Research Article

Reverse Genetic Identification of CRN1 and its Distinctive Role in Chlorophyll Degradation in *Arabidopsis*

Guodong Ren[1][†], Qian Zhou[1][†], Shouxin Wu[1], Yufan Zhang[1], Lingang Zhang[2], Jirong Huang[2], Zhenfei Sun[1] and Benke Kuai[1*]

[1]State Key Laboratory of Genetic Engineering, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200433, China

[2]National Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China

[†]Both authors contributed equally to this paper

[*]Corresponding author

Tel(Fax): +86 21 6564 2648; E-mail: bkkuai@fudan.edu.cn

Abstract

Recent identification of *NYE1*/*SGR1* brought up a new era for the exploration of the regulatory mechanism of Chlorophyll (Chl) degradation. Cluster analysis of senescence associated genes with putative chloroplast targeting sequences revealed several genes sharing a similar expression pattern with *NYE1*. Further characterization of available T-DNA insertion lines led to the discovery of a novel stay-green gene *CRN1* (*Co-regulated with NYE1*). Chl breakdown was significantly restrained in *crn1-1* under diversified senescence scenarios, which is comparable with that in *acd1-20*, but much more severe than that in *nye1-1*. Notably, various Chl binding proteins, especially trimeric LHCP II, were markedly retained in *crn1-1* four days after dark-treatment, possibly due to a lesion in disassociation of protein-pigment complex. Nevertheless, the photochemical efficiency of PSII in *crn1-1* declined, even more rapidly, two days after dark-treatment, compared to those in Col-0 and *nye1-1*. Our results suggest that *CRN1* plays a crucial role in Chl degradation, and that loss of its function produces various side-effects, including those on the breakdown of Ch-protein complex and the maintenance of the residual photosynthetic capability during leaf senescence.

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Introduction

Chlorophyll (Chl) breakdown is the remarkable phenomenon that occurs during leaf senescence in autumn and during fruit ripening. It is also initiated under various stressful conditions. Disassembly of Chl from its protein complex is regarded as the prior step before further enzymatic catabolism. Chl *b* is proposed to be transformed to Chl *a* by Chl *b* and 7[1]-OH-Chl *a* reductases respectively, before being further degraded. Recent reports show that NYC1/NOL is responsible for this reaction and loss of NYC1 function in *Oryza Sativa* exhibits

the stay-green phenotype, mainly due to the accumulation of Chl *b* during leaf senescence (Kusaba et al. 2007; Sato et al. 2009). According to the proposed biochemical pathway of Chl degradation, the phytol chain was considered to be removed initially from Chl *a* by Chlorophyllase (Chlase). Some of the encoding gene candidates were cloned from citrus and *Arabidopsis* (Jacob-Wilk et al. 1999; Tsuchiya et al. 1999). However, our recent analyses as well as Schenk et al.'s work provide a strong evidence that AtCLH1 and AtCLH2 may not be involved in senescence-linked Chl degradation (Liao et al. 2007; Schenk et al. 2007; Zhou et al. 2007). After that,

magnesium in the porphyrin ring is removed by a putative Mg-dechelatase or Mg-dechelating substance, which is yet to be identified. The resultant pheophorbide *a* (Pheide *a*) is subsequently converted to a tetrapyrrole product, named as primary fluorescent chlorophyll catabolites (pFCCs) through the sequential functioning of two enzymes: pheophorbide *a* oxygenase (PaO) and red chlorophyll catabolite reductase (RCCR). pFCCs are modified and transported out of the chloroplast and finally converted to nonfluorescent chlorophyll catabolites (NCCs) in the vacuole (Hörtensteiner 2006).

