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Review

Thiamine in plants: Aspects of its metabolism and functions

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ABSTRACT

Thiamine diphosphate (vitamin B_1) plays a fundamental role as an enzymatic cofactor in universal metabolic pathways including glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle. In addition, thiamine diphosphate has recently been shown to have functions other than as a cofactor in response to abiotic and biotic stress in plants. Recently, several steps of the plant thiamine biosynthetic pathway have been characterized, and a mechanism of feedback regulation of thiamine biosynthesis *via* riboswitch has been unraveled. This review focuses on these most recent advances made in our understanding of thiamine metabolism and functions in plants. Phenotypes of plant mutants affected in thiamine biosynthesis are described, and genomics, proteomics, and metabolomics data that have increased further our knowledge of plant thiamine metabolic pathways and functions are summarized. Aspects of thiamine metabolism such as catabolism, salvage, and transport in plants are discussed.

Contents

1. Introduction

Thiamine (vitamin B_1) (**9**) (Fig. 1) was first isolated as the "anti-beriberi factor" from rice polishings in 1926 (Jansen and Donath, 1926). Its biochemical action in treating beriberi, a lethal disease

Abbreviations: HMP, 4-amino-2-methyl-5-hydroxymethylpyrimidine; HET, 4-methyl-5-β-hydroxyethylthiazole.

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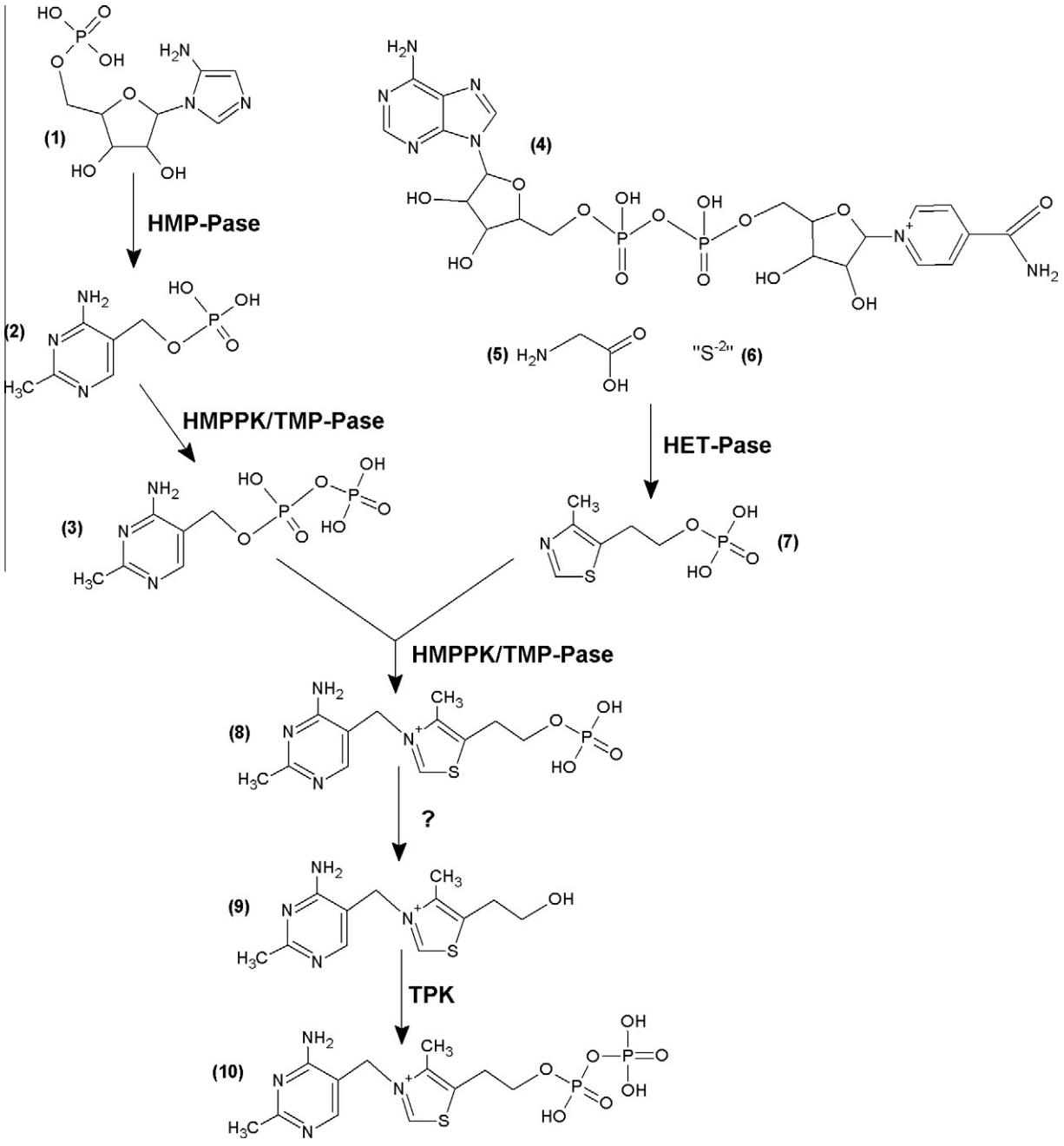

Fig. 1. Scheme of proposed *de novo* thiamine diphosphate biosynthesis in plants. (1) 5-Aminoimidazole ribonucleotide (AIR), (2) 4-amino-2-methyl-5-hydroxymethylpyrimidine monophosphate (HMP-P), (3) 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate (HMP-PP), (4) nicotinamide adenine dinucleotide (NAD), (5) glycine, (6) sulfur donor, (7) 4-methyl-5-β-hydroxyethylthiazole phosphate (HET-P), (8) thiamine monophosphate (TMP), (9) thiamine, and (10) thiamine diphosphate (TDP).

which is common in developing countries where the main food source is low in thiamine (**9**) and high in carbohydrates (Lonsdale, 2006; Rindi, 1996), was discovered in the 1930s (Peters, 1936). Since these early findings, extensive efforts have been devoted to resolve thiamine (**9**) metabolism and its functions in living organisms. In plants, it is only in recent years that much progress has been made.

