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CND41, a chloroplast DNA-binding protease, is involved in Rubisco degradation

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Introduction

CND41 is a 41 kDa chloroplast DNA-binding protease isolated from cultured cells [1,2]. Our previous studies indicated that purified CND41 from cultured tobacco cells had strong proteolytic activity *in vitro* at acidic pH [3], and CND41 antisense tobacco with reduced CND41 (low CND41 tobacco) showed enhanced chloroplast gene expression, dwarf phenotype and retarded senescence[2]. These results suggested that CND41 has multi-functions as DNA-binding protease. To make the functional relationship of protease and phenotype of low CND41, we focused on the degradation of proteins in chloroplast during the senescence because low CND41 tobacco clearly showed retarded senescence. For this purpose, we examined the effect of nitrogen-depletion-induced senescence on the chloroplast protein degradation in low CND41 plant (R22) using SDS-PAGE and immuno-blot analyses. Our results clearly indicated that CND41 was involved in Rubisco degradation in senescent leaves under both normal and nitrogen-depletion conditions. To evaluate the indirect/direct effects of stunted growth on the retarded senescence and Rubisco degradation, we treated low CND41 tobacco with gibberellic acid (GA_3), to recover the growth and examined the protein degradation. GA treatment confirmed the primary role of CND41 in Rubisco degradation in aged leaves. The regulation of protease activity of CND41 *in vivo* is also discussed with *in vitro* studies on Rubisco degradation by CND41.

Materials and Methods

Plant materials, nitrogen depletion and GA treatment

Transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN; line R22) with reduced amount of CND41, and control plant (X6) transformed with modified pBI121 vector were established and maintained as reported [2]. Low CND41 (R22) and control tobacco were prepared from auxiliary shoots maintained *in vitro* and cultured hydroponically in a quarter strength of Linsmaier and Skoog (LS) medium without sucrose under the continuous light condition (40μmol $m^{-2}s^{-1}$) at 28ºC. After 1 week of hydroponic culture, culture medium was changed to nitrogen-free medium containing 4.70 mM KCl instead of 5.15 mM NH_4NO_3 and 4.70 mM KNO_3 for 1 week. To examine the effect of gibberellic acid on the protein degradation, low CND41 tobacco was cultured in a quarter strength of LS medium with 10^{-6} M GA_3 under the same condition as described above. Culture medium was renewed every 2-3 days.

Protein extraction, SDS-PAGE and immuno-blot analyses

Leaves of each plant were harvested, numbered from bottom to top, and frozen in liquid nitrogen. The frozen leaves were powdered in liquid nitrogen and extracted in 10 times volume of extraction buffer (0.6 M sorbitol, 50 mM Tris-HCl (pH 8.0), 3 mM EDTA, 1 mM 2-mercaptoethanol). Total protein was extracted with an equal volume of phenol, which was saturated with 100mM Tris-HCl (pH8.0), and precipitated with 0.1M CH_3COONH_4/methanol. Total protein was washed, dried, and dissolved in 50mM Tris-HCl buffer (pH8.0) containing 2% SDS as described previously [4]. The total protein amounts were measured with Bio-Rad DC Protein Assay kit (Bio-Rad). Proteins were analyzed by SDS-PAGE and immuno-blotting with anti-Rubisco antibodies.

Results and discussion

To examine the possibility that CND41 is involved in the protein degradation in senescent leaves, we examined the effect of nitrogen-depletion on the induction of senescence and Rubisco degradation both in control (X6) and low CND41 tobacco (R22). Generally, the old leaves in control plant have lower content of proteins than young leaves. The senescence induced by the nitrogen-depletion decreased the protein content in old leaves, whereas high protein content was maintained in young leaves. In contrast, low CND41 tobacco showed much constant amount of protein throughout the entire plant under normal growth condition, and nitrogen-depletion did not affect this protein profile; retarded senescence in low CND41 tobacco was observed even under nitrogen-depletion condition (Fig. 1). SDS-PAGE analysis of protein extracts of both control and low CND41 tobacco clearly indicated that the change of Rubisco amount was the main cause of the differences of protein content in control and low CND41 tobacco (data not shown). Immuno-blot analyses confirmed that changes in Rubisco corresponded with changes in protein contents both in control and low CND41 plants. Furthermore, more careful observation of immuno-blot indicated that several Rubisco-degradation products, which were never found in control tobacco, were found in young leaves of low CND41 tobacco. These results confirmed that the remarkable decrease of protein in lower leaves in senescence of control plants including nitrogen-depletion was due to the degradation of Rubisco and CND41 was involved in the *in vivo* degradation of Rubisco.