Identification of stay-green mutants is considered to be one of the most efficient ways in exploring the regulatory mechanism of Chl degradation. Stay-green mutants can be classified as either functional or non-functional/cosmetic, depending on whether the other senescence processes, photosynthetic ability in particular, are affected (Thomas and Howarth 2000). The non-functional stay-green mutant is extremely useful for the analysis of Chl catabolism since the lesion of Chl breakdown in functional stay-green mutants may be an outgrowth of delayed leaf senescence. Through screening for non-functional stay-green mutants, a key regulatory gene responsible for Chl degradation during senescence was recently identified among various species, which was initially termed as *NYE1* in *Arabidopsis* in our laboratory, but it is now more commonly referred as *SGR* (Armstead et al. 2006; Armstead et al. 2007; Jiang et al. 2007; Park et al. 2007; Ren et al. 2007; Sato et al. 2007; Alós et al. 2008; Barry et al. 2008; Borovsky and Paran 2008). Over-expression of *NYE1* in *nye1-1* results in the whole spectrum of leaf yellowing phenotypes, the severity of which correlates with its expression level. To further explore its regulatory mechanism, we set out to identify novel components involved in this pathway by reverse genetic approach, and initially characterized those senescence associated genes sharing similar expression patterns with *NYE1*. Here we report the characterization of *CRN1* in *Arabidopsis*, a novel hydrolase protein (EC3.1.1) involved in the Chl catabolism pathway. One of its homologous T-DNA insertion mutants, referred as *crn1-1*, exhibited a severe non-yellowing phenotype during leaf senescence as well as a significant retention of Chl binding proteins, the trimeric LHCP II in particular, during dark induced senescence. Unexpectedly, the photochemical efficiency dropped even more rapidly in *crn1-1* than those in Col-0 and *nye1-1* during dark incubation, indicating that the malfunction of CRN1 also imposes a significant effect on maintaining the residual photosynthetic ability during leaf senescence.

Results

Cluster analysis of Co-regulated genes with *NYE1*

Co-regulated genes may be involved in the same biological event (Toufighi et al. 2005; Obayashi et al. 2007). An analysis of transcriptome in senescent leaves revealed that approximately 827 genes were induced in all three senescent scenarios (age-dependent, dark- and starvation-induced senescence) (Buchanan-Wollaston et al. 2005). The Gene Ontology (GO) analysis showed that 67 of them were targeted into the chloroplast, and the Expression Angler analysis further revealed that a list of co-regulated genes were grouped together with *NYE1* (**Figure 1**) (Toufighi et al. 2005). Coincidently, the *PaO*, encoding a key enzyme responsible for the ring-opening step on the Chl degradation pathway, was also closely grouped with *NYE1* (Pružinská et al. 2003). This result indicated not only the reliability of the analysis, but also the feasibility of utilization of chip-data based reverse-genetic tools to dig out novel components in a given pathway.

