RESEARCH PAPER

The rate of CO₂ assimilation controls the expression and activity of glutamine synthetase through sugar formation in sunflower (Helianthus annuus L.) leaves

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Abstract

The expression and activity of glutamine synthetase (GS, EC 6.3.1.2) were examined in relation to the rate of CO₂ assimilation in sunflower (Helianthus annuus L.) leaves. Intact plants were kept in the dark for 72 h and subsequently exposed to light under different atmospheric CO₂ concentrations (100, 400 and 1200 µl l⁻¹) for 6 h. The in vivo rates of net CO₂ assimilation correlated with atmospheric CO₂ concentrations. Stomatal conductances and transpiration rates remained largely unaffected by CO₂ levels. Exposure of the plants to increasing CO₂ concentrations in the light caused concomitant increases in the contents of starch and soluble sugars and a decrease in the nitrate content in leaves. Both cytosolic and chloroplastic (GS2) GS activities were higher at elevated CO₂. A greater accumulation of GS2 mRNA was also observed under high CO₂.

Exogenous supply of sucrose to detached leaves greatly increased the levels of GS enzyme activity and of mRNA for chloroplastic GS in the dark. These results indicate that GS expression and activity in sunflower leaves are modulated by the rate of CO₂ assimilation, and that photosynthesized sugars are presumably involved as regulatory metabolites.

Key words: Carbohydrates, CO₂ assimilation, gene expression and enzyme activity, glutamine synthetase, Helianthus annuus.

Introduction

Glutamine synthetase (GS) (EC 6.3.1.2), which catalyses the ATP-dependent synthesis of glutamine from glutamate and ammonium, is a key enzyme in the nitrogen metabolism of the plants. Glutamine synthetase exists as multiple isoforms that are either cytosolic (GS1) or plastidic (GS2) (McNally et al., 1983). These distinct isoforms of GS are encoded by a small family of nuclear genes whose expression is differentially regulated by light and nitrogen supply in a development- and tissue-specific manner (Edwards and Coruzzi, 1989; Cock et al., 1991; Li et al., 1993; Lam et al., 1996; Finnemann and Schjoerring, 1999). In leaves of C₃ plants, GS1 is expressed in phloem cells and it appears to be involved in the generation of glutamine for nitrogen transport (Kamachi et al., 1992; Pereira et al., 1992). GS2 is expressed in photosynthetic cells where it functions to assimilate ammonium produced from nitrate reduction and photorespiration (Wallsgrove et al., 1987; Cren and Hirel, 1999).

The effects of light and organic carbon supplementation on the expression of genes for chloroplastic GS2 or cytosolic GS1 have been tested by Oliveira and Coruzzi (1999) in Arabidopsis thaliana. They found that GS2 and GS1 were both induced by light or by carbon metabolites as sucrose. The existence of sugar-regulated gene expression in plants has become apparent in the past few years. Carbohydrate depletion up-regulates the expression of genes for photosynthesis and reserve mobilization, whereas high sugar levels enhance the expression of genes for carbon storage and utilization (Koch, 1996). Nitrogen assimilation by plants requires...
carbon skeletons derived from photosynthesized carbohydrates for the synthesis of amino acids (Huppe and Turpin, 1994). Nitrate assimilation proceeds at low rate in plants with low carbohydrate levels (Stitt and Schulze, 1994), whereas sucrose feeding increased rates of nitrate assimilation and amino acid synthesis (Morcuende et al., 1998). Addition of sugars in the dark to carbohydrate-depleted plants was shown to enhance nitrate reductase (NR)-gene expression (Cheng et al., 1992). Moreover, it has been reported (Larios et al., 2001) that a short exposure of the plant to elevated atmospheric CO₂ increased the rate of CO₂ assimilation and enhanced NR expression and activity.

The aim of the present work was to investigate whether GS, which links nitrogen to carbon metabolism, is regulated in vivo in sunflower leaves by the rate of CO₂ assimilation through sugar formation. For that purpose, intact plants were illuminated for 6 h under various atmospheric CO₂ concentrations, and CO₂ fixation rates, leaf carbohydrate contents, and expression and activity of GS were analysed. Moreover, the effects of sugar feeding to detached leaves from dark-treated (low carbohydrate-content) plants on GS expression and activity were tested.

