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RESEARCH PAPER

Inducible antisense suppression of glycolate oxidase reveals its strong regulation over photosynthesis in rice

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Abstract

Photorespiration is one of the most intensively studied topics in plant biology. While a number of mutants deficient in photorespiratory enzymes have been identified and characterized for their physiological functions, efforts on glycolate oxidase (GLO; EC 1.1.3.15) have not been so successful. This is a report about the generation of transgenic rice (*Oryza sativa* L.) plants carrying a GLO antisense gene driven by an estradiol-inducible promoter, which allowed for controllable suppressions of GLO and its detailed functional analyses. The GLO-suppressed plants showed typical photorespiration-deficient phenotypes. More intriguingly, it was found that a positive and linear correlation existed between GLO activities and the net photosynthetic rates (P_N), and photoinhibition subsequently occurred once P_N reduction surpassed 60%, indicating GLO can exert a strong regulation over photosynthesis. Various expression analyses identified that Rubisco activase was transcriptionally suppressed in the GLO-suppressed plants, consistent with the decreased Rubisco activation states. While the substrate glycolate accumulated substantially, few changes were observed for the product glyoxylate, and for some other downstream metabolites or genes as well in the transgenic plants. Further analyses revealed that isocitrate lyase and malate synthase, two key enzymes in the glyoxylate cycle, were highly up-regulated under GLO deficiency. Taken together, the results suggest that GLO is a typical photorespiratory enzyme and that it can exert a strong regulation over photosynthesis, possibly through a feed-back inhibition on Rubisco activase, and that the glyoxylate cycle may be partially activated to compensate for the photorespiratory glyoxylate when GLO is suppressed in rice.

Key words: Glycolate oxidase (GLO), glyoxylate cycle, inducible antisense, photorespiration, photosynthesis, rice.

Introduction

Glycolate oxidase (GLO) is a key enzyme for photorespiration that is metabolically coupled with photosynthetic CO_2 assimilation (i.e. the Calvin cycle). GLO catalyses the oxidation of glycolate with equimolar amounts of glyoxylate and hydrogen peroxide produced. As characterized by CO_2 release, photorespiration is apparently counter-productive to the Calvin cycle and may account for at least 20% loss of net CO_2 assimilation in C_3 plants (Peterson, 1983; Sharkey, 1988). However, despite its seemingly negative impact, this pathway is believed to play various

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Abbreviations: F_v/F_m, maximum efficiency of PSII photochemistry after dark-adaptation; GDC, glycine decarboxylase; GDC-H, H subunit of glycine decarboxylase; GLO, glycolate oxidase; HPR, hydropyruvate reductase; ICL, isocitrate lyase; MLS, malate synthase; PCR, polymerase chain reaction; PGLP, 2-phosphoglycolate phosphatase; P_N, the net photosynthetic rate; PSII, photosystem II; RCA, Rubisco activase; SGAT, serine:glyoxylate amino transferase; WT, wild type.

functional roles in plants. First, from an evolutionary point of view, it may act as an ancillary metabolism to compensate for the futile withdrawal of ribulose-1,5-bisphosphate from the Calvin cycle under aerobic conditions by serving as a carbon recovery system reconverting 2-phosphoglycolate to 3-phosphoglycerate (Boldt *et al.*, 2005). More interestingly, a growing body of evidence has shown that photorespiration functions in amino acid metabolism (Keys, 2006), nitrate reduction (Rachmilevitch *et al.*, 2004), stress resistance (Wingler *et al.*, 2000; Moreno *et al.*, 2005), and signal transduction (Verslues *et al.*, 2007). The complete photorespiratory C_2 cycle needs a number of enzymes which are localized in three types of organelles, i.e. chloroplasts, peroxisomes, and mitochondria (Somerville, 2001).

In an early landmark study on photorespiration, Somerville and Ogren (see Somerville, 2001) established a brilliant and unique approach that led to the creation of *Arabidopsis* mutants which are deficient in various photorespiratory enzymes (Somerville and Ogren, 1979, 1980*a*, *b*, 1981, 1982*a*). Similar mutants were subsequently isolated from barley (Blackwell *et al.*, 1988; Kleczkowski *et al.*, 1990; Leegood *et al.*, 1995) and tobacco (McHale *et al.*, 1988). T-DNA insertional mutants deficient in activities of glycerate kinase and 2-phosphoglycolate phosphatase (PGLP) were also recently isolated, which displayed photorespiration-deficient phenotypes (Boldt *et al.*, 2005; Schwarte and Bauwe, 2007). However, the mutants deficient in GLO have not been identified by such a photorespiratory screen approach. Some years ago, Yamaguchi and Nishimura (2000) identified certain transformants with GLO activities co-suppressed when they tended to generate GLO-over-expressing tobacco plants. Functional analyses on these transformants showed that photoinhibition was elicited when GLO reduction was over a threshold of 60% relative to the wild type (WT) (Yamaguchi and Nishimura, 2000).

As described above, the biological significance for GLO remains to be further understood in plants. In this study, transgenic rice plants carrying an estradiol-inducible GLO antisense gene were generated, allowing GLO expression to be effectively controlled. The molecular and physiological analyses revealed that GLO is a typical photorespiratory enzyme and can exert a strong regulation over photosynthesis, possibly through a feed-back inhibition on Rubisco activase (RCA), and that the glyoxylate cycle may be partially activated to compensate for the photorespiratory glyoxylate when GLO is suppressed in rice.

Materials and methods

Construction of transgenic lines and induced down-regulation of GLO

Rice (*Oryza sativa* L. cv. Shishoubaimao) was used for the antisense gene transformation in this study. The estradiol-inducible gene expression vector pER8 was kindly provided by Dr Nam-Hai Chua (Rockefeller University, New York) (Zuo *et al.*, 2000). To generate the pER8-GLO antisense construct, the complete cDNA of *OsGLO1* (AK098878; Table S1 in Supplementary data available at *JXB* online) was cloned by reverse transcription-polymerase chain reaction (PCR), and then inserted into pER8 between *Spe*I and *Xho*I restriction sites. DNA sequencing confirmed both the correct orientation and the cDNA identities (100% identical to NM_001058022). The antisense gene was transformed into rice callus by *Agobacterium*-mediated infection (strain EHA105). Two cycles of hygromycin treatment (50 μg ml^{-1}) were applied for the primary selection since nopaline synthase promoter governing the hygromycin-resistance gene works less efficiently in monocots. This was followed by a PCR check of the hygromycin phosphotransferase marker gene. For the estradiol-inducibility test, the T_1 seeds were grown in Kimura B complete nutrient solution as detailed below. The three-leaf-old plants were treated with estradiol (5 μM) by adding it to the nutrient solution which was renewed every 3 d. GLO activity was determined 8 d after the treatment. The individuals with dramatic decreases in GLO activities were selected as inducibility-positive, and soon transferred to normal soil conditions for seed production. The T_2 heterozygous plants which originated from the two independent lines (GLO-AS-26 and GLO-AS-46) were used for the analysis in this study unless otherwise specified.