Identification of a novel stay-green mutant *crn1-1*

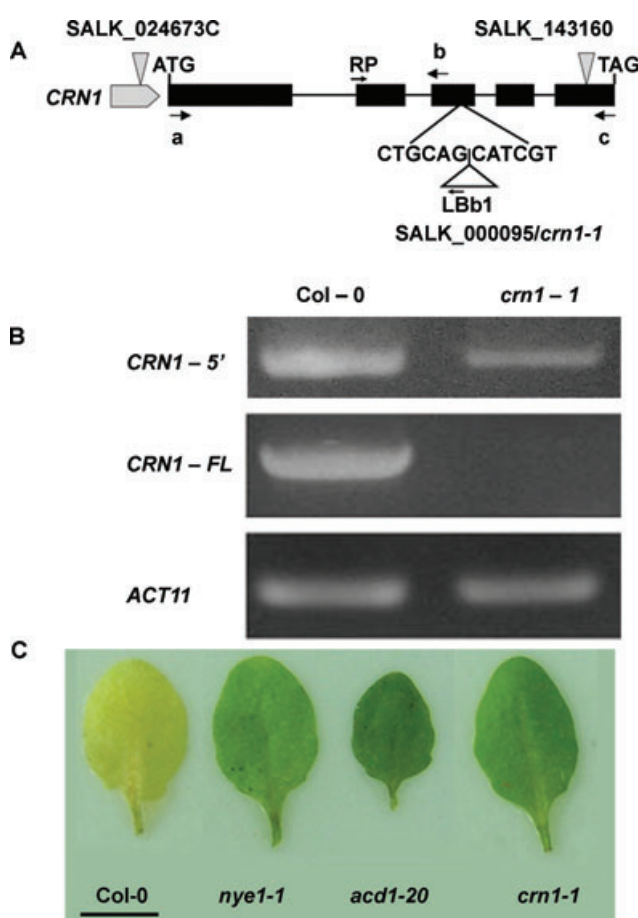T-DNA insertion mutants corresponding to the candidate genes screened above were ordered from Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org).
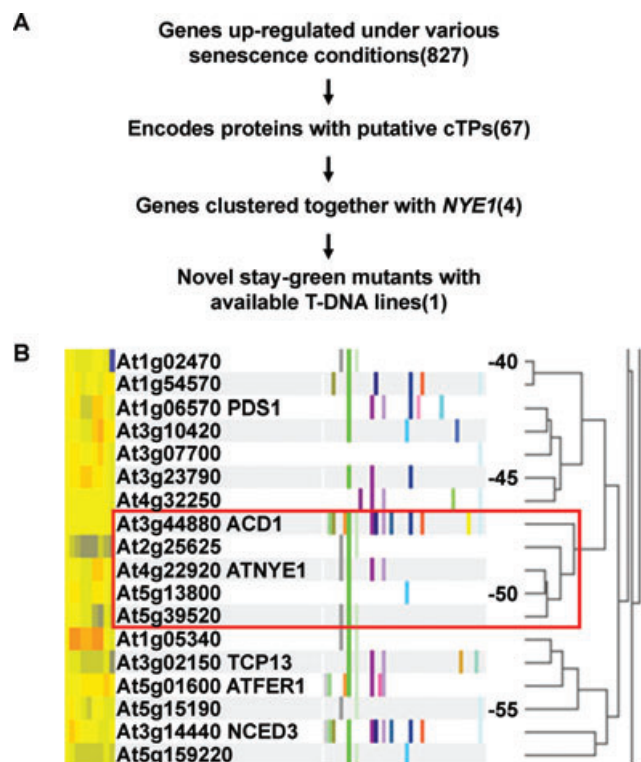
Figure 1. Screening of chloroplast targeted genes co-regulated with NYE1.

(A) A work flow chart. cTP, Chloroplast transit peptide.
(B) Cluster analysis of senescence inducible and chloroplast targeted genes.
Genes in the red box were clustered most closely with NYE1 and ACD1/PaO.

Homozygous mutants were identified as described in detail in Materials and Methods. To determine if they are involved in the regulation of Chl degradation during green organ senescence, the senescent phenotype of their detached leaves were initially examined at 6 d after darkness (DAD). While no obvious differences in the senescent phenotype were observed in all other mutants (No T-DNA insertion line available for At2g25625), a strong stay-green phenotype was detected with the homozygous T-DNA allele (SALK_000095) of At5g13800 (**Figure 2**). The stay-green phenotype was co-segregated with the homozygous status of the T-DNA insertion and all heterozygous/wild-type (HZ/WT) plants did

not stay green during dark-induced leaf senescence (data not shown), suggesting that the T-DNA allele conferred the recessive stay-green phenotype. The corresponding gene was tentatively named as *CRN1* (for Co-Regulated with *AtNYE1*) and SALK_000095 as *crn1-1*. Subsequently, the stay-green phenotype was rescued by over-expressing *CRN1* in *crn1-1* using a dexamethasone (DEX) inducible over-expression system during dark induced senescence (Aoyama and Chua 1997), indicating that the mutation in *CRN1* is surely responsible for the phenotype (**Figure 3D**). *CRN1* encodes an α/β hydrolyse with 484 amino acids and contains a putative chloroplast targeted peptide of 46 residues in its N-terminal, based on the analyses of ChloroP (http://www.cbs.dtu.dk/services/ChloroP/; Emanuelsson et al. 1999) and PPDB (http://ppdb.tc.cornell.edu/; Friso et al. 2004).

