**Rice CYO1, an ortholog of *Arabidopsis thaliana* cotyledon chloroplast
biogenesis factor AtCYO1, is expressed in leaves and involved in
photosynthetic performance**

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Summary

In the dicotyledonous plant *Arabidopsis thaliana*, the cotyledon chloroplast biogenesis factor AtCYO1 is crucial for the biogenesis of cotyledon chloroplasts. *Arabidopsis* mutants lacking *AtCYO1* have pale cotyledons but develop normal mature leaves. In the monocotyledonous plant *Oryza sativa*, the gene *OsCYO1* has high sequence identity to *AtCYO1*, but its function is unknown. We examined the role of *OsCYO1* in *O. sativa*. We first confirmed that transformation with *OsCYO1* could recover the phenotype of the *Arabidopsis cyo1* mutant. Similar to *AtCYO1*, recombinant *OsCYO1* has protein disulfide reductase (PDR) activity, which increased as a function of disulfide glutathione concentration with an apparent K_m of 3.2 μM and K_{cat} of 0.53 min^{-1} . The PDR activity was reduced when NADPH or NADH was used as an electron donor; however, PDR activity was observed with *OsCYO1* and glutathione, suggesting that glutathione may serve as a reducing agent for *OsCYO1 in vivo*. In *O. sativa*, the *OsCYO1* transcript level was higher in leaves compared with the coleoptile, which is the first leaf-like organ that forms during rice

embryogenesis. Many *OsCYO1* mutant lines defective in RNA interference had green leaves, however, three mutant lines had not only albino coleoptile but also albino leaves. Those having green leaves had reduced photosynthetic performance in leaves. Our results demonstrate that *OsCYO1* is enzymatically equivalent to *AtCYO1* but that the physiological role of *OsCYO1* in monocotyledonous plants may differ from that of *AtCYO1* in dicotyledonous plants.

Key words: cotyledon; coleoptile; leaf; CYO1; *Oryza sativa*; pulse amplitude modulation

Abbreviations

AtCYO1, *Arabidopsis thaliana* cotyledon chloroplast biogenesis factor;
Di-E-GSSG, dieosin glutathione disulfide; E-GSH, eosin glutathione; GSH, glutathione; *OsCYO1*, *Oryza sativa* CYO1; PDR, protein disulfide reductase; PAM, pulse amplitude modulation; PSI and PSII, photosystems I and II.

1. Introduction

In dicotyledonous plants such as *Arabidopsis thaliana*, the development of cotyledons (embryonic leaves formed during embryogenesis) is distinct from that of true leaves, which arise as the result of apical meristem activity. Cotyledons and true leaves both contain chloroplasts and are photosynthetically active; however, chloroplast differentiation follows distinct paths in these two organs (Deng and Gruissem, 1987; Mansfield and Briarty, 1996). In rice, which is a monocotyledon, the coleoptile is the first leaf-like organ that forms during embryogenesis, and it surrounds the embryonic shoot (Hirano, 2008). The coleoptile is believed to originate from a primordium common to that which produces the scutellum, but this remains controversial (Supplementary Fig. S1; (Hirano, 2008).

The *A. thaliana* cotyledon chloroplast biogenesis factor AtCYO1 localizes to the thylakoid membrane in *Arabidopsis* chloroplasts and interacts with the A1 and A2 subunits of photosystem I (PSI), the CP43 and CP47 subunits of PSII (Muranaka et al., 2012; Shimada et al., 2007), and the chlorophyll-binding LHCB

1 protein (Tanz et al., 2012). AtCYO1 catalyzes the formation, reduction, and
2 isomerization of disulfide bonds (Muranaka et al., 2012). It has a C₄-type zinc
3 finger domain (CXXCXGXG) similar to that of *Escherichia coli* DnaJ, which also
4 catalyzes the formation, reduction, and isomerization of disulfide bonds (de
5 Crouy-Chanel et al., 1995). This is similar to protein disulfide isomerase (EC
6 5.3.4.1), a molecular chaperone that catalyzes dithiol/disulfide interchange
7 reactions and promotes protein disulfide formation, isomerization, or reduction
8 depending on the redox potential and nature of the polypeptide substrate
9 (Freedman, 1989; Gilbert, 1990; Holmgren, 1979). The importance of CYO1 and
10 its homologs among different plant species is implied from the high level of
11 amino acid sequence identity in higher plants, including both dicotyledons and
12 monocotyledons (Supplementary Fig. S2 and S3); however, the function of
13 CYO1 in monocotyledons has not been analyzed. In this study, we demonstrate
14 that the monocotyledonous *Oryza sativa* gene Os09g0458400 is an ortholog of
15 *AtCYO1*, and characterize the expression of the *OsCYO1* transcript as well as
16 the enzymatic activity and physiological function of the *OsCYO1* protein.

