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Orchestration of Thiamin Biosynthesis and Central Metabolism by Combined Action of the Thiamin Pyrophosphate Riboswitch and the Circadian Clock in *Arabidopsis*[C][W]

Samuel E. Bocobza,[a] Sergey Malitsky,[a] Wagner L. Araújo,[b,1] Adriano Nunes-Nesi,[b,1] Sagit Meir,[a] Michal Shapira,[c] Alisdair R. Fernie,[b] and Asaph Aharoni[a,2]

[a] Department of Plant Sciences, Weizmann Institute of Science, 76100 Rehovot, Israel
[b] Max-Planck-Institut fur Molekulare Pflanzenphysiologie, 14476 Potsdam-Golm, Germany
[c] Department of Life Sciences, Ben Gurion University at the Negev, 84105 Beer Sheva, Israel

Riboswitches are natural RNA elements that posttranscriptionally regulate gene expression by binding small molecules and thereby autonomously control intracellular levels of these metabolites. Although riboswitch-based mechanisms have been examined extensively, the integration of their activity with global physiology and metabolism has been largely overlooked. Here, we explored the regulation of thiamin biosynthesis and the consequences of thiamin pyrophosphate riboswitch deficiency on metabolism in *Arabidopsis thaliana*. Our results show that thiamin biosynthesis is largely regulated by the circadian clock via the activity of the *THIAMIN C SYNTHASE (THIC)* promoter, while the riboswitch located at the 3′ untranslated region of this gene controls overall thiamin biosynthesis. Surprisingly, the results also indicate that the rate of thiamin biosynthesis directs the activity of thiamin-requiring enzymes and consecutively determines the rate of carbohydrate oxidation via the tricarboxylic acid cycle and pentose-phosphate pathway. Our model suggests that in *Arabidopsis*, the *THIC* promoter and the thiamin-pyrophosphate riboswitch act simultaneously to tightly regulate thiamin biosynthesis in a circadian manner and consequently sense and control vital points of core cellular metabolism.

INTRODUCTION

Riboswitches are pre-mRNA elements that regulate gene expression in response to intracellular concentration of specific metabolite ligands. Typically, direct binding of a ligand to the RNA molecule induces RNA structural rearrangements that affect expression of genes involved in the biosynthesis, catabolism, and transport of this ligand (Mironov et al., 2002; Mandal and Breaker, 2004). Thus, riboswitch-mediated gene expression is directly determined by the intracellular concentration of the ligand and by the affinity of the riboswitch for this ligand (Winkler et al., 2002; Mandal et al., 2003). This allows autotrophic organisms to tightly control the levels of essential metabolites without protein involvement. While riboswitches are widespread in bacteria, only a single thiamin pyrophosphate (TPP) binding riboswitch has been reported in eukaryotes (Bocobza et al., 2007; Cheah et al., 2007; Croft et al., 2007; Wachter et al., 2007). In bacteria, TPP binding to the TPP riboswitch represses the thiamin operon at the transcriptional (Mironov et al., 2002) and translational (Winkler et al., 2002) levels. In fungi, algae, and plants, the TPP riboswitch regulates gene expression through alternative splicing of the pre-mRNA (Bocobza et al., 2007; Cheah et al., 2007; Croft et al., 2007; Wachter et al., 2007). Notably, in bacteria, the genes involved in thiamin metabolism and transport are located in operons, each of which are regulated by TPP riboswitches, but in higher plants the TPP riboswitch has evolved to regulate just the *THIAMIN C SYNTHASE* (*THIC*) gene (Bocobza and Aharoni, 2008).

In *Arabidopsis thaliana*, three enzymes participate together in the synthesize of thiamin monophosphate (TMP), namely, TH1 (Ajjawi et al., 2007a), THI1 (Belanger et al., 1995; Machado et al., 1996), and the TPP riboswitch–regulated THIC (Bocobza et al., 2007; Raschke et al., 2007; Wachter et al., 2007; Kong et al., 2008). Unlike in prokaryotes where TMP can be directly converted into TPP (Begley et al., 1999), in *Arabidopsis*, TMP is subsequently dephosphorylated into thiamin (Komeda et al., 1988), which is then pyrophosphorylated into TPP by the thiamin pyrophosphokinases (TPKs), namely, TPK1 and TPK2 (Ajjawi et al., 2007b).

TPP is the active form of thiamin and is also the most abundant intracellular form of this vitamin in yeast (Schweingruber et al., 1991), plants (Ajjawi et al., 2007a), and animal tissues (Rindi and De Giuseppel, 1961). It is an essential coenzyme for the enzyme complexes pyruvate dehydrogenase (PDH) and 2-oxoglutarate dehydrogenase (2-OGDH) of the tricarboxylic acid (TCA) cycle and for the α-ketose transketolase (TK) homodimer of the pentose phosphate pathway (PPP; Frank et al., 2007).

[1] Current address: Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Viçosa, Brazil.
[2] Address correspondence to asaph.aharoni@weizmann.ac.il.
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Intriguingly, these enzyme complexes appear to be the controlling steps of the pathways they are involved in (Berthon et al., 1992; Wang et al., 1997a, 1997b; Henkes et al., 2001; Araújo et al., 2008), and TPP is additionally limiting for their assembly (Singleton and Martin, 2001; Tovar-Méndez et al., 2003; Bunik and Fernie, 2009). Altogether, the above facts emphasize the importance of TPP for the proper functioning of the TCA cycle and the PPP and consequently for general energy production in all cells.

Thus far, extensive research efforts have been devoted to the determination of the molecular mechanisms that underlie the capacity of riboswitches to regulate gene expression. Yet, very little is known about the integration of these RNA regulatory elements with the physiology and metabolism of the organism, including the physiological consequence(s) of riboswitch malfunction on the fitness of multicellular organisms. Here, we investigated in detail how the 5′ (containing the promoter) and 3′ (containing the riboswitch) regions of the *Arabidopsis THIC* gene participate in the regulation of *THIC* expression. The former exposed the role of the biological clock in the transcriptional regulation of thiamin metabolism, while the latter revealed the role of the TPP riboswitch and its ligand in plant core metabolism. Interestingly, we observed that riboswitch-deficient plants displayed increased activities of the thiamin-requiring enzymes. As a consequence, carbohydrate oxidation through the TCA cycle and the PPP was enhanced, and amino acids accumulated. Given the universality of the thiamin-requiring enzyme complexes and their associated metabolic pathways, these results further suggest a crucial role of TPP in the regulation of core, cellular metabolism in all living organisms.

RESULTS

THIC Is Regulated in a Circadian Manner and the CCA1 and LATE ELONGATED HYPOCOTYL Clock Proteins Bind the Evening Element in Its Promoter

To investigate the regulatory mechanism that controls thiamin biosynthesis and the involvement of the TPP riboswitch in this process, we first examined the changes in the expression of the thiamin biosynthetic genes and especially those of the *Arabidopsis THIC* gene and its splicing products, throughout the day period. The two splicing variants in the *THIC* 3′ untranslated region (UTR) were evaluated since their levels were found previously to be riboswitch dependent and to respond to altered cellular TPP concentrations (i.e., the unstable intron-spliced variant increases and the stable intron-retained variant decreases when levels of the TPP ligand rise and vice versa; Bocobza et al., 2007; Wachter et al., 2007). In a circadian assay, we found that *THIC* and its splice variants displayed significant circadian oscillations (Figure 1A). The significance of oscillations of transcripts and metabolites were determined using the N-model (Haus and Touitow, 1992) and cosinor (Refinetti et al., 2007) programs. The highest relative transcript levels occurred at the end of the expected light period and the lowest at the end of the expected period of darkness. In contrast with *THIC*, the expression of other thiamin pathway genes (i.e., *THI1*, *TH1*, *TPK1*, and *TPK2*) did not display oscillations (Figure 1A).

These results raised the question whether the TPP riboswitch takes part in the circadian regulation of *THIC* or whether additional regulatory elements (e.g., the promoter) are directly responsible for these oscillations. To separate between these two modes of gene control (i.e., the promoter and the riboswitch), a dual reporter assay was developed that allowed us to observe simultaneously the in vivo activity of both elements. Expression of one reporter gene (*yellow fluorescent protein* [*YFP*]) was driven by a constitutive promoter (35S *cauliflower mosaic virus* [CaMV]) and fused to the *THIC* 3′ UTR, and a second reporter (*red fluorescent protein* [*RFP*]) was placed under the control of the native *THIC* promoter and fused to the *NOPALINE SYNTHASE* (*NOS*) terminator (see Supplemental Figure 1A online). These two reporters were introduced into the *Arabidopsis* wild-type genetic background and their expression monitored in a circadian manner. We found that, in these plants, both the *THIC* transcript and the *RFP* reporter (driven by the *THIC* promoter) displayed significant circadian oscillations, identical to those of *THIC*, but *YFP* expression (fused to the riboswitch-containing *THIC* 3′ UTR) remained constant (Figure 1B). This result indicated that the circadian oscillations observed in *THIC* expression are merely the direct consequence of promoter activity and are not the result of riboswitch action.

In order to determine whether the circadian oscillations described above were a consequence of biological clock activity, we followed the expression of the thiamin biosynthetic genes in transgenic plants altered in their biological clock. In this experiment, we used *Arabidopsis* plants overexpressing the *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) gene (Wang and Tobin, 1998; i.e., *ox-CCA1*). In a circadian assay, we observed that in this genotype, the circadian expression of *THIC* and its splice variants were altered compared with the wild type. In addition, we performed this assay using the *prr9-11 prr7-10 prr5-1* triple mutant (*d975*), which is affected in the expression of its *PSEUDO-RESPONSE REGULATOR* genes (Nakamichi et al., 2009). We found that expression of *THIC* and its splice variants was particularly altered in this line (Figure 1C; see Supplemental Figure 1B online; the *GRP7* gene [Heintzen et al., 1997] was used as a positive control).

Interestingly, the relative transcript levels of *THIC* and *CCA1* oscillate in an opposite pattern (Figure 1A). Since CCA1 is a transcriptional repressor that shares overlapping functions with LATE ELONGATED HYPOCOTYL (LHY) (Mizoguchi et al., 2002), we asked whether a direct regulation of *THIC* by CCA1 or LHY could occur. This was even more relevant since an evening element present in the *THIC* promoter (AAAATATCT, 128 bp upstream of the *THIC* 5′ UTR) was found to be a requirement for the evening-phased expression of genes repressed by CCA1 (Alabadí et al., 2001; Covington et al., 2008). Thus, we performed an electrophoretic mobility shift assay (EMSA) using purified CCA1 or LHY proteins and a double-stranded oligomer that corresponds to the *THIC* promoter region that contains the evening element present in the *THIC* promoter. We found that both the CCA1 and the LHY proteins were able to bind this evening element in vitro (Figure 1D). This interaction was confirmed by competition experiments using unlabeled native or mutated oligomers.

