Characterization of a novel allele encoding pheophorbide a oxygenase in rice

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ABSTRACT

We identified a rapid cell death 2 (rcd2) mutant from an indica cultivar Zhongjian100 mutant bank. The red-brown lesions appeared firstly on young seedling leaves, then gradually merged and the leaves completely withered at the late tillering stage. rcd2 displayed apparent cell death at/around the lesions, accumulation of superoxide anion (O_2^-) and disturbed ROS scavenging system, impaired photosynthetic capacity with significantly reduced chlorophyll content. The lesion formation was controlled by a single recessive nuclear gene and induced by natural light as well as mechanical wounding. A single base mutation (A1726T) at the 6th exon of OsMh-D3G0040800 resulted in I576F substitution in the encoding protein, pheophorbide a oxygenase (PAO). Functional complementation could rescue the mutant phenotype and PAO-knockout lines exhibited the similar phenotype to rcd2. The activity of PAO decreased significantly while the content of PAO substrate, pheophorbide a, increased apparently in rcd2. The expression of chlorophyll synthesis/degradation-related genes and the contents of metabolic intermediates were largely changed. Furthermore, the level of chlorophyllide a, the product of chlorophyllase, increased significantly, indicating chlorophyllase might play a role in chlorophyll degradation in rice. Our results suggested that the I576F substitution disrupted PAO function, leading to O_2^- accumulation and chlorophyll degradation breakdown in rice.

Introduction

One of the most obvious signs of plant senescence is the loss of green color, that is, the degradation of chlorophyll (Chl), which is of great significance for plant growth, development and reproduction. Chl degradation is catalyzed by a series of Chl catabolic enzymes (CCES) among which pheophorbide a oxygenase (PAO) is the key enzyme in the pathway termed the "PAO" pathway.1

During the process, the intermediate, pheophorbide (Pheide) a, is further converted into red Chl catabolite (RCC) by the catalysis of PAO and begin to the next step of degradation.2,3 It has been commonly observed that PAO mutants from multiple plant species including the maize lethal leaf spot 1 (lls1), Arabidopsis accelerated cell death 1 (acd1), rice early senescence 1 (eas1), sorghum dropdead1(ded1), tomato virus-induced gene silencing PAO mutants, and wheat PAO ortholog knockdown mutants result in necrotic lesion formation.4-10 The protein sequence are highly conserved with a similarity up to 70% manifested by a conserved N-terminal chloroplast transit peptide sequence among plant species such as Arabidopsis, tomato, corn and rapeseed.11 In rice, EAS1 is 68% identical to Arabidopsis ACD1. Unlike ACD1 that contains a single transit peptide, EAS1 contains two putative transit peptides in the N-terminus, suggesting that the targeting of EAS1 to chloroplasts is likely mediated by a putative bipartite transit peptide.2 Whether it is a unique mechanism for PAO transport into the chloroplasts in monocot has yet to be clarified.

So far, it is generally considered that the reason for lesion formation in PAO mutants is conserved across plant species due to a common fact that the metabolic defect universally induces the burst of reactive oxygen species (ROS) and subsequent cell death.10 It has been noticed that the blocking of PAO activity in these mutants may accumulate a phototoxic catabolic intermediate that produces singlet oxygen in light [Mach. 2001]. In maize, LL1S1 is a chloroplast-localized protein and may act to prevent ROS formation or serve to remove a cell death mediator so as to maintain chloroplast integrity and cell survival.12,13 In Arabidopsis, the delayed cell death phenotype of acd1 in darkness may be in part explained by a lower production of H_2O_2 or other ROS in the mutant plants that are expected to exhibit reduced photorespiration and the Mehler reaction.2 In sorghum, the lack of superoxide anion and H_2O_2 production at/around lesions suggests these two ROSs are not responsible for lesion formation in ded1; instead, singlet oxygen (O_2^·) might be the direct cause but has yet to be clarified.10 Besides, the lesions of lls1, acd1 and ded1 all appear on the leaves at the late growing stage, conferring severe impacts on plant reproductive growth. Overall, the direct cause for lesion formation in many lesion mimic/spotted-leaf mutants is largely controversial and required further studies.

