Analysis of *Chlamydomonas* thiamin metabolism in vivo reveals riboswitch plasticity

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Edited by Robert Haselkorn, The University of Chicago, Chicago, IL, and approved July 23, 2013 (received for review April 24, 2013)

Thiamin (vitamin B_1) is an essential micronutrient needed as a cofactor for many central metabolic enzymes. Animals must have thiamin in their diet, whereas bacteria, fungi, and plants can biosynthesize it de novo from the condensation of a thiazole and a pyrimidine moiety. Although the routes to biosynthesize these two heterocycles are not conserved in different organisms, in all cases exogenous thiamin represses expression of one or more of the biosynthetic pathway genes. One important mechanism for this control is via thiamin-pyrophosphate (TPP) riboswitches, regions of the mRNA to which TPP can bind directly, thus facilitating fine-tuning to maintain homeostasis. However, there is little information on how modulation of riboswitches affects thiamin metabolism in vivo. Here we use the green alga, *Chlamydomonas reinhardtii,* which regulates both thiazole and pyrimidine biosynthesis with riboswitches in the *THI4* (Thiamin 4) and *THIC* (Thiamin C) genes, respectively, to investigate this question. Our study reveals that regulation of thiamin metabolism is not the simple dogma of negative feedback control. Specifically, balancing the provision of both of the heterocycles of TPP appears to be an important requirement. Furthermore, we show that the *Chlamydomonas THIC* riboswitch is controlled by hydroxymethylpyrimidine pyrophosphate, as well as TPP, but with an identical alternative splicing mechanism. Similarly, the *THI4* gene is responsive to thiazole. The study not only provides insight into the plasticity of the TPP riboswitches but also shows that their maintenance is likely to be a consequence of evolutionary need as a function of the organisms' environment and the particular pathway used.

eukaryotic riboswitch | gene expression coordination | metabolic regulation | cross-talk

Thiamin (vitamin B_1) plays a vital role in all organisms as a cofactor for enzymes of glycolysis, the Krebs cycle, the pentose phosphate pathway, and the Calvin cycle in photosynthetic organisms. Animals are dependent on a source of thiamin in their diet, whereas microorganisms and plants can perform biosynthesis de novo. The biosynthesis of thiamin has two branches that culminate in the production of the heterocyclic moieties, hydroxyethylthiazole phosphate (HET-P) and hydroxymethylpyrimidine pyrophosphate (HMP-PP) (Scheme 1). These moieties are condensed to form thiamin monophosphate (TMP), which in eukaryotes is dephosphorylated by a phosphatase to form thiamin and is subsequently pyrophosphorylated by a pyrophosphokinase to the active cofactor thiamin pyrophosphate (TPP) (2, 3). Despite the importance of thiamin, regulation of its metabolism is only beginning to be unraveled in eukaryotes.

In bacteria, fungi, algae, and plants, exogenous thiamin has been shown to regulate expression of some of the thiamin biosynthesis genes via sequences in the mRNA called riboswitches (4–9). Riboswitches comprise both an evolutionarily conserved metabolite sensing, or aptamer, domain and an expression platform that carries the gene regulatory signal (9). Numerous studies have demonstrated that the cofactor form of vitamin B_1, TPP, binds directly to the aptamer, inducing a conformational change that interferes with gene expression (9–13). Several mechanisms for the modulation of gene expression have been described that reflect the diversity of control. For example, in bacteria, riboswitches are found in the 5′ untranslated region (UTR) of the corresponding mRNAs (14), where either transcription termination or translation is affected in the presence of the metabolite. In eukaryotes, in contrast, expression is modulated through alternative splicing. Demonstrated examples of the latter include the fungi, *Aspergillus oryzae* (15) and *Neurospora crassa* (5); the green alga *Chlamydomonas reinhardtii* (6); and the plant *Arabidopsis thaliana* (4, 8, 16). Interestingly, the location of the riboswitch varies in these organisms from being in an intron in the 5′-UTR, for example, in *ThiA* of *Aspergillus* (15) and *THI4* of *Chlamydomonas* (6), to being in an internal intron in *Chlamydomonas THIC* (6), to being in an intron in the 3′-UTR in *THIC* of *Arabidopsis* (17).

Intriguingly, with the discovery of thiamin riboswitches, it has emerged that there are no strict rules as to how many and which genes in the metabolic pathways are coupled to this RNA sensor. Our own analysis (based on a search at http://rfam.sanger.ac.uk) has revealed that many bacterial species can have from relatively few (e.g., two in *Listeria monocytogenes*) to several (e.g., *Bacillus* species are predicted to have eight) thiamin riboswitches, most of which are associated with genes involved in biosynthesis and transport. Eukaryotic organisms also vary; certain algae can have either one or two riboswitches (e.g., *Thalassiosira pseudonana* and *Chlamydomonas*, respectively) (6). Ancient plant taxa (e.g., bryophytes and lycophytes) also appear to have two riboswitches, whereas only one has been found in higher plants to date (4, 17). In contrast, yeast has no riboswitches. Further complexity results from the fact that the biosynthesis pathways differ between organisms. Bacteria use intermediates derived from purine and isoprenoid biosynthesis for the pyrimidine and thiazole branches, respectively (18), whereas the pathway in yeast uses B vitamin derivatives as precursors [specifically pyridoxal 5′-phosphate and nicotinamide adenine dinucleotide (NAD^+)] (1, 19–20). Plants and *Chlamydomonas* use the bacterial pyrimidine route and make thiazole from NAD^+ and glycine (as shown in Scheme 1), and there is subcellular partitioning of the biosynthesis steps (2). It has therefore been suggested that the riboswitch has been primarily acquired and maintained through a mechanism involving horizontal gene transfer (21, 22).

