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DIFFERENTIAL REGULATION OF GS-GOGAT GENE EXPRESSION BY PLANT GROWTH REGULATORS IN *ARABIDOPSIS* SEEDLINGS

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Abstract: Primary and secondary ammonium assimilation is catalyzed by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway in plants. The *Arabidopsis* genome contains five cytosolic GS1 genes (*GLN1;1 – GLN1;5*), one nuclear gene for chloroplastic GS2 isoform (*GLN2*), two Fd-GOGAT genes (*GLU1* and *GLU2*) and a *GLT1* gene coding for NADH-GOGAT. Even though the regulation of GS and GOGAT isoforms has been extensively studied in response to various environmental and metabolic cues in many plant species, little is known about the effects of phytohormones on their regulation. The objective of this study was to investigate the impact of representative plant growth regulators, kinetin (KIN), abscisic acid (ABA), gibberellic acid (GA_3) and 2,4-dichlorophenoxyacetic acid (2,4-D), on the expression of *A. thaliana* GS and GOGAT genes. The obtained results indicate that GS and GOGAT genes are differentially regulated by growth regulators in shoots and roots. KIN and 2,4-D repressed GS and GOGAT expression in roots, with little effect on transcript levels in shoots. KIN affected all tested genes; 2,4-D was apparently more selective and less potent. ABA induced the expression of *GLN1;1* and *GLU2* in whole seedlings, while GA_3 enhanced the expression of all tested genes in shoots, except *GLU2*. The observed expression patterns are discussed in relation to physiological roles of investigated plant growth regulators and N-assimilating enzymes.

Key words: Glutamine synthetase; glutamate synthase; abscisic acid; kinetin; gibberellic acid

INTRODUCTION

The glutamine synthetase-glutamate synthase (GS-GOGAT) cycle provides an entry route for reduced inorganic nitrogen into all organic nitrogenous compounds in plants. GS (EC 6.3.1.2) catalyzes ATP-dependent ligation of ammonia and glutamate to yield glutamine, while GOGAT catalyzes the redox transfer of the glutamine amide group to α-ketoglutarate, forming two glutamate molecules. Apart from assimilation of ammonia from primary sources such as nitrate reduction, nitrogen fixation and ammonia uptake, the GS-GOGAT cycle is active during secondary assimilation of ammonia derived from different metabolic processes such as photorespiration, amino acid catabolism and the phenylpropanoid pathway [1]. Consistent with the diversity of metabolic roles, GS and GOGAT are present in most plants as several isoforms. Higher plants contain one nuclear gene for a plastidic GS isoform, GS2, and a small gene family encoding different cytosolic GS1 isoforms [2]. The *Arabidopsis* genome contains one GS2 gene *(GLN2)* and five GS1 genes (*GLN1;1 – GLN1;5*) with specific spatio-temporal regulation [3], different kinetic properties and differential regulation of expression by external N supply [4], suggesting non-redundant functions [4-6]. Two types of GOGAT isoforms, Fd-GOGAT (EC 1.4.7.1) and NADH-GOGAT (EC 1.4.1.14), are localized in the plastids [7]. *Arabidopsis* contains two Fd-GOGAT genes, *GLU1* and *GLU2*, and one NADH-GOGAT – *GLT1*, which were also shown to have non-overlapping roles in distinct processes [8].

Given the importance of the GS-GOGAT cycle in providing and recycling organic nitrogen, and the fact that nitrogen availability and its utilization efficiency are limiting factors in plant productivity [1], the effort to understand the regulation and roles of various GS and GOGAT isoforms is not surprising. The key regulators of GS and GOGAT gene expression are light, nitrate, ammonium, amino acids and sugars [4,8-11]. Recently, several classes of phytohormones, namely cytokinins, auxins and abscisic acid (ABA) have been implicated in the regulation of nitrogen transport, assimilation and distribution [12], while evidence of the effects of gibberellins is mostly indirect [13-14]. Here we present a study of the effects of four representative growth regulators on the expression of GS and GOGAT isoforms in *Arabidopsis* seedlings grown in liquid culture.

MATERIALS AND METHODS

Plant growth conditions and growth regulator treatment

Arabidopsis thaliana ecotype Columbia (NASC ID: N60000) seedling liquid culture was established *in vitro,* as previously described [15]. The seedlings, cultivated for one month submerged in growth regulator-free liquid MS medium [16] supplemented with 3% sucrose, were transferred to fresh medium containing varying concentrations (10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M) of 2,4-dichlorophenoxyacetic acid (2,4-D), ABA, kinetin (KIN) or gibberellic acid (GA_3). Plant harvesting was carried out 24 h after hormone application; roots and shoots were separated and stored at -70°C.

