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Regulation of Nodule Glutamine Synthetase by CO_2 Levels in Bean (*Phaseolus vulgaris* L.)[1]

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ABSTRACT

Nodulated bean (*Phaseolus vulgaris*) plants were grown for 17 days after infection in normal (0.02%) CO_2 and from day 8 to 17 in high (0.1%) CO_2 in order to increase nitrogen fixation and define how nodule glutamine synthetase (GS) isoforms are regulated by the ammonia derived from the bacteroid. Nitrogenase activity was detected by day 10, and by day 17 activity was over twofold higher in 0.1% of CO_2 compared with plants grown in 0.02% CO_2 and inoculated with *Rhizobium* wild-type strain CE3. Likewise, plant fresh weight increased in response to increased CO_2, particularly in plants inoculated with the *Rhizobium phaseoli* mutant strain CFN037. Glutamine synthetase specific activity increased 2.5- to 6.5-fold from day 11 to 17. However, increased CO_2 did not appear to have an effect on GS specific activity. Analysis of the nodule GS polypeptide composition revealed that the γ polypeptide was significantly reduced in response to high CO_2, whereas the β polypeptide was not affected. The significance of this result in relation to the regulation of GS isoforms and their role in the assimilation of ammonia in the nodule is discussed in this paper.

GS[2] in bean (*Phaseolus vulgaris*) root-nodules is expressed as two different isoforms designated GS_{n-1} and GS_{n-2} (3). GS_{n-1} is expressed during nodule development concomitantly with nitrogenase activity (8). The nodule GS_{n-1} is composed of a polypeptide called γ, and the GS_{n-2} isoform is composed of the β polypeptide, which is also expressed in roots and leaves (9). Initial expression of GS-γ polypeptide, as other nodulins (5), is independent of nitrogenase activity (12), but its optimal induction requires normal nitrogen fixation. Studies on the expression of the γ (Gln-γ) and β (Gln-β) GS genes in *Lotus corniculatus* transgenic plants demonstrated that Gln-γ is expressed in the infected zone of mature nodules where ammonia assimilation takes places, and Gln-β is expressed in the nodule cortex (7). These results indicate that the GS_{n-1} (γ) is responsible for the ammonia assimilation in bean nodules. Furthermore, in bean plants grown in argon (Ar) in which nitrogen fixation is inhibited, the γ polypeptide is reduced, suggesting that ammonia regulates its expression (2). Recently, it has been reported that ammonia induces soybean GS gene expression in transgenic *L. corniculatus* roots (10). Here, we report differences in the synthesis of GS isoforms when higher nitrogenase activity is induced by increasing CO_2 concentration.

MATERIALS AND METHODS

Plant Material

Bean (*Phaseolus vulgaris* L. cv Negro Jamapa) inoculated with *Rhizobium leguminosarum* bv *phaseoli* wild-type strain CE3 or a $TMPD^{2+}$ mutant CFN037 (13) was grown in a controlled-environment growth chamber programed for 12-h photoperiod at 25°C day/night temperature; the PPFD during the photoperiod was 906 μE $m^{-2}s^{-1}$. At day 8 after inoculation, CO_2 was raised to 0.1% during the photoperiod and held at that level until day 17. Chamber CO_2 concentration was maintained with a Conviron CMP3246 CO_2 controller (Controlled Environments Inc.). CO_2 concentration was monitored by the nondispersive infrared absorptiometry method. Plants were harvested daily for fresh weight and nitrogenase determination. The data presented per day are the average of at least 20 plants.

Enzyme Activity

GS activity was measured by the transferase assay (6). One unit of activity represents 1 μmol of γ-glutamyl-hydroxamate formed per min at 30°C. Nitrogenase activity was measured by acetylene reduction as described by Dart *et al.* (4). Protein was determined by the Bradford procedure with BSA as a standard (1).