Growth conditions and treatments of plants

Pre-germinated seeds were grown first in Kimura B complete nutrient solution (Yoshida *et al.*, 1976) in a greenhouse under natural conditions. Various treatments of the three-leaf-old seedlings were conducted in a growth chamber under a cycle of 14 h (06:00–20:00) light/10 h dark (30/25 °C) at 350 or 800 μmol m^{-2} s^{-1}. To induce GLO down-regulation, estradiol (5 μM) was applied to the nutrient solution and renewed every 3 d. For high CO_2 (0.5%) treatment, pure CO_2 gas was introduced into the growth chamber and the concentration was controlled by a rotameter and monitored by an infrared gas analyser.

Southern, northern, and western blots, transcript analysis, and enzyme activity assays

Southern blot: Five micrograms of the genomic DNA was digested independently with *Bgl*II restriction enzymes (Takara), separated on 0.8% agarose gel, and blotted onto Hybond-N^+ nylon membrane (Amersham). Further analysis was performed by standard methods. The hygromycin phosphotransferase probe was labelled with ^{32}P by random priming using the Random Primer DNA Labeling Kit (Takara).

Northern blot: Total RNA was extracted from rice leaves according to Logemann *et al.* (1987). Northern hybridization was after Sambrook *et al.* (1989). Thirty micrograms of total RNA were size-fractionated on a formaldehyde agarose gel and transferred to Hybond-N nylon membrane (Amersham). The probes were labelled the same as for the Southern blot. All of the primers used in this study are

listed in Table S2 in Supplementary data available at *JXB* online.

Semi-quantitative PCR and real-time PCR: The RNA isolated above was further treated with DNase I (RNase free). RNA quality and quantity was assessed by denaturing RNA agarose gel electrophoresis (Sambrook *et al.*, 1989) and spectrophotometric detection at 260 nm and 280 nm. cDNA synthesis was done using ReverTra Ace (Toyobo Co. Ltd, Osaka, Japan) with random hexamers according to the manufacturer's instructions. Semi-quantitative PCR was performed on PTC-200 (Bio-Rad, Hercules, CA, USA). PCR products were separated on 1% agarose gels and visualized by staining. For real-time PCR analysis, the specific primers were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, Canada). The PCR reaction consisted of 10 μl of 2× SYBR Green PCR Master Mix (Toyobo Co.), 200 nM primers, and 2 μl of 1:40-diluted template cDNA in a total volume of 20 μl. No template controls were set for each primer pair. Real-time PCR was performed employing the DNA Engine Option 2 Real-Time PCR Detection system and Opticon Monitor software (Bio-Rad). The specificity of amplications was verified by melting curve analysis (70–95 °C) after 40 cycles and agarose gel eletrophoresis. Three technical replicates of each sample were used.

Western blot: Proteins were extracted by homogenizing 0.5 g of fresh leaves in 4 ml 20 mM phosphate buffer (pH 8.0). The homogenate was centrifuged at 15 000 *g* for 15 min. Equally loaded proteins (15 μg) were fractionated on a 4–20% gradient SDS–PAGE, and then electroblotted onto a nitrocellulose membrane using a Mini Trans-Blot cell (Bio-Rad). GLO protein was detected using a rabbit polyclonal GLO antibody (1:1000). The antibody was prepared by expressing the complete *OsGLO1* cDNA (inserted into a pET23d vector; Novagen) in *Escherichia coli* (BL21) after Dumbroff and Gepstein (1993). The expressed GLO protein induced by isopropyl-β-D-thiogalactopyranoside was purified on 4–20% gradient SDS–PAGE and then injected into a rabbit. The serum was withdrawn as the antibody.

GLO activity was assayed according to Hall *et al.* (1985) with some modifications (Xu *et al.*, 2006). Rubisco activity was determined after Ward and Keys (1989). The activation state of Rubisco was calculated as the relative ratio of initial to total Rubisco activities (Perchorowicz *et al.*, 1981).

Gas exchange measurements and chlorophyll fluorescence analysis

The net photosynthetic rate (P_N) was measured with a portable photosynthesis system (LI-6400, LI-COR). The youngest fully expanded leaf on each plant was used for the determination. The measurement conditions were set as follows: leaf temperature 25 °C, photon flux density 800 μmol m^{-2} s^{-1}, humidity 65%, and CO_2 concentration 0.038%. The chlorophyll fluorescence was measured with a PAM 2000 portable chlorophyll fluorometer (Heinz Walz GmbH) according to Murchie *et al.* (1999). The F_v/F_m was assayed after the leaves were dark adapted for 15 min. All of the above measurements were conducted between 11:00 and 13:00 on the measuring day.

Extraction and quantification of organic acids and free amino acids

Glycolate and glyoxylate were determined according to Petrarulo *et al.* (1989, 1990) with some modifications (Ji *et al.*, 2005). The youngest fully expanded leaves (0.1 g) were harvested between 17:00 and 18:00, then immediately frozen in liquid N_2 and stored at –80 °C for subsequent measurements. The samples were homogenized in 1 ml of 0.5 N HCl. The homogenate was heated at 80 °C for 8–10 min with intermittent shaking. Distilled water was added to the homogenate to a volume of 5 ml. One millilitre of the diluted homogenate was withdrawn and centrifuged at 12 000 *g* for 10 min. Then 0.5 ml of the supernatant was filtered through a 0.45 μm membrane. For HPLC (high-performance liquid chromatography) analysis, glyoxylate in the filtrate was first derivatized by phenylhydrazine to form phenylhydrazone. The derivative was separated and quantified by reversed-phase HPLC analysis with an Alliance 2695 reversed-phase system (Waters, Wexford, Ireland), and a Waters 2487 UV detector set at 324 nm. Ten microlitres of each sample were injected into a reversed-phase column (Sun Fire™, C_{18} column, 5 μm, 4.6×250 mm; Waters). The mobile phase consisted of 5% methanol and 95% phosphate buffer (13 mM potassium biphosphate; 1 mM potassium phosphate dibasic, pH 6.0). Analytes were quantified from the ratio of their respective peak areas to the peak area of the standard curve. Glycolate was determined by oxidizing glycolate into glyoxylate with purified GLO, then following the same procedure as described above for determining glyoxylate content.