2. Materials and methods

2.1. Plant materials and growth conditions

O. sativa L. ssp. Japonica (cv. Nipponbare) was grown at 27°C under a 16-h light/8-h dark cycle on Murashige and Skoog (MS) medium plates (Murashige and Skoog, 1962) or in soil. The *Arabidopsis cyo1* mutant (Shimada et al., 2007) was grown at 23°C under a 16-h light/8-h dark cycle on 1/2 MS medium plates.

2.2. Expression and purification of OsCYO1 protein

An expression vector containing a DNA fragment encoding amino acid residues 74–205 of OsCYO1 (Os09g0458400) was constructed as described (Shimada et al., 2007). The cDNA encoding this polypeptide was amplified by reverse transcription (RT)-PCR using primers containing recognition sites for *Nde*I (OsCYO1-Nde-5') and *Xho*I (OsCYO1-Xho-3') (Supplementary Table 1). *E. coli* strain BL-21-codonPlus (DE3; Agilent Technology, USA) was used for expression of a recombinant OsCYO1 fusion protein containing a 6×His tag. The

protein was expressed and purified as described (Shimada et al., 2007) .

2.3. Protein disulfide reductase (PDR) assay

Dieosin glutathione disulfide (Di-E-GSSG) was prepared as described (Raturi and Mutus, 2007), and the PDR assay was conducted in protein disulfide isomerase assay buffer (100 mM potassium phosphate, pH 7.0) containing OsCYO1 (100 nM), Di-E-GSSG (100–20,000 nM), and dithiothreitol (DTT, 5 μ M). The increase in fluorescence at 545 nm was monitored with excitation at 525 nm (Muranaka et al., 2012). Kinetic parameters were calculated using KaleidaGraph software (Synergy Software, USA).

2.4. RNA expression analysis by RT-coupled quantitative PCR (qPCR)

NucleoSpin[®] RNA kit (Takara, Japan) and ReverTra Ace[®] qPCR RT Master Mix (TOYOBO, Japan) were used for preparation of total RNA and cDNA from rice, respectively. All qPCR reactions were analyzed with an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA),

and data were analyzed using SDS 2.2.1 software (Applied Biosystems). qPCR was carried out using the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Boston, MA, USA) with gene-specific primers for *OsCYO1* (OsQ1F and OsQ1R) and *eEF-1* (eEF-F and eEF-R; Supplementary Table 1; (Caldana et al., 2007).

2.5. Construction of transformation vectors and transformation of plants

The plasmid used for *Arabidopsis* transformation was constructed using vector pGWB2 (Nakagawa et al., 2007). The full-length cDNA fragment of *OsCYO1* was amplified by RT-PCR using primers OsCYO1-ATG and OsCYO1-cDNA-3 (Supplementary Table 1). The plasmid vectors used for *O. sativa* transformation were constructed according to a previous method (Miki and Shimamoto, 2004). Primers Rcyo1-500F and Rcyo1-R were used for amplification of the cDNA fragment between 500 and 962 bp, and primers Rcyo1-600 and Rcyo1-R were used for amplification of the cDNA fragment between 600 and 962 bp. Transgenic rice plants were produced by *Agrobacterium*-mediated transformation of rice calli according to a published

protocol (Hiei et al., 1994).

2.6. Chlorophyll contents and immunoblot analysis

Chlorophyll content and immunoblot analyses were performed according to protocols described by Shimada *et al.* (2007). Antibodies against PsaA and PsaB (Agrisera, Vannas, Sweden) were used for immunoblot analysis.