Much has been deciphered of the molecular mechanism of thiamin riboswitches from analyses in vitro of TPP binding to the reconstituted aptamer domain (9–13). However, the requirement for these RNA sensors and their specific control over particular components of a thiamin metabolic pathway remains to be unraveled. In this context, *Chlamydomonas* provides a particularly amenable example because both the thiazole and pyrimidine branches are under riboswitch control, with riboswitches

Author contributions: M.M., A.G.S., and T.B.F. designed research; M.M., G.T.D.T.N., and M.A.S. performed research; M.M. and M.A.S. contributed new reagents/analytic tools; M.M., G.T.D.T.N., M.A.S., A.G.S., and T.B.F. analyzed data; and M.M., A.G.S., and T.B.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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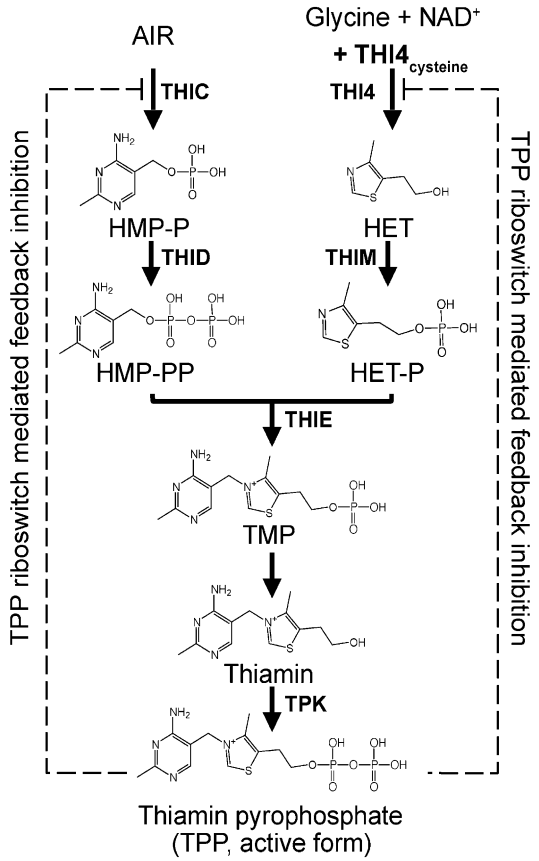
This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1307741110/-/DCSupplemental.



Scheme 1. The thiamin biosynthesis pathway in *Chlamydomonas*. As in all organisms, TMP is generated from the condensation of HET-P and HMP-PP by the action of TMP synthase. THI4 catalyzes the formation of the thiazole moiety from NAD^+, glycine, and the sulfur of a backbone cysteine residue (1). THIM, which is a salvage enzyme in bacteria, is essential for thiazole production in *Chlamydomonas*. The pyrimidine moiety is biosynthesized from aminoimidazole ribonucleotide (AIR) via the action of THIC and THID. THID and TMP synthase enzyme activities are found in a single, bifunctional enzyme equivalent to TH1 in higher plants (3). To generate the active cofactor, TPP, TMP is first dephosphorylated by an unknown phosphatase and then subsequently pyrophosphorylated by thiamin pyrophosphokinase (TPK). Both the *THI4* and *THIC* genes are regulated through a TPP riboswitch (dashed line).

associated with the *THI4* and *THIC* genes, respectively (6). We have taken advantage of this system to perform an analysis in vivo of the response of thiamin biosynthesis in this organism upon perturbation of the pathway. Our results reveal a mechanism whereby balancing the provision of the heterocycle precursors emerges as a necessity for regulating thiamin metabolism in this organism. Furthermore, insight is revealed into the plasticity of the TPP riboswitch as a function of the particular pathway used.

Results

A Single Point Mutation in the *THI4* Riboswitch Causes Thiamin Overaccumulation. To assess the effect of perturbation of the regulation of the thiamin biosynthesis pathway, we used the *pyr1* mutant of *Chlamydomonas*, which is resistant to pyrithiamin, an antimetabolite that binds to TPP riboswitches. *pyr1* has a single point mutation (C to U) in the P2 stem loop of the aptamer of the *THI4* riboswitch (ref. 6 and Fig. 1*A*), and this mutation has been shown to prevent TPP binding to the *THI4* riboswitch in vitro (6). Thus, this mutant serves as a tool for understanding how riboswitches regulate thiamin biosynthesis in *Chlamydomonas.* In the first instance, we measured the level of the B_1 vitamers (thiamin, TMP, and TPP) in *pyr1* relative to the wild-type strain. Interestingly, all were found to be substantially higher (5–10-fold increase) in *pyr1* compared with wild-type, with TPP being the most abundant vitamer (Fig. 1*B*). Therefore, inactivation of the *THI4* riboswitch results in the accumulation of considerably higher levels of B_1 vitamers. Furthermore, a quantitative analysis of the transcript level of *THI4* and *THIC* under normal growth conditions revealed that although *THI4* was more abundant, as expected in *pyr1* compared with wild-type, so was *THIC* (Fig. 1*C*). In other words, the presence of a functional riboswitch in *THIC* does not appear to respond to the overaccumulation of TPP observed in *pyr1*.