Analysis of GS Polypeptides

Nodule extracts were loaded on a 0.5 mL hydroxylapatite column equilibrated in 20 mM phosphate buffer, pH 7.8, washed with 4 mL of 80 mM phosphate, and GS activity was eluted with 400 mM phosphate buffer. Fractions of 200 μL were collected. Fractions containing GS activity were used for the polypeptide analysis. GS polypeptide composition was examined by two-dimensional PAGE (11), followed by western immunodetection (14) with an anti-GS antiserum raised against the purified enzyme from nodule (9) and an alkaline phosphatase-conjugated second antibody. The intensity of the

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[2] Abbreviations: GS, glutamine synthetase; β and γ, glutamine synthetase polypeptides; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

developed spots was quantified with a laser scanning densitometer (Biomed Instruments SLR-2D/1D). The GS-β/GS-γ polypeptide ratio was determined with an Autospover Videophoresis program (Biomed Instruments, Inc.).

RESULTS AND DISCUSSION

Plant Growth and Nitrogenase Activity

Plants were inoculated with two different *Rhizobium phaseoli* strains: the wild-type strain CE3 and the mutant strain CFN037 isolated by its increased ability to oxidase $TMPD^{2+}$, which confers earlier bacteroid development and nitrogenase induction (13). Plant fresh weight increased from day 10 to 17 after infection. High CO_2 (0.1%) stimulated plant growth, particularly in those plants inoculated with *Rhizobium* strain CFN037 (Fig. 1). Plants grown in 0.1% CO_2 also display higher nitrogenase activity than those grown in 0.02% CO_2. In normal CO_2 (0.02%), nitrogenase activity in plants inoculated with the CFN037 *Rhizobium* mutant was higher and remained higher from day 11 to 17 than in plants inoculated with the wild-type strain CE3, as previously reported (13) (Fig. 2). In high CO_2 (0.1%), no difference in nitrogenase activity was observed in plants inoculated with either the CFN037 or the CE3 *Rhizobium* strains. This result suggests that the *Rhizobium* strain CFN037 not only promotes early bacteroid development and nitrogenase induction (13), but also provides more fixed N to incorporate into plant fresh weight when CO_2 is increased.

Characterization of Glutamine Synthetase

Glutamine synthetase activity increased almost sevenfold from day 11 to day 17 in nodules from plants infected with

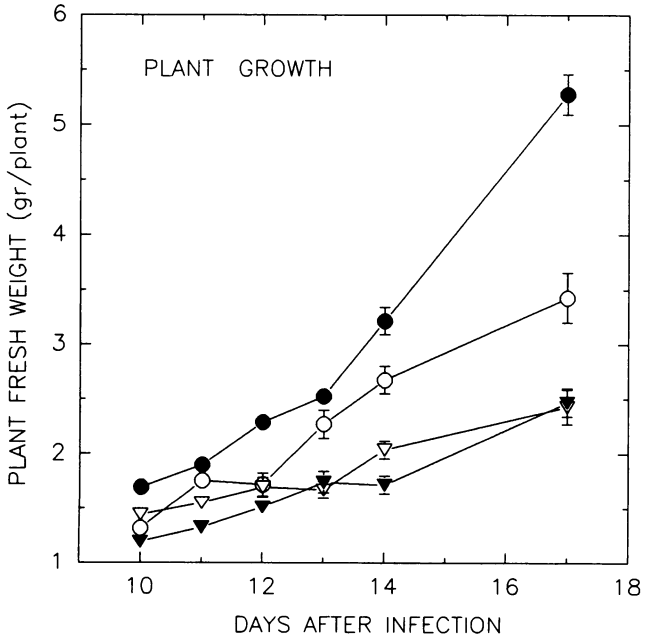

Figure 1. Effect of CO_2 on the increase in plant fresh weight of plants inoculated with *R. phaseoli* CE3 (○, ▽) and CFN037 (●, ▼). Plants were grown in 0.1% (circles) and 0.02% (triangles) CO_2. Vertical bars are ± SE.

