Reduction to below Threshold Levels of Glycolate Oxidase Activities in Transgenic Tobacco Enhances Photoinhibition during Irradiation

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The effects of decreased flux in the glycolate pathway on photoinhibition was investigated in transgenic tobacco (Nicotiana tabacum L. cv. SR1) plants. These plants harbored a pumpkin cDNA for glycolate oxidase (GO), an enzyme in the glycolate pathway, under the control of the cauliflower mosaic virus 35S promoter. Some transformants showed both reduced amounts and reduced activities of GO. The decrease of GO was enhanced at a later growth stage of these transformants, whereas no changes were observed in the amounts of other enzymes in the glycolate pathway, such as hydroxypyruvate reductase and serine glyoxylate aminotransferase. The phenotype grown under a low light condition (30 μE s⁻¹ m⁻²) resembled that of the wild type. Transformants with about 35% lower GO activity than wild type, had a lower Fv/Fm under 500 μE s⁻¹ m⁻² irradiation for 8 h. After 60 μE s⁻¹ m⁻² irradiation for 8 h, Fv/Fm was lowered in some transformants with less than 20% of the GO activity of the wild type. These results suggest that photosynthesis was susceptible to photoinhibition with reduction to below threshold levels of GO activities and that higher activities of GO are required under a higher irradiation. The increase in the electron transport rate (ETR) with increased irradiation was suppressed only in transformants that had GO activity one-third less than the wild type, suggesting that the regeneration of the substrate for the Calvin cycle was decreased only when there was an extreme reduction of GO. These results also suggest that the photosystem was disturbed when the concentration of the substrate for the Calvin cycle decreased until it became insufficient to receive the excess photon energy generated in each light environment.

Key words: Calvin cycle — Glycolate oxidase — Glycolate pathway — Hydroxypyruvate reductase — Photoinhibition — Photosrespiration.

Abbreviations: ETR, electron transport rate; Fd, ferredoxin; GO, glycolate oxidase; HPR, hydroxypyruvate reductase; RuBP, ribulose1,5-bisphosphate; SGAT, serine glyoxylate aminotransferase.

Introduction

In higher plants, microbodies are classified into three categories, glyoxysomes, leaf peroxisomes and non-specialized microbodies. Glyoxysomes contain enzymes of the fatty acid β-oxidation pathway and glyoxylate cycle (Huang et al. 1983). With irradiation, cotyledons of fatty seedlings undergo greening, and there is a functional transition in microbodies, namely, glyoxysomes are converted to leaf peroxisomes (Nishimura et al. 1986, Titus and Becker 1985). The composition of microbody proteins changes drastically during the microbody transition (Mori and Nishimura 1989, Nishimura et al. 1998, Yamaguchi et al. 1995). Leaf peroxisomes function in photorespiration, which is assumed to be a mechanism that, along with the xanthophyll cycle and Mehler-ascorbate peroxidase reaction, protects against oxidative stress in stressful environments (Asada 1994, Pfendel and Bilger 1994). In particular, photorespiration is thought to function to maintain the supply of CO₂ in chloroplasts under CO₂ limited environments such as drought conditions (Kozaki and Takeba 1996, Osmond et al. 1997). Photorespiration involves a cooperative interaction among enzymes localized in chloroplasts, mitochondria and leaf peroxisomes, and is performed by the glycolate pathway. Three of the members of the glycolate pathway, glycolate oxidase (GO), hydroxypyruvate reductase (HPR) and serine glyoxylate aminotransferase (SGAT) are localized in leaf peroxisomes (Tolbert et al. 1968). In this organelle, GO is the first enzyme of this pathway, and produces glyoxylate and hydrogen peroxide from glycolate and oxygen.

Purification of GO from pumpkin and cloning of its cDNA were previously performed in our laboratory (Nishimura et al. 1983a, Nishimura et al. 1983b, Tsugeki et al. 1993). It has been shown that the expression of photorespiratory enzymes such as GO and HPR is induced during greening in the light (Bertoni and Becker 1996, Greenler et al. 1989, Hayashi et al. 1996, Mano et al. 1999, Tsugeki et al. 1993). Both the amount of messenger RNA and the amount of protein synthesis are increased during greening.

