RESEARCH COMMUNICATION

Riboswitch-dependent gene regulation and its evolution in the plant kingdom

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Riboswitches are natural RNA sensors that affect gene control via their capacity to bind small molecules. Their prevalence in higher eukaryotes is unclear. We discovered a post-transcriptional mechanism in plants that uses a riboswitch to control a metabolic feedback loop through differential processing of the precursor RNA 3' terminus. When cellular thiamin pyrophosphate (TPP) levels rise, metabolite sensing by the riboswitch located in TPP biosynthesis genes directs formation of an unstable splicing product, and consequently TPP levels drop. When transformed in plants, engineered TPP riboswitches can act autonomously to modulate gene expression. In an evolutionary perspective, a TPP riboswitch is also present in ancient plant taxa, suggesting that this mechanism is active since vascular plants emerged 400 million years ago.

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Riboswitches are regions in mRNA that act as metabolite sensors. These selectively bind small molecule targets such as vitamins, amino acids, and nucleotides through a conserved sensor domain. Upon substrate binding, the conformation of a variable "expression platform" coupled to the sensor domain is changed, and this can affect different modes of gene control including transcription termination, translation initiation, or mRNA processing (Mandal and Breaker 2004; Nudler and Mironov 2004; Coppins et al. 2007). Notably, riboswitches exert their functions without the need for protein cofactors (Mironov et al. 2002; Winkler et al. 2002). In most cases, they act in feedback regulation mechanisms; once the level of an end product in a metabolic pathway rises, riboswitch binding occurs, triggering a repression of gene expression in the same pathway. The substrate specificity of riboswitches is extremely high, allowing them to perform their activity amid the presence of numerous

related compounds (Sudarsan et al. 2005). In prokaryotes, genetic control mediated by riboswitches is a prevalent phenomenon, and the dozen riboswitches identified to date regulate >3% of all bacterial genes (Nudler 2006).

Thiamin pyrophosphate (TPP) is a coenzyme derived from vitamin B1 (thiamin) that is synthesized by bacteria, fungi, and plants, and serves as a dietary requirement for humans and other animals. It is an obligatory cofactor that plays an important role in amino acid and carbohydrate metabolism; thus, fine control of its levels is essential for the viability of all living cells. TPP-binding riboswitches, first identified in *Bacillus subtilis* and *Escherichia coli* (Mironov et al. 2002; Winkler et al. 2002), exist in the genomes of species belonging to most bacterial phyla (Rodionov et al. 2002). Binding of TPP by the riboswitch down-regulates expression of thiamin biosynthesis genes by inducing either the formation of a transcription terminator hairpin or the formation of a Shine-Dalgarno sequester hairpin (Mironov et al. 2002; Rodionov et al. 2002; Winkler et al. 2002). Much less is known about riboswitch-mediated gene control in eukaryotic organisms, and TPP-binding riboswitches are the only ones identified to date (Kubodera et al. 2003; Sudarsan et al. 2003). Very recently, Cheah et al. (2007) demonstrated that a TPP riboswitch controls expression of the *THI4* and *NMT1* genes in *Neurospora crassa* by directing the splicing of an intron located in the 5' untranslated region (UTR). Intron retention results in the appearance of upstream and out-of-frame initiation codons, whereas intron splicing generates a complete and correct open reading frame. While the prokaryotic and fungi riboswitches are located in the 5' UTR, a plant TPP riboswitch located in the 3' UTR of the *Arabidopsis* *THIAMINE C SYNTHASE* gene (*AtTHIC*) was recently identified (Sudarsan et al. 2003). This difference in location suggests a unique mode of action for the plant riboswitch. Yet, the mechanisms mediated by riboswitch activity in higher eukaryotes, particularly in the plant kingdom, remain to be elucidated. Here we propose a unique prevalent model for TPP riboswitch-controlled gene expression in all flowering plants, according to which TPP binding to *THIC* pre-mRNA engenders alternative splicing that leads to the generation of an unstable transcript, which in turn lowers TPP biosynthesis.