Materials and methods

Plant material and growth

Seeds of sunflower (Helianthus annuus L.) from the isogenic cultivar HA-89 (Semillas Cargill SA, Sevilla, Spain) were surface-sterilized in 1% (v/v) hypochlorite solution for 15 min. After rinsing in distilled water, seeds were imbibed for 3 h and then sown in plastic trays containing a 1:1 (v/v) mixture of perlite and vermiculite. Seeds were germinated and plants grown in a growth chamber with a 16 h photoperiod (400 µmol m⁻² s⁻¹ of photosynthetically active radiation provided by ‘cool white’ fluorescent lamps supplemented by incandescent bulbs) and a day/night temperature and relative humidity regimes of 25/19 °C and 70/80%, respectively. Plants were irrigated daily with a nutrient solution containing 10 mM KNO₃ (Hewitt, 1966).

Plants were grown under the above conditions for 19 d and then under continuous darkness for 72 h to minimize sugar accumulation. The plants were transferred to different controlled-environment cabinets (Sanyo Gallenkamp Fitotron, Leicester, UK) equipped with an ADC 2000 CO₂ gas monitor. The plants were maintained for 6 h in the light (400 µmol m⁻² s⁻¹) at 25 °C and 80% relative humidity, but with different atmospheric CO₂ concentrations (100, 400 and 1200 µmol l⁻¹). CO₂ of high purity was supplied from a compressed gas cylinder (Air Liquide, Sevilla, Spain). Before and after CO₂ treatments, young leaves were excised, frozen immediately in liquid N₂ and stored at -80 °C. The frozen plant material was ground to a fine powder in a mortar precooled with liquid N₂ and the powder was distributed into small vials, which were stored at -80 °C for extraction and analysis of enzyme, transcripts and metabolites.

Net CO₂ fixation, transpiration and stomatal conductance were measured on attached leaves, using a portable infrared gas analyser (ADC LCA-3) with a PLC-3 chamber (The Analytical Development Co., Hoddesdon, UK), under the atmospheric CO₂ concentration of each treatment.

Sucrose feeding to detached leaves

Young leaves were excised from 19-d-old plants that were subsequently kept in the dark for 72 h, as indicated above. Detached leaves were placed into test tubes containing 90 mM sucrose or distilled water as control. They were incubated for 24 h in the dark. After rinsing with distilled water, leaves were frozen in liquid N₂ and ground as indicated above for analyses.

GS extraction

Frozen material was homogenized with cold extraction medium (4 ml g⁻¹) consisting of 100 mM TRIS-HCl buffer (pH 7.6), 10 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM MgCl₂, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µM leupeptin, 25 mg ml⁻¹ insoluble polyvinylpolypyrrolidone (PVPP), and 5 mg ml⁻¹ streptomycin sulphate. The homogenate was filtered through four layers of gauze and then centrifuged at 40 000 g for 30 min at 4 °C. The clear supernatants were desalted on PD-10 Sephadex G-25 columns equilibrated with 10 mM TRIS-HCl buffer (pH 7.6), 1 mM EDTA, and 1 mM MgCl₂.

Separation of GS isoenzymes

The desalted homogenate was loaded onto a diethylaminoethyl (DEAE)-Sephacel (Pharmacia, Uppsala) column (10×1 cm) previously equilibrated with 10 mM TRIS-HCl buffer (pH 7.6) and 1 mM MgCl₂. The column was washed with the same buffer until no protein was detectable in the eluate. Elution of adsorbed proteins was carried protein with a linear gradient of 0–0.5 M NaCl dissolved in 100 ml of the equilibrating buffer. The flow rate was maintained at 20 ml h⁻¹ and 2 ml fractions were collected. All the above procedures were carried out at 4 °C. Collected fractions were assayed for GS activity as described by de la Haba et al. (1992). Typical chromatography elution profiles of GS from sunflower have been previously shown (Cabello et al., 1991; de la Haba et al., 1992). The level of activity of each isoenzyme was estimated from the area of the corresponding elution profile after fractionation. About 80% of the total GS activity present in crude extracts was recovered after chromatographic separation.