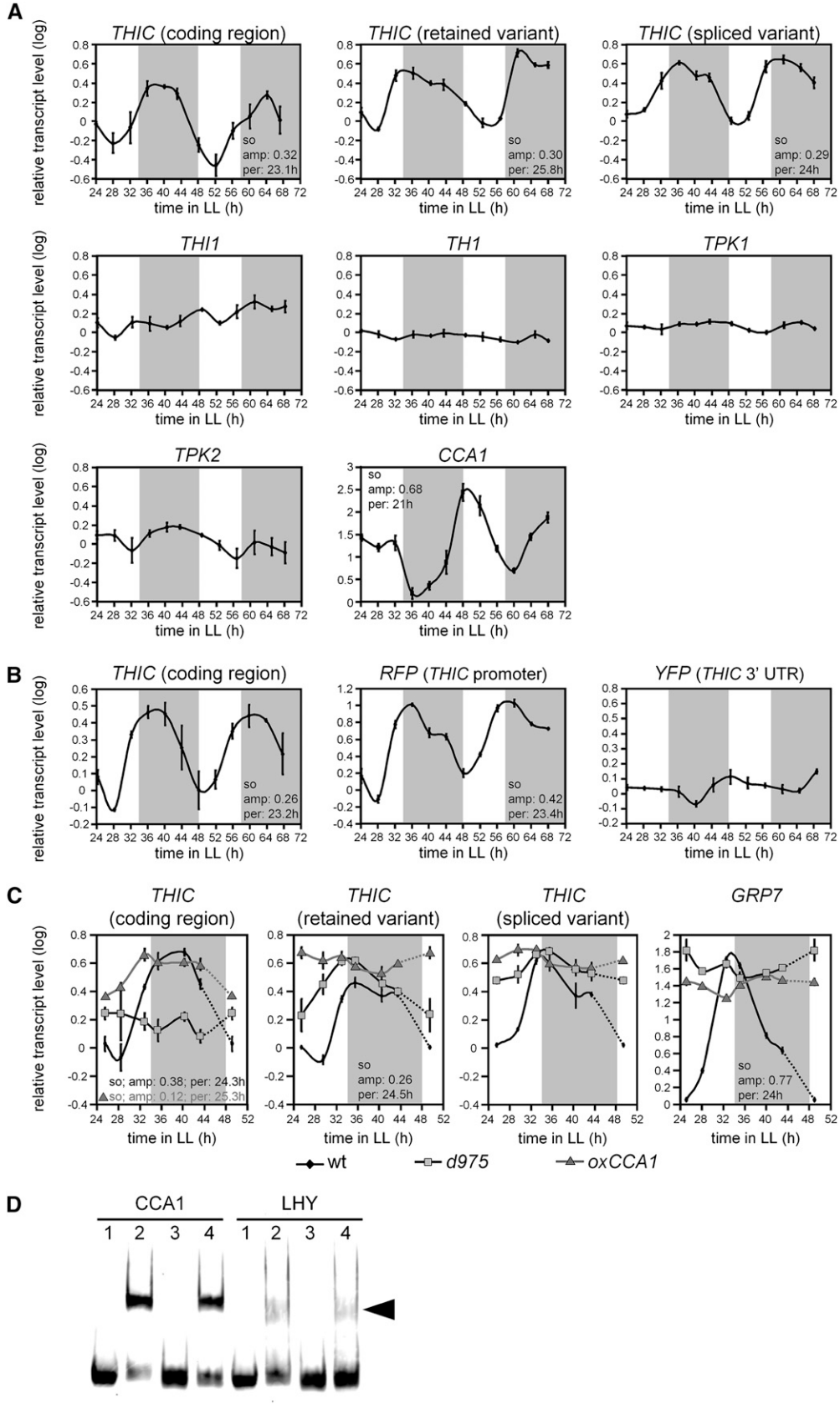

Figure 1. Circadian Regulation of the Thiamin Biosynthetic Gene *THIC*.

(A) Circadian expression of the thiamin biosynthetic genes measured by qPCR (values are presented as means ± SE using three independent biological replicates; $n = 3$) in *Arabidopsis* plants grown under short-day conditions for 21 d, prior to being transferred to constant light (LL) for the indicated time. The expected light and dark periods are indicated by white and gray backgrounds, respectively.

These observations suggested that the levels of thiamin metabolites (i.e., TMP, thiamin, and TPP) could also be modulated in a circadian manner. Following the levels of thiamin esters up to 44 h in exposure to constant light (see Methods), we observed that TMP levels displayed a significant oscillation, although detected only in a single period, and TPP levels displayed only a mild oscillation (Figure 2A; thiamin could not be detected in this assay). To clarify whether the oscillations of TMP levels were the consequence of the biological clock activity, we measured TMP in the *ox-CCA1* line. We observed that TMP oscillations were altered in this genotype, but TPP levels were not affected (Figure 2A). A similar result was obtained using *d975* mutant plants. Together, these results provided evidence that thiamin biosynthesis is regulated by the circadian clock.

The *THIC* Promoter Directs Gene Expression in Green Tissues, While the TPP Riboswitch Regulates Gene Expression in a TPP-Dependent Manner

We took advantage of the dual reporter system to elucidate how the *THIC* promoter and 3′ UTR, which contains the TPP riboswitch, act jointly to adjust *THIC* expression. In this assay, expression of one reporter gene (*YFP*) was driven by a constitutive promoter (35S CaMV) and fused to the *THIC* 3′ UTR, while a second reporter (*RFP*) was placed under the control of the native *THIC* promoter and fused to the *NOS* terminator (see Supplemental Figure 1A online). We observed that in fully grown plants (in the wild-type background), the *THIC* promoter directs *RFP* expression in all green tissues, but not in roots, seeds, and petals. By contrast, the *THIC* 3′ UTR represses *YFP* expression in green tissues, probably due to endogenous TPP levels (see Supplemental Figure 2A online). In order to determine if either the *THIC* promoter and/or the 3′ UTR containing the riboswitch element respond to altered TPP levels, wild-type plants harboring the two reporters were exposed to increasing TPP concentrations. In this experiment, increasing TPP concentration caused a moderate reduction in *YFP* expression but did not affect *RFP* expression. The moderate reduction in *YFP* expression indicated that endogenous TPP levels present in wild-type plants are, at least partially, sufficient to mask the effect of the exogenous TPP applied (see Supplemental Figure 2B online). To clarify this, we used *Arabidopsis thi1* mutant plants, which are deficient in thiamin biosynthesis and whose phenotype can be rescued by the supplementation of thiamin, TMP, or TPP. When the reporter genes were cointroduced into *thi1* mutant plants, the *THIC* promoter did not respond to exogenous TPP levels, as observed in the wild-type background. By contrast, the *THIC* 3′ UTR induced a strong reduction in *YFP* expression upon high TPP concentration (Figures 2B and 2C; untransformed plants did not display autofluorescence; see Supplemental Figure 2C online). Overall, these results suggested that the *THIC* promoter directs gene expression in a time- and organ-specific manner but is not TPP dose responsive, but the riboswitch-containing *THIC* 3′ UTR regulates gene expression in response to TPP, in a concentration-dependent manner.

Disruption of the TPP Riboswitch Activity and Its Effects on the Plant Life Cycle

To determine the significance and the role of the TPP riboswitch during the plant life cycle, we generated transgenic lines that are specifically altered in their TPP riboswitch function. To this end, we engineered transgenic *Arabidopsis* plants that display TPP riboswitch deficiency. This was achieved by generating two *THIC* expression cassettes, containing the promoter, the gene, and the 3′ region (containing the TPP riboswitch) of *THIC*, with or without an A-to-G point mutation in the TPP riboswitch (i.e., A515G, relative to the stop codon) that reduces its activity (Sudarsan et al., 2005; see Supplemental Figure 3A online). These cassettes were introduced independently into the background of an *Arabidopsis* mutant in which a T-DNA insertion abolishes *THIC* expression (Kong et al., 2008). Interestingly, while plants harboring the native TPP riboswitch (thereafter referred as control plants) did not display any particular phenotype under short-day conditions (i.e., 10 h/14 h, day/night), those harboring the deficient TPP riboswitch exhibited chlorosis, growth retardation, and delayed flowering (observed in 10 independent transformed lines; Figure 3A; see Supplemental Figure 3B online). Interestingly, chlorosis and the delayed flowering were less apparent if plants were grown under or transferred to long-day conditions (i.e., 18 h/6 h, day/night; see Supplemental Figure 3C online). We further assessed the starch levels of 8-week-old mature plants, grown under short-day conditions, at the beginning and the end of the light photoperiod. We

Figure 1. (continued).

(B) Circadian expression of *RFP*, *YFP*, and *THIC*, observed in *Arabidopsis* plants harboring the double reporter gene system (see Supplemental Figure 1A online), resolved by qPCR ($n = 3$; SE). *RFP* expression is directed by the *THIC* promoter, and *YFP* expression is controlled by the CaMV 35S promoter and is fused to the *THIC* 3′ UTR (containing the riboswitch).

(C) Circadian expression of the *THIC* gene, its splice variants, and the *GRP7* gene measured by qPCR ($n = 3$; SE) observed in *Arabidopsis d975* mutants (square), *CCA1* overexpressers (triangle), and wild-type (wt; black) plants.

(D) Binding of the CCA1 and LHY proteins to the evening element located in the promoter of the *THIC* gene detected by an EMSA. Fluorescent-labeled oligonucleotides were separated on a 7% Tris-Gly PAGE without additional treatment (lane 1) or with 3 μg of the respective purified protein (lanes 2 to 4). Competition experiments were performed using a 100-fold native or mutated cold probe (lanes 3 and 4, respectively). The arrow indicates the band corresponding to the LHY probe complex.

Note: "so" (for significant oscillation) indicates that the oscillation displayed by a given graph (in **[A]** to **[C]**) is significant (P value < 0.05) according to both N-model and cosinor analyses (see Methods). The amplitude (amp) and the period (per) of the significant oscillations are indicated in the same color as the corresponding graph.

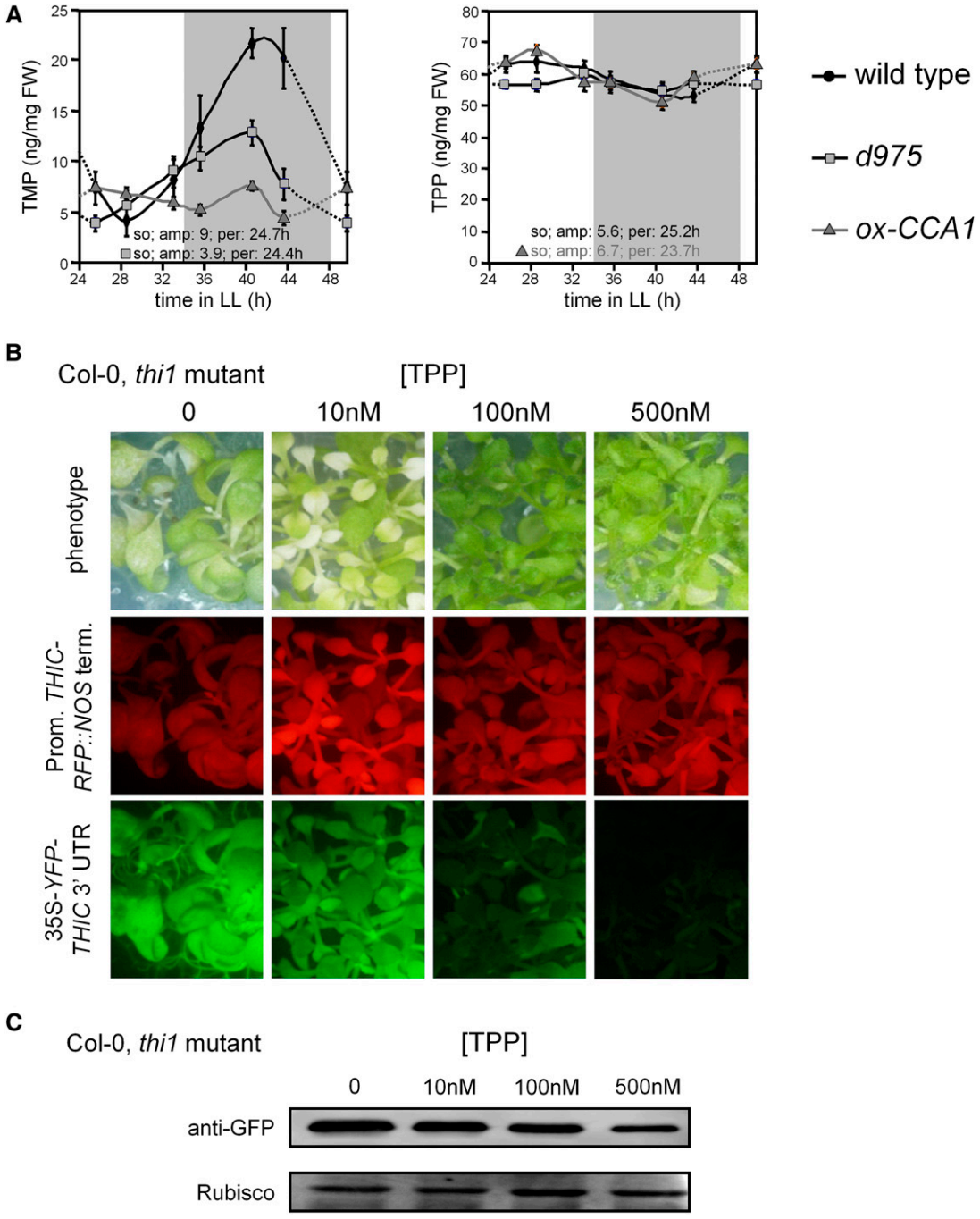

Figure 2. Circadian Oscillations of Thiamin Esters and Response of the *THIC* Promoter and 3′ UTR to Exogenous TPP Application.