There were another two putative T-DNA insertion lines in this locus in the ABRC seed stock (**Figure 2A**). Homozygotes of SALK_024673C with insertion position in *CRN1* promoter region did not show the stay-green phenotype. RT-PCR analysis showed that the full-length of CRN1 was normally expressed, indicating a normal function of *CRN1* in this line (data not shown). Puzzlingly, the third line (SALK_143160) with putative insertion position in C-terminal region of *CRN1* could barely germinate and all the survived seedlings were WT (data not shown).

Chl breakdown was also significantly delayed in *crn1-1* during developmental senescence (**Figure 3A**) as well as upon ethylene treatment of adult plants (**Figure 3B**), implying that the fundamental step in Chl degradation pathway(s) might be compromised in *crn1-1*, as in *nye1-1*. Furthermore, Chl breakdown rate was delayed seemly more significantly in *crn1-1* than that in *nye1-1* during dark-treatment, but less significantly than that in *acd1-20* (a knock out allele of PaO) (**Figures 2C**, **3C**). The ratio of Chl *a* / Chl *b* was found to decline in *crn1-1*, while it rose in Col-0 and *nye1-1* during the first four days in darkness (**Figure 3C**).

Figure 2. Identification of *crn1-1* as a stay-green mutant.

(A) Schematic structure of CRN1 gene, its putative T-DNA insertion lines and the positions of primers used in panel B (arrows with lowercase a, b and c).

(B) RT-PCR characterization of *crn1-1* mutant. The full-length coding sequence (FL) used primers a and c, and the 5′ segment used primers a and b.

(C) Age-matched leaves of Col-0, *nye1-1*, *acd1-20* and *crn1-1* were cut and incubated in permanent darkness for 6 d. Bar = 1 cm.

crn1-1 is a cosmetic(non-functional) stay-green mutant

To assess whether the functional deterioration of *crn1-1* leaves was also delayed during senescence, the photochemical efficiency of its PSII (Fv/Fm) at different time points after incubation in darkness was determined by using PAM-IMAGING (WALZ, Germany). It was shown that the ratio began to decrease rapidly at 2 DAD, suggesting that *crn1-1* was a non-functional stay-green mutant. Unexpectedly, it decreased even more rapidly than that in Col-0 at 2DAD, while the decline rate in *nye1-1* was not significantly different from that in Col-0 (**Figure 4A**). The decline was mainly due to a decrease in Fm rather than Fo (data not shown). Expressions of *Ribulose Bisphosphate Carboxylase/Oxygenase Small Subunit* (*RBCS*) and *Chlorophyll a/b binding Protein* (*CAB*) genes, both of which
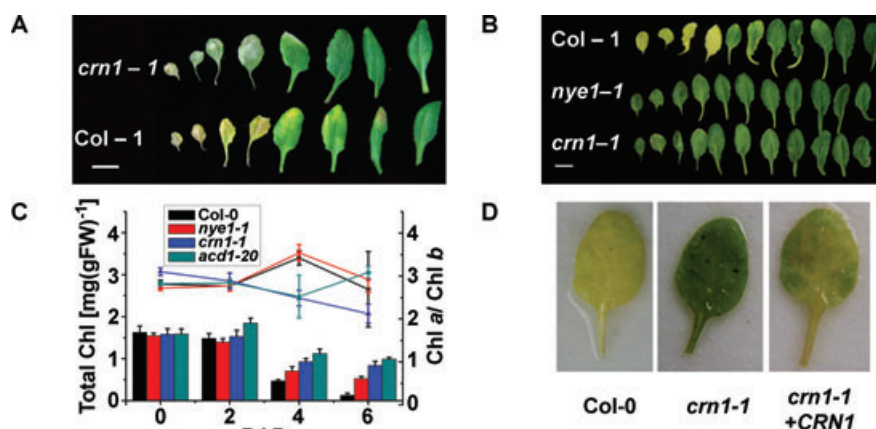
Figure 3. Phenotypic characterization of *crn1-1*.

(A) The bottom leaves of 5-week old plant were cut and photographed. Bar = 1 cm.

(B) Plants when the 1st silique appeared were transferred to ethylene treatment (50 ppm) and photographed 3 d after the treatment. Bar = 1 cm

(C) Total Chl content (column, left Y axis) and Chl *a*/*b* (line, right Y axis) in Col-0, *nye1-1*, *crn1-1* and *acd1-20* during dark-induced senescence, Error bars represent standard deviation ($n = 3$).

