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Photorespiration: metabolic pathways and their role in stress protection

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Photorespiration results from the oxygenase reaction catalysed by ribulose-1,5-bisphosphate carboxylase/oxygenase. In this reaction glycollate-2-phosphate is produced and subsequently metabolized in the photorespiratory pathway to form the Calvin cycle intermediate glycerate-3-phosphate. During this metabolic process, CO_2 and NH_3 are produced and ATP and reducing equivalents are consumed, thus making photorespiration a wasteful process. However, precisely because of this inefficiency, photorespiration could serve as an energy sink preventing the overreduction of the photosynthetic electron transport chain and photoinhibition, especially under stress conditions that lead to reduced rates of photosynthetic CO_2 assimilation. Furthermore, photorespiration provides metabolites for other metabolic processes, e.g. glycine for the synthesis of glutathione, which is also involved in stress protection. In this review, we describe the use of photorespiratory mutants to study the control and regulation of photorespiratory pathways. In addition, we discuss the possible role of photorespiration under stress conditions, such as drought, high salt concentrations and high light intensities encountered by alpine plants.

Keywords: drought stress; glutamate synthase; glutamine synthetase; glycine decarboxylase; hydroxypyruvate reductase; serine:glyoxylate aminotransferase

1. THE ORIGINS OF PHOTORESPIRATION

Photorespiration is a consequence of the oxygenation of ribulose-1,5-bisphosphate (RuBP) catalysed by RuBP carboxylase/oxygenase (rubisco). The ratio of the carboxylation rate (v_c) to the oxygenation rate (v_o) is dependent on the CO_2 and O_2 concentrations, the Michaelis constants for these gases (K_c and K_o) and the maximal velocities (V_c and V_o) (Farquhar *et al.* 1980): $v_c/v_o = V_c K_o/V_o K_c$ ([CO_2/O_2]), with the term $V_c K_o/V_o K_c$ defining the specificity factor of rubisco.

Using rubisco kinetics, it is possible to calculate the ratio of v_c to v_o and thereby estimate the rate of photorespiration at different CO_2 concentrations (Sharkey 1988). In order to calculate the rate of photorespiratory CO_2 release, it has to be taken into account that one CO_2 is released for every two oxygenation reactions (figure 1). Sharkey (1988) estimated that under ambient conditions, the rate of photorespiratory CO_2 release is about 25% of the rate of net CO_2 assimilation (A). With increasing temperatures, the specificity of rubisco for CO_2 decreases (Brooks & Farquhar 1985) and the solubility of CO_2 decreases relative to that of O_2, resulting in enhanced rates of photorespiration at high temperatures. Due to the high rates of photorespiratory CO_2 release, photorespiration is a wasteful process imposing a strong carbon drain on plants. When the rate of photorespiration becomes too high, for example, when the O_2 concentration is increased above the O_2 compensation point for a

given CO_2 concentration (Tolbert *et al.* 1995), photorespiration leads to a depletion of carbohydrates and to accelerated senescence. On the other hand, long-term growth in low O_2 (2 kPa) to suppress photorespiration appears to be detrimental to plants and results in decreased rates of photosynthetic CO_2 assimilation (measured in air), poor plant growth and alterations in the chloroplast structure (Migge *et al.* 1999). It has been suggested that photorespiration is important for energy dissipation to prevent photoinhibition (Osmond 1981; Osmond & Grace 1995; Osmond *et al.* 1997; Kozaki & Takeba 1996; Wu *et al.* 1991). In addition, photorespiration can generate metabolites, such as serine and glycine, which can be exported out of the leaf (Madore & Grodzinksi 1984) or used in other metabolic pathways, for example, provision of glycine for the synthesis of glutathione (Noctor *et al.* 1997, 1998, 1999). Since glutathione is a component of the antioxidative system in plants (Noctor & Foyer 1998), photorespiration may provide additional protection against oxidative damage in high light by supplying glycine. Thus, photorespiration, in addition to being wasteful, may also be a useful process in plants.

We have used a range of barley mutants with reduced photorespiratory enzyme activities to study the following aspects of photorespiratory metabolism: (i) the control exerted by photorespiratory enzymes on photosynthetic flux; (ii) the effect of photorespiratory metabolites on photosynthetic metabolism; (iii) regulation of the expression of photorespiratory enzymes; (iv) the occurrence of alternative photorespiratory pathways; and (v) the significance of photorespiration under stress conditions.
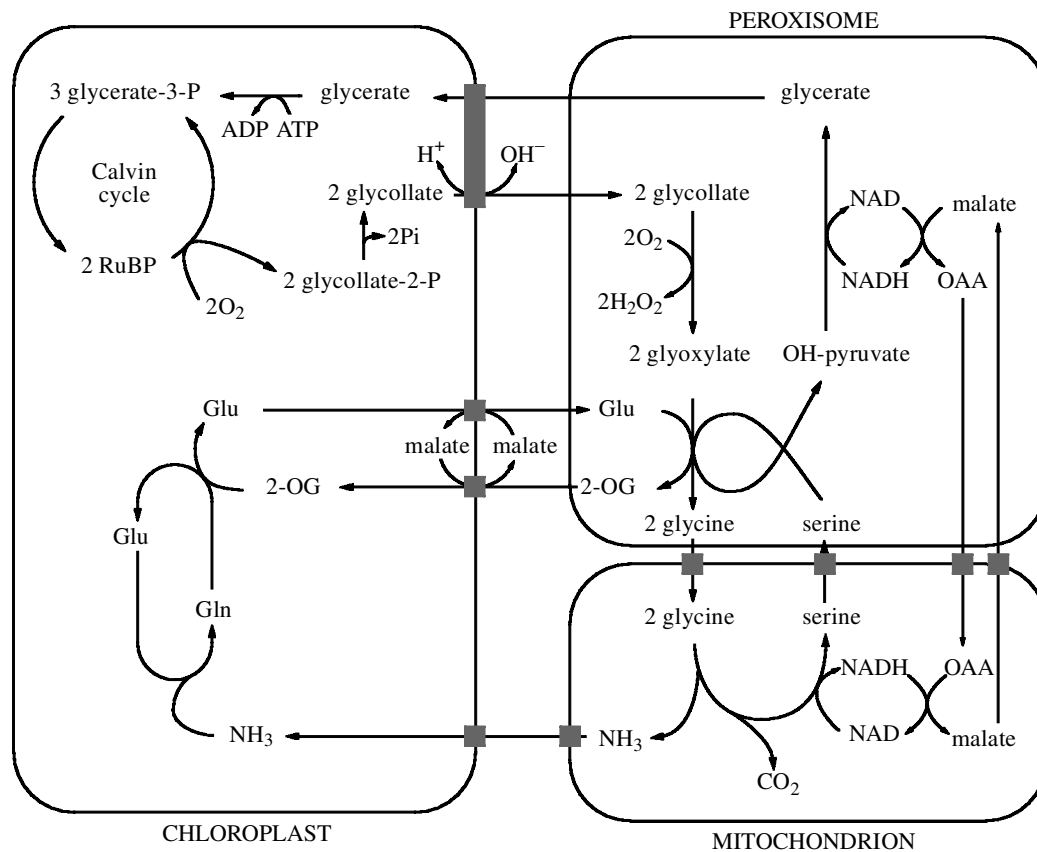
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Figure 1. Photorespiratory metabolism, shown with oxygenation of two molecules of RuBP. This oxygenation gives rise to two molecules of glycerate-3-P (C3) and two molecules of glycollate-2-P (C2). The latter are converted into a further molecule of glycerate-3-P in the photorespiratory cycle. Since one molecule of CO_2 is liberated by GDC, only nine carbons of the original ten carbons in the two molecules of RuBP (C5) are recycled in the Calvin cycle. Metabolite transporters are indicated by solid rectangles, though in certain cases these are speculative, as for NH_3 transport between organelles (although see Gazzarrini *et al.* 1999) and for glycine and serine transport across the mitochondrial membrane. Glu, glutamate; Gln, glutamine; 2-OG, 2-oxoglutarate; OAA, oxaloacetate.

