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ORIGINAL ARTICLE

Yusuke Kato · Yumiko Yamamoto
Shinya Murakami · Fumihiko Sato

Post-translational regulation of CND41 protease activity in senescent tobacco leaves

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Abstract The degradation of chloroplast proteins is an important occurrence in the mobilization of nutrients from senescing leaves to reproductive organs during senescence. Recently, we proved that tobacco CND41 protease is involved in Rubisco degradation and the translocation of nitrogen during senescence. In this study, we show the post-translational regulation of CND41 protease. Using very specific antibodies that were prepared against CND41-specific peptide (anti-Val 186 to Ser 206), immunoblot analysis clearly indicated a change in the accumulation and processing of CND41 during the maturation of leaves in whole plants. The developmental modification of CND41 was also observed in transgenic tobacco with constitutive expression of CND41 under cauliflower mosaic virus 35S promoter. Further studies of seedlings under senescence induced by combined treatment with nitrogen-starvation and high sucrose confirmed that the processing of CND41 was important for protease activity and senescence. A possible mechanism for the regulation of CND41 activity is discussed.

Keywords Chloroplast · Aspartic protease · Senescence · Nitrogen-depletion · Post-translational regulation · Rubisco

Y. Kato · Y. Yamamoto · F. Sato (✉)
Division of Integrated Life Science,
Graduate School of Biostudies,
Kyoto University, Kyoto 606-8502, Japan
E-mail: fumihiko@kais.kyoto-u.ac.jp
Tel.: +81-75-7536380
Fax: +81-75-7536398

S. Murakami · F. Sato
Division of Applied Life Sciences,
Graduate School of Agriculture,
Kyoto University, Kyoto 606-8502, Japan

S. Murakami
Boyce Thompson Institute for Plant Research,
Cornell University, Tower Road, Ithaca,
NY 14853, USA

Abbreviations LS: Linsmaier and Skoog medium · MOPS: 3-Morpholinopropanesulfonic acid buffer · SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis · Rubisco: Ribulose 1,5-bisphosphate carboxylase/oxygenase · CND41: Chloroplast Nucleoid DNA-binding protein · PCR: Polymerase chain reaction · PBST: Phosphate-buffered saline with 0.1% Tween20 · PVDF: Polyvinylidene fluoride membrane · GS: Glutamine synthetase · OEC33: Extrinsic 33-kDa protein in the oxygen-evolving complex · CBB: Coomassie brilliant blue

Introduction

Senescence in plants is considered the final stage of leaf development. It is not merely a degenerative process, but is also a recycling process that involves the remobilization of nutrients from senescing leaves to reproductive organs or younger leaves (Buchanan-Wollaston 1997; Gan and Amasino 1997; Nam 1997; Bleecker 1998; Hortensteiner and Feller 2002). During senescence, leaf cells undergo dramatic changes in gene expression and the sequential degeneration of cellular structures (Lee et al. 2001; Gepstein et al. 2003; Lin and Wu 2004). In this degeneration process, chloroplasts that hold most of the leaf proteins are broken down in the early phase of senescence, whereas mitochondria and the nucleus remain intact until the final phase (Nooden 1988; Buchanan-Wollaston 1997; Gan and Amasino 1997; Nam 1997; Bleecker 1998; Hortensteiner and Feller 2002). However, the biochemical and molecular mechanisms that control chloroplast degradation are not yet known.

Although the key regulatory factor during senescence has not yet been identified, we found that a chloroplastic aspartic protease, i.e., CND41 in tobacco, plays a role in the regulation of senescence (Kato et al. 2004). CND41 was shown to have strong proteolytic activity at acidic pH (pH2–4) (Murakami et al. 2000), and could degrade denatured Rubisco at physiological pH with increased expression during leaf aging (Kato et al. 2004). The

characterization of transgenic tobacco with low CND41 supported the notion that CND41 plays a role in the in vivo degradation of Rubisco protein during senescence (Kato et al. 2001, 2004).