Carbohydrate determinations

Carbohydrates were extracted from the powdered frozen tissue by successive steps of extraction with different ethanol/water solutions, as described by Scheible et al. (1997). Supernatants from each centrifugation were collected and combined for the analysis of soluble sugars, and the pellets were reserved for starch determination. Sucrose was analysed as in Outlaw and Tarczynski (1984), glucose as in Kunst et al. (1984), and fructose as in Beuler (1984). The pellets were resuspended in water and incubated at 100 °C for 5 h. Glucose was then released by incubation with α-amylase and amyloglucosidase and assayed enzymatically, as described above.

Nitrate determination

The nitrate content of the leaf material was determined in the same ethanol/water extracts used for sugar determinations. 10 µl aliquots were injected into an HPLC system. Separation was carried out on a Nucleosil 100–10SB column (250 mm long, 4 mm i.d.) and eluted with 125 mM potassium phosphate (pH 4.0) at a flow rate of 1 ml min⁻¹. The nitrate peak was detected at 210 nm and quantified by a computing integrator. Nitrate concentration was calculated by comparison with a calibration curve obtained by injecting known amounts of analytical-grade potassium nitrate.

Isolation of RNA and northern analyses

Total RNA was isolated from leaves by using the TRI-REAGENT (Molecular Research Center, Cincinnati, USA), according to the method of Chomczynski (1993). Pellets of RNA were dissolved in...
Regulation of glutamine synthetase in sunflower leaves

Results

Influence of atmospheric CO₂ concentration on leaf GS activity

Sunflower plants grown under ambient CO₂ and kept in continuous darkness for 72 h were transferred to light under different atmospheric CO₂ concentrations for 6 h. Exposure of the plants to light increased GS activity, this increase being markedly dependent on CO₂ concentration from 100 to 1200 μl l⁻¹ CO₂ (Table 1). The total leaf GS activity was more than six times higher under 1200 μl l⁻¹ CO₂. Cytosolic GS1 and chloroplastic GS2 isoforms were both positively affected by CO₂ concentration.

Assimilation of CO₂ and leaf carbohydrate and nitrate contents under different atmospheric CO₂ concentrations

Carbon dioxide fixation rates, transpiration and stomatal conductance were measured on attached leaves after 72 h of continuous darkness (control) and after a subsequent 6 h exposure to light under 100, 400 and 1200 μl l⁻¹ CO₂. Increasing atmospheric CO₂ concentrations led to increasing rates of photosynthetic CO₂ assimilation (Table 2). Stomatal conductances and transpiration rates were not significantly affected by CO₂ levels.

Exposure of plants to increasing CO₂ concentrations in the light also promoted concomitant increases in soluble sugars (glucose, fructose and sucrose) and starch in the leaf (Table 3). By contrast with sugars, increasing CO₂ concentrations lowered leaf nitrate content (Fig. 1).

Effects of atmospheric CO₂ concentration and sucrose feeding to leaves on GS gene expression

Results presented above showed that increasing rates of CO₂ fixation enhanced sugar formation and levels of GS activity in sunflower leaves. These observations prompted an examination of the effects of CO₂ on GS at the transcriptional level. GS mRNA levels were determined by northern blot analysis using a cDNA probe isolated from sunflower corresponding to the chloroplastic (GS2) isoform. This is the most abundant molecular form of GS in sunflower leaves (Cabello et al., 1991), and plays a major role in ammonium assimilation and in nitrogen recycling during photorespiration (Wallsgrove et al., 1987). Northern analyses of GS2 mRNA revealed a greater abundance of transcripts after 6 h in high CO₂ than in low CO₂ (Fig. 2A), indicating that CO₂ enhanced GS2-gene expression. The CO₂ effect was dependent on light, as inferred from the observation that, under 400 μl l⁻¹ CO₂, accumulation of GS2 mRNA was higher in the light than in the dark. These results suggest that the positive effect of CO₂ on GS expression requires its assimilation by the plant and, therefore, it could be mediated by photosynthesized sugars.

