Rice 7-Hydroxymethyl Chlorophyll a Reductase Is Involved in the Promotion of Chlorophyll Degradation and Modulates Cell Death Signaling

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The loss of green coloration via chlorophyll (Chl) degradation typically occurs during leaf senescence. To date, many Chl catabolic enzymes have been identified and shown to interact with light harvesting complex II to form a Chl degradation complex in senescing chloroplasts; this complex might metabolically channel phototoxic Chl catabolic intermediates to prevent oxidative damage to cells. The Chl catabolic enzyme 7-hydroxymethyl Chl a reductase (HCAR) converts 7-hydroxymethyl Chl a (7-HMC a) to Chl a. The rice (Oryza sativa) genome contains a single HCAR homolog (OshCAR), but its exact role remains unknown. Here, we show that an oshcar knockout mutant exhibits persistent green leaves during both dark-induced and natural senescence, and accumulates 7-HMC a and pheophorbide a (Pheo a) in green leaf blades. Interestingly, both rice and Arabidopsis hcar mutants exhibit severe cell death at the vegetative stage; this cell death largely occurs in a light intensity-dependent manner. In addition, 7-HMC a treatment led to the generation of singlet oxygen (¹O₂) in Arabidopsis and rice protoplasts in the light. Under herbicide-induced oxidative stress conditions, leaf necrosis was more severe in hcar plants than in wild type, and HCAR-overexpressing plants were more tolerant to reactive oxygen species than wild type. Therefore, in addition to functioning in the conversion of 7-HMC a to Chl a in senescent leaves, HCAR may play a critical role in protecting plants from high light-induced damage by preventing the accumulation of 7-HMC a and Pheo a in developing and mature leaves at the vegetative stage.

Keywords: 7-hydroxymethyl chlorophyll a reductase (HCAR), cell death, chlorophyll, chlorophyll catabolic enzyme, rice

INTRODUCTION

Leaf senescence is characterized by the gradual loss of green coloration, mainly due to chlorophyll (Chl) degradation. Since free Chl and its catabolic intermediates are highly phototoxic, they must be degraded rapidly and completely, along with photosynthetic proteins and other macromolecules. During this process, Chl is ultimately converted to non-phototoxic colorless catabolites, termed phyllobilins, through the PAO/phyllobilin pathway (Hörtensteiner and Krautler, 2011); this reaction requires seven Chl catabolic enzymes (CCes), which function in senescing chloroplasts (Hörtensteiner, 2013). Six CCE genes have been characterized in rice and/or Arabidopsis, including NON-YELLOW COLORING1 (NYC1, Horie et al., 2009; Kusaba et al., 2007) and NYCTLIKE (NOl, Horie et al., 2009; Sato et al., 2009), along with genes encoding Chl b reductases, 7-hydroxymethyl Chl a reductase (HCAR, Meguro et al., 2011), pheophytinase (PPH, Schelbert et al., 2009), pheophorbide a oxygenase (PAO,
Pružińska et al., 2003), and red Chl catabolite reductase (RCGR, Pružińska et al., 2007). Most mutants of these CCEs show a stay-green phenotype during natural senescence and/or artificially induced senescence, including dark- and phytohormone-induced leaf senescence, due to an impaired Chl degradation pathway (Horie et al., 2009; Hörtensteiner, 2009; Kusaba et al., 2007; Schellbert et al., 2009).

In addition to these CCEs, STAY-GREEN1 (SGR1, also termed NONYELLOWING1, NYE1) homologs have been isolated in various plant species, where they function as key positive regulators of Chl degradation; sgr mutants typically show a strong stay-green phenotype during both natural and dark-induced senescence (DIS) (Barry et al., 2008; Park et al., 2007; Ren et al., 2007; Sakuraba et al., 2015a). We previously showed that Arabidopsis SGR1/NYE1 physically interacts with the six CCEs and LHCl, forming a multi-protein complex that is likely important for rapid detoxification of Chl catabolic intermediates in senescing chloroplasts (Sakuraba et al., 2012, 2013).

In addition to SGR1, all plants contain an additional SGR subgroup, SGR-LIKE (SGRL). The C-terminal sequence of SGRL differs considerably from that of SGR (Hörtensteiner, 2009; Sakuraba et al., 2015a). OsSGRL-overexpressing plants show a premature leaf yellowing phenotype (Rong et al., 2013). Similarly, overexpression of AtSGRL accelerates leaf yellowing under abiotic stresses, including high salinity and heat stress, at the vegetative stage (Sakuraba et al., 2014b), indicating that SGRL mainly contributes to Chl degradation during vegetative growth. Very recently, Shimoda et al. (2016) reported that SGR and SGRL homologs have Mg-dechelatase activity for Chl a and chlorophyllide a, respectively, representing the last of the seven CCEs in the PAO/phyllobilin pathway.

Cell death is a common process that occurs in response to pathogen attack or abiotic environmental stress. Cell death, often referred to as the “hypersensitive response (HR)”, has important functions in protecting plants from advancing pathogen infection (Greenberg et al., 1994). A few CCEs play an important role in inhibiting HR, because Chl catabolic intermediates act as strong photosensitizers in plants. Thus, some mutants of CCEs show an accelerated cell death phenotype. In Arabidopsis, accelerated cell death 1 (acd1) and acd2 are null mutants of PAO and RCCR, respectively. Under normal photoperiodic growth conditions, acd1 and acd2 accumulate phophorhible a (Pheo a) and RCC in their green leaves, respectively, causing excessive accumulation of reactive oxygen species (ROS). Consequently, acd1 and acd2 show a necrotic phenotype and growth retardation (Pružińska et al., 2003; 2007). Cell death symptoms were also observed in RNAi-mediated knockdown mutants of PAO and RCCR in rice (Tang et al., 2011). Interestingly, RCCR-overexpressing Arabidopsis plants exhibit increased tolerance to oxidative-stress-induced cell death (Yao and Greenberg, 2006), indicating that some CCEs have the potential for controlling cell death mechanisms, possibly through the metabolic channeling of phototoxic Chl intermediates.

