Glutamine and \(\alpha\)-ketoglutarate are metabolite signals involved in nitrate reductase gene transcription in untransformed and transformed tobacco plants deficient in ferredoxin-glutamine-\(\alpha\)-ketoglutarate aminotransferase

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Received: 26 July 2000 / Accepted: 2 November 2000 / Published online: 17 February 2001
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Abstract Transformed tobacco (Nicotiana tabacum L.) plants with varying activities of the key enzyme of ammonia assimilation, ferredoxin-glutamine-\(\alpha\)-ketoglutarate aminotransferase (Fd-GOGAT; EC 1.4.7.1), were used to examine the roles of ammonium, glutamine (Gln) and \(\alpha\)-ketoglutarate (\(\alpha\)-KG) in the regulation of nitrate reductase (NR; EC 1.6.6.1) transcript abundance. In wild-type leaf discs, NR mRNA abundance was increased following feeding with \(\text{NO}_3^\text{-}\), sucrose and \(\alpha\)-KG and decreased by feeding Gln. In air, leaves with decreased GOGAT accumulated Gln and \(\alpha\)-KG simultaneously; this was accompanied by increased NR transcripts. The inhibition of NR transcription by Gln observed in leaf-disc experiments was therefore not observed in the low-Fd-GOGAT plants that accumulate Gln in vivo. The results suggest that the negative effect of Gln on NR transcript abundance was offset by high \(\alpha\)-KG and that the relative amounts of \(\alpha\)-KG and Gln are more important in controlling NR gene transcription than the concentration of either metabolite alone.

Keywords \(\alpha\)-Ketoglutarate · Ferredoxin-glutamate synthase · Glutamine · Nicotiana (nitrate reductase) · Nitrate reductase

Abbreviations Fd: ferredoxin · Gln: glutamine · GOGAT: glutamate synthase · GS: glutamine synthetase · \(\alpha\)-KG: \(\alpha\)-ketoglutarate · NR: nitrate reductase · PEG: polyethylene glycol · WT: wild type

Introduction

Effective metabolic cross-talk co-ordinating the pathways of carbon and nitrogen assimilation in plants requires the concerted action of a repertoire of signals to allow graded molecular and physiological responses. Despite intensive research efforts in this area, relatively few signal molecules have been identified and fundamental questions regarding mechanism remain unanswered. Recently, nitrate accumulation in the shoot was implicated in the regulation of shoot-root allocation (Scheible et al. 1997a) and in the modulation of carbon partitioning in tobacco (Scheible et al. 1997b).

Transformed plants modified in nitrate reductase (NR) activity have been shown to be invaluable tools in elucidating the role of nitrate in signal transduction and its role in regulating numerous enzymes of carbon and nitrogen metabolism (Scheible et al. 1997b; Stitt and Krapp 1999). Regulatory roles for sugars such as sucrose and glucose on transcriptional and post-translational regulation of nitrate assimilation, amino acid metabolism and photosynthesis have been demonstrated (Krapp et al. 1993; Kaiser and Huber 1994; Morcuende et al. 1998). These metabolites convey information on the carbohydrate and nitrogen status of the plant to allow rapid and appropriate adaptive responses to environmental and metabolic stimuli.

Nitrate, sugars and amino acids such as glutamine (Gln), contribute to the regulation of the transcription of NR and other genes coding for enzymes of carbon and nitrogen assimilation (Hoff et al. 1994; Oliveira and Coruzzi 1999). Nitrate and sucrose are known to be inducers of NR gene transcription and Gln is an inhibitor (Hoff et al. 1994). However, in Arabidopsis mutants that are deficient in ferredoxin-glutamine-\(\alpha\)-ketoglutarate aminotransferase (Fd-GOGAT) and therefore accumulate Gln to high concentrations, NR gene transcription was similar to that of the wild type.
This indicates that high Gln alone does not exert negative control of the NR gene. The plants used in the experiments described here were transformed with 35S-antisense-Fd-GOGAT and selected for low Fd-GOGAT content (10–90% of the Fd-GOGAT activity of the wild type; Ferrario-Méry et al. 2000). The glutamine synthetase (GS)/GOGAT cycle is considered to be the major pathway of ammonium assimilation (Lea and Miflin 1974). The major isoforms of GS and GOGAT are localised in the plastids in both leaves and roots. Fd-GOGAT is the major isoform in photosynthetic cells but NADH-dependent GOGAT isoforms have also been identified in leaves as well as in roots (Suzuki and Rothstein 1997). The Fd-GOGAT catalyses transfer of the amido group of Gln to α-ketoglutarate (α-KG) to form glutamate. The transition from CO₂ enrichment (where photorespiration is inhibited) to air (where photorespiration is a major process of ammonium production in leaves) was used to probe the regulation of NR gene transcription in vivo in tobacco plants transformed with 35S-antisense-Fd-GOGAT.

In air, the leaves containing low Fd-GOGAT activity accumulated more foliar Gln and α-KG than the untransformed controls (wild type, WT) and the photorespiration-dependent increases in foliar Gln and α-KG were proportional to the decreases in foliar Fd-GOGAT (Ferrario-Méry et al. 2000). The results obtained in the present study on these transformed plants provide evidence suggesting that α-KG and Gln participate antagonistically in signal transduction. In addition, feeding α-KG greatly increased NR gene expression in untransformed tobacco leaf discs while Gln supply decreased NR gene transcription.

**Materials and methods**

Production of plant material

The antisense construct used for transformation of tobacco (*Nicotiana tabacum* L., cv. Xanthi; INRA, Versailles, France) was produced from a partial tobacco cDNA encoding Fd-glutamate synthase and was fused to the CaMV-3S5 promoter, as described previously (Ferrario-Méry et al. 2000). Transformed plants were obtained by transformation with *Agrobacterium tumefaciens*, as described previously (Ferrario-Méry et al. 2000).