(A) Circadian levels of TMP and TPP, observed in the aerial parts of 21-d-old *d975* mutants (square), *CCA1* overexpressers (triangle), and wild-type (black) *Arabidopsis* plants, were monitored by HPLC analysis (n = 5; SE). "so" (significant oscillation) indicates that the oscillation displayed by a given graph is significant (P value < 0.05) according to both N-model and cosinor analyses (see Methods). The amplitude (amp) and the period (per) of the significant oscillations are indicated in the same color as the corresponding graph. FW, fresh weight.

(B) *RFP* and *YFP* reporter genes expression monitor the activities of the *THIC* promoter (Prom.) and the *THIC* 3′ UTR (containing the riboswitch), respectively. The experiments were performed in the *Arabidopsis thi1* mutant background harboring the double reporter gene system (see Supplemental Figure 1A online), grown for 10 d in vitro and supplied with the indicated TPP concentrations. Untransformed plants do not display autofluorescence (see Supplemental Figure 2C online). Comparable results were obtained with two additional independent transgenic lines. term., terminator.

observed lighter iodine staining (i.e., reduced starch levels) at dawn in plants harboring a deficient riboswitch (Figure 3B). Examination of the leaf chloroplast ultrastructure in these plants using transmission electron microscopy revealed that chloroplasts derived from plants deficient in riboswitch activity were amorphous. They also possessed altered starch grain structure (Figure 3C), suggesting an effect on starch metabolism.

Riboswitch deficiency had a significant effect on *THIC* gene expression. Its total mRNA level and that of the intron-retained variant were upregulated in the transgenic lines harboring a deficient TPP riboswitch compared with wild-type and control plants. Reversely, expression of the intron-spliced variant was downregulated in the riboswitch-deficient plants (Figure 3D). It should be noted that since the A515G mutation of the TPP riboswitch prevents intron splicing (Bocobza et al., 2007; Wachter et al., 2007), the intron-spliced variant measured in this analysis is likely derived from the poorly expressed endogenous *THIC* gene rather than from the transgene. One cannot differentiate experimentally between the two variants in this case.

We also performed a circadian assay in these transgenic plants and observed that *THIC* and its retained variant oscillate in a circadian manner. Notably, in the plants harboring a deficient riboswitch, the *THIC* gene and its intron-retained variant were upregulated during the whole period of measurement compared with control plants (Figure 3E). These results indicated that TPP riboswitch deficiency elicited intron retention and thereby triggered the overexpression of *THIC*, by elevating the level of the more stable retention variant.

We next evaluated whether the levels of TMP, thiamin, and TPP, which are synthesized natively in this order, were altered due to riboswitch deficiency. The results showed that plants carrying a deficient riboswitch contained am approximately threefold increase in TMP levels compared with the plants carrying a functional riboswitch and wild-type plants (Figure 4A), whereas TPP contents were only moderately elevated (Figure 4B). We also measured the levels of thiamin esters in seeds and detected that riboswitch-deficient plants accumulated ~20% more thiamin in seeds but did not accumulate TMP or TPP, which are normally absent in this sink tissue (Molin et al., 1980; Figure 4B).

To assess whether the overexpression of *THIC* caused by riboswitch deficiency was the sole cause of increased TMP levels, we measured the transcript levels of the other thiamin biosynthetic genes in plants deficient in riboswitch activity. We found that in these plants, the transcript levels of *TH1* and *TPK1* were significantly but moderately increased, while levels of *THI1* and *TPK2* remained unchanged (see Supplemental Figure 4A online). Since overexpression of *TH1* does not increase TMP levels (Ajjawi et al., 2007a), this result suggested that the reaction performed by THIC may be rate limiting for TMP biosynthesis and that riboswitch deficiency could directly increase thiamin biosynthesis at the posttranscriptional level. Furthermore, to determine whether the deficient riboswitch could in turn alter the circadian clock, the relative transcript level of the *GRP7* gene was measured in a circadian assay. We found that riboswitch deficiency did not alter the significant circadian oscillations of this transcript compared with wild-type and control plants (see Supplemental Figure 4B online).

Further examination of the transgenic plants deficient in riboswitch activity revealed that TMP levels displayed an oscillatory trend and its levels were highest during the period of expected darkness (Figure 4C). However, in the control plants, TMP circadian oscillations were less obvious. Notably, no obvious oscillations were observed for the TPP levels of plants harboring the defective riboswitch and that of control plants (Figure 4C). Furthermore, the levels of TMP and TPP were higher in the riboswitch-deficient plants during the entire sequence of this experiment, as compared with control plants, corroborating the evidence that thiamin biosynthesis was increased by riboswitch deficiency.

To confirm that *THIC* overexpression itself will result in increased TMP levels, we expressed the *THIC* coding sequence under the control of the *Arabidopsis UBIQUITIN1* promoter and terminator, which does not contain a riboswitch (Callis et al., 1990). Two of the several independent *THIC* overexpression lines exhibited a chlorotic phenotype, while elevation of *THIC* expression and an increase in TMP and TPP levels were observed in five independent lines (Figures 4E and 4F). Thus, *THIC* overexpression, whether it was caused by riboswitch deficiency or by a transgene, increased TMP and TPP levels resulting in a chlorotic phenotype.

Alteration of Thiamin Metabolism Due to Riboswitch Deficiency Enhances the Activity of Thiamin-Requiring Enzymes and Augments Carbohydrate Oxidation

TMP and thiamin are the precursors for TPP biosynthesis, which serves as an obligatory ligand for the key enzymes involved in both the TCA cycle and the PPP (Frank et al., 2007; see Supplemental Figure 5A online). Since we observed that TPP riboswitch disruption resulted in an approximately threefold increase in TMP but only in a slight, ~20%, increase in TPP levels, we hypothesized that the TPP turnover was augmented in these plants, as it was used by the thiamin-requiring enzymes. To evaluate this, enzymatic activities of the three thiamin-requiring enzymes, PDH, 2-OGDH, and TK, were measured in extracts of plants harboring the defective riboswitch and compared with those harboring the construct with a native riboswitch and to

Figure 2. (continued).

(C) Protein levels of YFP detected by protein gel blot. Soluble proteins were extracted from *Arabidopsis thi1* mutant background harboring the double reporter gene system (see Supplemental Figure 1A online), grown for 10 d in vitro and supplied with the indicated TPP concentrations. Proteins were separated on 10% PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane, which was subsequently immunodetected with anti-GFP antibodies. Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.
[See online article for color version of this figure.]

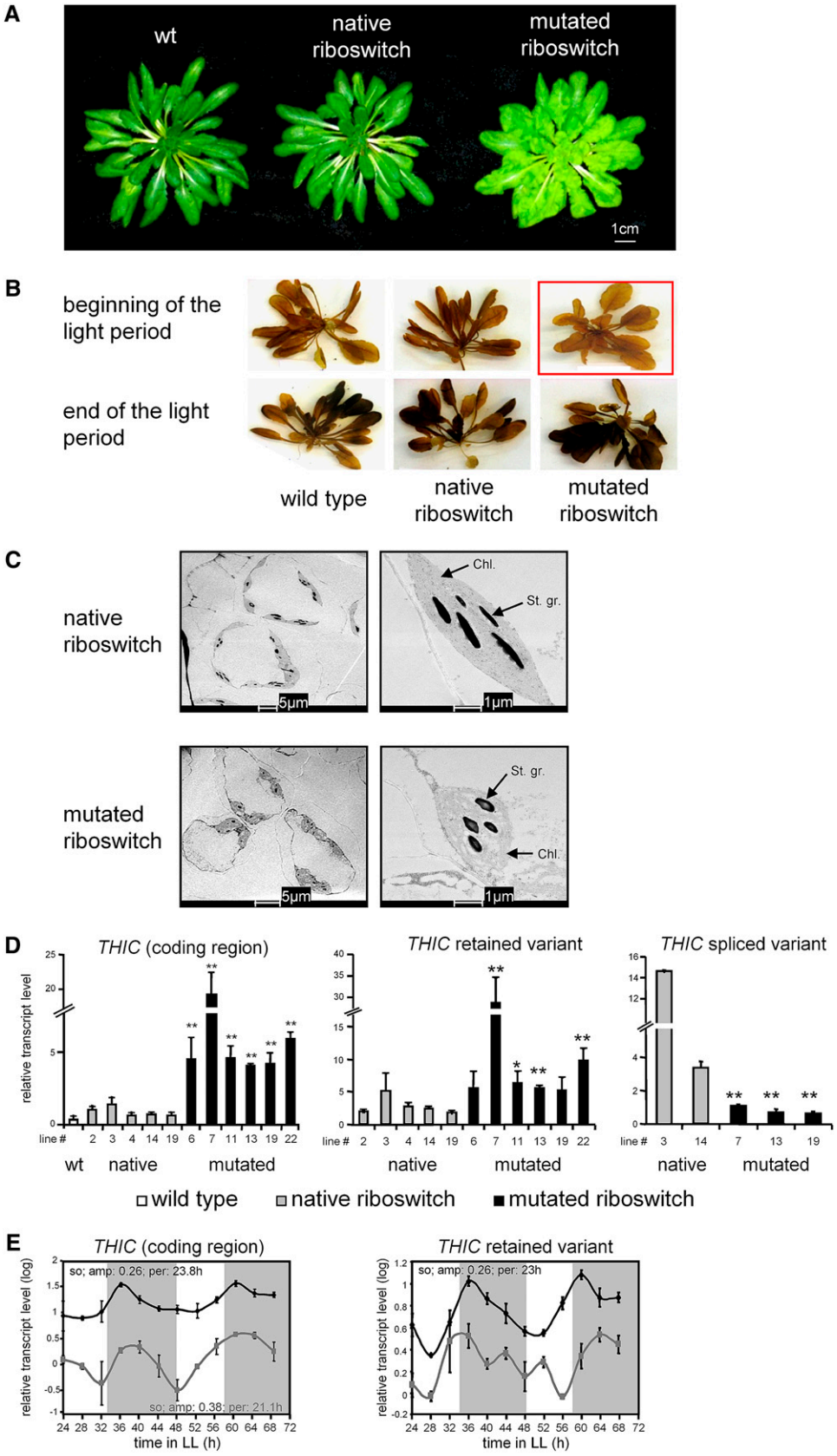

Figure 3. Effects of TPP Riboswitch Deficiency on *Arabidopsis* Phenotype and on *THIC* Expression.

wild-type plants. This assay was performed in vitro, using desalted protein extracts where TPP was removed, by supplementing increasing TPP levels. Interestingly, thiamin-requiring enzymes extracted from plants deficient in riboswitch activity displayed in vitro higher enzymatic activities in the presence of increasing TPP concentrations compared with the control and wild-type plants (Figure 5A). The increased activity of thiamin-requiring enzymes was not the result of higher protein levels, as PDH and TK levels were not increased in these plants, both at the beginning and at the end of the light period (Figure 5B). Additionally, plants deficient in riboswitch activity exhibited an increase in glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme activity compared with control and wild-type plants (see Supplemental Table 1 online), but the activities of ADP-Glc pyrophosphorylase and isocitrate dehydrogenase (NAD and NADP dependent) remained unchanged.