There Is a Natural Imbalance in *THI4* and *THIC* Gene Expression. It has previously been shown that supplementation of *Chlamydomonas* cultures with thiamin results in an alternative splicing event that causes a decrease in the level of the functional *THIC* and *THI4* transcripts (annotated $THIC_S$ and $THI4_S$, respectively) (6). We compared the sensitivity of the *THIC* and *THI4* riboswitches by monitoring the response of the functional transcripts to supplementation with thiamin in the wild-type strain, using real-time quantitative PCR (qPCR). Even with low nanomolar quantities of thiamin, both *THIC* and *THI4* responded to a similar extent (Fig. 2*A*). Moreover, the response of *THIC* in *pyr1* was observed to be at least similar, if not more sensitive, to that of the wild-type under the same conditions (Fig. 2*B*). As expected, *THI4* did not respond to thiamin supplementation in *pyr1*. Given this, we were intrigued as to why $THIC_S$ expression was not decreased in *pyr1*. One explanation could be that *THIC* expression is controlled by an additional unknown factor when the thiazole branch, or at least the *THI4* gene, is overexpressed, which would allow a good balance between the thiazole and the pyrimidine branches. Alternatively, the accumulated TPP in *pyr1* is sequestered, preventing interaction with *THIC* pre-mRNA. However, we surmised that the *THIC* transcript level increases in *pyr1* to balance the increased level of *THI4*; that is, both branches of the pathway are required to facilitate the biosynthesis of TPP. In this case, a higher level of TPP would be required to down-regulate

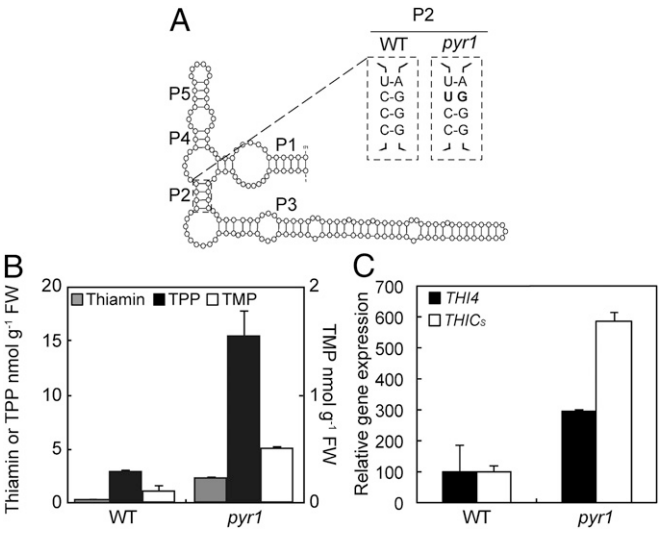


Fig. 1. A single nucleotide exchange in the *THI4* riboswitch permits overaccumulation of TPP and does not down-regulate *THIC*. (*A*) Representation of the *Chlamydomonas THI4* aptamer. In the *pyr1* mutant of *Chlamydomonas*, a single base mutation (C→U) alters base pairing in stem P2 and confers resistance to the antimetabolite pyrithiamin. (*B*) B_1 vitamer content in wild-type and *pyr1 Chlamydomonas* strains grown for 3 d in TAP medium: gray, thiamin; black, TPP; white, TMP. (*C*) qPCR of *THI4* (black) and *THIC* (white) from wild-type or *pyr1* grown for 3 d in TAP medium. In all supplementation cases, 10 μM of the respective compound was added. For *B* and *C*, the values shown are the mean ± SD from three or more independent experiments.

