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Barley Mutants Lacking Chloroplast Glutamine Synthetase—Biochemical and Genetic Analysis

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ROGER M. WALLSGROVE*, JANICE C. TURNER, NIGEL P. HALL, ALAN C. KENDALL, AND SIMON W. J. BRIGHT
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

ABSTRACT

Eight mutants of barley (*Hordeum vulgare* cv Maris Mink) lacking the chloroplast isozyme of glutamine synthetase (EC 6.3.1.2.) were isolated by their inability to grow under photorespiratory conditions. The cytoplasmic isozyme of glutamine synthetase was present in the leaves of all the mutants, with activities comparable to the wild-type (10–12 nanokatals per gram fresh weight). The mutant plants developed normally and were fully fertile under conditions that minimize photorespiration. In 1% O_2 the rate of CO_2 fixation in leaves of one of the mutants, RPr 83/32, was the same as the wild-type, but in air this rate declined to 60% of the wild-type after 30 minutes. During this time the ammonia concentration in leaves of the mutant rose from 1 to 50 micromoles per gram fresh weight. Such ammonia accumulation in air was found in all the mutant lines. In back-crosses with the parent line, F_1 plants were viable in air. In the F_2 generation, nonviability in air and the lack of chloroplast glutamine synthetase co-segregated, in both the lines tested. These two lines and four others proved to be allelic; we designate them *gln 2a–f*. The characteristics of these mutants conclusively demonstrate the major role of chloroplast glutamine synthetase in photorespiration and its associated nitrogen recycling.

The isolation of higher plant mutants with defects in photorespiratory metabolism has proved extremely valuable in the study of this apparently wasteful process. Mutants at seven steps in the pathway have been isolated in either *Arabidopsis* or barley (5, 15). During photorespiration ammonia is released and reassimilated in a photorespiratory nitrogen cycle (7): mutants lacking Fd-glutamate synthase and chloroplast dicarboxylate transport have confirmed the importance of this cycle in both *Arabidopsis* (15) and barley (5, 6, 21).

GS[1] catalyzes the first step in ammonia assimilation. This enzyme is present in all tissues of higher plants, and up to four isozymic forms may be present, as judged by subunit composition and differential expression (2, 10, 11). In barley leaves, a cytosolic isozyme, GS_1, is present, together with a chloroplast enzyme, GS_2 (11). The relative contributions of GS_1 and GS_2 to photorespiratory nitrogen cycling have been a matter of debate (20). We report here the first isolation of mutants lacking chloroplast GS_2, thus demonstrating the prime importance of this isozyme in photorespiratory nitrogen cycling. A preliminary report of some of this work has been published (5).

Inhibition of GS by specific chemical inhibitors markedly inhibits CO_2 fixation in leaves of both C_3 and C_4 plants (3, 4, 9, 13, 17), but it has not been possible to relate this inhibition directly to the accompanying increase in ammonia concentration (3, 4, 16). Doubts have been raised concerning the source of NH_3 in such inhibited leaves (4, 9, 13). The mutants described here, lacking GS_2, have allowed us to reassess the interaction of ammonia accumulation and CO_2 fixation.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* L. cv Maris Mink) mutants derived from M_2 seed after azide mutagenesis were selected and grown as previously described (5, 6).

Gas Exchange Analysis. CO_2-fixation by leaf segments was analyzed as previously described (6). For experiments using attached leaves, an IR gas analyzer (LCA2) and hand-held leaf chamber (PLC) (Analytical Development Co., Hoddesdon, Herts, U.K.) were employed. Photon flux density was 400 μmol quanta m^{-2} s^{-1}.

Enzyme Extraction and Assay. GS, glutamate dehydrogenase (EC 1.4.1.2) and glutamate synthase (EC 1.4.7.1) were extracted and assayed as previously described (6). For GS, the transferase assay (1) was also used.

Ion-Exchange Chromatography. An aliquot (500 μl) of desalted crude leaf extract was applied to a Pharmacia Mono Q anion exchange column (Pharmacia FPLC system) equilibrated with 25 mM Tris/HCl (pH 7.6), 1 mM $MgSO_4$, 1 mM DTE, 10% (v/v) ethanediol, 50 mM KCl. Enzyme was eluted with a linear gradient (20 ml) from 0.05 to 0.45 M KCl, at a flow rate of 0.75 ml min^{-1}. Fractions (1 ml) were collected and assayed for GS (transferase).

