INTRODUCTION

All life forms have an absolute requirement for vitamin B₁. Whereas microorganisms and plants have the capacity to biosynthesize this vitamin de novo, animals including humans must obtain it through dietary means. In humans, a nutritional deficiency in vitamin B1 can have major negative impacts on the nervous system including peripheral polyneuritis or irreversible lesions in the mid-brain (Haas, 1988). While the association of vitamin B1 deficiencies with disease has been well documented, vitamin B₁ supplementation has been shown to have substantial beneficial effects with regard to neurodegenerative diseases including Alzheimer’s and Parkinson’s (Luong & Nguyen, 2013, Gibson et al., 2016). In plants, a deficiency in vitamin B₁ also impacts general fitness, leading to chlorosis, and general deterioration (Papini-Terzi et al., 2003, Ajjawi et al., 2007, Raschke et al., 2007). Such symptoms can be rescued by supplementation. However, too much vitamin B₁ can also negatively impact plant fitness (Bocobza et al., 2013, Rosado-Souza et al., 2019). Therefore, it is important to monitor vitamin B₁ levels and ensure that supply meets demand such that the right balance is achieved, i.e. vitamin B₁ homeostasis.

In this context, it is important to realize that vitamin B₁ is a family of molecules, the most renowned member of which is diphosphorylated thiamine (TDP)—a coenzyme vital for the activity of key enzymes of energy metabolism. Triphosphorylated thiamine derivatives also exist within this family, specifically thiamine triphosphate (TTP) and adenosine thiamine triphosphate (ATTP). They have been investigated primarily in mammalian cells and are thought to act as metabolic messengers but have not received much attention in plants. In this study, we set out to examine for the presence of these triphosphorylated thiamine derivatives in Arabidopsis. We could find TTP in Arabidopsis under standard growth conditions, but we could not detect ATTP. Interestingly, TTP is found primarily in shoot tissue. Drivers of TTP synthesis are light intensity, the proton motive force, as well as TDP content. In plants, TTP accumulates in the organelar powerhouses, the plastids, and mitochondria. Furthermore, in contrast to other B₁ vitamers, there are strong oscillations in tissue levels of TTP levels over diel periods peaking early during the light period. The elevation of TTP levels during the day appears to be coupled to a photosynthesis-driven process. We propose that TTP may signify TDP sufficiency, particularly in the organelar powerhouses, and discuss our findings in relation to its role.

KEYWORDS
Arabidopsis, metabolism, plastids, proton motive force, thiamine phosphates, vitamin B₁

Abstract

Vitamin B₁ is a family of molecules, the most renowned member of which is diphosphorylated thiamine (TDP)—a coenzyme vital for the activity of key enzymes of energy metabolism. Triphosphorylated thiamine derivatives also exist within this family, specifically thiamine triphosphate (TTP) and adenosine thiamine triphosphate (ATTP). They have been investigated primarily in mammalian cells and are thought to act as metabolic messengers but have not received much attention in plants. In this study, we set out to examine for the presence of these triphosphorylated thiamine derivatives in Arabidopsis. We could find TTP in Arabidopsis under standard growth conditions, but we could not detect ATTP. Interestingly, TTP is found primarily in shoot tissue. Drivers of TTP synthesis are light intensity, the proton motive force, as well as TDP content. In plants, TTP accumulates in the organelar powerhouses, the plastids, and mitochondria. Furthermore, in contrast to other B₁ vitamers, there are strong oscillations in tissue levels of TTP levels over diel periods peaking early during the light period. The elevation of TTP levels during the day appears to be coupled to a photosynthesis-driven process. We propose that TTP may signify TDP sufficiency, particularly in the organelar powerhouses, and discuss our findings in relation to its role.
2 | MATERIALS AND METHODS

2.1 | Plant growth material

Arabidopsis thaliana (ecotype Columbia 0) was used as wild type. The THIC THI1 overexpressing line (Dong et al., 2015) was kindly provided by Aymeric Goyer (Oregon State University). Seeds were cultivated in sterile culture and were surface sterilized in 70% ethanol (v/v) and dried prior to plating on half-strength MS medium without vitamins (Murashige & Skoog, 1962) containing 0.55% agar (w/v) in Petri dishes. Unless stated otherwise, seeds were stratified for four days at 4°C in the dark before transfer to a growth incubator (CLF Climatics CU-22L) under the following conditions: 120–150 μmol photons m⁻² s⁻¹ generated by fluorescent lamps [Philips Master T-D Super 80 18W/180] for 12 h followed by 12 h of darkness at constant 20°C, 60% relative humidity, and ambient CO₂. For treatment with 3-(3′,4′-dichlorophenyl)-1,1-dimethylyurea (DCMU) Arabidopsis seedlings (22–24 seedlings per plate) were grown in sterile culture for 14 days after germination under the conditions described above and sprayed with ~300 μl of 50 μM DCMU (50 μl of 10 mM DCMU, dissolved in ethanol, was diluted in 10 ml dH₂O) or 0.5% ethanol (mock) at the beginning of the night period on the 15th day. Plant material for vitamer analysis was sampled at the end of the night period in the darkness (0 h light) and 4 h in the light period (4 h light). Similar material was prepared for treatment with carbonyl cyanide m-chlorophenyl hydrazone (CCCP), only that seedlings were grown on mesh in sterile culture and transferred (on the mesh) to a Petri dish containing 30 ml of 100 μM CCCP in 1% ethanol, or 1% ethanol (mock) on the morning of the 15th day and incubated for 30 min before the onset of light, after which they were blotted dry, returned to sterile culture medium and harvested in the dark (0 h light) as well as 4 h in the light period (4 h light).