To determine whether sugars could have a direct role in the regulation of GS-gene expression, plants were placed in the dark for 72 h to lower the leaf carbohydrate content and, afterwards, leaves were excised and incubated in the dark for 24 h with water as control or with 90 mM sucrose. Results show (Fig. 2B) that exogenously supplied sucrose greatly increased the levels of GS2 mRNA in the absence of light. Sucrose treatment could also induce the levels of

Table 1. Effects of light and atmospheric CO₂ concentration on total GS activity and on the activities of GS1 and GS2 isoforms in sunflower leaves

Plants kept in the dark for 72 h were subsequently exposed to light under the indicated atmospheric CO₂ concentrations for 6 h. At the end of the dark period (control) and after the 6 h light treatment, leaves were excised for GS extraction, isoenzyme separation, and activity determination, as described in the Materials and methods. The relative proportion of each isoenzyme is expressed as a percentage of the total GS activity. Data are means ±SD of duplicate determinations from three separate experiments.

<table>
<thead>
<tr>
<th>CO₂ (μl l⁻¹)</th>
<th>Total GS (nkat g⁻¹ FW)</th>
<th>GS1 (nkat g⁻¹ FW) (%)</th>
<th>GS2 (nkat g FW⁻¹) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.4±2.3</td>
<td>3.5±0.1</td>
<td>14.0</td>
</tr>
<tr>
<td>100</td>
<td>43.8±3.7</td>
<td>6.1±0.3</td>
<td>13.8</td>
</tr>
<tr>
<td>400</td>
<td>117.6±6.7</td>
<td>17.9±0.2</td>
<td>15.3</td>
</tr>
<tr>
<td>1200</td>
<td>278±9.8</td>
<td>58.2±0.4</td>
<td>20.9</td>
</tr>
</tbody>
</table>

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enzyme activity of the cytosolic GS1 and the chloroplastic GS2 isoforms (Table 4).

Discussion

The enzyme GS is responsible for the primary assimilation of ammonium generated in nitrate reduction and dinitrogen fixation, and is also involved in the reassimilation of ammonium released in photorespiration and amino acid catabolism (Lea et al., 1990). The leaves of sunflower plants contain both cytosolic (GS1) and chloroplastic (GS2) glutamine synthetases, the latter being the most abundant isoform (Cabello et al., 1991).

Evidence has accumulated recently that demonstrates a close interaction between carbon and nitrogen metabolism in higher plants. In particular, NR, which catalyses the first step of the nitrate assimilation process, has been shown to be up-regulated at both transcriptional and post-translational levels by CO2 assimilation products (Cheng et al., 1992; Vincentz et al., 1993; Larios et al., 2001). A direct relationship between the rate of photosynthetic CO2 assimilation by intact sunflower plants and the expression and activity of the GS isoforms in leaves is reported here.

A short exposure (6 h) of the plants to increasing CO2 concentrations (up to 1200 μl l−1) in the light elicited concomitant increases in the rate of CO2 fixation and in the amount of starch and soluble sugars in the leaf, and induced GS both at the level of gene transcription and that of enzyme activity. Exogenous supply of sucrose to carbohydrate-depleted leaves also enhanced both GS-gene expression and GS-enzyme activity in the dark, which appears to indicate that sugars derived from CO2 assimilation act as positive regulatory metabolites of GS in sunflower plants. GS in A. thaliana has also been reported to be induced by sucrose, whereas amino acids were shown to antagonize the sucrose induction of GS (Oliveira and Coruzzi, 1999). Hence, it was suggested that GS expression is metabolically regulated by the relative abundance of carbon skeletons versus amino acids.

Sunflower plants were grown on nitrate as the external nitrogen source. Nitrate assimilation for amino acid synthesis is dependent on carbon assimilation for the supply of carbon skeletons. It was found that increased rates of CO2 assimilation at high CO2 concentration (Table 2), and concomitant higher carbohydrate availability (Table 3), enhanced the expression and activity of NR (Larios et al., 2001) and GS (Table 1; Fig. 2). In fact, nitrate assimilation was shown to proceed at a low rate in plants with low carbohydrate levels (Stitt and Schulze, 1994). This was also supported by the finding that elevated atmospheric CO2 concentration resulted in a lower accumulation of nitrate in leaves (Fig. 1). The observation that plants exposed to 100 μl l−1 CO2 in the light accumulated more nitrate in their leaves than plants kept in the dark, in spite of their higher leaf carbohydrate content (Table 3), may be due to a higher rate of nitrate transport from roots to leaves in the light as a result of a higher transpiration rate (Table 2).