The enhanced activities of the thiamin-requiring enzymes observed in plants deficient in riboswitch activity suggested an alteration in the metabolic fluxes through the TCA cycle and the PPP. To directly assess the effect of a defective riboswitch on the plant respiratory rate, we estimated the fluxes in those pathways on the basis of $^{14}CO_2$ evolution. This was achieved by incubating leaf discs (isolated during the dark period) with [1-^{14}C]-Glc, [3,4-^{14}C]-Glc, or [6-^{14}C]-Glc over a period of 6 h. The consequent $^{14}CO_2$ emissions were then measured at hourly intervals. We observed that the release of $^{14}CO_2$ from all labeled Glcs were significantly higher in plants deficient in riboswitch activity compared with the control and wild-type plants, which were very similar to each other (Figure 5C). However, the ratio of $^{14}CO_2$ evolution from the C1 or the C6 position of Glc to that from the C3,4 positions were similar in riboswitch-deficient plants and in control plants (see Supplemental Figure 5B online), providing evidence that riboswitch deficiency resulted in a general increase in respiration rate.

Deficiency in TPP Riboswitch Activity Leads to Altered Primary/Central Metabolism

Since we established that riboswitch disruption resulted in increased fluxes through the TCA cycle and the PPP, we further investigated to what extent these alterations would affect the steady state levels of plant primary/central metabolites throughout the day. For this purpose, we harvested 4-week-old plants at four time points and measured the levels of primary metabolites of interest using colorimetric protocols (see Methods). We observed that plants deficient in riboswitch activity displayed elevated total free amino acid content during the dark period compared with the control and wild-type plants, which were very similar throughout the 24-h cycle (Figure 6A). However, the levels of Glc, Fru, Suc, protein, and nitrate remained practically unchanged compared with the control and wild-type plants (see Supplemental Figure 6A online).

To further characterize the metabolic alterations (largely in core metabolism) that occurred in leaves of riboswitch-deficient plants, an established gas chromatography–mass spectrometry–based metabolic profiling (Lisec et al., 2006) was performed during a diurnal period (four time points, start and middle of both the light and dark periods). It appeared that the metabolic network was strongly affected. Notably, the steady state levels of 18 metabolites, out of 43 identified, differed significantly in the plants harboring a deficient riboswitch in at least one time point (Figure 6B; see Supplemental Figure 6B online). We found that riboswitch deficiency caused the accumulation of six amino acids (Ala, β-Ala, Asp, Thr, Pro, and Trp) and the reduction of seven other amino acids (γ-aminobutyric acid [GABA], Gly, Met, His, Gln, Tyr, and Phe; Figure 6B). Furthermore, riboswitch deficiency also triggered a higher steady state level of amino acid precursors, such as isocitrate, during the dark period and a lower steady state level of precursors, such as succinate, during the entire day period. Interestingly, the steady state levels of Gln and GABA were decreased, most probably because these two compounds serve as alternative carbon donors for the TCA cycle. In addition, a significant increase in spermine and tyramine levels was observed, suggesting an augmentation of both β-Ala and hydroxycinnamic acid tyramine amide biosynthetic pathways.

To further characterize how riboswitch malfunction affected the fluxes through the TCA cycle, we performed isotope labeling experiments. We evaluated the relative isotope redistribution in leaf discs that were fed with [U-^{13}C]-Glc or [U-^{13}C]-pyruvate and further processed using a gas chromatography–mass spectrometry

Figure 3. (continued).

(A) Phenotype of wild-type (wt; left) and transgenic plants harboring the native (middle, line #4) or the mutated (right, line #7) TPP riboswitch, grown for 8 weeks under short-day conditions. Similar phenotypes were obtained in five additional independent transgenic lines.

(B) Iodine staining shows starch accumulation in 8-week-old *Arabidopsis* wild-type and transgenic plants harboring the native (line #4) or the mutated (line #7) TPP riboswitch observed at the beginning and the end of the light period. Reduced starch accumulation is observed in plants harboring the mutated riboswitch at the beginning of the light period (bordered in black). Similar results were obtained using additional plants.

(C) Transmission electron microscopy of leaves derived from 3-week-old transgenic plants harboring the native (line #4) or the mutated (line #7) riboswitch. Chloroplast (Chl.) and starch granules (St. gr.) are shown.

(D) Transcript levels of the *THIC* coding region and retained and spliced variants were resolved by qPCR ($n = 3$; SE). Student's *t* test indicates significant changes from wild-type plants (left) or between a sample and line #2 (middle) or line #3 (right), which harbor the native riboswitch: *P value < 0.05; **P value < 0.01. Wild-type (21 d old; white) and transgenic *Arabidopsis* harboring the native (gray) or the mutated (black) riboswitch were used. Line numbers indicate independent transgenic lines.

(E) Circadian expression of *THIC* and its intron-retained variant resolved by qPCR ($n = 3$; SE) in transgenic *Arabidopsis* harboring the native (gray, line #4) or the mutated (black, line #7) riboswitch, grown under short-day conditions for 21 d, prior to being transferred to constant light (LL) for the indicated time. The expected light and dark periods are indicated by white and gray backgrounds, respectively. "so" (significant oscillation) indicates that the oscillation displayed by a given graph is significant (P value < 0.05) according to both N-model and cosinor analyses (see Methods). The amplitude (amp) and the period (per) of the significant oscillations are indicated in the same color as the corresponding graph.

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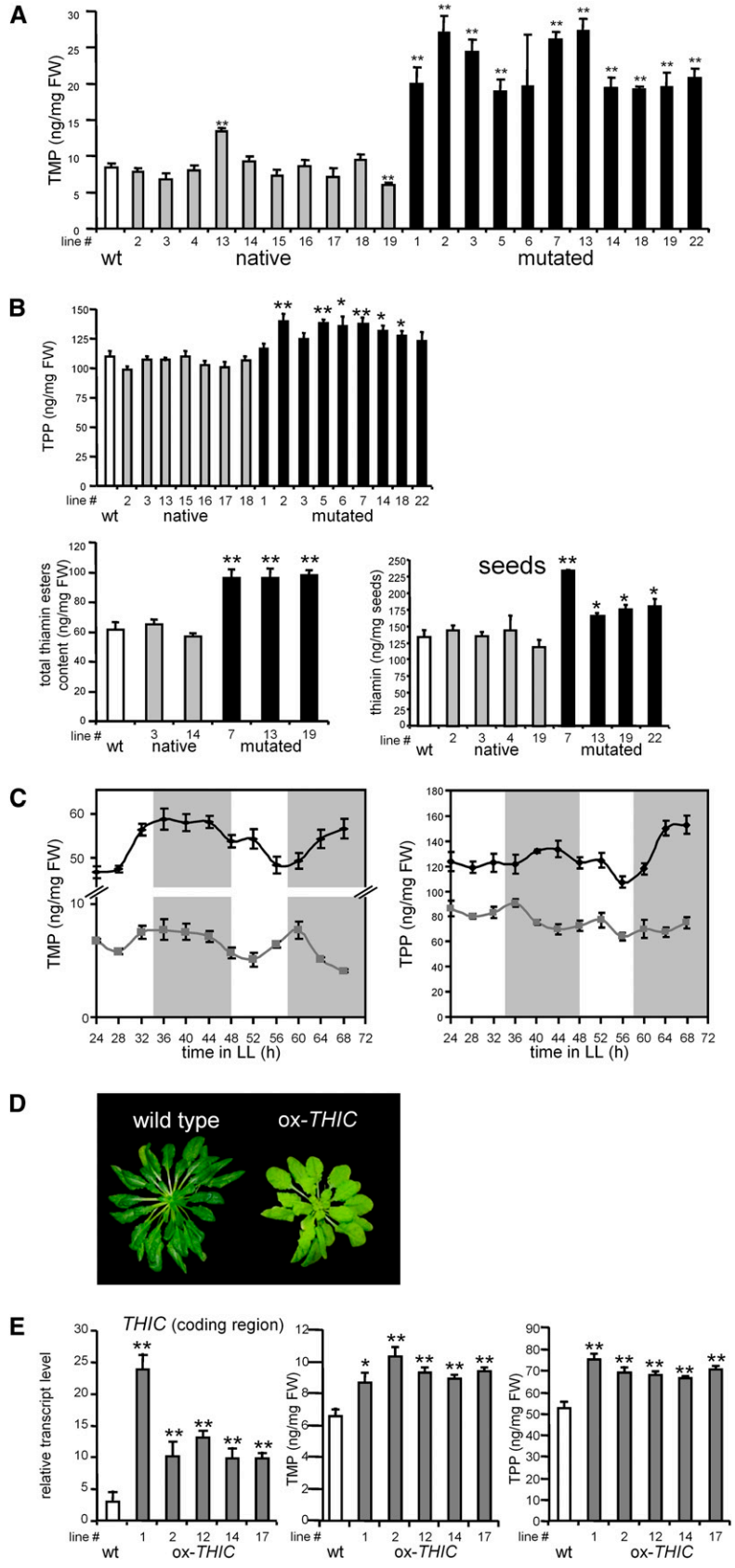

Figure 4. Effects of TPP Riboswitch Deficiency or *THIC* Overexpression on Thiamin Biosynthesis.

approach that facilitates isotope tracing (Roessner-Tunali et al., 2004). Interestingly, an increase in label redistribution to most amino acids was observed (Asp, Asn, Ile, Ala, β-Ala, Pro, Phe, and Ser; Figure 7; see Supplemental Table 2 online). Additionally, we noticed an augmentation in label redistribution of the TCA cycle intermediates citrate and fumarate as well as Glu. By contrast, a reduction in label redistribution was observed for malate, Gln, and GABA metabolites.

Deficiency in Riboswitch Activity Impinges on Isoprenoid Metabolism, Photosynthetic Activity, and Specialized Metabolism

Since plants deficient in TPP riboswitch activity displayed phenotypes of chlorosis, we performed metabolic profiling of compounds belonging to the isoprenoid pathway using an established high-performance liquid chromatography, photo-diode array detection (HPLC-PDA)–based protocol (Fraser et al., 2000). Riboswitch-deficient plants accumulated significantly less isoprenoids, including chlorophyll *a* and *b*, δ- and γ-tocopherol, β-cryptoxanthin, violexanthin and neoxanthin compared with control plants (see Supplemental Figure 7A online). The lower chlorophyll content observed in these plants led us to investigate whether these plants also exhibited altered photosynthetic rates. The results indicated a reduced photosynthetic rate of the plants harboring a defective riboswitch, while stomatal conductance, electron transfer rate, and transpiration rate were unaffected (see Supplemental Figure 7B online), suggesting that the effect on photosynthesis was due to the lower chlorophyll content.

To evaluate the broader consequences of altering riboswitch activity in plants, we performed metabolomics assays using high-resolution liquid chromatography–mass spectrometry (Malitsky et al., 2008). This system is optimized to detect mainly semipolar, specialized (i.e., secondary) metabolites (Mintz-Oron et al., 2008). Comparing the metabolite profiles of plants harboring a defective riboswitch to those harboring a functional one and the wild type revealed that riboswitch deficiency dramatically altered secondary metabolism. We also observed larger abundance of differential mass signals in the middle and in the end of the dark photoperiod (see Supplemental Table 3 and Supplemental Figure 7C online), in accordance with our previous finding that the metabolic phenotype caused by riboswitch deficiency was more pronounced during the dark photoperiod (Figure 6).