In a series of studies, Somerville and Ogren used Arabidopsis mutants to demonstrate the physiological importance of the glycolate pathway (Somerville and Ogren 1982a). Each of these mutants was deficient in phosphoglycolate phosphatase (Somerville and Ogren 1979), SGAT (Somerville and Ogren 1980b), glycine decarboxylase (Somerville and Ogren 1982b), serine hydroxymethyl transferase (Somerville and Ogren 1981), glutamate synthase (Somerville and Ogren 1980a) and dicarboxylate transporter (Somerville and Ogren 1983). These
mutants were viable in carbon dioxide enriched conditions, indicating that they could grow under non-photorespiratory conditions. Wingler et al. (1997) produced oscurant mutants in barley by crossing the wild type with a mutant (LaPr 87/30) that was completely defective in glycine decarboxylase, and investigated their growth in the presence of various concentrations of carbon dioxide. However, it is unknown how changes in the flux of the glycolate pathway affect the photosynthesis apparatus under natural air conditions.

In the present study, we obtained transformants of tobacco with decreased GO activity by cosuppression. These transformants were deficient in GO activity to various degrees in individual lines, and their GO deficiency increased with development. Therefore, these transformants can be used as a ‘transformant series’ with continuous levels of GO activities, and they can be used as probes to investigate phenomena that are induced by a limitation of the metabolic flux in the glycolate pathway. This point is a noticeable difference from report-
ed mutants of Arabidopsis (Somerville and Ogren 1982a) and barley (Blackwell et al. 1990). We analyzed the relation between the amounts of GO and the degree of photoinhibition using these transformants, and determined the amount of GO that was required to maintain normal photosynthesis under different light conditions.

Materials and Methods

Construction of the GO sense cDNA for transformation

The full-length cDNA encoding pumpkin GO (Tsugeki et al. 1993) was inserted between the Smal and KpnI sites of the Ti vector pBI121-Hm-multi, a derivative of pBI121. A blunted HindIII fragment of pLAN101MHYG and multi-cloning sites containing Xhol, XhoI, Smal, SpeI, KpnI and SacI were introduced into a blunted EcoRI site and XbaI-SacI site of pBI121, respectively. The inserted cDNA was expressed by the cauliflower mosaic virus 35S promoter.

Plant transformation and regeneration

Plant transformation vectors were transferred to Agrobacterium tumefaciens (strain EHA101) by electroporation. The construct was introduced into tobacco (Nicotiana tabacum cv. SR1) by the leaf disk method (Ohta et al. 1990). Primary transformants were designated T0 plants. T1 seeds, harvested from T0 plants by self-pollination, were selected as one-locus transformants of T-DNA. T2 homogenic plants. T1 seeds, harvested from T0 plants by self-pollination, were se-
lected in plates containing 300 μg/ml kanamycin and 75 μg/ml hygromycin. T1 lines with a 3 : 1 ratio of resistant:sensitive to antibiotics were selected as one-focus transformants of T-DNA. T2 homogenic lines were generated and were used for the following experiments.

Growth conditions of plants

Tobacco seeds were germinated in soil, and were grown at 25°C under various intensities of continuous light as described in the legends of the figures.

SDS-PAGE and immunoblotting

Tobacco leaves were homogenized in extraction buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 M LiCl, 20% glycerol, 10 mM 2-mercaptoethanol and 1% lithium dodecylsulfate), and the homogenate was centrifuged at 15,000×g for 10 min, and the supernatant was subjected to SDS-PAGE. SDS-PAGE (12.5% acrylamide) was performed by the method of Laemmli (Laemmli 1970). The separated proteins on the gels were electrophoretically blotted onto cellulose nitrate mem-

brane (Schleicher & Schuell, Dassel, Germany). Immuno-reactions were detected by monitoring the activity of horseradish peroxidase coupled with antibodies against rabbit IgG (ECL system; Amersham Pharmacia, Japan). Preparation of antibodies against GO of pumpkin was described previously (Tsugeki et al. 1993). The amount of protein was quantified with a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo) with bovine gamma albumin as a standard.