Chloroplast Isolation. Chloroplasts were isolated from protoplasts as previously described (19), using first leaves of 6- to 10-d-old seedlings.

Ammonia Determination. Ammonia was assayed as previously described (6).

Immunological Analysis (Western blot). Young leaves were extracted as for enzyme analysis and, after heating in SDS, 40 and 80 μl aliquots (corresponding to 10 and 20 mg FW) were subjected to SDS-PAGE. The separated polypeptides were transferred to nitrocellulose by electro-blotting (Bio-Rad Transblot, 4 h at 60 V). After washing the nitrocellulose in buffer (20 mM Tris HCl [pH 7.5], 0.5 M NaCl) containing 3% BSA, it was incubated with buffer containing 450 μl of antiserum (1 mg protein ml^{-1}) raised against *Phaseolus* nodule GS (a gift from R. Saarelainen), for 1.5 h at room temperature. After a further wash, the filter was probed with Protein A-peroxidase (Sigma) for 1 h at room temperature, and washed. Peroxidase activity was visualized by incubation with 60 mg 4-chloro-1-napthol and 40 μl H_2O_2 in a total of 60 ml. After 10 min the nitrocellulose was washed with water to stop the reaction.

[1] Abbreviations: GS, glutamine synthetase; M_2, selfed progeny of mutagenized seed; FW, fresh weight; kat, katal (1 mol s^{-1}).

RESULTS

Seedlings derived from azide-mutagenized barley were selected for their ability to thrive in 0.8% CO_2 but not in air. From 1.2 $\times 10^5$ M_2 seeds, 50 such plants were isolated which, upon selfing, produced air-sensitive progeny (5). Eight of those mutant lines (designated RPr 83/20, 83/31, 83/32, 84/12, 84/34, 84/50, 84/83, and 84/84) produced similar symptoms after exposure to air (severe chlorosis after 2- or 3-d exposure, without pronounced necrotic lesions). They had similar patterns of photosynthetic inhibition following transfer from nonphotorespiratory to photorespiratory conditions (5). Their most striking characteristic, however, was the very high concentration (up to 50 μmol g^{-1} FW) of ammonia present in leaves after relatively short exposure to air.

The transferase assay for GS indicated the mutants had only 20 to 27% of the wild-type activity (Table I). Use of the biosynthetic assay at appropriate pH values allowed the activity of each GS isozyme to be measured separately. At pH 7.8 (optimum for GS_2), young leaves of the mutants contained 3 to 5 nkat g^{-1} FW GS (*cf.* 40–50 nkat g^{-1} FW for Maris Mink). At pH 7.2 (optimum for GS_1) mutant leaf extracts contained 10 to 12 nkat g^{-1} FW (*cf.* 8 to 12 nkat g^{-1} FW for Maris Mink). Table I illustrates the results obtained with this assay for one of the mutants, RPr 83/32. Activities of Fd-glutamate synthase (30–40 nkat g^{-1} FW) and glutamate dehydrogenase (1–2 nkat g^{-1} FW) were similar in both mutant and wild-type leaves.

Figure 1 shows the separation of GS isozymes by ion-exchange chromatography, which confirmed the results obtained using the biosynthetic assay. Two peaks of activity were found in extracts from Maris Mink, GS_1 eluting at 0.23 M KCl and GS_2 at 0.37 M KCl. In RPr 83/32 only the first isozyme was detectable. For all the other mutants except RPr 84/12 a similar result was obtained—for this line 3% of the total GS activity eluted at 0.37 M KCl. Chloroplasts from leaves of RPr 83/32 and 84/34 contained no detectable GS activity, whereas chloroplasts from Maris Mink contained 70 to 80% of the GS activity (and 90–95% of the Chl) found in the protoplasts.

As a preliminary step in the immunological investigation of GS proteins present in these mutants, Western blots were run on samples from leaves of RPr 83/32 and 84/34 (Fig. 2). The antiserum used, raised against *Phaseolus* nodule GS, cross-reacts with both GS_1 and GS_2 from Maris Mink. Extracts from the mutants had an apparently normal band corresponding to GS_1, but only very faint bands appeared in the region corresponding to GS_2.