RNA Extraction and RT-qPCR

Total RNA was isolated from roots and shoots using Trizol (Invitorgen), quantified spectrophotometrically and its integrity was checked by gel electrophoresis. For reverse transcription, 1 μg of total RNA was pretreated with DNase I (Fermentas) followed by reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (Fermentas), with oligo-dT primers, at 42°C, according to the manufacturer's instructions. Real-time PCR reactions were set with Maxima SYBR Green mix (Fermentas) using cDNA corresponding to 50 ng RNA and 0.3 mM primers (Table 1), in 25 μl total volume. The amplification was carried out on an ABI PRISM 7000 SDS thermal cycler (Applied Biosystems). The qPCR program included initial denaturation (95°C/10 min), 40 cycles of denaturation (95°C/15 s), annealing (58.5°C/20 s) and extension (72°C/30 s), followed by melting curve analyses. Standards for absolute quantification were prepared from PCR products purified by gel electrophoresis and extracted with a GeneJET Gel Extraction Kit (Fermentas) according to the manufacturer's protocol. Constitutive expression of 18S rRNA was confirmed in parallel using the same poly$(dT)_{18}$-primed RT mixture [17].

Table 1. Sequences of primers used for qPCR.

gene	GenBank	**forward (5′→3′)**	**reverse (5′→3′)**
GLN1;1	NM_123119	AGAAGTCATGCCGGGTCAGT	GTCAGCAGTCTCGTGGTGTC
GLN1;2	NM_105291	ACGGGACACCATGAAACTGC	GGCAGTGTCAACCGGTACAA
GLN1;3	NM_112663	TCGGCCCTGTTGAGGGTATT	CACGTCCCACTCTCACTGAC
GLN1;4	NM_121663	GGAGTTCCAAGTCGGTCCCA	CGGTTTGCCACACCCCATAA
GLN1;5	NM_103743	CATGCCTGGACAATGGGAGT	CACCGATGCTCCACGATCC
GLN2	NM_122954	ATGCCTGGACAGTGGGAGTT	GTCTCGTGCTTTCCGGTCAA
GLU1	NM_180432	CTTGTGGTCGTGTTGCTGGT	CTCCAGCTTTGCCTCTAGCG
GLU2	NM_129687	CCCTGTTGGGAAGGTTGAGC	TGACACCAAAACGCCCTGAG
GLT1	NM_124725	TCGAGCTGCGTTGAACCTTC	CACTTGAGCAGACCCTCACG
18S*rRNA*	X16077	TGACGGAGAATTAGGGTTCG	CAATGGATCCTCGTTAAGGG

RESULTS AND DISCUSSION

The expression data in control plants (Table 2) showed that the GS1 genes generally have slightly higher expression in roots compared to shoots, and that *GLN1;2* transcripts are the most abundant in both tissue samples. The expression of *GLN1;4* was below the detection threshold in shoots, while *GLN1;5* transcripts were not detected in seedlings. These results are consistent with previously published data for plants grown under ample nitrogen supply [4-5,15]. As expected, based on the proposed roles in photorespiration [8-11,18], the expression of the chloroplastic *GLN2* was higher in shoots, while Fd-GOGAT *GLU1* transcripts were found in shoots only. The GOGAT genes *GLU2* and *GLT1* were dominantly expressed in roots (Table 2). Previously it was shown that *GLT1* is mainly involved in primary assimilation and remobilization of nitrogen [8, 19], while it was proposed that *GLU2* encoded enzyme serves to supply a constitutive level of glutamate for the maintenance of a basal level of protein synthesis [8].

The tested plant growth regulators differentially modulated the expression of GS (Fig. 1) and GOGAT (Fig. 2) genes in roots and shoots. KIN had a higher impact on gene expression in roots, where significant repression was found for all GS and GOGAT genes, while the effect on expression in shoots was less pronounced, usually at higher KIN concentrations. This result is consistent with the postulated role of cytokinins, which act in signaling routes that communicate the internal and external nitrogen status of the plant. Cytokinin synthesis is upregulated during N abundance, while high cytokinin levels cause suppression of N-uptake and primary assimilation [12,20].