Results and Discussion

The concentration of TPP controls alternative splicing in the 3' UTR of flowering plant THIC genes

The conservation of the TPP-binding riboswitch in the plant kingdom was examined by computational screening of plant EST libraries (<http://compbio.dfci.harvard.edu/tgi>). We discovered that a TPP riboswitch is present in the 3' UTR of *THIAMINE C SYNTHASE* (*THIC*) genes of 13 different plant species (Supplementary Table S2). Moreover, in eight of them, two alternatively spliced variants were detected in which the second intron of the 3' UTR (int2) was either retained or spliced out, without affecting the coding sequence (Fig. 1A; Supplementary Table S2). In all cases, the 3' splice site of int2 was located inside the riboswitch P2 box. Previous studies showed that RNA folding at 3' splice sites may alter the

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Figure 1. TPP-dependent alternative splicing in the 3' UTR of *Arabidopsis* and tomato *THIC* genes. (A) Schematic representation of the *THIC* pre-mRNA and the two RNA splicing products, marking the TPP riboswitch, introns 1 and 2 (dark gray), and exons (light gray). Numbers indicate oligonucleotides used for experiments described in B (see Supplemental Material). (B) Transcript levels in TPP-treated *Atthi1* mutant seedlings resolved by qPCR experiments ($n = 3$; $P < 0.01$; SEM; see Materials and Methods for details). (var.) Variant. The oligonucleotides (see A) used were #21 and #22 (coding seq.), #23 and #24 (intron-retention var.), and #25 and #26 (intron-spliced var.), and *AtACTIN2* was used as endogenous control (oligonucleotides #34 and #35). (C) End-point analysis RT-PCR products that monitor the levels of the *THIC* RNA splicing products in the tomato thiamin auxotroph mutant strain grown under increasing TPP concentrations. The two splicing variants were amplified with specific oligonucleotides (#8 and #9, and #8 and #10), and the coding region was amplified with oligonucleotides derived from conserved regions (#8 and #11). The intron-retention variant, the intron-spliced variant, and the coding sequence were amplified for 31, 35, and 33 cycles, respectively. The effect of TPP addition on whole plants is shown at the bottom of each panel. (D) Stability of the *AtTHIC* splice variants and coding sequence during variable time points after the addition of the transcription inhibitor actinomycin D to *Arabidopsis* cell suspension culture supplemented with 1 mM TPP, as resolved by qPCR using the same oligonucleotides as in B ($n = 3$; $P < 0.01$; SEM). *AtUBIQUITIN* was used as the endogenous control (oligonucleotides #27 and #28). The decay of the intron-spliced variant was on average 63% per hour.

splicing process (Buratti and Baralle 2004). We therefore predicted that a conformation change due to ligand binding by the riboswitch would alter the splicing of this intron.

To test the prediction that ligand binding affects alternative splicing of *THIC*, we analyzed the expression of the two RNA splicing products in *Arabidopsis* (*At*) plants supplemented with increasing TPP concentrations. When such experiments were conducted with wild-type *Arabidopsis* plants (containing endogenous TPP), the effect of adding exogenous TPP on *AtTHIC* splicing was barely detectable (Supplementary Fig. S1B). Therefore, a thiamin auxotroph mutant strain of *Arabidopsis* (*Atthi1*) (Supplementary Fig. S1A; Li and Redei 1969) that contains very low levels of endogenous TPP was used. The results showed a fourfold decrease in the expression of the intron-retention variant in the presence of rising TPP concentrations (from 0 to 10 μ M), while expression of the intron-spliced variant increased sevenfold (Fig. 1B). Furthermore, testing the expression of the total *THIC* mRNA population (using oligonucleotides driven from the coding sequence) revealed a 3.75-fold decrease with the same rising TPP concentrations (Fig. 1B). For all three transcripts, the statistical signifi-

cant change in expression was detected between 100 nM and 1 μ M TPP (Fig. 1B). This finding is in correspondence with the Kd of the *Arabidopsis* TPP riboswitch observed in our in-line probing assay (~500 nM) (Fig. 4C, below). In order to assess the universality of the TPP effect on alternative splicing, similar experiments were performed using a tomato (*Solanum lycopersicon*) thiamin auxotroph mutant (Boynton 1966). Indeed, as detected for *AtTHIC*, the tomato *THIC* RNA splicing products showed a TPP concentration-dependent expression pattern (Fig. 1C). These results, from two different plant species, provide evidence that the TPP level determines the pattern of RNA splicing of the *THIC* precursor mRNA.

