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The Folylpolyglutamate Synthetase Plastidial Isoform is Required for Postembryonic Root Development in *Arabidopsis* [1] [W]

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A recessive Arabidopsis mutant with short primary roots and root hairs was identified from a forward genetic screen. The disrupted gene in the mutant encoded the plastidial isoform of folylpolyglutamate synthetase (FPGS) previously designated as AtDFB, an enzyme that catalyzes the addition of glutamate residues to the folate molecule to form folylpolyglutamates. The short primary root of *atdfb* was associated with a disorganized quiescent center (QC), dissipated auxin gradient in the root cap, bundled actin cytoskeleton, and reduced cell division and expansion. The accumulation of monoglutamylated forms of some folate classes in *atdfb* was consistent with impaired FPGS function. The observed cellular defects in roots of *atdfb* underscore the essential role of folylpolyglutamates in the highly compartmentalized one carbon transfer reactions (C1 metabolism) that lead to the biosynthesis of compounds required for metabolically active cells found in the growing root apex. Indeed, metabolic profiling uncovered a depletion of several amino acids and nucleotides in *atdfb* indicative of broad alterations in metabolism. Methionine and purines, which are synthesized *de novo* in plastids via C1 enzymatic reactions, were particularly depleted. The root growth and QC defects of *atdfb* were rescued by exogenous application of 5-formyl-tetrahydrofolate (5-CHO-THF), a stable folate that was readily converted to metabolically active folates. Collectively, our results indicate that AtDFB is the predominant FPGS isoform that generates polyglutamylated folate cofactors to support C1 metabolism required for meristem maintenance and cell expansion during postembryonic root development in Arabidopsis.

The growth and development of a root is dictated by the coordinated activity of cell division, cell expansion and cell differentiation. Forward genetic screens for root mutants in the model plant *Arabidopsis thaliana* have uncovered complex developmental networks that orchestrate patterns of cell division in the meristem and trigger the onset or extent of longitudinal cell expansion in the elongation zone. Mutant studies also have led to the discovery of signaling pathways that specify the position of root hair emergence, which typically accompany the maturation of cells in the zone of differentiation, or define the polarity of root hair growth (Benfey et al., 2010). Components of these signaling and developmental networks include a dynamic cytoskeleton and vesicular trafficking system, both of which play pivotal roles in regulating membrane recycling and cell wall deposition to sustain normal root growth (Bernal et al., 2008; Yoo et al., 2008; Beck et al., 2010). Also part of the intricate developmental networks that govern root growth are a collection of transcription factors and an array of chemical messengers including sugars, nutrients, amino acids, and most of the major plant hormones (Benková and Hejátko, 2009; Forde and Walch-Liu, 2009; Iyer-Pascuzzi et al., 2009).

Although plant hormones continue to dominate studies of chemical signals that regulate root development, there is accumulating evidence for the involvement of other endogenous metabolites such as vitamins in the maintenance of normal root morphology. For example, pyridoxine (vitamin B6) has been linked to the control of Arabidopsis root growth since a mutant in a gene that encodes a pyridoxine synthase 1 (*pdx1*), exhibited shorter primary roots compared to wild type (Chen and Xiong, 2005; Wagner et al., 2006). In addition to their protective roles against oxidative stress, vitamins serve as cofactors for several enzymes that metabolize compounds required for normal cellular function. Thus, the root growth defects reported in Arabidopsis *pdx1* mutants could be the result of alterations in metabolic pathways that are critical for the biosynthesis of compounds required for normal cell physiology. For example, the stunted root development in the *pdx1* mutant was associated with impaired local auxin biosynthesis and a broad disruption in metabolism that led to altered levels of amino acids, sugars and various organic acids (Wagner et al., 2006; Chen and Xiong, 2009).

Tetrahydrofolate (THF) and its derivatives, collectively referred to as folates, constitute a group of B vitamins (vitamin B9) that are essential for several major metabolic pathways in the cell because they mediate the addition and removal of one-carbon (C1) units in a set of reactions commonly referred to as C1 metabolism. The products of these C1 transfer reactions include purines, methionine, thymidylate, and pantothenate (vitamin B5), all of which are crucial for the normal function of living cells (Hanson et al., 2000; Sahr et al., 2005). In 7 day old pea seedlings, it was shown that the root meristem contained 5-fold more folates than the mature root. The elevated level of folates in the root meristem was strongly correlated with high mRNA and protein expression of dihydropterin pyrophosphokinase-dihydropteroate synthase (HPPK-DHPS), an enzyme that catalyzes reactions specific to THF synthesis (Jabrin et al., 2003). In maize roots, expression of the gene encoding the bifunctional dihydrofolate reductase (DHFR) and thymidylate synthase (TS), which is involved in *de novo* generation of folate and thymidylate (Cossins, 2000; Fig. 1), was highest in the meristem but barely detectable in the elongation zone (Cox et al., 1999). These studies indicate that a steady pool of folate cofactors is essential to sustain actively dividing cells in the root tip. Indeed, pharmacological induction of folate deficiency in plants caused a reduction in cell division in Arabidopsis suspension cells and an inhibition of seedling growth (Loizeau et al., 2007; Loizeau et al., 2008; Raichaudhuri et al., 2009). In plants, both folate biosynthetic and folate-dependent enzymes are distributed among three cellular compartments: plastids, mitochondria and the cytosol (Appling, 1991; Rébeillé et al., 2006; Loizeau et al., 2008; Fig. 1). However, the significance of folate compartmentation to plant development is not yet fully understood.

Although much progress has been made toward understanding the biochemistry of plant enzymes involved in folate biosynthesis and turnover (Ravanel et al., 2001; Hanson and Gregory, 2002), genetic evidence for the *in planta* functions of these enzymes is limited. In one study, the Arabidopsis *globular arrest 1 (gla1)* mutant, which exhibited defective embryo development was shown to be disrupted in the *AtDFA* gene (Ishikawa et al., 2003). In Arabidopsis, *AtDFA* is a single gene that encodes a functional mitochondrial matrix-localized dihydrofolate synthetase (DHFS), which catalyzes the addition of the first glutamyl side chain to dihydropteroate to form dihydrofolate (Ravanel et al., 2001). Downstream of DHFS is folylpolyglutamate synthetase (FPGS), an enzyme that catalyzes the sequential conjugation of additional glutamate residues to the folate molecule to form folylpolyglutamates, the preferred substrates for several enzymes involved in C1 metabolism (Cossins and Chen, 1997; Ravanel et al., 2001; Fig. 1). Polyglutamylation is also essential for retention of folates within a given cellular compartment since organellar folate transporters are more specific to monoglutamylated folates (Bedhomme et al., 2005; Klaus et al., 2005; Raichaudhuri et al., 2009). In Arabidopsis, three genes namely *AtDFB*, *AtDFC* and *AtDFD* have been reported to encode the plastidial, mitochondrial and cytoplasmic FPGS isoforms, respectively (Ravanel et al., 2001; Fig. 1). In a recent reverse genetics study, different double mutant combinations to these Arabidopsis FPGS isoforms resulted in dramatic developmental phenotypes ranging from embryo lethality, seedling lethality, late flowering, and dwarf plants with various reproductive defects (Mehrshahi et al., 2010). However, phenotypes were only reported in double *FPGS* mutants but not in single mutants leading to the conclusion of redundancy in compartmentalized FPGS activity (Mehrshahi et al., 2010). Also in Arabidopsis, double mutants to genes encoding two 10-formyl THF (10-CHO-THF) deformylases (PurU) that metabolize 10-CHO-THF to formate and THF, were smaller and paler compared to wild type plants. In addition, double *PurU* mutants exhibited delayed embryo development and shriveled non-viable seeds (Collakova et al., 2008).

In this study, a forward genetic screen led to the identification of a recessive Arabidopsis mutant that exhibited stunted primary root and root hair growth. The mutant contained a T-DNA insertion in the *At5g05980* gene, which encodes the plastidial FPGS isoform designated as AtDFB for *Arabidopsis thaliana* DHS-FPGS isoform B (Ravanel et al., 2001) and recently renamed FPGS1 by Mehrshahi et al. (2010). The short primary root of *atdfb* was associated with a disorganized quiescent center (QC), reduced cell division and expansion, an extensively bundled actin cytoskeleton, and dissipation of the auxin gradient in the root cap. The *AtDFB* mutation led to changes in the glutamylation status of some folate classes that was coupled to a general depletion of amino acids and nucleotides, indicative of broad alterations in metabolism. The disorganized QC in *atdfb* was consistent with previous transcript profiling studies that showed strong *AtDFB* expression in the QC (Nawy et al., 2005) and therefore point to a role for metabolically active folates in maintaining QC function. No obvious defects in root development were observed in single mutants to *AtDFC* and *AtDFD.* Our data indicate that AtDFB is the predominant FPGS isoform that generates physiologically active folate cofactors required to sustain postembryonic root growth in developing Arabidopsis seedlings.

RESULTS

AtDFB Loss-of-Function Mutants are Impaired in Primary Root Growth

We isolated a mutant originally designated as *drh2* from a T-DNA activation tagged population with root hairs that were wavy and short (Supplemental Fig. S1). It was also found that the mutant had significantly shorter primary roots than wild type (Fig. 2A). The F2 generation segregated in a 3:1 (wild type to mutant) ratio after crossing the mutant to wild type consistent with a single recessive mutation. Closer examination of the primary roots of 7 day old seedlings showed a shorter growth zone in *drh2* as evident from the emergence of root hairs very close to the root tip (Fig. 2A). In 11 day old seedlings, roots of wild type were more than 8-fold longer than *drh2* and formed about 2 adventitious roots (Fig. 2B). At 3 to 8 days, primary root growth rate of *drh2* was only 1.67±0.09 mm/day but increased to 4.40±0.31 mm/day by 15-17 days (Fig. 2C). In wild type seedlings, primary root growth rate was 8.35±0.46 mm/day in 3-8 day old seedlings and decreased to 7.02±0.37 mm/day in 15-17 day old seedlings (values are means of 20 roots ± S.E.).

TAIL-PCR of the *drh2* mutant identified a T-DNA insert in the 5th intron of the *At5g05980* gene, which encodes AtDFB (Ravanel et al., 2001). SAIL_556_G08 and SALK_015472 were identified as additional lines with T-DNA insertions in the 6th intron and 8th exon of *At5g05980* respectively and both lines exhibited root phenotypes similar to those of *drh2* indicating that the defective primary root and root hair growth exhibited by the *drh2* seedlings were due to mutations in the *AtDFB* gene. *Drh2*, SAIL_556_G08 and SALK_015472 were therefore designated as *atdfb-1*, *atdfb-2* and *atdfb-3* respectively (Fig. 3A, B). Reverse transcription (RT)-PCR analysis showed that *atdfb-1*, *atdfb-2* and *atdfb-3* had no detectable transcript using primers flanking the T-DNA insertion (Fig. 3C). Despite the short primary root of *atdfb*, no obvious defects in the development of the above ground organs were observed (Supplemental Fig. S2).