2.2 | Bacterial growth and analysis

Escherichia coli strain BL21 (DE3) pLysS (F ompT hsdS b (r g- m g-) gal dcm (DE3) pLysS (CamRI) was used. After growth for 16 h (37°C, 180 rpm rotation) in lysogeny broth (LB) medium (BD Difco), cell paste from 350-ml cultures was washed and resuspended in 40 ml of M9 minimal medium (16.8 mM Na₂HPO₄∙12H₂O, 22.0 mM KH₂PO₄, 18.7 mM NH₄Cl, 8.8 mM NaCl, 20.4 μM CaCl₂, 2H₂O, 1 mM MgSO₄∙7 H₂O, pH 7.0 adjusted with 1 M KOH) or M9 minimal medium containing glucose (10 mM). After incubation for 3 h (37°C, 180 rpm rotation), 5 ml of the bacterial cultures were taken and cells were collected by centrifugation (5 min, 5500 g, 22°C) and resuspended in 12% trichloroacetic acid (TCA) for 30 min on ice. The precipitated fraction was removed by centrifugation (10 min, 16100 g at 22°C) and 1 volume of supernatant (100 μl) was treated with 3 volumes of diethyl ether to remove TCA. Ten μl of supernatant was diluted in 5 volumes of H₂O and derivatized for B₁ vitamer analysis by HPLC (2 μl injection), as outlined below.
2.3 | Heterologous expression and purification of recombinant MmTTPase

The synthetic and codon-optimized Mus Musculus thiamine triphosphatase (Uniprot ID Q8JZL3, MmTTPase) in pMH-HT as described in Martínez et al. (2015) was used for heterologous expression in E. coli. Protein expression was carried out in BL21 (DE3) RIL cells (Invitrogen) by induction with 0.25 mM isopropyl β-D-1-thiogalactopyranoside in LB medium (BD Difco) at 19°C for 16 h. After collection by centrifugation at 6000 g, cells were washed in 80 ml of phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 10 mM sodium hydrogen phosphate, 2.0 mM potassium dihydrogen phosphate) and the pellet was snap-frozen in liquid nitrogen. For protein purification, the cell pellet was resuspended in lysis buffer (50 mM sodium phosphate, pH 8, containing 300 mM NaCl, 10 mM imidazole, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, lysozyme (0.5 mg/ml, w/v), and complete protease inhibitor cocktail tablet (Roche), homogenized (Emulsiflex C-3, Avestin) with bovine deoxyribonuclease 1 (Sigma), followed by centrifugation at 6000 g to remove insoluble material. The MmTTPase protein was purified from the supernatant by Ni-NTA chromatography following the manufacturers’ instructions (Macherey-Nagel) using sodium phosphate buffer, pH 8.0 containing 300 mM NaCl, and 10 mM imidazole for washing, and eluted with the same buffer but containing 250 mM imidazole. Protein concentration was measured by infrared absorbance using the Direct Detect system (Merck Millipore).

2.4 | Analysis of B1 vitamers by HPLC

B1 vitamers (free and protein bound) were extracted from Arabidopsis material (~100–150 mg) ground in liquid nitrogen 2 × 10 s using 2-mm glass beads and a tissue lyser and subsequently vortexed for 30 min at 22°C in 200 μl of 1% TCA per 100 mg tissue. The samples were centrifuged at 16100 g for 10 min at 22°C and 50 μl of supernatant were transferred into two separate tubes for treatment with or without MmTTPase or MmTTPase E157A E159A, as indicated, (8 μM) in 50 mM Tris HCl, pH 9.0 containing 5 mM MgCl2 followed by incubation at 37°C for 30 min. Samples were derivatized by adding 7 μl of 46 mM potassium ferricyanide (dissolved in 23% NaOH) and incubation in the dark for 10 min at 22°C. Finally, 12 μl of 1 M NaOH and 12 μl of methanol were added and the solution was centrifuged at 16100 g for 10 min at 22°C. The supernatant was analyzed by HPLC on an Agilent 1260 Infinity system (Agilent Technologies) equipped with a Cosmosil X-NAP column, 2.0ID × 150 mm, 5-μm particle size (Nacalai Tesque). Ten μl of the derivatized samples was chromatographed using a solvent system developed at a flow rate of 0.5 ml/min with a gradient from 89% A (50 mM KPO4, pH 7.2) and 11% B (methanol) to 68% A within 9.5 min, then to 40% A within 3.5 min and to 100% B within 0.5 min, held for 2 min and returned to the initial conditions within 0.5 min. The final conditions were maintained for 6 min. B1 vitamers were detected using a fluorescence detector with an excitation wavelength at 375 nm and an emission wavelength at 450 nm. Quantification of thiamine, TMP, and TDP was performed by comparison to standard curves of commercially available standards. Quantification of TTP (or aTTP) was performed in a similar manner with chemically synthesized compounds kindly provided either by Lucien Bettendorff (University of Liège, Belgium) and/or synthesized according to Bettendorff et al. (2003), Frédéric et al. (2009), and Hofer et al. (2016) by Hennig Jessen (University of Freiburg, Germany) and compared to samples treated with MmTTPase.