The F_2 seedlings from a backcross between Maris Mink and RPr 83/32 were tested for leaf GS activity and viability in air. Out of 60 seedlings, 16 had less than 25% of the wild-type GS (transferase assay), and all these plants developed symptoms in air characteristic of RPr 83/32. This distribution suggests the segregation of a single recessive nuclear gene. However, 32 of the 60 seedlings had intermediate GS activity (40–60% of wild-type)

Table I. *Glutamine Synthetase Activities in Wild-type and Mutant Barley*

The data are from single experiments (duplicate assays) using young expanding leaves, carefully matched for size and developmental age. All the mutant were assayed several times, with results in close agreement with those shown.

Transferase Assay		Biosynthetic Assay	
	$\Delta A\ min^{-1}\ g^{-1}\ FW$		$nkat\ g^{-1}\ FW$
Maris Mink	26.6		
RPr 83/20	6.1	(A) At pH 7.8	
83/31	7.0	Maris Mink	45
83/32	7.1	RPr 83/32	3.7
84/12	6.2		
84/34	6.0	(B) At pH 7.2	
84/50	5.3	Maris Mink	8.6
84/83	5.7	RPr 83/32	11.5
84/84	5.4		

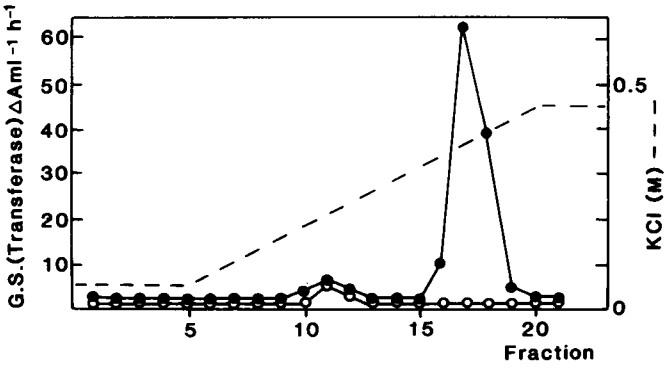

FIG. 1. Separation of GS isozymes by ion exchange chromatography. Desalted extracts from young, expanding leaves were applied to a Pharmacia Mono Q column and eluted with a linear 0.05 to 0.45 M KCl gradient. (●), Maris Mink; (○), RPr 83/32.