Real time quantitative (q) RT-PCR analysis revealed that *AtDFB* was expressed in both shoots and roots but expression in roots was higher compared to shoots (Fig. 4A). Whole mount *in situ* hybridization using gene specific probes confirmed stronger expression of *AtDFB* in the root apex compared to shoots (Fig. 4B-D). Furthermore, previous transcript profiling of GFP-marked root cell types in Arabidopsis revealed that *AtDFB* was strongly expressed in the QC

(Nawy et al., 2005). In agreement with these results, distinct *AtDFB* expression was observed in QC region of the root tip (Fig. 4C).

AtDFC and *AtDFD* encode two other FPGS isoforms that have been reported to localize to the mitochondria and cytoplasm respectively (Ravanel et al., 2001; Fig. 1). No obvious defects in root development were observed in single mutants to these other FPGS isoforms (Supplemental Fig. S3). The higher root expression of *AtDFB* compared to *AtDFC* and *AtDFD* as shown by publicly available Arabidopsis microarray expression data sets from Genevestigator (Supplemental Fig. S4; Zimmermann et al., 2004) supports our findings that AtDFB plays a more important role in root development than the other two FPGS isoforms.

The Short Root Phenotype of *atdfb* is the Result of Reduced Cell Division and Cell Expansion

We next determined whether the short primary root of *atdfb* was the result of reduced cell division and/or expansion. Differential interference contrast (DIC) images of the epidermal cells from the root hair region were acquired and their lengths measured. From the DIC images, it was obvious that epidermal cells of wild type roots were two times longer than epidermal cells of *atdfb-1* roots (Fig. 5A, B). The average epidermal cell length of wild type seedlings was 154.4±34.8 μm compared to 74.1±2.4 μm in *atdfb-1*(Fig. 5C) (values are means ± SE of 40-50 cells from 10-12 seedlings).

Because the actin cytoskeleton is an important regulator of cell expansion (Blancaflor et al., 2006), we asked if the short primary roots of *atdfb-1* was impaired in filamentous actin (F-actin) organization. In wild type seedlings, cells in the root elongation zone consisted of randomly organized fine F-actin arrays, a typical feature of actin organization in wild type roots (Fig. 5D; Wang et al., 2008). On the other hand, F-actin in the shorter epidermal cells of *atdfb-1* roots was extensively bundled (Fig. 5E). Bundling and fluorescent aggregates were also observed in the interphase cells within the root meristem of *atdfb-1* but not in wild type (Fig. 5F, G). In addition, cells that were undergoing cytokinesis in wild type roots could be easily identified by the presence of GFP-labeled F-actin in the phragmoplast (Fig. 5F). F-actin labeled phragmoplasts were rarely observed in the meristem of *atdfb-1* roots (Fig. 5G).

The scarcity of F-actin labeled phragmoplasts (Fig. 5G) in *atdfb-1* roots strongly suggested that cell division was impaired. Consistent with the F-actin labeling result, the average number of mitotic cells in 5 day old DAPI-stained roots of *atdfb-1* seedlings was significantly less than in wild type (Fig. 5H). Taken together, these cellular assays indicated that the reduced primary root growth of *atdfb-1* can be attributed to defects in both cell division and cell expansion.

Postembryonic Quiescent Center Organization and Auxin Gradients are Altered in Roots of *atdfb*

Because the QC plays a crucial role in root meristem maintenance by serving as a source for replenishing expiring cell initials (van den Berg et al., 1997), it is possible that altered QC function is one cause of the impaired cell division in *atdfb-1* given the strong expression of *AtDFB* in the QC (Nawy et al., 2005; Fig. 4C). Indeed, it was found that unlike wild type roots, which clearly showed the two to four cell sized QC typically observed in median confocal sections of PI stained roots (Nawy et al., 2005; Fig. 6A), cells in the root apex of 5 day old *atdfb-1* seedlings that corresponded to the QC region of wild type roots were disorganized (Fig. 6B). However, no gross differences in the organization of cells in the QC region of mature embryos were observed between wild type and *atdfb* (Fig. 6C, D), which appeared to persist for up to 3-4 days post-germination (Fig. 6E, F). Clear differences in QC organization between wild type and *atdfb-1* only became obvious in seedlings older than 4-5 days.

Another important regulator of root development is the plant hormone auxin. In Arabidopsis roots, local auxin concentration gradients have been shown to direct patterns of cell division, expansion and differentiation in the root apex (Overvoorde et al., 2010). We therefore investigated whether the root defects of *atdfb* were associated with altered local auxin gradients. For these studies we used the synthetic auxin responsive reporter, *DR5:GFPm*, which indirectly reports patterns of local auxin accumulation in Arabidopsis roots (Ottenschläger et al., 2003). Roots from 8 day old wild type seedlings showed *DR5:GFPm* expression in both the QC and central cells of the columella (Fig. 7A). In contrast, the expression of *DR5:GFPm* in roots of *atdfb-1* was confined to only a few cells in the columella (Fig. 7B). The number of cells expressing *DR5:GFPm* in median confocal sections of the root cap of 8 day old *atdfb-1* seedlings was about 3-fold less than that of wild type (Fig. 7C).

Another function of the QC is the generation of cell autonomous signals that suppress differentiation and therefore maintain the adjacent stem cells (van den Berg et al., 1997). In Arabidopsis roots where the QC was removed by laser ablation or mutants that were unable to specify a QC, the ability to maintain the surrounding columella initials was compromised (van den Berg et al., 1997; Sabatini et al., 2003). In the root cap, differentiation into columella cells is marked by the formation of starch filled amyloplasts that can be readily imaged by Lugol staining. In this and other studies, it was shown that columella initials of wild type roots proximal to the QC typically lacked amyloplasts (Fig. 7D; Sabatini et al., 2003). However, similar to roots with a defective QC (Sabatini et al., 2003), cells immediately below the disorganized QC region of *atdfb* acquired the amyloplast differentiation markers that are typically only expressed in mature columella cells (Fig. 7E). Furthermore, real time qRT-PCR of the *AGL42* gene, which is a marker for the QC (Nawy et al., 2005) was significantly reduced in root tips of *atdfb-1* (Fig. 7F).

5-Formyl Tetrahydrofolate Rescues the Root Growth Defects of *atdfb*

We next tested whether the exogenous application of a stable form of THF such as 5-CHO-THF could rescue the *atdfb* root defects. 5-CHO-THF has been used to rescue the inability of cultured *gla1* embryos, which are defective in the gene encoding *AtDFA*, to form calli (Ishikawa et al., 2003) and partially rescue the developmental defects of Arabidopsis FPGS double mutants (Mehrshahi et al., 2010). When all three mutant alleles of *AtDFB* were planted on media supplemented with 500 μM 5-CHO-THF, their primary roots were restored to wild type lengths (Fig. 8A, B). The restoration of root growth of *atdfb-1* to wild type upon exposure to 5-CHO-THF was also accompanied by the reformation of wild type QC organization (Fig. 8C) and a corresponding increase in the number of mitotic cells (Supplemental Fig. S5A). In addition, the average root hair length of *atdfb* increased significantly compared to untreated seedlings and was almost restored to wild type lengths (Supplemental Fig. S5B, C).

To better understand why 5-CHO-THF, which is not a C1 donor, was able to chemically complement the root phenotype of *atdfb*, we analyzed total folates in roots of the rescued seedlings. It was found that a massive (>2300 fold) increase in total folate occurred in 5-CHO-THF supplemented roots compared to non-supplemented roots in both wild type and *atdfb-1* seedlings. Individual folate classes accumulated in 5-CHO-THF-treated seedling roots showed similar contributions to the folate pool than those observed in solvent controls with 5-CH_3-THF as the most predominant folate that accumulated (Supplemental Fig. S5D).

The Folate Glutamylation Profile is Altered in *atdfb* Seedlings

To determine the impact of the *AtDFB* mutation on folate metabolism, folate analyses were performed on shoots and roots of 15 day old seedlings. Total folate content did not significantly change between wild type and *atdfb-1* in both shoots and roots. However, we did find differences in the accumulation patterns of some folate classes and general changes in the contribution of each folate class to the total folate pool (Supplemental Table S1).

Because AtDFB catalyzes the extension of the glutamate tail of the folate molecule (Ravanel et al., 2001; Fig. 1), differences in the glutamylation state of folates between wild type and *atdfb-1* seedlings might be predicted rather than differences in total folate content. Indeed, a considerable increase in total monoglutamylated folates in *atdfb* roots when compared to wild type roots was found (Fig. 9B). This difference was not observed in shoots (Fig. 9A) and polyglutamylated total folate content was not altered in either tissue (Fig. 9C, D). However, when we looked at the individual folate classes, the glutamylation state of some folate classes was significantly different between wild type and *atdfb-1* seedlings (Fig. 9A-D). The most significant difference was an increase in monoglutamylated 5-CH_3-THF from 9% of the total 5-CH_3-THF pool in wild type roots to 53% in *atdfb-1* roots (Student's t-test, $\alpha=0.05$, $p\leq0.0006$; Fig. 9B). The root glutamylation profile of 5,10-methenyl-THF (5,10-CH=THF), which is the sum of the conversion of 10-CHO-THF at acidic pH and the existing 5,10-CH=THF pool (Quinlivan et al., 2006), was also impacted by the *AtDFB* mutation. Roots of *atdfb-1* had 77% of the 5,10-CH=THF pool in the monoglutamylated form as opposed to wild type, which had only 44% of the 5,10-CH=THF pool in the monoglutamylated form (Fig. 9B).

Although the level of monoglutamylated 5-CH_3-THF in both shoots and roots of *atdfb-1* was generally higher than wild type (Fig. 9A, B), the polyglutamylation level of this folate class was not significantly different between wild type and *atdfb-1* (Fig. 9C, D). However, polyglutamylation levels of 5,10-CH=THF were significantly lower in both shoots and roots of *atdfb-1* compared to wild-type (Fig. 9C, D). A 7-fold increase in levels of 5-CHO-THF was also detected in *atdfb-1* roots compared to wild type with more than 90% of this increase in the polyglutamylated form (Fig. 9D).

Amino Acids and Nucleotides are Depleted in Whole Seedlings of *atdfb*

Non-targeted metabolic profiling of 7 day old whole Arabidopsis seedlings was performed using GC-MS to determine how the *AtDFB* mutation impacted overall metabolism. A total of 776 polar and 415 non-polar compounds were detected in *atdfb-1* and wild type seedlings respectively, of which 193 polar compounds and 92 non-polar compounds could be assigned a chemical structure based on EI-MS (electron ionization mass spectrometry) spectral matching to authentic compounds (Supplemental Table S2 and S3).