Table 2. Absolute expression of GS and GOGAT genes in roots and shoots of control (hormone untreated) plants. The values represent the average copy number per μg of total RNA for three biological repetitions (n = 3) of 10 plants each ± standard error (SE), nd – not detected.

gene	root	shoot
GLN1;1	$2.65 \cdot 10^6 \pm 4.27 \cdot 10^5$	$1.38 \cdot 10^6 \pm 2.46 \cdot 10^5$
GLN1;2	$5.31 \cdot 10^7 \pm 6.57 \cdot 10^6$	$2.27 \cdot 10^7 \pm 3.29 \cdot 10^6$
GLN1;3	$6.49 \cdot 10^5 \pm 5.28 \cdot 10^4$	$5.85 \cdot 10^5 \pm 1.02 \cdot 10^5$
GLN1;4	$3.07 \cdot 10^4 \pm 4.30 \cdot 10^3$	nd
GLN1;5	nd	nd
GLN2	$7.42 \cdot 10^5 \pm 5.21 \cdot 10^4$	$2.29 \cdot 10^6 \pm 3.10 \cdot 10^5$
GLU1	nd	$9.43 \cdot 10^5 \pm 4.23 \cdot 10^4$
GLU2	$1.87 \cdot 10^6 \pm 4.64 \cdot 10^5$	$2.85 \cdot 10^5 \pm 2.98 \cdot 10^4$
GLT1	$6.49 \cdot 10^5 \pm 4.37 \cdot 10^4$	$7.41 \cdot 10^4 \pm 3.88 \cdot 10^3$

Treatment with 2,4-D had a more pronounced effect on gene expression in roots, where it downregulated the most abundantly expressed GS1 gene – *GLN1;2*, plastidic *GLN2* (Fig. 1), as well as the two GOGAT genes – *GLU2* and *GLT1* (Fig. 2). The only gene whose expression was enhanced in both roots and shoots in the presence of high 2,4-D concentrations was *GLN1;3.* Based on this it can be concluded that 2,4-D and KIN have somewhat overlapping effects on primary N-assimilation; however, 2,4-D shows higher specificity and less potency in the repression of GS1 gene expression. Similar results were obtained when the effect of cytokinins and auxins on the expression of *Arabidopsis* root-type nitrate transporter genes (*AtNRT*) was evaluated [12]. Namely, trans-Zeatin showed non-selective repression of all root *AtNRT* genes, while the effect of indole-3-acetic acid was less pronounced and apparently selective.

Treatment with ABA significantly induced *GLN1;1, GLN1;3* and *GLU2* genes in entire seedlings, as well as *GLN1;4* and *GLN2* expression in roots (Figs. 1 and 2). The most affected genes were *GLN1;1,* whose expression in roots was increased almost 4-fold, and *GLU2,* which was induced 5.5-fold in shoots. Since ABA is considered one of the main hormonal regulators during abiotic stress responses [21], the ABA-responsive *GLN1;1* and *GLU2* genes can be considered as stress-responsive genes. Indeed, *GLN1;1* is upregulated during cold [22] and salinity stress [23]. Furthermore, among all *A. thaliana* GS1 single knockout mutants, only *GLN1;1*ko had an increased stress sensitivity phenotype [22]. The role of *GLU2* is not well defined, but it seems that *GLU2* is involved neither in primary nitrogen assimilation nor in photorespiratory ammonium removal [8]. Interestingly, *Arabidopsis* abiotic stress expression maps [24] indicate that *GLU2* transcripts accumulate in shoots of plants under osmotic and salt stress.