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**Table 2. Stomatal conductances, transpiration and CO2 fixation rates in leaves of sunflower plants exposed to different atmospheric CO2 concentrations**

Plants kept in the dark for 72 h were subsequently exposed to light under the indicated atmospheric CO2 concentrations for 6 h. Measurements were performed at the end of the dark period (control) and after the 6 h light treatment. Data are means ±SD of measured values on leaves of ten plants randomly selected.

<table>
<thead>
<tr>
<th>CO2 (μl l−1)</th>
<th>Stomatal conductance (mol H2O m−2 s−1)</th>
<th>Transpiration (mmol H2O m−2 s−1)</th>
<th>CO2 fixation (μmol CO2 m−2 s−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.02±0.005</td>
<td>0.20±0.005</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>100</td>
<td>0.31±0.02</td>
<td>1.97±0.09</td>
<td>2.80±0.17</td>
</tr>
<tr>
<td>400</td>
<td>0.28±0.03</td>
<td>1.78±0.12</td>
<td>4.65±0.28</td>
</tr>
<tr>
<td>1200</td>
<td>0.30±0.01</td>
<td>2.10±0.10</td>
<td>8.12±0.64</td>
</tr>
</tbody>
</table>

**Table 3. Contents of glucose, fructose, sucrose, and starch in sunflower leaves as affected by atmospheric CO2 concentration**

Carbohydrate contents were analysed in leaves of sunflower plants after 72 h in continuous darkness (control) and after the subsequent exposure to light under the indicated atmospheric CO2 concentrations for 6 h. Data are means ±SD of duplicate determinations from three separate experiments.

<table>
<thead>
<tr>
<th>CO2 (μl l−1)</th>
<th>Carbohydrate content (μmol g−1 FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose  Fructose  Sucrose  Starch</td>
</tr>
<tr>
<td>Control</td>
<td>0.40±0.15  0.24±0.08  0.61±0.08  1.36±0.13</td>
</tr>
<tr>
<td>100</td>
<td>1.36±0.46  0.41±0.04  2.18±0.13  2.41±0.47</td>
</tr>
<tr>
<td>400</td>
<td>15.75±0.51 13.62±0.29 10.17±0.72  49.20±5.22</td>
</tr>
<tr>
<td>1200</td>
<td>25.05±0.17 20.56±0.04 14.02±0.15 66.72±4.41</td>
</tr>
</tbody>
</table>
Besides being involved in the assimilation of the ammonium produced during nitrate reduction, the chloroplastic GS isoform (GS2) also participates in the refixation of the ammonium generated in illuminated leaves during photorespiration (Wallsgrove et al., 1987). Photorespiration has been estimated to produce at least a 10-fold higher flux of ammonium than nitrate reduction (Givan et al., 1988). This has raised the question as to whether photorespiration is able to control the expression of GS2. The rates of photosynthesis and photorespiration are determined by the kinetic properties of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme and the relative proportion of its alternative substrates, and also the competitive inhibitors CO$_2$ and O$_2$ (Woodrow and Berry, 1988). Then, the possible regulation of GS2 by the rate of photorespiration has been analysed in leaves of C$_3$ plants grown in a CO$_2$-enriched atmosphere to suppress photorespiration. A positive control of photorespiration on GS2-gene expression has been suggested by the result that the GS2-gene expression in leaves of pea or bean grown at a very high atmospheric CO$_2$ concentration was lower than in leaves of the plants grown in air (Edwards and Coruzzi, 1989; Cock et al., 1991). However, suppression of photorespiration during growth of A. thaliana and tobacco in an atmosphere with 3000 $\mu$l $^{-1}$ CO$_2$ had no effect on the leaf GS2 transcript level (Beckmann et al., 1997; Migge et al., 1997). Hence, it was suggested (Beckmann et al., 1997) that photorespiration does not exert a metabolic control on GS2-gene expression, and that the lower than normal GS2 mRNA levels found in leaves of high CO$_2$-grown pea or bean may have resulted from a metabolic acclimation of the leaves to the very high (20 ml $^{-1}$) levels of CO$_2$ that were applied to suppress photorespiration in that study. Plants exposed to high CO$_2$ for long periods have a reduced capacity to assimilate CO$_2$ as result of acclimation of photosynthesis (Drake et al., 1997). These results show that acclimation of photosynthesis did not occur in sunflower during the short (6 h) exposure of the plant to a moderately high (1200 $\mu$l $^{-1}$) CO$_2$ concentration, as inferred from the higher capacity of the leaves to synthesize carbohydrates, which also points out that photorespiration was decreased. Under these conditions, GS2-gene expression and the corresponding enzyme activity increased, suggesting that sunflower leaf GS2 is positively modulated by CO$_2$ assimilation, through formed sugars, and not by high photorespiration. Recent work (Finnemann and Schjoerring, 2000; Riedel et al., 2001) has

