Variation in the Activity of Some Enzymes of Photorespiratory Metabolism in C₄ Grasses

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INTRODUCTION

In C₃ plants, about 30% of fixed CO₂ is released by photorespiration. This results from the reaction of oxygen with ribulose-1,5-bisphosphate (RuBP) catalysed by the oxygenase reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which generates glycolate-2-phosphate; this is metabolized to glycerate-3-phosphate in the glycolate metabolic pathway, involving three organelles (the chloroplasts, peroxisomes and mitochondria). During metabolism, CO₂ is released by the oxidation of glycine catalysed by the glycine decarboxylase (GDC) complex in the mitochondria (Douce and Heldt, 2000; Wringler et al., 2000). However, some plants have evolved mechanisms that reduce the magnitude of photorespiration. In the leaves of C₄ plants, for example, the photosynthetic function is a cooperative process between two cell types—mesophyll and bundle sheath cells. In the mesophyll cells, atmospheric CO₂ is primarily fixed into C₄ acids by phosphoenolpyruvate carboxylase. Subsequently, the C₄ acids are transferred into the bundle sheath cells and enzymatically decarboxylated there. Released CO₂ is refixed by Rubisco in the bundle sheath chloroplasts. Thus, the C₄ pathway functions as a mechanism for concentrating CO₂ for the C₃ cycle in the bundle sheath cells. As a result, the oxygenase reaction of Rubisco is greatly decreased, and photorespiration is reduced (Leegood and Walker, 1999; Edwards et al., 2001).

It is thought that C₄ plants evolved from C₃ plants primarily in response to a reduction in atmospheric CO₂ concentration (Ehleringer et al., 1997), and there is some evidence that photorespiration occurs in C₄ leaves, but at a slower rate than in C₃ leaves. For example, C₄ leaves have activities of enzymes involved in the glycolate pathway, though at lower rates than in C₃ leaves (e.g. Osmond and Harris, 1971; Ohnishi and Kanai, 1983). Incorporation of ¹⁴C- and ¹⁸O-labelled CO₂ and O₂ into intermediates of the glycolate pathway was observed in C₄ leaves (Lawlor and Fock, 1978; Farinera et al., 1984; de Veau and Burris, 1989). Using ¹⁸O₂ and mass spectrometry, it was demonstrated that C₄ leaves show considerable light-dependent O₂ uptake (Furbank and Badger, 1982, 1983). Simultaneous gas exchange and chlorophyll fluorescence measurements (Dai et al., 1993, 1995; Yoshimura et al., 2001) and studies on mutant C₄ plants with altered photo-synthesis (Lacuesta et al., 1997; Maroco et al., 1998) also demonstrated the capacity for photorespiration in C₄ leaves.

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However, it is still unclear how much photorespiration occurs in C₄ leaves because it is difficult to assess the photorespiratory capacity from measurements of carbon fluxes.

During evolution from C₃ plants to C₄ plants, some enzymes involved in the glycolate pathway were localized in specific cells, accompanied by the differentiation of tissues into Kranz anatomy (Leegood and Walker, 1999; Sage, 2004). In C₄ plants, the GDC complex is localized in the bundle sheath mitochondria (Ohnishi and Kanai, 1983; Farineau et al., 1984; Ueno, 2001), whereas glycerate kinase occurs only in the mesophyll chloroplasts (Usuda and Edwards, 1980). The peroxisomal enzymes of the glycolate pathway are distributed in both cell types, but unevenly (Ohnishi and Kanai, 1985; Popov et al., 2003).