2. PHOTORESPIRATORY METABOLISM

In the oxygenase reaction catalysed by rubisco, one molecule each of glycerate-3-phosphate and glycollate-2-phosphate are formed (figure 1). Seventy-five per cent of the carbon of glycollate-2-phosphate is recycled in the photorespiratory pathway (Leegood *et al.* 1995). In this pathway, glycollate-2-phosphate is first hydrolysed to glycollate by a chloroplastic phosphoglycollate phosphatase. After transport into the peroxisomes, glycollate is oxidized to glyoxylate by glycollate oxidase. Glyoxylate can be transaminated to glycine by serine:glyoxylate aminotransferase (SGAT) or by glutamate:glyoxylate aminotransferase. Half of the glycine molecules are converted to N^5,N^{10}-methylene tetrahydrofolate (THF) in the reaction catalysed by glycine decarboxylase (GDC) in the mitochondria. In this reaction, CO_2 and NH_3 are released. The other half of the glycine molecules can react with N^5,N^{10}-methylene THF in the serine hydroxymethyl-transferase (SHMT) reaction to form serine. After transport from the mitochondria to the peroxisomes, serine is converted by SGAT to hydroxypyruvate, which is reduced to glycerate by hydroxypyruvate reductase (HPR). Glycerate is then phosphorylated by glycerate kinase in the chloroplasts and the resulting glycerate-3-phosphate is converted to RuBP in the Calvin cycle. Due to the transamination of glyoxylate to glycine and the formation of NH_3 in the GDC reaction, photorespiratory carbon metabolism is intimately linked to nitrogen metabolism in the leaf. Since photorespiration proceeds at very high rates, it has been estimated that the production of NH_3 by photorespiration is an order of magnitude greater than the primary assimilation of nitrogen resulting from nitrate reduction (Keys *et al.* 1978). Therefore, the reassimilation of photorespired NH_3 by plastidic gluta-mine synthetase (GS-2) and ferredoxin-dependent gluta-mate synthase (Fd-GOGAT) is essential for maintaining the nitrogen status in plants. This process is necessarily very efficient: the rate of NH_3 emission in wild-type barley is about 0.01% of the rate of photorespiratory NH_3 release (Mattson *et al.* 1997), assuming a rate of photo-respiratory CO_2 release of 25% of *A*.

(a) *The use of mutants to study the control of photorespiratory metabolism*

The involvement of most of the enzymes in photo-respiratory metabolism has been confirmed by work with the respective mutants. A mutant screen was first devised by Somerville & Ogren (1979) to isolate photorespiratory mutants of *Arabidopsis*. It is based on the fact that

photorespiratory mutants are conditional lethals. In high (> 0.2%) CO_2, photorespiration is suppressed and the growth of the photorespiratory mutants is indistinguishable from the wild-type. However, when the mutants are transferred from high CO_2 into air, they show severe symptoms of stress, such as chlorosis. After transferring the plants back into high CO_2, photorespiratory mutants recover. This method was also used to isolate photorespiratory mutants of barley (Kendall *et al*. 1983). Over the years, *Arabidopsis*, barley, tobacco and pea mutants with mutations in a large range of photorespiratory enzymes and transporters (phosphoglycollate phosphatase, catalase, SGAT, GDC, SHMT, NADH-dependent HPR, GS-2, Fd-GOGAT, dicarboxylate transport) have been isolated (for reviews, see Somerville 1986; Blackwell *et al*. 1988; Leegood *et al*. 1995).

While, in the long term, the homozygous photorespiratory mutants are not viable at ambient CO_2 concentrations, heterozygotes can be grown in air. We have used heterozygous mutants of GS-2, Fd-GOGAT, GDC and SGAT to study the control exerted by photorespiratory enzymes on photosynthetic and photorespiratory metabolism (Häusler *et al*. 1994*a,b*, 1996; Wingler *et al*. 1997, 1999*a,b*). The concept of control theory was developed by Kacser & Burns (1973). They showed that the control of flux through a metabolic pathway is shared by the enzymes of the pathway and that for each enzyme a control coefficient (the fractional change in the flux through a pathway divided by the fractional change in the amount of enzyme) can be calculated. As it is difficult to quantify the rate of photorespiration, the control exerted by photorespiratory enzymes on photorespiration cannot easily be determined. Accumulation of substrates of the respective reactions can, however, serve as a first indication for an impairment of flux through the photorespiratory pathway. In addition, restrictions on oxygenation of RuBP should also affect carboxylation, so that photosynthetic flux should be decreased in a manner similar to the photorespiratory flux. The effects of reduced photorespiratory enzyme activities can be expected to be most severe under conditions that lead to a high rate of oxygenation of RuBP. Such conditions are high light, low external CO_2 concentrations, high external O_2 concentrations, high temperatures and stress conditions that lead to stomatal closure and a decline in intercellular CO_2 concentrations (C_i). We, therefore, studied the effect of a variety of conditions on the performance of photorespiratory barley mutants.

In contrast to the homozygous mutants, heterozygotes show only minor effects of the lowered enzyme activities on photosynthesis. Of the heterozygous mutants studied, the decrease in photosynthesis was most severe in plants with reduced activities of Fd-GOGAT (Häusler *et al*. 1994*b*). Even in moderate light and in ambient CO_2, these plants exhibited reduced rates of CO_2 assimilation. In plants with reduced activities of GDC (Wingler *et al*. 1997) and GS-2 (Häusler *et al*. 1994*b*), the effect on CO_2 assimilation was negligible in ambient CO_2 but became more severe in low CO_2. Plants with reduced activities of SGAT, on the other hand, did not show a significant reduction in CO_2 assimilation, even when photosynthesis was measured under conditions of high rates of photorespiration (high light and low CO_2; Wingler *et al*. 1999*a*).

However, the rates of CO_2 assimilation were reduced compared with the wild-type, when the stomata closed during moderate drought stress (Wingler *et al*. 1999*b*). Therefore, photorespiratory enzymes, such as SGAT and GDC, that are 'in excess' under normal growth conditions, can, in the long term, exert appreciable control under stress conditions that lead to increased rates of photorespiration.

In plants with reduced photorespiratory enzyme activities, the following alterations could lead to reduced rates of photosynthesis: (i) an impairment of the recycling of the carbon in the photorespiratory pathway could result in a depletion of Calvin cycle metabolites; (ii) an impairment of photorespiratory nitrogen reassimilation could result in a decline in the nitrogen status of the leaf and a reduction in the amount of photosynthetic proteins; and (iii) accumulation of photorespiratory metabolites could have a feedback effect on Calvin cycle activity.