Arabidopsis HCAR, which catalyzes the reduction of 7-hydroxymethyl Chl a (7-HMC a) to Chl a, is a CCE that functions in senescing chloroplasts (Meguro et al., 2011). HCAR is a homolog of cyanobacterial divinyl reductases, which participate in Chl biosynthesis (Ito et al., 2008). The Arabidopsis hcar (athcar) mutant shows a stay-green phenotype during DIS, accompanied by strong accumulation of 7-HMC a and Pheo a (Meguro et al., 2011). Furthermore, HCAR physically interacts with other CCEs, such as SGR1/NYE1, NYC1, NOL, RCCR, and LHCl, indicating that HCAR is a component of the Chl degradation complex (Sakuraba et al., 2013). The rice genome encodes a homolog of HCAR (hereafter OsHCAR, Supplementary Fig. S1), but its physiological role remains unknown.

In this study, we performed a functional analysis of OsHCAR using a T-DNA insertion knockout mutant. The oshcar mutant showed a stay-green phenotype during DIS, along with strong accumulation of the Chl catabolic intermediates 7-HMC a and Pheo a, indicating that OsHCAR is a component of the Chl degradation complex in rice. In addition, both the oshcar and athcar mutants showed an accelerated cell death phenotype due to excessive accumulation of singlet oxygen (1O2). Furthermore, 7-HMC a-treated protoplasts produced large amounts of 1O2, indicating that rice and Arabidopsis HCAR play an important role in protecting pre-senescent leaf cells from cell death. We discuss the possible roles played by HCAR in Chl degradation and in protecting plants from cell death.

MATERIALS AND METHODS

Plant materials and growth conditions

The oshcar mutant and its parental wild-type japonica rice cultivar ‘Hwayoung’ were grown in a growth chamber under long days (LD, 14.5 h light, 30℃/9.5 h dark, 24℃, 300 μmol m−2s−1) or in a paddy field under natural LD (>14-h light/day) in Suwon, Korea (37°N latitude). The T-DNA insertion oshcar mutant (stock number: PFG_2A-00576) was obtained from the Crop Biotech Institute at Kyung Hee University, Korea (Jeon et al., 2000). For DIS, detached or attached leaves of 3-week-old plants were incubated in complete darkness. The Arabidopsis thaliana (At) T-DNA insertion athcar mutant (SALK_018790C) and the AtHCAR-overexpressing (ATHCAR-OX) line were described previously (Sakuraba et al., 2013), and they were grown under LD (16 h light/day, 70 μmol m−2s−1).

Plant transformation

To produce Arabidopsis lines ectopically expressing OsHCAR, pMDC43 harboring 35S:OsHCAR was transformed into Agrobacterium tumefaciens stain GV3101. Agrobacterium-mediated transformation of the athcar mutant was performed using the floral-dip method (Zhang et al., 2006). Transformants were confirmed by genomic PCR using OsHCAR-specific primers (Supplementary Table 1).

Chl quantification

To measure total Chl concentrations, pigments were extracted from leaf tissues with 80% ice-cold acetone. Chl concentrations were determined by spectrophotometry, as described previously (Porra et al., 1989).
SDS-PAGE and immunoblot analysis
Total protein extracts were prepared from leaf tissues, using the middle section of the third leaf in the main culm of each 2-month-old rice plant grown under LD. Leaf tissues were ground in liquid nitrogen, and 10 mg aliquots were homogenized with 100 μl of sample buffer (50 mM Tris, pH 6.8, 2 mM EDTA, 10% glycerol, 2% SDS, and 6% 2-mercaptoethanol). The homogenates were centrifuged at 10,000 × g for 3 min, and the supernatants were denatured at 80°C for 5 min. A 4 μl aliquot of each sample was subjected to 12% (w/v) SDS-PAGE, followed by electroblotting onto a Hybond-p membrane (GE Healthcare). Antibodies against photosystem proteins Lhcα1, Lhcα2, Lhcβ1, Lhcβ2, Lhcβ4, Lhcβ5, CP43, and PsαA (Agrisera, Sweden) were used for immunoblot analysis, and RbcL was visualized by Coomassie Brilliant Blue (CBB) staining. The level of each protein was examined using the ECL system with WESTSAVE (AbFRONTIER, Korea) according to the manufacturer’s protocol.

Measurement of Fv/Fm ratios
The Fv/Fm ratios of wild type and oshcar plants grown in the paddy field were measured using an OS-30p instrument (OptiSciences, USA), as previously described (Sakuraba et al., 2015b). The middle section of each flag leaf was adapted in the dark for 5 min to complete oxidation of QA. After dark treatment, the Fv/Fm ratio was measured in the paddy field. For both experiments, more than three experimental replicates per plant were conducted.

RNA isolation and quantitative reverse-transcription PCR (qPCR) analysis
For the RT reactions, total RNA was extracted from rice leaf blades and other tissues using an RNA Extraction Kit (Macrogen, Korea). First-strand cDNA was prepared with 2 μg total RNA using M-MLV reverse transcriptase and oligod(T)18 primer (Promega) in a total volume of 25 μl and diluted with 75 μl water. For quantitative reverse-transcription PCR (qPCR), a 20 μl mixture was prepared including first-strand cDNA equivalent to 2 μl total RNA, 10 μl 2× GoTaq master mix (Promega), 6 μl distilled water, and gene-specific forward and reverse primers (Supplementary Table 1). The qPCR was performed using a LightCycler 480 (Roche Diagnostics). Rice UbiquitinS (UBQS) or GAPDH (encoding glyceraldehyde phosphate dehydrogenase) was used as an internal control. The relative expression level of each gene was calculated using the 2^(-ΔΔCT) method, as previously described (Livak and Schmittgen, 2001).