The two RNA splicing variants of the *THIC* gene differ in their stability

Elevated levels of TPP results in the generation of a transcript in which the second intron (int2) of the 3' UTR is spliced out. We found that the *THIC* intron-spliced variant is different from the intron-retention variant in two major features. The intron-retention variant has a single consensus polyadenylation [poly(A) formation] signal, and polyadenylation occurs at one accurate site (U370 in the 3' UTR, detected by 3' Rapid Amplification of cDNA Ends [RACE] experiment) (Supplementary Fig. S2). However, in the intron-spliced variant, the consensus polyadenylation signal is eliminated, and polyadenylation occurs at a range of positions along the 3' UTR (from U531 to C945, detected by 3' RACE experiments). Another feature that distinguishes between the two RNA splicing products is that the 3' UTR transcript is longer when int2 is spliced out due to the absence of a consensus poly(A) signal (Fig. 1A; Supplementary Fig. S2). It is documented that the selection of polyadenylation sites and 3' UTR length may result in different intrinsic stabilities of a transcript (Maret et al. 2004). The observation that the overall transcript level of the *THIC* gene (the sum of RNA splicing products) is reduced while the level of the intron-spliced variant is increased (under elevated TPP concentrations) suggests reduced stability of the intron-spliced variant.

To evaluate the difference in stability between the two RNA splicing products, we examined their decay rate following incubation of *Arabidopsis* cell suspension cultures supplemented with the transcription inhibitor actinomycin D (Fig. 1D; Supplementary Fig. S1C). The results showed that the relative stability of the intron-spliced variant is much lower than that of the intron-retention variant. During the 2 h following the actinomycin D treatment, the intron-spliced variant exhibited an average decay rate of 63% per hour, while the intron-retention variant and the overall transcript level of the *AtTHIC* gene did not show a significant decay (Fig. 1D). The rapid decay of the intron-spliced variant detected here suggests that our analysis of its expression levels (e.g., Fig. 1B), particularly under high TPP concentration, is an underestimation of its genuine production rate.

The results show that the two alternatively spliced transcripts formed as a result of riboswitch activity differ in their stability, the intron-spliced variant retaining a much higher decay rate (compared with the intron-retention variant).

Figure 2. TPP riboswitch-dependent alternative splicing in transgenic *Arabidopsis* plants. (A) Schematic representation of the constructs used to generate transgenic *Arabidopsis* plants and protoplasts expressing a *YFP* reporter gene that was transcriptionally fused to the native *AtTHIC* 3' UTR (i), the same 3' UTR but with a point mutation disabling riboswitch ligand binding (A515G) (ii), the same 3' UTR but with a point mutation in the 5' splice (spl.) site of int2 that prevents splicing in this region (iii) (Fig. 1A), and *YFP* transcriptionally fused to the *NOPALINE SYNTHASE* (NOS) terminator (iv). (B) RT-PCR end-point analysis experiments in which the transcript levels of the *YFP* RNA splicing products and the coding region were monitored in transgenic plants grown with or without additional TPP. The intron-retention variant, the intron-spliced variant, and the coding sequence were amplified for 18, 22, and 18 cycles, respectively. (C) RT-PCR end-point analysis experiments in which transcript levels of the *YFP* RNA splicing products and the coding region were detected in *Arabidopsis* protoplasts transiently transformed with the corresponding constructs, and incubated with or without additional TPP. The intron-retention variant, the intron-spliced variant, and the coding sequence were amplified for 22, 22, and 27 cycles, respectively. (D) *YFP* expression in transgenic *Arabidopsis* plants determined by fluorescence and Western blot analyses.

