Plant Signaling & Behavior 6:5, 1-4; May 2011; © 2011 Landes Bioscience

The plastidial folylpolyglutamate synthetase and root apical meristem maintenance

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Folylpolyglutamate synthetase (FPGS) catalyzes the attachment of glutamate residues to the folate molecule in plants. Three isoforms of FPGS have been identified in Arabidopsis and these are localized in the plastid (AtDFB), mitochondria (AtDFC) and cytosol (AtDFD). We recently determined that mutants in the *AtDFB* (At5G05980) gene disrupt primary root development in *Arabidopsis thaliana* seedlings. Transient expression of AtDFB-green fluorescent protein (GFP) fusion under the control of the native *AtDFB* promoter in *Nicotiana tabacum* leaf epidermal cells verified the plastid localization of AtDFB. Furthermore, low concentrations of methotrexate (MTX), a compound commonly used as a folate antagonist in plant and mammalian cells induced primary root defects in wild type seedlings that were similar to *atdfb*. In addition, *atdfb* seedlings were more sensitive to MTX when compared to wild type. Quantitative (q) RT-PCR showed lower transcript levels of the mitochondrial and cytosolic *FPGS* in roots of 7-day-old *atdfb* seedling suggesting feedback regulation of *AtDFB* on the expression of other FPGS isoforms during early seedling development. The primary root defects of *atdfb*, which can be traced in part to altered quiescent center (QC) identity, pave the way for future studies that could link cell type specific folate and FPGS isoform requirements to whole organ development.

Key words: *Arabidopsis thaliana*, carbon one metabolism, chloroplast, folate biosynthesis, quiescent center, root development

Submitted: 03/07/11

Accepted: 03/07/11

DOI:

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Addendum to: Srivastava AC, Ramos-Parra PA, Bedair M, Robledo-Hernández AL, Tang Y, Sumner LW, et al. The folylpolyglutamate synthetase plastidial isoform is required for postembryonic root development in Arabidopsis. Plant Physiology 2011; 155:1237–51; PMID: 21233333; DOI:10.1104/pp.110.168278.

It is a well known fact that vitamin deficiency in animals, which lack the complete enzymatic machinery for de novo synthesis of vitamins, results in various physiological, developmental and neurological abnormalities.[1] In plants, vitamin deficiency also can have profound effects on development. For example, an Arabidopsis mutant disrupted in a gene encoding a Guanosine DiPhosphate (GDP)-mannose pyrophosphorylase (*vtc1*), which is involved in ascorbic acid (vitamin C) biosynthesis, exhibited reduced primary root elongation.[2,3] Similarly, Arabidopsis mutants with altered pyridoxine (vitamin B6) biosynthesis displayed reduced seedling growth.[4]

Folates (vitamin B9) comprise another group of vitamins that are well documented to have adverse effects on development when depleted because of their involvement as cofactors in carbon one (C1) metabolism, a metabolic pathway for the biosynthesis of essential amino and nucleic acids. In animals, consequences of inadequate supply of folates in the diet include anemia, fetal neural tube defects and cardiovascular disease.[1,5] Like in animals, folate deficiency in plants can be induced by the exogenous application of compounds such as methotrexate (MTX) or sulfanilamide, which interfere with folic acid biosynthesis. Application of these compounds caused a reduction in cell division in Arabidopsis suspension cells, an inhibition of Arabidopsis seedling growth and a decline in the rate of chlorophyll synthesis in pea leaves.[6-9]

An important component of folate biosynthesis is the generation of polyglutamylated folates, which are the preferred