HPLC analysis
Leaves were weighed and pulverized in acetone using a Shake Master grinding apparatus (BioMedical Science), and the extracts were centrifuged for 15 min at 22,000 × g. The pigments were separated on a Symmetry C8 column (150 × 4.6 mm; Waters) as described previously (Zapata et al., 2000). The elution profiles were monitored by measuring the absorbance at 653 nm (SPD-M10A: Shimadzu), and pigments were identified based on their retention times and spectral patterns. Pigment quantification was performed based on the areas of the peaks.

Reactive oxygen species detection
For singlet oxygen (¹O₂) detection, Singlet Oxygen Sensor Green (SOSG; Invitrogen) reagent was used, as previously described (Han et al., 2012). Leaves of 2-week-old plants were treated with 50 mM SOSG in 10 mM sodium phosphate buffer (pH 7.5). After 30 min incubation, fluorescence emission following excitation at 480 nm was imaged using a laser scanning confocal microscope (LSM510, Carl Zeiss-LSM510). The red autofluorescence from Chl was also detected following excitation at 543 nm. Detection of hydrogen peroxide (H₂O₂) and superoxide (O₂⁻) was carried out as previously described (Li et al., 2010), with minor modifications. Hydrogen peroxide (H₂O₂) and superoxide (O₂⁻) were detected using 3,3-diaminobenzidine (DAB) and nitroblue tetrazolium chloride (NBT), respectively. Leaves of 2-month-old plants grown in a paddy field were sampled and incubated in 0.1% DAB (Sigma) or 0.05% NBT (Duchefa) in 50 mM sodium phosphate buffer (pH 7.5) at room temperature overnight with gentle shaking. Chlorophyll was completely removed by incubation in 90% ethanol at 80°C.

Yeast two-hybrid analysis
The full-length cDNAs of rice HCAR, SGR, NYC1, NOL, PPX, PAO, and RCCR in entry vectors were inserted into destination vectors pDEST32 (bait) and pDEST22 (prey) (Invitrogen). Yeast strain MaV203 was used for cotransformation of bait and prey clones, and β-galactosidase activity was measured via a liquid assay using chlorophenol red-β-β-galactoside (Roche Applied Science) according to the Yeast Protocols Handbook (Clontech).

Trypan blue staining
Trypan blue staining was performed as described by Koch and Slusarenko (1990) with minor modifications. Arabidopsis leaves and rice leaf discs exposed to herbicide-induced oxidative stress were incubated overnight in lactophenol-trypsin blue solution (10 ml lactic acid, 10 ml glycerol, 10 g phenol, and 10 mg trypan blue dissolved in 10 ml distilled water). The stained leaves were boiled for 1 min and decolorized in 60% glycerol solution.

Oxidative stress assay
For MV treatment, one-month-old rice plants were sprayed with 50 μM methyl viologen dichloride (MV, Sigma), and three-week-old Arabidopsis plants were sprayed with 10 μM MV. The plants were incubated under continuous light conditions for the indicated times.

Protoplast isolation and light treatment
Rice leaf protoplasts were isolated from 15- to 20-d-old plants as described previously (Liang et al., 2003). Arabidopsis protoplasts were isolated from 3-week-old rosette leaves as described previously (Wu et al., 2009). The protoplasts were resuspended with protoplast incubation solution (500 mM mannitol, 20 mM KCl, 4 mM MES [pH5.8]), and incubated in the dark (control), under moderate light (200 μmol m⁻² s⁻¹), or under high light (500 μmol m⁻² s⁻¹), without or with 10 μM Chl b (Sigma-Aldrich), Pheo a (Sigma-Aldrich), and 7-HMC a, at 22°C under fluorescent light. ¹O₂ produc-
Rice HCAR Modulates Cell Death Signaling
Weilan Piao et al.

Fig. 1. Green leaf color persists much longer during DIS in the hcar mutant than in wild type.
(A) The T-DNA insertion in the tenth exon of HCAR in Korean japonica rice cultivar ‘Dongjin’.
(B) The absence of HCAR transcripts in the hcar mutant was confirmed by RT-PCR. Total RNA was extracted from the second leaf blades of one-month-old plants. GAPDH was used as an internal control. (C) Whole wild-type (WT) and hcar plants grown for one month under LD (14 h light/day) were transferred to darkness at 28℃ for 14 days (14 DDI). (D-G) Changes in color (D), total Chl level (E), photosystem protein level (F), and Chl a/b ratio (G) in WT and hcar leaf discs during dark incubation. Leaves from nyc1 were used as a stay-green control. The leaf discs were incubated in 3 mM MES (pH 5.8) buffer abaxial side-up at 28℃ in darkness and sampled at different DDI for each experiment. (F) Antibodies against PSII antenna proteins (Lhcb1, Lhcb2, Lhcb4, and Lhcb5), PSI antenna proteins (Lhca1 and Lhca2), PSII core proteins (CP43), and PSI core protein (Psaa) were used for immunoblot analysis. RbcL proteins were visualized by Coomassie Brilliant Blue staining. (E, G) Mean and SD values were obtained from more than three biological samples. Asterisks indicate significant difference compared to WT (Student’s t-test, *P < 0.05, **P < 0.01). DDI, day(s) of dark incubation.