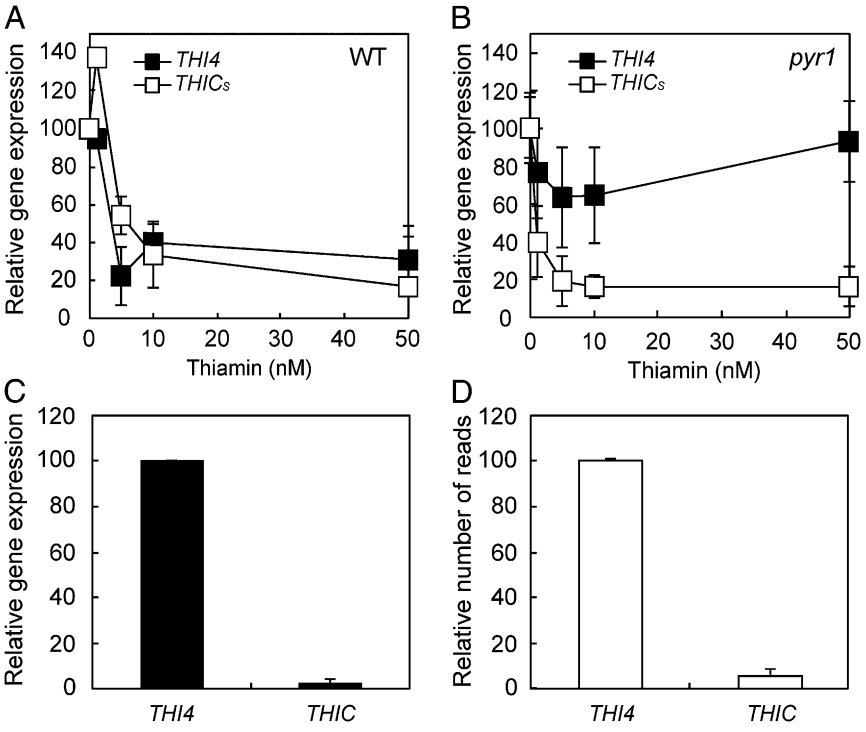


Fig. 2. Imbalance in the *THI4* and *THIC* transcripts. (*A* and *B*) qPCR of *THI4* (■) and *THIC* (□) from wild-type (*A*) or *pyr1* (*B*) grown for 6 h in the presence of different levels of thiamin supplementation. (*C*) qPCR of the level of *THIC* transcripts compared with *THI4* in *Chlamydomonas* grown for 3 d on TAP medium. (*D*) Relative reads number of *THIC* transcripts compared with *THI4* found in publicly available RNAseq data. For *A*–*C*, the values shown are the mean ± SD from three or more independent experiments.

THIC, as there is more present. A further implication is that there is cross-talk between the two branches of the pathway.

We then questioned whether both branches of the thiamin biosynthesis pathway are equally expressed. qPCR analysis of the level of *THI4* and *THIC* gene expression in *Chlamydomonas* cells grown without supplementation demonstrated that *THI4* is much more abundant (~20-fold) than *THIC* (Fig. 2*C*). This result is corroborated by several sets of publicly available RNAseq data (23–25), in which the number of reads indicates a similarly disproportionate level in the abundance of the *THI4* and *THIC* transcripts (Fig. 2*D*).

Thiamin Precursor Intermediates Play an Active Part in Regulating the Biosynthesis Pathway. These observations prompted us to probe the effect of the heterocyclic precursors on thiamin biosynthesis and growth of *Chlamydomonas* in more detail. Supplementation with either HET or HMP did not significantly change the B_1 vitamer profile of the wild-type culture (Fig. 3*A*). As it is difficult to measure the precursors directly, we took advantage of thiamin-requiring mutants that are exclusively dependent on one or the other of the heterocycles for growth (26) to validate their uptake by *Chlamydomonas*. All mutants analyzed could grow in the presence of thiamin supplementation, but two mutants were restored to wild-type levels of growth by either HET (CC24) or HMP (CC25) supplementation alone (Fig. 3*B*). As this confirmed that the precursors could be taken up, the effect of the precursors on expression of *THIC* and *THI4* in the wild-type strain was assessed. Supplementation with HET significantly down-regulated expression of *THI4* almost twofold (Fig. 3*C*). A tendency for up-regulation of *THIC* was observed under the same conditions; however, it was not statistically significant compared with the nonsupplemented culture (Fig. 3*C*). In contrast, supplementation with HMP caused a dramatic down-regulation of *THIC* expression (12-fold), whereas *THI4* expression was not significantly changed (Fig. 3*C*).

The alternative splicing of *Chlamydomonas THIC* pre-mRNA caused by thiamin supplementation results in a longer version of *THIC* mRNA ($THIC_L$) harboring a premature stop codon (Fig. 3*D*, *Upper*), which prevents expression of a full-length and functional THIC protein (6). In the context of these experiments, we checked whether a similar event occurs on supplementation with HMP. We observed a strong accumulation of the nonfunctional $THIC_L$ splicing variant in the presence of HMP (Fig. 3*D*, *Lower*), and indeed, under our conditions, the amount of $THIC_L$ was more pronounced with HMP than with thiamin supplementation (Fig. 3*D*). There was no significant change observed on HET supplementation. This implies that HMP supplementation induces a similar alternative splicing event to that observed with thiamin. Moreover, sequencing of the *THIC* splicing variant formed on HMP supplementation confirmed that it corresponds to $THIC_L$ (Fig. S1). Furthermore, an immunochemical analysis using an antibody raised against *Arabidopsis* THIC demonstrated loss of the full-length THIC protein from *Chlamydomonas* on supplementation with HMP (or thiamin), but not with HET (Fig. 3*E*). The higher mobility band observed with this antibody is specific for THIC, whereas the lower mobility band is nonspecific (Fig. 3*E*). As the steady-state levels of TPP do not change when HMP is added to the algal culture (Fig. 3*A*), these data suggest that *THIC* is responding directly to HMP.

HMP Specifically Regulates *THIC* Gene Expression Through the TPP Riboswitch. To compare the response of *THIC* to HMP and thiamin supplementation, we performed a time course of expression on treatment with low nanomolar quantities of HMP; that is, levels that had been shown to induce a response with thiamin (Fig. 2 *A* and *B*). The expression of *THI4* was monitored as a control. *THIC* responded to HMP within the first hour of treatment, similar to the response observed with thiamin (Fig. 4*A*). In contrast, although the expression of *THI4* was repressed by thiamin treatment, there was no effect on treatment with HMP under these conditions (Fig. 4*B*). As it had previously been reported that HMP is not a ligand for the TPP riboswitch (27), we sought to test whether HMP supplementation regulates *THIC* promoter activity. Therefore, we fused the 1,200-bp region immediately upstream of the start codon of *THIC* to the luciferase gene as a reporter construct and introduced this into *Chlamydomonas*. However, no significant effect on the luminescence of three independent transformed lines was observed in the presence of HMP (Fig. S2). This was also the case for cultures grown in the presence of HET and thiamin as controls (Fig. S2).