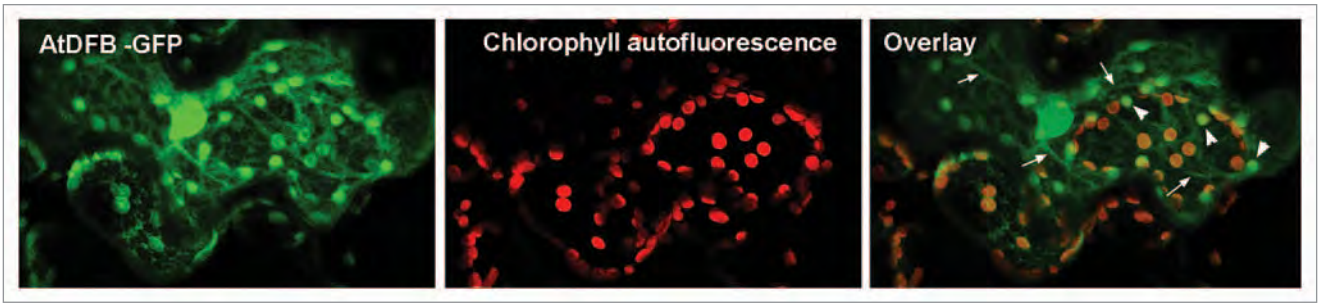

Figure 1. *pAtDFB-AtDFB-GFP* transient expression in *Nicotiana tabacum* leaf epidermal cells. AtDFB is localized in the chloroplasts (arrowheads) and cytosolic strands (arrows).

cofactors for enzymes in C1 metabolism.[10] This reaction is catalyzed by FPGS with three isoforms identified in Arabidopsis.[11] AtDFB was previously reported to localize in chloroplasts by western blotting of isolated chloroplasts fractions using AtDFB antibodies and transient expression of *35S::AtDFB-GFP* in protoplasts.[11] Here, we expanded on the aforementioned study by creating an AtDFB-GFP fusion under the control of the native *AtDFB* promoter (*pAtDFB*). The *pAtDFB::AtDFB-GFP* construct was transiently expressed by syringe inoculation of *Nicotiana tabacum* leaves using the hypervirulent LBA4404 strain of *Agrobacterium tumefaciens*.[12] Leaves were examined with a confocal microscope and in agreement with Ravanel et al. (2001), *pAtDFB::AtDFB-GFP* was associated with chloroplasts. However, in addition to chloroplasts, GFP signal was observed in the cytosol (**Fig. 1**). It was recently proposed based on double mutant studies and organelle folate analysis that the different FPGS isoforms may be targeted to more than one cell compartment.[13] The localization of AtDFB in both the chloroplast and cytosol support this hypothesis. The clear reduction in root growth of *AtDFB* mutant seedlings[14] paves the way for in depth studies to test the significance of FPGS targeting in root development and meristem maintenance.

The antifolate MTX is a dihydrofolate analog, which inhibits tetrahydrofolate (THF) synthesis through its effects on dihydrofolate reductase (DHFR), the enzyme that catalyzes the step leading to the formation of THF.[15] DHFR is immediately upstream of FPGS in the biosynthesis of folylpolyglutamates[16] and inhibition of this enzyme by MTX has been shown to induce severe developmental defects in Arabidopsis seedlings.[8] We therefore examined whether treating wild-type seedlings with MTX could phenocopy the root defects of the *atdfb* mutants. When wild-type seeds were germinated on media supplemented with 5 nM MTX, we observed a dramatic inhibition of root growth. The effect of 5 nM MTX on roots of wild-type seedlings somewhat mirrored the short root phenotype of *atdfb-1* mutants grown on media supplemented with solvent control solution (**Fig. 2A**). Furthermore, we observed that seedlings of *atdfb-1* mutants were more sensitive to MTX compared to wild-type. At 5 nM MTX, *atdfb-1* mutants not only had stunted root growth but also displayed significantly smaller leaves, cotyledons and hypocotyls compared to wild-type seedlings (**Fig. 2B**). At 25 nM MTX, wild-type seedlings still grew but were clearly stunted compared to untreated seedlings. On the other hand, the inhibition of overall seedling growth at 25 nM MTX was more severe in *atdfb-1* mutants. Aerial organs and roots of *atdfb-1* seedlings were barely able to expand at 25 nM MTX and the severely stunted seedlings of the mutant exhibited extensive brown discoloration (**Fig. 2C**). Quantification of primary root length clearly showed that *atdfb-1* was more sensitive than wild-type to the growth inhibitory effects of nanomolar concentrations of MTX (**Fig. 2D**).

The root tip is a physiologically active region of the plant characterized by major cellular processes that involve C1 metabolism. Within this region, products of C1 metabolism such as amino acids and nucleotides are needed to sustain both cell division and expansion that drive the overall growth of the root. Thus, a steady pool of metabolically active folate cofactors is essential to maintain efficient transfer of C1 units in the biochemically active cells of the root tip[14,17] and depletion in the folate pools by the antifolate drug MTX, could inhibit cell division in cell cultures.[7] The observation that wild type seedlings treated with low nanomolar concentrations of MTX caused primary root defects that mirrored the *atdfb* mutant is consistent with MTX and *AtDFB* acting on the downstream steps of the biosynthetic pathway leading to the formation of folypolyglutamates.[5]