In this study, we further characterized the regulation of Rubisco degradation by CND41; we examined the transcriptional and post-transcriptional regulation of degradation. For this purpose, we prepared specific antibodies against CND41-specific peptide (anti-Val 186 to Ser 206) to distinguish CND41 from other aspartic proteases, which are very common in senescent plants. These newly prepared antibodies specifically reacted with CND41. Aspartic proteases are synthesized as inactive precursors (zymogens), which prevent undesirable protein degradation (Koelsch et al. 1994; Khan and James 1998; Kervinen et al. 1999). Since random chloroplast protein degradation is very harmful for living cells, we expected that the protease activity of CND41 should be strictly regulated. To evaluate this hypothesis, we also prepared transgenic tobacco that overexpressed CND41 and found specific conditions that induced leaf senescence, which included combined treatment with nitrogen starvation and high sucrose in the light. Immunoblot analysis of leaves clearly indicated that the accumulation of CND41 was not sufficient for the degradation of Rubisco; i.e., the specific accumulation of processed CND41 was essential for Rubisco degradation. These results further support the importance of CND41 in leaf senescence with regard to both developmental and stress (nitrogen-starvation)-induced degradation of Rubisco. A possible mechanism that regulates the processing of CND41 is also discussed.

Material and methods

Vector construction

To construct the CND41-overexpression vector, the entire CND41 coding sequence was amplified by PCR using the specific primers 5′-GCTCTAGATGGAACATTCACTAAT-3′ and 5′-CCGCTCGAGAGCCTGTAGCTGAGATTCATTTG-3′. The PCR products were cloned into the pBluescript KSII(-) vector using *Xho*I and *Xba*I restriction sites. The sequence identity was confirmed by nucleotide sequencing of cloned PCR. The cloned fragments were digested with *Xho*I and *Xba*I and inserted into the *Xho*I and *Xba*I sites of the binary vector pBIE6X. Transgenic plants were obtained by *Agrobacterium*-mediated transformation as described previously (Nakano et al. 1997).

Plant material and growth conditions

Tobacco plants (*Nicotiana tabacum* cv. Samsun NN; wild-type and transgenic plants with the over-expression vector CNDsense03, CNDsense09) were grown in soil at 28 ± 1°C under continuous light (100 $\mu E/m^2/s$). The seeds of homozygous T2 generation were hydroponically cultured in half-strength Linsmaier and Skoog (LS) medium (Linsmaier and Skoog 1965) without sucrose under continuous light (40 $\mu E/mm^2/s$) at constant temperature (28 ± 1°C) for 2 weeks. After germination, nitrogen-depletion treatment of 2-week-old seedlings was performed in hydroponic culture with half-strength LS medium containing 4.7 mM KCl instead of 5.15 mM NH_4NO_3 and 4.7 mM KNO_3. Cultured cells of tobacco (*Nicotiana tabacum* cv. Samsun NN; cell line NII) were maintained in modified LS liquid media as described elsewhere (Takeda et al. 1990; Nakano et al. 1997).

Preparation of specific antibodies against CND41

CND41-specific peptide bearing the sequence VKSCYAQQQPIFDPSTSKTYS was synthesized, linked with keyhole limpet hemocyanin and used to produce antibodies with rabbit (Sawady Technology Co., Ltd., Japan). The Mac Vector program (Cosomo Bio Co., Ltd., Japan) was used to determine the exposed peptide surface of CND41. Whole serum was used for antibodies.

Protein extraction, SDS-PAGE and immunoblot analysis

Leaves of tobacco plants (wild-type and transgenic plants) were numbered from bottom to top and harvested. Harvested leaves and seedlings were frozen in liquid nitrogen and stored at −70°C until use. The frozen leaves were powdered in liquid nitrogen with a mortar and extracted to 5× its volume with extraction buffer (50 mM NaH_2PO_4 pH 7.2, 10 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 M NaCl). After centrifugation at 15,000*g*, the supernatants were used for protein assay according to the method of Bradford (1976) using a Bio-Rad Protein Assay (Bio-Rad). For immunoblot analysis, soluble proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% acrylamide gel, and blotted onto a PVDF membrane (Millipore, U.S.A.). PVDF membranes were then incubated with 5% skim milk in PBST buffer for 1 h. After being washed twice with PBST buffer, the membrane was incubated with anti-Rubisco antibodies, anti-OEC33 antibodies (gifted by Dr. A. Watanabe at University of Tokyo), anti-glutamine synthetase antibodies (gifted by Prof. T. Yamaya at Tohoku University, Yamaya et al. 1992) or anti-CND41 antibodies, and then detected by secondary antibodies (goat anti-rabbit IgG) with the enhanced chemiluminescence (ECL) method (PerkinElmer Life Sciences Co., Ltd., U.S.A.).