Processing of *THIC* pre-mRNA is dependent on TPP riboswitch activity, and engineered riboswitches can act autonomously to modulate gene expression

We further investigated whether the alteration in splicing under high TPP levels is independently mediated by the TPP riboswitch activity. These experiments were performed in planta, with the following constructs (Fig. 2A): (i) the *YELLOW FLUORESCENCE PROTEIN* (*YFP*) transcriptionally fused to the native *AtTHIC* 3' UTR (35S:*YFP-THIC3'*); (ii) the same construct but with a single point mutation (A515G relative to the stop codon) in the 3' UTR that prevents the riboswitch from binding TPP (35S:*YFP-THIC-Mu3'*) (Sudarsan et al. 2003, 2005; Thore et al. 2006); (iii) the same construct but with a mutated 5' splice site of int2 (Fig. 1A) that prevents splicing of this intron (35S:*YFP-THIC-NoSpl3'*); and (iv) the same construct but with the *YFP* fused to the *NOPALINE SYNTHASE* terminator (35S:*YFP-NOS3'*).

The expression of the two RNA splicing products and the *YFP* reporter was examined in two plant systems. In the first system, stably transformed *Arabidopsis* plants harboring the constructs 35S:*YFP-THIC3'*, 35S:*YFP-THIC-Mu3'*, and 35S:*YFP-NOS3'* were used (Fig. 2B,D). The second system used *Arabidopsis* protoplasts transiently transformed with all four constructs (Fig. 2C). In every experiment, reporter expression was analyzed with and without supplemental TPP. Remarkably, under high TPP levels, expression of the intron-retention variant in 35S:*YFP-THIC3'* decreased and that of the intron-spliced

variant increased (Fig. 2B,C). No expression of the intron-spliced variant was detected in both systems harboring either the mutated riboswitch (35S:*YFP-THIC-Mu3'*) or the mutated 5' splice site of int2 (35S:*YFP-THIC-NoSpl3'*). Expression of the retention variant in transgenic plants harboring the 35S:*YFP-THIC-Mu3'* was slightly reduced (Fig. 2B), suggesting that the mutation introduced in our construct *THIC-Mu3'* reduces the riboswitch affinity to TPP but does not abolish its activity completely (Sudarsan et al. 2005). Expression of the retention variant in transgenic plants harboring the 35S:*YFP-THIC-NoSpl3'* was not affected by additional TPP (Fig. 2C).

We also examined the level of *YFP* gene expression in the different transformed lines (using oligonucleotides specific to its coding sequence). Addition of TPP reduced the expression of *YFP* in 35S:*YFP-THIC3'* plants but had no such effect in plants or protoplasts harboring 35S:*YFP-THIC-Mu3'* or 35S:*YFP-THIC-NoSpl3'*, as well as in the control 35S:*YFP-NOS3'* plants (Fig. 2B,C). The fact that disabling splicing of int2 prevented the decrease in overall *YFP* expression demonstrated that alternative splicing is essential for riboswitch controlled expression. At the protein level, *YFP* accumulation (measured either by Western blotting or by *YFP* fluorescence analysis) was greatly reduced in 35S:*YFP-THIC3'* plants supplemented with TPP, while its expression was not altered in 35S:*YFP-THIC-Mu3'* as well as in 35S:*YFP-NOS3'* (Fig. 2D).

Taken together, these experiments show that when transformed in plants, engineered TPP riboswitch can act autonomously to modulate gene expression. They also demonstrate that alternative splicing in the 3' UTR of the transgene is dependent on riboswitch activity. Furthermore, under high TPP concentration, alternative splicing is essential for the reduction in mRNA levels.

The TPP riboswitch is present in the *THIC* gene of all plant taxa, and an additional TPP riboswitch, lost during gymnosperm evolution, is present in the *THI1* gene of ancient plants

To trace the evolutionary origin of plant riboswitches, we searched for homologous sequences of the TPP riboswitch across the plant kingdom. Homologous sequences were identified in the *THIC* 3' UTR of all ex-

Figure 3. The TPP riboswitch is present in two genes of the thiamin biosynthesis pathway in ancient plants. The presence of the TPP riboswitch in the 3' UTR of the *THIC* and *THI1* genes across the plant kingdom is depicted. (np) Not present, based on analysis of whole genome sequences; (nd) not detected in PCR assays.