Free amino acids were determined referring to Masclaux-Daubresse *et al.* (2006). The youngest fully expanded leaves were sampled as described above at 0.2 g each. First, three replicate samples were pooled together (total 0.6 g) and homogenized in 3 ml of 4% (w/v) sulphosalicylic acid. The homogenate was kept at room temperature for 2 h, then centrifuged at 15 000 *g* for 20 min. Free amino acids in the supernatant were analysed by a high-speed automatic amino acid analyser (Hitachi 835-50; Tokyo, Japan).

Microarray analysis

The youngest fully expanded leaves from WT and transgenic individuals with 30% and 90% reductions in GLO activity were pooled together in liquid N_2 for microarray analysis. RNA was isolated using Trizol Reagent (Invitrogen). Affymetrix GeneChip Rice Genome Arrays were used, and the Affymetrix chip analyses were performed at CaptalBio Corporation (Beijing, China). The transcripts which were changed by at least 2-fold were defined to be differentially expressed. Results for gene transcripts changed by at least 2-fold can be found in Table S3 in Supplementary data available at *JXB* online. The list of probes present in the arrays is given on the manufacturer's Web site (http://www.affymetrix.com).

Results

Generation of inducible antisense plants

Bioinfomatic analysis shows there are at least five putative GLO gene members in the rice genome, which are located on chromosome 3, 4, and 7, respectively (Table S1 in Supplementary data available at *JXB* online). The identities both at the cDNA level and at the protein level are shown in Table S4 in Supplementary data. A fragment on chromosome 8 is also annotated as GLO but currently no start code can be identified. In this study, the whole cDNA of *OsGLO1* (Table S1 in Supplementary data) was used to construct the transgenic plants, because years ago when this work started *OsGLO1* was the only sequence in the GenBank for the enzyme in rice. The cDNA and protein, respectively share 72% and 86% identities with the enzyme from spinach (Volokita and Somerville, 1987). Bioinformatic analysis suggested OsGLO1, 3, and 5 are peroxisomal, 2 and 4 are cytosolic. The antisense gene was inserted behind an estradiol-inducible promoter and the resulting construct was used to transform rice. Consequently, >40 independent hygromycin-resistant plantlets harbouring GLO antisense constructs were created. Further activity assays identified two independent lines (designated as GLO-AS-26 and GLO-AS-46) whose GLO activities could be dramatically decreased after estradiol application. GLO activities were suppressed by >90% when 5 μM estradiol was applied to GLO-AS-26 for 10 d (Fig. S1A in Supplementary data available at *JXB* online). Under the same conditions GLO-AS-46 showed about 80% reduction (Fig. S1B in Supplementary data). Various levels of GLO activity were seen among different T_1 plants of both transgenic lines (Fig. S1 in Supplementary data). Southern blot analysis showed that the fusion fragment was inserted into the genome in a multi-copy fashion (Fig. S1C, D in Supplementary data).

The suppression effect was dependent on the concentration of estradiol. A reduction of about 60% was observed when 1 μM estradiol was applied for 10 d, and the maximal reduction (>90%) occurred at 5 μM (Fig. 1A). Thus, 5 μM of estradiol was used both for testing the time-course responses and for the functional analyses. At 5 μM, significant reductions in GLO activity were seen at day 4, and over 90% reduction occurred at day 10 after the treatment (Fig. 1B). Without estradiol, no enzymatic differences could be observed between the transgenics and WT, and the activities of WT were not affected even under estradiol treatment (Fig. 1A, B).

Two groups of estradiol-treated plants from GLO-AS-26 were used to analyse GLO transcripts and proteins. One group had about 60% reduction in GLO activity and the other around 90%. Western blot analyses showed that the protein abundance was correspondingly reduced (Fig. 1C).

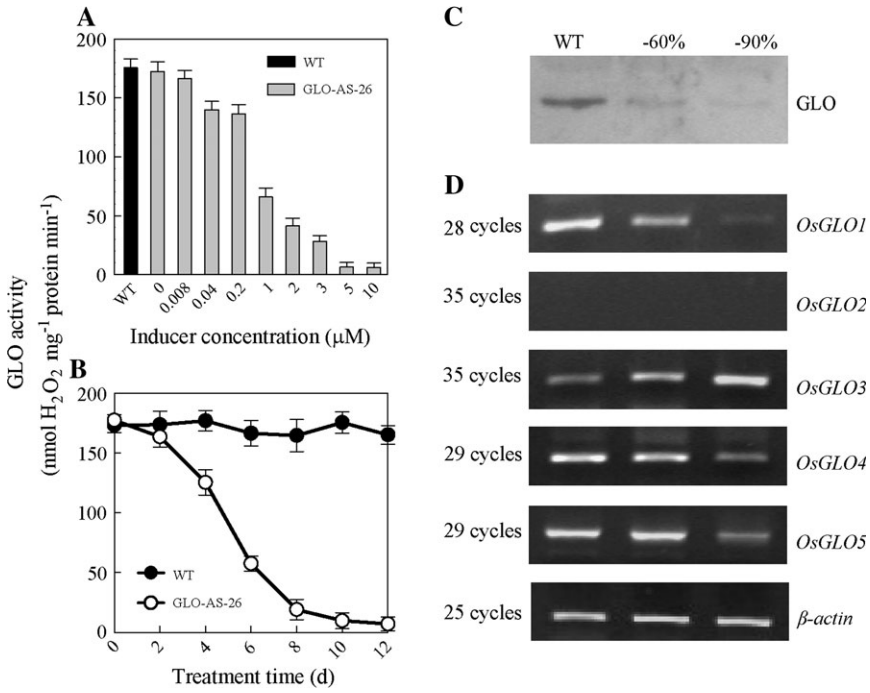

Fig. 1. Activities and expressions of glycolate oxidase (GLO) in the antisense plants. At the three-leaf stage, T_2 plants from GLO-AS-26 were treated in a controlled environment chamber with various concentrations of estradiol for 10 d (A), or with 5 μM of estradiol for various days (B). At each measuring point, six individual plants showing the strongest inhibition were used to calculate the average and the standard errors. The youngest fully expanded leaf from each individual was sampled between 17:00 and 18:00 on the day. The T_2 plants were treated with estradiol for 12 d. Then 2–3 cm tips from the youngest fully expanded leaves were detached between 17:00 and 18:00 to determine GLO activities first, and, then, according to the activities determined, those individual plants with about 60% or 90% reduction were pooled into two groups. Protein and RNA were subsequently isolated from the grouped samples for western blot (C) and semi-quantitative reverse transcription-PCR (D) analysis.

