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Nitrogen and metabolic regulation of the expression of plastidic glutamine synthetase in alfalfa (*Medicago sativa*)

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Abstract

We have identified a single putative plastidic glutamine synthetase (GS_2), isolated from *Medicago sativa* (alfalfa) leaf. Analysis of organ/tissue specific expression of the GS_2 gene in this study has shown that it is expressed in all green tissues. We show that the alfalfa GS_2 gene is also expressed in nitrogen fixing root nodules where its expression is not regulated by fixed nitrogen. Treatment with nitrate (NO_3^-) results in the induction of GS_2 in the roots and leaves of alfalfa, but the signaling mechanism in the two organs is different. In the roots NO_3^- appears to act as a direct signal for the induction of GS_2 whereas in the leaves secondary metabolites of NO_3^- probably act as the signal. We also demonstrate that 2-oxoglutarate (2-OG), in combination with NO_3^-, appears to significantly induce GS_2 expression, pointing to 2-OG as a potential primary metabolic inducer of alfalfa GS_2. Treatment with glutamine or sucrose, in combination with NO_3^-, also appears to induce GS_2 in the roots, but there is a lag in the induction when compared to the 2-OG/NO_3^- combination. Our interest ultimately lies in dissecting how carbon:nitrogen status modulates the expression of GS at transcriptional and post-transcriptional levels.

Keywords: 2-Oxoglutarate; Glutamine synthetase; *Medicago sativa*; Nitrate; Plastid; Regulation

1. Introduction

Glutamine synthetase (GS) (EC 6.3.1.2) is a key enzyme in plant growth because of its involvement in the first major step of ammonia assimilation [1]. GS catalyzes the ATP-dependent formation of glutamine from ammonium and glutamate. Glutamine is subsequently deaminated by the enzyme glutamate synthase (GOGAT) (EC 1.4.1.14) to produce two molecules of glutamate. One of the molecules of glutamate can then be recycled back into the GS/GOGAT cycle, while the other may be used as the substrate for transaminating enzymes [2].

Plant GS is an octameric protein with a molecular weight of 350–400 kD [3]. Two major isoforms of GS are found in plants: GS_1 or the cytosolic isoform and GS_2 or the chloroplastic/plastidic isoform. The two isoforms have non-overlapping functions in the assimilation of ammonium (NH_4^+) [4]. Although encoded by homologous but different genes, the primary amino acid sequence of the chloroplastic and cytoplasmic GS subunits is very similar, differing by additional 60 and 16 residues located at the N- and C-terminus, respectively, of the GS_2 enzyme. The GS_2 polypeptide is synthesized on cytoplasmic ribosomes as a precursor protein with a 50 amino acid N-terminal transit peptide, which targets the protein to the chloroplast [5].

GS_2 is primarily expressed in the mesophyll cells of leaves, where photorespiratory ammonia is released. The analysis of barley mutants deficient in GS_2 showed that the main role of this enzyme is in the reassimilation of ammonia from photorespiration [6–9]. GS_2 has also been implicated in the assimilation of NH_4^+ reduced from nitrate and nitrite [10]. GS_2 cDNAs have been cloned from a number of legumes [11–16], and non-legumes [17–23]. In all plants tested, a single nuclear gene has been identified for GS_2.

Nitrate (NO_3^-) is the major source of nitrogen in most plants and nitrate applications have been shown to induce the expression of many C- and N-metabolic genes [24–27].

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Both GS_2 and GS_1 genes are also regulated by external nitrogen applications, but the extent of this regulation depends on the plant species, nitrogen source and plant organ/tissue [28]. Regulatory effects of N assimilation, NH_4^+ and/or NO_3^- on gene expression have been reported for *Medicago truncatula* [11], rice [29], maize [19,30], tobacco [31,32], tomato [33], sunflower [34] and mustard [35]. In some cases, both NO_3^- and NH_4^+ increased GS activity or transcript levels. In other cases, either NH_4^+ or NO_3^- was found to have an effect. However, more study is needed to determine if signals for regulation are derived from nitrate itself, or from metabolites formed during nitrate assimilation [36].

Along with nitrogen, GS_2 expression is also regulated by both light and metabolic factors associated with light, such as sucrose and carbon substrates. Light affects GS_2 expression by acting directly via phytochrome [37]. Red light induces GS_2 mRNA levels in dark-adapted Arabidopsis plants, and this induction is reversed by a subsequent pulse of far-red light in a typical phytochrome-dependent response [37]. The GS_2 gene, however, differs from other light regulated genes in that it is also expressed in non-green tissues, such as roots, where it helps in NH_4^+ assimilation [38].