(D) Complementation of *crn1-1* by pTA7002-CRN1-FLAG in permanent darkness with 30 μM DEX inducer for 4 d.

were widely used as the molecular markers of photosynthetic capacity, were also examined by quantitative real-time PCR (qPCR). Both of them were down regulated dramatically in the dark-induced senescent leaves of *crn1-1*, just as those in the age-matched leaves of the wild-type treated in the same way. Somehow, higher background expressions of both the genes were detected in *crn1-1*, which is yet to be explored and explained (**Figure 4B**). *SEN1*, a senescence associated gene, was dramatically up-regulated during dark-induced senescence in *crn1-1*, indicating that the senescence process was largely not affected by the loss of *CRN1* function (**Figure 4C**).

Increased stability of Chl binding proteins in *crn1-1* during dark-induced senescence

Since the photochemical efficiency declined greatly in *crn1-1*, we wondered whether its photosynthesis related proteins were also degraded in a kinetically coordinated way. For all protein analyses, comparisons were made with reference to the fresh weight of the leaves, since both protein and Chl are degraded during dark-treatment. SDS-PAGE as well as western blot assay revealed that the degradation of Rubisco, the most abundant as well as the key photosynthesis related protein, was not significantly delayed in *crn1-1* (**Figure 5A**, S2). In contrast, the CAB proteins were significantly retained in *crn1-1*, as well as in *nye1-1* (**Figure 5A**, S2). For a detailed characterization, we employed immunoblotting analysis to analyze the degradation

of various photosynthesis related proteins. Degradation of all Lhcas and Lhcbs examined were inhibited in both *crn1-1* and *nye1-1* at 4DAD. Somehow, the breakdown of Lhcb1 was less inhibited in *crn1-1* than in *nye1-1* (**Figure 5A**). Blue-native PAGE was employed to further characterize the retardation of photosynthetic protein degradation. It was shown that trimeric LHCPII was predominantly retained in *crn1-1*, compared to that both trimeric LHCP II and PSI/ dimeric PSII were retained to some extent in *nye1-1* (**Figure 5B**).

Discussion

The biochemical pathway of Chl degradation during senescence has been substantially established in the past few years, but its genetic regulatory mechanism is still largely unknown. Identifying more related genes would certainly facilitate our exploration of the mechanism. By combining chip-data based analysis and molecular approach, we identified a novel stay-green gene, *CRN1*, which shared a similar expression pattern with *NYE1*, a key regulator of Chl degradation during senescence, initially identified by our laboratory. There are two additional proteins sharing low similarity with CRN1 in *Arabidopsis* (Figure S1). However, phylogenetic analysis revealed that they differentiated before the divergence of plant species, indicating that CRN1 is likely a single copy gene in Arabidopsis. Its orthologous gene in rice was also identified recently (Morita et al. 2009). In a parallel study in *Arabidopsis*,
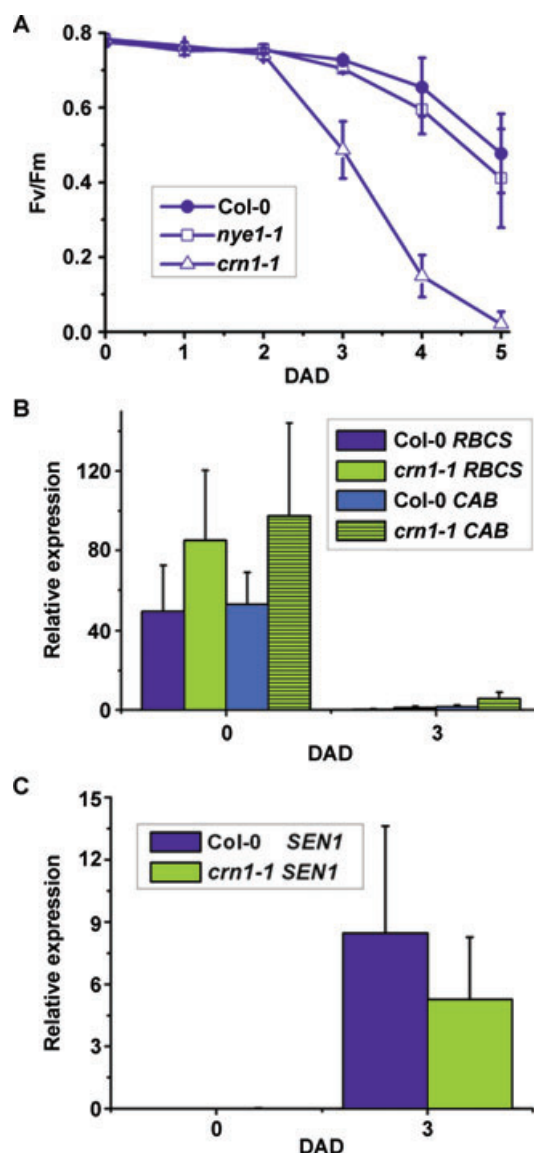
Figure 4. Photosynthetic capacity and senescence-associated processes in *crn1-1*.