Chlorophyll quantitation

Chlorophyll content was determined with 80% acetone-extract of powdered frozen leaves. The chlorophyll

concentration was determined according to Porra et al. (1989).

Northern blot analysis

For Northern blot analysis, total RNA was isolated from frozen samples using the RNeasy Plant Mini Kit (Qiagen Inc.). Total RNA was then electrophoresed on a formaldehyde-denaturing-agarose gel in 1× Mops buffer (20 mM Mops-KOH pH7.0, 5 mM Na acetate an 1 mM EDTA) and blotted onto a Hybond N+ charged nylon membrane (Amersham Pharmacia). Hybridization and detection were performed according to standard protocols (Sambrook et al. 1989), with a ^{32}P-labeled full-length fragment of CND41 cDNA (Nakano et al. 1997) as a probe.

Results

Possible post-translational regulation of CND41 in senescent leaves

Transgenic tobaccos that overexpressed CND41 under the control of cauliflower mosaic virus 35S promoter were established to examine the effect of the ectopic expression of CND41 on senescence. After *Agrobacterium*-mediated transformation, we obtained nine kanamycin-tolerant tobacco lines. Further Northern analysis identified three transgenic tobacco lines that showed a high expression of CND41 (Fig. 1).

These transgenic tobacco plants that overexpressed CND41 showed enhanced senescence in lower leaves in the flowering period compared to wild-type tobacco, whereas these transformants and wild-type showed the induction of yellowing at a similar age. Thus, tobacco that overexpressed CND41 showed more rapid senescence after the induction of yellowing and died, whereas pale green or yellow leaves were still seen in wild-type tobacco at a later stage (Fig. 2). The levels of both total chlorophyll and soluble proteins also showed that leaf senescence proceeded rapidly in lower leaves of tobacco that overproduced CND41 (Fig. 2). These results supported our previous report that CND41 plays an important role in leaf senescence (Kato et al. 2004).

On the other hand, the phenotypes of young and mature leaves in CND41-overproducing tobacco were not distinguishable from the wild-type, whereas some experiments showed enhanced greening in young leaves in transformants, as shown in Fig. 2. Furthermore, the protein levels in young and mature leaves were similar in wild-type and CND41-overproducing tobacco plants. That is, no progression of protein degradation in young or mature leaves of CND41-overproducing tobacco occurred, whereas these wild-type leaves showed a high expression of CND41 (Fig. 1).

Detection of CND41 processing in senescent leaves with specific antibodies

To characterize the in vivo role of CND41 more carefully through the use of CND41 in leaf senescence, we prepared specific antibodies against a unique peptide fragment of CND41, since aspartic proteases are very common in senescent leaves; we used a synthetic peptide, with the sequence VKSCYAQQQPIFDPSTSKTYS, to prepare antibodies. This sequence in CND41 showed low homology to other aspartic proteases and was predicted to be exposed to the surface of protein based on an analysis of its amino acid content (Fig. 3). Immunoblot analysis using these specific peptide antibodies showed strong and specific reactivity with CND41 (Fig. 3).

We next examined the accumulation of CND41 in wild-type and CND41-overproducing tobacco by immunoblot analysis using specific antibodies against CND41 peptide. Immunoblot analysis of leaves at various developmental stages clearly showed that CND41 protein was accumulated only in senescent leaves in control tobacco (Fig. 4). On the other hand, CND41-overproducing tobacco showed the accumulation of mature-size CND41 even in young leaves as well as senescent leaves (Fig. 4), whereas no detectable Rubisco degradation was observed in young leaves. This result suggested that mature-size CND41 in young leaves of CND41-overproducing tobacco would be inactive as a protease.