RESULTS

Rice hcar mutants exhibit a stay-green phenotype during leaf senescence

The Oryza sativa genome contains only a single HCAR homolog (OsHCAR; LOC_Os04g25400), and the amino acid sequence of OsHCAR is highly similar to those of HCAR homologs in other plant species (Supplementary Fig. 1). To elucidate the function of HCAR in leaf senescence in rice, we obtained the T-DNA insertion line PFG_2A-00576, which contains a single T-DNA fragment inserted into the fifth exon of HCAR (Fig. 1A) and does not accumulate HCAR transcripts in senescing leaves (Fig. 1B), indicating that this mutant harbors a knockout allele of HCAR (hereafter oshcar).

Like most CCE mutants in Arabidopsis, athcar mutants show a stay-green phenotype during DIS (Meguro et al., 2011; Sakuraba et al., 2013). Thus, we examined the phenotype of the oshcar mutant during DIS. At the vegetative stage, the leaf color of oshcar was almost the same as that of wild type. However, the entire plant showed a stay-green phenotype after 14 days of dark incubation (14 DDI; Fig. 1C). We also confirmed that detached leaf segments of oshcar also showed stay-green phenotype after 6 DDI (Fig. 1D). The stay-green phenotype was observed in the OsHCAR/oshcar heterozygous progenies that were segregated from the
T-DNA heterozygous (OsHCAR/oshcarr) plants, similar to the oshcar homozygous progenies (Supplementary Fig. 2). We used detached leaf segments for further phenotypic characterization. To characterize the photosynthetic parameters in the mutant, we compared oshcar with a nonfunctional stay-green mutant, nyc1, which is impaired in Chl b reductase activity (Kusaba et al., 2007). Consistent with its visible phenotype, Chl was highly retained in hcar during DIS (Fig. 1E). We also investigated the levels of photosystem proteins during DIS. As shown in Fig. 1F, only LHC proteins, such as Lhcb1, Lhcb2, Lhcb4, Lhcb5, and Lhca1, were highly retained in the nyc1 mutant, whereas all types of photosystem proteins were highly retained in the oshcar mutant after dark incubation (2 and 4 DDI), when all photosystem proteins were undetectable in wild type. Similarly, the Chl a/b ratios were unchanged in oshcar (Fig. 1G), while nyc1 retained more Chl b, primarily in senescing leaves. In this study, we used the oshcar T3 seeds that were harvested in 2014. In addition, we also confirmed that T4 and T5 generations of oshcar mutants (harvested in 2015 and 2016, respectively) showed stay-green phenotype with highly retaining of Chl (Supplementary Fig. 3).

![Diagram](attachment:image.png)

**Fig. 2. Accumulation of 7-HMC a in the rice hcar mutant.** (A) Chl b (Cb) is converted to 7-HMC a (7HMC) by Chl b reductase, which is then converted to Chl a (Ca) by HCAR. (B-D) HPLC profiles of Chls and 7-HMC a in WT and hcar leaves before (B) and after 4 DDI (C). Pigments were extracted from the leaves of one-month-old WT and hcar plants. (D) The identity of 7-HMC a, Chl a, and Chl b was confirmed by fluorescence spectroscopy from 250 to 800 nm. L, lutein; UC, unknown carotenoid; B, β-carotene. DDI, day(s) of dark incubation.
Rice HCAR Modulates Cell Death Signaling
Weilan Piao et al.

We then examined the senescence phenotype of *oshcar* during natural senescence. To this end, we grew the plants in a paddy field under natural LD conditions (~14 h light/day at 37° N latitude, Suwon, Korea). During grain filling, *oshcar* exhibited delayed senescence (Supplementary Fig. 4A) while retaining higher levels of Chl (Supplementary Fig. 4B) than wild type, as well as a high Fv/Fm ratio (Supplementary Fig. 4C), indicating that rice HCAR is involved in Chl degradation during both DIS and natural senescence. Some stay-green rice plants exhibit increased crop yields (Liang et al., 2014; Sakuraba et al., 2015b). Therefore, we measured several agronomic traits in the *oshcar* mutant, such as panicle length, plant height, number of grains per panicle, grain weight, and fertility. These traits were significantly lower in the *oshcar* mutant than in wild type (Supplementary Fig. 5), indicating that the stay-green trait of *oshcar* does not lead to increased grain yields.

To examine whether *OshCAR* could recover the defects in the *athcar* mutant, we developed transgenic plants overexpressing *OshCAR* in the *athcar* background (35S:OshCAR/*athcar*). We evaluated the expression of *OshCAR* in three independent transgenic lines by RT-PCR (Supplementary Fig. 6A). The *athcar* mutant stayed green after dark treatment, as previously reported (Meguro et al., 2011); in contrast, plants from the three transgenic lines senesced normally during dark treatment (Supplementary Figs. 6B and 6C), indicating that the role of HCAR in Chl degradation is conserved between Arabidopsis and rice.

**Rice HCAR is enzymatically equivalent to Arabidopsis HCAR**

HCAR is a CCE that converts 7-HMC a to Chl a (Fig. 2A). In Arabidopsis, 7-HMC a accumulates in the hcar mutant, although it is detected only under DIS conditions (Meguro et al., 2011). To examine whether 7-HMC a accumulates in the *oshcar* mutant, we performed HPLC analysis. Chl and its intermediates were extracted from green leaves (0 DDI) and dark-treated leaves (5 DDI) from wild type and *oshcar* plants. The substrate of HCAR, 7-HMC a, was undetectable in wild-type leaves, but it accumulated to levels high enough to detect in *hcar* leaves before and after dark treatment (Figs. 2B and 2C), which also occurs in the *athcar* mutant (Supplementary Fig. 7; Meguro et al., 2011), indicating that HCAR is also essential for the reduction of 7-HMC a to Chl a in rice.