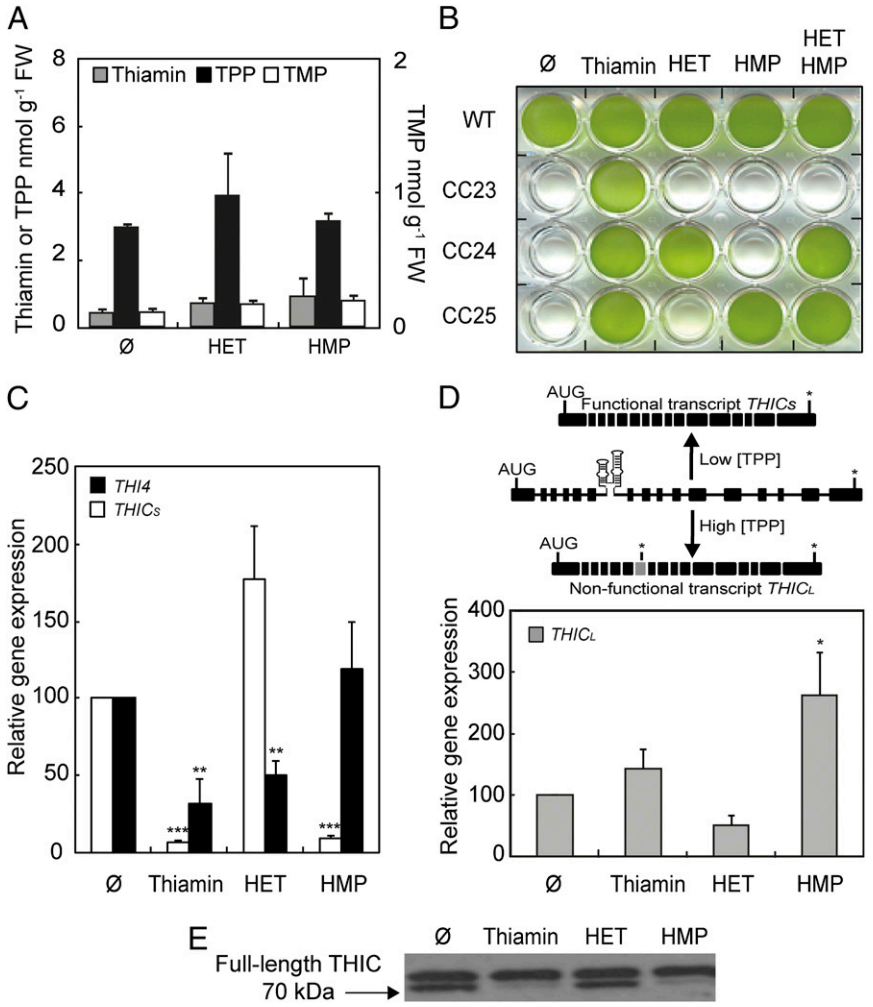


Fig. 3. HMP supplementation affects the *THIC* riboswitch. (*A*) B_1 vitamer content of wild-type *Chlamydomonas* grown for 3 d in the presence or absence of thiamin, HET, or HMP. Gray, thiamin; black, TPP; white, TMP. (*B*) Analysis of the rescue of thiamin-requiring mutants on supplementation with thiamin, HET, HMP, or HET and HMP. Algae were grown for 3 d. Note, CC24 and CC25 can be rescued by supplementation with only HET or HMP, respectively. (*C*) qPCR of *THI4* (black) and *THIC* (white) of wild-type strains grown for 3 d in the presence or absence of thiamin, HET, or HMP. The data were normalized to *actin*. (*D, Upper*) a scheme of *THIC* splicing events. The riboswitch is located in the intron between exon 6 and 7 (black boxes). Under low levels of TPP, a *THIC* transcript ($THIC_S$) leading to functional protein is produced. In the presence of high levels of TPP, a long splice variant is produced ($THIC_L$), which has a premature stop codon (*). (*Lower*) qPCR data showing relative expression of $THIC_L$ in wild-type cells grown for 3 d in the presence or absence of thiamin, HET, or HMP. The data were normalized to *actin*. (*E*) Immunochemical analysis of THIC on total protein extracted from wild-type cells grown for 3 d in the presence or the absence of thiamin, HET, or HMP. The arrow indicates the full-length THIC protein. In all supplementation cases, 10 μM of the respective compound was added. Values shown are the mean ± SD from three or more independent experiments. The asterisks indicate significant changes compared with no treatment and according to a Student *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