Figure 4. (A) Schematic representations of the TPP-binding domain of *P. patens* (*Pp*) located in the 3' UTRs of the *PpTHIC* and *PpTHI1-2* genes. Exon and intron sequences are indicated by uppercase and lowercase letters, respectively. Arrows indicate the adenine base that forms the crucial assembly platform of the TPP riboswitch (Serganov et al. 2006; Thore et al. 2006); the arrowhead marks the 3' splice site. Shadowed nucleotides are mostly conserved among TPP riboswitches (Sudarsan et al. 2003). (B) RT-PCR end-point analysis products that monitor the transcript levels of the *PpTHIC* and *PpTHI1* genes in *P. patens* (*Pp*) protomemata grown without or with additional TPP. (PDS) Phytoene desaturase transcript was used for calibration. *PpTHIC* and *PpTHI1* genes were amplified for 25 cycles. (C) Apparent K_d for TPP binding by the TPP riboswitch of *Arabidopsis* (*AtTHIC*) and *P. patens* (*PpTHI1-2*), determined by in-line probing (Sudarsan et al. 2003). (D) Fragmentation pattern of the in vitro-transcribed 80-bp RNA molecule that comprises the *P. patens* *THI1-2* TPP-riboswitch. The in-line probing assay was performed in the presence of increasing TPP concentrations (0–10 μ M). (NR) Non-reacted RNA; (OH⁻) RNA subjected to alkaline degradation; (T1) RNA submitted to RNase T1 digestion.

aminated species, ranging from the liverwort *Marchantia polymorpha* (bryophytes) to higher plants (angiosperms) (Fig. 3; Supplementary Fig. S3). However, when we examined the *THIC* gene of *Physcomitrella patens* (*P. patens* or *Pp*), we could not find evidence for splicing in the second intron of its 3' UTR, as detected for flowering plants (int2) (see Fig. 1A). Therefore, it seems that the *PpTHIC* gene is regulated by a different mechanism than the one used in flowering plants, which does not involve alternative splicing in its 3' UTR. This is in accordance with previous studies reporting that intron-containing genes and genes undergoing alternative splicing have increased considerably from ancestral to higher eukaryotes and that alternative splicing is a relatively advanced evolutionary process (Reddy 2007).

TPP biosynthesis in plants occurs by the condensation of thiazol and pyrimidine moieties (Supplementary Fig. S4). Interestingly, we also found that in *P. patens*, a putative TPP riboswitch sequence is present in the 3' UTR of the *THIAZOL BIOSYNTHESIS 1* (*THI1*; a *THI4* ortholog) gene family members, which encode a thiazol biosynthetic enzyme (Fig. 4A; Supplementary Fig. S5). This suggests that the TPP biosynthesis pathway is controlled by a system of double riboswitch check points. In

contrast to *Arabidopsis*, there are four *THI1* genes in *P. patens*, and three of them possess a TPP riboswitch in the 3' UTR (*PpTHI1-2*, *PpTHI1-3*, *PpTHI1-4*). In two of these genes (*PpTHI1-2*, *PpTHI1-3*), splicing at the 3' UTR occurs, and the intron 3' splice site is located at the P3 box of the TPP riboswitch. However, this intron seems to be constitutively spliced as we could not find, either experimentally or in public databases, evidence for the presence of an intron-retention variant (Fig. 4B; Supplementary Fig. S5). In contrast, no indication of splicing was evident in the 3' UTR of the third *P. patens* gene (*PpTHI1-4*) (Supplementary Fig. S5). *PpTHIC*, *PpTHI1-3*, and *PpTHI1-4* are down-regulated upon TPP supplementation (expression of *PpTHI1-1* and *PpTHI1-2* was barely detectable) (Fig. 4B). In-line probing analysis of the *PpTHI1* 80-base-pair (bp) TPP riboswitch revealed that it exhibits a TPP-dependent structural modulation that is comparable with the parallel bacterial and *Arabidopsis* TPP riboswitches (Fig. 4A,D; Sudarsan et al. 2003). The *PpTHI1* RNA binds TPP at a K_D of ~400 nM (Fig. 4C), which is comparable with the K_D value (~500 nM) obtained by our in-line probing assay using the *AtTHIC* riboswitch.