We previously used yeast two-hybrid and co-immunoprecipitation assays to show that in Arabidopsis, HCAR physically interacts with other CCEs (SGR1/NYE1, NYC1, NOL, PAO, PPH, and RCCR), directly or indirectly, at LHCII (Sakuraba et al., 2013). Thus, it is highly likely that HCAR also interacts with other CCEs in rice. To investigate this notion, we performed yeast two-hybrid assays to examine the pairwise interactions between HCAR and six other CCE genes (SGR, NYC1, NOL, PPH, PAO, and RCCR). HCAR interacted with five CCEs, whereas PPH failed to interact with HCAR (Fig. 3). Interestingly, HCAR interacted with itself, suggesting that it may form dimers that interact with other CCEs at LHCII and induce Chl degradation.

![Fig. 3. Rice HCAR directly interacts with other CCEs in yeast two-hybrid assays.](image)

The expression pattern of *OshCAR* differs from that of *AtHCAR* during leaf senescence

To investigate how *HCAR* transcription is regulated in rice, we used RT-qPCR to examine the expression levels of *HCAR* in different tissues, including root, leaf sheath, leaf blade, tiller, tiller base, and internode tissue. *HCAR* mRNA was highly abundant in leaf sheath, leaf blade, and internode tissue, which contain Chls (Supplementary Fig. 8), indicating that like *AtHCAR*, HCAR plays an important role in green tissues in rice.

We next examined whether *HCAR* expression is altered during leaf senescence. During DIS, the mRNA levels of *HCAR* and six other CCE genes (SGR, NYC1, NOL, PAO, PPH, and RCCR) significantly increased during DIS (Fig. 4A). Interestingly, the expression pattern of *OshCAR* was completely different from that of *AtHCAR*, which exhibits a rapid decrease in expression during senescence (Sakuraba et al., 2013). Similarly, during natural senescence, *HCAR* was expressed at high levels in the yellowing sector of the rice leaf blade (region ‘d’) but at significantly lower levels in the green sector (regions ‘a’ and ‘b’) (Fig. 4B), suggesting that the requirement phase of HCAR activity during leaf senescence in rice does somehow differ from that in Arabidopsis.
Fig. 4. Expression patterns of rice CCE genes during dark-induced and natural senescence. Expression of rice HCAR, NYC1, NOL, PPH, PAO, RCCR, and SGR during dark-induced (A) and natural senescence (B) was examined using the second leaf blades in the main culms of 70- and 120-d-old WT (Dongjin) plants, respectively. The relative expression levels were determined by RT-qPCR analysis and normalized to UBQ5 transcript levels. Mean and SD values were obtained from more than three biological replicates. DDI, day(s) of dark incubation; LS, leaf sector.

Cell death occurs in hcar leaves during vegetative growth
In both Arabidopsis and rice, knockout and/or knockdown mutants of PAO and RCCR show an accelerated cell death phenotype, even under normal growth conditions, due to excess accumulation of phototoxic Chl intermediates (Prużynska et al., 2003, 2007; Tang et al., 2011). Thus, it is possible that HCAR is involved in regulating cell death signaling by modulating the metabolic processes underlying Chl degradation.

At 80 d after seeding (DAS) in the paddy field, the oshcar mutant exhibited accelerated cell death, especially in the tip sectors of older leaves (Figs. 5A-5C). In addition, under long-day (LD) conditions in growth chambers, the oshcar mutant showed accelerated cell death symptoms under high light, while cell death was barely detectable under low light (Supplementary Fig. 9), indicating that the cell death phenotype in oshcar leaves is largely dependent on light intensity. Because variegation or accelerated cell death symptoms in rice leaves are closely associated with ROS accumulation (Han et al., 2012; Li et al., 2010; Wang et al., 2015), we measured the levels of three types of ROS, i.e., singlet oxygen (\( {\cdot}O_2 \)), hydrogen peroxide (H\(_2\)O\(_2\)), and superoxide (O\(_2\)\(^{-}\)) in oshcar leaves. We measured \( {\cdot}O_2 \) levels using Singlet Oxygen Sensor Green (SOSG), finding that SOSG signals were present in oshcar leaves but not in wild-type leaves (Fig. 5D). Similarly, H\(_2\)O\(_2\) and O\(_2\)\(^{-}\) levels, based on the intensity of DAB and NBT staining, respectively, were much higher in oshcar leaves than in wild-type leaves, primarily in older leaves (Figs. 5E and 5F). We then investigated whether cell death signaling-related genes are differentially expressed in oshcar. Genes that are upregulated during cell death, such as JAmyb, OsNAC4, and OsAPX1 (Han et al., 2012), were significantly upregulated in hcar leaves versus wild type, especially under high light (Supplementary Fig. 10). Taken together, these results indicate that HCAR plays an important role in the regulation of cell death in rice, as do PAO and RCCR (Tang et al., 2011).

The rice hcar mutant is susceptible to oxidative stress-induced cell death
It has previously been reported that the atrccr mutant, also known as acd2, is highly susceptible, whereas AtRCCR-OX plants are more tolerant to the treatment of methyl viologen, which induces oxidative stress by blocking the electron transport during photosynthesis in the chloroplast (Yao and Greenberg, 2006). To investigate whether the oshcar mutant also shows increased susceptibility to oxidative stress, we treated mutant and wild-type seedlings with 50 \( \mu \)M methyl viologen (MV). As expected, after 6 d of MV treatment, oshcar seedlings wilted much more quickly than WT (Figs. 6A and 6B). This leaf necrosis phenotype was also confirmed in oshcar heterozygous lines, while WT segregates did not show the phenotype, similar to Hwayoung WT (Supplementary Fig. 11). Cell death in the oshcar mutant was confirmed...
Rice HCAR Modulates Cell Death Signaling
Weilan Piao et al.