(i) First indications that reduced rates of photosynthesis in homozygous photorespiratory mutants are due to a depletion of metabolites were obtained by providing carbon and nitrogen in metabolites that cannot be formed at sufficient rates in the mutants. For example, the supply of glutamine to a GS-2 mutant of barley (Blackwell *et al*. 1987), of serine to a GDC mutant of barley (Blackwell *et al*. 1990) or of sucrose to cell cultures of an SGAT mutant of *Nicotiana sylvestris* (McHale *et al*. 1989) partially restored photosynthetic activity. However, the pools of RuBP, which should directly affect photosynthesis, appear to be very stable in the mutants. Transfer of the homozygous barley mutant lacking GS-2 from high CO_2 into air did not lead to a decline in RuBP content (Leegood *et al*. 1995), and heterozygous mutants with reduced activities of GS-2, SGAT or GDC did not contain less RuBP than wild-type plants (Wingler *et al*. 1999*b*). On the other hand, other metabolites of the Calvin cycle, such as fructose-1,6-bisphosphate did decrease in the mutants, indicating a feedback regulation of rubisco activity.

(ii) Since the photorespiratory pathway and nitrogen assimilation are closely linked, one might expect that reduced photorespiratory enzyme activities could lead to a depletion of metabolically available nitrogen. Clear alterations in nitrogen metabolism became apparent in the heterozygous photorespiratory mutants: in the Fd-GOGAT mutants, the content of glutamine increased while the content of glutamate decreased (Häusler *et al*. 1994*a*), in the GS-2 mutants NH_3 production increased while the content of glutamine decreased (Häusler *et al*. 1994*a*; Mattson *et al*. 1997), in the SGAT mutants the content of serine increased (Wingler *et al*. 1999*a,b*) (figure 2*c*), and in the GDC mutants the content of glycine increased under conditions of high photorespiratory flux (Wingler *et al*. 1997, 1999*b*). In plants with reduced activities of GS-2 and, more severely, in plants with reduced activities of Fd-GOGAT, the protein content in the leaves and the total activity of rubisco were reduced. It is, therefore, likely that the reduced rates of photosynthesis in plants with reduced Fd-GOGAT activities were partly caused by

Figure 3. Western blot for H-protein of the GDC complex. H-protein was detected using an antiserum against H-protein from wheat (provided by J. Lorang and T. Wolpert, Oregon State University, USA). For leaves of the homozygous GDC mutant of barley (gdc) and the roots of wild-type barley (wt root) 1 mg fresh weight was loaded, for leaves of wild-type barley (wt) 0.1 mg fresh weight (1:10) and 0.01 mg fresh weight (1:100) were loaded.
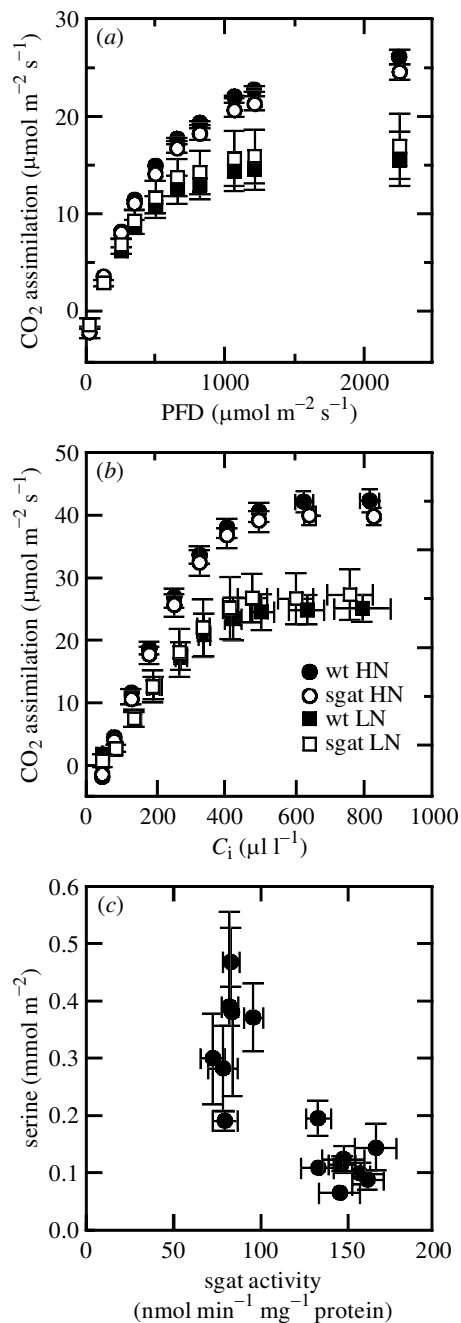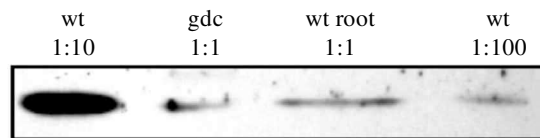
Figure 2. Effect of high and low nitrogen supply on wild-type barley (wt) and heterozygous mutants with reduced activities of SGAT (sgat). The plants were grown in a glasshouse with high ($5\,mM\ NO_3^-$, HN) or low ($0.5\,mM\ NO_3^-$, LN) nitrogen supply. (*a*) Relationship between the rate of CO_2 assimilation and photon flux density (PFD) measured in $350\,\mu l^{-1}\ CO_2$ ($n = 3$–4 lines $\pm$ s.e.). (*b*) Relationship between the rate of CO_2 assimilation and C_i measured at a PFD of $1213\,\mu mol\,m^{-2}\,s^{-1}$ ($n = 3$–4 lines $\pm$ s.e.). (*b*) Serine contents in plants grown with low ($0.5\,mM\ NO_3^-$) nitrogen supply ($n = 4$ leaves $\pm$ s.e.).

lower amounts of photosynthetic enzymes due to a reduced availability of nitrogen. Plants with reduced activities of SGAT or GDC, did not, however, exhibit changes in the total protein content. In order to increase the possibility of a depletion of physiologically available nitrogen, plants with reduced SGAT activities were grown with a low supply of nitrogen. Under these conditions, the protein content

was reduced to the same extent as in wild-type plants and there was no effect of reduced SGAT activities on photosynthesis (figure 2*a*,*b*), even though serine also accumulated under these conditions (figure 2*c*).