(A) Photochemical efficiency of PSII in *crn1-1* during dark induced leaf senescence. Fv/Fm, maximum quantum yield of PSII electron transport (maximum variable fluorescence/maximum yield of fluorescence), Error bars represent standard deviation ($n = 7$ leaves). **(B,C)** qPCR analysis of *RBCS*, *CAB* and *SEN1* with *ACTIN2* as the internal reference.
Error bars represent standard deviation ($n = 3$).

it was shown that the CRN1 had pheophytinase activity, which was accordingly named as PPH. The finding results in a re-proposed biochemical pathway of chlorophyll degradation during senescence (Schelbert et al. 2009).

A previous report claimed that the stay-green phenotype of *pao1/acd1-20* was due to a repression of *NYE1* (Park et al. 2007). In this study, we found that Chl degradation in *crn1-1*, as well as in *acd1-20*, was more severely retarded than that in *nye1-1* during dark induced senescence (**Figure 2C**). We here consider that there might exist additional upstream genetic pathway(s) independent of *NYE1*, which respond to the feedback of downstream impairment.

Unexpectedly but interestingly, the photochemical efficiency of PSII was reduced more sharply in *crn1-1* during dark induced senescence, while it was not significantly altered in *nye1-1* as compared with that in Col-0. This observation is in agreement with that reported by Schelbert et al. (Schelbert et al. 2009). This phenomenon indicated that the function of photochemical efficiency of PSII was not merely associated to the content of Chl and its binding proteins.

Almost all stay-green mutants identified to date were also compromised in the degradation of Chl-binding proteins, especially the LHCP II apoproteins. However, degradation patterns of Chl-binding proteins varied among different mutants. Previous reports showed that both monomeric and trimeric LHCP II, mainly Lhcb1 and Lhcb2 but not others, were retained in *nyc1-2/nol-1*, as well as in their single mutants (Kusaba et al. 2007; Sato et al. 2009). Almost all Chl-binding proteins examined were significantly retained in *crn1-1*, which is consistent with the observation made by Schelbert et al. (Schelbert et al. 2009), as well as in *nye1-1*, compared with those in Col-0 at 4 DAD. However, more D2 but less Lhcb1 were detected in *crn1-1* than those in *nye1-1*. Distinctively, an extremely high level of trimeric LHCPII protein was retained in *crn1-1* while significant levels of both trimeric LHCP II and PSI/ dimeric PSII in *nye1-1* (**Figure 5B**). It was suggested that the transition of trimeric LHCP II into the monomeric state was a prerequisite event for subsequent degradation, since monomeric LHCP II appears to be the main substrate of the protease (Yang et al. 2000). We propose therefore that the transition step might be severely restrained in *crn1-1* and that different stay-green genes may regulate Chl degradation during senescence through distinctive mechanisms. As we know, each LHCPII monomer contains 8 Chl *a* and 6 Chl *b*, with the normal Chl *a/b* ratio of 1.33 (Liu et al. 2004). We here consider that the failure in the breakdown of trimeric LHCP II was probably the causation of the reduction of Chl *a/b* in *crn1-1* during senescence, rather than a deficiency in Chl *b* reductase activity.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (ecotype Columbia-0), *nye1-1* and T-DNA insertion mutant *crn1-1* plants were grown under long-day condition, as described previously (Ren et al. 2007). For dark
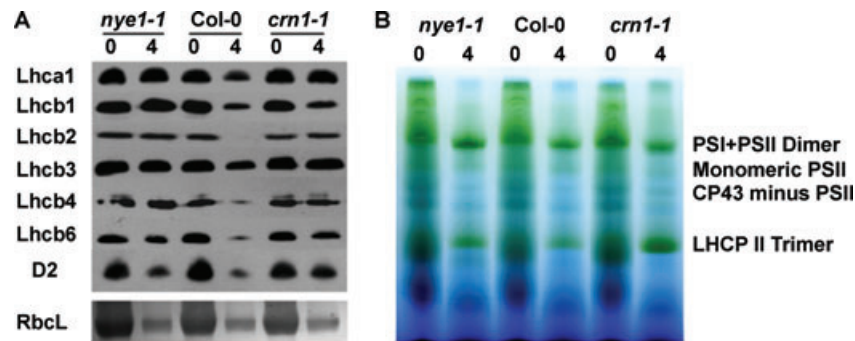
Figure 5. Degradation of photosynthetic proteins in *crn1-1* during dark induced senescence.

