Glutamate synthase in greening callus of *Bouvardia ternifolia* Schlecht

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Abstract. The distribution of the two glutamate-synthase (GOGAT) activities known to exist in higher plants (NADH dependent, EC 2.6.1.53; and ferredoxin dependent, EC 1.4.7.1) was studied in non-chlorophyllous and chlorophyllous cultured tissue as well as in young leaves of *Bouvardia ternifolia*. The NADH-GOGAT was present in all three tissues. Using a sucrose gradient we found it in both the soluble and the plastid fraction of non-chlorophyllous and chlorophyllous tissue, but exclusively in the chloroplast fraction of the leaves. Ferredoxin-GOGAT was found only in green tissues and was confined to the chloroplasts. Ferredoxin-GOGAT activity increased in parallel with the chlorophyll content of the callus during the greening process in Murashige-Skoog medium (nitrate and ammonium as the nitrogen sources), while NADH-GOGAT was not affected by the greening process in this medium. Furthermore, both activities were differentially affected by either nitrate or ammonium as the sole nitrogen source in the medium during this process. It is suggested that each GOGAT activity is a different entity or is differently regulated.

Key words: *Bouvardia* – Callus tissue – Chloroplast (glutamate synthase) – Ferredoxin – Glutamate synthase – Greening.

Introduction

Chloroplasts are important in nitrogen metabolism (for review, see e.g. Miflin and Lea 1980) since the two distinct glutamate-synthase (GOGAT) activities (NADH- and ferredoxin-dependent) which have been found in plants seem to be localized in these organelles (Wallsgrove et al. 1979; Matoh and Takahashi 1981). The physiological meaning of these activities is however not clearly understood. Ferredoxin-GOGAT has been related to ammonium reassimilation from the photorespiratory cycle (Somerville and Ogren 1980; Woo et al. 1982); however, the enzyme has also been found in non-chlorophyllous tissue (Matoh and Takahashi 1981; Susuki et al. 1982; Wallsgrove et al. 1982), leaving its role still open.

Callus culture is a useful material for the study of certain aspects of plant metabolism. A greening callus represents biochemical and cellular differentiation with relatively less tissue and organ differentiation; therefore, metabolic changes related to cellular differentiation can be examined in this system. The aim of the present work was to determine the changes in maximum catalytic activity, and the intracellular location, of NADH- and ferredoxin-GOGAT during the differentiation of plastids in tissue cultures. For comparative purposes young leaves were also included in this study.

Materials and methods

Tissue and plant culture. *Bouvardia ternifolia* Schlecht leaf callus was grown on MS medium (Murashige and Skoog 1962) supplemented with 1 mg l⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.005 mg l⁻¹ of kinetin (N⁶-furfurylaminopurine) as previously described (Fernández and Sánchez de Jiménez 1982). In order to induce greening, the tissue was placed on MS medium with a different set of hormones: 5 mg l⁻¹ of benzyladenine (N⁶-benzylaminopurine) and 0.005 mg l⁻¹ of α-naphthaleneacetic acid; sucrose was reduced from 3% to 1.5%. In some experiments the MS nitrogen source (20 mM KNO₃ and 20 mM NH₄NO₃) was replaced by either 10 mM NH₄Cl plus 10 mM potassium citrate, or 20 mM KNO₃. The calli were grown under continuous white light (Slimline 39; Solar lamp, México City; 20 W m⁻², measured with a LI-185B photometer,
LI-COR, Lincoln, Neb., USA) at 28°C. A CO₂-enriched atmosphere was generated in a closed chamber using NaHCO₃ and citric acid as a source of CO₂, to obtain a 0.1% concentration, calculated at atmospheric pressure and 28°C.

Young leaves of Bouvardia ternifolia (from 4 to 6 weeks) were obtained from stock plants grown in the University Botanical fields, under natural light, night-day temperatures ranging approximately from 12°C to 28°C.

**Protoplast preparation.** Protoplasts were obtained either from non-chlorophyllous callus induced from leaves, or from young leaves according to the method of Power and Cocking (1970) using cellulase (Onozuka R-10) and pectinase (Macerozyme R-10) leaves according to the method of Power and Cocking (1970) as described previously (Fernández and Sánchez de Jiménez 1982).

**Enzyme activities.** Calli were homogenized with a 0.05 M potassium-phosphate buffer (using KH₂PO₄ and K₂HPO₄), pH 7.5, 5 mM ethylenediaminetetraacetate (EDTA), 1 μM phenylmethylsulfonyl fluoride (PMFS), 0.1% β-mercaptoethanol (in the case of leaves and chlorophyllous callus 10%, w/v, polyvinyl pyrrolidone [PVP] was added) in a Polytron homogenizer (Kinematica, Luzern, Switzerland) at 20000 rpm for 20 s. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 20000 g for 15 min; the supernatant was used as the enzyme source. Measurement of NADH-GOGAT was as reported in Fernandez and Sánchez de Jiménez (1982); ferredoxin-GOGAT was measured according to Arima (1979). One unit of GOGAT activity is defined as 1 nm glutamate transformed per 1 min under the assay conditions. Ferredoxin was purified according to Buchanan and Arnon (1981). Protein content was measured by the biuret method (see Gornall et al. 1949), and chlorophyll content according to Arnon (1949).