DISCUSSION

To date, the research on riboswitches has focused essentially on their mechanisms of ligand binding and gene regulation in bacteria, while only limited research was dedicated to their biological functions, especially in eukaryotes. This study of the sole eukaryotic TPP riboswitch known to date facilitated a primary insight into how these elements can integrate the physiological and metabolic cycle of complex organisms. Here, we provide several lines of evidence suggesting that spatially and clock-directed transcriptional control over the *THIC* promoter acts in coordination with the TPP riboswitch to direct *THIC* expression and thereby control thiamin biosynthesis, which in turn mediates central metabolism daily.

Here, we described that *THIC* is the only circadian regulated gene of the currently known set of thiamin biosynthetic genes. As TMP oscillations could not be detected for more than one period, we cannot conclude that thiamin levels oscillate in a circadian manner, and further experiments will be required to determine the exact mechanisms underlying this phenomenon. Nevertheless, the circadian regulation of thiamin biosynthesis was substantiated by experiments using plants altered in their biological clock, showing that the circadian oscillations of *THIC* transcript levels were altered in these plants. Moreover, we found that the transcriptional repressors CCA1 and LHY were able to bind the evening element present in the *THIC* promoter region in vitro. Notably, an evening element was also found in the promoter regions of starch degradation–related genes that display circadian oscillations similar to those of *THIC* (Lu et al., 2005), suggesting that starch degradation and thiamin biosynthesis could be regulated by a common or similar mechanism. Since *CCA1* and *LHY* encode transcriptional repressors and oscillate in a circadian manner in an opposite behavior to that of *THIC*, they may directly inhibit *THIC* expression at the beginning of the light photoperiod and allow *THIC* circadian oscillations as we observed here. Following this model, we suggest that the *THIC* promoter could act as a circadian regulator of thiamin biosynthesis, while the TPP riboswitch, which directs adjacent gene expression in response to TPP levels

Figure 4. (continued).

(A) TMP levels observed in 21-d-old wild-type (wt; white) or transgenic plants harboring the native (gray) or the mutated (black) riboswitch grown under short-day conditions were monitored by HPLC analysis ($n = 5$, SE, Student's *t* test indicates significant changes from wild-type plants: *P value < 0.05; **P value < 0.01). Line numbers indicate independent transgenic lines. FW, fresh weight.

(B) Levels of thiamin, TPP, and total thiamin, observed in dry seeds or in the aerial parts of 21-d-old *Arabidopsis* wild-type (white) and transgenic plants harboring the native (gray) or the mutated (black) riboswitch grown in soil under short-day conditions, were monitored by HPLC analysis ($n = 5$, SE, Student's *t* test indicates significant changes from the wild type, *P value < 0.05; **P value < 0.01). Independent lines of transformation are depicted by the line numbers.

(C) Circadian levels of TMP and TPP observed in the aerial parts of 21-d-old transgenic plants harboring the native (gray, line #4) or the mutated (black, line #7) riboswitch grown under short-day conditions were monitored by HPLC analysis ($n = 5$, SE).

(D) Phenotypes of wild-type (left) and transgenic plants overexpressing the At *THIC* gene (right, line #1), grown for 8 weeks under short-day conditions.

(E) Transcript levels of the At *THIC* gene expression (detected by qPCR; $n = 3$; SE) and TMP and TPP levels (detected by HPLC analysis; $n = 4$, SE) were monitored in the aerial part of 21-d-old wild-type (white) and transgenic *Arabidopsis* overexpressing the At *THIC* coding sequence (gray) grown in soil under short-day conditions. Line numbers indicate independent transgenic lines. Student's *t* test indicates significant changes from the wild type: *P value < 0.05; **P value < 0.01.

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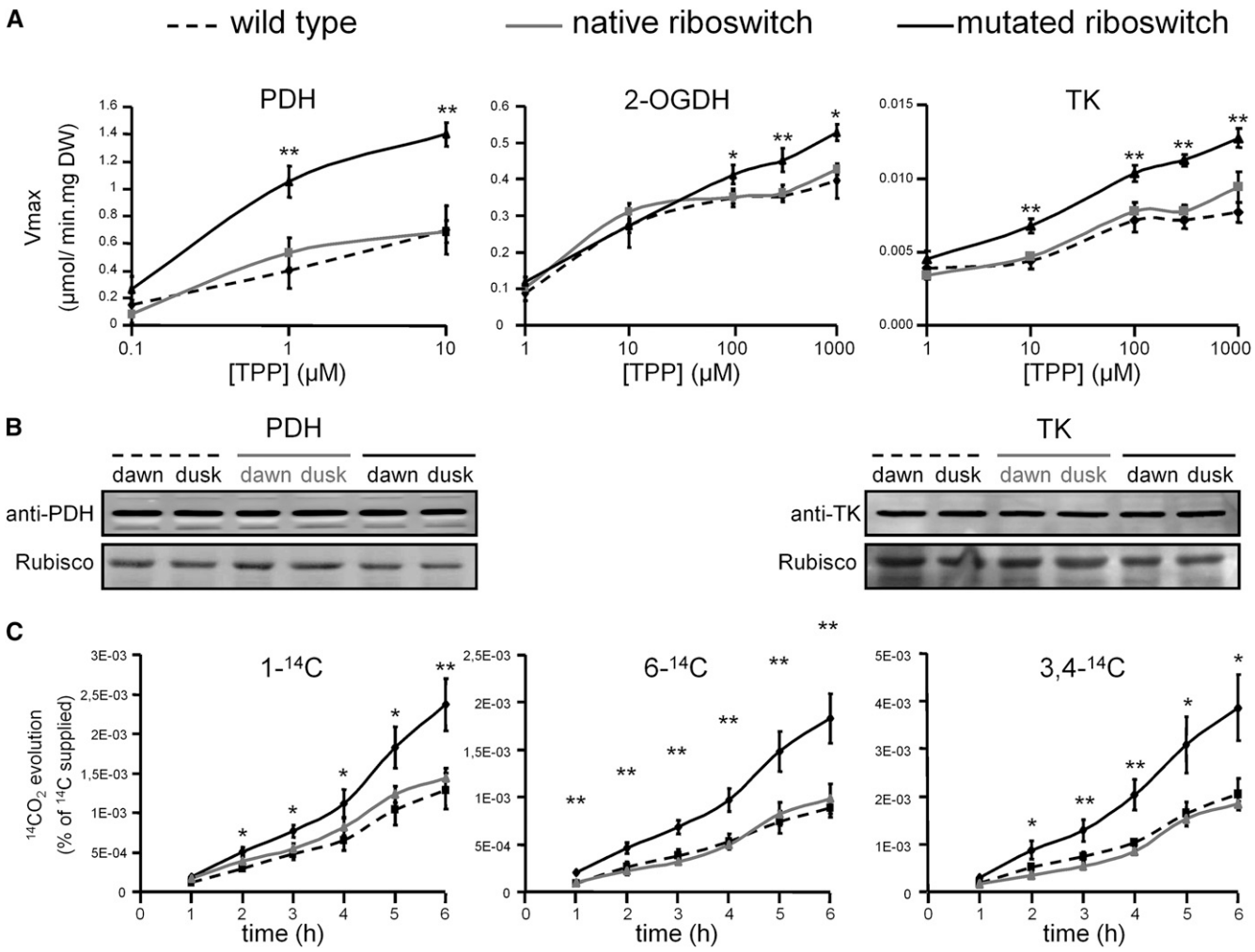

Figure 5. Riboswitch Deficiency Results in Enhanced Activities of Thiamin-Requiring Enzymes and Increased Carbohydrate Oxidation through the TCA Cycle and the PPP.

(A) Activities of PDH, 2-OGDH, and TK were determined in 30-d-old fully expanded leaves harvested in the middle of the light photoperiod. DW, dry weight.

(B) Protein levels of PDH and TK detected by a immunoblot in 21-d-old *Arabidopsis* at the beginning (dawn) and the end (dusk) of the light period. Soluble proteins were extracted from *Arabidopsis* plants, grown for 14 d in soil. Proteins were separated on 10% PAGE, transferred to a polyvinylidene difluoride membrane, which was subsequently immunodetected with either anti-PDH or anti-TK antibodies. Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

(C) Evolution of $^{14}CO_2$ released from isolated leaf discs incubated with [1-^{14}C]-, [3,4-^{14}C]-, or [6-^{14}C]-Glc. The $^{14}CO_2$ liberated was captured (at hourly intervals) in a KOH trap, and the amount of $^{14b}CO_2$ released was subsequently quantified by liquid scintillation counting.

Measurements were performed using wild-type (dashed) and transgenic *Arabidopsis* plants harboring the native (gray, line #4) or the mutated (black, line #7) riboswitch. Values are presented as means ±SE of determinations using six **(A)** or three **(C)** independent biological replicates per genotype. Student's *t* test indicates significant changes from wild-type plants: *P value < 0.05; **P value < 0.01.

(Bocobza et al., 2007; Wachter et al., 2007), may serve as a homeostatic buffer mechanism to prevent thiamin deficiency or overdose. The complexity of thiamin biosynthesis regulation emphasizes the potentially deleterious effect of inappropriate thiamin levels on plant fitness.

Interestingly, we also observed that riboswitch deficiency caused higher in vitro enzymatic activities of the three thiamin-requiring enzymes in the presence of increasing TPP concentrations. This can result either from a larger abundance of enzymes in the riboswitch-deficient plants or from a greater abundance of active enzymes in these plants. Notably, the protein levels of PDH and TK were not affected in the riboswitch-deficient plants, supporting the second hypothesis rather than an alteration in gene expression. This could well be explained by earlier findings showing that TPP binding is a rate-limiting step in the assembly of the PDH complex (Tovar-Méndez et al., 2003), the 2-OGDH complex (Bunik and Fernie, 2009), and the TK homodimer (Singleton and Martin, 2001). The increased activity of the thiamin-requiring enzymes can

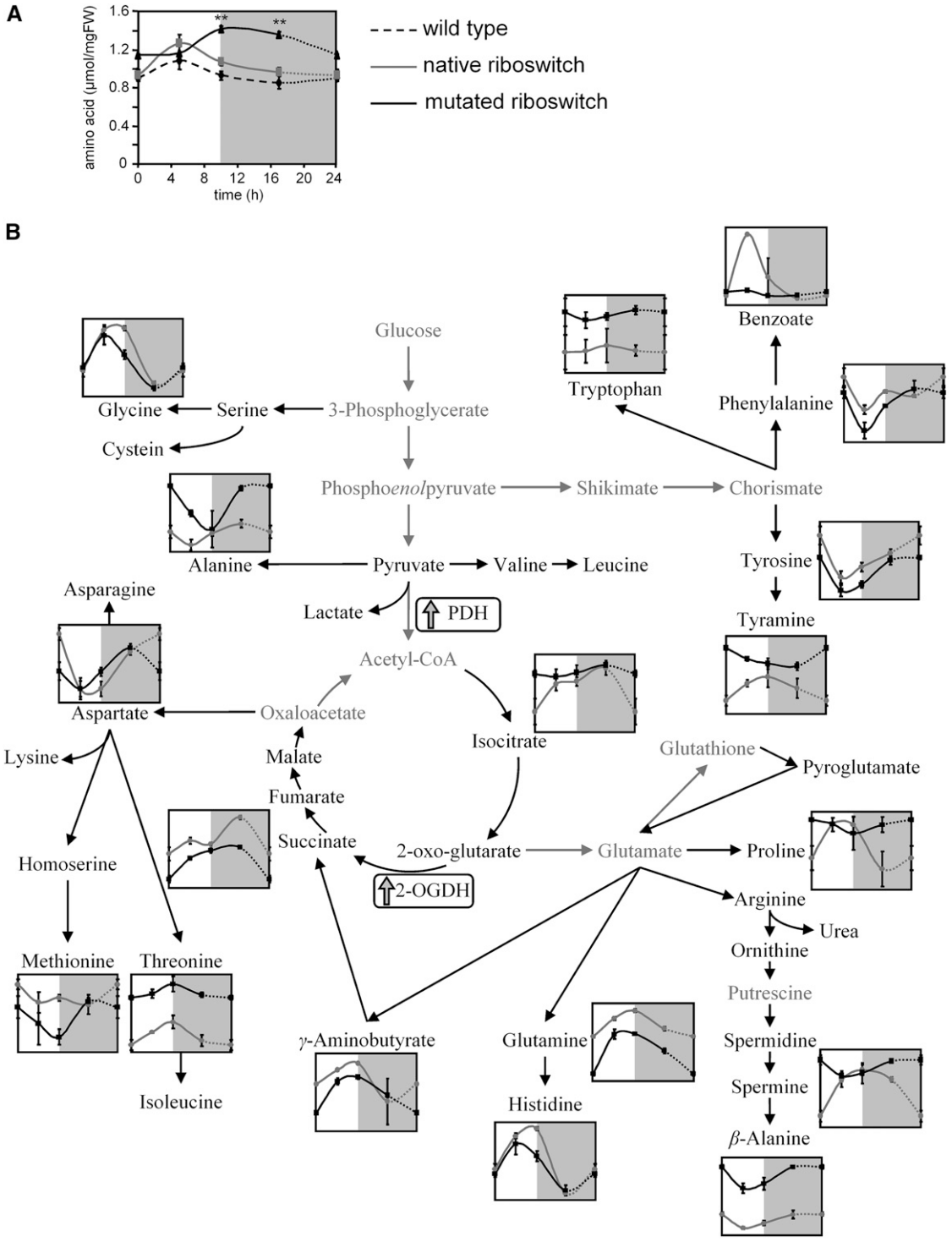

Figure 6. Riboswitch Deficiency Alters Steady State Core Metabolism.