Immunoblot analysis clearly indicated the accumulation of truncated fragments of CND41 in senescent leaves (leaf 4 in wild-type, leaves 3 and 4 in transformants in Fig. 4). Interestingly, the relative amount of truncated fragments of CND41 increased with age, whereas the total amount decreased. Our finding suggests that CND41 is also processed in senescent leaves and is activated as an active protease.

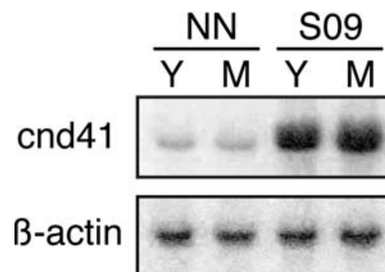

Fig. 1 Northern analysis of CND41 gene expression in transgenic tobacco Total RNAs (6 μg each) were extracted from young (Y) and mature (M) leaves in control plant (NN) and CND41-overexpressing tobacco (S09), and hybridized with ^{32}P-labeled CND41 cDNA and ß-actin probe. Northern analysis for other CND41-overexpressing tobacco showed a similar expression of the CND41 gene

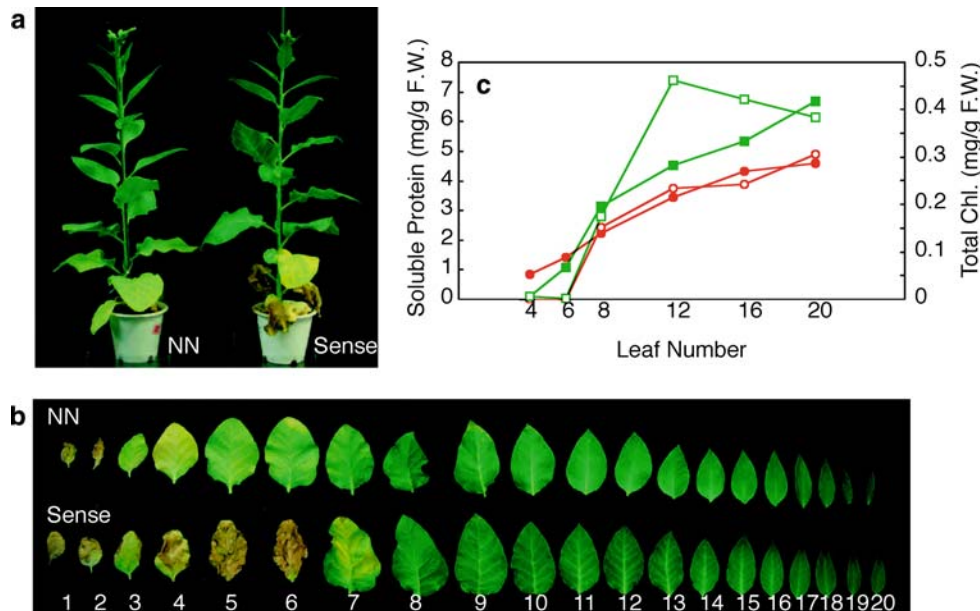

Fig. 2 Protein and chlorophyll content in CND41-overexpressing tobacco **a** Tobacco plant under normal growth conditions. Control tobacco (*Nicotiana tabacum cv*. Samsun NN) and CND41-overexpressing transgenic tobacco Sense03 were cultured for about 2 months under continuous light (100 $\mu E/m^2/s$) at 28 ± 1°C. Both plants showed similar growth kinetics and flowering. **b** Visual inspection of leaves. The leaves in the control and CND41-overexpressing tobacco were of a similar size. Early and rapid senescence was evident at leaf 6, whereas the control leaf at the same position retained a yellow color. **c** Protein and chlorophyll content. Whereas young leaves of both control and CND41-overexpressing tobacco showed a similar content of protein and chlorophyll, promoted senescence was observed in CND41-overexpressing tobacco in lower leaves compared to the control. Leaves were numbered from *bottom to top*. Soluble protein (control: *closed circle*, sense overexpressing tobacco: *opened circle*) and total chlorophyll contents (control: *closed square*, sense: *opened square*) were measured three times with powered leaves. Variation of measurement was less than 5%