It may thus be possible that HMP itself can bind to the *THIC* riboswitch in vivo. Indeed, a study probing TPP riboswitch ligand interactions with a series of analogs concluded that the riboswitch is most selective for the pyrimidine heterocycle (28), although HMP did not bind the aptamer domain in vitro (27). However, it is noteworthy that the pyrophosphorylated form of HMP shares the key chemical features (i.e., pyrimidine and pyrophosphate; Scheme 1) that are considered to be essential for recognition by the TPP riboswitch (12, 13). Therefore, we tested whether it can bind to the *Chlamydomonas* riboswitch directly employing equilibrium dialysis, using the aptamer domain of the *THIC* riboswitch (Fig. S3) and [^{3}H]-HMP-PP. The results clearly demonstrate that HMP-PP can bind to the *THIC* aptamer to the same extent as TPP (Fig. 4*C*). We also assessed whether HMP or HMP phosphate (HMP-P) could be better ligands than HMP-PP, using a competition assay by equilibrium dialysis. The results show that unlike TPP, neither HMP-P nor HMP could displace HMP-PP binding (Fig. S4). In contrast, HMP-PP did not bind with any significance to the *THI4* aptamer (Fig. 4*C*). This may be accounted for by subtle ribonucleotide differences between the *THI4* and *THIC* aptamer.

We sought to explore this further by testing the effect of the individual precursors on the *THI4* riboswitch in vivo. The *THI4* 5′-UTR has been shown to be sufficient to cause repression of a gene fused downstream by the addition of exogenous thiamin (6, 29). We generated *Chlamydomonas* lines expressing the *THI4* 5′-UTR fused to the bleomycin-resistance gene (30). These lines are resistant to the antibiotic zeocin but die in the presence of thiamin and zeocin (Fig. 4*D*). The addition of HMP had no effect, confirming the results from the equilibrium dialysis. Intriguingly, HET supplementation showed reduced zeocin resistance, which might indicate that this precursor had a direct effect on the *THI4* gene. Notably, a similar effect on the transcript levels of the endogenous *THI4* gene was observed earlier (Fig. 3*C*).

Discussion

Several interesting points emerge in relation to thiamin metabolism and TPP riboswitches from this study of *Chlamydomonas*. First, it appears that a single point mutation in the *THI4* riboswitch is sufficient to considerably increase B_1 vitamer levels in this alga. This is significant because a recent study in the model plant *Arabidopsis* showed that the equivalent mutation in the *THIC* riboswitch affects only the TMP levels, with a threefold change (31). In our study on *Chlamydomonas*, all three vitamers are increased 5–10-fold (Fig. 1*B*). Moreover, despite the substantial increases in the levels of the vitamers observed in the *pyr1* mutant, there is no negative effect on the growth of the organism (Fig. S5). Furthermore, supplementation with HMP and HET also increases the levels of the vitamers (Fig. S6) with no perturbation of growth observable (Fig. 3*B*). This is in contrast to the *Arabidopsis* study, in which mutation of the riboswitch negatively affected metabolic homeostasis, resulting in chlorosis of the leaves, growth retardation, and delayed flowering. As in our study, there was enhanced expression of *THIC* in the *Arabidopsis* riboswitch mutant (31). The authors suggest that this is because an increase in the activity levels of enzymes dependent on TPP leads to a stronger flux through central metabolism, resulting in higher carbohydrate oxidation that in turn severely

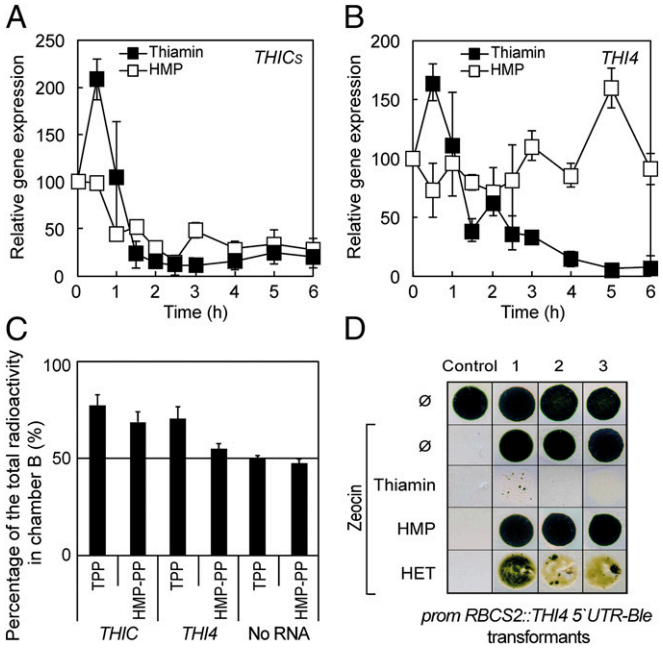


Fig. 4. HMP-PP binds to the *THIC* riboswitch. (*A* and *B*) qPCR of *THIC* (*A*) or *THI4* (*B*) over time after the addition of 50 nM thiamin (■) or 50 nM HMP (□). (*C*) Equilibrium dialysis using the *THI4* and *THIC* aptamers: 200 nM of radiolabeled ligand (TPP or HMP-PP) was introduced into chamber A, and 5 μM RNA was added to chamber B. The results are expressed as a percentage of the total radioactivity in chamber B. Detection of more than 50% of the radiolabeled ligand in chamber B indicates binding of the ligand. The details of the aptamer sequences used are given in Fig. S3. (*D*) Response of the *THI4* riboswitch to thiamin, HMP, or HET in vivo. Phenotypic response of three different *promRBCS2::THI4 5′-UTR-Ble* transformants and the host strain grown on TAP agarose plates in the presence of 50 μg/mL zeocin alone (Ø), or with 10 μM thiamin, HMP, or HET. The picture was taken 8 d postspotting. Values shown are the mean ± SD from three or more independent experiments.

influences plant physiology. However, it must be pointed out that considerable increases in the levels of TPP were not observed directly; rather, the maximal activity of certain TPP-dependent enzymes (i.e., pyruvate dehydrogenase and transketolase) was shown to increase (31). Although the physiological effect of enhanced thiamin levels in *Chlamydomonas* needs to be studied in more detail, it could provide an interesting alternative to plants in certain contexts in which the vitamin is required as a commodity for fortification by supplementation.