In an evolutionary perspective, we found that an additional TPP riboswitch is present in the *THI1* gene of ancient plant taxa (bryophytes, lycopphytes, and monili-forms), including in the most ancient land plants, the liverwort *Marchantia polymorpha*, but it was lost from this gene family during gymnosperm evolution (Fig. 3; Supplementary Fig. S3). We also discovered that alternative splicing in the 3' UTR of the *THIC* and *THI1* genes occurred already in lycopphytes (i.e., *Selaginella*), an ancient vascular plant family that appeared 150–200 million years before angiosperms such as *Arabidopsis* and rice (Fig. 3; Supplementary Fig. S3).

In this study, we report on a novel post-transcriptional regulatory mechanism for gene regulation in plants (Fig. 5). The findings reveal that a TPP riboswitch mediates feedback regulation in the thiamin biosynthesis pathway through modulation of alternative splicing in the 3' UTR region of a gene of this pathway. High levels of TPP in the cell promote ligand binding and a change in the riboswitch structural conformation. Consequently, an unstable intron-spliced variant is formed, which in turn results in a decrease of TPP levels. Alternatively, under

Figure 5. A model for TPP riboswitch-mediated regulation of gene expression via alternative splicing in vascular plants. The 3' splice site is highlighted with a star.

low cellular TPP levels, the conformation of the riboswitch dictates intron retention that generates a stable transcript, which in turn promotes an increase in TPP biosynthesis. An intriguing question that arises is: How does riboswitch conformational change direct alternative splicing? The structure of the *Arabidopsis* riboswitch-TPP complex was recently resolved by Thore et al. (2006). Examination of this complex structure reveals that in the ligand-bound state, the 3' splice site of the intron is exposed (Supplementary Fig. S6). We therefore hypothesize that ligand binding brings about conformational changes in the 3' region of the intron that promote splicing. On the other hand, riboswitch conformation in the unbound state could therefore hinder intron processing by preventing the splicing machinery from accessing this region (Buratti and Baralle 2004).

Riboswitch-mediated alternative splicing gene expression control is active in both ancient and modern vascular plants and hence might have appeared during their emergence 400 million years ago. The riboswitch loss in the thiazol branch of the thiamin pathway (in *THI1*) in the gymnosperm lineage suggests that a single point of riboswitch control may be sufficient, especially if new regulatory mechanisms have been adopted in parallel. The data reported here on the riboswitch-mediated control of both endogenous and foreign genes expression revealed a unique regulatory mechanism that acts autonomously in planta, independent of promoter activation or effects exerted by coding sequences or the 5' UTR. To what extent riboswitch-dependent processes are widespread in plants is hard to predict at this point, but we expect that this report will trigger additional findings of a similar nature. This study thus paves the way for future engineering of plant riboswitches, in an attempt to map metabolite concentrations and regulate pre-mRNA splicing of genes that are involved in their biosynthetic pathways.

Materials and methods

Oligonucleotides

All oligonucleotides used in our experiments are listed in Supplementary Table S1.

Plant material

See the Supplemental Material for all details regarding plant material.

Plant transformation

For the generation of transgenic *Arabidopsis*, the *AtTHIC* 3' UTR was amplified by PCR using *Arabidopsis* genomic DNA (ColO) with oligonucleotides #1 and #2 (Supplementary Table S1). 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 #20. The *AtTHIC* 3' UTR was then fused to the *YFP* reporter gene, and the fusion fragment was subsequently inserted downstream from the double 35S promoter of the Cauliflower Mosaic Virus (CaMV). The 5' splice site mutation was obtained by amplifying the cassette-containing plasmid with oligonucleotides #29 and #30, followed by self-ligation of the PCR product and transformation of the resulting plasmid into *Escherichia coli*. This resulted in one construct containing the following mutations: T263A, T267A, T276A, T279A, T287A, and T292A. The cassettes were then inserted into the pBinPlus binary vector containing the kanamycin resistance gene for the selection of transformants (van Engelen et al. 1995). *Arabidopsis* plants were transformed using the floral-dip method (Clough and Bent 1998), and kanamycin-resistant seedlings were then transferred to soil.