2.7. Analysis of photosynthetic performance

Chlorophyll fluorescence and gas exchange in leaves were measured using a Junior-PAM (Walz) and LI-6400XT (Li-Cor), respectively. Parameters were calculated with software supplied by the manufacturers. All measurements of maximum quantum yield (F_v/F_m) were performed after the leaves were adapted in the dark for 30 min. Gas exchange measurements were carried out at a leaf temperature of $30^\circ \pm 0.1^\circ\text{C}$, ambient CO_2 concentration of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, and irradiance of $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (photosynthetically active radiation)

(sufficiently saturating photosynthesis). To investigate the role of OsCYO1 in photosynthetic electron flow, chilling-induced photoinhibition and subsequent recovery were evaluated in plants exposed to 4°C and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. For F_v/F_m and quenching analyses, plants were placed in a chilled (4°C) growth chamber in the dark for 12 h (dark), then illuminated in the chilled chamber for 16 h (light), and then transferred to another chamber at room temperature (25°C) in dark for 12 h (recovery). Quenching analysis was performed with actinic irradiance of 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR.

3. Results

3.1. *OsCYO1 is an ortholog of AtCYO1*

Os09g0458400 of *O. sativa* encodes a protein with high amino acid sequence identity to AtCYO1 (Fig. 1A). The protein comprises 205 residues with a molecular mass of 23 kDa and contains a C₄-type zinc finger domain (Supplementary Fig. S4). To confirm that the Os09g0458400 gene is an ortholog of *AtCYO1*, the full-length cDNA (AK070055.1) was expressed in the

Arabidopsis cyo1 mutant (Shimada et al., 2007). The *cyo1* plants had albino cotyledons (Fig. 1B, left), whereas *cyo1* plants complemented with AK070055.1 cDNA had normal green cotyledons (Fig. 1B, right and Supplementary Fig. S5). Based on these results, we conclude that Os09g0458400 is an ortholog of *AtCYO1* and have designated the gene as *OsCYO1*.

3.2. PDR activity of *OsCYO1*

AtCYO1 has been shown to have PDR activity (Muranaka et al., 2012; Shimada et al., 2007). To elucidate the enzymatic characteristics of *OsCYO1*, we assessed its PDR activity using Di-E-GSSG as a substrate. Fig. 2A shows the Michaelis-Menten plot obtained from this analysis. The PDR activity of *OsCYO1* increased as a function of Di-E-GSSG, with a K_m of $3.2 \pm 0.9 \mu\text{M}$ and $53 \pm 5 \text{ nM min}^{-1}$. We estimated the K_{cat} and catalytic efficiency ($K_{cat}K_m^{-1}$) to be $0.53 \pm 0.05 \text{ min}^{-1}$ and $2.8 \times 10^3 \pm 0.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The K_{cat} of *OsCYO1* was similar to that of *AtCYO1* ($0.53 \pm 0.05 \text{ min}^{-1}$), but the K_m of *OsCYO1* was greater than that of *AtCYO1* ($824 \pm 87 \text{ nM}$), and therefore the

1 catalytic efficiency was lower than that of AtCYO1 ($10.7 \times 10^3 \pm 1.5 \times 10^3 \text{ M}^{-1}$
2 s^{-1}). The lower affinity of OsCYO1 for Di-E-GSSG compared with AtCYO1
3 suggests that the *in vivo* substrate may differ between OsCYO1 and AtCYO1.

4 To determine what biomolecules can function as electron donors for
5 OsCYO1 *in vivo*, we assessed the effect of reducing agents on OsCYO1 PDR
6 activity (Fig. 2B). Lower PDR activity of OsCYO1 was observed when NADPH or
7 NADH was used as an electron donor; however, higher PDR activity was
8 observed with OsCYO1 and GSH or DTT as the electron donor. These results
9 suggest that OsCYO1 utilizes GSH, which exists in large quantities in
10 chloroplasts (Koffler et al., 2013), as a reducing agent.

12 3.3. Expression analysis of OsCYO1

13 In dicotyledonous *Arabidopsis*, AtCYO1 is expressed only in cotyledons.
14 In monocotyledonous rice, the coleoptile is the first leaf-like organ that forms
15 during embryogenesis, and the first leaf develops after germination of the
16 coleoptile. To assess OsCYO1 expression in rice organs, we performed

RT-qPCR (Fig. 3). The *OsCYO1* transcript accumulated to a lesser extent in the coleoptile than in the first leaf; accumulation was greatest in 3-day-old first leaves. In contrast to *AtCYO1* in *Arabidopsis*, where *AtCYO1* is not expressed in true leaves (Shimada et al., 2007), the expression of *OsCYO1* was higher in younger first leaves than in the coleoptile.