Measurement of the activities of GO and HPR

Assays of enzymatic activity were performed at room temperature with a spectrophotometer (DU-7500; Beckman, Palo Alto, CA, U.S.A. or model U-2000; Hitachi, Tokyo, Japan). The basal medium for the preparation of extracts for enzyme assays contained 100 mM HEPES-KOH, pH 7.2, 1 mM EDTA, and 10 mM 2-mercaptoethanol. Samples were homogenized with 10 volumes of buffer (w/v) in a mortar with a pestle using liquid nitrogen. Enzymes were assayed according to the cited references with minor changes. The GO assay was per-
formed with 100 mM triethanol amine (pH 7.8), 3 mM EDTA, 0.75 mM oxidized glutathione, 4 mM phenyl hydradine and 2.3 mM sodium glycolate (Feierabend 1975). The reaction was started by add-
ing sodium glycolate and the absorbance at 324 nm was recorded for 5 min. HPR was assayed by monitoring the decrease in absorbance at 340 nm in a solution of 50 mM potassium phosphate (pH 7.0), 5 mM lithium hydroxypyruvate and 85 μM NADH (Lord and Beevers 1972).

Measurements of chlorophyll fluorescence

The chlorophyll fluorescence parameters (Genty et al. 1989, Krause and Weis 1991) were determined with MINI-PAM (Heinz Waltz, Effertrich, Germany). The Fv/Fm index and non-photochemi-
cal quenching (qN) index of chlorophyll fluorescence, were calculated as (Fm – Fo)/Fm and (Fm – Fm’)/Fm – Fo, respectively, where ini-
tial (Fo) and maximal fluorescence (Fm) were determined using dark-
adapted leaves. ETR was calculated as 0.84 × 0.5 × (Fm’ – Fs)/Fm’, where steady (Fs) and maximal fluorescence (Fm’) were determined under various light intensities. The leaves were exposed for 2 min un-
til the intensity of chlorophyll fluorescence became constant at each light intensity.

Results

Expression of GO in transgenic tobacco

A cDNA clone for GO of pumpkin was previously isolat-
ed by immuno-screening (Tsugeki et al. 1993). The cDNA for pumpkin GO was inserted in the sense direction downstream of the 35S promoter in the binary vector pBI121-Hm-multi which contains two genes that confer resistance to kanamycin and hy-
gromycin. This GO sense construct was introduced into Nicotiana tabacum (cv. SR1) by an Agrobacterium-mediated leaf-
disc transformation (Ohta et al. 1990). Twenty-two transform-
ant lines were independently selected by their resistance to both antibiotics.

Modification of the GO levels in transformants and their phenotypes

Typical transformants were analyzed by immunoblotting using antiserum against GO (Fig. 1A). Some lines such as 7 and 9 had slightly higher levels of GO protein, whereas other lines such as 1, 3 and 21 had lower levels. The decrease or dis-
Reduction of GO levels and photoinhibition

Appearance of GO seems to be due to cosuppression. A decrease of GO protein was observed in 9 out of 22 transformant lines (data not shown). HPR and SGAT are known to be localized in leaf peroxisomes, and cooperatively function in the glycolate pathway of photorespiration with GO (Tolbert et al. 1968). The amounts of HPR (Fig. 1A) and SGAT (data not shown) in all lines were not significantly different.