Hierarchical clustering analysis (HCA) using a green-black-red diagram was used to illustrate the distribution of normalized signal intensities of cellular metabolites in wild type and *atdfb-1*. This analysis showed that nucleotides and amino acids were generally depleted in *atdfb-1* (Fig. 10A). Amino acids that showed a statistically significant reduction in *atdfb-1* compared to wild type included cysteine, arginine, glutamine, asparagine, methionine, phenylalanine, lysine and tyrosine (Fig. 10B). Folates serve as cofactors for methionine biosynthesis and serine-glycine interconversion (Hanson and Roje, 2001; Fig. 1). However, we did not find any significant differences in serine and glycine levels between wild type and *atdfb-1* seedlings (Fig. 10C). For nucleotides, statistically significant reductions in the levels of guanosine, adenine, uridine, uridine monophosphate and 5-methyl pyrimidine were noted (Fig. 10D, E).

Exogenous Methionine and Guanosine Partially Alleviate the Root Growth Defects of *atdfb*

Metabolic profiling showed that endogenous methionine levels in *atdfb-1* seedlings were lower relative to wild type (Fig. 10B). In yeast, *met7*, a mutant disrupted in a gene encoding a cytosolic FPGS, requires a source of external methionine for growth (Cherest et al., 2000). Expressing the *AtDFB* gene in *met7* abolished its dependence on external methionine (Ravanel et al., 2001). We therefore tested whether the application of exogenous methionine could rescue the root defects of *atdfb*. It was found that methionine concentrations of 10-25 μM induced a more than 2 fold increase in root growth of *atdfb-1* compared to the non-treated *atdfb-1* controls (Supplemental Fig. 6A). Although root growth of *atdfb-1* seedlings was clearly promoted upon exposure to methionine, their roots remained significantly shorter than wild type roots grown with or without external methionine (Supplemental Fig. 6B).

We also tested whether supplying seedlings with external adenine or guanosine had any effect on roots of *atdfb-1* given the reduction in the levels of these metabolites in the mutant compared to wild type (Fig. 10E). Exogenous guanosine at 10 nM induced a slight but statistically significant increase in root length of *atdfb-1*. However, 10 µM external guanosine did not impact root growth of *atdfb-1* (Supplemental Fig. 6C). Unlike methionine and guanosine, adenine did not promote root growth of the mutant (Supplemental Fig. S6C). A combination of adenine, guanosine and methionine induced a statistically significant increase in *atdfb-1* root length compared to controls but the induction of growth was similar to either methionine or guanosine alone (Supplemental Fig. S6C).

DISCUSSION

Three genes in Arabidopsis encode FPGS isoforms that were previously shown to localize to different cellular compartments (Ravanel et al., 2001). In a recent reverse genetics study, various double mutant combinations to these Arabidopsis FPGS isoforms resulted in dramatic developmental phenotypes providing compelling evidence for the importance of polyglutamylated folates in plant development (Mehrshahi et al., 2010). However, because single FPGS mutants did not appear to have any obvious defects in the above ground organs of mature plants that were grown directly from soil, it was concluded that redundancy in compartmentalized FPGS activity is likely operating during plant development (Mehrshahi et al., 2010). In the present study, we show that disruption of a gene encoding a single FPGS isoform (*AtDFB*) resulted in a dramatic reduction in primary root elongation. Because single *AtDFC* and *AtDFD* mutants had no obvious primary root defects (Supplemental Fig. S3) our data indicate that *AtDFB* is the predominant FPGS isoform that generates bulk of the polyglutamylated folate cofactors for C1 metabolism required to sustain normal meristematic activity and cell expansion in actively growing root tips.

The importance of *AtDFB* for root development is supported by its strong expression in the root QC (Nawy et al., 2005; Fig. 4). Loss of *AtDFB* function could theoretically compromise regenerative cell divisions of the QC and explain the various root cell defects of *atdfb*. Indeed, it was found that cells in the QC region of *atdfb* were disorganized and this was coupled to altered gradients of the auxin sensitive *DR5:GFPm*, reduced expression of the QC-expressed gene, *AGL42* (Nawy et al., 2005), and premature formation of amyloplast differentiation markers. In this regard, it is noteworthy that genetic disruption of *DR5* expression and mutations that resulted in loss of QC identity led to short primary roots and cell patterning abnormalities reminiscent of *atdfb* defects (Sabatini et al., 1999; 2003). Furthermore, the adventitious root phenotype of *atdfb* mirrored that of *short root* (*shr*), which is consistent with the loss of root apical meristem activity (Lucas et al., 2010).

The lack of any obvious embryonic and shoot defects in *atdfb* support recent reverse genetic studies suggesting that *AtDFC* or *AtDFD* are able to compensate for the absence of *AtDFB* during embryo and above ground vegetative development (Mehrshahi et al., 2010). However, a novel finding from our studies is that these other FPGS isoforms do not always function redundantly at least within a specific window of postembryonic root development. During this developmental window, the QC-expressed AtDFB isoform plays a major role in maintaining QC identity to support normal meristematic function during postembryonic root growth. We cannot rule out the possibility that QC identity in *atdfb* is already impacted before defects in cell organization within the QC region become apparent. Future studies will examine the spatial and temporal expression of several QC identity markers (Sabatini et al., 2003; Nawy et al., 2005) in *atdfb* mutants at different stages of embryonic and postembryonic root development to better understand the dynamics of QC function in relation to folate metabolism. It also is not clear whether AtDFB acts exclusively within the QC since expression data suggest that it could be required for normal function of other cell types within the root meristem (Fig. 4; Supplemental Fig. S4).

Because folate monoglutamates that accumulated in *atdfb* cannot be efficiently utilized by folate requiring enzymes (Appling, 1991; Sahr et al., 2005), biochemical processes in the root apex that require C1 metabolites may not function optimally leading to the root defects of *atdfb*. On the other hand, differences in polyglutamylated folates between wild type and *atdfb*, which were absent in whole root folate profiling experiments, could be more pronounced in plastids where *AtDFB* was shown to localize (Ravanel et al., 2001). Indeed, Mehrshahi et al. (2010) found that chloroplasts in mature leaves of *atdfb* had lower polyglutamylated folates than wild type but even within this compartment, polyglutamylated folate content was not totally abolished in the mutants. This led to the proposal that different FPGS isoforms might be targeted to more than one compartment or that plants have a mechanism for transporting folate polyglutamates (Mehrshahi et al., 2010). Given that the root developmental defects of *atdfb* can be traced in part to a defined group of cells that comprise the QC, whole root folate quantification could dilute differences in the folate polyglutamylation profiles between wild type and *atdfb*. Thus, it will be necessary to develop sensitive methods to quantify folates from plastids of specific root cell types to better understand how metabolic changes within a defined group of cells translate into whole organ development.

Closer examination of individual folate classes revealed higher monoglutamylated and lower polyglutamylated 5,10-CH=THF in *atdfb-1* roots compared to wild type (Fig. 9B, D). In this regard it is worth noting that the main form of folate found in chloroplasts is polyglutamylated 5,10-CH=THF, which comprises both 5,10-CH=THF and 10-CHO-THF (Klaus et al., 2005; Orsomando et al., 2005, Mehrshahi et al., 2010). In Arabidopsis, *de novo* purine biosynthesis occurs in plastids and requires 10-CHO-THF in two transformylation steps (Zrenner et al., 2006). A reduction of polyglutamylated 10-CHO-THF could prevent its retention within plastids and thus hinder its efficiency to be used as a cofactor in reactions leading to the biosynthesis of purines (Zrenner et al., 2006; Fig. 1). Indeed, it was found that endogenous levels of adenine and guanosine were reduced in *atdfb* seedlings (Fig. 10).

We did not find significant changes in THF + 5,10-CH_2-THF polyglutamylation levels in *atdfb* roots compared to wild type (Fig. 9D). However, mutant shoots showed an increase in polyglutamylated forms of these folate classes (Fig. 9C) suggesting that THF and 5,10-CH_2-THF did not accumulate predominantly in plastids as previously shown (Orsomando et al., 2005; Mershahi et al., 2010). These two folate classes are involved in serine-glycine interconversion by the action of serine hydroxymethyltransferase (SHMT, Besson et al., 1995), which is present in different cell compartments, including plastids (Hanson and Roje, 2001; Zhang et al., 2010; Fig. 1). These observations are consistent with the maintenance of serine-glycine pool that we found in mutant whole seedlings (Fig. 10C), which could be due to other SHMT isoforms acting outside plastids.

We also found a 7-fold increase of 5-CHO-THF in *atdfb* roots with more than 90% of this increase in the polyglutamylated form (Fig. 9D). Polyglutamylated 5-CHO-THF was found to be a major folate in leaf mitochondria (Orsomando et al., 2005; Goyer et al., 2005). Thus, it is possible that reduction of polyglutamylation in plastids induced a redirection of folate flux, which then caused an accumulation of this folate class in roots. In fact, the contribution of each folate class to the total folate pool changed in the mutant in both shoots and roots (Supplemental Table S1), suggesting that the absence of AtDFB generates changes in the distribution and interconversion of folate pools within the cell. In this respect, previous work in Chinese hamster ovary cells showed that changes in mitochondrial and cytosolic FPGS activities caused clear folate redistribution between cell compartments (Lin et al., 1993; Lowe et al., 1993; Qi et al., 1999). 5-CHO-THF does not participate in C1 reactions and its role *in planta* is not very well understood. In fact this folate species has been shown to inhibit the activity of many folate utilizing enzymes (Stover and Schirch, 1993; Roje et al., 2002). Therefore, it was interesting to find that exogenous 5-CHO-THF rescued the root defects of *atdfb* (Fig. 8). When we analyzed these chemically complemented mutant seedlings, we found that they accumulated more than 1,900 nmol/g of folates, which is by far above all natural folate levels reported (Supplemental Fig. S5D). This indicates that the exogenously supplied 5-CHO-THF was effectively taken up and converted into metabolic active folates by 5-formyltetrahydrofolate cycloligase (5-FCL, Goyer et al., 2005, Fig. 1). This conversion occurred quite efficiently because 5-CHO-THF represented less than 18% of the accumulated folates with 5-CH_3-THF being the most predominant folate class in the total folate pool (Supplemental Fig. S5D). Thus, the recovery of root growth in *atdfb* treated with 5-CHO-THF was most likely due to the excess of folates in the monoglutamylated form, which might be able to complement the mutation by accomplishing the same functions as a small amount of polyglutamlylated folates. Additionally, these results demonstrate the high capacity of the seedling to metabolize and accumulate large amounts of folates without apparent negative effects.