2.5 | Construction of transgenic lines and confocal fluorescence microscopy

The consensus coding sequence of the MmTTPase (CCDS27108.1, NCBI) fused at the 5′-end to either the coding sequence of the ATPase γ-subunit (pFAγ, (Lee et al., 2012)) or the E1α-subunit of pyruvate dehydrogenase (E1α, (Lee et al., 2009)) was synthesized by Eurofins Genomics (Eurofins Scientific). The synthesized products were cloned into the pENTR/D-TOPO vector using the pENTR/D-TOPO cloning kit (Life Technologies) to generate pENTR/D-TOPO-pFAγ-MmTTPase and pENTR/D-TOPO-E1α-MmTTPase plasmids. The MmTTPase without transit peptide was amplified from pENTR/D-TOPO-pFAγ-MmTTPase using primer sequences specified in Supplemental Table S1 and cloned into the pENTR/D-TOPO vector as above to generate pENTR/D-TOPO-MmTTPase. Each respective MmTTPase construct was then cloned into the Gateway destination vector pUBC-YFP-DEST (Greifen et al., 2010) using the LR clonase enzyme mix II (Life Technologies) to generate pUBC-pFAγ-MmTTPase-YFP, pUBC-E1α-MmTTPase-YFP, and pUBC-MmTTPase-YFP plasmids. Site-specific mutations, i.e. E157A and E159A, were introduced by PCR using Pfu Turbo DNA polymerase AD (Agilent Technologies) and primer sequences specified in Supplemental Table S1. The destination constructs were introduced into Agrobacterium tumefaciens strain C58 and used to transform Arabidopsis (Col-0) plants by the floral dip method (Clough & Bent, 1998). Transformants were selected by BASTA treatment due to the presence of the BAR gene in each construct. Homozygous lines were selected from the T3 generation according to their segregation ratio for BASTA resistance and used for analysis. Confocal fluorescence microscopy was performed on 3-day-old plants grown in sterile culture as specified above with a SP5 laser-scanning confocal microscope (Leica), equipped with a 63x oil NA 1.4 PlanApo objective and with zoom and pinhole settings giving voxel sizes around 120x120x600 nm. When relevant, samples were incubated in 500 nM MitoTracker Red CMXRos (Invitrogen) for 30 min in water, then rinsed in water and (for all samples) mounted in water between slide and coverslip with a double-sided scotch tape spacer. A 514-nm laser line was used to excite YFP and (when relevant) chlorophyll and to generate transmission images. YFP fluorescence was collected by a HyD detector between 519 and 560 nm, and chlorophyll fluorescence was collected by a PMT between 650 and 800 nm. MitoTracker Red CMXRos was excited sequentially at 579 nm and a HyD detector collected its fluorescence between 595 and 634 nm. Image analysis
was performed using the software Fiji (Schindelin et al., 2012). A Gaussian blur of radius 0.6 pixels was applied to all z slices, and maximum intensity projections were applied and displayed.

2.6 | Gene expression analysis by quantitative real-time reverse transcription PCR

Total RNA was extracted from Arabidopsis seedlings, grown in sterile culture as described above, using the RNA Nucleospin Plant kit (Macherey-Nagel) and treated with DNaseI (Invitrogen) to remove DNA contamination according to the manufacturers’ instructions. One µg of RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase (200 units, Invitrogen) and oligo(dT)$_{20}$ primers (50 ng) (Promega), according to the manufacturers’ recommendations. Real-time quantitative reverse transcription PCR (qPCR) was performed in 384 well plates on a QuantStudio5 Real-Time PCR system (Applied Biosystems) using Power SYBR Green master mix (Applied Biosystems) and the following amplification program: 10 min denaturation at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were analyzed using the comparative CT method ($2^{-ΔΔCT}$) normalized to the reference gene UBC21 (At5g25760). Primers used are listed in Supplemental Table S1. Each experiment was performed with three biological and three technical replicates.

2.7 | Accession numbers

Sequence data from this article can be found in either the EMBL or GenBank data libraries under the following accession numbers: At1g76410 (ATL8), At5g32470 (pale1), At5g54770 (THII1), At2g29630 (THIC), or MGC38198 (MmTTPase).

3 | RESULTS

3.1 | Detection of TTP in Arabidopsis seedlings

As the TTP and ATTP derivatives of thiamine are reported to be present in very low amounts in vivo, it is essential to be able to reliably measure levels with a sensitive and specific assay, in order to get to the precise role of these compounds. To this goal, we modified an existing HPLC method (Moulin et al., 2013) achieving separation and detection of all relevant thiamine derivatives using the commercially available compounds and chemically synthesized TTP and ATTP as standards (kindly provided by Lucien Bettendorff, University of Liège and/or synthesized according to (Bettendorff et al., 2003, Frédérical et al., 2009, Hofer et al., 2016) (Figure 1A). To validate the method, we replicated the previous groundbreaking experiments performed with E. coli (Bettendorff et al., 2007, Lakaye et al., 2004). Growth of E. coli in M9 minimal medium devoid of amino acids led to the detection of ATTP (ca. 190 pmol g$^{-1}$ bacterial paste) (Figure 1A). Addition of glucose led to the disappearance of ATTP and the appearance of TTP (ca. 190 pmol g$^{-1}$ bacterial paste) (Figure 1A). This suggests that these thiamine derivatives appear under different environmental conditions with ATTP detected when amino acids and a carbon source are limiting and TTP detected upon supplementation of the medium with a carbon source, corroborating earlier studies (Bettendorff et al., 2007). The coenzyme form TDP could be detected under both conditions in similar quantities, whereas neither TMP nor thiamine was detected under either condition with any significance (Figure 1A).