Second, our data strongly suggest that HMP-PP itself binds to the *THIC* riboswitch, which has major implications not only for our understanding of the regulation of thiamin metabolism in general but also for the dogma associated with the mechanism behind this riboswitch. Several pieces of evidence collected within this study corroborate this statement: Supplementation with HMP causes a dramatic down-regulation of *THIC* expression, whereas it has no effect on *THI4* expression under the same conditions, and moreover, the levels of TPP do not change from that without supplementation. Interestingly, the same alternative splicing event occurs on either HMP or thiamin supplementation, where the nonfunctional $THIC_L$ splicing variant accumulates with a corresponding loss in the level of the THIC protein (Fig. 3 *D* and *E*). However, in vitro studies have shown that HMP itself does not bind to the riboswitch (27), and our own studies here indicate that neither HMP nor HMP-P competes with HMP-PP binding. If we therefore assume that this also applies in vivo, then it is highly plausible that HMP is pyrophosphorylated by THID (32) and can bind to the riboswitch in a similar manner to TPP. Direct evidence that HMP-PP binds to the *THIC* riboswitch is provided by equilibrium dialysis (Fig. 4*C*). The question now arises as to how selectivity for either HMP-PP or TPP arises in vivo. In this context, it is expected that both branches of the TPP biosynthesis pathway would be balanced to produce TPP efficiently. HMP-PP would only accumulate under circumstances in which there is an imbalance in the provision of HET-P. This statement is corroborated by our observations with the *pyr1* mutant, in which the up-regulation of *THI4* (as a consequence of the impaired riboswitch) is balanced with a concomitant up-regulation of *THIC*, resulting in higher levels of the thiamin vitamers. Notably, the accumulation of HET under these conditions could induce the up-regulation of *THIC* by decreasing the negative control of TPP, the mechanism of which needs further investigation. However, it is important to note that it has recently been demonstrated that the homolog of *THI4* from *Saccharomyces cerevisiae* (THI4p) is a suicidal enzyme (1). The HET moiety is assembled from glycine, NAD^+, and sulfur from a backbone cysteine (Cys205) of the THI4p protein (1). This cysteine and several other key catalytic residues are conserved in the *Chlamydomonas* THI4 (Fig. S7), and therefore, a similar mechanism can be anticipated. Under these conditions, as THI4 can only catalyze a single turnover, its levels would be expected to be higher than those of THIC. Indeed, our own analysis here and the RNA-seq data of others (Fig. 2 *C* and *D*) allow us to conclude that the *THI4* transcript is in fact more abundant than that of *THIC*. Moreover, a previous proteomic study of *Chlamydomonas* was able to identify only THI4 among the thiamin biosynthetic enzymes, suggesting it was more abundant (33). We propose, therefore, that in *Chlamydomonas*, the *THIC* riboswitch can recognize both HMP-PP and TPP as ligands because of the nature of its thiamin biosynthesis pathway. When both branches of the pathway are balanced, it is the build-up of TPP that will switch both branches off. However, if the production of HMP-PP by THIC is not balanced by an equal production of HET, then the recognition of HMP-PP by the riboswitch provides an additional checkpoint to switch off *THIC* expression. As THI4 is used up anyway in the provision of HET, such a short-circuit may not be required for this branch of the pathway. On the other hand, the effect of HET supplementation on the transcript levels of *THI4* (Fig. 3*C*) and activity of the transgenic riboswitch (Fig. 4*D*), albeit not as dramatic as with HMP on *THIC*, might argue for a similar feedback loop. In this context, a previous study using a WaterLOGSY (Ligand Observed via Gradient SpectroscopY) NMR provided evidence for weak binding of HET to the *Escherichia coli thiM* riboswitch (27). Alternatively, the observed effect on *THI4* expression in this study might be mediated by subtle alterations in metabolite levels. It is noteworthy that plants also use a homolog of *THI4* (named *THI1*) for the biosynthesis of the HET moiety (Fig. S7). However, although lower plants have a riboswitch controlling each branch of the pathway, similar to *Chlamydomonas*, higher plants have evolved to use just one riboswitch (i.e., that in *THIC*) to regulate the entire pathway. It must also be mentioned that the up-regulation of *THIC* in the *pyr1* mutant, if as a consequence of the imbalance in the provision of HET, suggests crosstalk between both branches of the pathway.

In summary, we believe that this study of thiamin metabolism in vivo has revealed the plasticity of riboswitches and that the regulation of the biosynthesis of this compound is more complex than originally thought. The use of different pathways by different organisms for thiamin biosynthesis and the corresponding divergence in the number and mechanism of riboswitch controls provides an illuminating example of how this particular RNA sensor has evolved and adapted to the specific environment in which it operates. Future studies on the in vivo effect on modulation of riboswitches may further reveal their plasticity as a function of the particular environment and pathway used.