To determine the relation between protein amounts and activities, the specific activities of GO and HPR of the transformants were measured 5 weeks after sowing. In the wild type, the specific activities of GO and HPR were 0.037±0.004 and 0.910±0.100 μmol min⁻¹ (mg soluble protein)⁻¹, respectively (Fig. 1B, C). The GO activity of the transformants varied from 0.012±0.003 μmol⁻¹ min⁻¹ (mg soluble protein)⁻¹ (line 21) to 0.044±0.006 μmol min⁻¹ (mg soluble protein)⁻¹ (line 7) (Fig. 1B), while the specific activity of HPR was indistinguishable from that of the wild type (Fig. 1C). The decrease in the amount of GO protein did not cause variations in the amount of other photorespiratory enzymes, HPR (Fig. 1A) and SGAT (data not shown). The phenotypes of the wild type and some transformants grown in a phytotron under natural light are shown in Figure 2. The transformants with decreased GO activity (lines 3 and 21) showed photo-bleaching of leaf color and drastic growth inhibition. The transformants with decreased GO activity (line 1) showed sectional photo-bleaching. However, other lines were not apparently different from the wild type.

Decrease of GO in transformants with aging causes severe damage to photo-apparatus

We used these cosuppressed lines in the following experiments. Plants were first grown under low irradiation (30 μE s⁻¹ m⁻²). This light condition had no effect on the growth rate or phenotype of any of the transformants including line 21, in which GO activity was suppressed (Fig. 3). The GO and HPR activities of the wild type and suppressed transformant line 21 were measured at various developmental stages (Fig. 4A). In wild-type plants, GO activity increased slowly with development. In transformant line 21, GO activity decreased, reaching 20% of that of the wild type 5 weeks after germination. HPR activities were the same in the wild type and line 21. To characterize the decrease in GO activity, the protein amounts of GO, HPR and SGAT were analyzed by immunoblotting using specific antibodies (Fig. 4B). In transformant 21, only the amount of GO decreased during development. These results showed that the decrease in GO activity corresponds to that of protein. The amounts of the other photorespiratory proteins remained constant or slightly increased with the stage of development, as they did in the wild type.

To investigate the effect of a decrease in the amount of GO under excess photon energy conditions, the sensitivity of the transformants to high irradiation was analyzed. The Fv/Fm index of chlorophyll fluorescence was used to monitor photoinhibition, because PSII is most susceptible to photoinhibition. Fv/Fm values measured before and after high irradiation treatments at different stages of development (2 weeks to 5 weeks) in line 21 and the wild type are shown in Figure 4C. The wild type and the transformants showed the same values before high irradiation. After irradiation at 500 μE s⁻¹ m⁻² for 8 h, Fv/Fm values decreased in both the wild type and transformants. In the wild-type plants, the decline was relatively small at all stages. In line 21, Fv/Fm values after the treatment were lower at later stages in comparison with the wild type.

In order to better understand the correlation between GO activity and sensitivity to high irradiation, wild type and five...
lines of suppressed transformants were analyzed. The trans-
formants were grown under 30 μE s⁻¹ m⁻² irradiation for 5
weeks after sowing, and then irradiated at 500 μE s⁻¹ m⁻² for
8 h. The GO activity in each transformant was expressed rela-
tive to that of the wild type, which was defined as 100 (Fig.
5A). The GO activity in the transformants ranged from 36 in
line 3 to 91 in line 6. As shown in Figure 5B, after irradiation
at 500 μE s⁻¹ m⁻² for 8 h, Fv/Fm values clearly decreased only
in line 3. Fv/Fm in line 4 (54) also showed a significant de-
crease, although not as much as in line 3, but in the other trans-
formants Fv/Fm was indistinguishable from that in the wild
type. In transformants having 20% GO activity relative to the
wild type, Fv/Fm was not different from that in the wild type
under 100 μE s⁻¹ m⁻² irradiation in 1% CO₂ non-photorespira-

Fig. 2 Phenotype of wild type and transformants grown in a phytotron under natural light. Plants were grown on soil for 5 weeks under natural
light at 25°C. WT represents a wild-type plant and GO1, 3, 7, 9, 13, 20 and 21 represent independent transformants.