C₄ plants are divided into three biochemical subtypes on the basis of differences in the mechanisms of decarboxylation of the C₄ acids: the NADP-malic enzyme (NADP-ME) subtype, the NAD-malic enzyme (NAD-ME) subtype, and the phosphoenolpyruvate carboxykinase (PCK) subtype (Leegood and Walker, 1999; Edwards et al., 2001). The grass family includes C₄ species of the three C₄ subtypes (Prendergast et al., 1987). The photorespiratory capacity of these C₄ subtypes has, however, not been investigated sufficiently, because the number of C₄ species previously examined for photorespiration is limited. Recently, Yoshimura et al. (2004) investigated whether there are specific quantitative differences in organelles involved in photorespiration and in the amounts of the P-protein of the GDC complex in the bundle sheath cells. There are differences in the abundance of mitochondria and peroxisomes in the bundle sheath cells relative to the mesophyll cells; both organelles were less abundant in NADP-ME species and more abundant in NAD-ME and PCK species with well-developed grana. Interestingly, there is a high positive correlation between the granal index (ratio of the length of appressed thylakoid membranes to the total length of all thylakoid membranes) in the bundle sheath plastids and the amount of GDC P-protein in the bundle sheath (Yoshimura et al., 2004). Chloroplast grana are the site where O₂ is produced through photosystem II (PSII) (Malkin and Niyogi, 2000). Thus, the degree of O₂ evolution from PSII and the O₂ and CO₂ partial pressures in the bundle sheath cells may differ among C₄ species. This suggests that variation among C₄ grasses in the structural and biochemical features involved in photorespiration might reflect differences in the O₂ and CO₂ partial pressures and in the potential photorespiratory capacity of the bundle sheath cells (Yoshimura et al., 2004).

It is hypothesized here that there are differences among various C₄ grasses in the activities of the peroxisomal enzymes of the glycolate pathway, glycolate oxidase (GO), hydroxypyruvate reductase (HPR) and catalase, related to the granal index. In this paper it is shown that C₄ grasses with reduced granal development in the bundle sheath plastids have smaller GO activities than C₄ grasses with well-developed grana, suggesting differences in photorespiration rates.

MATERIALS AND METHODS

Plant materials

To test the hypothesis that grana are related to potential for photorespiration, peroxisomal enzymes of the glycolate pathway, GO, HPR and catalase, were assayed in 28 C₄ species in the Poaceae: 11 NADP-ME species, seven NAD-ME species and ten PCK species (Table 1). Seven C₃ grass species were also examined for comparison. The C₄ species (excluding Arthraxon hispidus) were examined by electron microscopy. Shoots of Arundinella hirta and the two Zoysia species were collected in a field near Tsukuba, Japan. Shoots of the two Cynodon and four Brachia species were provided by Dr Y. Kawamoto (University of the Ryukyus, Okinawa, Japan). Shoots were planted in pots with 4.5-L of commercial soil mix for vegetables (Iseki, Tokyo, Japan). The other species were grown from seed in 4.5-L pots filled with the same soil. Plants were grown in a growth chamber at approx. 27°C during the light period (14 h) and 20°C in the dark (10 h) for 1–1.5 months and watered daily. Illumination was provided by metal-halide lamps at about 400 μmol m⁻² s⁻¹ (400–700 nm). Recently expanded mature leaves were used in the experiments.

Ultrastructural observation of the leaves

Two leaves were collected 4 h after the beginning of the light period. Segments from the middle of the leaf blades were immediately fixed in 3 % (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 1.5 h, then washed with phosphate buffer and post-fixed in 2 % (v/v) OsO₄ in phosphate buffer for 2 h. Subsequently, they were dehydrated through an acetone series and embedded in Spurr’s resin. Transverse ultrathin sections of the leaves were stained with lead citrate and observed under an electron microscope (Hitachi H-7100; Hitachi, Tokyo, Japan) at 75 kV.

To evaluate the degree of granal development, the granal index was measured for several chloroplasts of the bundle sheath cells from two leaves on electron micrographs at ×47000. The granal index value was divided into four ranks: <3 %, between 5 and 10 %, between 15 and 25 % and >45 % (−, +, ++ and +++ in Table 1, respectively). A preliminary examination was conducted to find out if there is a difference in the granal index between leaf positions in some C₄ grasses. No difference was found over the range of ranks between leaf positions.