Molecular biology and microscopy

Unless specified, all molecular biology manipulations were performed as described in Sambrook et al. (1989). RNA extractions were all performed

using the RNeasy kit (Qiagen) and DNA extractions were performed using the CTAB method (Doyle and Doyle 1987).

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 oligonucleotide sequences are provided in Supplementary Table S1 (#21–#26), and their efficiency standard curves are depicted in Supplementary Figure S1D. *AtACTIN2* was used as an endogenous control for the expression analysis of each *AtTHIC* variant, and *AtUBIQUITIN C* was used as an endogenous control for the relative stability experiment (with oligonucleotides #34–#35 and #27–#28, respectively). A fixed amount of DNase-treated total RNA was reverse-transcribed using AMV Reverse Transcriptase (EurX Ltd.). RT-PCR reactions were tracked on an ABI 7300 instrument (Applied Biosystems) using the PlatinumR SYBR SuperMix (Invitrogen). Each sample was PCR-amplified from the same amount of cDNA template in triplicate reactions. Following an initial step in the thermal cycler for 15 min at 95°C, PCR amplification proceeded for 45 cycles of 15 sec at 95°C and 30 sec at 60°C, and was completed by melting curve analysis to confirm specificity of the PCR products. The baseline and threshold values were adjusted according to the manufacturers' instructions.

RT-PCR end-point analysis was performed as described in Aharoni et al. (2004a) with oligonucleotides #3–#7 (Supplementary Table S1). In tomato, the intron-retention variant was detected with oligonucleotides #8 and #9, the intron-spliced variant was detected with #8 and #10, and the *SITHIC* coding region transcripts were detected with #8 and #11 (Supplementary Table S1). The *TUBULIN* or *UBIQUITIN C* genes (for *Arabidopsis*) and *ACTIN* gene (for tomato) were used for calibration with oligonucleotides #17–#18, #28–#33, and #12–#13, respectively. The *YFP* mRNA transcript levels were determined using oligonucleotides #16 and #4 for the intron-retention variant and #16 and #5 for the intron-spliced variant, and the *YFP*-coding region transcripts were determined with #14 and #15. For all other plant species, DNA or RNA fragments corresponding to the 3' region of the *THIC* and *THI1* genes were amplified with the forward oligonucleotides #8 and #10, respectively, and reverse #9. All splicing products were confirmed by sequencing.

For RNA stability determination, actinomycin D (Sigma, catalog no. A1410) was added to *Arabidopsis* suspension cell culture to a final concentration of 50 µg/mL. Cells supplemented with 1 mM TPP were harvested at three or four time points (0, 1 h, 2 h, 4 h), followed by RNA extraction and quantitative real-time experiments with the specific oligonucleotides #21–#28 as described above (Fig. 1D). RNA stability was also determined by RT-PCR end-point analysis with the variant-specific oligonucleotides (#3–#7) (Supplementary Fig. S1C). All splicing products were confirmed by sequencing. 3' RACE experiments were performed as described by Aharoni et al. (2004b), with oligonucleotides #8, #19, #31, or #32. The *YFP* signal was detected by a standard fluorescence microscope.

In-line probing

The in-line probing experiment was conducted as described previously by Sudarsan et al. (2003). Briefly, RNA was synthesized in vitro from DNA templates by T7 polymerase, 5'-end-labeled with ³²P, and gel-purified. The radiolabeled RNA was subjected to in-line probing by incubation for 40 h at 25°C in 20 mM MgCl₂, 100 mM KCl, and 50 mM Tris-HCl (pH 8.3), in the presence of increasing TPP concentrations from 0 to 10 µM. The partially cleaved fragments were resolved on a denaturing gel (8 M urea–8% polyacrylamide), which was further subjected to a Phosphor-Imager analysis. Kd values were estimated as described in Cheah et al. (2007).

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