We next tested extracts of whole Arabidopsis seedlings grown in sterile culture under standard equinoctial (12-h light, 12-h dark) laboratory conditions for the presence of the compounds. Indeed, a small peak corresponding to the retention time of TTP could be detected but no peak corresponding to ATTP (Figure 1B). To rule out interference from other compounds, the extract was spiked with authentic TTP and led to the expected increase in fluorescence (Figure 1B). To corroborate the identity of TTP in particular, we made use of recombinant Mus musculus (mouse) thiamine triphosphatase (MmTTPase) that is highly efficient and specifically hydrolyzes TTP (Delvaux et al., 2013). Treatment of the plant extract with MmTTPase resulted in disappearance of the peak corresponding to TTP (Figure 1B). Furthermore, we exchanged two glutamate residues (E157 and E159) to alalines in the active site of MmTTPase (Figure 1C). These negatively charged residues are essential for coordination of the metal group (Mg$^{2+}$) necessary for catalytic activity; thus their exchange to uncharged alalines renders the enzyme inactive (Delvaux et al., 2013, Martinez et al., 2015). Treatment of the plant extract with MmTTPase E157A E159A did not impact the peak corresponding to TTP, confirming the loss of activity in MmTTPase E157A E159A and moreover, validating the identity of TTP peak (Figure 1B). Therefore, TTP can be detected in extracts of Arabidopsis seedlings grown under standard laboratory conditions. This is significant because the only other study to date on TTP in Arabidopsis states that it was not detectable in plants grown under standard conditions. Rather, TTP was only detected when plants were allowed to wither (and presumably suffering a water deficit, i.e. stressed) (Makarchikov et al., 2003). Furthermore, treatment of a mix of the B$_1$ vitamer standards with MmTTPase led to complete disappearance of the peak corresponding to TTP (Figure 1B). This suggests that the MmTTPase hydrolyzes TTP to TDP and inorganic phosphate.

A previous study reports that ATTP can be detected in roots of soil grown samples (Bettendorff et al., 2007), although the conditions for growth and processing of the plant samples were not given. As we could not detect ATTP in whole seedlings as described above, we probed for the presence of thiamine derivatives in more detail in Arabidopsis tissue by analyzing pooled samples of separated shoots and roots of seedlings. Interestingly, TTP was clearly measurable in shoot tissue but not in root tissue (Figure 1F). In this context, we
calculated a limit of detection for TTP based on linear regression and set the lower limit for reliable detection of this compound with this HPLC method at 6.4 pmol g⁻¹ FW. TDP, TMP, and thiamine were also clearly detectable in both tissues, with TDP being the most abundant vitamer by far and moreover, significantly more abundant in shoot rather than root tissue (Figure 1F). However, we were unable to detect ATTP in either tissue, suggesting that it is either below the limit of detection under standard conditions, or is induced under specific conditions yet to be identified.

Taken together, we conclude that TTP can be detected in Arabidopsis shoots under standard growth conditions. However, we could not detect ATTP in Arabidopsis tissue under such conditions.
This is in contrast to the earlier studies reporting that TTP can only be detected when plants are subjected to stress (wilting) (Makarchikov et al., 2003) and that ATTP can be detected in root tissue under standard conditions (Bettendorff et al., 2007).

3.2 Evidence for regulation of TTP levels in Arabidopsis shoots as a function of light

Next, in an initial effort to primarily detect ATTP in Arabidopsis, we attempted to mimic the conditions for appearance of this compound in E. coli (minimal medium devoid of amino acids and a carbon source Figure 1A), by subjecting plants to starvation conditions. More specifically, during the process of photosynthesis in plants, light energy is used to fix carbon dioxide into organic carbon compounds such as soluble sugars and the insoluble storage form starch (Smith & Zeeman, 2020). The sugars provide essential carbon skeletons for assimilation of nitrogen into amino acids and other essential nitrogenous compounds. In the absence of light, stored starch is solubilized to maintain metabolism and provide energy equivalents for vital cell processes such as respiration in the dark. Under 24-h daily light/dark cycles, these processes take place in a precise manner regulated by the circadian clock with the linear biosynthesis of starch during the light being precisely followed by its linear degradation in the dark, such that almost 95% of the stored starch is consumed at the end of the dark period (Smith & Zeeman, 2020). Extension of the dark period beyond that anticipated by the plant and/or subjection of the plants to an unanticipated early night leads to starvation (Graf et al., 2010). Accordingly, we entrained plants to equinoctial conditions for 14 days; on the 15th day, we subjected plants to an early night (after 8 h of light) and furthermore, extended the night by an additional 4 h (20 h dark in total); this was followed by re-exposure of the plants to light for 4 h (see scheme in Figure 2A). We could confirm that the seedlings were suffering from starvation during the extended night based on the induction of the starvation reporter gene at locus At1g76410 (ATL8) (Graf et al., 2010) (Figure 2B). Moreover, At1g76410 transcript abundance decreased upon re-exposure of the plants to light, in line with the onset of photosynthesis and the biosynthesis of sugars, whereas levels of the transcript remained high in plants kept in the dark (Figure 2B). Under these conditions, we could still detect TTP at the selected time points, as before, but were unable to detect ATTP (Figure 2C). During this set of experiments, we were struck by the observation that the levels of TTP were low during the dark period and became much more abundant upon re-exposure to light (Figure 2C). Indeed, we could demonstrate that the level of accumulation of TTP was dependent on the light intensity, and increased concomitant with an increase in photon irradiance (Figure 2D). Among the other B1 vitamers, we noted that TMP levels decreased during the dark period and increased again during the light period, whereas the level of induction was less pronounced than for TTP (Figure 5A). As TMP is biosynthesized through the de novo pathway, which relies on reducing equivalents from photosynthesis at least in shoots (Martinez-Gomez & Downs, 2008), this may account for this behavior. Notably, TDP levels were not significantly changed over the course of the experiment, whereas thiamine levels appeared to increase during the dark period and decrease during the light (Figure 5A). The increase in abundance of a B1 vitamer as a function of light intensity was exclusive to TTP, as none of the other vitamer levels were significantly affected under the conditions used (Figure 51b).