(iii) An accumulation of metabolites could also directly lead to a feedback regulation of photosynthetic activity. This topic has been extensively discussed by Leegood *et al.* (1995, 1996). There is little evidence that NH_3 accumulating in the GS-2 mutants directly inhibits photosynthesis by uncoupling of photosynthetic electron transport. This was shown by feeding glutamate to homozygous GS-2 mutants (Blackwell *et al.* 1987). Supply of external glutamate increased the accumulation of NH_3, while, at the same time, it restored photosynthesis to wild-type rates. Accumulation of serine is also unlikely to inhibit photosynthesis. In cell cultures of the *Nicotiana sylvestris* mutant lacking SGAT, an almost ninefold increase in serine had no effect on photosynthesis when sucrose was supplied as a carbon source to prevent the depletion of carbon stores (McHale *et al.* 1989). Of the metabolites we measured in the mutants, glyoxylate is the most likely to exert a feedback effect on photosynthesis. In *in vitro* studies, it had been shown that glyoxylate can inhibit the activation of rubisco (Campbell & Ogren 1990). Using heterozygous GS-2 mutants, Häusler *et al.* (1996) have shown that there is a negative relationship between the glyoxylate content in the leaves and the activation state of rubisco, indicating that glyoxylate can act as a feedback inhibitor of photosynthesis *in vivo*. Other photorespiratory metabolites that have been shown to inhibit enzymes of the Calvin cycle are glycerate, which inhibits fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase (Schimkat *et al.* 1990), and glycollate-2-phosphate, which inhibits triose-phosphate isomerase (Anderson 1971). In addition to inhibiting activities of certain enzymes, it has been proposed that photorespiratory metabolites might also act as signals in the regulation of the expression of photorespiratory and other enzymes.

3. REGULATION OF THE EXPRESSION OF PHOTORESPIRATORY ENZYMES

Expression of most of the photorespiratory enzymes, i.e. glycollate oxidase, catalase, HPR, SGAT, P-, H- and
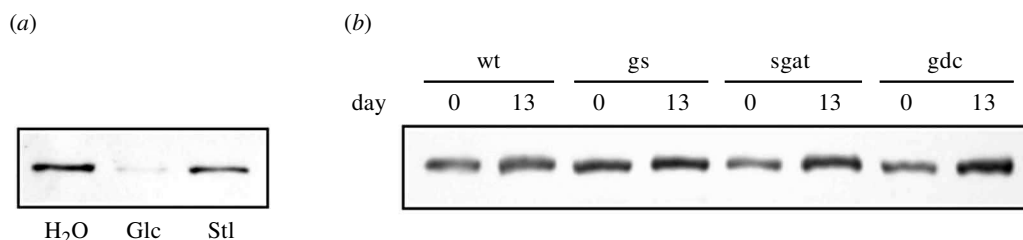
Figure 4. Western blots for NADH-dependent HPR. NADH-dependent HPR was detected using an antiserum against the NADH-dependent HPR of spinach (Kleczkowski *et al.* 1990). (*a*) Effect of glucose on the amount of NADH-dependent HPR. Discs of tobacco leaves were incubated for ten days floating on water, 50 mM glucose (Glc) or 50 mM sorbitol (Stl). They were kept at $23\,^{\circ}\mathrm{C}$ and cycles of 16 h of light ($20\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$) and 8 h of darkness. A leaf area of $3.3\,\mathrm{mm^2}$ was loaded per lane. (*b*) Effect of drought stress on the amount of NADH-dependent HPR in wild-type barley (wt) and heterozygous mutants with reduced activities of plastidic GS (gs), SGAT (sgat) or GDC (gdc), 0 and 13 days after withholding water. The plants were grown in cycles of 12 h light ($460\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$) at $24\,^{\circ}\mathrm{C}$ and 12 h darkness at $20\,^{\circ}\mathrm{C}$. Eight micrograms of protein were loaded per lane.

T-proteins of the GDC complex and SHMT, is induced by light (Raman & Oliver 1997). Expression of the sub-units of the GDC complex has been studied in detail. GDC is a mitochondrial multi-enzyme complex cata-lysing the conversion of glycine, NAD and THF into CO_2, NH_3, NADH and N^5,N^{10}-methylene THF (Douce & Neuburger 1999). The GDC complex is formed from P-, H-, T- and L-proteins with an approximate stoichiometry of 2 P-protein dimers:27 H-protein monomers:9 T-protein monomers:1 L-protein dimer. In wheat leaves, the amounts of P-, H- and T-proteins were highest in the leaf tip and declined towards the base (Rogers *et al.* 1991), showing that their expression is under developmental control. Only the L-protein, which is also a component of the pyruvate dehydrogenase complex, was already present in the leaf base. In pea, the amounts of P-, H- and T-proteins increased when etiolated seedlings were transferred to the light (Turner *et al.* 1993). During the development of pea leaves, the expression of the genes encoding the P-, H- and T-proteins of the GDC complex was coordinated with the expression of the rubisco *rbcS* genes (Vauclare *et al.* 1996). However, the mRNAs encoding the GDC proteins peaked before the GDC complex was formed while, at this stage, rubisco was already active. This implies that post-transcriptional regulation controls the formation of the GDC complex, possibly with a photorespiratory metabolite, such as glycine, acting as a signal. In mutants with reduced activities of GDC, we have shown that the amount of P-protein was reduced in plants that had a content of H-protein that was lower than 60% of wild-type contents, while the amounts of T- and L-proteins were normal (Blackwell *et al.* 1990; Wingler *et al.* 1997). This indicates that the mutation in this GDC mutant is probably in a gene encoding H-protein and that the synthesis of P-protein is also regulated downwards, when the formation of functional GDC complexes is limited by the avail-ability of H-protein. After very long exposure, H-protein was also visible in the leaves of the GDC mutant on Western blots (figure 3). Dilution of extracts from wild-type leaves revealed that the amount in the mutant was about 1% of that in the wild-type. There was no differ-ence in the content of H-protein in the roots of the GDC mutant compared with the wild-type. These results suggest that, in addition to the photorespiratory gene for

H-protein, barley contains a second gene which is constitutively expressed in roots and leaves, but its func-tion is unknown.

The other enzyme whose expression has been inten-sively analysed is NADH-dependent HPR. Induction of the expression of the HPR gene in cucumber by light involves a phytochrome-dependent component (Bertoni & Becker 1993). In the dark, expression of the HPR gene can be induced by cytokinin (Chen & Leisner 1985; Andersen *et al.* 1996). This induction has been shown to be, at least in part, due to transcriptional regulation. It has also been suggested that photorespiratory metabolites have an effect on the expression of the HPR gene. When photorespiration in cucumber plants was suppressed in high CO_2, the HPR mRNA decreased (Bertoni & Becker 1996). There was, however, no effect of high CO_2 on HPR activity in pea (Thibaud *et al.* 1995).

In tobacco, we found that cytokinin can prevent the senescence-dependent decline in HPR protein (Wingler *et al.* 1998). This was observed in naturally senescing plants, which endogenously produced cytokinin by over-expression of isopentenyl transferase, as well as in leaf discs floating on cytokinin-containing solutions. Glucose, on the other hand, accelerated the decline in HPR protein (figure 4*a*), and it also overrode the effect of cyto-kinin. Thus, the reduced expression of the HPR gene observed in high CO_2 (Bertoni & Becker 1996) could be part of the response of plants to sugar accumulation described as sugar-mediated gene expression (Koch 1996). In contrast, the increase in sugar contents observed in drought-stressed barley (Wingler *et al.* 1999*b*) did not lead to a decline in HPR protein. Instead, the amount of HPR protein increased in drought-stressed leaves (figure 4*b*). This was also the case in the SGAT and the GDC mutants, suggesting that either a general drought-related signal or a metabolite formed in the photorespira-tory pathway before the GDC reaction (e.g. glycollate) could have acted as the signal.