Thiamine (**9**) is composed of a pyrimidine (4-amino-2-methyl-5-pyrimidyl) ring linked to a thiazole (4-methyl-5-β-hydroxyethylthiazolium) ring by a methylene bridge. It can be phosphorylated on its hydroxyl group to form monophosphate (TMP), diphosphate (TDP), or triphosphate (TTP) esters. In plants, the thiamine (**9**) biosynthetic pathway has recently been characterized, and been shown to feature similarities to both bacterial and yeast pathways. The feedback regulation of plant TDP biosynthesis *via* a riboswitch has also recently been unraveled (Bocobza et al., 2007; Wachter et al., 2007).

TDP, the active cofactor form, is required for various enzymes of carbohydrate and amino acid metabolism in all living organisms. For instance, TDP serves as a cofactor of mitochondrial pyruvate dehydrogenase (PDH) during glycolysis (LeClere et al., 2004), and of acetolactate synthase in the synthesis of the branched-chain amino acids valine, leucine and isoleucine (Chang and Duggleby, 1997; Roux et al., 1996; Singh et al., 1988; Wu et al., 1994). Recently, TDP has also been shown to play an important role other than as a cofactor in resistance against abiotic and biotic stress in

plants (Ahn et al., 2005; Sayed and Gadallah, 2002; Tunc-Ozdemir et al., 2009).

This review will provide an overview and update on thiamine (**9**) metabolism and functions in plants. New findings that have resulted from the tremendous advances in genomics, proteomics, metabolomics, and the use of reverse genetics are emphasized.

2. Metabolism

2.1. Biosynthetic pathways

In plants, yeast, and bacteria, TMP is synthesized by condensation of HMP-PP (**3**) and HET-P (**7**) (Fig. 1) (Begley et al., 1999; Nosaka, 2006; Roje, 2007).

In plants, the pyrimidine moiety of thiamine (**9**) is very likely synthesized *via* a pathway identical to that of bacteria in which the first committed step involves a complex chemical rearrangement of 5-aminoimidazole ribonucleotide (AIR) (**1**) to HMP-P (**2**) which is catalyzed by HMP-P synthase (THIC) and requires *S*-adenosylmethionine (SAM) and reduced nicotinamide. Indeed, a homolog of bacterial *THIC* was recently characterized in Arabidopsis (Kong et al., 2008; Raschke et al., 2007). The Arabidopsis *THIC* gene complemented *Escherichia coli thiC* mutant (Kong et al., 2008). Expression studies of fusion proteins showed that THIC is localized in the stroma of chloroplasts (Kong et al., 2008; Raschke et al., 2007). Seedlings of *thiC* knockdown mutants had reduced thiamine (**9**) content, exhibited a chlorotic phenotype, and did not develop beyond the cotyledon stage, but could be rescued by supplementing thiamine (**9**) in the medium. Metabolite profiling of Arabidopsis seedlings showed a dramatic increase in the level of certain amino acids and certain constituents of the tricarboxylic acid cycle in the *thiC* mutant, revealing the importance of THIC in primary metabolism (Raschke et al., 2007). The recombinant Arabidopsis THIC protein featured UV–visible spectra characteristic of [Fe–S] cluster proteins (Raschke et al., 2007). This observation was later reinforced by the presence of one [4Fe–4S] cluster per subunit of bacterial THIC (Chatterjee et al., 2008a; Martinez-Gomez and Downs, 2008). Interestingly, silencing the *CpnifS* gene of Arabidopsis, which encodes the chloroplastic cysteine desulfurase which supplies the sulfur for [Fe–S] clusters, led to a reduction in THIC protein accumulation and a reduction of total thiamine (**9**) content (Raschke et al., 2007). In bacteria, THIC is a member of the radical SAM family (Chatterjee et al., 2008a). The [4Fe–4S] cluster of bacterial THIC reduces SAM to give an adenosyl radical. This radical then participates in the isomerization of AIR (**1**) to HMP-P (**2**). The presence of [Fe–S] clusters in plant THIC suggests that a similar reaction may occur in plants.

The HET-P (**7**) biosynthesis pathway in plants seems to be similar to that of yeast, in which HET-P synthase catalyzes the formation of HET-P (**7**) from NAD, glycine, and a yet-to-be-identified sulfur donor (Chatterjee et al., 2007, 2008b). HET-P synthase is the only enzyme involved in thiazole biosynthesis which has been identified so far in eukaryotes. Supporting a yeast-like pathway in plants, sequence homologs of the yeast HET-P synthase (THI4) were found in *Zea mays* (Belanger et al., 1995), *Alnus glutinosa* (Ribeiro et al., 1996), *Arabidopsis thaliana* (Machado et al., 1996), and *Oryza sativa* (Wang et al., 2006). HET-P synthase cDNAs from *Z. mays*, *A. glutinosa*, and *A. thaliana* could complement the *thi4* yeast mutant deficient in HET-P synthase. In addition, Arabidopsis *tz* (thiazole requirement) mutants are chlorotic and die early during development unless supplemented with thiazole or thiamine (**9**) (Feenstra, 1964; Redei, 1965). Later studies showed that the Arabidopsis *THI1* (or *ara6*) gene encoding a putative HET-P synthase homologous to yeast THI4 mapped in chromosome V in the *tz* locus (Ribeiro et al., 1996). A point mutation in the *THI1* gene that causes an amino acid substitution in a highly conserved region among eukaryotic *THI1* homologs was identified in a *tz* mutant line (Papini-Terzi et al., 2003). These results suggest that the *tz* locus corresponds to the Arabidopsis structural gene *THI1* and that the encoded HET-P synthase is essential for thiamine (**9**) biosynthesis. Supporting this observation, transgenic rice plants with reduced expression of the putative HET-P synthase (OsDR8) had lower thiamine (**9**) content compared to wild-type plants (Wang et al., 2006). The three-dimensional structure of the THI1 protein from Arabidopsis was recently resolved (Godoi et al., 2006) and this established that the protein assembles as an octamer, combines with a potential intermediate of the thiazole biosynthesis in eukaryotes, and that NAD probably acts as a substrate in the formation of this intermediate as has been demonstrated in yeast (Chatterjee et al., 2007). Supporting earlier studies showing that thiazole synthesis takes place in plastids (Julliard and Douce, 1991), HET-P synthases from *Z. mays* (Belanger et al., 1995) and Arabidopsis (Chabregas et al., 2001) were detected in chloroplasts by immunogold labeling. HET-P synthase was also detected in mitochondria in Arabidopsis (Chabregas et al., 2001, 2003), and the authors showed that differential usage of two in-frame translational start codons regulates subcellular localization of HET-P synthase in Arabidopsis. In addition to their role in thiamine (**9**) biosynthesis, HET-P synthases from Arabidopsis and yeast were shown to be involved in mitochondrial DNA damage tolerance (Machado et al., 1996, 1997). Therefore, the dual targeting of HET-P synthase would enable this enzyme to function in protection against DNA damage when targeted to mitochondria and to function in thiamine (**9**) biosynthesis when targeted to chloroplasts (Ajjawi et al., 2007b).