(A) Western blot analysis of representative proteins of LHCI, LHCII, PSII reaction center and RbcL.

(B) Blue-native PAGE analysis of photosynthetic protein complexes.

treatment, leaves 4 to 6 from ∼25 d old *Arabidopsis* plants were excised and incubated on wet filter paper in darkness in culture room for various periods, as indicated. For dexamethasone (DEX) treatment, filter paper were soaked with 30 µM DEX and 0.01% Tween-20 in de-ionized water.

Characterization of T-DNA insertion lines

T-DNA mutants were obtained from the T-DNA-transformed Arabidopsis collection (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso et al. 2003) from ABRC . The status of T-DNA insertion was characterized by PCR analysis of the products using the T-DNA primer LBb1/LBa1 and gene-specific primers (LP, RP).

Primer sequences used were as follows:

SALK_000095
LP: 5′CTACCAATCCTGGACTCCTCC 3′;
RP: 5′ TGTACAGGTTATCGGTGAGCC 3′

SALK_143160
LP: 5′ ACCGTGGAACTGACAAAAATG 3′;
RP: 5′ TCTGCGTGATTGGTGTTATTG 3′

SALK_024673C
LP: 5′ CCTAATTGCTTCCCAAGAACC 3′;
RP: 5′ AAGTAACCACAGAGCACTGGG 3′

SALK_137085 (AT5G39520)
LP: 5′ TCAAAACGGTGGAGAGTGAAG 3′
RP: 5′ TGGTTCCATTACTCGGTCAAG 3′
LBa1: 5′ TGGTTCACGTAGTGGGCCATCG 3′
LBb1: 5′ GCGTGGACCGCTTGCTGCAACT 3′

Plasmid construction and plant transformation

For inducible over-expression of CRN1, the full length coding sequence of CRN1 was amplified with a pair of primers (5′ CATCTCGAGATGGAGATAATCTCACTGAACG

TTGTG 3′ and 5′ CCACTAGTCTA<u>CTTATCGTCGTCATCCTTGTAATC</u>TGCAGACTTCCCTCCAAACACCCT 3′, the underlined sequence is a FLAG tag). The PCR product was cloned into pMD 19-T vector (TaKaRa Biotechnology Co. Ltd. Dalian, China) and sequenced. After digested with Xho I and Spe I, the released fragment was subcloned into pTA7002 to produce pTA7002-CRN1-FLAG. It was then introduced into the *Agrobacterium tumefaciens* strain GV3101. GV3101 cells containing either pTA7002 control vector or pTA7002-CRN1-FLAG were used to transform *crn1-1 via* the floral-dipping method (Clough and Bent 1998). Transgenic plants were selected on plates containing 25 mg L^{-1} Hygromycin.