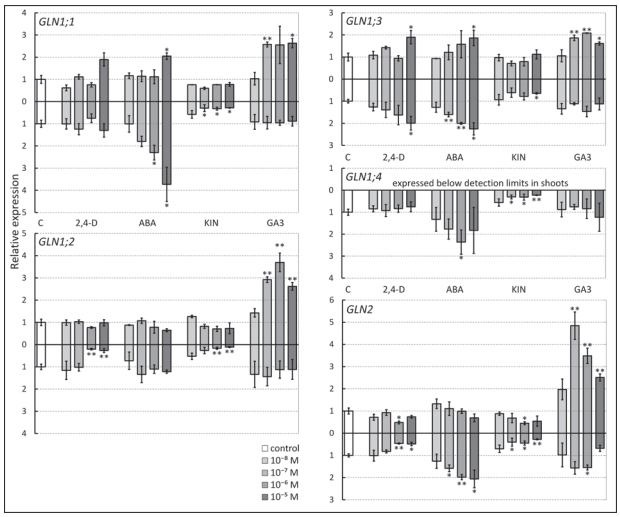

Fig. 1. Expression of GS genes in growth regulator treated Arabidopsis seedlings. Relative expression (normalized to controls) of five *A. thaliana* GS genes (*GLN1;1-1;4* and *GLN2*) in shoots (above the X axis) and roots (below the X axis) of plants treated with 10^{-8}-10^{-5} M concentrations of growth regulators for 24 h: C – control; 2,4-D – 2,4-dichlorophenoxyacetic acid; ABA – abscisic acid; KIN – kinetin; GA3 – gibberellic acid. Expression of *GLN1;5* is not shown because it was below detection limits in all samples. Means and standard errors (SE) are for three biological repetitions (n=3) of 10 plants each. Significant differences estimated using Student t test are indicated by *P < 0.05 and **P < 0.01.

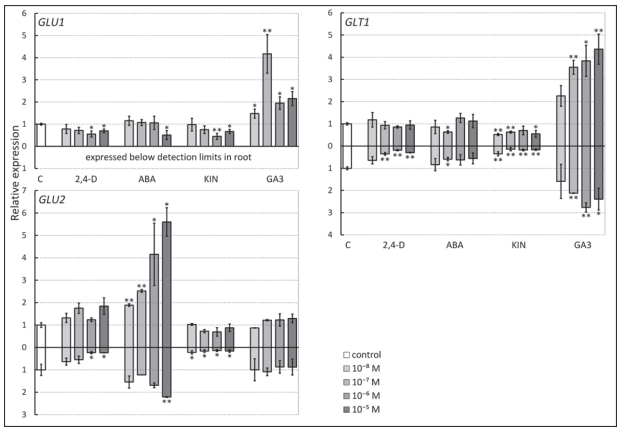

Fig. 2. Expression of GOGAT genes in growth regulator treated Arabidopsis seedlings. Relative expression (normalized to controls) of three *A. thaliana* GOGAT genes (*GLU1, GLU2* and *GLT1*) in shoots (above the X axis) and roots (below the X axis) of plants treated with 10^{-8}-10^{-5} M concentrations of growth regulators for 24 h: C – control; 2,4-D – 2,4-dichlorophenoxyacetic acid; ABA – abscisic acid; KIN – kinetin; GA3 – gibberellic acid. Means and standard errors (SE) are for three biological repetitions (n=3) of 10 plants each. Significant differences estimated using Student t test are indicated by *P < 0.05 and **P < 0.01.

Previously it was shown that during abiotic stress responses a large portion of Glu synthesized by the GS-GOGAT pathway is used for proline production [25], which is a common osmoprotectant.

GA_3 treatment significantly induced the expression of all studied genes in the shoots except *GLU2,* while its effect on expression in roots was minor or absent. This result indicates that GA_3 does not regulate primary assimilation of ammonium in roots, but is involved in ammonium (re)assimilation in shoots. Little is known about the effects of gibberellins on nitrogen metabolism. This class of hormones has been shown to induce the expression of cytosolic GS in pine [26] and to reduce glutamate content in *Arabidopsis* shoots [14], probably by stimulating its utilization for growth. In view of this, the observed stimulation of GS and GOGAT expression in shoots by GA_3 might serve to provide enhanced Glu production, to support gibberellin-stimulated shoot growth.

We have shown that the expression of GS and GOGAT genes in *Arabidopsis* seedlings grown submerged in liquid culture is comparable to literature data on the expression profiles of these isoforms in seedlings grown under standard conditions. We found liquid culture convenient for investigation of the effects of plant growth regulators on gene expression in different organs of *Arabidopsis* seedlings, since this system allows for the uniform application of constant concentrations of the investigated substances, without consideration of internal transport issues or concentration fluctuations imposed by foliar or other types of applications. All of the GS-GOGAT genes were found responsive to at least one of the four tested growth regulators, indicating that N assimilation is modulated not only by N supply, availability of carbon skeletons, light and other factors broadly investigated in literature, but also by phytohormones. The results obtained concur well with the available literature data on the specific functions of different GS and GOGAT isoforms. The established system and the obtained results provide a good basis for further investigation into the role of phytohormones in the regulation of N assimilation.

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Conflict of interest disclosure: The authors declare that they have no competing interests.

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