Phosphorylation of HMP-P (**2**) to HMP-PP (**3**) and condensation of HMP-PP (**3**) and HET-P (**7**) to form TMP is catalyzed by a bifunctional enzyme in plants. Indeed, cDNAs from *Brassica napus* (*BTH1*), *Z. mays* (*THI3*), and Arabidopsis (*TH1*) were shown to encode proteins which have sequence similarity to many bacterial HMP-P kinases (HMPPK) at the N-terminus (phosphorylation of HMP-P) and to bacterial TMP pyrophosphorylases (TMP-PPase) at the C-terminus (condensation of HMP-PP (**3**) and HET-P (**7**)) (Ajjawi et al., 2007b; Kim et al., 1998; Rapala-Kozik et al., 2007). The *B. napus* and Arabidopsis cDNAs could complement an *E. coli* mutant deficient in TMP-PPase activity as well as *E. coli* and *Salmonella* mutants deficient in HMPPK activity (Ajjawi et al., 2007b; Kim et al., 1998). The Arabidopsis *th1* mutant is a thiamine nutritional auxotroph that possesses a bleached phenotype and eventually dies when grown on media devoid of thiamine (**9**) (Li and Redei, 1969), and leaf extracts from this mutant were unable to form TMP when provided with HMP and HET (Komeda et al., 1988). Interestingly, the Arabidopsis *TH1* cDNA complemented the thiamine (**9**) auxotrophy of the *th1* mutant, showing that the *th1* locus corresponds to the structural gene *TH1* and that the encoded HMPPK/TMP-PPase is essential for thiamine (**9**) biosynthesis (Ajjawi et al., 2007b). The recombinant THI3 protein from *Z. mays* was purified and shown to possess both HMP kinase and HMP-P kinase activities, as well as TMP-PPase activity (Rapala-Kozik et al., 2007). The TMP-PPase activity of THI3 required the presence of magnesium ions in the reaction mixture and was inhibited by excess of HMP-PP (**3**) and ATP. Site-directed mutagenesis experiments showed the essential roles of certain amino acid residues for HMPPK and TMP-PPase activities. All plant HMPPK/TMP-PPases contain an N-terminal extension with characteristics of an organellar targeting peptide (Rapala-Kozik et al., 2007), and fusion proteins experiments showed that the Arabidopsis TH1 protein is addressed to chloroplasts (Ajjawi et al., 2007b).

In plants and yeast, TMP (**8**) formed by condensation of HMP-PP (**3**) and HET-P (**7**) is dephosphorylated to thiamine (**9**). Then free thiamine (**9**) is pyrophosphorylated to TDP (**10**). Plants contain

numerous phosphatases amongst which some possess hydrolase activities towards TMP (**8**) and TDP (**10**) (and other substrates) *in vitro* (Rapala-Kozik et al., 2008, 2009). However, hydrolases specific of TMP (**8**) have never been characterized and it is currently believed that TMP (**8**) dephosphorylation is catalyzed by unspecific hydrolases. Thiamine pyrophosphokinase (TPK) catalyzes the conversion of free thiamine (**9**) to TDP (**10**). Recently, two genes encoding proteins with sequence similarities to animal and fungal TPKs were characterized in Arabidopsis (Ajjawi et al., 2007a). Expression of both Arabidopsis cDNAs in *E. coli* which lacks TPK activity conferred bacterial cells the ability to convert thiamine (**9**) to TDP (**10**) in the presence of magnesium and ATP. Biochemical characterization of the purified recombinant proteins showed that both proteins were catalytically very similar to each other. T-DNA insertion mutant lines for each Arabidopsis TPK gene were analyzed. The single TPK gene knockouts were similar in appearance to the wild-type plants and possessed similar thiamine (**9**) profiles compared to the wild-type. However, the double knockout mutant was auxotrophic for thiamine (**9**) and had a seedling lethal phenotype; it accumulated free thiamine (**9**), and was almost completely depleted of TDP (**10**). This mutant could be rescued by misting the seedlings with TDP (**10**), but not thiamine or TMP, showing that the TPKs are the primary route for TDP (**10**) formation in Arabidopsis. Subcellular localization studies showed that TPKs are located exclusively in the cytosol.

The results reviewed here show that plants have evolved a thiamine (**9**) biosynthetic pathway that has similarities with both bacterial and yeast pathways. While most thiamine (**9**) biosynthetic genes have now been isolated in plants, future studies should emphasize on the biochemical activities of the initial enzymes of the pyrimidine and thiazole branches of the pathway.

2.2. Regulation of biosynthesis

Thiamine (**9**) biosynthesis must be regulated in order to perfectly match the production to the demand for the cofactor. Below is summarized what is known about the regulation of thiamine (**9**) biosynthetic genes/proteins in plants.

