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Arabidopsis plastidial folylpolyglutamate synthetase is required for nitrogen metabolism under nitrate-limited condition in darkness

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ABSTRACT

Folates play an important role in plant metabolism. Here we report a T-DNA insertion mutant (*atdfb-3*) of the plastidial folylpolyglutamate synthetase gene (*AtDFB*) was defective in folate metabolism and nitrogen metabolism under nitrate-limited conditions in darkness. Exogenous applied 5-formyl-tetrahydrofolate (5-F-THF) completely restored nitrogen content, soluble protein, total amino acids, individual amino acids including Glu, Gln, Asp, Asn, Pro, Arg and Met, nitrate, and endogenous 5-F-THF in *atdfb-3* to the wild-type level. At the same time the application of 5-F-THF partially restored the content of Ser and nitrite in the mutant. Taken together, these results indicated that intact folate metabolism was necessary for nitrogen metabolism in *Arabidopsis thaliana* under nitrate-limited condition in darkness, providing novel insights into function of folate.

1. Introduction

Tetrahydrofolate and its derivatives are collectively called folates. Folate derivatives are tripartite molecules containing pteridine, ρ-aminobenzoate, and glutamate moieties with a short γ-linked chain of glutamyl residues attached to the first glutamate. Folylpolyglutamate folate derivatives are central cofactors of folate-dependent enzymes involved in purines and thymidylate synthesis, methionine, serine and glycine synthesis, panthothenate (vitamin B5) synthesis, and methylation reactions [1–7].

Mutants of Arabidopsis folylpolyglutamate synthetase (FPGS) isoforms, an enzyme that catalyzes the sequential conjugation of additional glutamate residues to the folate molecule to form folylpolyglutamates, are defective in embryo developing, post-germinative growth and development, stunted growth and reduced fertility [8]. A mutation in the *AtDFB* gene, which encodes the plastidial FPGS isoform, caused changes in the glutamylation status of some folate classes coupled to a general depletion of amino acids and nucleotides, broad alterations in metabolism and resulted in plants with short primary roots containing a disorganized quiescent center [3,9]. In our previous study, defects in hypocotyl elongation and nitrogen metabolism were observed in *atdfb-3* in darkness under nitrate-sufficient conditions, and defective hypocotyl was further enhanced under nitrate-limited conditions. Application of 5-formyl-tetrahydrofolate (5-F-THF) effectively restored the hypocotyl length of dark-grown *atdfb-3* seedlings to wild-type level. It is interesting to explore whether the nitrogen metabolism was recovered by the application of exogenous 5-F-THF under nitrate-limited conditions [10]. The mutation of *AtDFC*, the mitochondrial FPGS, was characterized for its altered nitrogen metabolism and enhanced phenotypes to low nitrogen stress, supporting insight into folate biosynthesis and nitrogen utilization during early seedling development in the light condition [11]. Taken together, the above findings suggest FPGS is required for nitrogen metabolism under both dark and light conditions.

Nitrogen, an essential macronutrient for plant growth and development, is not only a constituent of key cell molecules, but is also the pivotal regulator of numerous biological processes [12–16]. Nitrate taken up by transport systems in root of higher plants is reduced into nitrite catalysed in the cytosol by the enzyme nitrate reductase (NR), and then nitrite is reduced to ammonium by the second enzyme nitrite reductase (NiR) [17–21]. Ammonium is mainly assimilated in the plastid/chloroplast by the so-called glutamine synthetase (GS)/glutamine 2-oxoglutarate amino transferase (GOGAT) cycle [22]. Nitrogen compounds are synthesized by incorporating ammonium into the carbon skeletons to produce various amino acids and subsequently proteins. Amino acids,

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Abbreviations

LC-MS	liquid chromatography-mass spectrometry
5-F-THF	5-formyl-THF
5-M-THF	5-methyl-THF
5,10-CH=THF	5,10-methenyl-THF
10-CHO-THF	10-formyl-THF
5,10-CH_2-THF	5,10-methylene THF
THF	tetrahydrofolate
DHF	dihydrofolate
NRT	nitrate transporter
NIA2	nitrate reductase 2
NR	nitrate reductase
NiR	nitrite reductase
GS	glutamine synthetase
GOGAT	glutamine 2-oxoglutarate amino transferase
FPGS	folylpolyglutamate synthetase
AtDFB	dihydrofolate synthetase folylpolyglutamate synthetase (DHFS-FPGS) homolog B
AtDFC	dihydrofolate synthetase folylpolyglutamate synthetase (DHFS-FPGS) homolog C
ADCL	aminodeoxychorismate lyase
FDF2	formytetrahydrofolate deformylase 2
5-FCL	5-formyl-THF cycloligase
THFS	10-formyl-THF synthetase
GGH	GTP cyclohydrolase I
MTHFR2	5,10-CH2-THF reductase 2
GTPCHI	GTP cyclohydrolase I

proteins, and particularly enzymes, are essential for almost all cellular activities, including amino acid metabolism, and carbon metabolism [23–26]. In recent report, nitrogen metabolism is also regulated by light/dark cycle [27].