In contrast to their cytosolic counterparts, the expres-sion of the plastidic isoforms of GS and GOGAT (GS-2 and Fd-GOGAT) is not strongly regulated by nitrogen supply but is highly responsive to light (Hecht *et al.* 1988; Migge *et al.* 1996; Migge & Becker 1996). An involvement of photorespiratory signals in the regulation of the expression of GS-2 has been suggested by Edwards &

Coruzzi (1989), because suppression of photorespiration in 2% CO_2 led to a decrease in the GS-2 mRNA in pea. Cock *et al.* (1991), however, were not able to detect any short-term effect on the GS-2 mRNA, when *Phaseolus vulgaris* plants grown in high CO_2 were transferred into air, as would have been expected if the expression was under photorespiratory control. Only in the long term, did plants growing in high CO_2 show a lower expression of GS-2 than air-grown plants. Growth of *Arabidopsis* or tobacco plants at a CO_2 concentration of 0.3%, which is probably high enough to suppress photorespiration, did not affect the amount of GS-2 mRNA compared with plants grown in air (Beckmann *et al.* 1997; Migge *et al.* 1997). Equally, there was no effect on Fd-GOGAT mRNA in tobacco. An involvement of photorespiratory metabolites in the expression of GS-2 or Fd-GOGAT is therefore questionable.

4. PHOTORESPIRATION IN C_4 PLANTS

In C_4 plants, CO_2 is first fixed as bicarbonate by phospho*enol*pyruvate carboxylase (PEPC) in the mesophyll cells, resulting in the formation of the C_4 acids malate or aspartate. After transport of these acids to the bundle sheath cells, where rubisco is located, CO_2 is released in reactions catalysed by NADP-malic enzyme, NAD-malic enzyme or phospho*enol*pyrurate carboxylunase. Because of the high resistance of the walls of bundle sheath cells to the diffusion of CO_2, the CO_2-pumping mechanism of C_4 plants results in a high CO_2 concentration at the site of rubisco and, consequently, in a strongly reduced rate of RuBP oxygenation. There are, however, indications that photorespiration also occurs in C_4 plants. It has been shown that high concentrations of O_2 can inhibit photosynthesis, and this inhibition becomes more severe when the external CO_2 concentration is reduced below ambient. The optimum partial pressure of O_2 for photosynthesis in C_4 plants is, however, higher (5–10 kPa) (Dai *et al.* 1993, 1995, 1996; Maroco *et al.* 1997) than in C_3 plants (about 2 kPa) due to a greater demand for ATP. The oxygen sensitivity of C_4 photosynthesis is dependent on the plant species and on the leaf age. Dai *et al.* (1995) showed that photorespiration, as indicated by the inhibition of photosynthesis at supraoptimal O_2 concentrations, is higher in young and senescing than in mature leaves of maize. Within the NADP-malic enzyme and the NAD-malic enzyme subtypes of C_4 plants, the dicotyledon species tend to have higher rates of photorespiration than the monocotyledon species (Maroco *et al.* 1997). This is probably due to a higher degree of bundle sheath leakiness for CO_2 in the dicotyledons. For the NAD-malic enzyme type *Amaranthus edulis*, the rate of photorespiration was estimated by measuring NH_3 production in the presence of the GS inhibitor phosphinothricin in air compared with 0.7% CO_2 (Lacuesta *et al.* 1997). It was calculated that the rate of photorespiration accounted for 6% of net photosynthesis.

By isolating photorespiratory mutants of *A. edulis*, Dever *et al.* (1995) obtained further evidence that photorespiration occurs to a significant extent in this C_4 species. In a screen similar to that devised by Somerville & Ogren (1979) for the isolation of photorespiratory mutant of *Arabidopsis*, it was possible to isolate *A. edulis*

mutants of the C_4 photosynthetic pathway, as well as mutants lacking the ability to metabolize photorespiratory glycine. Similar to photorespiratory mutants of C_3 plants, these glycine accumulators are only viable in high CO_2. In air, they did not only accumulate high concentrations of glycine, but also its precursor in photorespiratory metabolism, glyoxylate (Wingler *et al.* 1999c). Furthermore, the production of photorespiratory NH_3, which is formed in the GDC reaction, was strongly reduced in these mutants (Lacuesta *et al.* 1997). On the other hand, the rate of photorespiration calculated from the rate of NH_3 production was increased to 48% of net photosynthesis in an *A. edulis* mutant lacking the C_4 enzyme PEPC (Lacuesta *et al.* 1997), probably because CO_2 was directly assimilated by rubisco. An increased sensitivity to O_2 (Maroco *et al.* 1998a) and a lower efficiency of electron transport for CO_2 assimilation (Maroco *et al.* 1998b) also indicated an increased rate of photorespiration in the PEPC mutant. The results obtained for the *A. edulis* mutants clearly demonstrate that, even though C_4 cycle activity leads to a reduction in the rate of photorespiration, this process is not completely abolished in C_4 plants.

5. THE FLEXIBILITY OF PHOTORESPIRATORY METABOLISM

The photorespiratory carbon and nitrogen cycles are not completely closed (Givan *et al.* 1988) and photorespiratory metabolites can be provided as substrates for other processes (Keys 1999). Glycine produced in the photorespiratory pathway can, for example, be used for the synthesis of glutathione (Noctor *et al.* 1997, 1998, 1999) or be exported out of the leaves (Madore & Grodzinski 1984). Some flexibility in photorespiratory nitrogen metabolism is achieved by the use of alternative amino donors. In addition to glutamate and serine, alanine and asparagine provide amino nitrogen for the synthesis of glycine (Betsche 1983; Ta & Joy 1986). This is due to the lack of specificity of the respective aminotransferases. Glutamate:glyoxylate aminotransferase also uses alanine (Nakamura & Tolbert 1983), and SGAT also catalyses asparagine:glyoxylate and serine:pyruvate aminotransferase reactions (Murray *et al.* 1987).

There are several possible pathways for the metabolism of glyoxylate (Igamberdiev 1989). For example, glyoxylate can be reduced back to glycollate by an NADPH-dependent HPR or an NADPH-dependent glyoxylate reductase (Tolbert *et al.* 1970; Kleczkowski *et al.* 1986, 1990) or oxidized to oxalate (Richardson & Tolbert 1961; Halliwell & Butt 1974). Glyoxylate can also be oxidatively decarboxylated to formate (Zelitch 1972; Igamberdiev *et al.* 1999). This decarboxylation of glyoxylate is probably a non-enzymatic reaction involving the H_2O_2 formed in the glycollate oxidase reaction (Halliwell & Butt 1974; Grodzinski 1978; Oliver 1979; Walton & Butt 1981). Since H_2O_2 is usually rapidly degraded by catalase, the rate of CO_2 formation by glyoxylate decarboxylation is likely to be low compared with the rate of CO_2 formation by GDC (Oliver 1981; Walton 1982; Yokota *et al.* 1985a), but assumes importance in mutants lacking SGAT (Murray *et al.* 1987), SHMT (Somerville & Ogren 1981) or in plants with decreased catalase activities (Brisson *et al.* 1998).