Unlike PAO, the role of chlorophyllase (CLH), which could catalyze the hydrolysis of Chl to chlorophyllide (Chlide) and phytol, has long been considered to participate in the first step of Chl degradation [Takamiya et al. 2000; Harpaz-Saad et al. 2007],13,14 However, some CLHs do not have a chloroplast transit peptide, indicating the involvement of other enzymes rather than CLH or presence of alternative pathways outside the chloroplasts.13,15 In addition, Arabidopsis mutants with interrupted expression of either AtCLH1 and AtCLH2 or both of them are able to degrade Chl during senescence. Both CLHs localize to the cytosol when tagged with the green fluorescent protein, suggesting that CLHs are not essential for Chl degradation in Arabidopsis.16 In contrast, antisense-suppression of broccoli CLH could delay the he
ad yellowing rate after harvest.\textsuperscript{17} The lemon CLH is localized to the chloroplast as shown by in situ immunofluorescence and is co-purified with chloroplast membranes after heterologous expression in tobacco mesophyll protoplasts.\textsuperscript{2} Furthermore, it has been shown that CLH participates in Chl breakdown during fruit ripening in Citrus species as the cellular Chl quantity is negatively correlated with plastid CLH accumulation, i.e. plastids with reduced Chl content contains significant levels of Chl, while plasts containing still-intact chlorophyll lack any CLH signal.\textsuperscript{14,18} Therefore, the role of CLH in Chl degradation remains to be clarified in plants. As one of the most important monocot species, the role of CLH in Chl degradation remains elusive in rice.

We report here the identification of a rapid cell death 2 (rcd2) mutant with deficient PAO resulting from a single amino acid substitution at position 576 (I/F). Furthermore, we found that the accumulation of superoxide anion (O$_2^\cdot$) instead of hydrogen peroxide (H$_2$O$_2$) is likely responsible for the cell death in rcd2 and CLHs might play a role in Chl degradation in rice.

### Materials and methods

#### Rice materials

The rcd2 mutant was originated from ethane methyl sulfonate (EMS) induced indica cultivar Zhongjian100 (wild type, WT). The rapid cell death phenotype has been stably inherited over multiple generations under the field and greenhouse conditions in Fuyang, Hangzhou, Zhejiang Province, China and Lingshui, Hainan Province, China. The mutant was used as the female parent and crossed, respectively, with the normal green leaf variety 80A90YR72 and Nipponbare to generate two F$_2$ populations. The parents and the F$_2$ individuals were grown in the paddy field at the China National Rice Research Institute (CNRRI) for genetic analysis and gene mapping. The complementary lines and the CRISPR/Cas9-mediated knockout lines derived from Kitaake were grown in the greenhouse at CNRRI.

#### Agronomic trait evaluation

The mutant rcd2 and the wild-type were grown in the paddy field at CNRRI from May to October 2018 with a conventional management of water and fertilizer. The major agronomic traits including plant height, internode length, number of tillers per plant, panicle length, number of filled grains per panicle, seed-setting rate and 1000-grain weight were measured from three-randomly chosen individual plants at full maturity. The means from three replicates were used for analysis by Student’s t-test with the Excel 2016 software.

#### Histochemical analysis

The WT leaves and rcd2 leaves with lesions at the tillering stage were collected for histochemical assay. Cell death was detected by trypan blue staining according to Yin et al.\textsuperscript{19} Furthermore, the leaf samples were also used for a terminal deoxyribo-nucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay by using a Fluorescein in situ Cell Death Detection Kit following the manufacturer’s instructions (Roche, Basel, Switzerland). Samples were photographed using a confocal laser scanning microscope (Ceise, Jena, Germany). The hydrogen peroxide (H$_2$O$_2$) accumulation was detected by 3,3’-diaminobenzidine (DAB) staining.\textsuperscript{20} Superoxide anion (O$_2^\cdot$) accumulation was detected by nitroblue tetrazolium (NBT) staining according to the method described by Qiao et al.\textsuperscript{21} The pictures were recorded using a scanner (HP scanner jet 4010, Shanghai, China).