Since multiple GLO genes exist in the rice genome as mentioned above, it is interesting to know whether these genes respond differentially in the antisense plants. Semi-quantitative PCR analyses using the specific primer pairs (Table S2 in Supplementary data available at *JXB* online) showed that *OsGLO1*, *OsGLO4*, and *OsGLO5* were highly expressed in WT plants and obviously suppressed in the antisense plants (Fig. 1D). *OsGLO3* expression was very low in WT and even up-regulated in the antisense plants (Fig. 1D). *OsGLO2* expression was hardly detected in either WT or antisense plants (Fig. 1D).

Phenotypes of GLO-suppressed plants under air and high CO_2

Consistent with the activity responses as addressed above, no phenotypic differences were observed between the antisense and WT plants when no estradiol was applied, and the phenotypes of WT were not affected even under the inducer treatment (data not shown). However, both transgenic lines were severely stunted after treatment with estradiol (Fig. 2A, C, D; data not shown for GLO-AS-46). Such inhibited phenotypes could be prevented under high CO_2 (0.5%) (Fig. 2B; data not shown for GLO-AS-46). Since the two transgenic lines showed the same phenotypes and similar decreases in P_N (Fig. 4; Fig. S2 in Supplementary data available at *JXB* online), only the progenies from GLO-AS-26 were used for the detailed functional analysis.

Metabolic analyses in response to GLO suppression

Since GLO activity decreased dramatically in transgenic plants after treatment with estradiol, it is interesting to know how the photorespiratory metabolites were changed. The metabolic analyses found that GLO substrate glycolate accumulated sensitively in response to the decreased GLO activities. The plants with 30%, 60%, and 90% fewer GLO activities accumulated, respectively, 40-, 60-, and 130-fold more glycolate than WT (Fig. 3A). This result reminds us that the GLO-catalysed glycolate oxidation is a predominant way to metabolize glycolate, at least in rice leaves. However, the product glyoxylate and some other downstream metabolites, such as glycine and serine, were not reduced under GLO deficiency (Fig. 3B–D). The data imply the existence of an alternative pathway for glyoxylate production and regulation of the photorespiratory pathway.

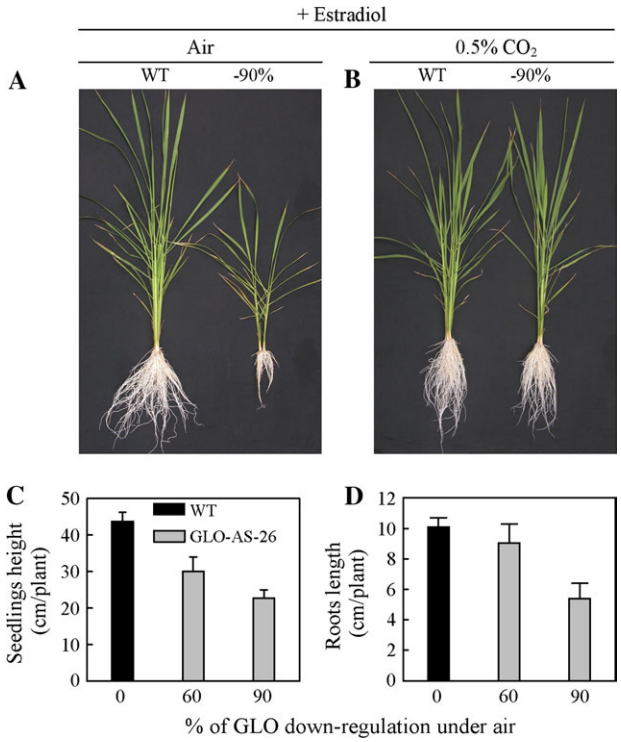

Fig. 2. Phenotypes of the antisense plants. Wild-type (WT) and T_2 plants (GLO-AS-26) were initially grown in a greenhouse under natural growth conditions, and then the three-leaf-old seedlings were treated with estradiol (5 μM) in two environment-controlled chambers under various conditions as specified in Materials and methods. Plants were treated under either normal air or high CO_2 (0.5%) conditions at 800 μmol m^{-2} s^{-1} irradiation. The photographs were taken at day 12 after the treatments (A, B). Seedling height (C) and root length (D) were determined under normal air conditions.

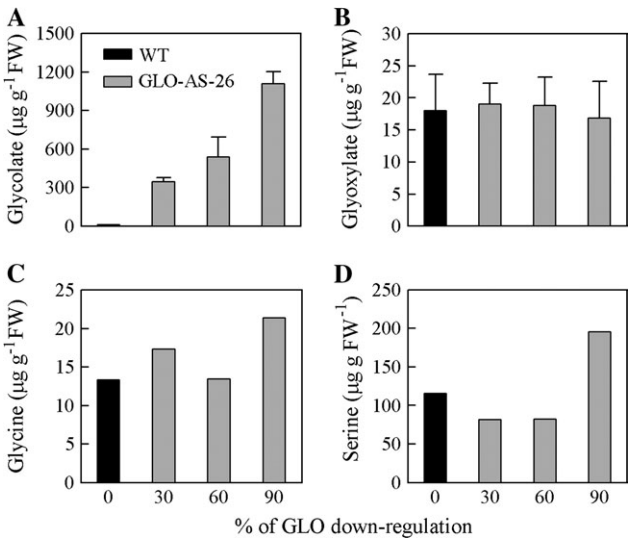

Fig. 3. Effects of glycolate oxidase (GLO) suppression on photorespiratory metabolites under air. T_2 plants were treated with estradiol under air at 350 μmol m^{-2} s^{-1} irradiation for 12 d. Then 2–3 cm tips from the youngest fully expanded leaves were detached between 17:00 and 18:00 on the day to assay GLO activities first, and, then, according to the activities determined, those individual plants, with about 30%, 60%, or 90% reduction, were pooled into three groups. Various metabolites (A, glycolate; B, glyoxylate; C, glycine; D, serine) were measured. Data for glycolate and glyoxylate represent means ±SE of three biological replicates. For analysis of glycine and serine, three biological replicates were pooled first and homogenized. The supernatant was loaded onto a high speed automatic amino acid analyser (for details, see Materials and methods).