Fig. 3 Phenotype of wild type (back row) and transformant 21 (front row) under low irradiation. Plants were grown on soil under 30 μE s⁻¹ m⁻²
irradiation at 25°C. Plants are (from left to right) 2, 3, 4, 5 and 6 weeks, of age.
Fig. 4  GO levels and the degree of photoinhibition at various stages. (A) Changes in activities of GO and HPR during stage development are indicated. Wild type (closed bars) and transformant 21 (open bars) were grown under 30 μE s⁻¹ m⁻² irradiation for 2, 3, 4 and 5 weeks, respectively. (B) Immunoblot analysis of GO, HPR and SGAT were performed. Plants were grown as in (A). Equal amounts of protein were loaded. The results for the wild type are also shown in the left panel and the results for the transformant are shown in the right panel. (C) The effect of high irradiation on Fv/Fm of chlorophyll fluorescence at various stages was investigated. Plants were grown as in (A) and Fv/Fm values were measured before (open bars) and after (closed bars) treatment with high irradiation (500 μE s⁻¹ m⁻²) for 8 h. The means±SE (n=8) are shown.
Under the same light intensity for 6 h in air, which allowed photorespiration, these transformants showed a lower Fv/Fm value (wild type, 0.791 ± 0.014; transformant, 0.721 ± 0.050). This indicates that the decrease of GO activity is a limiting factor of photorespiration and repression of photorespiration enhances photoinhibition.

**Different amounts of GO are required under different light irradiation conditions**

The results shown in the previous section indicate that the decrease in GO activity relative to the wild type (100%) is closely related to photosensitivity. Although the final levels of GO protein were different in each line, they were nearly the same among the plants in the same line. However, in the same line, GO decreased differently with the plant during the developmental stage. Such aspect is a phenomenon observed as a pattern of expression decrease by cosuppression (Palauqui et al. 1996, Zhao et al. 2000). Therefore, we analyzed the correlation between the decrease of GO activity and the photoinhibition levels in some independent transformant lines at different stages. The photosensitivity under different light intensity was analyzed using 5 or 6 plants each from lines 1, 3, 4, 6 and 20. In the transformants having at least 35–40% GO activity, Fv/Fm values were similar to those in the wild type under these conditions (Fig. 6A), while the transformants that had less than 30% GO activity had lower Fv/Fm value. Data from seven transformants having less than 40% of GO activity converge on the regression line significantly (Fig. 6). These results suggest that a GO activity of 35–40% might be the threshold value for maintenance of the steady state of photosynthesis. Under lower light intensities such as at 60 μE s⁻¹ m⁻² irradiation, only...
Reduction of GO levels and photoinhibition

Some transformants having less than 20% GO activity showed reduction of Fv/Fm (Fig. 6B). These results indicate that high irradiation requires a higher GO activity than does low irradiation for maintenance of photosynthesis.

ETR was measured under various light intensities. Figure 7A shows the light-ETR curves of transformants having different GO activities relative to the wild type (100%). The light-ETR curves in transformants having GO activity of more than 53% were indistinguishable from those in wild type. In transformants having 34%, 20% and 10% activities, the maximum ETRs were around 50, 45 and 30, respectively, and the ETRs were saturated at 400, 300 and 150 μE s⁻¹ m⁻², respectively. These results showed that ETR was limited by only a moderate reduction of GO activity.

Figure 7B shows the light–non-photochemical quenching curves, which strongly reflect the light–ETR curves in Figure 7A. Saturation of ETR is closely related to the increase of non-photochemical quenching. That is, in transformants with ETR values saturated at a lower light intensity, qN increased at a lower light intensity. A part of the increase of qN is due to the fact that the thylakoid membrane is energized by the depression of release of proton gradient by inhibition of the Calvin cycle. These results indicate that progression of the Calvin cycle was inhibited under lower irradiation by the deficiency of GO.