3.3 Evidence that TTP accumulation is linked to the proton motive force with free TDP as substrate in plants

Based on observations in animals and in bacteria, it has been demonstrated that TTP is synthesized through a chemiosmotically driven mechanism from TDP and inorganic phosphate (P i) in an ATP synthase-like manner involving the proton motive force (pmf) (Lakaye et al., 2004, Gangolf et al., 2010, Gigliobianco et al., 2013). Unlike either animals or bacteria, plants are unique in that there are two compartments in which electron transfer can generate a pmf; the mitochondria and plastids, respectively. To test if production of TTP is driven by the pmf in these organelles, we subjected seedlings to treatment with the general protonophore carbonyl cyanide m-chlorophenyl cyanide (CCCp), which impedes the pmf in both organelles. Indeed,
the light-dependent increase in TTP accumulation was strongly reduced—to the limit of detection, in the presence of CCCP (Figure 3a).

On the other hand, there was no significant change in TDP levels although levels tended to decrease, whereas TMP and thiamine levels significantly increased as a function of CCCP in the light (Figure S2). Studies in rat brain and *E. coli* demonstrate that the TDP vitamer acts as substrate for TTP synthesis (Gangolf et al., 2010, Gigliobianco et al., 2013). Therefore, if a similar mechanism is in place in plants,
plants with altered TDP levels should correspondingly affect TTP levels. Transgenic lines overexpressing THI1 (At5g54770) and THIC (At2g29630) (THIC THI1 OE) have enhanced TDP content in leaves (Dong et al., 2015). By contrast, the pale green 1 (pale1) mutant, impaired in the TMP phosphatase, TH2 (At5g32470), is deficient in leaf TDP (Mimura et al., 2016, Hsieh et al., 2017). We examined the B₁ vitamer profile of these lines and could confirm that TDP was increased in THIC THI1 OE but decreased in pale1 (Figure 3B).
Importantly, parallel changes in TTP levels were observed, with a 6-fold increase in THIC THI1 OE whereas TTP was below the detection limit in pale1 (Figure 3C). Notably, there was also a strong increase in thiamine levels in THIC THI1 OE whereas it could not be detected in pale1 (Figure 3D). However, thiamine is not considered to be a precursor of TTP. On the other hand, TMP levels were increased in both THIC THI1 OE and pale1 (Figure 3E) and therefore, do not correlate with alterations in TTP levels. Furthermore, supplementation of wild-type plants with thiamine led to increased TDP content as well as TTP (Figure 3F).

Thus, it appears that TTP synthesis in plants is associated with the pmf and correlates with TDP levels—the assumed precursor.

3.4 | TTP accumulates in the organellar powerhouses

Given that TTP levels were strongly impacted by uncoupling of the pmf, we were next prompted to provide evidence of where it accumulates at the cellular level. However, subcellular fractionation methods are not suitable for determining the distribution of very low abundance soluble metabolites with any level of accuracy (Dietz, 2017). Thus, to investigate the subcellular location of TTP biosynthesis in plants, we made use of the only enzyme known to be specifically involved in TTP metabolism—the mammalian TTP phosphatase (TTPase) (Makarchikov & Chernikevich, 1992). In particular, we engineered Arabidopsis to express the Mus musculus (mouse) TTPase (MmTTPase) as described above. Lines were engineered to target MmTTPase to either the mitochondria or plastids, or to retain the protein in the cytosol. The prerequisite of the Arabidopsis ATPase-synthase γ-subunit (pFAγ) (Lee et al., 2012) was used to guide the MmTTPase into the mitochondria (MmTTPase_mito), whereas the E1α-subunit of pyruvate dehydrogenase (Lee et al., 2009) was used for plastid targeting (MmTTPase_plastid). MmTTPase without any targeting peptide was expected to lead to retention in the cytosol (MmTTPase_cytosol). To facilitate visualization of the intended subcellular localization, YFP was fused to the C-terminus of each MmTTPase (see Figure 4A for a scheme of each construct). We performed confocal fluorescence microscopy on the generated transgenic lines to validate the subcellular localization. In the case of MmTTPase_mito, a punctate pattern typical for localization to the mitochondria was observed in representative lines isolated to homozygosity (Figure 4B). Moreover, infiltration with MitoTracker® Red CMXRos that specifically stains mitochondria showed a clear overlap of the fluorescence signals, confirming the targeting of MmTTPase_mito to this organelle (Figure 4B). In the case of MmTTPase_plastid, the pattern of fluorescence of representative transgenic lines overlapped with chlorophyll autofluorescence indicating localization to chloroplasts (Figure 4C).