Effects of GLO suppression on photosynthesis under air and high CO_2

After 5–6 d of estradiol treatment in air, P_N was not reduced until the GLO suppression percentage surpassed about 60% at both low (350 μmol m^{-2} s^{-1}; Fig. 4A) and high (800 μmol m^{-2} s^{-1}; data not show) irradiations. The correlation became linear as the induction treatment was prolonged (Fig. 4B). The induction time required to reach linearity depended on light intensity. At 350 μmol m^{-2} s^{-1}, linearity was established around 12 d after estradiol treatment (Fig. 4B: y=–0.230x+24.552, R^2=0.925, n=91, P <0.001), while it required only 8 d under a higher light intensity (800 μmol m^{-2} s^{-1}; data not show). Such a linear correlation indicates that GLO can exert a strong regulation over photosynthesis.

Photosynthesis requires coordination of light and dark reactions. Since P_N was shown to be linearly correlated with GLO activities (Fig. 4B), it is interesting to know further how the light reactions are affected. The maximum efficiency of photosystem II (PSII) photochemistry after dark-adaptation (F_v/F_m), an indicator of photoinhibition, was determined. The result showed that the F_v/F_m remained constant at first and then dropped sharply when the GLO activity was reduced to a threshold (Fig. 5A). The threshold value finally stabilized at ~60% reduction of GLO activity (Fig. 5A). Similar patterns were observed for some other parameters such as non-photochemical quenching of chlorophyll fluorescence and quantum efficiencies (ϕPSII) (Fig. 5B, C). It can be estimated further that photoinhibition was elicited after P_N reduction was over about 60% (Fig. 5D), demonstrating more quantitatively that photoinhibition occurs as a result of a reduction in P_N.

Similar to the phenotypic responses, reduced P_N and F_v/F_m could be prevented in the GLO-suppressed plants under high CO_2 (0.5%) at either 350 or 800 μmol m^{-2} s^{-1} irradiation (Fig. 6; data not shown for 800 μmol m^{-2} s^{-1}). It was noticed, however, that high CO_2 lowered the absolute values of both P_N and Fv/Fm (Fig. 6 compared with Figs 4, 5). Similar results have been reported previously by Reuveni and Bugbee (1997).

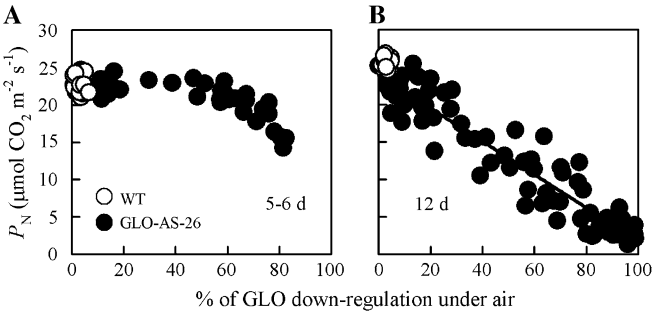

Fig. 4. Effects of glycolate oxidase (GLO) suppression on the net photosynthetic rates (P_N) in air at 350 μmol m^{-2} s^{-1} irradiation. On the indicated day after treatment, P_N was first determined between 11:00 and 13:00 on the day, using the youngest fully expanded leaves. After measuring P_N, the same leaf was detached to assay GLO activities. Then the P_N data and the corresponding GLO activities were collected for correlation analysis. (A) 5–6 d; (B) 12 d. Regression for (B): y=–0.230x+24.552, R^2=0.925, n=91, P <0.001. The result is representative of three independent experiments.

Transcriptomic responses in GLO-suppressed plants

As described above, GLO exerted a strong regulation over photosynthesis. To understand the possible mechanism further, a genome-wide expression was analysed and contrasted

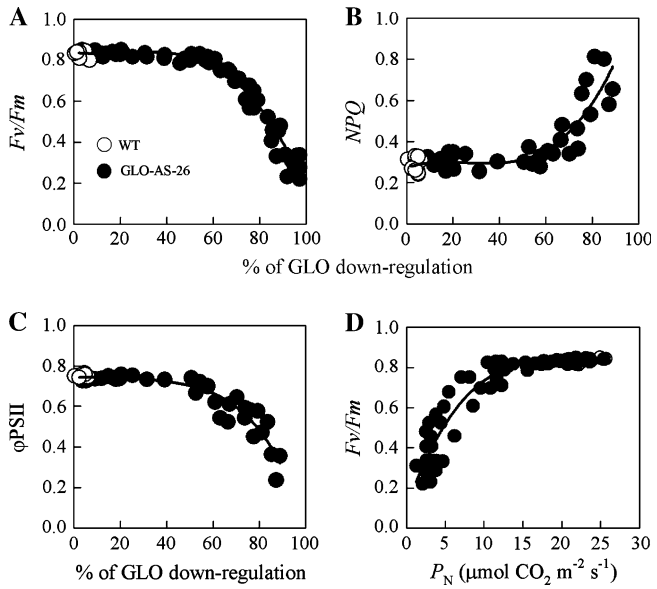

Fig. 5. Effects of glycolate oxidase (GLO) suppression on chlorophyll fluorescence parameters and the relationship between F_v/F_m and P_N at 350 μmol m^{-2} s^{-1} irradiation. The same individual plants were used as in Fig. 4 to measure chlorophyll fluorescence. The maximum efficiency of PSII photochemistry (F_v/F_m, A), non-photochemical quenching of chlorophyll fluorescence (NPQ, B), and quantum efficiencies (ϕPSII, C) were determined under air at 350 μmol m^{-2} s^{-1} on day 12; F_v/F_m against P_N at 350 μmol m^{-2} s^{-1} on day 12 (D). The results are representative of three independent experiments.