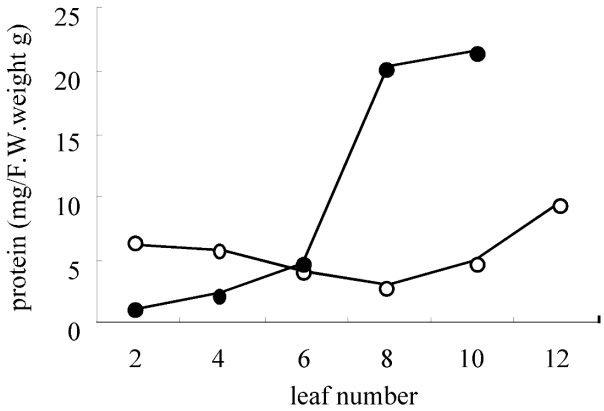

Fig. 1. Effect of nitrogen-depletion on the soluble protein contents in low CND41 tobacco. Leaves were numbered from bottom to top. These plants were cultured in nitrogen-depleted medium. Protein in control plant (close-circle) and low CND41 tobacco (open-circle) .

To get more direct evidence that CND41 can degrade Rubisco, *in vitro* degradation of Rubisco at physiological pH (pH 7.5) was examined. Whereas CND41 did not degrade native Rubisco at physiological pH[3], denaturation of Rubisco increased the degradation by CND41

at pH 7.5 (data not shown). The activation of Rubisco with Mg^{2+} and HCO_3^- made Rubisco more tolerant to denaturation and degradation, whereas the actual denaturation mechanism is not clear. It is highly probable that senescence can induce the denaturation of Rubisco.

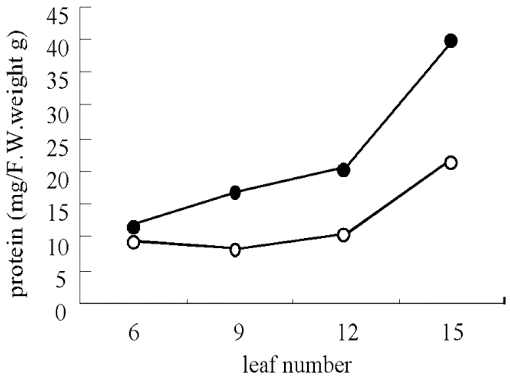

Fig. 2. Effect of GA3 on the protein contents in low CND41 tobacco. These plants were cultured in normal (close-circle) or nitrogen-depleted (open-circle) medium with 10 μM GA3.

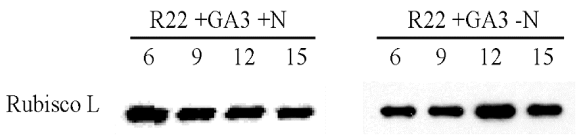

Fig. 3. Immuno-blotting analysis of Rubisco L subunit in low CND41 tobacco under nitrogen-depletion condition. Low CND41 transformants grown under normal (+N) and nitrogen-depleted (-N) medium with 10 μM GA3. Leaves numbered from bottom to top. Each lane contains 0.2 μg of total protein.

On the other hand, low CND41 tobacco also showed stunted growth, and recent investigation revealed that this growth retardation was due to the reduction of active gibberellic acid in low CND41 tobacco (Nakano et al, in preparation). Because the stunted growth may delay the induction of senescence, we treated low CND41 tobacco with GA_3 to restore the growth and examined the effect of nitrogen-depletion on the protein content. Whereas the growth of low CND41 tobacco was restored by the GA_3 treatment, fairly constant protein content throughout entire plant was maintained in low CND41 tobacco even under nitrogen-depleted condition (Fig. 2 & 3). This result clearly indicated that reduction of GA concentration was not the primary cause of decrease in Rubisco degradation, and CND41 amount itself was the main determinant of Rubisco degradation and leaf senescence.

To examine the role of CND41 during the senescence more in detail, we measured the protein profile in senescent leaves. Immuno-blot analyses using anti-OEC33, anti-PEPC and chloroplast RNA binding protein of 29 kDa antibodies showed that these protein levels were constant in both control and low CND41 tobacco. Whereas the number of proteins analyzed was limited, these results suggested that CND41 was rather specific for the Rubisco degradation. Importance of Rubisco degradation for the induction of senescence is under investigation.

It is important to understand how Rubisco degradation by CND41 is regulated, because Rubisco is an important enzyme in photosynthesis and the degradation must be regulated. CND41 has dual functions, protease activity and DNA-binding function. Then, we expected that DNA-binding function would be involved in the regulation of protease activity in senescence as well as the induction of denaturation of Rubisco. Analysis of *in vitro* CND41 protease activity showed that the addition of DNA inhibited the protease activity of CND41 at physiological pH. Damage or degradation of chloroplast DNA during senescence may induce the activation of CND41 protease. Chloroplast DNA amount and nucleoid structure in senescent leaves should be investigated.

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