Assay of enzyme activities

The middle of the leaf blades was collected from four plants 5–6 h after the beginning of the light period and immediately frozen in liquid nitrogen. The leaf samples (0.25 g f. wt) were ground with a pestle in a mortar (on ice) containing 0.5 g sea sand, 25 mg polyvinylpyrrolidone and 1 mL of grinding medium of 50 mM HEPES–KOH (pH 7.5), 0.2 mM EDTA, 2.5 mM MgCl₂, 2.5 mM MnCl₂, 5 mM dithiothreitol and 0.7 % (w/v) bovine serum albumin. Homogenates were filtered through gauze. The filtrates were centrifuged at 10000 g for 5 min at 4°C, and the supernatants used for the enzymatic assays.
All enzymes were assayed spectrophotometrically in 1 mL of reaction mixture at 25°C. The reaction mixture for the GO assay (EC 1.1.3.1) contained 33 mM TRIS–HCl (pH 7.8), 2.7 mM EDTA, 0.008% (v/v) Triton X-100, 3.3 mM phenylhydrazine, 0.67 mM oxidized glutathione-Na, 0.2 mM FMN and 5 mM glycine acid (Feierabend and Beevers, 1972). The reaction mixture for the HPR assay (EC 1.1.1.29) contained 25 mM sodium phosphate buffer (pH 5.8), 0.001% (v/v) Triton X-100, 0.2 mM NADH, 1 mM dithiothreitol and 20 mM sodium glyoxylate (Feierabend and Beevers, 1972). The reaction mixture for the catalase assay (EC 1.11.1.6) contained 50 mM potassium phosphate buffer (pH 7.2) and 10 mM H$_2$O$_2$ (Luck, 1965). The chlorophyll content was determined by the method of Arnon (1949).

### Table 1. Degree of granal development in the bundle sheath chloroplasts and activities of photorespiratory enzymes in the leaves of $C_3$ and $C_4$ grasses