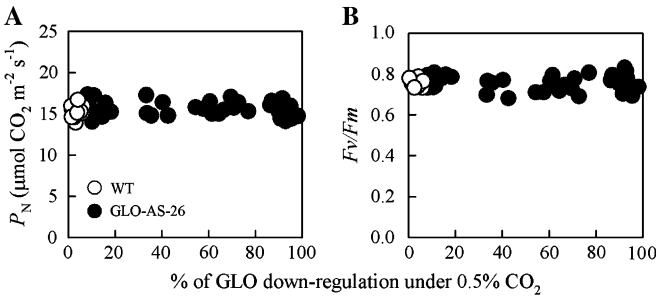

Fig. 6. Effects of glycolate oxidase (GLO) suppression on both the net photosynthetic rates (P_N) and maximum efficiency of PSII photochemistry after dark-adaptation (F_v/F_m) under high CO_2 (0.5%) at 350 μmol m^{-2} s^{-1}. T_2 plants were treated with estradiol under high CO_2 for 12 d. The other details are the same as described in Fig. 4.

between WT and the transgenic plants. The results revealed a number of genes whose expression was significantly altered in the antisense plants (Tables S3, S5 in Supplementary data available at *JXB* online). In addition to the antisense gene *GLO*, another photorespiratory gene *PGLP* was also suppressed in the antisense plants (Table S5 in Supplementary data). Besides, isocitrate lyase (*ICL*) and malate synthase (*MLS*), two key genes for the glyoxylate cycle, were very highly up-regulated as GLO activities were suppressed (Table S5 in Supplementary data). Among the photosynthetic genes detected, *RCA* was obviously suppressed, while the others were little affected (Table S5 in Supplementary data).

To validate the changes in certain genes of interest from the microarray analysis, northern blot and real-time PCR analysis were used further. The transcripts of both *ICL* and *MLS* were indeed largely increased as GLO was repressed (Fig. 7A, B). Some GLO downstream enzyme genes, such as serine:glyoxylate amino transferase (*SGAT*), H subunit of glycine decarboxylase (*GDC-H*), and hydropyruvate reductase (*HPR*), were altered little in the antisense plants (Fig. 7C–E). In addition, both northern blot and real-time PCR analyses further confirmed that *RCA* transcripts were truly reduced in response to GLO suppression under the air condition (Fig. 8A, B), and high CO_2 reduced its transcripts in WT but increased them in the transgenic plants (Fig. 8E, F). Further enzyme assays showed that the Rubisco activity and Rubisco activation states were correspondingly reduced in the antisense plants (Fig. 8C, D), and high CO_2 almost abolished the differences between the GLO-suppressed and WT plants (Fig. 8G, H).

Discussion

GLO is a typical photorespiratory enzyme

A number of mutants deficient in various photorespiratory enzymes have been identified and characterized in plants, ultimately from *Arabidopsis*, barley, and tobacco based on a specific protocol developed by Somerville and Ogren (McHale *et al.*, 1988; Leegood *et al.*, 1995; Somerville, 2001; Eckardt, 2005). Most of these mutants which were lethal in air (photorespiratory condition), but were viable under high CO_2 (non-photorespiratory condition), are referred to as photorespiration-deficient phenotypes. Such a phenotype indicates that the mutated gene is indispensable for photorespiration (Somerville and Ogren, 1982*b*; Somerville, 2001). While GLO is a key player in photorespiration, the mutants did not turn up during these extensive screens. To interpret the failure, it was once inferred that GLO could have a second essential role beyond the photorespiratory pathway (Somerville and Ogren, 1982*b*), like Rubisco. Nowadays, this failure may also be explained by the presence of multiple genes in plants, since there are at least five *GLO* gene members in the rice genome, and six in *Arabidopsis* (Table S1 in Supplementary data available at *JXB* online). The semi-quantitative PCR and microarray analyses verified that, in WT plants, of the five GLO genes *OsGLO1*, *OsGLO4*, and *OsGLO5* were highly expressed, especially *OsGLO1* and *OsGLO5* (Fig. 1D; Table S5 in Supplementary data). Certain tobacco transformants with GLO activities co-suppressed were identified and studied by Yamaguchi and Nishimura (2000), but the photorespiratory

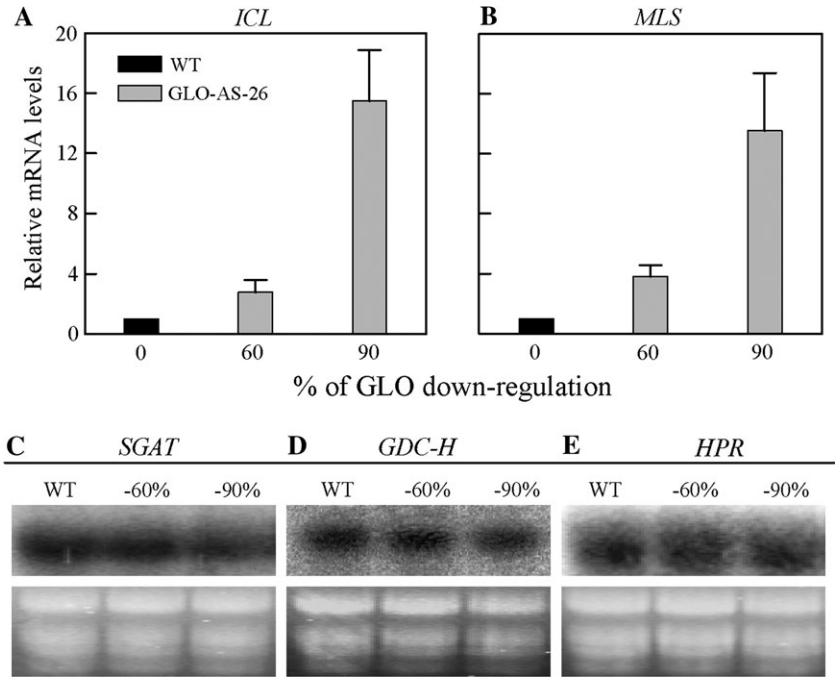

Fig. 7. Expression of some genes related to the glyoxylate cycle and photorespiratory C_2 cycle in response to glycolate oxidase (GLO) suppression. Sampling and RNA isolation were made in the same manner as described in Fig. 1. (A, B) Expressions of isocitrate lyase (*ICL*) and malate synthase (*MLS*) detected by real-time PCR; (C–E) transcripts for serine:glyoxylate amino transferase (*SGAT*), H subunit of glycine decarboxylase *(GDC-H)*, and hydropyruvate reductase (*HPR*) analysed by northern blot.