CND41 activation and Rubisco degradation in chloroplast degeneration under nitrogen-starved and high sucrose conditions

To evaluate the importance of the post-translational processing of CND41 in Rubisco degradation and chloroplast degeneration, we examined the role of CND41 under other conditions in chloroplast degeneration. We used seedlings and nitrogen starvation in the presence of sugar, since the low-CND41 transformant maintained green leaves and constant protein levels, especially ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), throughout the whole plant, whereas wild-type tobacco showed marked senescence and a decrease in protein levels in the lower leaves under nitrogen starvation (Kato et al. 2004). Northern analysis showed that CND41 expression was dependent on combined treatment with nitrogen starvation, high sucrose and light (Fig. 5), whereas each treatment showed considerably different phenotypic changes; while marked senescence was observed in combined treatment with nitrogen starvation, high sucrose and light, seedlings were etiolated in the dark (Fig. 5).

SDS-PAGE analysis clearly showed that remarkable protein degradation only occurred under the combined treatment conditions (Fig. 5). Immunoblot analysis using CND41-specific antibodies also showed the accumulation of processed CND41 under the same conditions as were used for protein degradation, especially for Rubisco (Fig. 5), whereas small amounts of mature-size CND41 were accumulated in either nitrogen-starved or high sucrose-treated seedlings. The fact that CND41 processing and Rubisco degradation occurred concurrently suggested that the processing of CND41 was important for the initiation of the degradation of Rubisco. Additional immunoblot analyses using several chloroplast protein-specific antibodies, such as glutamine synthetase GS (Tobin and Yamaya 2001) and the extrinsic 33-kDa protein in the oxygen-evolving complex OEC33 (Yamamoto et al. 1998) confirmed that these protein levels were constant under the conditions that induced Rubisco degradation (Fig. 5), and CND41 is specific for Rubisco, as reported previously (Kato et al. 2004).

Discussion

Post-translational regulation of CND41 in senescent leaves

The degradation of chloroplasts is one of the key factors in leaf senescence for recycling and remobilizing the nutrients from senescing leaves to reproductive organs or younger leaves (Buchanan-Wollaston 1997; Gan and Amasino 1997; Nam 1997; Bleecker 1998; Hortensteiner and Feller 2002). Although the key factors in chloroplast degradation are not yet known, there are some reports that aspartic proteases are in-