Table 1. *Energy requirement of photosynthesis and photorespiration*

(Oxygenation plus carboxylation for $v_c = 1.25$, $v_o = 0.5$ and $A = 1$. $1.25(3\ \mathrm{ATP} + 2\ \mathrm{NADPH}) + 0.5\ (3.25\ \mathrm{ATP} + 2\ \mathrm{NADPH}) = 5.375\ \mathrm{ATP} + 3.5\ \mathrm{NADPH}$.)

	carboxylation	oxygenation
phosphorylation of glycerate	—	0.5 ATP
reassimilation of NH_3	—	0.5 ATP + 1 Fd_{red} (0.5 NADPH)
Calvin cycle	3 ATP + 2 NADPH	2.25 ATP + 1.5 NADPH
sum	3 ATP + 2 NADPH	3.25 ATP + 2 NADPH

Formate can be activated by reacting with THF in the Cl-THF synthase pathway, which provides one-carbon units for the synthesis of purines, thymidylate, methionine and formylmethionyl-tRNA (Cossins & Chen 1997). The enzymes involved in the Cl-THF synthase pathway in plants are a monofunctional N^{10}-formyl-THF synthetase (Nour & Rabinowitz 1991, 1992) and a bifunctional N^5,N^{10}-methylene-THF dehydrogenase:N^5,N^{10}-methenyl-THF cyclohydrolase (Kirk *et al.* 1995). The reactions catalysed by these enzymes result in the formation of N^5,N^{10}-methylene-THF from formate. Since N^5,N^{10}-methylene-THF is incorporated into serine in the SHMT reaction, formate, instead of the N^5,N^{10}-methylene-THF produced in the GDC reaction, can be used as an alternative substrate for the formation of serine (Gifford & Cossins 1982*a,b*; Prabhu *et al.* 1996). Together with the Cl-THF synthase/SHMT pathway, the oxidative decarboxylation of glyoxylate to formate could, therefore, form a GDC-independent bypass to the normal photorespiratory pathway, as shown for *Euglena gracilis* (Yokota *et al.* 1985*b*).

By using homozygous GDC mutants of barley and *A. edulis*, we studied the significance of such a bypass in higher plants (Wingler *et al.* 1999*c*). In contrast to wild-type plants, the mutants showed a light-dependent accumulation of glyoxylate and formate, which was suppressed in high (0.7%) CO_2. After growth in air, the activity and amount of N^{10}-formyl-THF synthetase were increased in the mutants compared with the wild-types. A similar increase in N^{10}-formyl-THF synthetase was induced when leaves were incubated with glycine under illumination, but not in the dark. In addition, the barley mutant was capable of incorporating [^{14}C]formate and [2-^{14}C]glycollate into serine. Since the GDC activity in the mutant (1% of wild-type activity; Wingler *et al.* 1997) (figure 3) was too low to support the rate of serine formation from glycollate, the formation of serine must have occurred via a GDC-independent pathway. Together, these results indicate that the mutants are able to bypass the normal photorespiratory pathway by oxidative decarboxylation of glyoxylate and formation of serine from formate, thereby partially compensating for the lack of GDC activity. The advantage of this alternative photorespiratory pathway is the absence of NH_3 release. In wild-type plants, the release of photorespiratory NH_3 is, however, very low ($< 0.5\ \mathrm{nmol\,m^{-2}\,s^{-1}}$) (Mattsson *et al.* 1997), because NH_3 is efficiently reassimilated by GS-2. In barley mutants with reduced activities of GS-2, on the other hand, the alternative pathway could be the mechanism by which the loss of nitrogen as NH_3 would be reduced (Häusler *et al.* 1996).

6. ENERGY REQUIREMENT OF PHOTORESPIRATORY METABOLISM

Oxygenation of 1 mol RuBP results in the formation of 1 mol glycerate-3-phosphate and 1 mol glycollate-2-phosphate, which is converted into 0.5 mol glycerate-3-phosphate in the photorespiratory pathway (figure 1). This conversion requires 0.5 mol ATP for the phosphorylation of glycerate to glycerate-3-phosphate and 0.5 mol ATP and 1 mol reduced Fd (Fd_{red}; equivalent to 0.5 mol NADPH) for the reassimilation of NH_3 in the GS-GOGAT cycle (table 1). Furthermore, the formation of RuBP from the 1.5 mol glycerate-3-phosphate formed per oxygenation requires 2.25 mol ATP and 1.5 mol NADPH. In total, 3.25 mol ATP and 2 mol NADPH are consumed per oxygenation. Since 0.5 mol CO_2 is released per mol of O_2 fixed and assuming a photorespiratory CO_2 release of 25% of A (Sharkey 1988), the v_o equals 0.5 when the v_c is 1.25 and when A is 1. In the presence of photorespiration, the consumption of ATP and reducing equivalents per CO_2 fixed (5.375 mol ATP and 3.5 mol NADPH) is clearly higher than in the absence of photorespiration (3 mol ATP and 2 mol NADPH). When photosynthetic CO_2 assimilation is reduced because of stomatal closure the ratio of v_o to v_c rises and the energy requirement for CO_2 assimilation becomes even higher. In addition, the CO_2 released during photorespiration can be partially reassimilated, which would result in an additional energy sink.

For the alternative photorespiratory pathway involving oxidative decarboxylation of glyoxylate and formation of serine from formate, the carbon balance is the same as in the normal pathway. In this pathway, 0.5 mol ATP are required in the 10-formyl-THF synthetase reaction, and 0.5 mol NADPH in the 5,10-methylene-THF dehydrogenase reaction. This requirement is equivalent to the consumption of 0.5 mol ATP and 1 mol Fd_{red} for the reassimilation of NH_3 in the normal photorespiratory pathway. In contrast to the normal pathway, the alternative pathway does not include the GDC reaction, which can provide the NADH for the reduction of hydroxypyruvate (Hanning & Heldt 1993), so that additional reducing equivalents are required in the HPR reaction.

7. PROTECTION AGAINST STRESS

Because of the high energy requirement of photorespiratory metabolism, it has been suggested that photorespiration is important for maintaining electron flow to prevent photoinhibition under stress conditions (Osmond

1981; Wu *et al.* 1991; Osmond & Grace 1995; Kozaki & Takeba 1996; Osmond *et al.* 1997). In the main, two different approaches have been employed to study the contribution of photorespiration to the protection of the photosynthetic apparatus. (i) Photorespiration has been reduced at the site of rubisco by reducing the O_2 concentration to between 1% and 2%. At these O_2 concentrations, the Mehler reaction is still active, while, due to its higher K_m, the oxygenase reaction of rubisco is strongly reduced (Osmond 1981). (ii) In other studies, the role of photorespiration has been analysed in plants in which photorespiratory enzyme activities were reduced by feeding inhibitors, by mutagenesis or by antisense transformation. Since photorespiration *per se* (i.e. oxygenation of RuBP and formation of glycollate-2-phosphate) is not directly affected by reducing the activities of enzymes of the photorespiratory pathway, it is not possible to conclude from such experiments whether or not the photosynthetic apparatus of plants is protected by photorespiration. Instead, an impairment of the flux through the photorespiratory pathway can lead to photoinhibition by inhibiting photosynthetic CO_2 assimilation caused by an insufficient regeneration of RuBP or by an accumulation of toxic metabolites. The best approach to study the role of photorespiration in photoprotection would be to alter the properties of rubisco (e.g. Bainbridge *et al.* 1995; Whitney *et al.* 1999) with the aim of abolishing the oxygenase activity without influencing the carboxylase activity. However, this goal has proved difficult to achieve.