#### Genetic analysis and gene mapping

The F$_1$ plants were grown in the paddy field at CNRRI for determining the dominant/recessive nature of the target gene(s). The F$_2$ individuals were used for segregation analysis and gene mapping. Bulk segregant analysis was first used to rapidly locate the mutati on on a chromosome and a physical linkage map was then constructed using additional markers surrounding the mutation. Equal amounts of leaves from 20 plants of 80A90YR72 (the male parent) and 20 rcd2 plants were collected for DNA extraction to form a wild-type DNA pool and a mutant-type DNA pool, respectively. The DNA of the parents and F$_2$ individuals with mutant phenotype were extracted following the method of Edwards et al.\textsuperscript{22} Simple sequence repeat (SSR) markers were obtained from the website (http://www.gramene.org/) while insertion/deletion (InDel) markers were designed using the Primer 5.0 software after comparison of the sequences between the japonica cultivar Nipponbare and the indica cultivar 93–11 on the website (http://plants.ensembl.org/index.html, http://gramene.org/ genome_browser/index.html). PCR was performed in a total volume of 10 µL reaction buffer containing 50 ng template DNA, 1.0 µmol/L each primer, 5.0 µL of 2× PCR mixture (TsingKe Biological Technology, Hangzhou, China). The reaction was performed on a Biometra Thermal Cycler (Hiomedizinische Analytik, Germany): Pre-denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR products were run on 6% non-denaturing polyacrylamide gel electrophoresis (PAGE) and detected using silver staining. The primers were synthesized by TsingKe Biological Technology (Hangzhou, China). The primers for gene mapping are listed in Supplemental Table 1.

#### Shading and dark treatment

To determine the effect of natural light on the initiation of lesions, the leaf shading experiment was carried out at the tillering stage. The top second leaves without lesions in rcd2 and WT were shaded, respectively, with a piece of 1–2 cm aluminum foil for 4 d under natural field conditions. Then, the foil was removed and

### Table 1. Comparison of agronomic traits between WT and rcd2.

| Material | Plant height (cm) | Panicle length (cm) | No. of tillers per plant | No. of filled grain per panicle | Seed-setting rate (%) | 1000-grain weight (g) |
|----------|-----------------|--------------------|--------------------------|-------------------------------|----------------------|----------------------|
| WT       | 116.7 ± 1.53    | 21.64 ± 3.08       | 14.5 ± 2.48              | 186.70 ± 17.69                | 85.45 ± 0.62         | 21.52 ± 0.42         |
| rcd2     | 78.0 ± 1.73**   | 20.51 ± 2.70*      | 11.0 ± 1.73**            | 101.67 ± 5.13**               | 76.38 ± 2.68**       | 17.99 ± 0.23**       |

Values are means ± SD; n = 3; * indicates significance at P ≤ 0.05; ** indicates high significance at P ≤ 0.01 by Student’s t-test.
light was reinstated for 3–7 d. Lesion development was photographed by a scanner (HP scanner jet 4010, Shanghai, China). To investigate the influence of dark on the leaf color and Chl content, the detached leaves of rcd2 without lesions and WT at the tillering stage were cut into 2 cm pieces, and placed in Petri dishes filled with water in dark at room temperature for 8 d. Means of Chl content from three replicates were used for analysis by Student’s t-test.

**RNA extraction and gene expression analysis**

Total RNA was extracted from leaves of rcd2 and WT using NucleoZOL Reagent Kit according to the manufacturer’s instructions (Macherey-Nagel, Düren, Germany). RNA was reverse-transcribed using the ReverTra Ace qPCR RT Master Mix with genomic DNA (gDNA) Remover Kit (Toyobo, Osaka, Japan). The quantitative real-time PCR (qRT-PCR) was carried out using the FastStar Essential DNA Green Master Kit (Roche, Basel, Switzerland) and performed on a Thermal Cycler Dice Real Time System II (Takara, Kusatsu, Japan), following the method described previously.22 The rice *Ubiquitin* was used as the internal control to normalize expression levels. The means from three replicates were used for analysis by Student’s t-test with the Excel 2016 software. All primers used in qRT-PCR are listed in Supplemental Table 1.