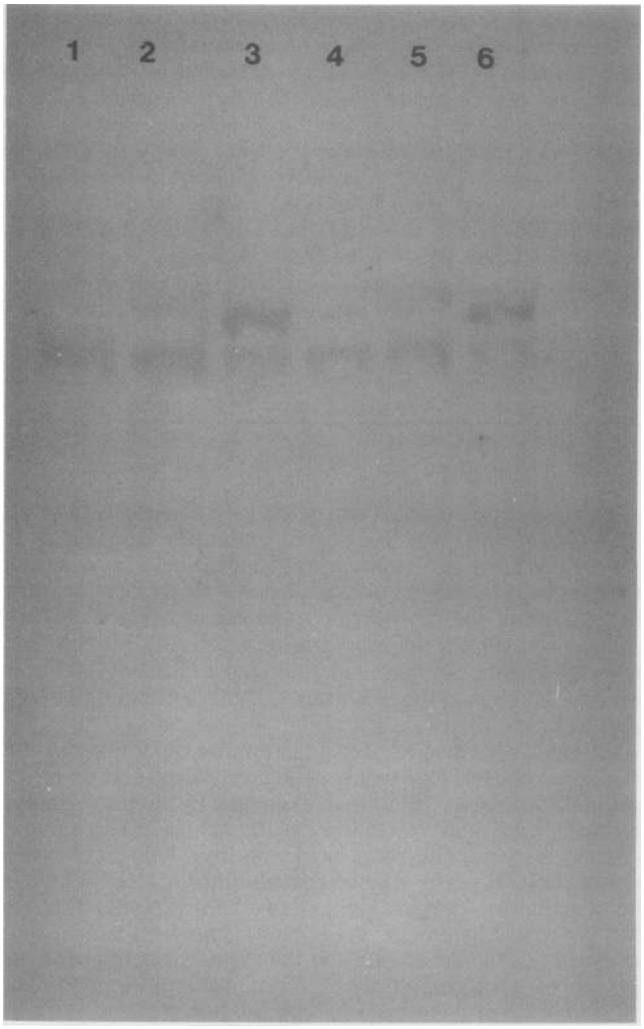

FIG. 2. Western blot of leaf extracts following SDS-PAGE. Bands cross-reacting to rabbit anti-GS serum were visualized using peroxidase linked to Protein A. (1 and 4), RPr 84/34 extract; (2 and 5), RPr 83/32 extracts; (3 and 6), Maris Mink extract. (1–3), 80 μl; (4–6), 40 μl.

and were viable in air. So for GS activity the segregation is close to the 1:2:1 ratio expected for a partially dominant gene. We designate the mutant gene *gln 2*, anticipating a comparable gene for GS_1 that would be *gln 1*. Crosses between the mutant lines RPr 83/20, 83/32, 84/12, 84/34, 84/83, and 84/84 gave, in each case, F_1 progeny that were all air sensitive; hence the mutations in these lines are allelic. We designate these alleles *gln 2a–f*, respectively. Two other mutants lacking GS (RPr 83/31, 84/50) are allelic to each other, but have yet to be crossed to the *gln 2* mutants.

Leaf segments from the mutants fixed CO_2 at wild-type rates in 1% O_2 (Fig. 3), but when the gas stream was changed to 21% O_2, each class of mutant showed a different response (5). In a glutamate synthase mutant (RPr 82/9 in this case) (6) the rate fell rapidly; in a chloroplast dicarboxylate uptake mutant (RPr 79/2) (21) the fall was initially slower. The GS mutant (RPr 83/32 in this experiment) gave a much slower decline in CO_2 fixation, such that after 30 min it was still more than half the wild-type rate. However, ammonia accumulated rapidly in leaves of RPr 83/32 under these conditions, showing a linear increase for at least 30 min. In the experiment shown in Figure 3, the initial ammonia concentrations in each leaf were less than 1 μmol g^{-1} FW, but after 30 min the concentrations were 50, 5, 1, and 1 μmol g^{-1} FW in RPr 83/32, RPr 82/9, RPr 79/2, and Maris Mink, respectively. This rapid ammonia accumulation was observed in all the GS mutants: its rate was increased by higher temperature and light intensity (data not shown).

The induction of CO_2-fixation after a 16 h dark period in attached leaves of mutant and wild-type barley is shown in Figure 4, together with the changes in ammonia concentration. For comparison with the GS-deficient mutant (RPr 83/20 in this experiment), data for RPr 84/13 (Fd-glutamate synthase deficient) (6) and RPr 79/2 (chloroplast dicarboxylate-uptake mutant) (21) are also shown. After a short lag period, the ammonia concentration in RPr 83/20 rose, and after 20 min there was a sustained increase. In RPr 84/13, the ammonia concentration increased much more slowly, such that after 60 min it was half that in RPr 83/20, whereas the ammonia concentration in RPr 79/2 increased by little more than that in the wild-type, though