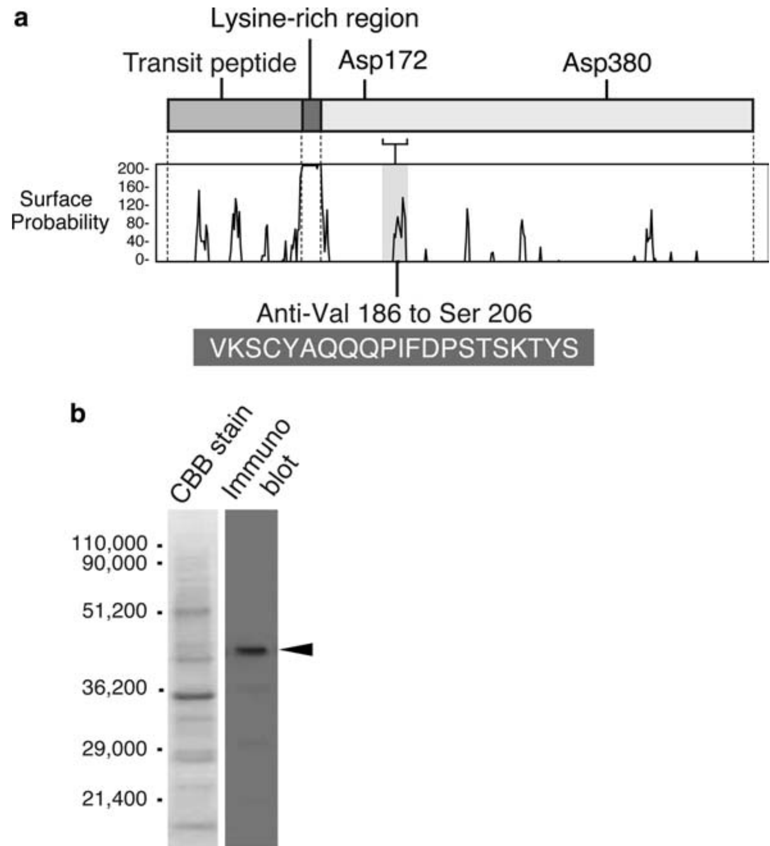

Fig. 3 Design of specific antibodies against CND41 **a** Site for the designed sequence in CND41. This peptide sequence (VKSCYAQQQ PIFDPSTSKTYS) showed a high probability to be localized at the surface of CND41. **b** Immunoblot analysis using anti-CND41 peptide-specific antibodies. Soluble proteins (10 μg) extracted from NII cultured tobacco cells were used for immunoblot analysis

volved in the degradation of chloroplast protein (Kuwabara and Suzuki 1995; Bhalerao et al. 2003). Our recent report on CND41 in chloroplasts provided the first molecular basis of Rubisco degradation in senescence (Kato et al. 2004, 2005).

In the previous report, it was unclear how CND41 protease activity is regulated in plants, since CND41 expression is observed before Rubisco degradation occurs. Our analysis of CND41-overproducing tobacco clarified that the expression of CND41 itself was not sufficient for inducing the degradation of Rubisco (Fig. 4). Rapid processing of senescence and the concomitant presence of the processed form of CND41 as well as Rubisco degradation suggest that post-translational processing of CND41 is important for the activation of CND41 protease. This speculation was further confirmed by an analysis of seedlings under nitrogen-starved and high sucrose conditions in the light-conditions under which chloroplasts in cotyledons are transformed to amyloplasts (data not shown). This combined treatment with nitrogen starvation, high sucrose and light induced the degradation of Rubisco and the accumulation of processed CND41. This finding clearly indicated that a post-translational process is involved in the activation of CND41 protease. Interestingly, CND41 was rather specific to Rubisco, since the levels of other chloroplast proteins such as glutamine synthetase and the extrinsic 33-kDa protein in the oxygen-evolving complex OEC33 were constant under the same conditions that induced Rubisco degradation (Fig. 5). These results coincided with our data using antisense CND41 tobacco (Kato et al. 2001, 2004).

In general, the processing of aspartic protease precursor is an important step in the generation of the active protease form (Davies 1990; Dunn 2002; Simoes and Faro 2004). Three-dimensional structural analyses have shown that pro-peptides of the N-terminus of aspartic proteases mask the substrate-binding cleft and prevent binding of the substrate to active sites (James and Sielecki 1986; Sielecki et al. 1991; Yang et al. 1997; Kervinen et al. 1999). Furthermore, ionic interactions play an important role in this mechanism for anchorage of the N-terminus in active sites (James and Sielecki 1986; Khan and James 1998; Kervinen et al. 1999); as an example, pepsinogen, a precursor of pepsin, has 13 positively charged residues in the prosegment-covered active site cleft (James and Sielecki 1986; Tanaka and Yada 2001). Similarly, we speculate that the lysine-rich N-terminal region of CND41 might interact with the active site. Whereas we could not directly detect the processing of the N-terminal region in truncated CND41 due to the difficulty of purifying this truncated form (data not shown), some enzymological properties of CND41 suggested that the N-terminal region is important for protease activity; the N-terminal region of CND41 is essential for DNA-binding activity (Nakano et al. 1997) and protease activity is inhibited by DNA (Kato et al. 2004).