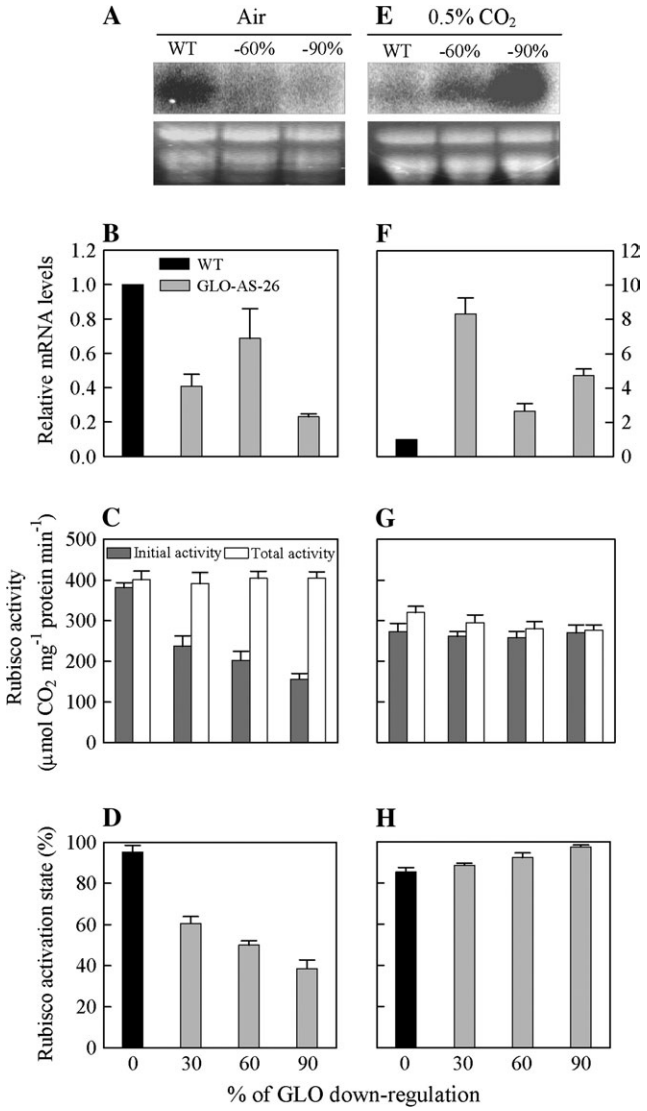

Fig. 8. Effects of glycolate oxidase (GLO) suppression on Rubisco activase (RCA) and Rubisco activities under air (A–D) or high CO_2 (E–H). T_2 plants were treated with estradiol under air or high CO_2 (0.5%) at 350 μmol m^{-2} s^{-1} irradiation for 12 d, and then 2–3 cm tips from the youngest fully expanded leaves were detached between 17:00 and 18:00 to first assay GLO activities. According to the activities determined, those individual plants with about 30%, 60%, or 90% reduction were pooled into three groups. RCA transcripts (A, E, northern blot; B, F, real-time PCR), Rubisco activity (C, G), and Rubisco activation states (D, H) were determined. Data represent means ±SE of three biological replicates.

phenotype and mechanistic basis were not determined in detail in this paper.

Here, an inducible antisense system was used to suppress GLO expression. This approach worked well with rice plants, and had clear advantages over the random mutagenesis approaches, particularly for those essential genes. Without the inducer treatment, no differences occurred between WT and transgenic plants, even under the air condition (Fig. 1A, B). Therefore, a high-CO_2 growth condition (i.e. a non-photorespiratory condition) is no longer needed for cultivating plants in the present case, which is otherwise an indispensable condition for photorespiratory mutants to survive (Somerville, 2001), More elegantly, this system enables various differential suppressions to be achieved which can be artificially controlled by adjusting inducer dose and/or treatment time (Fig. 1A, B). This tight inducer dependence also allows those unspecific phenotypes to be readily excluded; in other words, it is highly unlikely that the observed phenotypes and physiological responses would be due to nonspecific insertional effects in the transgenic lines. This is also the reason why only one independent line can be used for detailed functional analysis, as was also reported by Zuo *et al.* (2002). Such transgenic plants were successfully created and further analyses revealed that the estradiol-induced GLO suppression can phenocopy the mutants that are characterized as typical of photorespiration-deficiency (Fig. 2). Of all the photorespiratory mutants so far identified, most of them show photorespiratory phenotypes, including PGLP, SGAT, serine hydroxylmethyl transferase, glycerate kinase, and ferredoxin-dependent Glu synthase (Somerville and Ogren, 1979, 1980*a*, *b*, 1981, 1982*a*; Leegood *et al.*, 1995; Somerville, 2001; Boldt *et al.*, 2005; Schwarte and Bauwe, 2007), with GLO added in this study. Exceptionally, a recent study found that deletion of glycine decarboxylase (GDC) in *Arabidopsis* is lethal not only in air but also under high CO_2, pointing to a second essential role for GDC beyond photorespiration in *Arabidopsis* (Engel *et al.*, 2007). The present identification and characterization of GLO-deficient transgenic plants has clearly filled an important gap for analysis of series of the defective mutants along the whole photorespiratory pathway.

Regulation over photosynthesis by GLO

While photosynthetic inhibition has been observed for various photorespiratory mutants (Leegood *et al.*, 1995; Wingler *et al.*, 2000), few of them exhibit high control over photosynthesis (Wingler *et al.*, 2000). For instance, P_N was greatly reduced in PGLP mutants (Somerville and Ogren, 1979), but their heterozygous plants, with about 50% less PGLP activity, were indistinguishable from WT (Hall *et al.*, 1987). Similar results were observed for some other photorespiratory mutants including SGAT, GDC, HPR, and glutamine synthetase (Häusler *et al.*, 1994; Leegood *et al.*, 1995; Wingler *et al.*, 1997, 2000; Bauwe and Kolukisaoglu, 2003). The homozygous plants of the above-described mutants have usually lost almost all of the enzyme activities, so these mutants are not applicable for analysing the regulation of photorespiration and/or photosynthesis (Leegood *et al.*, 1995). The present inducible antisense plants may overcome these disadvantages. Various differential suppressions were readily obtained by adjusting either inducer concentrations or treatment time (Fig. 1A, B), which allowed metabolic regulation to be investigated.