On the other hand, the diffuse extra-organellar fluorescence pattern of representative lines harboring the MmTTPase_cytosol construct was typical of localization to the cytosol, with some also localized to the nucleus (Figure 4D). Furthermore, we also generated transgenic Arabidopsis lines, as above but carrying an inactive version of MmTTPase. In particular, each construct for organelle-targeted or cytosol retention expression in Arabidopsis was engineered to harbor the inactive MmTTPase E157A E159A as described above, yielding MmTTPase E157A E159A_mito, MmTTPase E157A E159A_plastid, and MmTTPase E157A E159A_cytosol. In each case, the intended subcellular localization was validated using confocal microscopy as for the active MmTTPase lines (Figure S3).

We then proceeded to measure the levels of TTP in leaf tissue from all transgenic lines. Interestingly, in the cases of MmTTPase_mito and MmTTPase_plastid lines, TTP levels were strongly reduced and below the limit of detection (Figure 4E). On the other hand, the levels of TTP in MmTTPase_cytosol approximated those seen in the wild type (Figure 4E) although the level in line 7.2 was significantly lower than wild type but considerably above the TTP level in the lines with organelle-targeted MmTTPase (Figure 4E). By contrast, lines expressing inactive MmTTPase had TTP levels approaching that of wild-type plants in general, although 2 lines had levels below that of wild type but were far above those of organelle-targeted active MmTTPase (Figure 4E). This provides very strong evidence for the functionality of the MmTTPase, i.e. it hydrolyzes TTP in plants. Moreover, the data indicate that TTP is localized to both the mitochondria and plastids in plants. However, we do not yet understand why TTP levels are depleted in both the MmTTPase_mito and MmTTPase_plastid lines. One explanation could be that a (small) proportion of each respective organelle-targeted MmTTPase is localized to the other organelle. This is corroborated by the fact that a proportion of proteins intentionally targeted to either the mitochondria or plastids can sometimes be found in the other organelle (Tanz et al., 2013). Given that MmTTPase is a highly efficient enzyme (K_m = 8.0 ± 2.0 μM, k_cat = 23.0 ± 3.0 s^-1 (Delvaux et al., 2013)), the localization of even a small proportion to the alternative organelle may facilitate TTP hydrolysis therein. However, we saw no evidence for mis-localization of the plastid- or mitochondria-targeted MmTTPase in the confocal fluorescence microscopy data. Notably, there was no corresponding change in the levels of TMP or TDP (Figure S4) indicating that MmTTPase acts exclusively on TTP. In addition, by contrast to the studies in vitro, we were not able to correlate the disappearance of TTP in vivo with the appearance of another vitamer, but this is not surprising given the low abundance of TTP in vivo (low picomoles) compared to TDP in particular (nanomoles)—i.e. the hydrolysis product seen upon treatment of the chemically synthesized standard (Figure 1D). Of note is that TMP and thiamine levels were significantly higher in some transgenic lines (Supplemental Figure S4), but there was no correlation with any other B1 vitamer, nor consistency among a particular set of transgenic lines.

Taken together, our data provide very strong evidence that TTP accumulates in the organellar powerhouses, i.e. plastids and mitochondria, in plants.

3.5 | TTP levels oscillate over a diel period

Two observations in the previous part of this study were intriguing with respect to TTP: 1) its abundance in shoots (rather than roots),
as well as 2) increased accumulation as a function of light. While TTP has been clearly identified in microorganisms and mammals since a long time, possible fluctuations of TTP levels over a diel period have not been examined in any organism. Therefore, prompted by the above observations, we monitored the levels of TTP for 72 h under equinoctial conditions, i.e. 12-h light, 12-h dark. Interestingly, we observed a strong diel oscillation in the abundance of TTP, repeatedly peaking 4 h into the light period followed by a decrease culminating...
at the end of the dark period (Figure 5A). It should be noted that we have recently demonstrated that none of the other well-characterized B1 vitamers (TDP, TMP, or thiamine) display an oscillation at the tissue level in plants (Noordally et al., 2020). As diurnal oscillations are frequently regulated by the genetically encoded circadian clock, we next examined if the circadian clock anticipated the oscillations. Seedlings were entrained under equinoctial conditions as before and then placed in a free-running period of constant light or constant darkness for 72 h. The diurnal oscillation of TTP was no longer detectable under both of these conditions (Figure 5b,c), indicating that either the circadian clock does not drive the rhythm of TTP or it is a low-amplitude rhythm below the limit of detection. Notably, under constant light conditions, the level of TTP was higher than under constant darkness conditions, further supporting its induction during the photoperiod. We also examined the levels of the other B1 vitamers under free-running periods of constant light or constant darkness for 72 h. We observed that while TDP levels did not change considerably over the period in constant light, there was a steady decrease upon prolonged incubation in constant darkness (Supplemental Figure S5a). On the other hand, while thiamine levels remained steady for the duration in constant light, the levels increased in constant darkness (Figure S5b). Interestingly, TMP levels showed a tendency to accumulate (4-fold) in constant light but remained steady for the duration in the dark (Figure S5c). In the first instance, the strong diel oscillation of TTP prompted us to examine if photosynthesis per se played an important role in the accumulation of this vitamer during the photoperiod. Photocynthesis can be blocked by treatment with the herbicide 3-(3′4′-dichlorophenyl)-1,1-dimethylurea (DCMU), which impedes electron transfer within photosystem II (Metz et al., 1986). This treatment repressed the induction of TTP levels seen in the light (Figure 5d), concomitant with the expected decrease in the quantum efficiency of photosynthesis. To dissect if this is specifically associated with a photosynthesis-derived sugar, we examined levels in the phosphoglucomutase mutant, pgm-1, which is unable to convert glucose 6-phosphate to glucose 1-phosphate (Caspar et al., 1985). As the latter is a precursor to starch biosynthesis in the plastid, the pgm-1 mutant lacks starch and accumulates higher than normal levels of soluble sugars throughout the day (sucrose, glucose) (Caspar et al., 1985, Pal et al., 2013). However, the levels and moreover, rhythmic oscillation of TTP in pgm-1 mirrored the pattern seen in wild type (Figure 5e). Thus, the data suggest that elevated TTP levels during the day are associated with a photosynthesis-driven process but not with specific sugar levels per se and remains to be dissected in future studies.