<table>
<thead>
<tr>
<th>Photosynthetic type and species</th>
<th>Granal index in bundle sheath chloroplasts</th>
<th>Glycolate oxidase ($\mu$mol mg$^{-1}$ Chl h$^{-1}$)</th>
<th>Hydroxypropionate reductase ($\mu$mol mg$^{-1}$ Chl h$^{-1}$)</th>
<th>Catalase ($\times10^3$ $\mu$mol mg$^{-1}$ Chl h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NADP-ME–grana grasses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthrasax hispidus</td>
<td>–*</td>
<td>4.4 ± 1.1</td>
<td>307 ± 32</td>
<td>17.8 ± 1.1</td>
</tr>
<tr>
<td>Arundo donax</td>
<td>–</td>
<td>3.4 ± 0.6</td>
<td>398 ± 76</td>
<td>36.8 ± 3.7</td>
</tr>
<tr>
<td>Cynodon dactylon</td>
<td>+++</td>
<td>17.7 ± 0.5</td>
<td>369 ± 55</td>
<td>65.8 ± 6.5</td>
</tr>
<tr>
<td>C. flexuosa</td>
<td>+++</td>
<td>10.1 ± 0.3</td>
<td>257 ± 52</td>
<td>26.5 ± 2.1</td>
</tr>
<tr>
<td>Eragrostis curvula</td>
<td>+++</td>
<td>13.5 ± 1.0</td>
<td>310 ± 46</td>
<td>29.2 ± 3.4</td>
</tr>
<tr>
<td>E. ferruginea</td>
<td>+++</td>
<td>13.9 ± 0.7</td>
<td>274 ± 22</td>
<td>40.9 ± 4.0</td>
</tr>
<tr>
<td>E. poaeoides</td>
<td>+++</td>
<td>16.6 ± 1.3</td>
<td>248 ± 63</td>
<td>50.1 ± 4.2</td>
</tr>
<tr>
<td>Panicum coloratum ‘Solai’</td>
<td>+++</td>
<td>13.0 ± 1.3</td>
<td>552 ± 43</td>
<td>21.4 ± 8.0</td>
</tr>
<tr>
<td>P. dichotomiflorum</td>
<td>+++</td>
<td>12.8 ± 1.1</td>
<td>430 ± 44</td>
<td>29.3 ± 2.4</td>
</tr>
<tr>
<td><strong>PCK grasses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brachiaria brizantha</td>
<td>+++</td>
<td>20.7 ± 1.7</td>
<td>526 ± 60</td>
<td>34.8 ± 1.9</td>
</tr>
<tr>
<td>B. decumbens</td>
<td>+++</td>
<td>24.1 ± 6.7</td>
<td>434 ± 13</td>
<td>42.8 ± 3.2</td>
</tr>
<tr>
<td>B. humidicola</td>
<td>+++</td>
<td>14.3 ± 2.8</td>
<td>389 ± 20</td>
<td>36.6 ± 5.1</td>
</tr>
<tr>
<td>B. mutica</td>
<td>+++</td>
<td>13.4 ± 3.2</td>
<td>471 ± 82</td>
<td>31.5 ± 8.7</td>
</tr>
<tr>
<td>Chloris gayana</td>
<td>+++</td>
<td>18.4 ± 2.6</td>
<td>393 ± 74</td>
<td>31.1 ± 5.4</td>
</tr>
<tr>
<td>Panicum maximum</td>
<td>+++</td>
<td>13.4 ± 2.4</td>
<td>250 ± 8</td>
<td>21.7 ± 5.1</td>
</tr>
<tr>
<td>Sporobolus indicus</td>
<td>+++</td>
<td>18.1 ± 2.8</td>
<td>448 ± 62</td>
<td>33.9 ± 1.3</td>
</tr>
<tr>
<td>Urochloa texana</td>
<td>+++</td>
<td>14.2 ± 3.0</td>
<td>431 ± 76</td>
<td>25.3 ± 5.9</td>
</tr>
<tr>
<td>Zoysia japonica</td>
<td>+++</td>
<td>13.1 ± 2.1</td>
<td>267 ± 36</td>
<td>58.2 ± 3.7</td>
</tr>
<tr>
<td>Z. tenafolia</td>
<td>+++</td>
<td>11.9 ± 1.0</td>
<td>279 ± 55</td>
<td>29.6 ± 3.2</td>
</tr>
<tr>
<td><strong>C$_4$ grasses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avena sativa</td>
<td>ND</td>
<td>62.5 ± 7.7</td>
<td>321 ± 51</td>
<td>84.3 ± 7.2</td>
</tr>
<tr>
<td>Dactylis glomerata</td>
<td>ND</td>
<td>78.2 ± 14.1</td>
<td>611 ± 89</td>
<td>122.2 ± 29.8</td>
</tr>
<tr>
<td>Lolium perenne</td>
<td>ND</td>
<td>70.7 ± 3.7</td>
<td>428 ± 44</td>
<td>151.9 ± 28.1</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td>ND</td>
<td>116.0 ± 14.1</td>
<td>718 ± 63</td>
<td>175.7 ± 31.1</td>
</tr>
<tr>
<td>Oroza sativa</td>
<td>ND</td>
<td>106.3 ± 9.9</td>
<td>348 ± 95</td>
<td>140.4 ± 14.8</td>
</tr>
<tr>
<td>Panicum bisalcatum</td>
<td>ND</td>
<td>50.5 ± 5.5</td>
<td>587 ± 91</td>
<td>80.5 ± 16.7</td>
</tr>
<tr>
<td>Triticum aestivum</td>
<td>ND</td>
<td>128.0 ± 40.4</td>
<td>754 ± 15</td>
<td>155.1 ± 25.1</td>
</tr>
</tbody>
</table>

Granal index in bundle sheath chloroplasts: –, ++ and +++ indicate <3%, between 5 and 10%, between 15 and 25% and >5%, respectively. ND, not determined.

Enzyme activities are given as the means ± standard deviation of four plants.

* Data adapted from Ueno (1995).

#### Statistical analysis

Activities of the photorespiratory enzymes were expressed per unit of chlorophyll and given as the means and standard deviations of four plants. The significance was tested at a probability of $P < 0.05$ of any differences in mean enzyme activities between the $C_3$ and $C_4$ types and between the $C_4$ subtypes using one-way ANOVA with the Tukey and Kramer HSD test.