3.4. Characterization of *OsCYO1* using RNA interference (RNAi)

To investigate the physiological functions of *OsCYO1* in rice, we generated transgenic rice lines using RNAi. Two *OsCYO1* RNAi constructs were generated using target sequences spanning 500–962 bp and 660–962 bp of *OsCYO1* cDNA (*OsCYO1* RNAi #500 and #600, respectively; Supplementary Fig. S5). More than 14 transgenic rice lines were obtained from each of two RNAi constructs. Many RNAi lines had normal green leaves; however, three T2 plants of *OsCYO1* RNAi #500 (#500-20, -21, and -26) showed an albino leaf phenotype (Fig. 4A). In T2 progeny, the hygromycin resistance and albino leaf phenotypes segregated ~1:2:1 (hygromycin-sensitive = 3,

hygromycin-resistant/green leaf = 13, and hygromycin-resistant/albino leaf = 4; Fisher's exact test, $P = 0.689$). These results indicate that the albino leaf phenotype co-segregated with the T-DNA for RNAi, suggesting that the homozygous genotype of T-DNA showed the albino leaf phenotype.

Albino plants could not grow after emergence of the first leaf in soil or on 1/2 MS plates without sucrose. *OsCYO1* expression in RNAi #500 and #600 lines having green leaves was ~35% and 50% of control, respectively, and expression in RNAi #500 plants having albino leaves was also significantly lower than in control plants (Fig. 4B). The chlorophyll *a* + *b* content in lines having green leaves did not differ significantly from control, whereas it was not analyzed because albino leaves generally lack detectable levels of chlorophyll (Fig. 4C).

To analyze the relative amounts of the PSI peptides PsaA and PsaB, we performed immunoblotting using specific antibodies. Interestingly, the relative amounts of PsaB were comparable in control and RNAi lines, whereas PsaA was significantly different relative to control (Fig. 4D). These results indicate that the *OsCYO1* mutation influenced the stoichiometric ratio of PsaA and PsaB in

1 the PSI complex.

2 To assess OsCYO1 function in photosynthetic performance, we first
3 measured light response curves using pulse amplitude modulation in 3-week-old
4 plants from *OsCYO1* RNAi lines having green leaves (Fig. 4). Compared with
5 control plants, the effective quantum yield of PSII ($Y(II)$) and photochemical
6 quenching (qP) were both reduced in RNAi lines, but the open quantum yield of
7 PSII ($F_v'/F_m' = Y(II)/qP$) was almost the same. The maximal quantum yield of
8 PSII (F_v/F_m) also did not differ between RNAi lines and control ($F_v/F_m = 0.82$). In
9 addition, gas exchange measurements suggested that the assimilation rates
10 decreased by 12% and 9% in *OsCYO1* RNAi #500 and #600, respectively (data
11 not shown), probably due to the diminished $Y(II)$. These results suggest that
12 *OsCYO1* affected the downstream activity of PSII, but did not affect PSII per se,
13 and that the downstream effects altered the redox state of the plastoquinone
14 pool.

15 We next exposed 1-week-old plants to a chilling temperature (4°C) with
16 weak illumination (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$), conditions under which PSI is known to be

more susceptible than PSII to photodamage (Zhang and Scheller, 2004). After exposure for 16 h, F_v/F_m was lower in the RNAi lines than in control (Fig. 6A). Moreover, F_v/F_m did not recover after plants were transferred to 25°C in the dark for 12 h, indicating that the chilling induced chronic damage to PSII (Fig. 6A). The reduction of F_v/F_m was greater in RNAi lines than in control. In accordance with this observation, quenching analysis revealed a markedly lower Y(II) in parallel with qP in both RNAi lines compared with control after recovery. The qP decreased to 58% and 71% of the initial value due to photodamage in RNAi #500 and #600 lines, respectively, while it remained intact in control (Fig. 6B). Accordingly, it is hypothesized that OsCYO1 acts downstream of the electron transport chain, perhaps in the PSI complex, which balances the redox state of the chloroplast. Excitation energy (in the form of reactive oxygen species) may be generated to excess in the absence of OsCYO1, and potentially damage the photosynthetic apparatus when the capacity of the Calvin cycle is limited.