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**Fig. 1.** Nitrate content in leaves of sunflower plants at the end of a 72 h dark period (hatched column) and after 6 h of light under the indicated atmospheric CO$_2$ concentrations (open columns). Data are means ±SD of duplicate determinations from three separate experiments.

**Fig. 2.** Effects of CO$_2$ fixation or sucrose supply on GS2 mRNA accumulation in sunflower leaves. (A) Plants were kept in the dark for 72 h and subsequently exposed to light under the indicated atmospheric CO$_2$ concentrations for 6 h. At the end of the dark period (control) and after the 6 h light treatment, leaves were excised and analysed for GS2 mRNA levels by northern blotting. (B) After keeping the plants in the dark for 72 h, leaves were excised and incubated either with water (control) or with 90 mM sucrose (Suc) for 24 h in the dark. Thereafter, GS2 mRNA levels were analysed in the leaves. Each lane was loaded with 20 $\mu$g of total RNA. Uniformity of RNA loading and RNA integrity were checked by ethidium bromide staining (lower panels in A and B) before blotting. For further details see the Materials and methods.
shown that GS, as previously found for NR (MacKintosh et al., 1995), is regulated post-translationally by reversible phosphorylation. Whether the increase of GS activity by CO₂ assimilation could also be the result of activation of existing GS remains to be determined.

Sugar-regulated gene expression in plants has become apparent in the past few years. Photosynthetic genes are typically up-regulated by sugar-depletion, whereas sugar abundance enhances the expression of genes for carbon storage and utilization (Koch, 1996). Nitrate assimilation, which includes nitrate reduction to ammonium and the subsequent ammonium assimilation, requires carbon skeletons derived from photosynthesized sugars for the synthesis of amino acids. Then, enhanced CO₂ fixation at elevated CO₂, and concomitant increased carbon availability, would stimulate nitrate utilization by enhancing the expression and activity of NR and nitrite reductase (see Introduction) and also that of chloroplastic GS (results presented here), thus maintaining an adequate C:N ratio in the plant.

Acknowledgements

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References


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**Table 4. Effects of sucrose feeding to detached sunflower leaves on leaf GS activity**

After keeping the plants in the dark for 72 h, young leaves were excised and incubated in the dark for 24 h either with water (control) or with 90 mM sucrose. Thereafter, GS was extracted, fractionated and assayed as indicated in the Materials and methods. Data are means ±SD of duplicate determinations from three separate experiments.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Total GS (nkat g⁻¹ FW)</th>
<th>GS1 (nkat g⁻¹ FW) (%)</th>
<th>GS2 (nkat g⁻¹ FW) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (control)</td>
<td>47.8 ± 2.4</td>
<td>15.3 ± 0.3</td>
<td>32.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>159.3 ± 5.7</td>
<td>56.0 ± 0.7</td>
<td>68.0</td>
</tr>
</tbody>
</table>

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assimilation into amino acids in higher plants. Annual Review of Plant Physiology and Plant Molecular Biology 47, 569–593.