The depleted levels of amino acids and nucleotides are likely contributors to the defective root growth of *atdfb*. However, because metabolic profiling was done on whole seedlings, only part of the reported metabolic changes in the mutant can be attributed to folate polyglutamylation defects in roots. Methionine in particular is an essential amino acid required for protein synthesis and formation of S-adenosylmethionine (AdoMet), the universal methyl donor in the methylation cycle (Ravanel et al., 2004; Rébeillé et al., 2007). *De novo* methionine biosynthesis in plants is known to occur in plastids and a critical step in the formation of this amino acid is the methylation of homocysteine, which is catalyzed by a plastid localized methionine synthase (Ravanel et al., 2004). The C1 unit transferred to homocysteine is donated by 5-CH_3-THF to form methionine (Rébeillé et al., 2006; Fig. 1). Because methionine synthase cannot efficiently utilize monoglutamate forms of 5-CH_3-THF (Ravanel et al., 2004), a substantial amount of the folate pool in the mutant (Fig. 9) cannot be used for methionine biosynthesis. Indeed, metabolic profiling showed that endogenous methionine levels were lower in *atdfb* seedlings compared to wild type (Fig. 10). A large percentage of the methionine flux goes to AdoMet synthesis, which is involved in methylation reactions for the metabolism of nucleic acids, proteins, lipids and various secondary metabolites. Thus, the lower levels of methionine in *atdfb* resulting from reduced FPGS activity could explain the alterations in a large number of cellular metabolites (Supplemental Table S2 and S3), and the cytoskeletal, cell division, and cell expansion defects in roots, as these processes are highly dependent on products of cellular methylation reactions. This notion is supported by the observation that exogenous application of methionine caused a partial restoration of root growth in *atdfb* (Supplemental Fig. S6).

The reduced levels of adenine and guanosine that may result from inadequate 10-CHO-THF polyglutamates, could also contribute to defective *atdfb* root growth given their important role as building blocks of DNA and as major energy donors in numerous metabolic reactions of the cell (Zrenner et al., 2006). However, only guanosine caused a promotion in root length of *atdfb*. It is possible that certain metabolites that are applied exogenously including methionine and guanosine, which were only able to partially restore root growth in the mutant, cannot reach a population of cells such as the QC where they are needed most. Alternatively, the applied compounds could be rapidly metabolized into forms that the cell cannot utilize for restoring complete biochemical function. Moreover, a combination of depleted levels of other metabolites combined with the toxic accumulation of others (e.g. adenosine) likely defines the overall root defects of *atdfb*. A more targeted analysis of metabolites within a defined population of cells particularly those that participate in C1 transfer reactions such as AdoMet and homocysteine will be needed to fully understand how changes in folate glutamylation, impact root development.

In conclusion, we provide genetic evidence that AtDFB plays a pivotal role in postembryonic primary root development in Arabidopsis. Consequences of mutating the *AtDFB* gene include the accumulation of monoglutamylated folates, broad alterations seedling metabolite levels, and various root cell defects that could be attributed in part to the loss of QC identity. Taken together, our results indicate that although redundancy in FPGS function is operating for certain plant developmental processes (Mehrshahi et al., 2010), a single FPGS isoform can play a critical role in the metabolism of a specific cell population to influence root growth within a defined window of development.

MATERIALS AND METHODS

Isolation of *atdfb* mutants

atdfb-1 (originally designated as *drh2 for defective root hair 2*; Supplemental Fig. S1) was identified through a forward genetic screen for mutants with abnormal root hairs (Yoo et al., 2008). Screening was done on a population of Arabidopsis (*Arabidopsis thaliana*) Col-0 Transfer DNA (T-DNA) mutagenized seed stock (CS31100) from the Arabidopsis Biological Resource Center (ABRC). *atdfb-1* was backcrossed to Col-0 at least two times to remove extraneous mutations. The location of T-DNA in the mutant was determined by thermal asymmetric interlaced (TAIL)-polymerase chain reaction (TAIL-PCR; Liu et al., 1995). Two additional lines with T-DNA insertions at the *AtDFB* locus (SALK_015472, and SAIL_556_G08) were obtained from the ABRC (Alonso et al., 2003). These lines were renamed *atdfb-2* and *atdfb-3* respectively after confirming the T-DNA insertion site and observing that the root phenotypes of these additional T-DNA lines were similar to *atdfb-1* (Fig. 3). Mutants to the *AtDFC* (SALK_008883) and *AtDFD* (SAIL_151_E09) gene were also obtained from ABRC and genotyping of these lines was essentially as described above (Supplemental Fig. S3).

Phenotypic Characterization of *atdfb*

For phenotypic analyses, seeds of wild type (Col-0) and mutants were surface-sterilized with 95% ethanol (v/v), 20% bleach (v/v) and washed extensively with sterile water before germinating these seeds on solid ½ Murashige and Skoog (MS) medium (pH 5.7) containing 1% (w/v) sucrose, phytagar (0.5-1 %), vitamins- pyridoxine-HCL and nicotinic acid (0.5 mg/mL), and thiamine (1 mg/mL). Plates were incubated vertically in a controlled-environment chamber at 120 to 140 μmol photons m^2/s, 14 hours of light at 22°C, and 10 hours of dark at 19°C. Digital photographs of primary roots and root hairs at various stages of seedling development were acquired, and their lengths measured using Image J.

For 5-CHO-THF [(6S)-5-formyl-5, 6, 7, 8-tetrahydrofolic acid, calcium salt; natural calcium folinate; Schircks Laboratories, Jona, Switzerland], methionine, adenine and guanosine (Sigma) supplementation experiments, stock solutions were applied to the growth medium to achieve the desired working concentration. Briefly, adenine and guanosine were dissolved in 1 ml deionized water with 2-3 drops of 1 M KOH. From this initial solution, a 10 mM stock solution was made by bringing the final volume to 10 ml with deionized water. For 5-CHO-THF and methionine, a stock solution of 10 mM was made using deionized water. Seeds were either planted directly or seedlings transplanted on the growth media and root growth assays were performed as described earlier.

Transcript Analysis and Whole Mount *in situ* Hybridization

Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) and reverse transcribed using a first strand cDNA kit with Oligodt^{20} and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). For semiquantitative PCR, first strand cDNA was amplified with Platinum Taq High Fidelity (Invitrogen). Cycling parameters were 94°C for 10 s, 60°C for 15 s, and 72°C for 50 s.

For quantitative two-step RT-PCR of *AtDFB* and *AGAMOUS-LIKE 42* (*AGL42*), 1 μg of total RNA from wild type and *atdfb-1* was reverse-transcribed to first-strand cDNA with the Qiagen cDNA synthesis kit (Qiagen). First-strand cDNA was used as a template for quantitative PCR using gene-specific primers. *Arabidopsis eukaryotic protein synthesis initiation factor 4A2* (*EIF4A2)* served as a control for constitutive gene expression in plants. Primers used are shown in Supplemental Table S4. Relative expression levels ($2^{-\Delta Ct}$) were calculated according to Ramakers et al., (2003). Expression levels of *AtDFB* were calculated based on the relative level of *EIF4A2* expression in each sample. Values are the means of three biological with three technical replicates for each.

Four day old seedlings were processed for *in situ* hybridization essentially as described by Hejátko et al., (2006) using *AtDFB* specific probes (Supplemental Table S4) Roots were examined with a Nikon Microphot FX compound microscope and images were acquired using a Nikon DXM 1200 camera running on ACT-1 software (Nikon Instruments, Melville, NY).

Cell biological Studies of Roots

The *atdfb-1* mutants were transformed with a green fluorescent protein (GFP) construct that binds to F-actin (GFP-ABD2-GFP; Wang et al., 2008) and our independently generated *DR5:GFPm*. The *DR5* auxin responsive element was a gift from Dr. Tom Guilfoyle, University of Missouri; Ulmasov et al., 1997). Seeds of wild type and *atdfb-1* expressing GFP-ABD2-GFP and *DR5:GFPm* were germinated on 64 x 48 mm cover glasses coated with growth media as described in Wang et al. (2008). Plates were incubated vertically for 4-8 d at 21^{o}C with a 14 h photoperiod. Primary roots of wild type and *atdfb-1* seedlings expressing the reporters were imaged with a Leica TCS SP2 AOBS confocal laser-scanning microscope. GFP was excited using the 488-nm line of the argon laser and emission was detected at 510 nm. QC organization was evaluated in confocal median longitudinal sections of roots stained for 5 minutes with 10 μM propidium iodide. In a parallel set of experiments, mature embryos from wild type and *atdfb-1* were dissected from seeds imbibed for 24 hours in water and the QC was imaged in aniline blue-stained embryos as described in Bougourd et al. (2000).

For evaluating mitosis, 4 d old seedlings were fixed in 1x PME (50 mM Pipes, 2 mM $MgCl_2$ and 10 mM EGTA; pH 7.0) buffer containing 2% formaldehyde and 0.5% glutaraldehyde as previously described (Heslop-Harrison, 1998). After repeated washing with PEMT (PEM+0.1% Triton X-100), fixed seedlings were digested with an enzyme solution containing 100 mg/ml Cellulase (Sigma) dissolved in 20 ml PEMT along with 1.5 g mannitol at room temperature for 20 min. After repeated rinsing in PME, roots were stained with stained with 4',6-diamidino-2-phenylindole (DAPI; 1:10 000 stock dilution) for 2 h.. DAPI was excited using a 405 blue diode laser and emission detected at 458 nm using a confocal microscope. The number of mitotic cells from the apical 100 μm of the root apex was counted from projected images of 26 confocal optical sections taken at 1 μm intervals.

To visualize amyloplasts, 7 day old seedlings were fixed in 4% formaldehyde and stained with potassium iodide (Lugol's solution) as described by Willemsen et al. (1998).

Folate Analysis

Shoots and roots from 15 day old wild type and mutant seedlings were analyzed for folates from one gram total tissue by high performance liquid chromatography (HPLC) as described previously (Goyer et al., 2005; Orsomando et al., 2005) with slight modifications: half of the folate extract was deglutamylated using a purified mixture of Arabidopsis recombinant gamma-glutamyl hydrolase (GGH) isoforms one and two (Orsomando et al., 2005) (0.1 mg each protein per gram of tissue, with 2 hour incubation time). The rest of the extract was used for profiling folate polyglutamylation. Purified extracts were separated by HPLC using an Atlantis dC18 column (150 x 4.6 mm, 5μm Waters, Milford, MA) and analyzed by a four-channel electrochemical detector (CoulArray Model 5600A, ESA, Chelmsford, MA) with potentials set at 100, 200, 300, and 400 mV. Detector response was calibrated using tetrahydrofolate (THF), 5-methyl-THF (5-CH_3-THF), 5-formyl-THF (5-CHO-THF), 5,10-methylene-THF standards (Schricks Labs, Jona, Switzerland).

Metabolite Analyses by Gas Chromatography-Mass Spectrometry

Metabolite analyses were carried out using a gas chromatography-mass spectrometry (GC-MS)-based method modified from Broeckling et al. (2005). Briefly, 7 day old seedlings were lyophilized, homogenized, vortexed and then incubated for 45 minutes at 50°C in glass vials after adding chloroform containing 10 μg/mL docosanol (internal standard). After samples equilibrated to room temperature, 1.5 mL of HPLC-grade water containing 25 μg/mL ribitol was added and samples were incubated for another 45 min at 50^{o}C. The biphasic solvent mixture was then centrifuged at 2900 g for 30 min. One mL of each layer was transferred to individual autosampler vials and dried under nitrogen for the nonpolar/chloroform aliquots, and in a vacuum centrifuge for polar/aqueous aliquots. The dried non-polar extracts were resuspended in chloroform and trans-esterified by adding HCl in methanol. Following trans-esterification for 4 h at 50 °C, samples were evaporated under nitrogen, resuspended in pyridine and derivatized using (MSTFA)+1%TMCS (Pierce Biotechnology, Rockford, IL, USA) for 1 h at 50 °C. The samples were equilibrated to room temperature, transferred to glass inserts, and analyzed by GC-MS.