#### Results

**Ultrastructure of the bundle sheath cells in $C_4$ grasses**

It was confirmed that the general features of the bundle sheath structure in leaves of the $C_4$ grasses are as reported...
by Hattersley and Browning (1981), Prendergast et al. (1987) and Yoshimura et al. (2004) (reviewed in Dengler and Nelson, 1999). All the NADP-ME grasses had centrifugally located chloroplasts in the bundle sheath cells (data not shown). Of these six (A. hispidus, A. hirta, Coix lacryma-jobi, Saccharum officinarum, Sorghum bicolor and Zea mays) had chloroplasts with none (agranal) or only a few grana, and the granal indexes were <3% (the NADP-ME/grana grasses in Table 1 and Fig. 1A). In comparison, the other five NADP-ME grasses had relatively well-developed grana in the bundle sheath chloroplasts (the NADP-ME/+grana grasses in Table 1 and Fig. 1B and C). The granal indexes of Digitaria sanguinalis and Setaria italica were between 5 and 10%, and those of the remaining species were between 15 and 25%. Suberized lamellae occurred in their bundle sheath cell walls (Fig. 1B), except in Eriachne aristidea, in which the bundle sheath cell walls lacked this structure (Fig. 1C).

The NAD-ME grasses had centripetally located chloroplasts and PCK grasses had centrifugally located chloroplasts in the bundle sheath cells, together with abundant mitochondria (data not shown). In these grasses, the bundle sheath chloroplasts had well-developed grana, and the granal indexes were >45% (Table 1 and Fig. 1D). Lamellae in the bundle sheath cell walls of the PCK grasses (Fig. 1D) were suberized but not the NAD-ME grasses (data not shown).
Activities of the photorespiratory enzymes

Activities of the three photorespiratory enzymes assayed are shown for each species in Table 1. Table 2 shows the mean values of enzyme activities in the C4 and C3 grasses and the four C4 groups (including two in the NADP-ME subtype). Overall, the C4 grasses had significantly less GO activity than the C3 grasses (Table 2). The GO activities differed between the C4 grasses (Table 2). The NADP-ME–grana grasses had significantly lower GO activities than did the NADP-ME/+grana, NAD-ME and PCK grasses. In the NADP-ME/+grana grasses, E. aristidea had most GO activity (Table 1). HPR activities in the C4 and C3 grasses did not differ significantly, nor did the C4 groups (Table 2). Catalase activities were significantly lower in the C4 than in the C3 grasses but there was no significant difference between the C4 groups, although the mean catalase activity of the NADP-ME–grana grasses was somewhat lower than those of the other C4 groups (Table 2).

The C3 grasses showed considerably large species differences in the photorespiratory enzymes (Table 1). Oryza sativa and Panicum bisulcatum originated from warm and subtropical regions, whereas other C3 grasses are from temperate regions. However, no differences in the enzyme activities were found between the two groups.

DISCUSSION

C4 plants have generally been believed to have smaller photorespiratory capacity than C3 plants. Consequently, their photorespiratory enzymes have not been extensively investigated, although activities of some enzymes in a few C4 species have been measured (e.g. Osmond and Harris, 1971; Devi and Raghavendra, 1993). It was hypothesized that NADP-ME C4 grasses with reduced granal development in the bundle sheath chloroplasts, which have low PSII activity and O2/C02 partial pressure in the bundle sheath cells, might have reduced activities of the peroxisomal enzymes of the glycolate pathway. Thus, the activities of three peroxisomal enzymes in 28 C4 grasses were examined. In the peroxisomes, GO is involved in the conversion of glycolate to glyoxylate, accompanied by the formation of H2O2, which is decomposed by catalase (Douce and Heldt, 2000). Activities of these enzymes are smaller in C4 plants than in C3 plants (Devi and Raghavendra, 1993; Li et al., 2001), and this is confirmed by the present study. HPR is involved in the reduction of hydroxypropionate to glycerate in the glycolate pathway (Douce and Heldt, 2000). No significant difference in HPR activities was found between C3 and C4 grasses, although there was tendency for the activities of C4 grasses to be somewhat lower than those of C3 grasses, as reported by previous investigators (Kleczkowski et al., 1988; Devi and Raghavendra, 1993).