(A) Diurnal changes in amino acid levels measured in leaves of 30-d-old wild-type (dashed) and transgenic *Arabidopsis* plants harboring the functional (gray, line #4) or the mutated (black, line #7) riboswitch, using a colorimetric method. The data presented are means ± SE of measurements from six individual biological replicates per genotype. Student's *t* test indicates significant changes from wild-type plants: **P value < 0.01. The light and dark periods are indicated by white and gray backgrounds, respectively. FW, fresh weight.

(B) Diurnal changes in steady states levels of polar and semipolar metabolites resolved using GC-TOF-MS. The data presented in black are means ± SE of measurements from three independent lines of transformation harboring the mutated riboswitch, and the data in gray are means ± SE of the wild type

in turn cause an increased TPP utilization that prevented steady state TPP accumulation in these plants, in a similar manner to what is observed in wild-type plants during the dark photoperiod. These findings indicate that the activation of the thiamin biosynthetic pathway observed in riboswitch-deficient plants, outlined by TMP accumulation, increased the formation of active enzymes complexes (for which it is rate limiting), which was characterized by an increased TPP responsiveness of these enzymes.

It is not clear at this point how the regulation of TPP availability links with the previously known allosteric mechanisms, largely investigated in nonplant systems, that regulate the activity of the thiamin-requiring enzymes and the rate of carbohydrate oxidation. The thiamin-requiring enzymes are inhibited by their products (e.g., acetyl-CoA, succinyl CoA, and NADH) and by the phosphorylation of the E1 subunit by PDH kinase (Tovar-Méndez et al., 2003; Bunik and Fernie, 2009). However, evidence suggests that in plants, these mechanisms might not be as prevalent as in other systems as (1) transgenic plants with decreased expression of the mitochondrial PDH kinase displayed very minor effects in their respiration rate (Marillia et al., 2003); (2) although E1 phosphorylation has been clearly demonstrated in vitro (Tovar-Méndez et al., 2003), convincing demonstration of its in vivo function is still lacking in plants; and (3) control of plant respiration by ATP/AMP level is generally desensitized at the allosteric level in comparison to nonplant systems (Fernie et al., 2004). Experiments using illuminated leaves revealed that the TCA cycle is apparently reduced by up to 95% with respect to flux through the PDH reaction and by 27% in total (Tcherkez et al., 2005). However, this experiment was likely performed during the light photoperiod, when thiamin biosynthesis is lower. We therefore suggest that under low TPP availability, allosteric regulation of the thiamin-requiring enzymes may be the main control over thiamin-requiring enzymes' activities, but during the dark photoperiod, high TPP availability becomes a major point of control of these enzymes.

Since the thiamin-requiring enzymes are also key control steps of the TCA cycle and the PPP (Berthon et al., 1992; Wang et al., 1997a, 1997b; Henkes et al., 2001; Araújo et al., 2008), we determined the relative Glc oxidation rate on the basis of $^{14}CO_2$ evolution of plants harboring a defective riboswitch. It was observed that riboswitch deficiency and the consequent activation of thiamin biosynthesis also caused the augmentation of the metabolic fluxes through the TCA cycle and the PPP. The elevated carbohydrate oxidation rate measured in riboswitch-deficient plants raised the question whether the plant primary metabolism was affected by riboswitch deficiency. Results obtained through metabolite profiling and ^{13}C labeling experiments confirmed the alteration of central metabolism and the augmentation in the carbon flux through the TCA cycle observed by means of $^{14}CO_2$ emissions. In addition, riboswitch deficiency increased amino acid biosynthesis. The reason for this could be that the TCA cycle produces building blocks for amino acids synthesis; thus, a stronger flux in this pathway would increase the availability of these precursors and increase amino acid production.

Furthermore, we observed that riboswitch deficiency caused the reduction in label redistribution for malate, Gln, and GABA. Since these compounds are carbon donors for the TCA cycle, an increased flux in this pathway would deplete these metabolites. In addition, we observed increased GAPDH activity in plants harboring a deficient riboswitch, which also suggests a stronger carbon flux through the TCA cycle in such plants. The overall increased carbohydrate oxidation rate detected in riboswitch-deficient plants can also explain our observation that these plants contained less starch than control and wild-type plants at dawn, as an increased carbohydrate oxidation rate would exhaust the carbohydrate reserved before the beginning of the light period. While insufficient TPP levels have been extensively reported to alter carbohydrate oxidation (e.g., in the case of the Beriberi disease), our findings suggest that increased TPP availability can directly augment carbohydrate oxidation rate.

Plants altered in riboswitch activity displayed chlorosis, reduced photosynthesis rate, growth retardation, and late flowering under short-day conditions. These phenotypes, particularly the effect on level of photosynthetic pigments (chlorophylls and carotenoids), might directly cause profound changes in carbon metabolism. Thus, it is difficult to determine without doubt whether altered thiamin metabolism or reduced photosynthesis (or both) was the cause for the changes in core metabolism observed. Interestingly, the chlorotic phenotype was more pronounced when plants were grown under short- rather than long-day conditions. Given that starch mutants have stronger phenotypes under short-day conditions, it suggests that the chlorotic phenotype of the riboswitch mutant is starch related (Caspar et al., 1991). Similarly, riboswitch-deficient plants displayed delayed flowering only under short-day conditions. This might result from alteration in their circadian clock (Imaizumi and Kay, 2006) or alternatively from perturbation in carbohydrate metabolism (Bernier et al., 1993; Levy and Dean, 1998; Yu et al., 2000). Given that the circadian clock of the riboswitch-deficient plants was not affected (illustrated by oscillations of *GRP7*), the aberrant starch metabolism of the riboswitch-deficient plants was likely the cause of the flowering retardation phenotype. Finally, we observed increased steady state levels of amino acids and additional core metabolism

Figure 6. (continued).

and two independent lines harboring the native riboswitch. Six individual biological replicates were measured for each genotype. A total of 43 compounds could be identified, among which 18 (depicted as graphs) exhibited differential levels in plants defective in riboswitch activity compared with plants harboring a functional riboswitch and to the wild type at least at one time point (P value < 0.05). The data presented are $\log_{10}$ (means) of the measurements. Graphs depicting the levels of all 43 metabolites identified in all transgenic and wild-type plants are shown in Supplemental Figure 6B online. Metabolites noted in black were detected, while those noted in gray were not. The increased activities observed for PDH and for 2-OGDH are represented by an upward arrow. Arrows represent either single or multiple steps. White and gray backgrounds in the graphs indicate the light and dark periods, respectively.

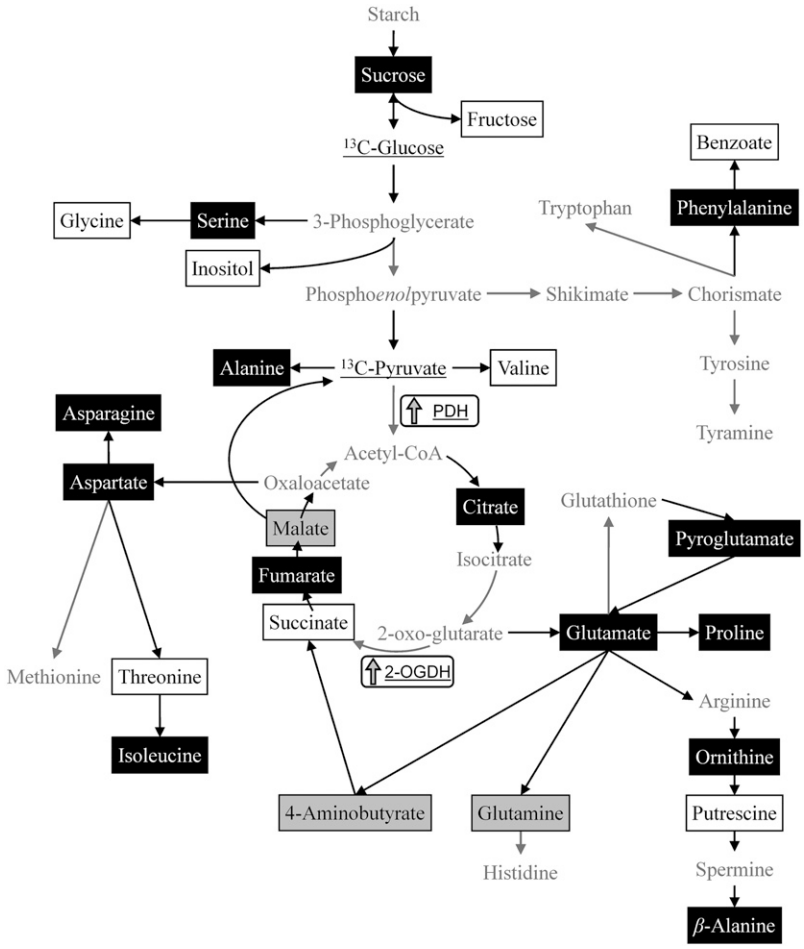

Figure 7. Redirection of Fluxes in Core Metabolism Mediated by Riboswitch Deficiency.

Discs of 10-week-old wild-type and transgenic *Arabidopsis* plants harboring a functional (line #4) or the mutated (line #7) riboswitch were fed with ^{13}C-pyruvate or ^{13}C-Glc and subjected to metabolic profiling by means of GC-TOF-MS. Changes in metabolite labeling are mapped on the metabolic network. Metabolites shown in a black background are more labeled, while those in gray background are less labeled in plants deficient in riboswitch activity compared with plants harboring a functional riboswitch and to wild-type plants (according to a Student's *t* test, P value < 0.05, $n = 6$). Metabolites shown in white background were detected but unchanged in this assay, and metabolites noted in gray were not detected. Arrows represent either single or multiple steps. The increased activities observed for PDH and for 2-OGDH are represented by an upward arrow. The complete results of the flux assay are presented in Supplemental Table 2 online.

intermediates; these observations are difficult to explain by reduced photosynthetic rates and isoprenoid levels.