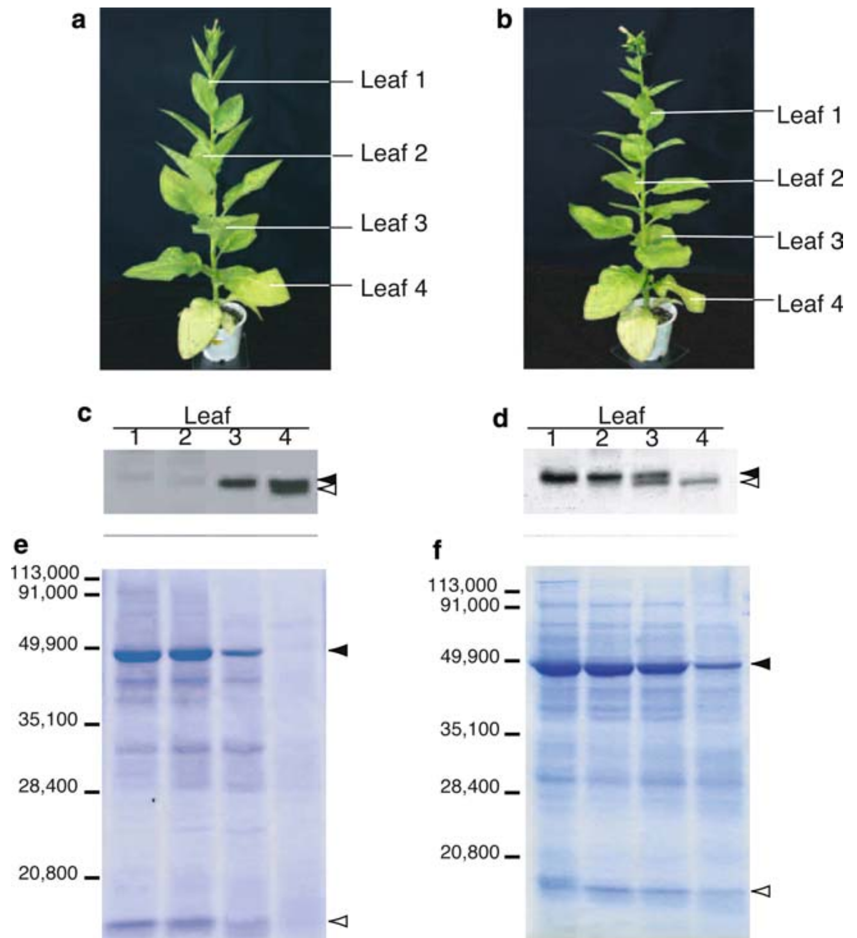

Fig. 4 Post-translational processing of CND41 and protein degradation **a** Control (*Nicotiana tabacum* cv. Samsun NN) and **b** CND41-overexpressing tobacco were cultured for about 2 months under continuous light (100 μE/m^2/s) at 28 ± 1°C. Leaves were numbered as shown; Leaf 1 (young leaf), Leaf 2 (mature leaf), Leaf 3 (early senescent leaf), Leaf 4 (late senescent leaf). Soluble proteins (20 μg) extracted from leaves were separated on 12.5% SDS polyacrylamide gels. Immunoblot analysis was conducted with anti-CND41 peptide-specific antibodies **c** Control tobacco, **d** CND41-overexpressing tobacco. *Black arrowhead* indicates the mature form of CND41 and *white arrowhead* indicates the truncated form of CND41. SDS-PAGE profiles in **e** Control tobacco and **f** CND41-overexpressing tobacco. *Arrowheads* indicate large and small subunits of Rubisco

Possible mechanisms for the regulation of CND41 protease

Another interesting question is how the activation of CND41 is triggered. One possible trigger is an increase in denatured Rubisco by oxidative stress in chloroplast. There have been several reports that the degradation of Rubisco was accelerated under oxidative stress conditions either in leaves or isolated chloroplasts (Mehta et al. 1992; Mitsuhashi et al. 1992; Eckardt and Pell 1995; Desimone et al. 1996). Under such stress conditions, reactive oxygen species cause the denaturation of Rubisco (Ishida et al. 1997, 1999), the main substrate of CND41 (Kato et al. 2004). Therefore, we examined cold treatment as a method for generating reactive oxygen in seedlings. We could not find either processed CND41 or degraded Rubisco under our cold condition, however, the level of CND41 mRNA slightly increased (data not shown). Another preliminary analysis of PsbP-silenced tobacco (Ifuku et al. 2003), in which oxygen-evolving complex in PSII was made unstable and reactive oxygen species were generated at a donor site, also showed no activation of CND41 expression or degradation of Rubisco. Although the denaturation of Rubisco by reactive oxygen species is an attractive hypothesis, more detailed studies on the relationship between reactive oxygen species and the activation of CND41 are required.