No reports of studies that extensively investigated the activities of photorespiratory enzymes in species in different C4 subtypes have been found. A previous study (Yoshimura et al., 2004) compared the amounts of GDC P-protein that occurs in the bundle sheath in seven species of C4 grasses, and it varied greatly depending on the granal index in the bundle sheath chloroplasts. Thus, it was suggested that there might be differences in the potential photorespiration capacity among the C4 grasses. It is now demonstrated that species with few or no grana in the bundle sheath chloroplasts (NADP-ME–/–grana) tended to have lower GO activities than C4 species with well-developed grana. This is probably related to the production of O2 and its use in Rubisco oxygenase activity in the bundle sheath: PSII is located in the grana, where O2 from photosynthesis is released (Malkin and Niyogi, 2000). Thus, these data support the suggestion (Yoshimura et al., 2004) that photorespiratory capacity might differ among C4 plants as a function of the CO2 and O2 partial pressures within the bundle sheath cells. Further investigation would be required to elucidate whether photorespiration has physiological significance in C4 plants (Lawlor and Fock, 1978), as has been found in C3 plants under stress conditions that depress internal CO2 concentration (Osmond and Grace, 1995; Wingler et al., 2000).

It is interesting to note that E. aristidea was the C4 grass with the highest GO activity. It has been classified in the NADP-ME subtype, but has unique structures in its bundle sheath cells (Prendergast et al., 1987; Taniguchi et al., 2003). The granal index of the bundle sheath chloroplasts of E. aristidea was the highest in the NADP-ME C4 grasses studied previously (Yoshimura et al., 2004). The chloroplasts are located centrifugally within the bundle sheath cells, as is the case in other NADP-ME grasses, but unlike other species in this subtype (Hattersley and Browning, 1981), there is no suberized lamella in the cell walls of the bundle sheath cells (Fig. 1C). Eriachne aristidea accumulates relatively large amounts of GDC P-protein in its bundle sheath mitochondria (Yoshimura et al., 2004).
Because suberized lamellae probably prevent leakage of CO$_2$ from the bundle sheath cells (Hattersley and Browning, 1981), those of $E. aristidea$ may be more ‘leaky’ for CO$_2$ than those of other NADP-ME grasses. It would be interesting to investigate whether this $C_4$ grass has a higher photorespiratory capacity than other NADP-ME $C_4$ grasses.

In $C_4$ grasses, peroxisomes are distributed in both the bundle sheath and mesophyll cells. In both PCK and NADP-ME grasses, however, peroxisomes occur more abundantly in the bundle sheath than mesophyll cells (Yoshimura et al., 2004). Ohnishi and Kanai (1985) reported that Panicum miliaceum (NAD-ME) has a higher GO activity in the bundle sheath peroxisomes than in the mesophyll peroxisomes. Popov et al. (2003) showed that in maize ($Z. mays$, NADP-ME), 21% and 79% of GO activity occurs in the mesophyll and bundle sheath cells, respectively. In addition, there are three GO isoenzymes in maize leaves: one specific to the bundle sheath cells, one specific to the mesophyll cells, and one in both cell types. Moreover, the kinetic properties differed between the isoenzymes specific to the bundle sheath and mesophyll cells (Popov et al., 2003). Leaves contain the cytosolic NADPH-dependent HPR as well as the peroxisomal NADH-dependent HPR examined in this study. The former HPR has been suggested to serve as a back-up reaction in the glycolate pathway recycling hydroxypyruvate lost from peroxisomes (Kleczkowski et al., 1988). In maize leaves ($C_4$), both HPRs are predominately localized in the bundle sheath cells, and high activity of NDPH-HPR, which is the upper range of values reported for $C_3$ leaves, is found in them (Kleczkowski and Edwards, 1989). Further comparative studies of GO and HPR would be required for $C_4$ grasses that differ in their photorespiratory capacity.

The present study demonstrates that there are specific differences in the activity of the peroxisomal enzyme GO as well as in the amount of the mitochondrial enzyme GDC among $C_4$ grasses. These differences are correlated with the degree of granal development in the bundle sheath chloroplasts, probably reflecting differences in the $O_2/CO_2$ partial pressure and the potential photorespiratory capacity in the bundle sheath cells.

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LITERATURE CITED