2.2.1. Riboswitch-dependent gene regulation

In both prokaryotes and eukaryotes, TDP (**10**) was shown to bind to metabolite binding domains within certain messenger RNAs called riboswitches (Bocobza and Aharoni, 2008; Narberhaus, 2010; Smith et al., 2010; Sudarsan et al., 2003; Wachter, 2010), triggering allosteric rearrangements of mRNA structures that result in modulation of gene expression and protein production. A TDP-sensing riboswitch is present in the 3′ UTR of the pyrimidine biosynthetic gene *THIC* of species across the plant kingdom (Bocobza et al., 2007; Wachter et al., 2007), and in the thiazole biosynthetic gene *THI1* in ancient plant taxa (Bocobza et al., 2007). The *THIC* riboswitch controls splicing and alternative 3′ end processing of *THIC* RNA transcripts. Three major types of *THIC* RNA transcripts with varying 3′ UTR lengths can be detected in Arabidopsis, tomato, and other plant species. *THIC* pre mRNA (or type I) represents the unprocessed *THIC* precursor transcript. Two spliced variants, intron-retention variant (or type II) and intron-spliced variant (or type III), in which the second intron of the 3′ UTR is either retained or spliced out, are the alternative processing fates of the *THIC-I* precursor transcript. *THIC* transcript types respond differently to changes in thiamine levels. After growing wild-type Arabidopsis seedlings (Wachter et al., 2007) or thiamine auxotroph mutant strains of Arabidopsis and tomato (Bocobza et al., 2007) on medium containing thiamine (**9**), *THIC-II* transcript levels decreased 4- to 5-fold whereas expression of *THIC-III* increased up to 7-fold and *THIC-I* transcript levels showed little change. The total amount of *THIC* transcripts also decreased over 4-fold in the presence of thiamine (**9**). Wachter et al. (2007) also showed that 12-days-old Arabidopsis plants carrying a double knockout of TPK, which rapidly depletes its pool of TDP (**10**) within 2 weeks of germination and requires exogenous TDP (**10**) to complete its life cycle (Ajjawi et al., 2007a), had a pronounced decrease in *THIC-III* RNA transcripts compared to the wild-type, indicating that the formation of this RNA is triggered by TDP (**10**). Together, these results provided evidence that the TDP (**10**) level determines the pattern of RNA 3′ end processing of the *THIC-I* precursor. The *THIC-I* and *THIC-III* transcripts can extend by more than 1 kb in their 3′ ends, while the *THIC-II* transcript has shorter 3′ UTR lengths. Also, polyadenylation occurs at one accurate site in *THIC-II* transcripts while it occurs at a range of positions along the 3′ UTR in *THIC-III* transcripts. These two major differences between *THIC-II* and *THIC-III* transcripts suggested different intrinsic stability of the transcripts. Bocobza et al. (2007) confirmed this hypothesis by showing that the intron-spliced variant had much higher decay rate compared to the intron-retention variant. Experiments on transgenic Arabidopsis plants and protoplasts expressing reporter genes that were fused to the native or engineered Arabidopsis *THIC* 3′ UTR showed that TDP binding to the riboswitch is essential for the reduction of *THIC* mRNA levels under high TDP concentration (Bocobza et al., 2007; Wachter et al., 2007). These studies also demonstrated that TDP riboswitch can act autonomously (without *THIC* promoter) to modulate gene expression and that alternative splicing in the *THIC* 3′ UTR is dependent on riboswitch activity. Wachter et al. (2007) further investigated the mechanism by which the riboswitch regulates *THIC* 3′ UTR processing events in a TDP (**10**) dependent manner and proposed a model for TDP riboswitch regulation in plants. Under low TDP concentration, the riboswitch interacts with a 5′ splice site in the 3′ UTR and prevents splicing. The retained intron contains a major processing site that permits transcript cleavage and polyadenylation. Processing from this site produces *THIC-II* transcripts, which yield high expression of the *THIC* gene. When TDP (**10**) concentrations are high, its binding to the riboswitch makes accessible the 5′ splice site in the 3′ UTR; subsequently, a splicing event removes the major processing site and transcription can extend. This results in the formation of *THIC-III* transcripts that carry long 3′ UTRs.

In the photosynthetic organism *Chlamydomonas reinhardtii*, TDP-sensing riboswitches are present in the sixth intron of *THIC* and in the first intron in the 5′ UTR of the thiazole biosynthetic gene *THI4* (these riboswitches are conserved in the related alga *Volvox carteri*) (Croft et al., 2007). When *C. reinhardtii* cells are grown without thiamine (**9**), two *ThiC* transcripts, $ThiC_S$ (for short) and $ThiC_L$ (for long), can be detected. After the addition of thiamine (**9**) to the growth medium, the $ThiC_S$ transcript level declines, concomitant with the accumulation of thiamine (**9**) and its esters in the cell, whereas that for $ThiC_L$ remains more or less constant. Long *THIC* transcripts contain an additional exon which results in the insertion of an in-frame stop codon and prevents expression of a full-length functional protein of 637 residues normally expressed from the $ThiC_S$ transcript. The truncated protein translated from the $ThiC_L$ transcript is missing 385 amino acids, which include the majority of the conserved residues. Similarly, in the absence of thiamine (**9**) in the growth medium, short *THI4* transcripts ($THI4_S$) can be detected. After the addition of thiamine (**9**) to *C. reinhardtii* cultures, $THI4_S$ starts to decline, concomitant with the increase in intracellular TDP, and two additional transcripts are detected, $THI4_M$ and $THI4_L$. Both $THI4_M$ and $THI4_L$ transcripts contain an additional exon with an upstream start codon (also called upstream ORF) which drives the expression of a short 27 amino acids peptide. The authors showed that a *pyr1* mutant of *C. reinhardtii* which contains a single base change in the 5′ UTR riboswitch of *THI4* contains only short *THI4* transcripts whether or not it is

grown in the presence of thiamine, confirming the role of this riboswitch in the regulation of transcript splicing by thiamine.

All together, these data show that photosynthetic organisms have developed highly sophisticated mechanisms of gene regulation that allow fine-tuning of the thiamine (**9**) biosynthetic pathway.

2.2.2. Tissue specificity, stress dependence, and (post-)translational regulation

2.2.2.1. HMP-P synthase. Consistent with a chloroplastic localization, *THIC* transcripts were predominantly found in green tissues and were barely detectable in roots and stems, and were more abundant in light (Kong et al., 2008; Raschke et al., 2007). An increase of *THIC* transcripts was also observed under different stresses, e.g., oxidative, drought plus heat, and cold (Rizhsky et al., 2004; Tunc-Ozdemir et al., 2009; Wong et al., 2006). Putative stress related promoter elements were identified *in silico* in the 5′ upstream of the *THIC* gene (Tunc-Ozdemir et al., 2009).