Fig. 5. The rice hcar mutant shows cell death symptoms in mature leaves. (A) Phenotypes of WT and hcar plants at 80 d after sowing (DAS) in the paddy field. (b, c) Phenotypes (B) and cell death rates (C) in leaf blades from WT and hcar plants shown in (a). Cell-death rate was calculated by the percentage of leaves in which more than 80% of leaf area became necrosis. 1, first (youngest); 2, second; 3, third; 4, fourth leaf blades from the top. (D) Singlet oxygen ($^1$O$_2$) accumulation in the third leaf blades of 80 DAS WT and hcar plants. $^1$O$_2$ was detected using Singlet Oxygen Sensor Green (SOSG) reagent. (E, F) Hydrogen peroxide (H$_2$O$_2$) and superoxide anion radicals (O$_2^-$) in the leaf blades of 80 DAS WT and hcar plants. The accumulation of H$_2$O$_2$ and O$_2^-$ was visualized by staining with DAB (E) and NBT (F), respectively. DAB, 3,3-diaminobenzidine; NBT, nitroblue tetrazolium chloride.

by trypan blue staining (Fig. 6C) and by the observation that the ion leakage rate in the mutants was higher than that of wild type (Fig. 6D). Moreover, high levels of $^1$O$_2$ accumulated in oshcar leaves during MV treatment (Fig. 6D), which also occurred in variegated oshcar leaves in the paddy field (Fig. 4).

Arabidopsis HCAR is involved in cell death signaling
Whereas the oshcar mutant showed accelerated cell death symptoms in both the paddy field and growth chamber (Figs. 5 and 6), this phenotype has not been reported for the athcar mutant, as previous studies of this mutant have mainly focused on its senescence phenotype during DIS (Meguro et al., 2011; Sakuraba et al., 2013). We therefore examined whether cell death occurs in athcar under normal conditions. Like oshcar, the 4-week-old athcar plants showed an accelerated cell death phenotype when grown under normal light (100 μmol m$^{-2}$ s$^{-1}$) conditions (Fig. 7A), which was confirmed by trypan blue staining (Fig. 7B). Under high light (500 μmol m$^{-2}$ s$^{-1}$) conditions, the cell death phenotype was more severe in athcar than in wild type and was observed even in 3-week-old plants (Figs. 7A and 7C). Furthermore, athcar plants were clearly smaller than wild-type plants (Fig. 7A), probably because severe cell death inhibits vegetative growth.

We then examined whether the athcar mutant is also highly susceptible to MV-induced oxidative stress. At 3 d after MV treatment, 4-week-old athcar plants displayed more severe cell death symptoms than wild type, whereas AthCAR-OX plants were more tolerant to this treatment than wild type (Figs. 7D and 7E). The athcar mutant was also highly susceptible to oxyfluorfen-induced oxidative stress...
Fig. 6. The rice hcar mutant is susceptible to herbicide-induced cell death. (A) Phenotype of one-month-old WT and hcar plants during methyl viologen (MV, 50 μM) treatment. Phenotypes at 0, 4, and 6 days of MV treatment (DT) are shown, respectively. (B) Magnified image of WT and hcar leaves at 6 DT shown in (A). (C) Cell death in hcar leaves during MV treatment was confirmed by trypan blue staining. (D) Cell death rates of WT and hcar plants during MV treatment. Black and white bars indicate WT and hcar, respectively. Cell-death rate was calculated by the percentage of leaves in which more than 80% of leaf area became necrosis. Asterisks indicate significant difference compared to WT (Student's t-test, *P < 0.05, **P < 0.01). (E) Singlet oxygen (¹O₂) accumulation in the second leaf blades of WT and hcar at 4 DT. Chl, chlorophyll; SOSG, singlet oxygen sensor green reagent.

Fig. 7. Overexpression of AthCAR increases tolerance to herbicide-induced cell death in Arabidopsis. (A) Phenotypes of 4-week-old WT and athcar plants grown under low light (LL) conditions (80 μmol m⁻² s⁻¹, upper panel), and 3- and 4-week-old WT and athcar plants grown under high light (HL) conditions (300 μmol m⁻² s⁻¹, lower panels). (B) Cell death in the fourth or fifth rosette leaves of 4-week-old WT and athcar plants grown under LL conditions visualized by trypan blue staining. (C) Cell death rate in the third and fourth leaves under the conditions described in (A). (D, E) Phenotypes (D) and cell death rates (E) of 3-week-old WT, AthCAR-OX, and athcar plants under MV (10 μM) treatment. (F) Singlet oxygen (¹O₂) accumulation in the fourth leaves of WT and athcar plants after 1 day of MV treatment (1 DT). Asterisks indicate significant difference compared to WT (Student's t-test, *P < 0.05, **P < 0.01). DT, day(s) of MV treatment; SOSG, singlet oxygen sensor green reagent.
Rice HCAR Modulates Cell Death Signaling
Wei Lan Piao et al.

Fig. 8. 7-HMC a treatment induces $\cdot$O$_2$ production in rice protoplasts. Ten-day-old etiolated WT and hcar rice seedlings were irradiated for 15 h and used for protoplast isolation. (A-C) (A) WT and hcar protoplasts incubated in darkness (negative control), (B) under low light (LL; 50 μmol m$^{-2}$ s$^{-1}$) for 2 h (upper panels) and 4 h (lower panels), and (C) under high light (HL; 200 μmol m$^{-2}$ s$^{-1}$) for 2 h. (D) WT protoplasts were combined with 20 μM Pheo a, 7-HMC a, and Chl b and incubated under LL for 1 h. SOSG, singlet oxygen sensor green reagent.

Fig. 9. Pheo a accumulates in the rice hcar mutant during DIS. (A, B) HPLC profiles (A) and quantification (B) of Pheo a in WT and hcar leaves before (0 DDI) and after 4 DDI. (C) PAO transcript levels in WT and hcar leaves during dark incubation were determined by RT-qPCR analysis and normalized to GAPDH transcript levels. (D) PAO protein levels in WT and hcar leaves during DIS determined by immunoblot analysis using an anti-PAO antibody. (B, C) Black and white bars indicate WT and hcar, respectively. Mean and SD values were obtained from more than three biological replicates. DDI, day(s) of dark incubation.