Despite a substantial reduction in primary root growth, no obvious defects in overall shoot morphology of *AtDFB* mutants were observed. This was consistent with *AtDFB* being more strongly expressed in roots compared to shoots.[14] As noted earlier two genes encoding other FPGS isoforms namely *AtDFC* and *AtDFD* are present in the Arabidopsis genome.[11] Absence of a distinct phenotype in shoots of *atdfb* indicates that metabolically active folates that allow the mutant to maintain unimpeded shoot growth could originate from the activity of the AtDFC and AtDFD isoforms. This prompted us to check if transcripts of the other two FPGS isoforms changed in the *AtDFB* mutant particularly in young seedlings where root growth defects of *atdfb* are most prominent. Real time quantitative (q) RT-PCR of roots from 7-day-old *atdfb-1* seedlings revealed that expression of *AtDFC* and *AtDFD* was lower in *atdfb-1* compared to wild type (**Fig. 3**). This indicates that the mitochondrial and cytosolic FPGS isoforms do not compensate for the lack of *AtDFB* expression but their slightly

lower transcript levels could partly contribute to the early root developmental defects of *atdfb*. Nonetheless, the observation that single knockouts to *AtDFC* and *AtDFD* do not display any obvious abnormalities in root development[14] and the elevated expression of AtDFB in the root QC,[18] continue to support the pivotal role of AtDFB isoform in postembryonic root meristem maintenance compared to the other two FPGS isoforms. Experiments designed to redirect the targeting of *AtDFC* or *AtDFD* to root plastids in *atdfb* and continued monitoring of AtDFC and AtDFD transcript levels in *atdfb* at various stages of development should help clarify issues of redundancy in compartmentalized FPGS activities.

Acknowledgements

This work was supported by the Samuel Roberts Noble Foundation (to Y.T and E.B.B.), the BioEnergy Science Center (BESC to Y.T. and E.B.B.) and the Mexican National Council for Research and Technology (CONACyT, grant 80459 to R.I.D.G.). BESC is a US Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the Office of Science, US Department of Energy.

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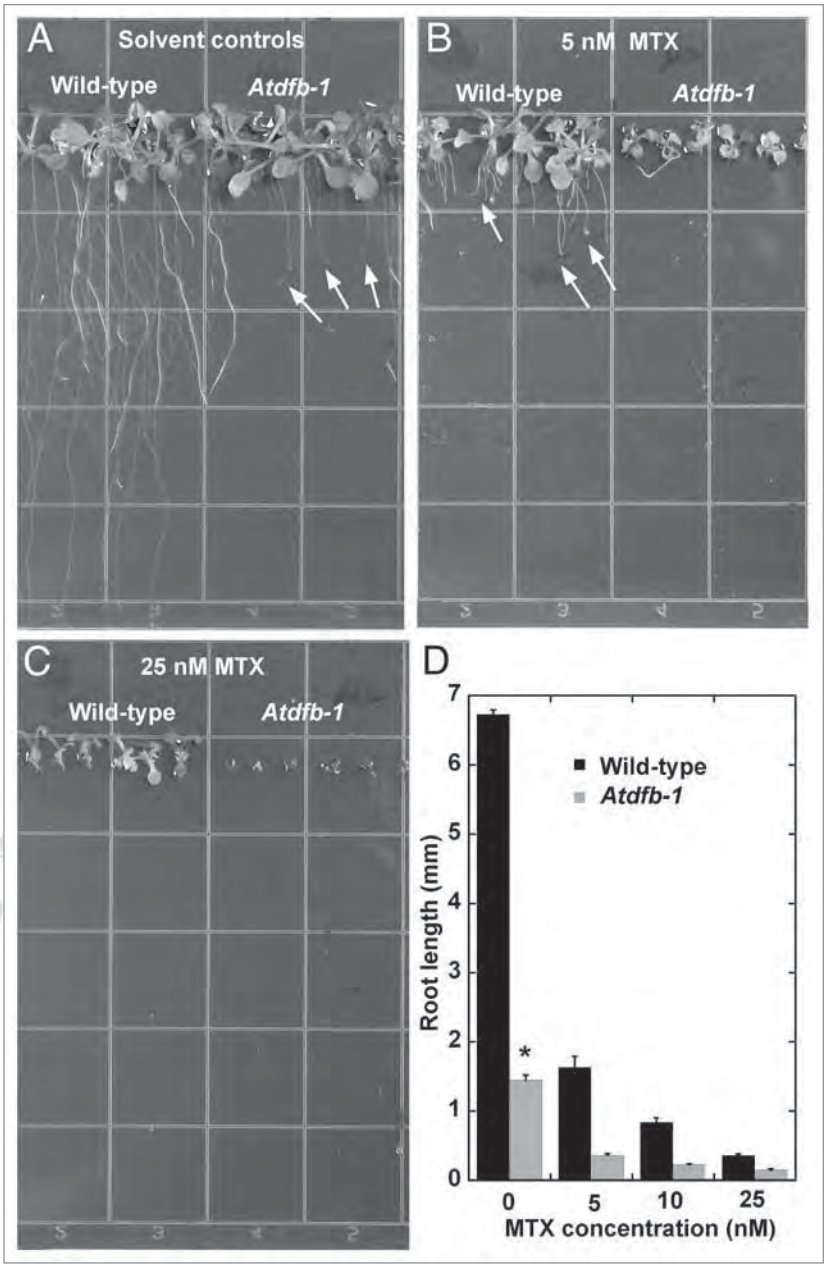