In addition, THIC protein contains a [Fe–S] cluster and it was suggested that the stability of the protein depends on this [Fe–S] cluster (Raschke et al., 2007). The authors suggested that to enable catalytic activity of THIC coupling with a reductant such as ferredoxin to reduce the [Fe–S] cluster must also occur. Proteomics studies showed that THIC is a potential target of thioredoxin in chloroplasts and contains nine conserved cysteines (Balmer et al., 2003). Chloroplastic thioredoxins could influence THIC protein activity, its oxidative regulation, and its assembly and folding.

2.2.2.2. HET-P synthase. Regulation of the HET-P synthase gene (*THI1*) in plants has been well documented. *THI1* transcripts were mostly found in green tissues and were found at much lower levels in roots, even when exposed to light, indicating that there might be tissue-specific transcription factors involved in regulation of *THI1* gene (Belanger et al., 1995; Papini-Terzi et al., 2003; Ribeiro et al., 1996). Ribeiro et al. (2005) suggested that this tissue specificity could be associated with an activation sequence-1 motif (Neuhaus et al., 1994) present in the *THI1* promoter. Expression studies using *THI1* promoter-GUS fusion constructs showed that *THI1* expression was restricted to vascular tissue in roots (Ribeiro et al., 2005).

THI1 transcripts accumulated under light conditions and decreased in the dark, and *in silico* promoter analyzes identified putative transcription binding motifs in this region related to light control (Ribeiro et al., 1996, 2005). Under oxidative stress conditions, *THI1* transcripts accumulated (Tunc-Ozdemir et al., 2009); the *THI1* promoter was also responsive to stress conditions such as sugar deprivation, high salinity, and hypoxia, as shown by *THI1* promoter-GUS fusion experiments, and several putative responsive motifs were identified *in silico* in *THI1* promoter (Ribeiro et al., 2005). Proteome profiling of *Populus euphratica* revealed an accumulation of HET-P synthase upon heat stress (Ferreira et al., 2006), suggesting that transcript and protein accumulations may correlate in plants subjected to stress. Expression of the thiazole kinase gene was induced in rice after pathogen inoculation (Wang et al., 2006).

THI1 contains two to three conserved cysteines and proteomics studies showed that THI1 is a potential target of chloroplastic thioredoxins (Balmer et al., 2003; Lemaire et al., 2004). Therefore, like THIC, thioredoxins could influence THI1 protein activity, its oxidative regulation, and its assembly and folding.

2.2.2.3. HMPPK/TMP-PPase. Transcripts of the HMPPK/TMP-PPase gene (*BTH1*) were detected in roots, stems, and leaves of 2-month-old *B. napus* plants, and expression was higher in leaves than in roots and stems (Kim et al., 1998). Expression of *BTH1* was repressed in all organs tested when plants were treated with 0.01% thiamine (**9**), but the mechanism by which it regulates *BTH1* expression is unknown. Also, oxidative stress was shown to induce the accumulation of HMPPK/TMP-PPase transcripts in Arabidopsis and to increase HMPPK/TMP-PPase activity in maize (Rapala-Kozik et al., 2008; Tunc-Ozdemir et al., 2009). The TMP-PPase activity of recombinant HMPPK/TMP-PPase from maize (THI3) was shown to be inhibited by excess of HMP-PP and ATP (Rapala-Kozik et al., 2007).

2.2.2.4. TPK. There are two genes encoding TPK in Arabidopsis: *AtTPK1* and *AtTPK2* (Ajjawi et al., 2007a). Both genes were expressed in roots, leaves, stems, siliques, and flowers, but at higher levels in leaves. *AtTPK1* was expressed at relatively high levels in roots while *AtTPK2* transcripts in roots were in low abundance. Like the other thiamine biosynthetic genes mentioned above, *AtTPK1* transcripts accumulated in response to oxidative stress (*AtTPK2* was not analyzed) (Tunc-Ozdemir et al., 2009), and TPK activity increased under water, salt, and oxidative stresses while it slightly decreased upon illumination in maize seedlings (Rapala-Kozik et al., 2008, 2009).

The data reviewed in this section show that thiamine (**9**) biosynthesis is differently regulated depending on plant tissues and environmental conditions, and that the regulation of its biosynthesis may occur at the gene, RNA, or protein levels.

2.3. Catabolism and salvage

Very little is known about thiamine (**9**) catabolism in plants and the possibility of a pathway for salvage of its degradation products has never been investigated in plants. However, recent characterization of Arabidopsis mutants affected in the thiamine (**9**) biosynthetic pathway enable us to estimate its breakdown in plants. Arabidopsis *thiC* mutants which were rescued on 100 μM thiamine (**9**) produced seeds with total thiamine content 91% of that of the wild-type. The progeny lost *ca.* 30% of total thiamine (**9**) content in 6 days, when grown on thiamine-deficient medium (Raschke et al., 2007). From these results one can estimate that Arabidopsis plants have a net thiamine breakdown of 5% per day and that the whole thiamine (**9**) pool could be depleted in 20 days in the absence of *de novo* thiamine biosynthesis.

How thiamine (**9**) degradation occurs, what its catabolism products are, and how thiamine catabolism products are salvaged remains to be elucidated. However, there is evidence that plants contain thiamine-degrading factors which were first discovered in green bracken and were shown to cause some change in the thiamine molecule resulting in the failure to detect the vitamin by the thiochrome assay (Evans, 1975). These factors have been classified into two categories: thermostable and thermolabile. Many thermostable "antithiamine" factors have been identified in various plants over the years and the most active chemicals were found to be *O*-dihydroxyphenols under aerobic conditions (Evans, 1975). Evans and colleagues investigated the products formed when thiamine (**9**) was incubated with caffeic acid, catechol, protocatechuic acid, and 4-methylcatechol. Depending on the nature of the phenolic compound, two main degradation compounds were found: thiamine disulfide and 4-amino-2-methyl-5-aminomethylpyrimidine. Thiaminases were identified as the so-called "thermolabile" factors. Thiaminase I catalyzes *in vitro* the cleavage of thiamine (**9**) by an exchange of the thiazole moiety with a variety of nucleophiles such as proline and cysteine (Evans, 1975); thiaminase II catalyzes the simple hydrolysis of thiamine (**9**) to HMP and HET. Thiaminase I and II activities were reported in a rather limited number of plants (Fujita, 1954; Meyer, 1989). The physiological significance of thiaminases within the organisms in which they are found has eluded investigators for many years. Interestingly, thiaminase II was recently shown to be involved in the regenera-

