Vitis 36 (4), 207–208 (1997)

Research Note

Evidence for ferredoxin glutamate synthase activity in grape shoot tissues

G. L. CREASY[1]) and P. J. BREEN[2])

Summary: Ferredoxin glutamate synthase (ferredoxin glutamine: oxoglutarate aminotransferase, or Fd-GOGAT, EC 1.4.7.1) activity was detected in grapevine (*Vitis vinifera* L.). Highest Fd-GOGAT activity was found in lamina tissue. Moderate levels of activity were found in petiole, tendril, rachis, and flower tissues. No activity was detected in extracts from pedicel tissue. Western blotting with rice Fd-GOGAT antibodies resulted in a positive reaction with single grape protein band of 73 kDa in all tissue types tested.

Key words: Fd-glutamate synthase, ammonium assimilation.

Introduction: Glutamate synthase (GOGAT) has been characterized in many herbaceous plants, but little research in woody perennials has been reported. Additionally, to avoid high levels of phenolics prevalent in these plant species, material used in earlier studies were either from seedlings or dark-grown suspension cultures.

In grape, ROUBELAKIS-ANGELAKIS and KLIEWER (1983) were unable to detect GOGAT activity in Chenin blanc root or leaf extracts, but JORDAN *et al.* (1992) reported NADH-GOGAT activity in crude grape leaf extracts from Cabernet Sauvignon. GU *et al.* (1994), working with Pinot noir shoot cuttings, used an inhibitor of GOGAT to infer that certain grape tissues had less GOGAT activity than others.

Here, we report the presence and activity of the most important form of GOGAT in photosynthetic tissues: that which utilizes Fd as the reductant.

Materials and methods: Grapevine tissue was obtained from both greenhouse and field-grown Pinot noir vines. Flower clusters were separated into flowers, pedicels, and rachis prior to freezing. Separation was done over ice in a cold room (4 °C) using fine-tipped scissors to clip first the flowers, then the pedicels from the inflorescence. Tissues were then frozen in liquid nitrogen and stored at -80 °C.

Frozen tissue for the western blots was ground in a mortar with 25 % (w/w) polyvinylpolypyrrolidone (PVPP), then mixed with 5 volumes buffer (50 mM phosphate, pH 7.6, with 5 mM ethylenediaminetetraacetic acid (EDTA), 1 % (v/v) Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 14 mM β-mercapto-ethanol, β-ME). After centrifugation, proteins were precipitated with 5 volumes of 0.1 M ammonium acetate in methanol at -20 °C overnight, washed twice with fresh 0.1 M ammonium acetate in methanol, then twice with -20 °C acetone.

The proteins were resuspended in sample buffer, their protein concentration determined (BRADFORD 1976), then loaded onto SDS mini gels (Mini-PROTEAN II apparatus, Bio-Rad Laboratories, Richmond, CA). Proteins were transferred to a nitrocellulose membrane using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. Membranes were probed with IgG anti-rice Fd-GOGAT (SUZUKI *et al.* 1982) and developed using a Bio-Rad Immun-Blot Assay Kit (AP goat anti-rabbit IgG).

For the enzyme assay, frozen tissues were ground with 75 % (w/w) PVPP, then added to 12 volumes 200 mM phosphate buffer, pH 7.6, containing 2.5 mM EDTA, 75 mM borax decahydrate, 28 mM β-ME, 5 mM dithiothreitol (DTT), 0.2 mM PMSF, and 75 % (w/w) PVPP. Ammonium sulfate precipitate between 25 % and 85 % saturation was resuspended in 2.0 ml buffer (10 mM phosphate, pH 7.6, with 2.5 mM DTT) and dialyzed (Spectra/Por MWCO: 6-8000 tubing, Spectrum Medical Industries, Inc., Los Angeles, CA) overnight at 4 °C. The protein concentration of the resulting solution was measured and then used for the assays, which were carried out at 30 °C for 2 min. The 1.2 ml reaction volume contained buffer (10 mM phosphate, pH 7.6, with 0.5 mM α-keto glutarate, 1.0 mM glutamine, 0.48 mM DTT, 20 mM KCl, and 0.4 mM EDTA), 0.05 mg ferredoxin, and 150 µl protein solution. The reaction was started with the addition of sodium hydrosulfite and stopped by immersion in boiling water. Glutamate in the reaction solution was separated and quantified using the method of MATOH *et al.* (1980).

Results: Distinct positive reactions occurred on the nitrocellulose membrane in lanes containing each grape tissue tested, though response was weaker for grape lamina and rachis. The major band for rice leaf corresponded to a molecular weight of 177 kDa, while that for grape tissues was approximately 73 kDa.

Analysis of free amino acid content of enzyme assay eluates showed glutamate as the only significant free amino acid present. Addition of 1 mM azaserine to the assay mixture effectively blocked enzyme activity, while addition of methionine sulfoximine (an inhibitor of glutamine synthetase) had no effect on glutamate production (CREASY 1996).

Fd-GOGAT activity was detected in all but one tissues tested (Table). Extracts from laminas showed the greatest activity, but no activity was found in the extract from pedicel tissue. Other tissues exhibited moderate, and similar, levels of activity.