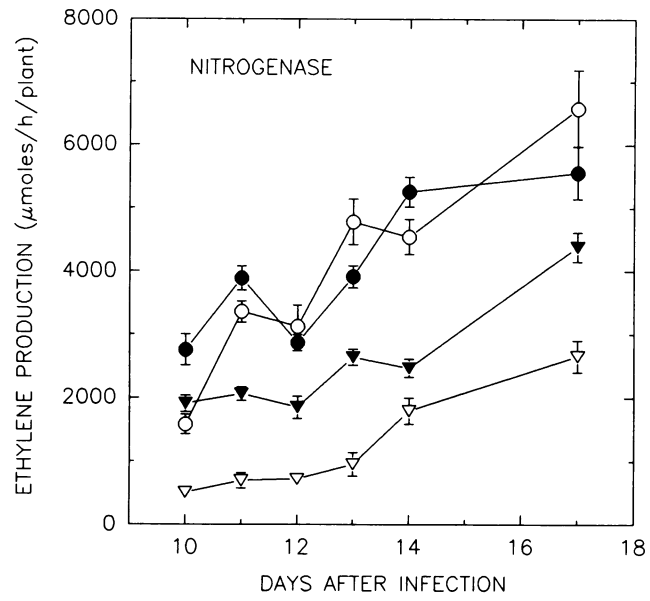

Figure 2. Effect of CO_2 on nitrogenase activity during nodule development. Plants inoculated with *R. phaseoli* (CE3 (○, ▽) and CFN037 (●, ▼) were grown in 0.1% (circles) and 0.02% (triangles) CO_2. Vertical bars are ± SE.

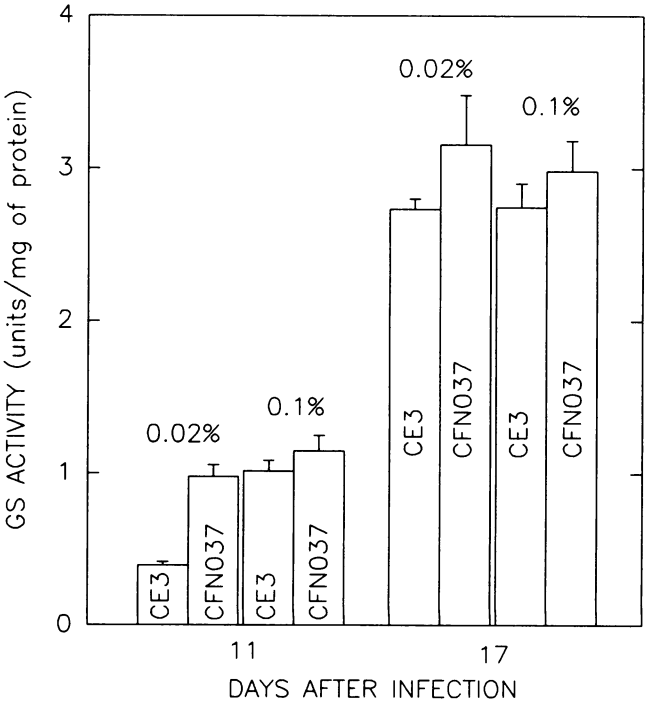

Figure 3. Nodule glutamine synthetase activity at day 11 and 17 after infection of plants inoculated with *R. phaseoli* strains CE3 and CFN037. Plants were grown in 0.1% and 0.02% CO_2. One unit of activity = 1 μmol of γ-glutamyl-hydroxamate formed per min at 30°C. Vertical bars are ± SD.