The dried polar extracts were methoximated in pyridine with methoxyamine–HCl, briefly sonicated, and incubated for 1h at 50 °C. Metabolites were then derivatized with MSTFA+1% TMCS for 1 h at 50 °C and analyzed by GC-MS. The GC system used was an Agilent 6890 coupled to a 5973 MSD quadrupole mass spectrometer scanning from m/z 50–650. Acquired mass spectra were deconvoluted using AMDIS software, and metabolite identifications were achieved by mass spectral matching to the Noble Foundation's in-house EIMS spectral library of authentic compounds, the publicly available GOLM library (http://csbdb.mpimp-golm.mpg.de/csbdb/dbma/msri.html), and the NIST08 library. Peak selection and alignment were performed using MET-IDEA software (Broeckling et al., 2006). The area of each peak was normalized against the area of the internal standard, and absolute quantification for selected metabolites was achieved using authentic standard calibration curves.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AtDFB (At) NP_196217; At5g05980.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *drh2 (atdfb-1)* has short and wavy root hairs.

Supplemental Figure S2. *AtDFB* knockouts have no obvious defects in shoot development when compared to wild type.

Supplemental Figure S3. Identification of mutants to *AtDFC* and *AtDFD*.

Supplemental Figure S4. *AtDFB* is more strongly expressed in roots compared to *AtDFC* and *AtDFD*.

Supplemental Figure S5. 5-formyl tetrahydrofolate (5-CHO-THF) rescues the cell division and root hair phenotypes of *atdfb*.

Supplemental Figure S6. Exogenous methionine and guanosine promotes root growth in *atdfb* seedlings.

Supplemental Table S1. Total folate content in shoots and roots of 15 day old Arabidopsis seedlings.

Supplemental Table S2. Polar metabolites from 7 day old wild-type and *atdfb-1* seedlings.

Supplemental Table S3. Non-polar metabolites from 7 day old wild-type and *atdfb-1* seedlings.

Supplemental Table S4. Primer sequences used for genotyping and gene expression analysis.

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Figure legends

Figure 1. Schematic representation of tetrahydrofolate production and one carbon metabolism and their distribution in different compartments of the plant cell (adapted from Ravanel et al., 2001; Loizeau et al., 2008). Arrows and numbers represent enzymatic reactions. Dashed arrows and gray squares represent folate transport and transporters respectively. 1. Dihydrofolate reductase/thymidylate synthase; 2. Folylpolyglutamate synthetase (AtDFB, AtDFC and AtDFD); 3. Serine hydroxymethyltransferase; 4. Glycine decarboxylase; 5. 5,10-Methylene-THF dehydrogenase/5,10-Methenyl-THF cyclohydrolase; 6. 10-Formyl-THF deformylase; 7. 5-Formyl-THF cycloligase; 8. Methionyl-tRNA formyltransferase; 9. Methionine synthase; 10. Glycinamide ribonucleotide (GAR) transformylase and aminoimidazole carboxamide ribonucleotide (AICAR) transformylase; 11. 5,10-Methylene-THF reductase; 12. *S*-adenosylmethionine synthetase; 13. Methionine *S*-methyltransferase; 14. *S*-adenosylhomocysteine hydrolase; 15. Ketopantoate hydroxymethyltransferase; 16. 10-Formyl-THF synthetase. Abbreviations used: standard abbreviations used for amino acids (Glu, Ser, Gly, Met, Hcy); THF tetrahydrofolate ; THF-Glu_n tetrahydropteroylpolyglutamate; THF and its derivatives: CH_3- (methyl), CH_2- (methylene), CH=(methenyl), 5-CHO-and 10-CHO- (formyl).

Figure 2. Primary root defects of *drh2*. A, 7 day old wild type and mutant seedlings. The primary root length of the mutant is about 4-fold less than that of wild type (arrowheads). The shorter primary root of *drh2* was due to a reduced growth zone as indicated by the emergence of root hairs closer to the root tip compared to wild type (white vertical bars; inset in A). B, In 11 day old seedlings, differences in root architecture between wild type and mutant become more apparent. In addition to the short primary root, *drh2* seedlings had about 2 adventitious roots (arrowheads, inset in B) while wild type seedlings had none. C, At 16 days, *drh2* primary roots started to elongate faster compared to the earlier stages of seedling development but were still significantly shorter than wild type. Bars = 200 µm (for insets in A and B).

Figure 3. A, Schematic diagram of the genome organization of *AtDFB. drh2* had a T-DNA insertion in the 5th intron of the *At5g05980* gene, which encodes AtDFB. Boxes indicate exons and lines indicate introns. The T-DNA insertion sites for each of the mutant alleles are indicated.

B, 12 day old seedlings of wild type, *atdfb-1*, *atdfb-2*, and *atdfb-3*. Note the severely reduced primary root length of all mutant alleles. C, Semi quatitative RT-PCR of *AtDFB* in wild type (WT), *atdfb-1*, *atdfb-2*, and *atdfb-3*. Total RNA was prepared from 7 day old seedlings and arrows in A indicate the positions of the primers used for RT-PCR. *EIF4A2* primers were used as a control.

Figure 4. Expression of *AtDFB* in 7 day old wild type Arabidopsis seedlings revealed by qRT-PCR and whole mount RNA *in situ* hybridization. A, qRT-PCR shows that *AtDFB* is more strongly expressed in roots compared to shoots. Values are means ± SE of 3 independent biological replicates (each replicate had 40 seedlings for RNA extraction). The asterisk indicates statistically significant differences according to a Student's t-test ($p<0.05$). *AtDFB* transcript is visible as a purple signal in brightfield images of the root apex hybridized with antisense but not with the sense probes (B, C). Note the strong purple staining in the quiescent center (QC). A purple precipitate was also observed in vascular tissues of the cotyledons and hypocotyls, and the first true leaves of 7 d old seedlings hybridized with antisense probes (arrows, D). Bars = 100 µm (B, D); 25 µm (C).

Figure 5. Cell expansion, cell division and F-actin organization are altered in roots of *atdfb-1*. Epidermal cells of wild type roots in the maturation zone (A) are longer than those of *atdfb* (B). Black vertical bars mark the end walls of a representative epidermal cell. C, Quantification of epidermal cell length in the maturation zone shows that wild type cells are more than 2-fold longer than mutant cells. Values are means ± SE of 40 epidermal cells from 10 roots. The actin cytoskeleton in the elongation zone (D, E) and meristem (F, G) of wild type and *atdfb-1*. Note the bundling of F-actin in mutant root cells (arrows in E and G). Meristem region of wild type roots are enriched in F-actin labeled phragmoplasts (arrowheads, F) but absent in *atdfb-1* (G). H, Number of mitotic cells is significantly reduced in roots of *atdfb* compared to wild type. Values are means ± SE of 5 root tips. Asterisks in C and H indicate statistically significant differences according to a Student's t-test ($p<0.01$). Bars = 20 µm (A, B); 10 µm (D-G).

Figure 6. The quiescent center (QC) is disorganized in postembryonic roots of *atdfb*. A, Median confocal image of a 5 day old propidium iodide stained wild type root clearly shows a well organized QC (arrows). B, In *atdfb-1* roots, cells in the region corresponding to the QC have a disorganized pattern (arrows). In mature embryos (C,D) and roots from 2 day old seedlings (E,F), QC organization in wild type (C, E) appears similar to *atdfb-1* (D, F). Bars= 10 µm.

Figure 7. *DR5:GFPm* expression and quiescent center (QC) identity are altered in *atdfb* roots. A, Root cap of an 8 day old wild type seedling shows *DR5:GFPm* expression in the QC and central columella cells (CC). B, In 8 day old *atdfb-1* seedlings, *DR5:GFPm* expression is confined to a few columella cells (arrows). Note the disorganized QC region in *atdfb-1* (asterisks). C, More cells in the root cap express *DR5:GFPm* in wild type seedlings compared to *atdfb-1*.Values are means from 15 roots ± SE. Asterisk indicates statistically significant difference (Student's t-test, $p<0.01$). D, Purple- stained amyloplasts are absent in columella initials (CI) of wild type seedlings. E, In roots of *atdfb-1*, amyloplasts (arrows) prematurely form close to the disorganized cells in the QC region (asterisks). F, qRT-PCR shows higher expression of the QC-expressed gene *AGL42* in 8 day old roots of wild type compared to *atdfb-1*. Values are means ± SE of 5 biological replicates. The asterisk indicates statistically significant differences according to a Student's t-test ($p<0.05$). Bars = 10 µm.

Figure 8. Exogenous 5-formyl tetrahydrofolate (5-CHO-THF) rescues the short primary root phenotype of *atdfb*. 7 day old seedlings of wild type and *atdfb* germinated in solvent control and 500 µM 5-CHO-THF Note that the primary root length of *atdfb* is restored to wild type (A, B). Data are means from 18 to 25 roots ± SE. Means with different letters are statistically significant (Tukey's test, $p < 0.05$). C, QC organization in *atdfb* is also restored to wild type patterns when grown on 500 µM 5-CHO-THF.

Figure 9. Folate glutamylation profile in shoots and roots of 15 day old Arabidopsis seedlings. Each folate class and total folates are presented as monoglutamates (A, B) or polyglutamates (C, D). Monoglutamyl contribution to each folate class was calculated by subtracting deglutamylated values from monoglutamyl quantification in polygutamylation profile analysis. Data are

means±SE of three independent preparations. Asterisks indicate statistically significant differences (Student's t-test, $p<0.05$*, $p<0.001$**). 5-CH3-THF (5-methyl-THF);10-CHO-THF(10-formyl-THF); 5,10-CH_2-THF (5,10-methylene THF) ; 5,10-CH=THF (5,10-methenyl-THF); 5-CHO-THF (5-formyl-THF).

Figure 10. Metabolite profiling in whole seedlings of wild type and *atdfb-1*. A, Heat map visualization of nucleotides and amino acids in 7 day old seedlings of *atdfb-1* and wild type (WT). Each biological replicate is visualized in a single column (average of n = 3). Red indicates high abundance, whereas metabolites in low abundance are in green. Alterations in mean amino acid levels (B, C) and mean nucleic acids (D, E) (Student's t-test, $p < 0.05$*) of *atdfb-1* compared to wild-type. For visual clarity, amino acids and nucleotides are presented in two groups based on their relative values.