Discussion: Western blots using anti-Fd-GOGAT IgG from rice leaf showed a positive reaction in grape tissues corresponding to a protein of approximately 73 kDa. The size of subunit Fd-GOGAT protein that has been reported in other plant species ranges from 115 kDa in rice (SUZUKI *et al.* 1982) to 165 kDa in pine seedling cotyledons (GARCÍA-GUTIÉRREZ *et al.* 1995). Fd-GOGAT from a variety of plant

[1]) National Wine and Grape Industry Centre, Charles Sturt University, P.O.Box 588, Wagga Wagga NSW 2678, Australia. Fax +61-2-6933 2107. E-mail: gcreasy@csu.edu.au

[2]) Department of Horticulture, Oregon State University, 4017 Agriculture & Life Sciences Bldg., Corvallis OR 97331-7304 USA

Table

GOGAT activity data (± standard error) from a variety of shoot tissues from Pinot noir grapevine. Assay conditions were as described in the text. The unit of enzyme activity (U) is defined as 1 µmol glutamate equivalent produced per minute of reaction time

Tissue	Source	Activity $U \cdot mg^{-1}$ protein	Activity $U \cdot g^{-1}$ fresh weight
Lamina	Greenhouse	0.431 ± 0.093	0.921 ± 0.180
Petiole 1	Field	0.177 ± 0.026	0.105 ± 0.015
Petiole 2	Field	0.165 ± 0.054	0.053 ± 0.017
Flower 1	Field	0.152 ± 0.053	0.180 ± 0.036
Flower 2	Field	0.129 ± 0.025	0.125 ± 0.143
Pedicel	Field	n.d.	n.d.
Rachis	Greenhouse	0.119 ± 0.009	0.161 ± 0.012
Tendril	Field	0.172 ± 0.029	0.132 ± 0.022

n.d.: not detectable.

species have been sequenced, and are fairly highly conserved (ZEHNACKER *et al.* 1992, GARCÍA-GUTIÉRREZ *et al.* 1995). Fd-GOGAT antibody from one species has successfully been used to probe other species, including monocotyledon to dicotyledon probes (GARCÍA-GUTIÉRREZ *et al.* 1995), so it is likely that the positive reaction reported here indicates a protein related to Fd-GOGAT in grape.

Grape lamina and rachis tissue had a lesser response in reaction to the antibody than other tissues, possibly because the enzyme may be expressed preferentially or to varying extents in tissues of different ages or types. In tobacco, high amounts of Fd-GOGAT protein were found in leaves, lesser amounts in pistils and anthers, and none in the corollas or stems (ZEHNACKER *et al.* 1992).

Gel filtration of the partially purified enzyme extract from grape leaves suggested that the native form Fd-GOGAT is a dimer or a trimer of the 73 kDa protein identified with the polyclonal antibody (CREASY 1996).

Leaf tissue had a level of Fd-GOGAT activity similar to published values, *e.g.* 0.195 $U \cdot mg^{-1}$ in rice (SUZUKI and GADAL 1982) and 2.64 $U \cdot mg^{-1}$ in tobacco (ZEHNACKER *et al.* 1992).

This is a preliminary study into the action of Fd-GOGAT in grape tissues. The recent isolation of DNA sequences encoding for Fd-GOGAT in grape (K. A. ROUBELAKIS-ANGELAKIS, personal communication) should do much to help elucidate ammonium cycling within grapevines.

The authors wish to thank Dr. AKIRA SUZUKI for the gift of IgG anti-rice Fd-GOGAT and Dr. D. ARP, Dr. S. PRICE, and Dr. M. KELLER for help in the preparation of this manuscript. Financial support provided in part by the Oregon Wine Advisory board is gratefully acknowledged.

BRADFORD, M. M.; 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248-254.

CREASY, G. L.; 1996: Inflorescence necrosis, ammonium, and evidence for ferredoxin-glutamate synthase activity in grape (*Vitis vinifera* L.). Ph. D. Thesis. Oregon State Univ., Corvallis, Oregon, USA.

GARCÍA-GUTIÉRREZ, A.; CANTÓN, F. R.; GALLARDO, F.; SÁNCHEZ-JIMÉNEZ, F.; CÁNOVAS, F. M.; 1995: Expression of ferredoxin-dependent glutamate synthase in dark-grown pine seedlings. Pl. Molec. Biol. **27**, 115-128.

GU, S.; LOMBARD, P. B.; PRICE, S. F.; 1994: Inflorescence necrosis induced from ammonium incubation and deterred by α-keto glutarate and ammonium assimilation in Pinot noir grapevines. Amer. J. Enol. Viticult. **45**, 155-160.

JORDAN, D.; PETERSON, C.; PEAKE, J.; CLARK, C.; 1992: Glutamate synthase activity in grape tissue. In: Proc. 4th Intern. Symp. Grapevine Physiology. San Michele and Torino, May 11-15, 1992, 469-472.

MATOH, T.; IDA, S.; TAKAHASHI, E.; 1980: A rapid and sensitive assay for ferredoxin-glutamate synthase. Bull. Res. Inst. Food Sci., Kyoto Univ. **43**, 1-6.

ROUBELAKIS-ANGELAKIS, K. A.; KLIEWER, W. M.; 1983: Ammonia assimilation in *Vitis vinifera* L.: II. Leaf and root glutamine synthetase. Vitis **22**, 299-305.

SUZUKI, A.; GADAL, P.; 1982: Glutamate synthase from rice leaves. Pl. Physiol. **69**, 848-852.

- -; VIDAL, J.; GADAL, P.; 1982: Glutamate synthase isoforms in rice. Immunological studies of enzymes in green leaf, etiolated leaf, and root tissues. Pl. Physiol. **70**, 827-832.

ZEHNACKER, C.; BECKER, T. W.; SUZUKI, A.; CARRAYOL, E.; CABOCHE, M.; HIREL, B.; 1992: Purification and properties of tobacco ferredoxin-dependent glutamate synthase, and isolation of corresponding cDNA clones. Planta **187**, 266-274.