4 | DISCUSSION

There is increasing awareness—supported by a large body of evidence—that certain metabolites can communicate aspects of cellular status for maintaining metabolic homeostasis (Dakik et al., 2019). Vitamin B1, in its form as the coenzyme TDP, plays a crucial role in core metabolic processes, such that without it, energy cannot be generated. In this study, we set out to examine for the presence of thiamine triphosphate derivatives in plants, which have been touted as potential molecular signaling components particularly in mammalian cells, but only narrowly investigated in plants. We could demonstrate clearly that triphosphorylated thiamine, TTP, can be found in Arabidopsis, primarily in shoot tissue. Under our conditions, however, we could not detect the adenylated thiamine triphosphate, ATTP. We therefore focused on characterizing features of TTP in Arabidopsis. We can conclude from our data that TTP levels are linked to the pmf and TDP content, the drivers of TTP biosynthesis. Interestingly in plants, TTP accumulates in the organelar powerhouses, the plastids and mitochondria. In contrast to other B1 vitamers, there are strong oscillations in tissue levels of TTP levels over diel periods peaking during the light period. The elevation of TTP levels during the day appears to be coupled to a photosynthesis-driven process. We discuss our findings below, while proposing that TTP may serve as a proxy for TDP-replete levels, particularly in the organelar powerhouses.

To date, the most comprehensive studies on TTP come from its characterization in mammalian tissues (Aleshin et al., 2019). In particular, brain tissue has received considerable attention and the molecule has been proposed to have a role in cellular energetics (Aleshin et al., 2019, Bettendorff et al., 2014). By contrast, TTP has not been studied in any detail in plants, although it was detected therein several decades ago (Kochibe et al., 1963, Yusa, 1961) and a more recent study reports observation of TTP under water-deficit conditions in Arabidopsis (Makarchikov et al., 2003). Here we detect
**FIGURE 5** Thiamine triphosphate levels oscillate over a diel period. (a–c) The upper panels show a scheme of the plant growth conditions. White and black boxes indicate the light and dark periods, respectively. The lower panels show the levels of thiamine triphosphate (TTP). In each case, seedlings of Arabidopsis wild type (Col-0) were grown in sterile culture under a light intensity of 125–135 µmol photons m⁻² s⁻¹ at 20°C. Seedlings were entrained for 14 days under equinoctial conditions (12 h light and 12 h dark) and were either maintained under those conditions (a), or transferred to constant light for 72 h (b), or transferred to constant darkness for 72 h (c). Shoot material was harvested every 4 h at the times indicated under the respective conditions (light/dark (a), constant light (b), or constant dark (c)). In the case of (b), white and hatched gray areas represent subjective day and night, respectively. While in (c), hatched gray and gray areas represent subjective day and night, respectively. Data of three individual biological replicates of pooled material (n = 20–25 per replicate) are shown with error bars representing SD. (d) TTP levels of Arabidopsis wild type (Col-0) in the absence (mock) or presence of the herbicide 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) at the end of the dark period (0 h light) and 4 h into the light period (4 h light). Statistical significance with and without DCMU was performed with a two-way ANOVA with multiple comparisons (Sidak's test), asterisks indicate p < .005. (e) The upper panel shows a scheme of the plant growth conditions and is as in (a). White and black boxes indicate the light and dark periods, respectively. The lower panel shows the level of TTP in seedlings of Arabidopsis wild type (dashed line) or the pgm mutant line (gray solid line). Shoot material was harvested every 4 h at the times indicated. Data of three individual biological replicates of pooled material (n = 20–25 per replicate) are shown with error bars representing SD. Statistical significance was performed with a two-way ANOVA with multiple comparisons (Sidak's test), asterisks indicate p < .001.
the compound under standard growth conditions in Arabidopsis. We find TTP primarily in shoot tissue, or at least it is more abundant in this tissue rather than roots, under our conditions. This is interesting in the context of what is known about thiamine compounds because the coenzyme TDP is biosynthesized in green tissue (i.e., shoots) (Colinas & Fitzpatrick, 2015). Moreover, previous studies in other organisms have demonstrated that TTP is the precursor of TTP (Gangolf et al., 2010, Gigliobianco et al., 2013). That TTP is derived from TDP is corroborated in this study, where we find increased levels in lines overexpressing enzymes of the TDP biosynthesis pathway and which have enhanced levels of this coenzyme. Furthermore, supplementation of wild-type plants with thiamine increases TTP levels, also coincident with increases in TDP. By contrast, we cannot detect TTP in the pale1 mutant, which is deficient in TDP. In this context, it is important to realize that the majority of biosynthesized TDP is expected to be protein bound rather than in free form, as has been recently deduced (Joshi et al., 2019). Here, we are measuring a combination of free and protein-bound TDP, although we have not included steps (e.g., enzyme digestion) to explicitly release protein-bound TDP. As TDP is assumed to be the precursor of TTP and as it will likely only be synthesized from free TDP (rather than protein bound), then TTP may reflect levels of free TDP, i.e., excess free TDP is converted to TTP. As free TDP has the capacity to bind to and boost the activity of enzymes dependent on it as coenzyme, conversion of free TDP into TTP may serve to remove it from the cellular milieu, such that it does not interfere with the corresponding enzyme-driven metabolic homeostasis.