Materials and Methods

Strains and Media. *C. reinhardtii* (wild-type strain 12) was a gift from Jean David Rochaix, University of Geneva. The mutant *pyr1* (CC1669) and thiamin-requiring mutants (CC23-CC25) were received from the *Chlamydomonas* Resource Center (http://chlamycollection.org), University of Minnesota

(St. Paul). *Chlamydomonas* strains were grown in Tris·acetate phosphate (TAP) medium (34) with or without thiamin (Sigma), HET (Sigma) or HMP (Exclusive Chemistry Ltd) supplementation at 10 μM unless otherwise indicated, under continuous light (60 μmol $m^{-2}s^{-1}$) at 25 °C and with shaking at 150 rpm. For growth on plates, 2% (wt/vol) agarose was added.

Determination of Thiamin and Its Esters. For analysis of the B_1 vitamer content, a 14-mL sample of midlog-phase *Chlamydomonas* culture was collected by centrifugation at 4,000 × *g* for 5 min and washed with distilled water three times and weighed. The cells were resuspended in 1 mL 1% (vol/vol) trichloroacetic acid. The mixture was centrifuged for 10 min at 10,000 × *g* and the supernatant decanted. Fifty microliters of sample were derivatized by the addition of 10 μL freshly prepared 30 mM potassium ferricyanide in 15% (wt/vol) sodium hydroxide, 15 μL 1 M sodium hydroxide, and 25 μL 100% methanol. After mixing, the samples were centrifuged for 10 min at 4,000 × *g* and transferred to vials. The extract was separated on a Cosmosil II-NAP column (150 × 4.2 mm, 3 μm pore size), using an Agilent 1200 HPLC. In each case, 20 μL of sample was chromatographed using a methanol (MeOH) gradient under the following conditions: 0–20 min, 5–90% MeOH; 20–21 min, 90–100% MeOH; 21–25 min, 100% MeOH; 25–26 min, 100–5% MeOH; and 26–40 min, 5% MeOH at a flow rate of 1 mL/min. The vitamers were detected by online fluorescence, using an excitation wavelength of 375 nm and emission wavelength of 450 nm, and quantified from standard curves. The data were normalized to the fresh weight of cells.

Plasmid Construction. The *promTHIC::LUC* construct was made from pCB740 (*Chlamydomonas* stock center) into which a cassette had been inserted encoding the *Ble* gene for selection on zeocin, to form pCBMTB. The *Gaussia princeps* luciferase gene (35) was cloned using the *SpeI* site of the multiple cloning site to generate pCBMTB-GLuc. The *THIC* promoter (1,200 bp) was amplified from *Chlamydomonas* genomic DNA with primers that included *NotI* and *SpeI* restriction sites (Table S1) and ligated into pCBMTB-GLuc to generate *promTHIC::LUC*. The *promRBCS2::THI4 5′-UTR-Ble* plasmid was constructed by Gibson assembly (36), using the isothermal method, at 50 °C for 1 h, from the following 5 different parts: promoter of RBCS2 (37), *THI4 5′-UTR* (6), *Ble exon 1-RBCS2 intron-Ble exon 2* (28), with the pUC18 backbone harboring the chloramphenicol resistant gene from pBAD28 (38). The primers (Table S1) for each part have 20–25-bp flanking ends, which are homologous to the adjoining parts to allow for Gibson assembly.

RNA Binding Studies. The equilibrium dialysis assays were performed using fast microequilibrium dialysers (Harvard Apparatus) in which two chambers (A and B) were separated by a 10,000 MWCO membrane. [^{3}H]-TPP (20 Ci/mmol) and [^{3}H]-HMP-PP (1 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. and Vitrax Inc., respectively. The final composition of buffer was 50 mM Tris·Cl at pH 8, containing 20 mM magnesium chloride. Five micromoles of *THIC* or *THI4* RNA aptamers transcribed in vitro (Fig. S3) were introduced into chamber B, and the assay initiated by the addition of either 200 nM [^{3}H]-TPP or [^{3}H]-HMP-PP (500 cpm·$pmol^{-1}$) to chamber A. For the displacement assays, 2 μM TPP, HMP-P, or HMP was added to chamber A in the presence of [^{3}H]-HMP-PP. After an overnight equilibration at 4 °C, a 20-μL aliquot from each chamber was diluted into Emulsifier Safe scintillation mixture (Perkin-Elmer) and quantified using a Beckman LS6500 liquid scintillation counter.

ACKNOWLEDGMENTS. We thank Prof. Jean-David Rochaix for his help and advice over the course of this study, as well as Dr. Finian J. Leeper for useful comments and suggestions on the manuscript. Dr. Zetty Norhana Balia-Yusof (University of Cambridge) is acknowledged for growth of the *pyr1* mutants. This work was funded by a Marie Curie Intra-European Fellowship within the Seventh European Community Framework Programme [IEF-2009-254907–RIBOREGAL (role of RIBOswitches in REGulating matabolism in ALgae); to M.M.], the Swiss National Science Foundation (Grant PP00A_119186 to T.B.F.), and the Ernst and Lucie Schmidheiny and the Mark Birkigt foundations (to T.B.F.). M.A.S. was supported by the UK Biotechnology and Biological Sciences Research Council.

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