The production of singlet oxygen in hcar mutant protoplasts is caused by the accumulation of 7-HMC a and Pheo a.

To analyze the role of HCAR in the cell death response in more detail, we monitored the production of singlet oxygen in protoplasts from wild-type and oshcar plants in rice. In the dark, neither wild-type nor oshcar protoplasts produced $\cdot$O$_2$ (Fig. 8A). After 2 h of low-light treatment, however, we detected green fluorescence corresponding to $\cdot$O$_2$ accumulation in wild-type protoplasts (Fig. 8B). Furthermore, we detected a much stronger fluorescence in oshcar protoplasts that were incubated for a longer period of time under low light (Fig. 8B). However, green fluorescence was barely detected in wild-type protoplasts under high light (Figs. 8B and 8C), indicating that oshcar protoplasts produce $\cdot$O$_2$ in a light-dependent manner. To examine whether the accumulation of 7-HMC a leads to $\cdot$O$_2$ production in oshcar protoplasts, we purified 7-HMC a from Chl b (Supplementary Fig. 15) and combined it with protoplasts from wild-type leaves. After 1 h of incubation under low light, we detected green fluorescence, although the fluorescence intensity was weaker compared with protoplasts incubated with Pheo a. In both cases, the fluorescence could be mainly observed at the outside of chloroplasts (probably at the outer membranes of chloroplasts and mitochondria), although a small portion of...
signal can be seen in the chloroplasts. The addition of Chl b hardly increase 1 O2 accumulation (Fig. 8D). We obtained similar results using wild type and hcar Arabidopsis protoplasts (Supplementary Fig. 16), indicating that like Pheo a and RCC, 7-HMC a also acts as a photosensitizer in vivo.

High levels of Pheo a accumulate in athcar plants during dark incubation (Meguro et al., 2011). We therefore investigated whether Pheo a also accumulates in the oshcar mutant. Before dark incubation, Pheo a was barely detected in wild-type and oshcar leaves. After 4 DD1, however, high levels of Pheo a accumulated in hcar leaves (Figs. 9A and 9B). We also investigated whether the levels of PAO mRNA and/or PAO protein are related to Pheo a accumulation in oshcar leaves. By RT-qPCR analysis, we found that the mRNA level of PAO in oshcar leaves was not significantly different to that of wild-type leaves (Fig. 9C), like the expression patterns of other CCE genes, such as SGR1, NYC1, NOL, PPH, and RCCR (Supplementary Fig. 17). Like the mRNA level, the protein level of PAO in the oshcar leaves was also similar to that of wild-type leaves (Fig. 9D), indicating that Pheo a accumulation in oshcar leaves is caused by an unknown mechanism independent of PAO protein level.

**DISCUSSION**

**Rice HCAR is a functional homolog of Arabidopsis HCAR involved in Chl breakdown**

ATHCAR is a key enzyme in the PAO/phytolobilin pathway for Chl degradation: it catalyzes the conversion of 7-HMC a to Chl a in vivo and in vitro, and the athcar mutant shows a stay-green phenotype during DIS (Meguro et al., 2011). In this study, we found that, like the athcar mutant, the oshcar mutant exhibits persistent leaf greenness much longer than wild type during DIS and natural senescence (Fig. 1; Supplementary Fig. 2), along with the accumulation of 7-HMC a and Pheo a (Fig. 2). Furthermore, overexpressing OsHCAR recovered the defects of the athcar mutant (Supplementary Fig. 4), strongly indicating that OsHCAR is a functional homolog of ATHCAR.

In Arabidopsis, HCAR interacts with other CCEs, such as SGR1/NYE1, NYC1, NOL, and RCCR, during leaf senescence (Sakuraba et al., 2013). Similarly, in rice, OsHCAR also interacted with SGR, NYC1, and NOL in yeast two-hybrid assays (Fig. 3). Interestingly, we also found that OsHCAR interacted with itself (Fig. 3), suggesting that OsHCAR can form homodimers or -trimers. The importance of the HCAR-HCAR interaction remains unclear. Very recently, Wang and Liu (2016) determined the crystal structure of AthHCAR and found that it has the potential to form trimers, which is likely important for its interaction with LHCI. Similarly, we previously showed that Arabidopsis SGR1/NYE1 forms a homodimer and/or hetero-dimer with other SGR homologs in Arabidopsis, i.e., SGR2 and SGR-LIKE (SGRL), possibly to help control the rate of Chl degradation (Sakuraba et al., 2014b; 2014c). In this respect, HCAR dimer (or trimer) formation is probably important for enhancing its functions, e.g., its interaction capacity with LHCI and other CCEs, similar to SGR/Mg-dechelatase proteins.