In plants, photosynthetic carbon fixation occurs during the day and results in starch accumulation. This in turn supports nocturnal metabolism and growth at night (Graf et al., 2010), and starch turnover is regulated so that carbohydrate reserves are almost completely exhausted at dawn (Stitt and Zeeman, 2012). Thus, higher TPP levels are likely required during the dark photoperiod when carbohydrate oxidation rate is higher (Tcherkez et al., 2005) to facilitate the elevated activities of the rate-limiting enzymes involved in this process (i.e., the thiamin-requiring enzymes) during this period. Thus, we wish to raise the hypothesis that regulation of TPP availability, through the action of both the *THIC* promoter and the TPP riboswitch, are involved in controlling the specific daily activities of the thiamin-requiring enzyme complexes to adjust carbohydrate metabolism.

Recent reports highlight the linkage between circadian rhythm and metabolism (Fukushima et al., 2009) as multiple clock genes were found to participate in metabolic homeostasis (Bass and Takahashi, 2010). Given the potency of TPP in the regulation of carbohydrate oxidation described here, it is conceivable that the circadian clock–driven thiamin biosynthetic gene (i.e., *THIC*) and its circadian oscillations participate in the daily regulation of carbohydrate oxidation and of the light/dark metabolic transition. The molecular timer that sets the rate of starch degradation in a circadian manner has been intensively pursued during the past years, and the involvement of a circadian mechanism was reported (Graf et al., 2010). We suggest that the circadian oscillations of thiamin biosynthesis, at least at the RNA level, contribute to this timer and assist the plant to anticipate dawn and sunset for optimal utilization of carbohydrate reserves.

To conclude, we provide here insight into the integration of riboswitch action, a noncoding RNA region mediated gene control mechanism, with physiological and metabolic processes that are vital for cellular activity. In a proposed model (Figure 8), the *THIC* promoter drives gene expression in a circadian manner to increase thiamin production during the dark period, while the TPP riboswitch directs the overall level of *THIC* expression. Consequently, high thiamin availability during the dark period enhances the activity of the thiamin-requiring enzymes, which in turn increases the carbon flux through the TCA cycle and through the PPP. In other words, the TPP riboswitch senses the TPP levels inside the nucleus and regulates thiamin biosynthesis accordingly. TPP reaches different subcellular compartments, including the chloroplast and the mitochondria to assist respiration and the nucleus to maintain its homeostasis throughout the cell. Thus, the TPP riboswitch acts as a regulator to prevent thiamin deficiency or overdose and together with the circadian clock adjusts TPP availability to control the rate of carbohydrate oxidation and central metabolism diurnally. Consequently, the riboswitch-directed thiamin biosynthesis tightly links the control over the TCA cycle and the PPP and balances primary/central metabolism and its associated downstream secondary metabolism.

This study investigated the regulation of thiamin biosynthesis in autotrophs and exposed a possible strategy to increase the thiamin content in crop plants by means of riboswitch alteration. Generating such crops can impact human populations suffering from malnutrition and thiamin deficiency. It will be important to take in consideration the potential phenotype that these engineered crop plants may have due to their higher thiamin production. Given

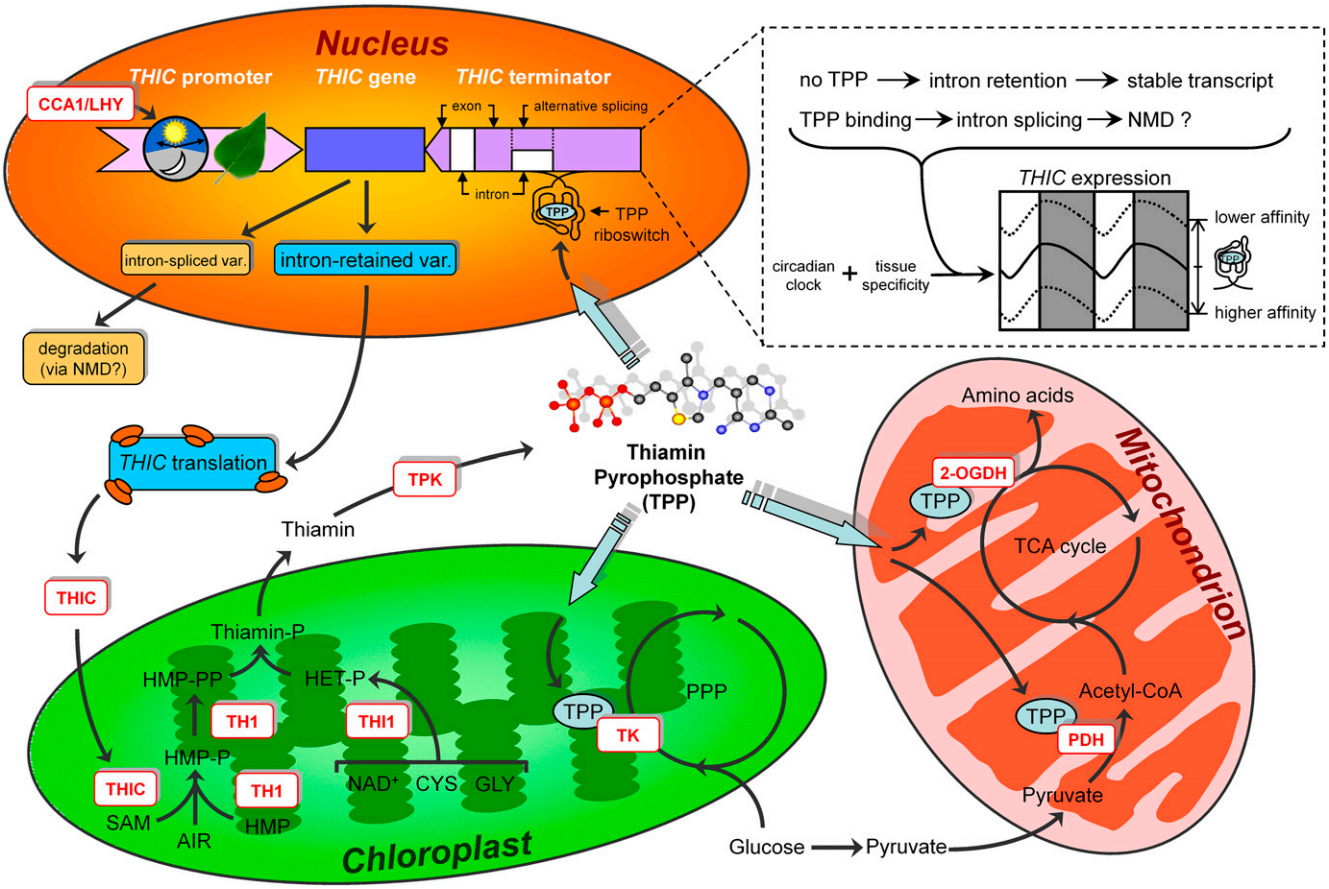

Figure 8. Model for TPP Riboswitch Action as a Pacesetter Orchestrating Central Metabolism in Thiamin Autotrophs.

The model represents multiple subcellular compartments, including the mitochondria, chloroplast, nuclei, and the cytosol. In the nucleus, the TPP riboswitch and the circadian clock regulate *THIC* expression together. In turn, THIC migrates to the chloroplast where it participates in thiamin biosynthesis. Thiamin is further pyrophosphorylated in the cytosol into TPP. Then, TPP reaches the nucleus to regulate *THIC* expression (via the TPP riboswitch) and the mitochondria and the chloroplast to assist enzymes of the TCA cycle and the PPP, respectively. AIR, 5-aminoimidazole ribonucleotide; DXP, 1-deoxy-D-xylulose-5-phosphate; HET-P, 4-methyl-5-(β-hydroxyethyl)thiazole phosphate; HMP, hydroxymethylpyrimidine; HMP-P, hydroxymethylpyrimidine phosphate; HMP-PP, hydroxymethylpyrimidine pyrophosphate; NAD, nicotinamide adenine dinucleotide; NMD, non-sense-mediated decay; SAM, *S*-adenosyl-L-Met; TH1, thiamin-monophosphate pyrophosphorylase; THI1, thiazole synthase; thiamin-P, TMP; TPK, thiamin pyrophosphokinase; var, variant.

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the strong conservation of the TPP riboswitch–regulated pathway for thiamin biosynthesis, the thiamin-requiring complexes, and their involvement in central metabolism, it is likely that our findings in plants will recur in bacteria, fungi, and algae.

METHODS

Plant Materials

Arabidopsis thaliana plants (ecotype Columbia-0) were grown on soil in climate rooms (22°C; 70% humidity; 18/6 h of light/dark for long-day conditions and 10/14 of h light/dark for short-day conditions). For all experiments, biological replicates were grown in different pots (or Petri dishes) but in identical conditions to ensure the reproducibility of the results. The *Arabidopsis thi1* and *thic* mutants were obtained from the European Arabidopsis Stock Center (http://Arabidopsis.info/; stock ID: N3375 and salk_011114, respectively). For experiments involving TPP supplementation, plants were grown for 14 d in Petri dishes on Murashige and Skoog media (basal salt mixture; Duchefa) with 1% Suc and 1% agar to which TPP (Sigma-Aldrich; catalog number C8754; water soluble) was added to the required concentrations.

Generation of DNA Constructs and Plant Transformation

For the generation of transgenic *Arabidopsis*, the *Arabidopsis THIC* 3′ UTR was amplified by PCR using *Arabidopsis* genomic DNA (Columbia-0) with the oligonucleotides #1 and #2 (see Supplemental Table 4 online). The mutation (A515G, starting from the stop codon) was introduced using the megaprimer-based mutagenesis strategy (Kammann et al., 1989) with oligonucleotides #1, #2, and #31. The *THIC* 3′ UTR was then fused to the *YFP* reporter gene and the fusion fragment was subsequently inserted downstream to the double 35S promoter of CaMV. In addition, the plasmids used to generate the transgenic plants deficient in riboswitch activity were obtained by amplifying the *THIC* promoter with the oligonucleotides #15 and #16, and the *THIC* genomic sequence with the oligonucleotides #17 and #18 and the resulting fragments were fused adjacent to the *THIC* 3′ UTRs in the plasmids used previously. Moreover, in an additional vector, the *THIC* promoter was amplified similarly and fused to the *RFP* reporter gene, which was adjacent to the *NOS* terminator. The cassettes were then inserted into the pGreenII vector containing the Basta resistance gene for selection of transformants. *Arabidopsis* plants were transformed using the floral dip method (Clough and Bent, 1998), and transgenic plants were selected in media containing 50 μg/mL kanamycin or directly on soil using 200 mg/L glufosinate ammonium.

Molecular Biology and Microscopy

Unless specified, all molecular biology manipulations were performed as described previously (Sambrook et al., 1989). Leaf samples were taken at the time point indicated, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis. Extraction was performed by rapid grinding of the tissue in liquid nitrogen and immediate addition of the appropriate extraction buffer. RNA extractions were all performed using the RNeasy kit (Qiagen) and DNA extractions using the hexadecyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). For gene amplification and cloning, all PCRs were performed with the GoTaq green mix (Promega) and the respective oligonucleotides. Amplicons were always sequenced prior to further cloning. Fluorescence of YFP and RFP signals was observed by an Olympus CLSM500 microscope with an argon laser at 488 nm for excitation and 505 to 525 nm for YFP emission and at 590 nm for RFP emission. No fluorescence was observed in plants that do not express either YFP or RFP (see Supplemental Figure 2C online). For transmission electron microscopy, leaves from 1-month-old plants were collected and processed using a standard protocol (Chuartzman et al., 2008). The Epon-embedded samples were sectioned (70 nm) using an ultramicrotome (Leica) and observed with a Technai T12 transmission electron microscope (FEI).

Gene Expression Analysis

Quantitative real-time PCR (qPCR) gene expression analysis was performed with three biological replicates using gene/variant-specific qPCR oligonucleotide pairs designed with Primer Express software (Applied Biosystems). Specific oligonucleotides sequences are provided in Supplemental Table 4 online. *UBIQUITIN C* was used as the endogenous control for all analysis. Fixed amount of DNase-treated total RNA was reverse transcribed using AMV reverse transcriptase (EurX). RT-PCRs were tracked on an ABI 7300 instrument (Applied Biosystems) using the Platinum SYBR SuperMix (Invitrogen). Each sample was PCR amplified from the same amount of cDNA template in triplicate reactions. Following an initial step of 10 min at 95°C, PCR amplification proceeded for 40 cycles of 15 s at 95°C and 60 s at 60°C and completed by melting curve analysis to confirm specificity of PCR products. All data analyses were performed using the 7300 system SDS software version 1.4.0.25 (Applied Biosystems) according to the manufacturer's instructions.