**Determination of enzyme activity and substrate content**

At the tillering stage, 0.5 g fresh weight of the top second leaves from WT and rcd2 under filed and dark conditions were used to determine the contents of enzymes and relevant substrates. The activity of enzymes associated with Chl degradation and the content of substrates were determined by the ELISA Kits (MEIMIAN, Suzhou, China) following the manufacturer’s instructions. The kits and catalog numbers are as follows: PAO content (MM-6255001), PAO activity (RJ25353), Pheide a content (MM-3297102), Chlide a content (MM-6256602), Phein a content (MM-6256402), CLH activity (MM-089001), PPH activity (MM-252601), RCCR activity (MM-6254801), MDCase activity (MM-6255001). The absorbance was collected by using a SpectraMax i3x multi-mode microplate reader (Molecular Devices).

**Malonaldehyde (MDA), soluble protein contents and ROS-related parameter measurement**

The leaf blades from WT and rcd2 at the tillering stage were sampled and immediately frozen in liquid nitrogen. The activities of reactive oxygen species (ROS) scavenging enzymes, including peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), as well as the contents of malonaldehyde (MDA), hydrogen peroxide (H₂O₂) and soluble proteins (SP), were determined following the manufacturer’s instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Functional complementation and CRISPR/Cas9 knockout**

The 6693 bp genomic DNA sequence containing a 4589 bp coding sequence, a 1600 bp upstream and a 504 bp downstream sequence was amplified from WT and inserted into the pCAMBIA1300 vector to generate a new construct pPAO-com for functional complementation. The CRISPR/Cas9 constructs were generated according to a previous report under the japonica cultivar ‘Kitaake’ background.23 The pPAO-com and CRISPR/Cas9 constructs were introduced into the *Agrobacterium tumefaciens* strain EHA105 by electroporation and transformed, respectively, into the embryogenic calli derived from rcd2 and Kitaake via *Agrobacterium tumefaciens*-mediated plant transformation.24 All primers used are listed in Supplemental Table 1.

**Results**

**Phenotypic characterization of rcd2**

Under the natural field conditions, rcd2 exhibited reddish-brown lesions at the bottom leaves approximately 14 d after sowing (Figure 1(a)). The new leaves were normal green similar to WT until 50d after sowing, and subsequently the reddish-brown lesions appeared from the bottom to the top on all the leaves. The lesions then merged and some leaves withered completely at both the tillering and heading stages (Figure 1(b,c)). Under the paddy field conditions, performance of agronomic traits including plant height, internode length, panicle length, number of tillers per plant, number of filled grains per panicle, seed-setting rate and 1000-grain weight were significantly decreased in rcd2 compared with WT (Table 1). The shortened plant height was due to significantly reduced lengths of the third and fourth internodes (Figure 1(d)). Taken together, the mutation resulted in lesion formation and the poor performance of agronomic traits.

**Physiological alterations of rcd2**

To determine the mutation effect on Chl metabolism, we firstly measured the Chl contents at the seedling and tillering stages. The results showed that the contents of Chl were similar between rcd2 and WT at the seedling stage (four-leaf stage) when the lesions appeared only at the bottommost leaves. In contrast, the content of Chl was significantly lower than that of WT at the tillering stage when the lesions appeared on all the leaves of rcd2 (Figure 1(e)). The results indicated that the formation of lesions significantly decreased the Chl level in rcd2.

We then examined the photosynthetic parameters and found that the net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) in rcd2 were significantly lower than those of WT while the intercellular CO₂ concentration (Ci) was apparently higher than that of WT in the uppermost leaves without lesions at the tillering stage (Table 2). The results indicated that the mutation resulted in significant decline of photosynthetic capacity in rcd2.

**ROS accumulation with perturbed ROS scavenging system and cell death in rcd2**

To determine the direct cause for the rapid cell death phenotype, we carried out histochemical staining with rcd2 and WT leaves. Trypan blue staining, an indicator of irreversible membrane damage or cell death,27 showed that blue stains were observed in rcd2 leaves, whereas no blue stains were observed on WT leaves (Figure 2(a)). To further confirm the membrane damage and cell death, we measured the levels of
malonaldehyde (MDA) and soluble proteins (SP), and carried out TUNEL assay. The results showed that the MDA level was apparently higher and the SP level was significantly lower than those of WT at the tillering stage (Figure 2(d,e)). In addition, a large number of labeled nuclei (green) were detected as TUNEL positive signals in rcd2, whereas a few number of labeled nuclei were found in WT (Figure 2(f)), indicating the presence of increased DNA fragmentation in rcd2. Taken together, these results revealed that cell death occurred in rcd2. Furthermore, to know whether the occurrence of cell death in rcd2 was associated with the burst of ROS, we firstly carried out DAB staining and measured the level of H$_2$O$_2$. The results indicated that no red brown precipitates were observed both on rcd2 and WT leaves at the tillering stage, and the H$_2$O$_2$ content of rcd2 was even lower than that of WT (Figure 2(b,g)). We then performed the NBT staining, an indicator of O$_2^-$ accumulation, and the results showed that a large number of blue stains were observed in rcd2 compared with WT (Figure 2(c)).
These results indicated that the occurrence of cell death was probably associated with the burst of $O_2^−$ in rcd2.