In addition to the energy consumption by photorespiration, energy can also be dissipated by electron transport to O_2 in the Mehler-peroxidase pathway or as heat by non-photochemical quenching. Non-photochemical quenching is linked to the formation of zeaxanthin in the xanthophyll cycle and an increased proton gradient across the thylakoid membrane (Ruban & Horton 1995). Since the Mehler-peroxidase pathway results in an increased acidification of the thylakoid lumen, it can also lead to increased energy dissipation by non-photochemical quenching (Schreiber & Neubauer 1990).

(a) *Drought stress*

In drought-stressed leaves, CO_2 assimilation is decreased, resulting in a reduced electron consumption by photosynthesis. Consequently, the importance of mechanisms protecting the photosynthetic apparatus is increased. Under conditions of mild to moderate drought stress, the decline in photosynthesis mainly results from lower C_i caused by stomatal closure (Kaiser 1987; Cornic & Briantais 1991; Quick *et al.* 1992; Lal *et al.* 1996; Sánchez-Rodríguez *et al.* 1999). When the stomatal control of photosynthesis is overcome, for example, by removing the lower epidermis or by increasing the external CO_2 concentration, CO_2 assimilation can be restored, suggesting that, at least during mild drought, photosynthesis is not inhibited due to damage to the photosynthetic apparatus (Cornic 2000). Under these conditions, extractable activities or activation states of photosynthetic enzymes, such as rubisco, chloroplast fructose-1,6-bisphosphatase or NADP-dependent malate dehydrogenase do not typically decrease (Sharkey & Seemann 1989; Lal *et al.* 1996; Sánchez-Rodríguez *et al.*

1999; Wingler *et al.* 1999*b*). In the long term, drought stress has, however, been shown to result in lower fructose-1,6-bisphosphatase activities in *Casuarina equisetifolia* (Sánchez-Rodríguez *et al.* 1999), and severe drought stress led to a decline in the amounts of sedoheptulose-1,7-bisphosphatase and NADP-dependent glyceraldehyde-3-phosphate dehydrogenase proteins in barley (Wingler *et al.* 1999*b*). The amounts of photorespiratory enzyme proteins (proteins of the GDC complex, GS-2, SGAT) were not affected by drought stress, and the amount of NADH-dependent HPR even increased (figure 4*b*). In combination, the decline in C_i and sustained activities of rubisco and photorespiratory enzymes are likely to result in increased rates of photorespiration—not only relative to photosynthesis but also in absolute terms. Therefore, photorespiration could serve as an important means to maintain electron flow.

Studies conducted with different species under a variety of conditions provide partly contradictory data on the role of photorespiration during drought stress. For example, Biehler & Fock (1996) showed that gross uptake of O_2 increased with increasing water deficit in wheat. The absolute rate of photorespiration, determined as the rate of glycollate formation, was, however, decreased at a water potential (ψ) of -2.6 MPa. This suggests that at this ψ, electron flow to O_2 was increased due to the Mehler reaction and not due to photorespiration. Since the drought stress in this study was quite severe, reactions of the Calvin cycle might have been inhibited, which could result in reduced contents of RuBP (Sharkey & Seemann 1989) and, consequently, in lower rates of RuBP oxygenation. Brestic *et al.* (1995) found that electron transport in the leaves of drought-stressed *Phaseolus vulgaris* plants was reduced by switching from 21% to 2% oxygen, demonstrating that photorespiration serves as an electron sink in dehydrated leaves. Incubation in 2% oxygen in the light, however, did not affect the parameter F_v/F_m measured after 20 min dark adaptation. Accordingly, it can be concluded that photorespiration is not essential for protecting the photosynthetic apparatus against photoinhibition under drought stress.

In our studies, we used heterozygous barley mutants of chlorophyll fluorescence which contained approximately 50% of wild-type activities of the photorespiratory enzymes, GS-2, GDC and SGAT, to study the role of photorespiration during drought stress (Wingler *et al.* 1999*b*). These mutants have normal rates of photosynthesis in moderate light and in ambient CO_2. In low CO_2, on the other hand, photosynthesis is reduced in the GS and GDC mutants. The rationale behind our study was that if photorespiration is increased in dehydrated leaves, photosynthesis should decline to a greater extent in the mutants than in the wild-type with increasing drought stress, and the control exerted by the photorespiratory enzymes on photosynthesis should increase. In addition, it appeared possible that a further down-regulation of photosynthetic CO_2 assimilation due to restrictions in the photorespiratory flux could lead to photoinhibition.

In well-watered plants, reduced activities of GS-2, GDC or SGAT did not affect photosynthesis. Drought stress was induced by withholding water over a period of two weeks, and the decrease in leaf ψ was monitored
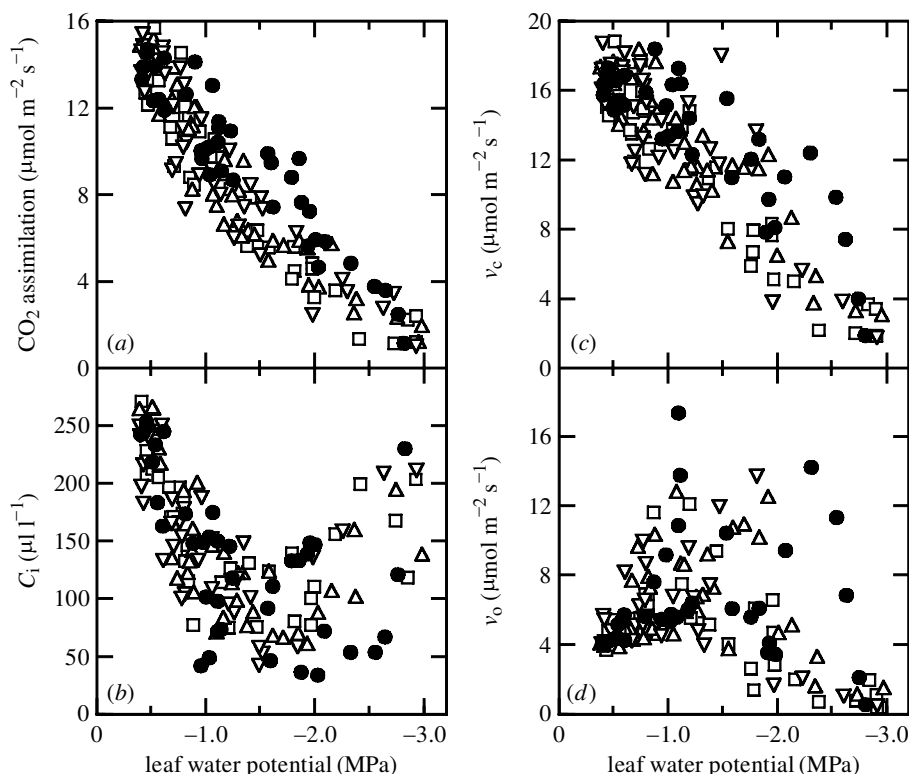
Figure 5. (a) A, (b) C_i, (c) v_c and (d) v_o with developing drought stress in wild-type barley (closed circles) and heterozygous mutants with reduced activities of plastidic GS (open squares), SGAT (open triangles) or GDC (inverted triangles). The plants were grown in cycles of 12 h light ($460\,\mu mol\,m^{-2}\,s^{-1}$) at 24 °C and 12 h darkness at 20 °C. The data were calculated according to Von Caemmerer & Farquhar (1981) assuming a day respiration of $0.45\,\mu mol\,m^{-2}\,s^{-1}$ and a CO_2 compensation point of $30.4\,\mu l\,l^{-1}$ (Häusler *et al.* 1996).

