Negative regulation of nitrate reductase gene expression by glutamine or asparagine accumulating in leaves of sulfur-deprived tobacco

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Abstract. Tobacco (Nicotiana tabacum L.) plants were subjected to a prolonged period of sulfur-deprivation to characterize molecular and metabolic mechanisms that permit control of primary N-metabolism under these conditions. Prior to the appearance of chlorotic lesions, sulfur-deprived tobacco leaves showed a strong decrease in the sulfate content and changes in foliar enzyme activities, mRNA accumulation, and amino-acid pools. The basic amino acids glutamine, asparagine and arginine accumulated in the leaves of sulfur-deprived plants, while the foliar concentrations of aspartate, glutamate, serine or alanine remained fairly unchanged. Maximal extractable nitrate reductase (NR; EC 1.6.6.1) activity decreased strongly in response to sulfur-deprivation. The decrease in maximal extractable NR activity was accompanied by a decline in NR transcripts while the mRNAs of the plastidic glutamine synthetase (EC 6.1.3.2) or the β-subunit of the mitochondrial ATP synthase were much less affected. Nitrate first accumulated in leaves of tobacco during sulfur-deprivation but then declined. An appreciable amount of nitrogen was, however, present in severely sulfur-depleted leaves. The repression of NR gene expression is, therefore, not related to the decrease in the leaf nitrogen level. However, glutamine- and/or asparagine-mediated repression of NR gene transcription is a possible mechanism of control in situations when glutamine and asparagine accumulate in leaves and provides a feasible explanation for the reduction in NR activity during sulfur-deprivation. The removal of reduced nitrogen from primary metabolism by redirection and storage as arginine, asparagine or glutamine combined with the down-regulation of nitrate reduction via glutamine- and/or asparagine-mediated repression of NR gene transcription may contribute to maintaining a normal N/S balance during sulfur-deprivation and indicate that the co-ordination of N- and S-metabolism is retained under these conditions.

Key words: Amino acid – Glutamine synthetase – Nitrate assimilation – Nitrate reductase – Nicotiana (sulfur-deprivation) – Sulfur-deprivation

Introduction

Protein biosynthesis in plant leaves requires both energy and inorganic carbon, nitrogen and sulfur. The reductive assimilation of these elements needs to be co-ordinated to prevent undesirable energy expenditure (Noctor et al. 1997). Photosynthesis and amino-acid biosynthesis are highly interdependent. Triose phosphate produced in the leaves as a result of photosynthetic carbon assimilation can be used for the synthesis of either carbohydrates or ketoacids (e.g. 2-oxoglutarate) via the anaplerotic pathway (Melzer and O'Leary 1987). The 2-oxoglutarate produced in the cytosol is imported into the chloroplasts where it may serve as the acceptor for the ammonium during amino-acid synthesis. Thus, amino-acid synthesis depends on the availability of carbon skeletons provided by photosynthesis (Huppe and Turpin 1994). To meet the needs of growth and development for both carbohydrates and amino acids, carbon partitioning is co-ordinated by a regulatory system involving the synchronous modulation of the activities of sucrose phosphate synthase and phosphoenolpyruvate carboxylase (Foyer et al. 1994; Huber et al. 1994). Sulfate reduction may compete with carbon and nitrogen assimilation for reducing equivalents. While sufficient quantities of sulfate must be reduced to support the requirements of protein biosynthesis, enough carbon and nitrogen must be made available for the synthesis of the precursors for the end-products of sulfate reduction, e.g. protein cysteine or methionine residues or low-molecular-weight thiols (Brunold 1993).
The species-specific nitrogen/sulfate balance maintained by plants in the leaves (Dijkshoorn and Van Wijk 1967) may be disturbed by sulfate-deprivation. Accumulation to sulfate-deprivation requires responses that allow essential reactions of primary metabolism to continue and enable the plant to tolerate sulfatedeficits. During prolonged sulfate-deprivation, changes in the pools of many metabolites followed by disturbances in nitrogen metabolism are observed (Friedrich and Schrader 1978; Prosser et al. 1997). For example, there is an inhibition of protein synthesis and an accumulation of amino acids (Klapheck et al. 1982). The physiological significance of these responses to sulfur-deprivation is not clear and little information is available on the co-ordination of nitrogen and sulfur assimilation in these circumstances (Stulen and De Kok 1993). A reciprocal regulatory coupling between the nitrate and sulfate assimilation pathways by metabolic control via O-acetylsereine, an intermediate of cytoeine biosynthesis, or by control of ATP-sulfurylase and nitrate reductase (NR) activity, has long been proposed (Reuveny et al. 1980; Barney and Bush 1985; Neuenwender et al. 1991).

The reduction of nitrate to nitrite catalyzed by NR represents the first enzymatic step of primary nitrogen assimilation. The regulation of NR gene expression is remarkably complex. The transcription of the NR gene nia is induced by nitrate (Galangau et al. 1988) and appears to be repressed by reduced forms of nitrogen (Faure et al. 1991). It is also induced by sugars (Cheng et al. 1992). Moreover, nia gene transcription is stimulated by light and a circadian rhythm in NR gene expression was observed (Becker et al. 1992). The NR activity is co-ordinated with the rate of photosynthesis and the availability of carbon skeletons by both transcriptional and post-translational controls (Huber et al. 1994; Foyer et al. 1996).

In the present study, we have followed the regulation of NR gene expression and of the major amino-acid pools through a prolonged period of sulfur-deprivation in young leaves of fully grown tobacco plants in order to characterize molecular and metabolic mechanisms that permit control of primary nitrogen metabolism under these conditions.