Tobacco seeds from transformed lines expressing the antisense Fd-GOGAT cDNA construct, were germinated on 0.8% solid agar plates containing 25 μg ml⁻¹ kanamycin. Kanamycin-resistant plants surviving after 2 weeks on this culture medium in a growth chamber supplemented with CO₂ (350 μl ¹⁻¹) under 150 μmol quanta m⁻² s⁻¹ irradiance with a 14-h (25 °C)/10-h (20 °C) day/night cycle, were used for further analysis. Tobacco seeds from untransformed lines were germinated on 0.8% solid agar plates without kanamycin. Two-week-old seedlings were placed in sand in pots and transferred immediately to a growth chamber with an atmosphere enriched in CO₂ (4,000 μl ¹⁻¹) in order to prevent photorespiration and allow plants with greatly decreased Fd-GOGAT activities to survive. The plants were watered three times per hour with 25 ml of a complete nutrient solution containing 10 mM NO₃⁻ and 2 mM NH₄⁺ (Coić and Leşjajt 1975).

The plants were provided with 350 μmol m⁻² s⁻¹ irradiance and a 16-h (23 °C)/8-h (18 °C) day/night cycle. Two-month-old plants were used in the following experiments. Leaf discs were harvested in the middle of the photoperiod from each of the 3 youngest mature leaves on 3 plants from each line (20 samples total per line). The atmospheric CO₂ content was then decreased from 4,000 μl ¹⁻¹ to 350 μl ¹⁻¹. Samples were harvested each day on the subsequent 3 days after transfer. Leaves were harvested at the same point in the photoperiod from the same three leaves harvested prior to transfer.

Net CO₂ assimilation was measured after the transfer of plants to photorespiratory conditions. At 72 h after the transfer to air the values for CO₂ assimilation in the WT were (mean ± SE) 6.9 ± 1.1 μmol m⁻² s⁻¹ compared with 7.22 ± 2.65 μmol m⁻² s⁻¹ in line 30, 7.11 ± 0.53 μmol m⁻² s⁻¹ in line 38 and 3.97 ± 1.98 μmol m⁻² s⁻¹ in line 15. While line 15 (with the lowest Fd-GOGAT activity) showed a clear tendency to lower rates of photosynthesis, the values were not significantly different from those of the other lines. Therefore, no significant differences were observed between the photosynthetic rates of the different lines. These observations indicate that the supply of reducing power and carbon skeletons necessary for the vigour of the tissues was not altered during the first 72 h after transfer to photorespiratory conditions in the transformed lines.

**Leaf-disc experiments**

Experiments were performed with fully expanded leaves of 10-week-old untransformed tobacco plants. These were grown in a greenhouse under natural lighting and temperature (20–25 °C). One week before the harvest, plants were transferred to a growth chamber under 350 μmol m⁻² s⁻¹ irradiance and a 16-h (23 °C)/8-h (18 °C) day/night cycle. Leaf discs (1 cm²) were cut from 12 tobacco plants, 3 h after the beginning of the light period. They were rinsed in distilled water and floated in petri dishes. Each dish contained 40 discs and 20 ml of buffer (40 mM KCl, 10 mM CaCl₂, 10 mM Mes, pH 6.5). These were incubated in the absence or presence of NO₃⁻ (100 mM), Gln (100 mM), α-KG (10 mM) or sucrose (200 mM) in a growth chamber (16-h photoperiod at 150 μmol quanta m⁻² s⁻¹ and 23 °C with an 8-h dark period at 18 °C in a day/night cycle). For each feeding solution, osmolality was maintained at 280 mmol/kg by adding polyethylene glycol (PEG; 200 MW). After 5 h of incubation (i.e., in the middle of the photoperiod) the leaf discs were rinsed with distilled water, dried and frozen in liquid nitrogen for metabolic and enzymatic analyses.

Net CO₂ assimilation rates were measured in order to verify that metabolism was not modified by the treatments. Photosynthesis was compared in intact leaves (5.46 ± 0.56 μmol m⁻² s⁻¹, mean ± SE), in excised leaf discs just before feeding with buffer alone (5.95 ± 1.28 μmol m⁻² s⁻¹), after 5 h of feeding with buffer alone (4.53 ± 0.74 μmol m⁻² s⁻¹) and after feeding metabolites in the presence of PEG. Leaf discs supplied with buffer alone in the presence of 3% PEG (5.09 ± 0.53 μmol m⁻² s⁻¹) or with buffer plus NO₃⁻ (4.3 ± 0.7 μmol m⁻² s⁻¹) had similar photosynthetic capacities. CO₂ assimilation rates were slightly decreased in leaf discs fed with buffer plus Gln (3.28 ± 0.63 μmol m⁻² s⁻¹) or α-KG in distilled water (3.34 ± 0.57 μmol m⁻² s⁻¹) in the presence of PEG (1 or 3%) but they were similar to the photosynthetic capacity of leaf discs fed with buffer plus NO₃⁻. Not surprisingly, CO₂ assimilation rates were reduced by half in leaf discs fed with sucrose (2.09 ± 0.55 μmol m⁻² s⁻¹).

**Extraction of RNA and Northern blotting**

Total RNA was extracted as described by Verwoerd et al. (1989) from plant material stored at –80 °C. Northern blot analysis was performed as described by Hiriol et al. (1987). The probes used for mRNA detection have been described previously: NR from *Nicotiana sylvestris* (Vaucleret et al. 1989) and pea 18S RNA (Deng et al. 1991). Relative mRNA amounts were determined by scanning densitometry with a Umax-Power look II (UMAX data Systems, Taiwan) and an advanced quantifier NIH Image program (National Institutes of Health, USA).