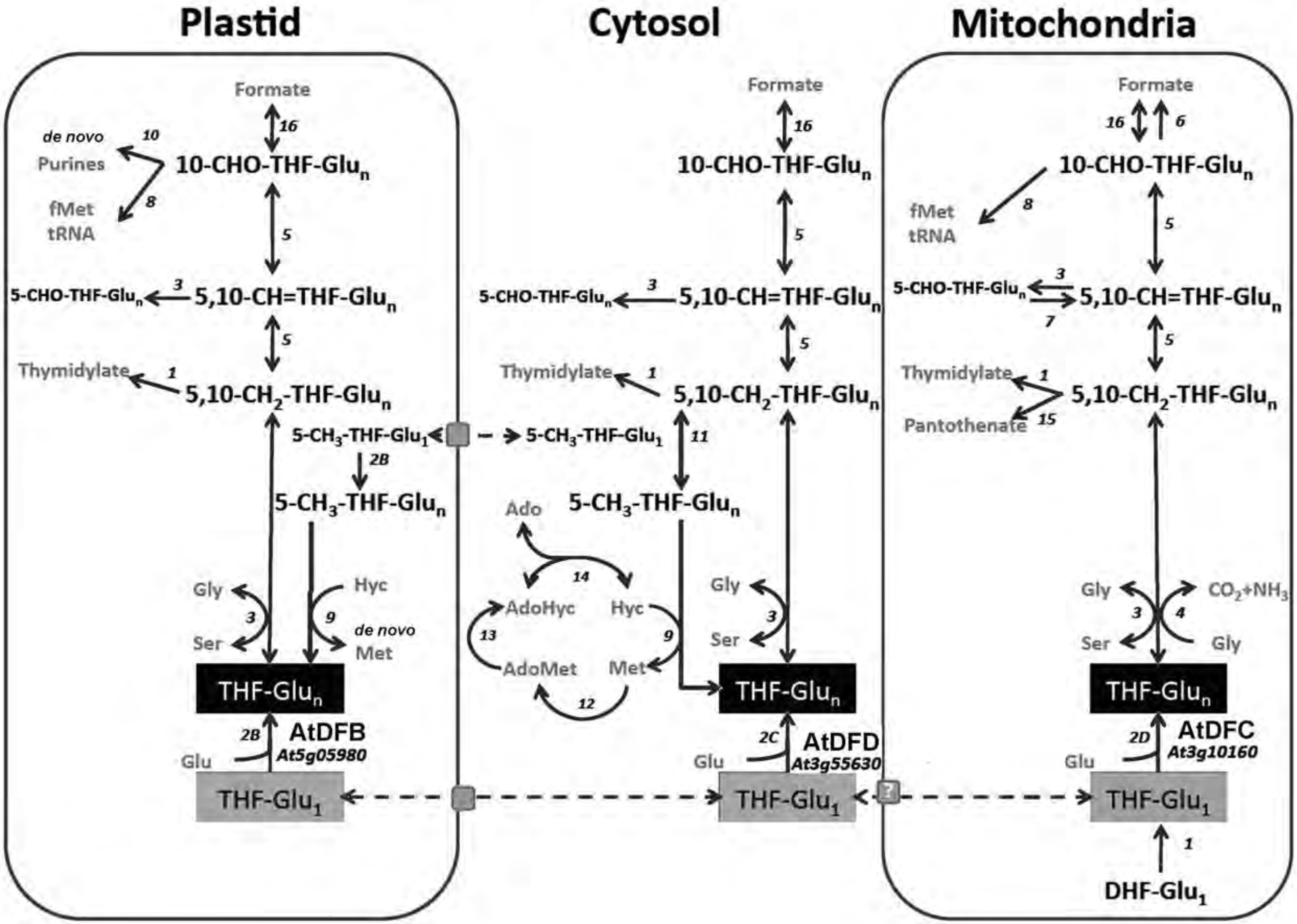
Supplemental Table Legends:

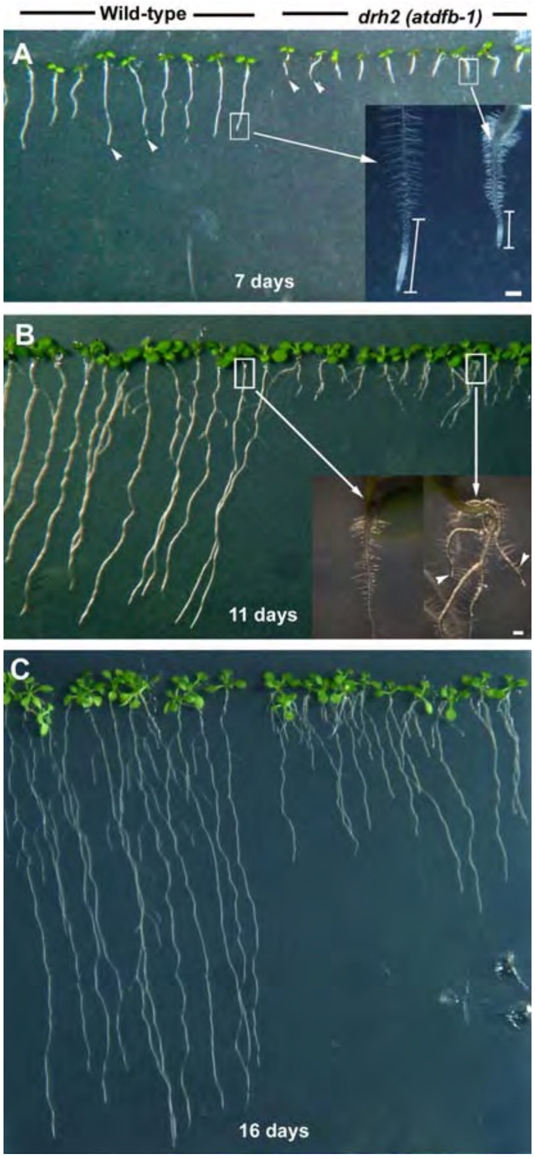
Supplemental Table S1. Total folate content in shoots and roots of 15 day old Arabidopsis seedlings. Values are means ± SE from three independent experiments. Values in bold represent statistically significant differences when wild type and *atdfb-1* where compared (student's t-test $\alpha=0.05$, $p \leq 0.01$*). Values in parenthesis are the percentage of each folate class related to total folate content.

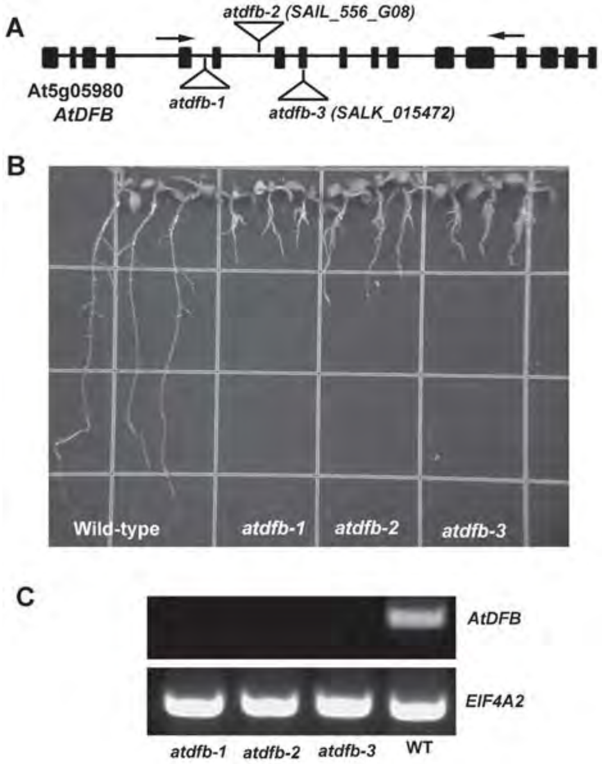
Supplemental Table S2. Polar metabolites from 7 day old wild type and *atdfb-1* seedlings. Rows that are highlighted indicate metabolites that are significantly different between wild type and *atdfb-1* (Student's t-test; $p<0.05$).

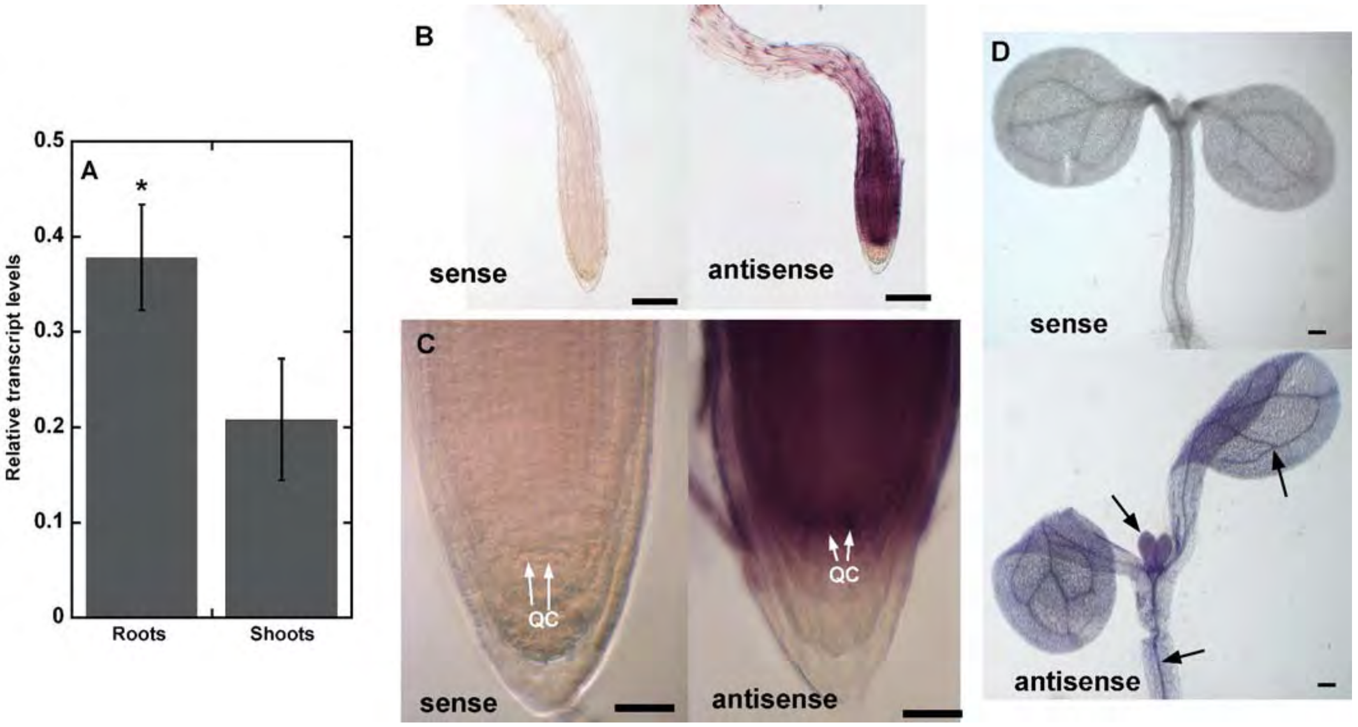
Supplemental Table S3. Non-polar metabolites from 7 day old wild type and *atdfb-1* seedlings. Rows that are highlighted indicate metabolites that are significantly different between wild type and *atdfb-1* (Student's t-test; $p<0.05$).