Figure 2. *atdfb* is hypersensitive to methotrexate (MTX). 15-day-old seedlings grown on solvent controls (A), 5 nM (B) and 25 nM (C) methotrexate. Note that roots of wild-type seedlings on 5 nM methotrexate (B, arrows) are almost similar in length to roots of *atdfb*-1 mutants grown on the solvent control solution (A, arrows). *AtDFB* mutants have more severe growth developmental defects compared to wild-type when grown on exogenous MTX (B and C, arrowheads). (D) Quantification of primary root length in wild-type and *atdfb* mutants grown on MTX. Asterisks indicate statistically significant differences in *atdfb*-1 root length compared to wild-type according to a Student's t-test ($p < 0.01$). Values are means ± SE

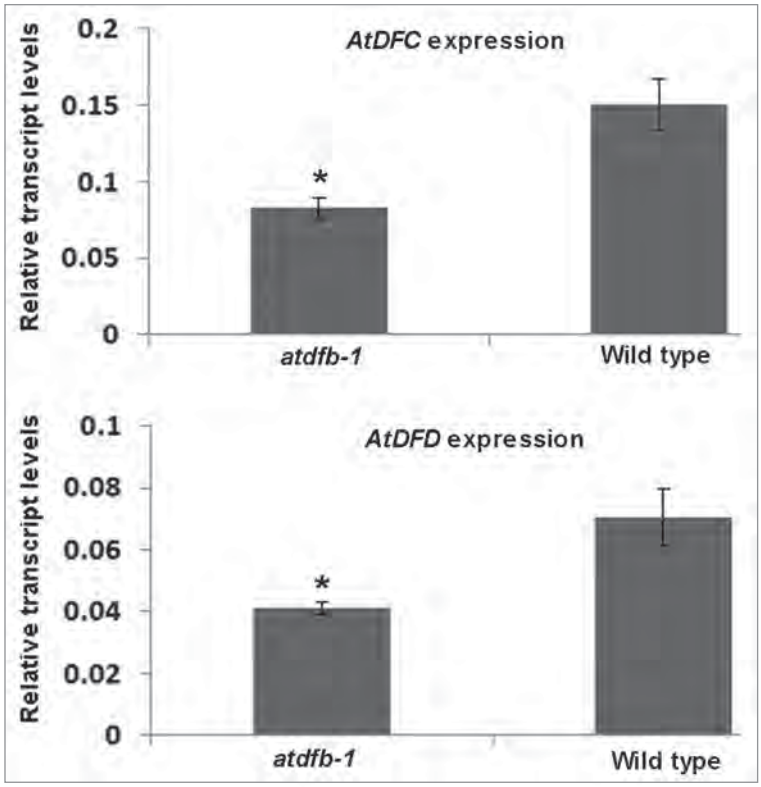

Figure 3. Expression of *AtDFC* and *AtDFD* in roots of 7-d old wild type and *atdfb* seedlings. qRT-PCR revealed that relative transcripts level of *AtDFC* and *AtDFD* is lower in *atdfb* compared to wild type seedlings. Values are means ± SE of 5 independent biological replicates (each replicate had 40 roots for RNA extraction). The asterisk indicates statistically significant differences according to a Student's t-test ($p < 0.05$).

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