molecular and Cellular Biology, Kyoto University for their continuous encouragement and for providing a stimulating laboratory environment.

Finally, I would like to acknowledge my parents for their kind encouragement and strong support.

March, 2 000

村上真也

Shinya Murakami