tion of pyrimidine rather than in thiamine (**9**) degradation in *Bacillus subtilis* and yeast (TenA in *Bacillus* and THI20 in yeast) (Haas et al., 2005; Jenkins et al., 2007; Onozuka et al., 2008). In this salvage pathway, a thiazole-degraded thiamine such as an *N*-formyl-5-aminomethyl derivative of 4-amino-2-methylpyrimidine is deformylated by an amidohydrolase and the resultant 4-amino-2-methyl-5-aminomethylpyrimidine is hydrolyzed to HMP by the newly discovered activity of thiaminase II. HMP can then enter the *de novo* biosynthetic pathway by phosphorylating HMP to give HMP-P (**2**) *via* HMP kinase. Three observations give us clues about thiamine (**9**) degradation and salvage in plants. First, the substrate 4-amino-2-methyl-5-aminomethylpyrimidine of thiaminase II salvage activities identified in *Bacillus* and yeast is also a thiamine degradation product when thiamine (**9**) is incubated with plant phenolic compounds *in vitro*. Second, genome analyses of *A. thaliana* and *Populus trichocarpa* reveal the presence of genes encoding proteins with amino acid sequence similarities to TenA. Although thiaminase activity has never been reported from those plants, these genomic data indicate that thiaminases may be more widespread in the plant kingdom than previously believed. Third, the bifunctional HMPPK/TMP-PPase from maize was shown to phosphorylate both HMP and HMP-P (**2**) *in vitro* (Rapala-Kozik et al., 2007), suggesting that plants can potentially salvage HMP for thiamine (**9**) biosynthesis. Thus, although thiamine (**9**) degradation and the identity of thiamine-degradation products *in planta* remain to be elucidated, the fact that plants are rich in phenol compounds which can possibly catabolize thiamine (**9**) and that plants contain thiaminase II genes/proteins suggest that plants may possess all of the elements of a salvage pathway similar to that found in microorganisms. This hypothesis will require further investigation.

2.4. Localization and transport

Biosynthesis and condensation of pyrimidine and thiazole moieties of thiamine (**9**) seem to take place in chloroplasts, while the final pyrophosphorylation step of thiamine (**9**) to TDP (**10**) apparently takes place exclusively in the cytosol. TDP (**10**) serves as a cofactor in enzymatic reactions which occur in cytosol, mitochondria, and chloroplasts. Therefore, unless alternative routes from free thiamine (**9**) to TDP (**10**) are discovered in mitochondria and chloroplasts, TDP (**10**) must be imported to organelles to participate in these enzymatic reactions. To date, only the yeast TDP carrier Tpc1p, the human Tpc, and the *Drosophila melanogaster* DmTpc1p have been identified as being responsible for the mitochondrial transport of TDP (**10**) (Iacopetta et al., 2010; Lindhurst et al., 2006; Marobbio et al., 2002). These proteins belong to the mitochondrial carrier family which comprises 45 members in Arabidopsis (Millar and Heazlewood, 2003) amongst which a TDP (**10**) transporter may exist.

Condensation of HMP-PP (**3**) and HET-P (**7**) to TMP (**8**) in chloroplasts indicates that TMP (**8**) or thiamine (**9**), depending on whether TMP dephosphorylation takes place in chloroplasts or in the cytosol, must be exported from chloroplasts to enable activation to TDP (**10**) by cytosolic TPK. Analogous transporters to the yeast Tpc1p carrier, which catalyze the exchange between cytosolic TDP (**10**) and intramitochondrial TMP (**8**), could exist in chloroplastic membrane and enable to supply chloroplasts with TDP (**10**) and regeneration of TDP (**10**) from thiamine (**9**).

Early reports showed that excised plant roots cannot grow without supplying thiamine (**9**) in the medium, besides a few exceptions such as flax and clover whose roots, when isolated, were able to grow in the absence of exogenous thiamine (**9**) but at a relatively low rate (Bonner, 1940, 1942). These results indicated that plant roots are not able to synthesize thiamine (**9**) at a sufficient rate for growth, which is supported by recent findings that most thiamine (**9**) biosynthetic genes are barely expressed in roots as discussed earlier in this paper. Therefore roots seem to be dependent on transport of thiamine from green tissues where its biosynthetic pathway is active. Roots are also able to absorb thiamine (**9**) from the medium (Bonner, 1940; Mozafar and Oertli, 1992; Robbins and Bartley, 1937; White, 1937), and uptake of thiamine by plant roots from soil containing this vitamin may play a significant role in the thiamine (**9**) budget of the plant tissues or for the growth of roots at some stages of plant development or under certain soil or environmental conditions. It is also well documented that exogenous free thiamine (**9**) can penetrate plant tissues such as leaves and be translocated throughout the plants (Mateikene et al., 1988; Mozafar and Oertli, 1992, 1993). Thiamine (**9**) seems to be transported through xylem and phloem in both acropetal and basipetal directions (Bonner, 1942; Mozafar and Oertli, 1992, 1993). More recently, activation of plant disease resistance by foliar application of thiamine (**9**), TMP (**8**), and TDP (**10**) (Ahn et al., 2005, 2007), and complementation of the Arabidopsis TPK double mutant by foliar application of TDP (**10**) suggest that TMP (**8**) and TDP (**10**) may also be transported throughout the plants by the vascular system. It was suggested that TDP (**10**) may be able to enter the plant vascular system *via* an apoplastic route, and that a facilitative intercellular transport system of TDP (**10**) to allow crossing of plasma membrane may exist (Ajjawi et al., 2007a).

As the data reviewed here show, our current knowledge of thiamine (**9**) localization and transport in plants is very limited. Future research will be needed to characterize transport mechanisms between subcellular compartments, between cells, and throughout the plant.