Finally, to determine whether the accumulation of $O_2^−$ was associated with a perturbed ROS scavenging system, we measured the activities of ROS scavenging enzymes including peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT). The results indicated that the activities of SOD and POD in rcd2 leaves at the tillering stage were significantly higher than those of WT. In contrast, the activity of CAT was significantly lower than that of WT at the tillering stage (Figure 3(h–j)). The results suggested that the ROS scavenging system was apparently impaired and the excessive accumulation of $O_2^−$ that was likely responsible for the cell death in rcd2.

**Light-induced lesion formation and dark induced stay-green in rcd2**

Lesion initiation of many mutants is light-dependent.\textsuperscript{25} To determine the effect of light on rcd2 lesion formation under field conditions, the leaves of rcd2 without lesions and WT were covered with a piece of 2 cm aluminum foil for 4d. The results showed that the reddish-brown lesions did not appear in the shaded leaf area, while a mass of lesions emerged in the unshaded leaf area of rcd2. After removing the foil and reinstating the light for 5d, new reddish-brown lesions occurred in the shaded leaf area of rcd2 (Figure 3(a)). These results suggested that the lesion initiation and development in rcd2 were light-dependent. Furthermore, new reddish-brown lesions occurred in the leaves of rcd2 after mechanical wounding for 7d (Figure 3(b)), suggesting that the lesions could be induced by wounding as well.

It is well known that darkness could induce chlorosis and senescence in plants.\textsuperscript{36} To determine whether the mutation could accelerate or delay senescence in darkness, the detached leaves were treated in dark for 8 d. The results showed that the detached leaves of rcd2 could stay green longer than that of WT, and the Chl content in rcd2 declined much slower than that of WT (Figure 3(c,d)). The results clearly showed that rcd2 exhibited a retarded Chl degradation and “stay-green” phenotype in dark.

**Map-based isolation of rcd2**

To determine the inheritance pattern of the rcd2 phenotype, we crossed rcd2 with three normal green leaf cultivars, including the wild-type, Nipponbare and 80A90YR72. All F\textsubscript{1} plants derived from the three crosses exhibited the normal green phenotype similar to WT, suggesting that the mutation was recessive in nature. In the population derived from rcd2/ Nipponbare, 367 individuals exhibited normal phenotype, while 119 individuals exhibited mutant phenotype. The segregation of WT/mutant type fitted to the 3:1 Mendelian ratio ($\chi^2 = 0.57 < \chi^2_{0.05} = 3.84$). Similarly, in the other two populations, the WT/mutant type segregation ratios were all fitted to 3:1 (Table 3). The results indicated that the rcd2 phenotype was controlled by a single recessive gene. We then proceeded to map the mutation using the F\textsubscript{2} population derived from rcd2/
80A90YR72 by bulk segregation analysis. Two markers RM22 and RM14641 on chromosome 3 were identified to be linked to the mutation in an initial mapping (Figure 4(a)). To fine map the locus, 893 F2 individuals with rcd2 phenotype were genotyped and the mutation was finally delimited to a 145 kb interval between markers InDel11 and InDel16 (Figure 4(a)). The WT DNA and the rcd2 DNA were subjected to high throughput sequencing, and the results showed that a single nucleotide substitution from A to T at position 1726 was detected between WT and rcd2 in the 145 kb candidate region (Figure 4(a)). The A1726T substitution localizes to the 6th exon of OsMH_03G0040800, leading to the change from isoleucine to phenylalanine at position 576 in the putative coding protein (Figure 4(b)). The results indicated that OsMH_03G0040800 was likely the candidate gene responsible for the mutant trait.