The effect of feeding carbon substrates on the expression of GS_2 has not been well studied, although activation could be indirectly linked via changes in levels of carbon metabolites (photosynthesis). It has been shown that in dark-adapted, nitrate-fed, Arabidopsis seedlings, sucrose enhances the expression of the GS_2 gene, appearing to mimic the effect of light [37,39], which suggests that sucrose or a product of sugar metabolism plays a role in GS_2 gene expression.

This paper details the isolation and characterization of a GS_2 gene from *M. sativa* (alfalfa), with a focus on understanding the transcriptional regulation of this gene. All previous studies on GS in alfalfa have concentrated on the characterization of GS_1 genes [40,41]. The data presented here shows that GS_2 gene in alfalfa is regulated by light, but expressed in both green and non-green tissues, including nodule. Moreover, the NO_3^- mediated induction of GS_2 gene in alfalfa appears to follow a different expression pattern in the roots and leaves, suggesting differences in the signaling mechanisms between the two organs. Finally, we begin to dissect the regulation of GS_2 expression that is dependent on the presence and interaction of N and C substrates and metabolites.

2. Material and methods

2.1. Southern blot analysis

Genomic DNA was isolated from alfalfa according to the modified hexadecyltrimethyl ammonium bromide (CTAB) procedure [42]. Total genomic DNA from alfalfa leaves was digested with *Eco*RI (E), *Hind*III (H), or *Xba*I (X) separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. Southern blot analysis was carried out according to southern [43]. The Southern blot was hybridized to the ^{32}P-dCTP 3′ Ut region, 5′ Ut/transit peptide (TP) region and the exon 10 region from pGS2-1. [44]. The 3′ UTR probe was obtained by cutting the fragment out from pGS2-1 with *Xba*I/*Xho*I. The 5′ UTR/TP and the exon 10 fragments were obtained through PCR (plasmid pGS2-1 as template) using the following primers: for the 5′ UTR/TP, 5′-GAGGTTCAGTGACACAAGGCTA-3′ (forward primer) and 5′-CTCTGAATTTAGCAGAACGTGCAAC-3′ (reverse primer), and for the exon 10, 5′-CAGTACAAAGAGCATGAGGGAA-3′ (forward primer) and 5′-CAAGAAAATGTGTTGATGCTGGC-3′ (reverse primer). The nitrocellulose filters were hybridized with the probes in hybridization solution (5× SSC, 5× Denhardt, 50 mM phosphate buffer pH 7, 0.1% SDS, 0.1 mg ml^{-1} denatured salmon DNA, 50% formamide) at 42 °C for 24 h under constant shaking. Filters were washed with 2× SSC (75 mM NaCl, 82.5 mM sodium citrate pH 7), 0.1% SDS at 42 °C for 1 h, followed by washes in 0.2× SSC and 0.1% SDS at 42 °C for 1 h, and then dried and exposed to Kodak film at −80 °C.

2.2. RNA isolation and northern hybridization

RNA was prepared from alfalfa tissues using the LiCl precipitation method [45]. The RNA loadings were adjusted to the specified μg/lane (see figure legends) and separated by electrophoresis on 1.4% agarose gels containing formaldehyde, and blotted onto nitrocellulose membranes (VWR, West Chester, PA). The membranes were hybridized and washed as described for southern hybridization. Images were quantified with the KODAK Image Analysis Software (Kodak Scientific Imaging Systems, Rochester, NY).

2.3. Reverse-transcription polymerase chain reaction (RT-PCR)

Reverse transcription of total RNA was done with an oligo-(dT)18-anchor$_{20}$ (A_{20}-oligo-dT; 5′-GTGAACTTAGGTGACTGACGT$_{18}$-3′) using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) following the standard Invitrogen protocol. For amplification of GS_2 3′ UTRs, the forward primer was an oligonucleotide made from a sequence of the alfalfa GS_2 (pGS2-1) coding region close to its 3′ UTR (5′-GCAGAGAGTACATTATTCTGGG-3′) and the reverse primer was the A_{20}-oligo-dT. For amplification of the 5′ UTRs, the forward primer was 5′-GAGGTTCAGTGACACAAGGCTC-3′ selected from the beginning of the 5′ UTR of the pGS2-1 and the reverse primer was 5′-TTCACCAGGGGCTTGTCCAG-3′ selected from the coding region. The PCR products were purified from the gel and subcloned into the pGEM-T vector (Promega, Madison, WI) and transformed into *E. coli* strain DH5-α. Selected clones were submitted for automated Licor