3. Physiological functions of thiamine and its phosphate esters

3.1. Role as a cofactor

Thiamine diphosphate (TDP) (**10**), the main biologically active thiamine derivative, serves as a cofactor in enzymatic reactions in universal metabolic pathways including glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle (Krampitz, 1969). In plants, TDP (**10**) serves as a cofactor of both mitochondrial and chloroplastic pyruvate dehydrogenases (PDH). The mitochondrial PDH complex converts pyruvate to acetyl-coenzyme A, thereby linking glycolysis to the Krebs cycle (LeClere et al., 2004). The plastid PDH complex provides acetyl-coenzyme A and NADH for *de novo* fatty acid biosynthesis (Tovar-Mendez et al., 2003). TDP (**10**) also serves as a cofactor for pyruvate decarboxylase which catalyzes the irreversible conversion of pyruvate into acetaldehyde and CO_2 during ethanolic fermentation (Lee and Langston-Unkefer, 1985; Rivoal et al., 1990). Subsequently, acetaldehyde is reduced to ethanol with the concomitant oxidation of NADH to NAD^+ by alcohol dehydrogenase. Pyruvate decarboxylase was shown to be vital for energy production under anoxia in Arabidopsis (Gass et al., 2005) and during pollen tube elongation in *Petunia hybrida* (Kursteiner et al., 2003). Transketolase also utilizes TDP (**10**) as a cofactor (Schenk et al., 1998). This enzyme catalyzes the reversible transfer of a two-carbon glycoaldehyde fragment from keto-sugars to the C-1 aldehyde group of aldo-sugars. This protein is a key enzyme in the oxidative pentose phosphate pathway for the metabolism of glucose-6-phosphate (Kruger and von Schaewen, 2003). In photosynthetic organisms, this enzyme is also part of the Calvin cycle where ribulose-1,5-bisphosphate is regenerated from phosphoglycerate formed during fixation of CO_2 (Lindqvist et al., 1992; Teige et al., 1998). Henkes et al. (2001) identified plastid transketolase as a key determinant of plant metabolism. Indeed, they observed that a small reduction of plastid transketolase activity in antisense tobacco transformants had dramatic effects on photosynthesis and phenylpropanoid metabolism.

TDP (**10**) also serves as a cofactor for 2-oxoglutarate dehydrogenase which is an essential enzyme in energy production (Krebs cycle), nitrogen assimilation, amino acid metabolism, as well as having functions in signaling and glyoxylate utilization (Bunik and Fernie, 2009). Acetolactate synthase is the enzyme that catalyzes the first step in the synthesis of the branched-chain amino acids valine, leucine and isoleucine, and exhibits absolute dependence on TDP (**10**) for activity (Chang and Duggleby, 1997; Roux et al., 1996; Singh et al., 1988; Wu et al., 1994). TDP (**10**) is also a cofactor for DXP synthase which catalyzes the condensation of pyruvate and glyceraldehyde 3-phosphate to give 1-deoxy-D-xylulose-5-phosphate (DXP). DXP is required for the biosynthesis of isoprenoids *via* the mevalonate-independent pathway, pyridoxol (Eisenreich et al., 1998; Himmeldirk et al., 1996; Rohmer, 1999; Sprenger et al., 1997), and in bacteria, the thiazole moiety of thiamine (Begley et al., 1999; Himmeldirk et al., 1996; Sprenger et al., 1997).

3.2. Non-cofactor roles

3.2.1. Disease resistance

Thiamine (**9**), as well as its phosphate esters TMP (**8**) and TDP (**10**), has been known for many years to trigger defense responses in plants. Asselin et al. (1985) first reported that uptake of exogenous thiamine (**9**) induces the light-dependent expression of pathogenesis-related (PR) proteins in green tissues in *Nicotiana* species, which is an indication of active plant defenses. Malamy et al. (1996) showed that thiamine (**9**) induces *PR-1* gene expression and local acquired resistance in tobacco, but not systemic acquired resistance (SAR). This induction was greatly reduced in *NahG* transgenic plants which produce a bacterial salicylate hydroxylase and are unable to accumulate salicylic acid (SA), indicating that thiamine (**9**) induction of the defense responses is SA-dependent. More recently, thiamine (**9**) application was shown to induce SAR in rice, Arabidopsis, tobacco, and cucumber. Ahn et al. (2005, 2007) observed that plants challenged with virulent pathogens accumulated PR mRNAs more rapidly and in larger amounts if they were pre-treated with exogenous thiamine (**9**) suggesting that thiamine induces SAR through elicitation competency (Graham and Graham, 1994) or priming (Conrath et al., 2002). Similarly to the earlier reports, no *PR-1* gene expression was detected in Arabidopsis plants expressing *NahG*, or in the *npr-1* Arabidopsis mutant which does not accumulate *PR-1* in response to SA. Together, these suggest that the defense-related gene expression induced by thiamine (**9**) is SA-dependent. The authors also indirectly demonstrated that thiamine (**9**) induces SAR through Ca^{2+}-related signaling pathway. Hydrogen peroxide was also shown to be a fundamental part of the thiamine (**9**) response. Thiamine (**9**) treatment and subsequent pathogen invasion triggered hydrogen peroxide accumulation, callose induction, and *PR-1* and *PAL1* (phenylalanine ammonia-lyase 1) transcription activation in Arabidopsis. Cellular and molecular defense responses as well as SAR were almost completely abolished by removal of hydrogen peroxide by catalase, indicating that SAR triggered by thiamine (**9**) through priming requires hydrogen peroxide.

Several recent reports also indicate that the accumulation of endogenous thiamine (**9**) *in planta* may be controlled by a thiazole biosynthetic gene and may be essential for disease resistance in plants. The rice gene *OsDR8* (Os070529600) encodes a protein with high sequence identity to the maize genes *THI1-1* and *THI1-2* which are involved in the synthesis of the thiazole moiety of thiamine (**9**) (Belanger et al., 1995). *OsDR8* colocalizes with a QTL for blast resistance that explains 24% of the phenotypic variance of resistance (Wang et al., 1994; Wen et al., 2003) and *OsDR8* transcript levels increased after pathogen inoculation (Wen et al., 2003). To test the role of *OsDR8* more specifically, transgenic rice plants that repress expression of *OsDR8* were compared with control plants and found to have reduced resistance or susceptibility to bacterial blight and blast agents (Wang et al., 2006). The transgenic plants had significantly lower levels of thiamine (**9**) than the control plants (1.4–2.5-fold lower) and their compromised defense responses could be reversed by its exogenous application.

Together, these data show that thiamine (**9**) plays an important role as a signaling molecule in plant defense responses against pathogens. Further investigation will be necessary to fully characterize the cascade of events triggered by thiamine (**9**).