Circadian and Diurnal Assays

For circadian gene expression analysis, plants were grown in soil under normal short-day conditions (10/14 h of light/dark) for 3 weeks, followed by 3 d of constant light. Samples were harvested every 4 h during the last 2 d of constant light. For diurnal analysis, plants were grown in identical conditions, and samples were harvested during light/dark conditions for the indicated time, starting from the beginning of the first light period. Significance of oscillations of transcript levels and metabolites were determined using N-model (Haus and Touitow, 1992) and cosinor (Refinetti et al., 2007) analyses. Oscillations were considered significant only when P value < 0.05 in both tests.

Protein Purification and EMSA

The coding sequences for CCA1 and LHY were PCR amplified and subsequently introduced into pET28a vectors. Protein expression was performed using BL21(DE3) pLys and grown in Luria-Bertani until OD_{600} reached 0.5. Induction was then performed for 2 h using 1 mM isopropylthio-β-galactoside (IPTG). Proteins were purified using the Ni-NTA His bind resins (Novagen) according to manufacturer's instructions. The EMSA was performed by incubating 3 μg purified protein with the evening element, which consisted of a double-stranded fluorescent oligomer labeled using hexachlorofluorescein on the 5′ (5′HEX-AACGGACACCAATTTTCGAC**AAAATATCT**GAGAAAGA) in 20 mM HEPES, pH 7.4, 50 mM KCl, 3 mM $MgCl_2$, 5% glycerol, and 2 mM DTT. Competition experiments were performed using 100-fold unlabeled double-stranded oligomers of the same sequence or unlabeled double-stranded oligomers harboring mutations (5′-AACGGACACCAATTTTCGACA**CCCGATCT**GAGAAAGA-3′). DNA proteins complexes were separated on 7% Tris-Gly PAGE and visualized with Tyfoon 9410 (GE Healthcare).

HPLC Analysis of Thiamin Derivatives

Samples (100 mg) were harvested from the aerial parts of 3-week-old plants grown in short-day conditions at the end of the light period and immediately frozen in liquid nitrogen. The plant samples were then grinded followed by the addition of 400 μL of 0.1 M HCl and sonicated in a water bath for 30 min. The resulting extracts were centrifuged at 14,000

rpm (in a regular bench centrifuge) for 10 min. Samples of 300 μL of the supernatant were supplemented consecutively with 50 μL of freshly made 10 mM $K_4Fe(CN)_6$, which was dissolved in 3.7 N NaOH and 100 μL of methanol (HPLC grade). The samples were vigorously shaken, sonicated for 5 min, and centrifuged at 14,000 rpm (on a regular bench centrifuge) for 10 min. For measurements of dry seeds, 30 mg seeds was grinded and the following ratios used: 250 μL HCl, 150 μL of the supernatant supplemented with 25 μL $K_4Fe(CN)_6$, and 50 μL methanol. Following centrifugation, supernatants were then fractionated with a Capcell Pak NH_2 column (150 mm × 4.6 mm i.d.) (Shiseido) using a 4:6 (v/v) solution of 100 mM potassium phosphate buffer, pH 8.4, and acetonitrile as mobile phase. The HPLC analyses were performed using a Merck L7200 autosampler, a Merck L7360 column oven set at 25°C, a Merck pump Model L7100, and a Merck FL-detector L7480. A Merck D7000 interface module was used, and the chromatograms were integrated using the HSM software. The flow rate was 0.5 mL/min, and the volume injected was 5 μL for all samples. Thiochrome derivatives of thiamin, TMP, and TPP were detected by fluorescence at excitation 370 nm and emission 430 nm. Different concentrations of thiamin, TMP, and TPP standards were analyzed using the same extraction procedure and chromatographic conditions. Calibration curves were generated for each of the standards. For quantification of the samples, the peak areas of the samples were compared with the corresponding standard curve.

Analysis of Enzyme Activities

Enzyme activities were measured from 10 mg dry weight of 4-week-old *Arabidopsis* plants grown under short-day conditions and harvested at the middle of the light period. Soluble proteins were extracted as described previously (Gibon et al., 2004). This extraction process includes a desalting stage that separates between TPP and the proteins. Therefore, measurements of the activities of the thiamin-requiring enzymes were performed in the presence of TPP supplied to the indicated concentrations. PDH was assayed as described earlier (Randall et al., 1989), and 2-OGDH activity was measured following the protocol of Araújo et al. (2008). Transketolase and AGPase activities were determined as described previously (Gibon et al., 2004). Ribulose-1,5-bisphosphate carboxylase/oxygenase activity was monitored as described by Sharkey et al. (1991). Isocitrate dehydrogenase activity was determined as described by Nunes-Nesi et al. (2007), and GAPDH activity was measured according to Biemelt et al. (1999).

Measurements of Photosynthetic Parameters

Fluorescence emission was measured in vivo using a PAM fluorometer (Walz) on 5-week-old plants. Measurements were performed at the end of the light period. Prior to measurement of chlorophyll fluorescence yield and relative electron transfer rate, which were calculated using the WinControl software package (Walz), plants were maintained at fixed irradiance (0, 50, 100, 200, 400, 800, and 1000 μmol photons m^{-2} s^{-1}) for 30 min. Gas-exchange measurements were performed in a special custom-designed open system (Lytovchenko et al., 2002). The CO_2 response curves were measured at saturating irradiance with an open-flow gas exchange system (LI-COR model LI-6400).

Measurement of Respiratory Parameters

Estimations of the PPP and TCA cycle flux on the basis of $^{14}CO_2$ evolution were performed following incubation of leaf discs taken from 4-week-old plants, in 10 mM MES-KOH, pH 6.5, containing 0.3 mM Glc supplemented with 2.32 kBq mL^{-1} of [1-^{14}C]-, [3,4-^{14}C]-, or [6-^{14}C]-Glc. This was performed in the dark at the end of the light period. Evolved $^{14}CO_2$ was trapped in KOH and quantified by liquid scintillation counting (Nunes-Nesi et al., 2007). Notably, carbon dioxide can be released from the C1 and C6 positions by the action of enzymes associated with the PPP, while it can be released from the C3,4 positions of Glc by enzymes associated with mitochondrial respiration (Nunes-Nesi et al., 2005). Moreover, the ratio of $^{14}CO_2$ evolution from the C1 or the C6 position of Glc to that from the C3,4 positions of Glc provides an indication of the relative rate of the TCA cycle with respect to other processes of carbohydrate oxidation (such as glycolysis and the PPP).

Determination of Metabolite Levels

The levels of Suc, Fru, and Glc in the leaf tissue were determined as described previously (Fernie et al., 2001). Levels of proteins, amino acids, and nitrate were assayed as described previously (Tschoep et al., 2009; Sienkiewicz-Porzucek et al., 2010). All measurements were performed using 4-week-old *Arabidopsis* aerial parts (50 mg fresh weight), grown under short-day conditions, harvested diurnally at the beginning and the middle of the light and dark period, and six biological replicates were used. Additionally, metabolite profiling of 4-week-old wild-type and transgenic plants was determined from 100 mg of plant extracts using a gas chromatography–time of flight–mass spectrometry (GC-TOF-MS) apparatus as described previously (Lisec et al., 2006). In this experiment, metabolites from three independent lines of transformation harboring the defective riboswitch, two lines harboring the functional riboswitch, and wild-type plants were monitored. We considered metabolites as "altered" only when they differed in all three transgenic lines harboring the defective riboswitch from the control and wild-type plants.

Measurement of Isotope Redistribution

The fate of ^{13}C-labeled pyruvate and Glc was traced following feeding of isolated leaf discs from 6-week-old transgenic *Arabidopsis* plants deficient in riboswitch activity compared with the wild type and to the control plants grown under short-day conditions and incubated in the dark at the end of the light period, since amino acids levels were most affected during this period (Figure 6A), in a solution containing 10 mM MES-KOH, pH 6.5, and 10 mM [U-^{13}C]-pyruvate or 10 mM [U-^{13}C]-Glc for 2 and 4 h. Fractional enrichment of metabolite pools was determined exactly as described previously (Roessner-Tunali et al., 2004), and label redistribution was calculated as described previously (Studart-Guimarães et al., 2007). A Student's *t* test ($P < 0.05$, $n = 6$) was applied to assess the metabolites that were more or less radiolabeled in the plants harboring the deficient riboswitch compared with the wild type and to the control plants.

Isoprenoid Profiling

Isoprenoid content analysis was performed on 4-week-old plants grown under short-day conditions and harvested at the middle of the light period. Measurements were performed using an high-performance liquid chromatography, photo-diode array detection (HPLC-PDA) detector (Waters) and a YMC C30 column (YMC) as described by Fraser et al. (2000). Peak areas of the compounds were determined according to the spectral characteristic

Metabolomic Assays by Means of UPLC–Quantitative Time-of-Flight–Mass Spectrometry

Ultra performance liquid chromatography (UPLC) quantitative time-of-flight–mass spectrometry analysis of 4-week-old *Arabidopsis* plants grown under short-day conditions and harvested diurnally at the beginning and the middle of the light and dark period was performed according to Malitsky et al. (2008).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession

numbers: *THIC*, At2g29630; *CCA1*, At2g46830; *LHY*, At1g01060; *GRP7*, At2g21660; *UbiquitinC*, At5g25760; *Ubiquitin1*, At3g52590; *THI1*, At5g54770; *TH1*, At1g22940; *TPK1*, At1g02880; and *TPK2*, At2g44750.

Supplemental Data

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

Supplemental Figure 1. The Double Reporter Gene System and Circadian Expression of Thiamin Biosynthetic Genes in Transgenic Plants Altered in their Circadian Clock.

Supplemental Figure 2. The *THIC* Promoter Directs Adjacent Gene Expression in Green Tissues, While the *THIC* 3′ UTR Represses Gene Expression in a TPP-Dependent Manner.

Supplemental Figure 3. Plants Harboring a Deficient Riboswitch Display Growth Retardation, Chlorosis, and Reduced Starch Accumulation.

Supplemental Figure 4. Levels of *THIC* Gene Alternatively Spliced Variants, Thiamin Derivatives, Thiamin Biosynthetic Genes, and *GRP7* in TPP Riboswitch–Deficient Plants as Compared with Control Plants and Effects of *THIC* Overexpression.

Supplemental Figure 5. Riboswitch Deficiency Affects Carbohydrate Oxidation through the Tricarboxylic Acid Cycle and the Pentose Phosphate Pathway.

Supplemental Figure 6. Effects of TPP Riboswitch Deficiency on Core Metabolism.

Supplemental Figure 7. Riboswitch Deficiency Affects Isoprenoid Levels, Photosynthesis, and Specialized Metabolites.

Supplemental Table 1. Specific Activities of Non-Thiamin-Requiring Enzymes Involved in Primary Metabolism in Riboswitch-Deficient Plants.

Supplemental Table 2. Redirection of Fluxes in Core Metabolism Mediated by Riboswitch Deficiency.

Supplemental Table 3. Differential Mass Signals Detected in LC-MS–Based Metabolomics Assays of Riboswitch-Deficient Plants.

Supplemental Table 4. List of Oligonucleotides Used in This Study.

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AUTHOR CONTRIBUTIONS

A.A., S.E.B., and A.R.F. designed the experiments. S.E.B., Se.M., W.L.A, A.N.-N., and Sa.M performed the experiments and analyzed data. S.E.B. and A.A. wrote the article. All authors discussed the results and corrected the final article.

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