To confirm whether OsMH_03G0040800 was the target gene, we carried out functional complementation. The construct pPAO-com carrying the WT allele was introduced into the rcd2-derived embryogenic calli via Agrobacterium-mediated plant transformation. A total of 20 positive transformants were obtained and all of them displayed the normal green leaf phenotype similar to WT. In addition, the content of Chl of the complementation plants recovered to WT level (Figure 5(a,b)). Furthermore, we genotyped the complementary plants and confirmed the presence of the transgene manifested at the mutation site (Figure 5(c)). The results clearly demonstrated that OsMH_03G0040800 was the target gene responsible for the rapid cell death phenotype of rcd2.

We also performed the knockout of OsMH_03G0040800 to further validate its function under Kitaake genetic background, a total of 2 Kitaake-background knockout lines were obtained (Figure 5(d,e)). The knockout line Cas9-2 had a six base deletion on the 4th exon (Figure 5(f)), resulting in the frameshift mutation. Unlike rcd2, Cas9-2 exhibited similar reddish-brown lesions but more severe wilted leaf blades leading to seedling death. Based on the annotation at the NCBI databank, RCD2 or OsMH_03G0040800 encodes a putative phophorhod oxide phosphohydrolase (PAO) and plays a vital role in Chl degradation in plants.

### Table 3. Genetic analysis of rcd2.

| Cross            | Phenotype F₀ | F₂ Total no. of plants | No. of WT plants | No. of mutant-type plants | P (x2) | χ² (P<0.05) |
|------------------|--------------|------------------------|------------------|--------------------------|--------|------------|
| rcd2/Nipponbare  | Green leaf   | 486                    | 119              | 367                      | 0.79   | 0.07       |
| rcd2/80A90YR72   | Green leaf   | 2938                   | 748              | 2190                     | 0.57   | 0.33       |
| rcd2/wild type   | Green leaf   | 176                    | 133              | 43                       | 0.86   | 0.03       |

Figure 3. Response of rcd2 to light, wounding and darkness treatment. (a) Effect of light on lesion formation in rcd2, including before shading, shaded for 4 d and reinstated for 5 d. The red dotted line box indicates the shaded area. Bar = 1 cm; (b) effect of mechanical wounding on lesion formation in rcd2. Bar = 1 cm; (c) detached rcd2 leaves stayed green under dark conditions. Bar = 1 cm; (d) the chlorophyll (Chl) contents measured at 1–8 d after dark treatment. Values are means ± SD; n = 3.
Figure 4. Map-based cloning of RCD2. (a) The mutation is delimited to a 145 kb region by genotyping 893 mutant type F2 individuals from the cross rcd2/80A90YR72; (b) sequence analysis reveals a single nucleotide substitution from A to T in the 6th exon of OsMH_03G0040800. Arrow head indicates the mutation site.

Figure 5. Functional complementation of rcd2. (a) Phenotype of WT, rcd2, and complementation line (Com-rcd2), Bar = 10 cm; (b) Chl contents of wild type, rcd2 and complementation line. Values are means ± SD (n = 3). Different letters indicate significant differences by one-way ANOVA and Duncan’s test (P < .05); (c) sequencing analysis of WT, rcd2 and complementation line; (d) phenotype of Kitaake and Kitaake PAO-knockout line (Cas9-2) at four-leaf stage, Bar = 5 cm; (e) phenotype of Kitaake and Cas9-2 at the heading stage; (f) sequencing analysis of Kitaake and knockout line Cas9-2 that shows a 6 bp deletion in the fourth exon of PAO.
Enzymatic activities, intermediate contents and expression of Chl-metabolism-associated genes

Having known that RCD2 encoded a PAO, we then carried out PAO activity assay, PAO content and substrate measurements. The results showed that both the activity and content of PAO were significantly decreased in rcd2 compared with WT (Figure 6(a,b)). In contrast, the level of Pheide a, the substrate of PAO, was apparently increased in rcd2 compared with WT (Figure 6(c)). Pheide a is a phototoxic substance whose accumulation could destroy the mesophyll cells in light, leading to the lesion formation. Taken together, our results suggested that the 1576F mutation lead to impaired function of PAO and the accumulation of Pheide a in rcd2.