4. Discussion

In *Arabidopsis* leaves, LQY1 and PSA2, which harbor two zinc finger domains and have protein disulfide isomerase activity, function in the assembly and repair of PSII (Lu et al., 2011) and accumulation of PSI (Fristedt et al., 2014). Cotyledons in dicotyledonous plants switch from sink to source status during development in order to support chloroplast biogenesis in the leaf. Chloroplast biogenesis in cotyledons in dicotyledonous plants also occurs when energy is limited. AtCYO1 in *Arabidopsis* may be involved directly in the rapid synthesis and assembly of the photosynthetic thylakoid membrane system. In contrast, rice, as a monocotyledonous plant, has an endosperm to serve as a sink in seeds and during hypogeal germination. Our observations that OsCYO1 (1) is expressed in the leaf and not the coleoptile and (2) functions in the accumulation and/or assembly of PSI are consistent with the structural and functional differences between monocotyledonous and dicotyledonous plants. Furthermore, analysis of photosynthetic performance in *OsCYO1* RNAi lines suggests the potential involvement of OsCYO1 in regulation and/or maintenance of

1 photosynthetic electron transport, giving it an important role in resistance to
2 photodamage, especially in stressful environmental conditions.

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- 3
- 4

Figure legends

Fig. 1. Alignment of CYO1 and complementation of *cyo1* with *OsCYO1*. (A)

Alignment of amino acid sequences for *OsCYO1* (*Oryza*, Os09g0458400) and

AtCYO1 (*Arabidopsis*, At3g19220). Conserved residues are shown in black,

similar residues are shown in gray, asterisks indicate cysteine residues, and red

boxes represent zinc finger domains. (B) Five-day-old *Arabidopsis cyo1* mutant

(left) and *cyo1* mutant complemented with *OsCYO1* cDNA clone (right).

Fig. 2. PDR activity of *OsCYO1*. (A) Estimation of K_m and V_{max} . *OsCYO1* was

incubated with DTT and varying concentrations of Di-E-GSSG at 25°C, and

initial rates of eosin glutathione (E-GSH) formation were monitored. The

theoretical hyperbolic curve was plotted with a K_m value of 3.164 nM and V_{max} of

53 nM min⁻¹. Values represent mean ± S.E.M. ($n = 3$). (B) Effect of reducing

agents on PDR activity of *OsCYO1*. *OsCYO1* (100 nM) was incubated with

Di-E-GSSG (10 μM) and the indicated reducing agents (5 μM) at 25°C, and initial

rates of E-GSH formation were monitored. Values represent mean ± S.D. ($n = 3$).

Fig. 3. RT-qPCR analysis of *OsCYO1* expression. RNA was extracted from organs of control rice of various ages. Relative mRNA levels were determined by RT-qPCR using *eEF-1* expression as a reference and presented as values relative to that measured in the 1-day-old coleoptile. Values represent mean $\pm$ S.E.M. ($n = 3$).

Fig. 4. Phenotype of *OsCYO1* RNAi plants. (A) Appearance of 1-week-old plants. Scale bar, 3 cm. (B) RT-qPCR analysis of *OsCYO1*, (C) analysis of chlorophyll *a + b*, and (D) immunoblot analysis of PsaA and PsaB (4 mg fresh weight per lane) in 1-week-old control rice (1) and *OsCYO1* RNAi #500 having green leaves (2), #500 having white leaves (3), and #600 having green leaves (4). Values represent mean $\pm$ S.D. ($n = 3$). NA: no assay performed.

Fig. 5. Differences in photosynthetic properties of *OsCYO1* RNAi #500 and #600 transgenic plants having green leaves and control plants measured by

1 pulse amplitude modulation. Values represent mean $\pm$ SE ($n \geq 5$).

2

3 **Fig. 6.** Effects of chilling on PSII photochemistry in *OsCYO1* RNAi. (A)

4 Maximum quantum yield (F_v/F_m) of PSII in cold (4°C) dark-adapted leaves before

5 (dark) and after (light) illumination and after (recovery) in the dark at room

6 temperature (25°C). (B) Quantum yield of PSII (Y(II)) and photochemical

7 quenching (qP) in illuminated leaves after recovery relative to that in dark

8 conditions. Values are mean $\pm$ SD ($n = 6$).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6