Our findings that there is a strong diel regulation of tissue levels of TTP, peaking in accumulation early during the light period, as well as its dependence on light intensity, strongly indicate that TTP biosynthesis is a photosynthesis-driven process in plants, although it does not appear to be directly related to sugar levels. With this in mind, it is important to recognize that at the cellular level biosynthesis de novo of thiamine is a light-driven process that takes place in the plastids during the photoperiod, as it relies on redox equivalents derived from photosynthesis (Raschke et al., 2007). The final step of diphosphorylation of thiamine to TDP takes place in the cytosol (Ajawi et al., 2007). Transporters at the organelle membranes serve to import TDP to supply enzymes reliant on it as a coenzyme (Gerdes et al., 2012, Noordally et al., 2020). On the one hand, regulation of supply and demand is buffered at the genetic level in the nucleus by a riboswitch present in the thiamine biosynthesis gene THIC (Rosado-Souza et al., 2019). That is, an abundance of free TDP in the nucleus leads to downregulation of THIC transcripts through alternative splicing (Wachter et al., 2007, Bocobza et al., 2007). It has recently been demonstrated by us that at the cellular level (and in contrast to the tissue level), there is a diel rhythm of free TDP in the nucleus, with the peak in TDP levels occurring early in the day (Noordally et al., 2020). The rhythmic diel pattern of TTP, at the tissue level in shoots, matches very closely to that of nuclear free TDP at the cellular level, and because TTP is found in both the plastids and mitochondria as shown in this study, we propose that it may act as a proxy of free TDP levels in these organelles.

It is interesting that TTP accumulates in both the plastids and the mitochondria. Indeed, the detail of how TTP accumulates in the respective organelles requires further investigation but is beyond the scope of this study. Nonetheless, if it is assumed that free TDP is the precursor of TTP, and TTP is a proxy for organellar levels of free TDP, then, as a corollary, interconversion of organellar TDP and TTP may serve to regulate TDP levels in the organelle powerhouses on a daily basis. As TDP levels are maximal during the day (as evidenced indirectly from the performance of the riboswitch) (Noordally et al., 2020), conversion to TTP may dissipate excess levels in the plastids and mitochondria. In this context, it is relevant to consider that enzyme targets of TTP have been identified in mammalian brain cell extracts (Mkrtchyan et al., 2015). In particular, TTP has been shown to be a positive allosteric effector of glutamate dehydrogenase (GDH) but inhibits pyridoxal kinase (PLK) (Mkrtchyan et al., 2015). Both of these enzymes are involved (directly or indirectly) in amino acid biosynthesis. GDH is required for conversion of glutamate to α-ketoglutarate, which is a key molecule that integrates carbon and nitrogen metabolism (Forchhammer & Selim, 2020). On the other hand, PLK contributes to the production of pyridoxal 5'-phosphate, a coenzyme required for numerous enzymes predominantly involved in amino acid metabolism (Fitzpatrick, 2011). Notably, GDH and PLK (annotated SALT OVERLY SENSITIVE 4 (SOS4) in Arabidopsis (Colinas & Fitzpatrick, 2015)) are found in the mitochondria and plastids of plants, respectively (Rueschhoff et al., 2013, Becker et al., 2000), which would fit with the sites of accumulation of TTP. However, it remains to be determined if these enzymes are TTP targets in plants. As TDP is mainly required for carbohydrate metabolism (Rapala-Kozik et al., 2009), the association of TTP with amino acid metabolism through GDH and PLK may have a role to play in carbon/nitrogen balance. This notion is corroborated by the studies in bacteria, where TTP is transiently synthesized in amino acid-starved cultures supplied with glucose (Lakaye et al., 2004) and as shown here. Moreover, it has been shown in bacteria that TTP accumulates when pyruvate can be adequately oxidized through the TCA cycle and the respiratory chain, i.e. C-replete conditions (Mkrtchyan et al., 2015). Thus, TTP abundance may coincide with balancing N under C-replete conditions. In an analogous fashion in plants, TTP may accumulate coincident with the onset of N assimilation when there is no limitation of C skeletons, such as during photosynthesis under standard growth conditions, as well as after exposure of C-starved seedlings to light.

In summary, our study provides insight into the nature of the vitamin B1-derivative TTP in plants. We believe that the molecule deserves further attention, as it may be an important metabolic messenger that regulates the organelle levels of the coenzyme TDP, important for energy metabolism and in particular that at the interface of carbon and nitrogen balancing.

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CONFLICTS OF INTEREST
The authors declare no conflict of interest associated with the work described in this manuscript.

AUTHORS CONTRIBUTIONS
T.B.F. and M.H. designed the research. M.H. performed most of the research and S.L. contributed to fluorescence microscopy. T.B.F. and M.H. analyzed data. T.B.F. and M.H. wrote the article.

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