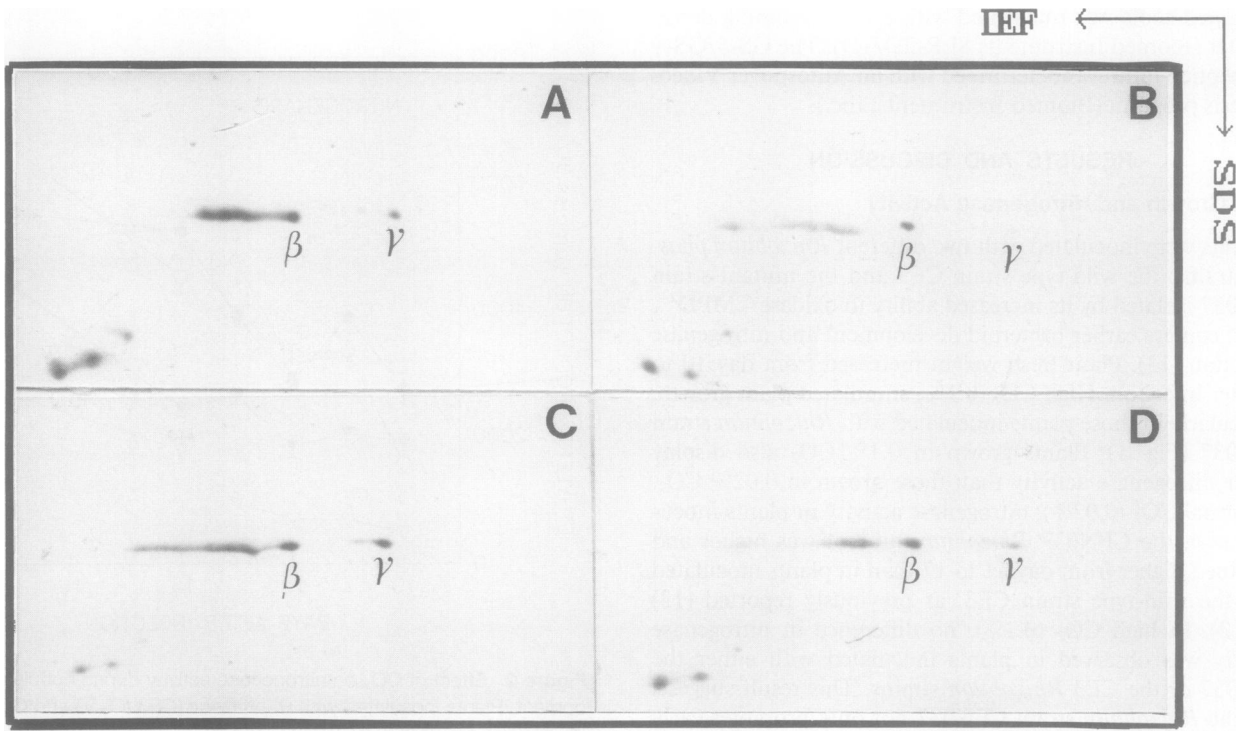

Figure 4. Glutamine synthetase polypeptide composition at day 17 after infection. Plants inoculated with *R. phaseoli* strains CE3 (A, B) and CFN037 (C, D) were grown in 0.02% (A, C) and 0.1% (B, D) CO_2. Forty micrograms of protein were applied to each gel.

the wild-type *Rhizobium* CE3 strain and grown in 0.02% CO_2 (Fig. 3). In the other conditions tested, GS activity increased 2.5- to 3-fold. No effect on GS activity by high CO_2 was found at day 17 under any of the conditions tested (Fig. 3). However, analysis of the GS polypeptide composition at day 17 after infection revealed that, in high CO_2, where nitrogenase activity is two- to threefold higher, the GS-γ polypeptide was significantly reduced, whereas the GS-β polypeptide was not affected (Fig. 4). In nodules infected with the wild type CE3 strain, the GS-β/GS-γ polypeptide ratio determined by scanning densitometry was 2.6 in normal CO_2 and 5.9 in high CO_2. In the nodules infected with the mutant CFN037 strain, the GS-β/GS-γ ratio was 1.2 and 2.1 in normal CO_2 and high CO_2, respectively. The GS-β/GS-γ ratio was twofold higher in 0.1% CO_2 compared with 0.02% CO_2 in both strains. The fact that the reduction in the GS-γ polypeptide does not reduce GS total activity requires further evaluation. In contrast with this result, recent studies (2) showed that, in nodulated root systems of beans grown in an Ar:O_2 atmosphere, the activity of the nodule GS-γ isoenzyme was reduced (85%) along with that of nitrogenase, whereas the GS-β isoenzyme was unaffected (2). Together these results suggest that both high and low nitrogenase activity can be correlated with a reduction of GS-γ. Because in both experimental procedures (excess of CO_2 or absence of N_2) one could expect a higher carbon/nitrogen (C/N) ratio, these data suggest that the C/N balance within the nodule and not the ammonia derived from the bacteroid *per se* may be a primary modulating factor of nodule GS-γ isoenzyme. Whether the reduction in the GS-γ is due to a reduced synthesis or to a posttranslational modification mechanism needs to be defined. If carbon availability plays an important role in GS regulation, this result could also explain why a gene encoding a cytosolic GS from soybean increased its expression with ammonia in *L. corniculatus* but not in transformed tobacco plants (10).

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