Semi-quantitative RT-PCR and qPCR

Total RNA was extracted from leaf samples using Trizol Reagent (Invitrogen), followed by chloroform extraction, isopropanol precipitation, and spectrophotometric quantification. After digestion with Rnase-free DNase (Promega), 2 µg total RNA was reverse transcribed with the Superscript reverse transcriptase (Shenenergy Biocolor). The products were diluted 5-fold with DEPC-treated water and subsequently used as templates for either semi-quantitative RT-PCR or qPCR analysis. For semi-quantitative RT-PCR, cDNA from 3 DAD leaf samples were used. All three transcripts were amplified by PCR with 28 cycles. Primers for semi-quantitative RT-PCR were as follows:

CRN1-5′:
Forward: 5′ ATGGAGATAATCTCACTGAACG 3′;
Reverse: 5′ CCTCCACAATACGTGAGAATAC 3′;

CRN1-FL:
Forward: 5′ ATGGAGATAATCTCACTGAACG 3′;
Reverse: 5′ CTATGCAGACTTCCCTCCAAAC 3′

ACT11:
Forward: 5′ GATTTGGCATCACACTTTCTACAATG 3′;
Reverse: 5′ GTTCCACCACTGAGCACAATG 3′;

The qPCR was performed using SYBR Green I PCR kit (Toyobo) on an iCycler (Bio-Rad) according to the manufacturer's suggestions with *ACT2* as a reference. Primers to respective genes were described previously (Ren et al. 2007).

Measurement of Chl Contents

For pigment quantification, leaves 4 to 6 (about 0.1 g fresh weight) from ∼25 d old plants were taken. After dark treatment, excised leaves were immediately frozen in liquid nitrogen and stored at −80 °C. Chl contents were quantified by spectrometer according to Benedetti and Arruda (Benedetti and Arruda 2002).

Determination of Chl fluorescence

Chl fluorescence of dark adapted leaves was measured using IMAGING-PAM (Walz, Germany) according to the manufacture's instructions. The photochemical efficiency of PSII was monitored by measuring Fv/Fm (the ratio of variable to maximum Chl fluorescence; a parameter of PSII photochemical efficiency).

Protein assay

For SDS-PAGE and protein gel-blot analysis, proteins were extracted from approximately 0.1 g fresh weight leaf samples with 0.5 ml 1× sample buffer (62.5 mM Tris, pH 6.8, 2.5% SDS, 5% β-mercaptoethanol, 10% glycerol). Antibodies against Lhca1, Lhcbs, D2 and RbcL were purchased from Agrisera (http://www.agrisera.com/). Antibodies were detected using a chemiluminescence detection system (ECL; Amersham Biosciences) according to the manufacturer's instructions.

For Blue Native (BN)-PAGE, 0.3 g fresh weight of leaves were homogenized in an ice-cold isolation buffer containing (400 mM sucrose, 50 mM HEPES, pH 7.6, 2 mM $MgCl_2$ and 10 mM NaCl with a chilled mortar and pestle and filtrated through two layers of nylon cloth. The filtrate was centrifuged at 5000 g for 10 min. The thylakoid pellets were washed with isolation buffer, re-centrifuged and re-suspended in isolation buffer. The resulting thylakoid membrane pellets were either used freshly or frozen in liquid nitrogen and stored at -80°C. Equal volumes of thylakoid membrane complexes corresponding to 10μg Chl in Col-0 0 d sample were loaded and separated by BN-PAGE (Cline and Mori 2001).

Accession Number: CRN1, NP_196884; NYE1, DQ437531; PaO/ACD1, NP_190074

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic analysis of proteins similar to CRN1. Numbers I~IV indicate for four clades. Arabidopsis protein for clade I, II and III were At5g13800 (CRN1), At4g36530 and At5g19850, respectively. For clade IV, no corresponding protein was found in Arabidopsis.

Figure S2. SDS-PAGE analysis of the degradation of large subunit of Rubisco (1) and chlorophyll binding proteins (2).

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