Discussion

Transformation of tobacco with the sense construct of GO cDNA induces suppression of GO

We transformed tobacco plants with a sense-constructed cDNA for GO of pumpkin. It is generally expected that transformation induces over-expression of the protein. However, we obtained no transformants which accumulated GO protein to a significant extent. On the contrary, nine transformants showed suppressed GO activity. Frequently, expression of transgenes and homologous genes present in a host genome can be reduced by sense-construction of the transgene. This phenomenon is known as cosuppression and has been reported for several transgenes, namely, chalcone synthase (Blokland et al. 1994), glucanase (de Carvalho et al. 1995), chitinase (Kunz et al. 1996), nitrate reductase (Vaucheret et al. 1997) and H⁺-ATPase (Zhao et al. 2000). The severe reduction in GO expression in the GO transformants was similar to that observed in a nitrate reductase transformant (Palaquii et al. 1996) and S-adenosyl-L-methionine transformant (Boerjan et al. 1994). These were defined as post-transcriptional gene silencing, and resulted in a strong reduction of mRNA accumulation in the cytoplasm without significant changes in the rate of transcriptional initiation in the nucleus. In Arabidopsis, two mutants, sgs1 and sgs2, were reported to repress post-transcriptional gene silencing and to inhibit cosuppression of nitrate reductase (Elmayan et al. 1998). This kind of mutant will be suitable for determining whether the suppression of the endogenous gene of tobacco that encodes GO is post-transcriptional gene silencing or not.

The suppression of GO does not affect other enzymes involved in photorespiration in leaf peroxisomes

We previously reported that the amounts of mRNA encoding GO and HPR as well as the amounts of GO and HPR proteins were increased by light (Mano et al. 1999). In photorespiratory metabolism, it is known that glycine decarboxylase in
mitochondria and Fd-GOGAT of plastids are also induced by light (Coschigano et al. 1998, Walker and Oliver 1986). Acquisition of the glycolate pathway during the transformation of glyoxysomes to leaf peroxisomes, which is induced by light, is the critical point in the establishment of photorespiratory mechanism. Therefore, it is reasonable that this mechanism is regulated by light. How the expression of photorespiratory genes is regulated remains unclear. Phytochrome is involved in the expression of HPR (Bertoni and Becker 1993), and cytokinin affects the expression of HPR (Wingler et al. 1998). Moreover, sucrose induces the expression of Fd-GOGAT (Coschigano et al. 1998) and HPR (Andersen et al. 1996). Although carbon dioxide in air does not affect the expression of the Fd-GOGAT gene (Coschigano et al. 1998), the HPR gene is down-regulated by an elevated concentration of carbon dioxide (Bertoni and Becker 1996). These findings indicate that some metabolites and environmental stimuli participate in the expression of glycolate pathway enzymes. Herein, the disturbance of glycolate metabolism by cosuppression of GO had no effect on other glycolate pathway enzymes localized in leaf peroxisomes, such as HPR and SGAT (Fig. 4B). These results suggest that acquisition of the capacity for photospiration might be regulated by more than one mechanism.

**Suppression of GO levels at different developmental stages of the plant**

None of the transformed plants showed any decrease in GO activity before 2 weeks after sowing. Starting at 2 weeks, the amounts of GO protein (Fig. 4B) and activity (Fig. 4A) of transformants decreased gradually, reaching a constant level at 5 weeks after sowing. The phenotype of these transformants exhibited a photoinhibition feature at 500 µE s⁻¹ m⁻² irradiation for 8 h (Fig. 4C).

**Reduction of GO below the threshold level caused drastic photoinhibition**

Arabidopsis mutants which have loss of the glycolate pathway have been reported (Somerville and Ogren 1979, Somerville and Ogren 1980a, Somerville and Ogren 1980b, Somerville and Ogren 1982a, Somerville and Ogren 1982b). These mutants could not survive under aerobic conditions which required the photorespiration pathway, and they were able to survive only under carbon dioxide-enriched conditions (with 2% CO₂).

In comparison with mutants that are almost completely lacking in some photosynthetic enzymes, partially defective mutants are useful tools for studying the control of metabolism and quantitative analysis of metabolic functions. These kinds of transformants, i.e. those with mutations in the Calvin cycle, have been obtained in tobacco by antisense transformation. These results help us understand the requirements of each enzyme in the Calvin cycle and how each enzyme is regulated. An osculant mutant was produced by crossing wild type barley and mutant (LaPr 87/30) that was completely defective in glycine decarboxylase, one of the photorespiratory enzymes. The osculant mutants had glycine decarboxylase activities that were 63% and 52% of the activity in the wild type, resulting in decreased rates of photosynthesis in high intensity light and in low CO₂ concentrations (Wingler et al. 1997).