daily. With decreasing ψ, rates of CO_2 assimilation declined almost linearly in the wild-type (figure 5a). In the mutants with reduced activities of photorespiratory enzymes, this decline was accelerated, resulting in lower rates of CO_2 assimilation at moderate drought stress. The control exerted by photorespiratory enzymes on photosynthesis was, therefore, increased in moderately drought-stressed leaves. However, under severe drought stress, the rates of CO_2 assimilation were equally low in the wild-type and in the mutants. The C_i-values determined by gas exchange measurements declined in all plants with increasing drought stress until a ψ of about $-1.5\,MPa$ was reached (figure 5b). When desiccation became more severe, the relationship between ψ and C_i broke down, and the estimated C_i-values rose again. This phenomenon, i.e. constant or even increased C_i-values calculated from gas exchange data, is often encountered in severely drought-stressed leaves (Cornic *et al.* 1989; Lal *et al.* 1996; Sánchez-Rodríguez *et al.* 1999). During severe drought stress, C_i-values are often overestimated due to the patchiness of stomatal closure (Sharkey & Seemann 1989) and altered lateral diffusion of CO_2 (Lal *et al.* 1996). v_c, as calculated from the C_i data and the CO_2 assimilation rates, decreased with increasing drought stress (figure 5c). As shown for the assimilation rates, v_c was higher in drought-stressed leaves of the wild-type than in leaves of the mutants. The calculated values for v_o rose until a ψ of $-1.5\,MPa$ was reached (figure 5d). As a consequence of the questionable C_i-values, the relationship between ψ

and v_o broke down when drought stress became more severe. Together with the lower rates of photosynthesis, the calculated values for v_o indicate that during moderate drought stress, when the calculation of C_i was probably still valid, photorespiration was increased. This was also confirmed by an increase in glycine contents in drought-stressed leaves of the GDC mutant (Wingler *et al.* 1999b).

The lower rates of photosynthesis in the heterozygous mutants were accompanied by decreased quantum efficiencies of photosystem (PS) II electron transport. This decreased electron consumption in photosynthesis and photorespiration in the mutants did not lead to a decline in F_v/F_m, which would have indicated chronic photoinhibition. Instead, energy dissipation by non-photochemical quenching increased. In the SGAT and GDC mutants, this was accompanied by a strong increase in the formation of zeaxanthin. As shown by Brestic *et al.* (1995) and Demmig *et al.* (1988), xanthophyll-cycle-dependent energy dissipation seems to be an important mechanism for protecting against the deleterious effect of light in drought-stressed leaves.

(b) *Salt stress*

Under conditions of high salinity, plants encounter similar problems to those during drought stress. Initially, photosynthesis is inhibited by closure of the stomata and the resulting decrease in C_i, while non-stomatal effects become limiting when the chloride concentration increases further (Walker *et al.* 1981; Downton *et al.* 1990;

Fedina *et al.* 1994). As for drought-stressed leaves, severe salt stress can result in non-uniform closure of the stomata and patchiness of photosynthetic CO_2 assimilation (Downton *et al.* 1990). Several lines of evidence suggest that stomatal closure in moderately salt-stressed leaves leads to enhanced rates of photorespiration. The following parameters, all indicative of higher rates of photorespiration, have been shown to increase: the CO_2 compensation point (Fedina *et al.* 1993, 1994), the light to dark ratio of CO_2 production (Rajmane & Karagde 1986), the stimulation of photosynthesis by lowering the O_2 concentration (Walker *et al.* 1981; Fedina *et al.* 1994), the activity of glycollate oxidase (Rajmane & Karadge 1986; Fedina *et al.* 1993, 1994) and the formation of photorespiratory metabolites, such as glycine, serine and glycollate (Downton 1977; Rajmane & Karadge 1986; Di Martino *et al.* 1999). The maintenance of considerable rates of electron transport in CO_2-free air also indicates a significant occurrence of photorespiration in salt-stressed leaves (Di Martino *et al.* 1999). In addition to sustained rates of electron transport due to photorespiration, the formation of zeaxanthin during salt stress probably also mitigates against photoinhibitory damage, although this protection by zeaxanthin is not complete in high light (Sharma & Hall 1992).

(c) *Light stress in alpine plants*

Plants are particularly prone to photoinhibition when they are exposed to high light in combination with drought or low temperatures, conditions mainly encountered by plants growing at high altitudes. These plant species are often highly resistant to photoinhibition and employ different protective strategies. For *Chenopodium bonus-henricus* grown in the Alps, Heber *et al.* (1996) found that photorespiration is essential for maintaining electron flow in high light, whereas the Mehler-peroxidase pathway is insufficient to prevent photoinactivation. Significant rates of electron flow were maintained when photosynthesis was inhibited by cutting the petioles or submerging the leaves in water. On the other hand, when photosynthesis and photorespiration were inhibited by feeding HCN or glyceraldehyde, the sensitivity of PS II to sunlight increased. At very low temperatures, photorespiratory metabolism is greatly reduced and cannot make a significant contribution to photoprotection. For the alpine plant *Geum montanum*, it was suggested that at low temperatures, cyclic electron transport around PS I results in a decrease in pH in the thylakoid lumen and, probably in combination with zeaxanthin formation, to increased energy dissipation at the site of PS II (Manuel *et al.* 1999). A strong dependence on the operation of the xanthophyll cycle in high light and at low temperatures has also been proposed for *Soldanella alpina* (Streb *et al.* 1998). *Ranunculus glacialis*, on the other hand, was less dependent on the formation of zeaxanthin, while feeding of the GS inhibitor phosphinothricin at low temperatures resulted in photoinhibition. This points to a significant flux through the photorespiratory pathway even at low temperatures. As photorespiration was, however, not blocked at the site of RuBP oxygenation, it cannot be established from this experiment that photorespiration itself has a protective function. Instead, accumulation of compounds such as NH_3 or glyoxylate or a depletion of

Calvin cycle intermediates could have led to secondary effects by, for example, reducing CO_2 assimilation. In addition to the mechanisms discussed above, an increased importance of the antioxidant system comprising ascorbate, α-tocopherol and glutathione has been proposed to protect alpine plants against photo-oxidation (Heber *et al.* 1996; Streb *et al.* 1997, 1998). Since the glycine required for the formation of glutathione is mainly provided by photorespiration, photorespiration could play an additional role in the protection of alpine plants.

8. CONCLUSION

Photorespiratory metabolism is not only a wasteful process inevitably resulting from the kinetic properties of rubisco, but, precisely because of this inefficiency, is also involved in stress protection. In addition, photorespiratory metabolism can generate metabolites, such as glycine, serine or one-carbon units for other processes in plants. Abolishing photorespiration by engineering rubisco may, therefore, not necessarily lead to improved plant performance, especially under unfavourable growth conditions.

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