Although the physiological role of OsHCAR is almost equivalent to that of AtHCAR, we found that the mRNA expression pattern of OsHCAR considerably differs from that of AthHCAR. Similar to AthNOL (Sakuraba et al., 2013), AthHCAR expression decreased during DIS, while the expression of OsHCAR and OsNOL increased under both DIS and natural senescence (Fig. 4). These results strongly suggest that the requirement for HCAR and NOL activity somehow differs in Arabidopsis and rice, especially in chloroplasts at the senescence phase. Indeed, the phenotypes of Arabidopsis and rice nol null mutants are quite different during leaf senescence: the rice nol plants display a stay-green phenotype (Sato et al., 2009), whereas the Arabidopsis nol plants undergo normal yellowing (Horie et al., 2009). Similarly, the oshcar mutant showed a strong stay-green phenotype during natural senescence in the paddy field (Supplementary Fig. 2), whereas the athcar mutant did not exhibit a stay-green phenotype during natural senescence, although both mutants remained green under DIS conditions (Fig. 1; Sakuraba et al., 2013). The different patterns of HCAR and NOL expression between Arabidopsis and rice further confirm that the transcriptional regulatory networks of these two genes are quite dissimilar. In Arabidopsis, several transcription factors, such as AB15/EEL, ORE1, EIN3, ANAC016, and ANAC072, directly activate the transcription of CCE genes, including SGR1/NYE1 (Li et al., 2016; Qiu et al., 2015; Sakuraba et al., 2014a; 2016), and a few directly activate more than two CCE genes. For example, EIN3 and ORE1 directly activate the transcription of SGR1/NYE1, NYC1, and PAO (Qiu et al., 2015): this co-activation is likely important because these genes function during the same phase. However, the expression patterns of ATHCAR and ATNOL suggest that these genes are regulated by transcription factors in different regulatory networks. In rice, two NAC TFs, OsNAP, and ONAC106, directly regulate the expression of CCEs, including SGR. OsNAP directly activates the transcription of SGR, NYC1, NYC3/PPH, and RCCR, whereas ONAC106 directly downregulates the expression of SGR and NYC1 during leaf senescence (Sakuraba et al., 2015b). The induction of CCE co-expression by a single transcription factor in rice suggests that HCAR and NOL are co-expressed with other CCEs by transcription factors in the same regulatory network. The upstream regulatory network of HCAR and NOL must be identified in both Arabidopsis and rice to evaluate the importance of the differential expression patterns of these genes in these plants.

**HCAR plays an important role in preventing cell death signaling in rice and Arabidopsis**

Under high-light conditions, Chl and its intermediates produce high levels of singlet oxygen, because their excited forms react with triplet oxygen, leading to the production of the highly reactive oxygen species 1 O2. Protoporphyrin IX (Proto IX) and protochlorophyllide (Pchlide) are strong photosensitizers in the Chl biosynthesis pathway (Jung et al., 2008; Nagata et al., 2005; op den Camp et al., 2003), whereas Pheo a and RCC are strong photosensitizers in the Chl degradation pathway: Arabidopsis pao and rccr mutants, also known as acd1 and acd2, respectively, show severe cell death symptoms, accompanied by the production of singlet oxygen.
oxygen (Pružinská et al., 2007). However, it was previously unclear whether other Chl intermediates can also have phototoxic effects.

In this study, we found that the oshcar mutant suffered from cell death symptoms in mature leaves under normal growth conditions in both the paddy field and growth chamber. We also found that oshcar mutant is also susceptible to herbicide-induced oxidative stress conditions (Fig. 5), and similar cell death symptoms in the Arabidopsis hcar mutant was also observed (Fig. 6). It has been known that MV blocks the electron transport during photosynthesis in the chloroplasts, leading to the production of ¹O₂. Produced ¹O₂ indirectly promotes the degradation of Chls, as well as photosystem apparatus, and further ¹O₂ is produced by the accumulation of 7-HMC a and Pheo a in hcar mutants, similar to rccr mutant (Yao and Greenberg, 2006). Furthermore, both rice and Arabidopsis protoplasts incubated with purified 7-HMC a produced large amounts of singlet oxygen (Fig. 8; Supplementary Fig. 13), indicating that 7-HMC a also acts as a phototoxic molecule. Pheo a also accumulates in hcar mutants, especially during DIS and under oxidative stress (Fig. 8; Meguro et al., 2011). Thus, the cell death phenotype of the hcar mutants is probably caused by the accumulation of both 7-HMC a and Pheo a. Like 7-HMC a and Pheo a, some Chl catabolic intermediates have the potential to act as phototoxic molecules. Both rice and Arabidopsis HCAR physically interact with other CCEs to form the Chl degradation complex at LHCII (Fig. 3: Sakuraba et al., 2013: 2015a); this complex formation is likely important for preventing the escape of such Chl catabolic intermediates from chloroplasts into the cytoplasm.

It is also possible that excessive accumulation of 7-HMC a changes the pigment composition in Chl-containing photosystem proteins in non-senescent green leaves. Indeed, in transgenic Arabidopsis plants expressing chlorophyllide a oxygenase (CAO-OX), high levels of Chl b accumulate in leaves, leading to changes in Chl a/b ratios in Chl-binding photosystem proteins, including LHCII and the subunits of core complexes (Sakuraba et al., 2009; Yamasato et al., 2005). Furthermore, CAO-OX plants exhibit cell death symptoms under high-light conditions, because their altered Chl composition ultimately changes their energy transfer capacity (Sakuraba et al., 2010), indicating that maintaining the proper balance of Chl composition in photosystem complexes is important for preventing cell death due to excessive light damage. Similarly, it is highly likely that the excessive 7-HMC a in the hcar mutants enters into the binding sites of Chls instead of photosystem proteins, leading to severe cell death symptoms (Figs. 5-7). Further biochemical studies, such as studies examining the Chl composition of photosystem proteins in hcar mutants, are needed to explore these possibilities.

Gene information

Sequence data from this article can be found in the National Center for Biotechnology Information (NCBI) database under the following accession numbers: for Arabidopsis, AtHCAR, At1g04620; for rice, GAPDH, Os06g45590; J Amyb, Os11g0684000; MTZb, Os05g0111300; NOL, Os03g0654600; NYC1, Os01g0227100; OsACS6; Os02g49880; OsHCAR, Os04g0320100; OsNAC4, Os01g60020; PAO, Os03g0146400; PPH, Os06g0354700; RCCR, Os10g0389200; SGR, Os09g0532000; SGR2, Os04g0692600; UBQ5, Os01g0328400.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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Weilan Piao et al.

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