**Cell fractionation.** Freshly prepared protoplasts (10⁴ cells ml⁻¹) were suspended in 10 mM KC1, 1 mM MgCl₂, 0.1% bovine serum albumin, 0.1 M Tricine \{N-[2-hydroxy-1-bis(hydroxy-methyl)ethyl]glycine\}, pH 7.5, at 4°C, passed several times through a No. 22 needle, and placed on a continuous-discontinuous sucrose gradient in 0.1 M Tricine buffer, pH 7.5, and 1 μM phenylmethylsulfonyl fluoride (PMSF). The gradient consisted of 4 ml of 60% sucrose, 6 ml of a 60–42% sucrose linear gradient, 5 ml 42% sucrose, 8 ml of a 42–25% sucrose linear gradient, and 3 ml of 25% sucrose. The gradients were centrifuged in a SW-27 Spinco rotor (Beckman Instruments, Palo Alto, Cal., USA) at 25000 rpm for 5 min, followed by 18000 rpm for 30 min. Fractions of 1.5 ml were collected and dialyzed against 0.01 M potassium-phosphate buffer, pH 7.5, 5 mM EDTA, 0.1% β-mercaptoethanol and 1 μM PMSF. Triton X-100 was added (0.1%, v/v) to each fraction, and enzyme activities were measured. Nitrite reductase was measured according to Losada and Paneque (1971), cytochrome-c oxidase according to Wharton and Tzagoloff (1967), alcohol dehydrogenase according to Dennis and Kaplan (1962), and catalase according to Arnon (1949).

**Differential centrifugation.** Freshly prepared protoplasts, broken as above, were centrifuged either at 5000 g (callus) or 2500 g (leaves) for 10 min. The supernatant was centrifuged at 20000 g for 10 min in a J2-21 centrifuge (Beckman Instruments at 4°C. The 2500-g, 5000-g and 20000-g pellet fractions were resuspended in 0.01 M potassium-phosphate buffer, pH 7.5, 5 mM EDTA, 0.1% β-mercaptoethanol, 1 μM PMSF and 0.1% Triton X-100. The supernatant was supplemented with EDTA and β-mercaptoethanol to have a final concentration of 5 mM and 0.1%, respectively; and enzyme activities were measured in each fraction.

**Chemicals.** Benzyladenine, EDTA, PMSF, Tricine, Triton X-100 and β-mercaptoethanol were obtained from Sigma Chemical Co., St Louis, Mo., USA., cellulase and pectinase from Yakult Biochemical Co., Nishinomiya, Japan.

**Results**

The changes in chlorophyll content, NADH-GOGAT and ferredoxin-GOGAT activities were followed in callus during the greening period in CO₂-enriched atmosphere (0.1%). Chlorophyll content and the activities of NADH- and ferredoxin-GOGAT were also measured in chlorophyllous callus and young green leaves. The specific activity of NADH-GOGAT was similar in all three tissues (leaf, non-chlorophyllous and chlorophyllous callus). In contrast, ferredoxin-GOGAT activity was much higher in leaf tissue than in chlorophyllous callus (Table 1). When ferredoxin-GOGAT activity was expressed as units per mg chlorophyll (10.5 mg chlorophyll g⁻¹ fresh tissue in green leaves, 1.5 mg chlorophyll g⁻¹ fresh weight in chlorophyllous callus), indicating that this enzyme activity is not linked to chlorophyll content (Table 1).

In the standard MS medium (nitrate and ammonium as nitrogen sources), chlorophyll in the chlorophyllous callus was detectable after 6 d of incubation and its level continued to rise until the end of the incubation period (20th day). Ferredoxin-GOGAT was not detectable until the eight day, and the activity reached a plateau at the end of the incubation period (20th day). However, NADH-GOGAT was present throughout the en-

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GOGAT activity (nmol glutamate min⁻¹ mg⁻¹ protein)</th>
<th>NADH-GOGAT (mg⁻¹ chlorophyll mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-chlorophyllous callus</td>
<td>0.0</td>
<td>5.4 ± 0.61*</td>
</tr>
<tr>
<td>Chlorophyllous callus</td>
<td>0.8±0.20</td>
<td>4.4±0.53</td>
</tr>
<tr>
<td>Leaves</td>
<td>4.5±0.33</td>
<td>4.2±0.21</td>
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* Each value represents the mean ± SD, n=4

Table 1. Ferredoxin- and NADH-GOGAT activities in young leaves, non-chlorophyllous and chlorophyllous callus of Bouvardia after 15 d of subculture.