Another possible trigger of CND41 activation is the chloroplast degeneration signal. Chloroplasts change their morphology and function to several different forms such as chromoplasts in flowers and fruits and amyloplasts in roots (Thomson and Whatley 1980). The high ▸

Fig. 5 Nitrogen starvation and high sucrose treatment in seedlings and processing of CND41 After cultivation on 1/2 LS medium without sucrose for 2 weeks, tobacco seedlings were hydroponically cultured for an additional 2 weeks under the indicated growth conditions (+N: normal 1/2LS medium, −N: nitrogen-free 1/2LS medium containing 4.7 mM KCl instead of 5.15 mM NH_4NO_3 and 4.7 mM KNO_3, +S: 1.5% (w/v) sucrose, −S: sucrose-free) under continuous light (100 μE/m^2/s) or dark at 28 ± 1°C. **a** Phenotype of tobacco seedlings under various conditions. **b** Total RNA (4 μg) extracted from seedlings was used for Northern blot analysis. **c** Quantification of the amount of mRNA observed in (**b**) using NIH-image. The amount of mRNA was determined by NIH-image, and the values were corrected by the amount of ß-actin. Values indicate the relative value of +N-S in the light condition (100). Duplicate experiments showed similar results. **d** Soluble proteins (10 μg) extracted from leaves, and separated on 12.5% SDS polyacrylamide gels. Immunoblot analysis using anti-CND41 peptide, anti-Rubisco, anti-glutamine synthetase (GS) and anti-OEC33 antibodies. *Black arrowhead* indicates the mature form of CND41 and *white arrowhead* indicates truncated CND41. **e** SDS-PAGE profiles. *Black and white arrowheads* indicate large and small subunits of Rubisco, respectively.

expression of a CND41 homologue in *Arabidopsis* was detected in flower tissues (Kato et al. 2005). Furthermore, we have observed ultrastructural changes in chloroplasts with a progressive loss of thylakoid integrity and the accumulation of starch under combined treatment with nitrogen starvation and high sucrose in the light (data not shown). However, etiolated seedlings in the dark did not show the degradation of Rubisco or the activation of CND41 processing under the same conditions except for light. This result suggests that additional signal(s) are needed in addition to the degeneration of chloroplasts; i.e. some signals induced by low nitrogen, high sucrose and light. Senescent leaves are known to have low levels of nitrogen (Delgado et al. 1994; Hortensteiner and Feller 2002), and the whole plant has a relatively high sugar content (Masclaux et al. 2000). Whereas the biochemical consequences of low nitrogen, high sucrose, and light are not yet clear, the glycation of Rubisco has been reported to increase the susceptibility to protease and such glycation is expected to proceed at a higher rate under light (Yamauchi et al. 2002).

Senescence is one of the most remarkable and unique steps in leaf development (Lim et al. 2003; Yoshida

2003). Our observation suggests that plant cells carefully control the degradation process in chloroplasts of senescent leaves to remobilize their nitrogen for developing chloroplasts in young leaves when growth conditions are favorable. It is interesting that Rubisco was specifically degraded at the beginning of senescence in leaves and in nitrogen-starved, high sucrose seedlings in the light. We are now examining the function of CND41 homologues in other plant species, such as *Arabidopsis*. Our preliminary results suggest that the *Arabidopsis* CND41 homologue At 5g 10770 protein might also be involved in the regulation of Rubisco turnover and leaf senescence (Kato et al. 2004). Although a truncated form of CND41, an unusual aspartic protease, was difficult to purify in an active form, further biochemical studies should provide unique insight into chloroplast protein degradation.

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