To determine the mutation effects on the expression of relevant genes and enzymatic activities in the PAO pathway, we performed qRT-PCR and ELISA analysis, respectively, on eight genes (PAO, RCCR1, SGR, NY3, CLH SGLR and CLHS) associated with Chl degradation, and two genes (CAO1 and CAO2) associated with Chl biosynthesis. Our results showed that the expressions of PAO, RCCR1, SGR, NY3, CLH SGLR and CAO1 were apparently downregulated, while the expression of CLHS was significantly upregulated in rcd2 at the late tillering stage compared with WT. The expression of CAO2 was similar between rcd2 and WT (Figure 6(d)). In addition, the accumulation of Chlide a, the product of CLH, and Phein a, the substrate of PPH, were significantly increased in rcd2 compared with WT (Figure 6(e,f)), suggesting that CLH might play a role in Chl degradation. Interestingly, the activity of CLH was apparently decreased in rcd2 although the level of Chlide a was increased (Figure 6(g)). Furthermore, the activities of RCCR and MDcase (SGR) were apparently increased while the activity of PPH was similar between rcd2 and WT (Figure 5(h–j)). Nevertheless, the results indicated that the mutation impaired the expression of nearly all genes associated with Chl degradation.

Altered enzymatic activities, intermediate contents and gene expression in dark

To determine the effect of darkness on the enzymatic activities, intermediate contents and expression of Chl metabolism-associated genes, we carried out analysis on the detached WT and rcd2 leaves without lesions at the early tillering stage. The results showed that the expression of PAO, RCCR1, SGR and CLHS were greatly upregulated, and the expressions of NY3, SGLR and CAO1 were apparently downregulated in rcd2 compared with WT while the expression of CAO2 and CLH was similar between rcd2 and WT before dark treatment (Figure 7(a)). Furthermore, the PAO content and activity in rcd2 were significantly lower than those of WT before dark treatment (Figure 7(c, d)). The level of Pheide a in rcd2 was apparently higher than that of WT before dark treatment (Figure 7(e)). The levels of Chlide a and Phein a were similar between rcd2 and WT before dark treatment (Figure 7(f,g)). The activity of CLH decreased obviously, and the

![Figure 6](https://example.com/figure6.png)

**Figure 6. Analysis of enzymatic activities, intermediate content and expression of relevant genes-associated with Chl metabolism.** (a) PAO content; (b) PAO activity and (c) Pheide a content of WT and rcd2 at the late tillering stage; (d) expression profile of relevant Chl metabolism genes in WT and rcd2 after lesion appeared; (e) Chlide a content; (f) Phein a content; (g–j) activities of CLH, PPH, RCCR and MDCase. Values are means ± SD; * indicates significance at P < .05; ** indicates significance at P < .01 by Student’s t-test. The top second leaves at the late tillering stage were used for analysis in all experiments.
activities of PPH, RCCR and MDCase were similar between rcd2 and WT before dark treatment (Figure 7(b–k)). The results indicated that the mutation affected Chl metabolism-associated gene expression before lesion formation.

After 4 d dark treatment, the expressions of PAO, RCCR1, SGR, NYC3, CLH, SGRL, CLHS and CAO1 were significantly down-regulated in rcd2 compared with WT except CAO2 which was similar between the two genotypes (Figure 7(b)). In addition, the level and activity of PAO were significantly decreased in rcd2 compared to WT (Figure 5(c,d)). The levels of Pheide a, Chlide a and Phein a were apparently lower than those of WT (Figure 7(e–g)). The activities of CLH, PPH and MDCase were increased obviously and the activity of RCCR1 was apparently decreased in rcd2 compared with WT (Figure 7(h–k)). Our results demonstrated that dark treatment inhibited significantly the expression of Chl metabolism-associated genes in the PAO pathway and retarded Chl degradation, leading to the stay-green phenotype in rcd2.