Nitrogen metabolism during seedling development is of great importance, and the disorder of nitrogen metabolism is a disaster to plant. Low inorganic nitrogen results in numerous perturbations in plant metabolism, such as decreases of chlorophyll, soluble protein, total amino acids, nitrate, Gln, malate, and fumarate content, a decrease of the Gln/Glu ratio, reduced NR and phosphoenolpyruvate carboxylase activities, higher GS and glutamate dehydrogenase activities, and a greater concentration of starch in Arabidopsis [10,11,23–25,28]. Especially, responses to low nitrogen stress are particularly striking in seedlings defective in folate metabolism, suggesting folates plays an important role in plant resisting low nitrogen stress [10,11].

In this report, a T-DNA insertion mutant in the plastidial folylpolyglutamate synthetase gene (*AtDFB*) named *atdfb-3* was defective in folate metabolism and nitrogen metabolism, and exogenous applied 5-F-THF completely restored nitrogen content, soluble protein, total amino acids, individual amino acids, nitrate, and endogenous 5-F-THF in *atdfb-3* to the wild-type level under nitrate-limited conditions in darkness. Meanwhile, 5-F-THF partially restored the content of Ser and nitrite in the mutant. These results indicate that intact folate metabolism is necessary for nitrogen metabolism in *Arabidopsis thaliana* under nitrate-limited condition in darkness, providing novel insights into function of folate.

2. Materials and methods

2.1. Plant materials and growth conditions

In this report, nitrate was used as the sole nitrogen source, 0.3 mM NO_3^- (0.3 N) was used as the nitrate-limited condition. For the 5-F-THF supplementation experiments, a stock solution was added to the growth medium to achieve the desired working concentration. Seeds of Arabidopsis wild-type (*Arabidopsis thaliana*, ecotype Columbia) and the T-DNA insertion mutant of *AtDFB* (SALK_015472, called *atdfb-3*) were sterilized, grown on the same plate, treated at 4 °C in the dark for 2 days, and then moved to a growth chamber at 22 °C under continuous dark conditions for 6 days [10].

2.2. Biochemical and folate profile analyses

The methods for sample preparation and metabolite measurement were described previously [10].

2.3. Accession numbers

Sequence data from this article can be found at the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AT5G05980 (*AtDFB*), AT3G18780 (*ACTIN2*), AT1G12110 (*NRT1.1*), AT1G37130 (*NIA2*), AT2G15620 (*NIR1*), AT5G37600 (*GS1.1*), AT5G16570 (*GS1.4*), AT5G35630 (*GS2*), AT5G53460 (*NADH-GOGAT*), AT5G04140 (*Fd-GOGAT*), AT3G10160 (*AtDFC*), AT5G57850 (*ADCL*), AT5G47435 (*FDF2*), AT5G13050 (*5-FCL*), AT1G50480 (*THFS*), AT1G78660 (*GGH1*), AT1G78680 (*GGH2*), AT2G44160 (*MTHFR2*), and AT3G07270 (*GTPCHI*).

3. Results and discussion

3.1. Altered nitrogen metabolism was recovered by the application of 5-F-THF

Our previous study showed that *atdfb-3* was characterized for its dramatically shortened hypocotyls, altered nitrogen and folate metabolism under nitrate-limited condition, and exogenous applied 5-F-THF completely restored hypocotyl length in *atdfb-3* seedlings with NO_3^- as the sole nitrogen source [10]. Whether the nitrogen and folate metabolism were also restored when 5-F-THF applied to nitrate-limited condition is worthy of exploring.

The N content in *atdfb-3* was 37% higher than that in the wild type under 0.3 N, and the application of 5-F-THF decreased N content in both genotypes, resulting in similar N content in *atdfb-3* and the wild type (Fig. 1A). The mutant accumulated more soluble protein, with 1.32-fold of that in the wild type. Similarly, 5-F-THF decreased the soluble protein content in both genotypes, and led to similar soluble protein content in the wild type and *atdfb-3* (Fig. 1B). Total free amino acids content in *atdfb-3* was 2.05-fold of that in the wild type, and 5-F-THF decreased the difference between the both genotypes (Fig. 1C). Ser in *atdfb-3* was 19.94-fold higher than that in the wild type, accounting for the main accumulation of the total amino acids. The application of 5-F-THF decreased the difference between the wild type and *atdfb-3*, and Ser content in *atdfb-3* was 4.27-fold of the wild type (Fig. 1D). The content of many free amino acids in *atdfb-3*, such as Gln, Asn, Pro, Arg, and Met was significantly higher than that in the wild type, and 5-F-THF restored the content of these amino acids in *atdfb-3* to the wild-type level. The level of Asp in *atdfb-3* was 41% less than the wild type, while the difference disappeared after adding 5-F-THF to the culture condition. There was also