In the present study, we examined the effects of decreased GO levels using transformants which showed various GO levels by cosuppression. We showed that transformed plants with a GO activity below the threshold level exhibited enhanced photoinhibition. These transformants were utilized to estimate the required amounts of GO under different light intensities. The threshold level of GO activity for enhancing photoinhibition was higher under 500 µE s⁻¹ m⁻² irradiation than under 60 µE s⁻¹ m⁻² (Fig. 6). Furthermore, photoinhibition in the transformant having 20% of the GO activity in the wild type was not affected under 30 µE s⁻¹ m⁻² (Fig. 3). These results suggest that the glycolate pathway was more important in the high intensity light condition.

It is possible that GO-defective transformants will become useful for estimating how the required GO levels vary with varying environmental parameters such as light intensity, temperature, water potential, concentration of carbon dioxide, nutrients and day length.

**Under an extremely low level of GO activity, ETR saturates under a lower light intensity and has a decrease maximum value**

We analyzed how changes in the level of GO activity affected ETR. A reduction of GO activity to one-third of that in the wild type did not affect the light-ETR curve of transformants grown under 30 µE s⁻¹ m⁻² irradiation. A further reduction of GO activity lowered the maximum value of ETR and also resulted in the saturation point of ETR occurring under lower irradiation. Schreiber and Nuebauer (1990) suggested that a part of non-photochemical quenching was related to the proton gradient on the thylakoid membrane. This proton gradient would be enhanced by a severe reduction in GO activity which would result from a decrease reaction of the Calvin cycle to receive excess photon energy from PSII (Fig. 7). That is, an extreme decrease of GO levels could become a control point for the regeneration of the substrate for the Calvin cycle. When plants produce more photon energy under a higher light intensity, they need to scavenge more photon energy. These results provide a direct evidence that the glycolate pathway functions for maintenance of metabolites for the Calvin cycle after the oxygenase reaction of RuBisCO under low CO₂ concentration in mesophyll cells. Whether or not photoinhibition is enhanced is thought to be determined by the balance between the pool of the substrate for the Calvin cycle and the amount of photon energy from the photosystem, which is determined by the light intensity. ETR was not affected in the range between 500 and 800 µE s⁻¹ m⁻² irradiation (Fig. 7A). Under 800 µE s⁻¹ m⁻² irradiation, no relation between depression of GO activity and photoinhibition was observed (data not shown). These data sug-
gest that, under 800 μE s⁻¹ m⁻² irradiation, the limiting factor was not the GO activity, but a low ETR for the plants grown in weak light (30 μE s⁻¹ m⁻²).

Why does RuBisCO have oxygenase activity in plants? There is much discussion on the function of photorespiration. The oxygenase reaction of RuBisCO depends on the oxygen/ carbon dioxide ratio in mesophyll cells. Therefore, regeneration of the substrate for the Calvin cycle by the glycolate pathway is essential for the maintenance of the Calvin cycle. When each enzyme in the glycolate pathway decreases below a threshold level, an insufficiency of the substrate for the Calvin cycle causes photoinhibition. An inevitable consequence of the kinetic properties of RuBisCO leads to an increase of O₂ uptake with respect to the gas environments in mesophyll cells, and a decrease of the substrate for the Calvin cycle due to the decreased flux in the glycolate pathway. Our 'transformant series' can be used for further analyses of phenomena induced by depression of the glycolate pathway.

We are interested in the molecular mechanism responsible for microbody transformation from glyoxyosomes to leaf peroxisomes. The results obtained with the damaged phenotypes used in this study indicate that transformation to leaf peroxisomes at an appropriate time is a critical factor in plant growth and survival.

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