Discussion

Cell death mutants have been widely reported in a number of plant species. These mutants usually produce necrotic leaf lesions similar to those caused by hypersensitive response (HR), a type of programmed cell death (PCD) induced by pathogen invasion. A group of cell death mutant is associated with deficiencies in the PAO pathway. For example, the Arabidopsis-accelerated cell death (acd2) mutant generates lesion mimic phenotype resulting from
A mutation in ACD2 that encodes a red chlorophyll catabolite reductase.29 In the present study, we identified a rapid cell death mutant rcd2 and isolated the responsible gene RCD2 encoding pheophorbide a oxygenase, the key enzyme in the PAO pathway. The PAO proteins are highly conserved in plants. Substitution of any amino acid residue would result in impaired functions of the enzyme. For example, the T255I in EASI disrupted the PAO activity and function in rice.9 In our case, the isoleucine at position 576 is also highly conserved and its substitution by phenylalanine resulted in decreased activity and content of PAO.

Like many cell death mutants or lesion mimic mutant with cell death,30–32 rcd2 leaves undergo a series of biochemical/physiological changes such as decreased chlorophyll and solution protein content, increased MDA content and declined photosynthetic capacity. Besides, rcd2 also exhibited accumulation of ROS and altered activities of ROS scavenging enzymes including CAT, POD and SOD. In plants, the excessive amount of ROS is thought to be the cause for necrotic leaf lesions, but which type of ROS responsible for cell death varies. For example, the rapid ROS production generated from the accumulation of Pheide a in Arabidopsis acd1 is responsible for the propagation of cell death.7 In sorghum dead1, the lack of superoxide and H2O2 production in/around lesions suggests these two ROSs are not responsible for lesion formation; instead, singlet oxygen (1O2) derived from Pheide a might be the direct cause but has yet to be clarified.40 In the present study, the accumulation of 1O2 rather than H2O2 is likely responsible for the rapid cell death in rcd2.

In plants, phototoxic substances are responsible for necrotic lesions of some mutants associated with the defective in PAO or the PAO pathway, such as Pheide a of lls1 in maize and Pheide a of acd1 in Arabidopsis and RCC of acd2 in Arabidopsis. In Arabidopsis, acd2 develops a cell death phenotype which highly correlates with the accumulation of RCC and RCC-like pigments.33 In rcd2, we found similar necrotic lesions and the increased level of Pheide a due to the significantly decreased gene expression, activity and content of PAO. The lesion formation is also light-dependent, suggesting that the accumulation of Pheide a could damage the mesophyll cells in light and lead to the lesion formation in rcd2. Furthermore, some PAO pathway-defective mutants such as Arabidopsis pph-1 and rice NYC334,35 show “stay-green” phenotype due to the breakdown of Chl degradation. In the present study, rcd2 also showed a “stay-green” phenotype after dark treatment, suggesting the mutation of RCD2 delayed the degradation of chlorophylls in dark.

So far, the role of CLHs in Chl degradation is controversial. In the present study, rcd2 displayed significantly increased accumulation of Phein a, which was derived from Chl after the removal of Mg2+ and would be dephytylated by pheophytin pheophorbide hydrolase (pheophytinase, PPH) in Arabidopsis or non-yellow coloring 3 (NYC3) in rice.34–36 Phein a, the certain intermediate metabolite of Chl degradation, was highly accumulated in the detached senescent leaves of WT after 4 d dark treatment, suggesting that PPH played a vital role for Chl degradation. Interestingly, we also observed appa rent accumulation of Chlide a, a product from Chl by the catalysis of CLHs, implying that Chlide a may also be an intermediate metabolite of Chl degradation in dark. This was supported by the upregulated expression of chlorophyllase gene CLHS, which can hydrolyze Chl to Chlide. Unexpectedly, the activity of CLHs was apparently increased in rcd2 although the level of Chlde a was decreased compared with WT after 4d dark treatment, suggesting that over-accumulation of Chlde a might feedback inhibited the activity of CLHs. There fore, we assumed that CLHs might involve in senescence-related Chl degradation in rice although further validation by knockout of CLHs-related genes would be helpful.

In conclusion, the A1726T mutation of the key gene PAO in the Chl degradation pathway could seriously impact the normal growth and reproduction in rice. The accumulation of O2− instead of H2O2 in rcd2 could be the direct cause for the lesion formation in rcd2. Furthermore, CLHs might play a role in Chl degradation in rice.

Author contributions
Conceptualization, ZZ and J-LW; Investigation, ZZ, YH, LL, YS, XX, and XZ; Supervision, J-L W; Writing – original draft, Z Z; Writing – review & editing, J-LW.

Disclosure of Potential Conflicts of Interest
The authors declare no conflict of interest.

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