Materials and methods

Plants and growth conditions. Tobacco (Nicotiana tabacum L. cv. Xanthi; INRA, Versailles, France) was grown in sand in a growth cabinet under white light from fluorescent lamps providing a photosynthetically active irradiance of 120 μmol photons m⁻² s⁻¹ for 16 h each day. The temperature was 25 °C/18 °C during the day/night cycle. The plants were supplied daily with nutrient solution A [3.9 mM KNO₃, 0.2 mM NaCl, 3 mM Ca(NO₃)₂, 0.05 mM Mg(NO₃)₂, 1.1 mM KH₂PO₄, 0.75 mM MgSO₄] supplemented with microelements (6 μM Na₂B₄O₇, 4.5 μM MnCl₂, 3.5 μM ZnCl₂, 1 μM CuCl₂, 1 μM CoCl₂, 0.28 μM Na₂MoO₄ and 1.4 μM FeSO₄·EDTA) according to Coic and Lesaint (1975).

Imposition of sulfur-deprivation. Tobacco plants that had been grown for 4 weeks on the complete nutrient solution A were transferred to one of the following conditions of sulfur-availability.

For 8 weeks, half of the plants received nutrient solution A daily (sulfur-replete), while the remaining plants were irrigated daily for 8 weeks with the sulfur-deficient medium B (sulfur-deprived). This medium consisted of 3.9 mM KNO₃, 0.2 mM NaCl, 3 mM Ca(NO₃)₂, 0.05 mM Mg(NO₃)₂, 1.1 mM K₂HPO₄ and 0.75 mM MgCl₂ and was supplemented with the same amounts of microelements as medium A, except that FeSO₄·EDTA was replaced by FeCl₃·EDTA. The green algae Chlorella vulgaris was unable to grow in medium B, demonstrating that medium B was essentially sulfur-deficient.

Sampling. Our attempt to compare levels of different cellular components between plants under different growth conditions makes standardization both critical and difficult. In each sampling, therefore, multiple leaves from separate plants were collected: 1 h after the beginning of the photoperiod of the days of the experiment indicated on the figures, three young leaves (see Results) were harvested from each of four separate sulfur-replete or sulfur-deprived tobacco plants. The leaves collected in each sampling were cut into small pieces. The leaf fragments were pooled and then divided into three portions. Each portion was weighed prior to immersion in liquid nitrogen. The frozen portions were subsequently used for RNA extraction, enzyme assays (which included soluble protein and total chlorophyll determinations) and the analysis of nitrate and sulfate, respectively. Replicates represent experiments done as described on separate plants.

Enzyme assays. Total soluble tobacco leaf protein was extracted at 0 °C using a potassium phosphate buffer (200 mM, pH 7.5) supplemented with 5 mM EDTA, 12.5 mM 2-mercaptoethanol and 2 mM phenylmethylsulfonyl fluoride.

The activity of NR was calculated from the increase of nitrite during the assay. The nitrite was colorimetrically determined at 540 nm after azocoupling with sulfanilamide and naphthylethylenediamine dihydrochloride (Galangau et al. 1988). Our reaction mixture contained 125 μM potassium phosphate (100 mM, pH 7.5), 50 μM KNO₃ (100 mM), 125 μM NaNO₃ (100 mM), 100 μM H₂O. The reaction was started by adding 50 μl protein extract and terminated after incubation for 15 min at 30 °C by adding 250 μl sulfanilamide [1% (w/v) in 1.5 N HCl] and 250 μl naphthylethylenediamine dihydrochloride [0.02% (w/v)].

In the semi-biosynthetic glutamine synthetase (GS) assay (Wallsgrove et al. 1979), the physiological substrate ammonium is replaced by hydroxyamine. The GS enzyme catalyses the formation of γ-glutamylhydroxamic acid (GHA) from hydroxylamine and glutamate. The GHA was colorimetrically determined at 540 nm after complexation with acidified ferric chloride. Our reaction mixture contained 100 μl imidazol buffer (450 mM, pH 7.2), 100 μl MgCl₂ (450 mM), 100 μl NH₄OH·HCl (60 mM), 100 μl ATP (80 mM), 100 μl l-glutamate (870 mM) and 300 μl H₂O. The reaction was started by adding 50 μl protein extract and terminated after incubation for 15 min at 30 °C by adding 850 μl of a solution containing 0.37 mM FeCl₃, 0.2 M trichloroacetic acid and 0.67 N HCI.

Isolation of RNA and northern blot analysis. Ribonucleic acid was extracted from frozen leaf tissue as previously described (Migge and Becker 1996). The isolated RNA was precipitated twice with 4 M LiCl for 60 min at 0 °C to remove traces of DNA and small RNA species. Denatured total RNA samples were fractionated by formaldehyde-agarose gel electrophoresis [1.5% (w/v) agarose] and then transferred onto “Zeta Probe” blotting membranes (Bio-Rad). The hybridizations with a ³²P-labelled 18S rRNA probe of pea (Deng et al. 1991) or a ³²P-labelled cDNAs encoding NR (Calza et al. 1986) or plastidic GS (GS-2) (Becker et al. 1992) of tobacco or the β-subunit of the mitochondrial ATP synthase (β-ATPase) of Nicotiana plumbaginifolia (Boury and Chua 1985) were performed as previously described (Migge and Becker 1996). The signals visible after autoradiography were quantified by densitometric scanning (Power Look II Scanner from Umax, Düsseldorf, Germany; software by Bio Image, Ann Arbor, Mich., USA).