The positive and linear correlation between GLO activity and P_N (Fig. 4B) implies that GLO can exert an intimate

regulation over photosynthesis, but it remains to be understood why photosynthesis needs to be reduced in such a sensitive manner in response to GLO suppression. Meanwhile it was noticed that photoinhibition was triggered after ~60% reduction in GLO activities (Fig. 5A), which agrees well with the result of Yamaguchi and Nishimura (2000). It is further estimated that photoinhibition elicited after P_N reduction was over about 60% (Fig. 5D). As far as is known, such a quantitative relationship between P_N reduction and photoinhibition has not been reported before, despite photoinhibition being also detected in the other photorespiratory enzyme mutants (Leegood *et al.*, 1995; Wingler *et al.*, 2000; Takahashi *et al.*, 2007).

How photosynthesis is inhibited in various photorespiratory mutants has not been well understood. Wingler *et al.* (2000) proposed two alternatives: (i) impairment of the carbon recycling and/or nitrogen re-assimilation causes reductions in the Calvin cycle metabolites and photosynthetic proteins; (ii) disruption of the photorespiratory pathway results in accumulation of the photorespiratory metabolites, which would confer negative feedback inhibition on the Calvin cycle. The current results showed that, as GLO was suppressed, the substrate glycolate substantially accumulated but the main downstream metabolites were not reduced (Fig. 3). Thus, the first possibility seems to be less important in the present case, such that elevated glycolate becomes the potential factor responsible for photosynthetic inhibition. A similar notion has been proposed in other studies (Chastain and Ogren, 1985; Leegood *et al.*, 1995; González-Moro *et al.*, 2003), leading us to query which metabolic point(s) in the Calvin cycle is regulated resulting in photosynthetic inhibition. The expression analyses and enzyme activity assays revealed that RCA and Rubisco activation states were depressed in the antisense plants (Table S5 in Supplementary data available at *JXB* online; Fig. 8A–D), indicating that the reductions in the Rubisco activation state, via RCA suppression, may be responsible for photosynthetic inhibition. The analysis of mutants with defects in GDC or further along the photorespiratory pathway has also shown a deactivation of Rubisco (Chastain and Ogren, 1985; Leegood *et al.*, 1995). High CO_2 (0.5%) reduced the transcription of RCA and Rubisco activation states in WT; but reversed the reduced transcription of RCA and Rubisco activation states in the antisense plants (Fig. 8E–H), consistent with the phenotypic (Fig. 2B) and physiological responses (P_N and F_v/F_m; Fig. 6). The data support the suggestion that high CO_2 is able to activate Rubisco independent of RCA. Actually, the discovery of RCA came out of a photorespiratory screen which is related to Rubisco activation under high CO_2 in the absence of RCA (Somerville *et al.*, 1982; Salvucci *et al.*, 1986). It is well documented how photoinhibition occurs when capacity for carbon assimilation is reduced. The main point is that the interruption of the Calvin cycle may reduce consumption of the photochemical energy (e.g. ATP and NADPH), resulting in an imbalance between production of photochemical energy and its consumption in photosynthesis, particularly under high light. In this case, the electrons originating from water oxidation at PSII are transferred to oxygen at PSI, producing reactive oxygen species (Asada, 2006). The increased reactive oxygen species would inhibit the *de novo* synthesis of D1 protein in PSII, thereby causing photoinhibition (Takahashi *et al.*, 2007).

The compensation of glyoxylate for photorespiration during GLO suppression

It was noticed first that glyoxylate could be compensated through certain anaplerotic reactions as GLO was suppressed: (i) the downstream metabolites, such as glyoxylate, glycine, and serine, were not reduced in the GLO-suppressed plants (Fig. 3B–D); (ii) the downstream enzyme genes, such as *SGAT*, *GDC-H*, and *HPR*, were not suppressed in the antisense plants (Table S5 in Supplementary data available at *JXB* online; Fig. 7C–E). Yamaguchi and Nishimura (2000) had also observed that the GLO downstream enzymes were little affected in the GLO-suppressed tobacco plants, and thus they considered photorespiration could be regulated by more than one mechanism. At first it was thought that the glycolate dehydrogenase, as recently reported in *Arabidopsis* (Bari *et al.*, 2004), could have contributed to the glyoxylate compensation in rice. But this hypothesis was clearly not supported by the fact that glycolate still accumulated in the antisense plants (Fig. 3A). Further expression analyses found that *ICL* and *MLS*, two key enzyme genes for the glyoxylate cycle, were remarkably induced in response to GLO suppression (Table S5 in Supplementary data; Fig. 7A, B), implying that the glyoxylate cycle may be involved in the compensation of glyoxylate as GLO activities were suppressed. Actually such an alternative source of glyoxylate/glycolate formation was noticed long ago by Zelitch (1973, 1988) and once highly debated (see also Somerville, 2001). More recently, Cornah *et al.* (2004) noticed that glyoxylate from the glyoxylate cycle can step into the photorespiratory pathway when *MLS* was mutated. It is not surprising that both *ICL* and *MLS* were together up-regulated because *ICL* and *MLS* are known to be always coordinately expressed in plants (Comai *et al.*, 1989; Rylott *et al.*, 2001), but it is currently hard to understand why glyoxylate should be compensated when *MLS* was induced as it also utilizes glyoxylate as a substrate. We consider that the *in situ* availability of the other substrate for MLS, i.e. acetyl-CoA, might be the limiting factor for converting glyoxylate into malate in the current case.

Supplementary data

The following can be found at *JXB* online.

Fig. S1. Suppression of glycolate oxidase (GLO) activities in the antisense lines.

Fig. S2. Photosynthetic response of GLO-AS-46 at 350 μmol m^{-2} s^{-1} under air at day 12.

Table S1. GLO gene members and their chromosomal localization in genomes of rice and *Arabidopsis*.

Table S2. The primer sequences for amplification, semi-quantitative PCR and real-time PCR analysis.

Table S3. Transcripts at least 2-fold changed in glycolate oxidase (GLO) down-regulated plants compared with WT plants.

Table S4. Identities of rice GLO gene members at levels of cDNA and protein.

Table S5. Microarray analysis of of gene expressions in response to glycolate oxidase (GLO) suppression.

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