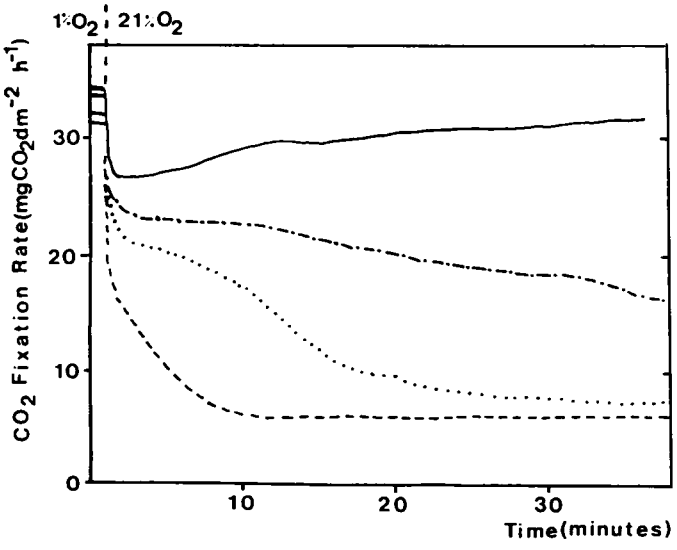

FIG. 3. CO_2-fixation in detached leaf sections of mutant and wild-type barley. Leaf sections were illuminated (1000 μmol quanta m^{-2} s^{-1}) for 1 h in 1% O_2, 350 μl L^{-1} CO_2, and the gas stream then switched to 21% O_2, 350 μl L^{-1} CO_2. (——), Maris Mink; (-·-·-), RPr 83/32 (deficient in GS_2); (·····), RPr 79/2 (deficient in chloroplast dicarboxylate uptake); (– – –), RPr 82/9 (deficient in Fd-glutamate synthase).

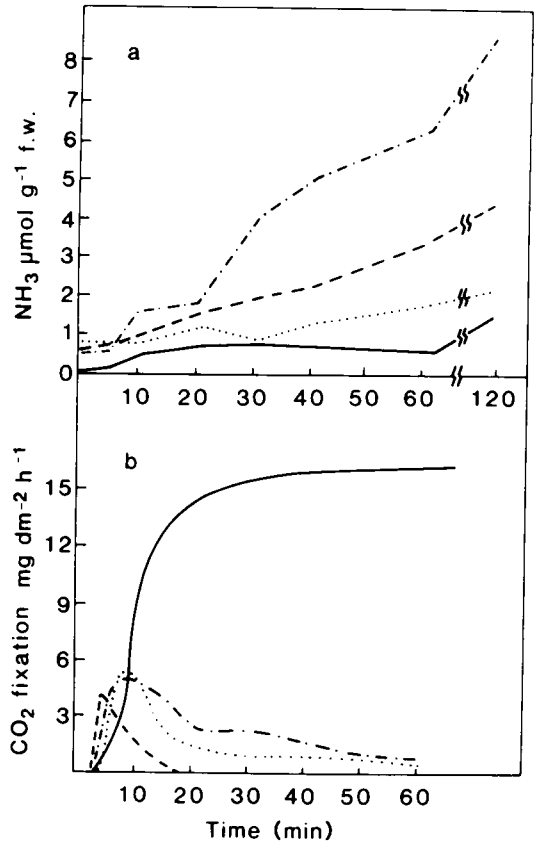

FIG. 4. CO_2-fixation rate and ammonia accumulation in attached barley leaves. After 16 h in the dark, plants were illuminated (400 μmol quanta m^{-2} s^{-1}) in air at 20°C. CO_2 fixation was measured using a clamp-on leaf chamber; equivalent leaf samples were taken at intervals for ammonia estimation. (——), Maris Mink; (-·-·-), RPr 83/20 (deficient in GS_2); (·····), RPr 79/2 (deficient in chloroplast dicarboxylate uptake); (– – –), RPr 84/13 (deficient in Fd-glutamate synthase).

the initial concentration was higher. By 10 min, CO_2 fixation in all the mutants had reached a maximum and begun to decline, rapidly in the case of RPr 84/13 and more slowly for RPr 79/2 and RPr 83/20.

DISCUSSION

The barley mutants described here contained no effective chloroplast GS (GS_2) but the activity of the cytoplasmic isozyme (GS_1) was identical to the parent line. As plants from all eight lines were healthy, vigorous, of normal appearance, and fully fertile when grown in high CO_2, we can conclude that GS_2 is not necessary for growth and development under conditions that suppress or prevent photorespiration. The major, and perhaps only, role of this enzyme must be the reassimilation of ammonia released during photorespiration. The magnitude of this release can be judged by the increase in free ammonia in the leaves of these mutants when exposed to light and air. In the wild-type leaf, GS_2 is almost certainly also involved in the metabolism of ammonia from primary assimilation (via NO_3^- and NO_2^-), as the chloroplast is the sole location for nitrite reductase (18): GS_2 is clearly not essential for this process.