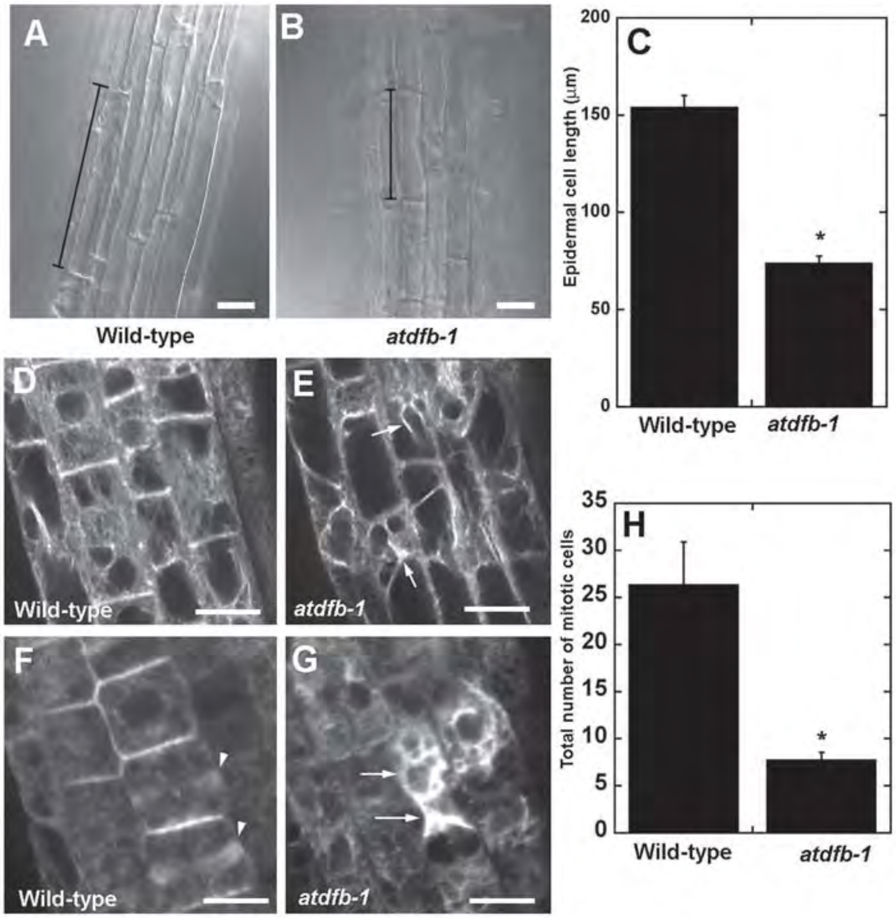
Supplemental Table S4. List of primers used for experiments.

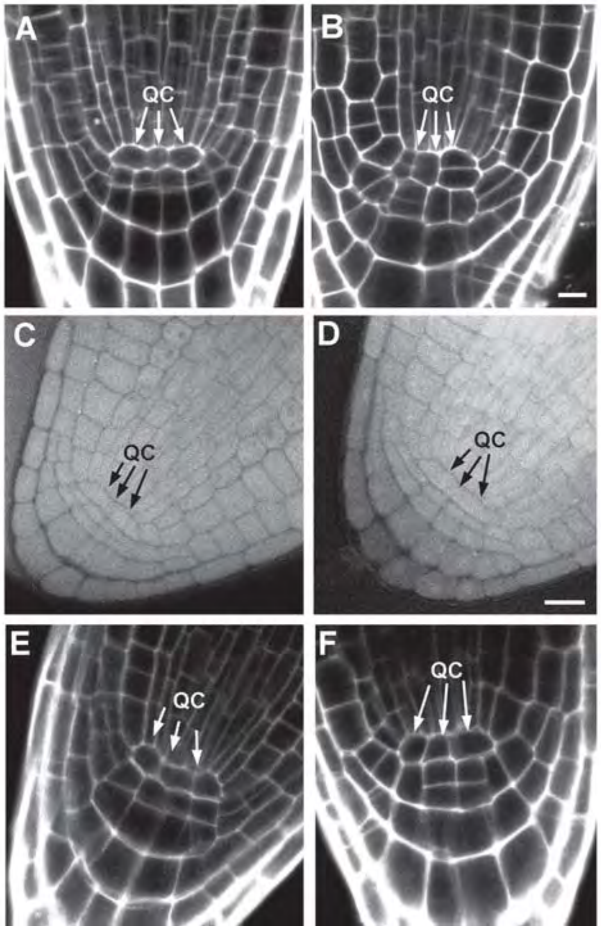

Plastid
Cytosol
Mitochondria
Formate
16
10
de novo
Purines
8
fMet
tRNA
10-CHO-THF-Glu$_n$
5
3
5-CHO-THF-Glu$_n$
5,10-CH=THF-Glu$_n$
5
1
Thymidylate
5,10-CH$_2$-THF-Glu$_n$
5-CH$_3$-THF-Glu$_1$
2B
5-CH$_3$-THF-Glu$_n$
Gly
3
Ser
Hyc
9
de novo
Met
THF-Glu$_n$
2B
AtDFB
At5g05980
Glu
THF-Glu$_1$
Formate
16
10-CHO-THF-Glu$_n$
5
3
5-CHO-THF-Glu$_n$
5,10-CH=THF-Glu$_n$
5
1
Thymidylate
5,10-CH$_2$-THF-Glu$_n$
5-CH$_3$-THF-Glu$_1$
11
5-CH$_3$-THF-Glu$_n$
Ado
14
AdoHyc
Hyc
13
9
AdoMet
Met
12
Gly
3
Ser
THF-Glu$_n$
2C
AtDFD
At3g55630
Glu
THF-Glu$_1$
?
Formate
16
6
10-CHO-THF-Glu$_n$
8
fMet
tRNA
5
3
5-CHO-THF-Glu$_n$
7
5,10-CH=THF-Glu$_n$
5
Thymidylate
1
Pantothenate
15
5,10-CH$_2$-THF-Glu$_n$
Gly
3
4
CO$_2$+NH$_3$
Ser
Gly
THF-Glu$_n$
2D
AtDFC
At3g10160
Glu
THF-Glu$_1$
1
DHF-Glu$_1$

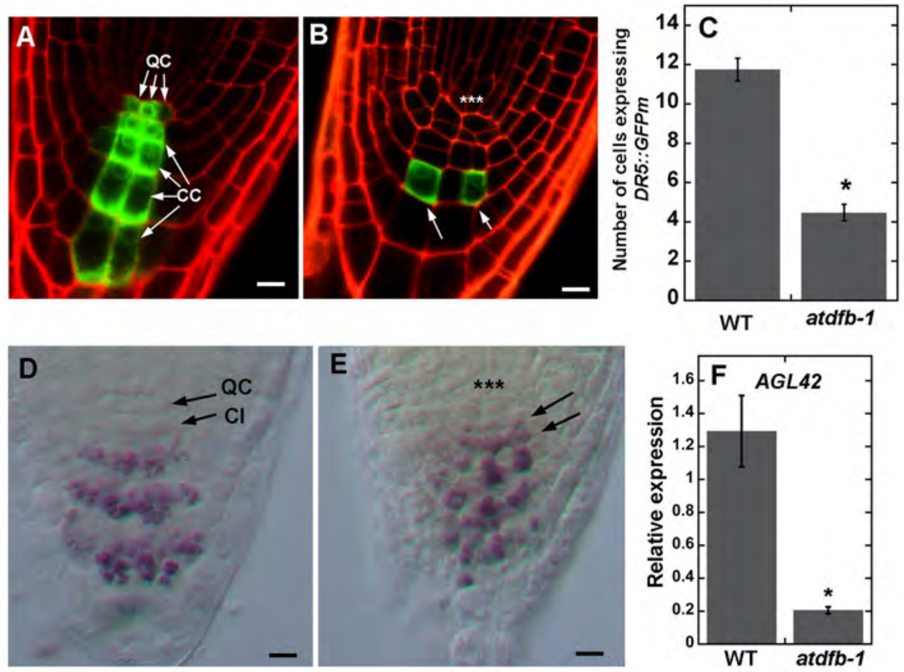

Wild-type
drh2 (atdfb-1)
A
7 days
B
11 days
C
16 days

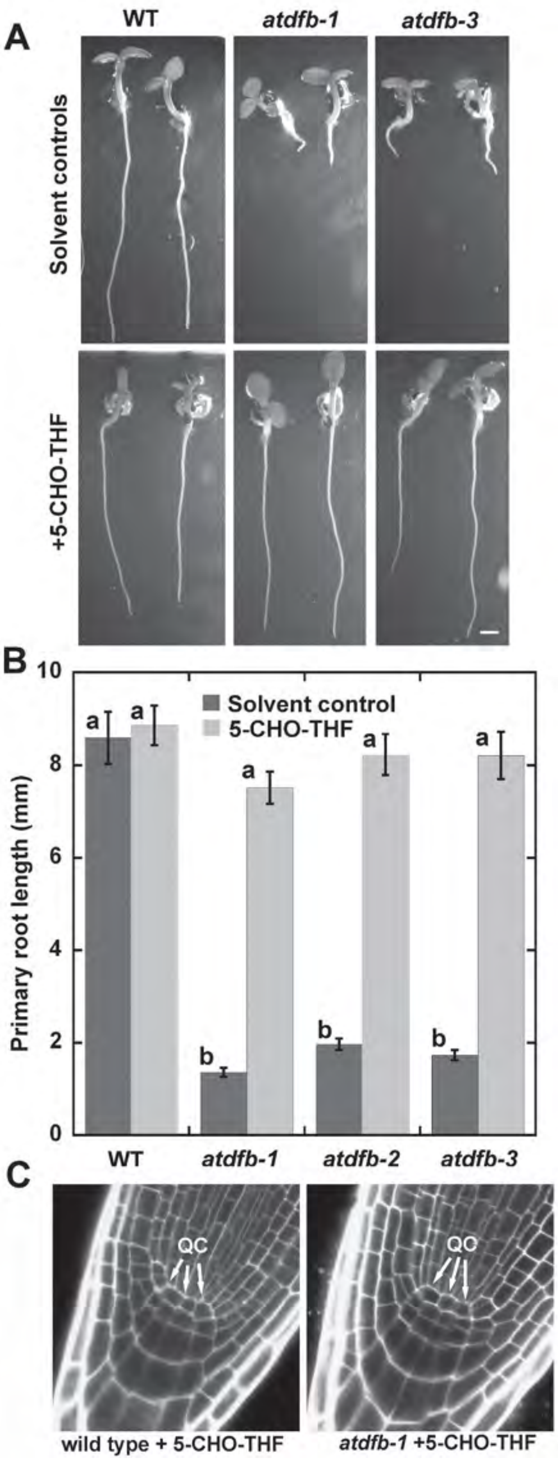

A
atdfb-2 (SAIL_556_G08)
At5g05980
AtDFB
atdfb-1
atdfb-3 (SALK_015472)
B
Wild-type
atdfb-1
atdfb-2
atdfb-3
C
AtDFB
EIF4A2
atdfb-1
atdfb-2
atdfb-3
WT

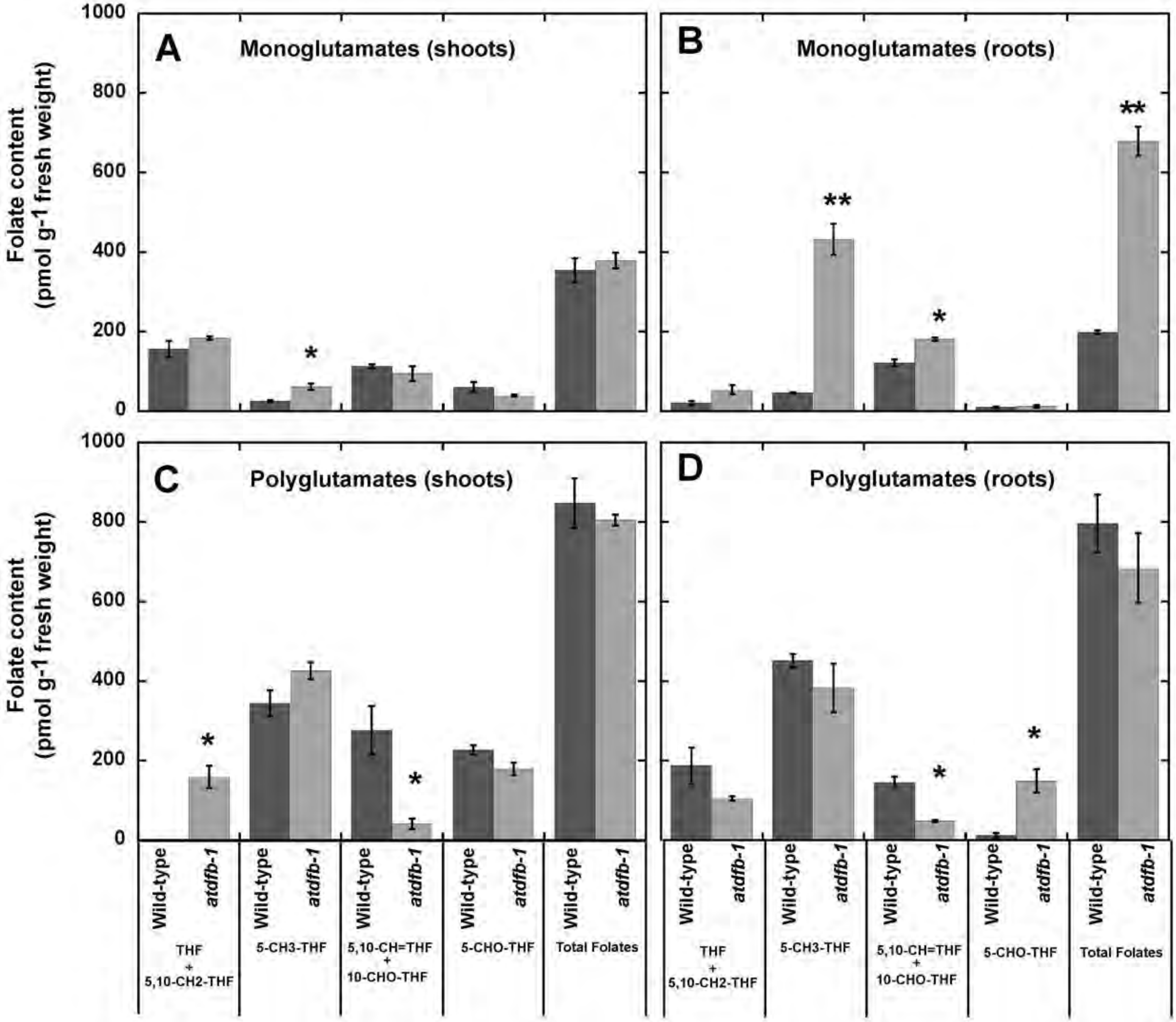

A
Relative transcript levels
0.5
0.4
0.3
0.2
0.1
0
*
Roots
Shoots
B
sense
antisense
C
QC
sense
QC
antisense
D
sense
antisense

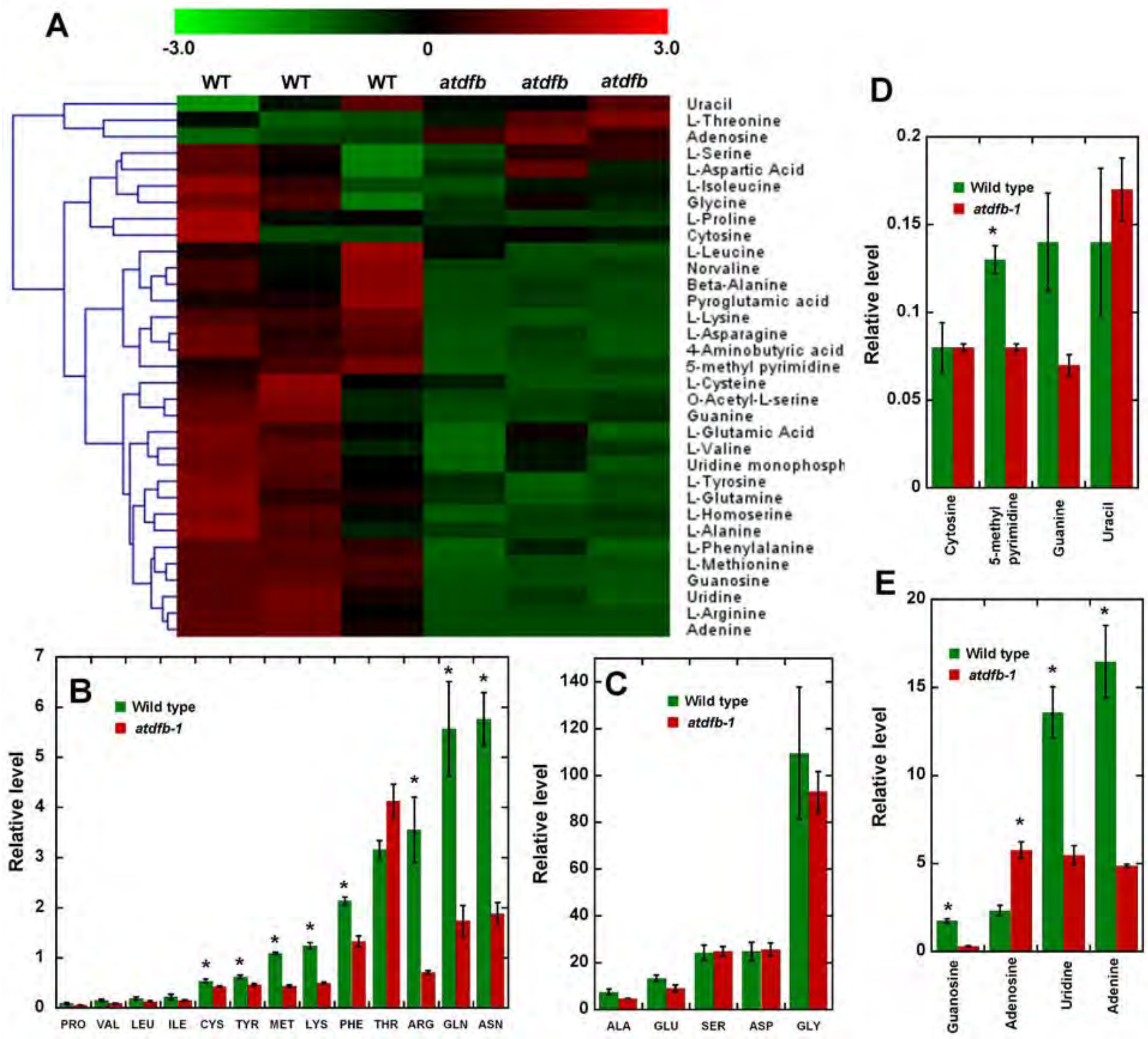

A
B
Wild-type
atdfb-1
C
Epidermal cell length (μm)
200
150
100
50
0
*
Wild-type
atdfb-1
D
Wild-type
E
atdfb-1
F
Wild-type
G
atdfb-1
H
Total number of mitotic cells
35
30
25
20
15
10
5
0
*
Wild-type
atdfb-1


A
B
C
D
E
F
QC
QC
QC
QC
QC
QC


A
QC
CC
B
***
C
Number of cells expressing DR5::GFPm
14
12
10
8
6
4
2
0
*
WT
atdfb-1
D
QC
CI
E
***
F
AGL42
Relative expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WT
atdfb-1


A
WT
atdfb-1
atdfb-3
Solvent controls
+5-CHO-THF
B
Solvent control
5-CHO-THF
Primary root length (mm)
0
2
4
6
8
10
a
a
b
a
b
a
b
a
WT
atdfb-1
atdfb-2
atdfb-3
C
QC
QC
wild type + 5-CHO-THF
atdfb-1 +5-CHO-THF


A
Monoglutamates (shoots)
B
Monoglutamates (roots)
C
Polyglutamates (shoots)
D
Polyglutamates (roots)
Folate content
(pmol g-1 fresh weight)
0
200
400
600
800
1000
Wild-type
atdfb-1
THF + 5,10-CH2-THF
5-CH3-THF
5,10-CH=THF + 10-CHO-THF
5-CHO-THF
Total Folates
*
**


A
-3.0
0
3.0
WT
WT
WT
atdfb
atdfb
atdfb
Uracil
L-Threonine
Adenosine
L-Serine
L-Aspartic Acid
L-Isoleucine
Glycine
L-Proline
Cytosine
L-Leucine
Norvaline
Beta-Alanine
Pyroglutamic acid
L-Lysine
L-Asparagine
4-Aminobutyric acid
5-methyl pyrimidine
L-Cysteine
O-Acetyl-L-serine
Guanine
L-Glutamic Acid
L-Valine
Uridine monophosph
L-Tyrosine
L-Glutamine
L-Homoserine
L-Alanine
L-Phenylalanine
L-Methionine
Guanosine
Uridine
L-Arginine
Adenine
B
Wild type
atdfb-1
Relative level
0
1
2
3
4
5
6
7
PRO
VAL
LEU
ILE
CYS
TYR
MET
LYS
PHE
THR
ARG
GLN
ASN
C
Wild type
atdfb-1
Relative level
0
20
40
60
80
100
120
140
ALA
GLU
SER
ASP
GLY
D
Wild type
atdfb-1
Relative level
0
0.05
0.1
0.15
0.2
Cytosine
5-methyl pyrimidine
Guanine
Uracil
E
Wild type
atdfb-1
Relative level
0
5
10
15
20
Guanosine
Adenosine
Uridine
Adenine