The observed increase in ammonia in the leaves of the mutants in air reached 50 μmol g^{-1} FW after 30 min, suggesting an intracellular concentration of about 50 mM. Such a concentration should completely uncouple photophosphorylation (8), but the gas exchange measurements suggested that this did not occur, at least in the short term. As seen in Figure 3, 30 min after transfer

from 1% O_2 to air, CO_2 fixation in the *gln 2* mutant was still 50% of the wild-type rate, whereas in a glutamate synthase (*glu S*) (6) mutant it was much lower, despite an ammonia concentration only one-tenth as high. The explanation for these differences may be found in the different abilities of the various mutants to trap ammonia in organic form and to generate glutamate as a substrate for this reaction. The *glu S* mutant is effectively incapable of synthesizing glutamate (the activity of NADH-glutamate synthase being very low compared to the rate of ammonia release): all available glutamate is rapidly metabolized to glutamine or used for glyoxylate transamination. In the absence of transamination equivalents the photorespiratory cycle will be blocked, so no further glycine (and NH_3) can be produced. We assume that a rapid depletion of Calvin cycle intermediates explains the dramatic decline in CO_2 fixation.

In contrast, the *gln 2* mutants retain considerable ability to assimilate ammonia and to regenerate glutamate. Heterozygous *gln 2/Gln 2* plants are phenotypically normal in air, so the homozygous *gln 2* plant may have as much as one-third to one-half the required activity of GS. Therefore, considerable recycling is possible (although not enough to cope with the maximum rate of ammonia production), allowing the photorespiratory cycle to return much of its carbon to the Calvin cycle: in this case CO_2 fixation can continue for much longer. It would seem that all available N is drawn into this cycle, much of it appearing as NH_3, in a way that is impossible when glutamate synthesis (rather than glutamine synthesis) is restricted. If the ammonia accumulating is not uncoupling the chloroplasts, presumably its effective concentration in chloroplast and cytosol is lower than the overall content implies. Given the steep pH gradient across the tonoplast membrane, a preferential accumulation in the acid environment of the vacuole may occur.

The rates of CO_2-fixation in photosynthetic tissues treated with GS inhibitors correspond much more closely to the behavior of the *glu S* mutants than to the *gln 2* lines (3, 4, 9, 13, 16). Such inhibitor-treated tissues lack all ability to reassimilate ammonia and hence to resynthesize glutamate. The observed rapid decline in CO_2-fixation is presumably, as with the *glu S* mutants, a consequence of the block in photorespiratory cycling, causing a depletion of Calvin cycle intermediates. Several groups have commented that the measured ammonia levels in such tissues could not explain the observed effects on CO_2-fixation (3, 4, 16), though the commonest inhibitor, methionine sulphoximine, has no effect on CO_2-fixation by isolated chloroplasts (12). The failure to observe differences between C_3 and C_4 plants (9, 13) and between high and low CO_2 atmospheres (4) has led to suggestions that photorespiration could not explain the results. For our mutants we can demonstrate that high rates of ammonia production are dependent on photorespiratory conditions: when photorespiration is suppressed in barley there is no evidence for significant ammonia release or recycling. The methodological problems associated with inhibitor studies may account for some of the discrepancies noted in such work. Comparing the rates of NH_3 release and net CO_2 fixation in detached leaf sections (Fig. 3), ammonia accumulation approximates to 40% of the rate of CO_2 fixation. This is a crude measure of the photorespiration rate, but will be an underestimate because of recycling via GS_1 (which we cannot measure).

The phenotype of air-sensitivity in these mutants behaves as a single recessive nuclear gene. There is evidence, however, of a gene-dose effect in the measurements of GS activity in the *gln 2/Gln 2* heterozygotes. Six of the eight lines so far isolated are allelic; data for the other two are not yet available. Four types of subunit have been reported for GS_2 from *Phaseolus* (separated by 2-dimensional electrophoresis) (10), but we have no comparable data on the barley enzyme. It is clear that a single gene mutation leads to loss of all GS_2 activity; preliminary immunological analysis suggests that these mutants contain little or no cross-reacting material corresponding to GS_2.

GS and Fd-glutamate synthase are absolutely necessary for reassimilation of ammonia released during photorespiration in higher plants. Although some authors still suggest a role for glutamate dehydrogenase in this process (14, 22), our results indicate that this must be of only minor significance.

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