3.2.2. Abiotic stress

Recent studies showed that pools of thiamine (**9**) and its phosphate esters TMP (**8**) and TDP (**10**) are modulated by environmental stresses. Indeed, the total thiamine (**9**) content increased in *Z. mays* and Arabidopsis seedlings subjected to osmotic, salt, and oxidative stress conditions (Rapala-Kozik et al., 2008; Tunc-Ozdemir et al., 2009), and in Arabidopsis seedlings subjected to cold, heat, and high light conditions (Tunc-Ozdemir et al., 2009). The largest increase in total thiamine (**9**) content was observed when maize seedlings were treated with hydrogen peroxide (~3-fold) and Arabidopsis seedlings subjected to high light (2.9-fold). All three thiamine forms (thiamine (**9**), TMP (**8**), and TDP (**10**)) increased approximately to the same degree relative to each other in all stresses in Arabidopsis seedlings and TDP (**10**) was the predominant thiamine form under all conditions (accounted for over ~80% of the total thiamine content) (Tunc-Ozdemir et al., 2009). In *Z. mays*, the largest increase was observed for free thiamine (**9**) (~2- to 3-fold) under all stress conditions, while TMP (**8**) increased mostly under oxidative stress (~2-fold) and TDP (**10**) levels dropped (Rapala-Kozik et al., 2008). Free thiamine (**9**) represented over 75% of the total thiamine content under all growth conditions. In Arabidopsis seedlings, the increase of thiamine (**9**) under oxidative stress conditions correlated with an increase in the levels of mRNA transcripts of four biosynthetic genes (HET-P synthase, HMP-P synthase, HMPPK/TMP-PPase, and TPK) (Tunc-Ozdemir et al., 2009). Transcriptomics studies have also reported on the accumulation of HMP-P synthase transcripts under a combination of drought and heat stresses conditions in Arabidopsis (Rizhsky et al., 2004) and after cold treatment in *Thellungiella* (Wong et al., 2006). Proteome profiling of *P. euphratica* also indicated an accumulation of HET-P synthase upon heat stress (Ferreira et al., 2006). It is noteworthy that this enzyme, in addition to its thiazole synthase activity, was shown to complement bacterial defects in DNA repair (Machado et al., 1996); DNA repair genes are commonly induced under various stresses. These suggest that HET-P synthase may be involved in DNA damage tolerance in plant cells. In maize seedlings, enzymatic activities of TPK significantly increased under water, salt, and oxidative stress conditions, as well as activities of unspecific TDP hydrolases under water and salt stresses (Rapala-Kozik et al., 2008).

In addition to the accumulation of thiamine (**9**) in plants subjected to various abiotic stresses, its exogenous application conferred some degree of resistance to salt and oxidative stresses. Indeed, Sayed and Gadallah (2002) showed that thiamine (**9**), either sprayed on shoots or applied to roots, counteracted the harmful effects of salinity on shoot and root growth in sunflower plants. Thiamine-treated plants subjected to salt stress had more chlorophyll, higher relative water content, lower leaf water potential, higher soluble sugars and total free amino acids contents, lower Na^+, Ca^{2+}, and Cl^- contents and higher K^+ content compared to untreated plants. Similarly, Tunc-Ozdemir et al. (2009) observed that growth retardation of roots of Arabidopsis wild-type plants or *apx1-1* mutant plants, which is deficient in cytosolic ascorbate peroxidase 1 and highly susceptible to reactive oxygen species (ROS), by treatment with paraquat (an herbicide which induces

oxidative stress *via* the production of ROS) could partially be alleviated by supplying thiamine (**9**) in the medium. In addition, proteome analysis showed that the *apx1-1* mutant accumulated HET-P synthase, suggesting that at least part of the thiamine (**9**) biosynthetic pathway was activated in the mutant plants. The authors observed that thiamine (**9**) treatment increases plant tolerance to paraquat through the reduction of ROS accumulation and subsequent oxidative damage. They also showed that thiamine (**9**) conferred paraquat tolerance in *sid2* mutant deficient in SA synthesis, indicating that SA signaling is not required for oxidative protection by thiamine. Interestingly, the Arabidopsis mutants *py* and *tz* which are affected in pyrimidine and thiazole moieties synthesis and are auxotrophic for thiamine (**9**) were more tolerant to paraquat and more responsive to thiamine (**9**) treatment than the wild-type. These mutants also accumulated mRNA transcripts of the major ROS-protective enzymes, ascorbate peroxidase, catalase, Fe-superoxide dismutase, and copper/zinc-superoxide dismutase to higher levels than the wild-type. The authors suggested that thiamine (**9**) deficiency in these mutants cause a continuous state of oxidative stress and subsequently the activation of different antioxidative mechanisms.

The increase in endogenous thiamine (**9**) content, either by activation of the biosynthetic pathway or by uptake of thiamine (**9**) applied exogenously, could answer the increased demand for TDP (**10**) dependent enzymes such as transketolase under stress conditions. Transketolase is a crucial enzyme of the oxidative pentose phosphate pathway (Kruger and von Schaewen, 2003) which produces NADPH required for the regeneration of various antioxidants such as glutathione and ascorbate. Interestingly, Rapala-Kozik et al. (2008) showed that transketolase activity increased in maize seedlings subjected to water, salt, and oxidative stresses.

Together, these data indicate that modulation of thiamine (**9**) metabolism is important to maintain normal functioning of plants subjected to water, osmotic, salt, light, oxidative, and heat stress. They also suggest a possible role for thiamine (**9**) as a signaling molecule in adaptation mechanisms to abiotic stress.

3.3. Other forms of thiamine and their possible roles in plants

3.3.1. Thiamine triphosphate

Thiamine triphosphate (TTP) has been detected in several plants. Yusa (1961) reported the presence of TTP in germinating seeds of maize, pea, and soybean, but absolute quantification was not performed. TTP was also detected in bean germs (20 pmol/g FW – 0.4% of total thiamine) and leaves of *Sphagnum palustei* (47 pmol/g FW – 5.5% of total thiamine (**9**)), as well as in wilting Arabidopsis plants (Makarchikov et al., 2007). The biological role of TTP in plants remains unknown.

3.3.2. Adenosine thiamine triphosphate

Recently Bettendorff et al. (2007) reported the discovery of the first adenine nucleotide containing thiamine (**9**), named adenosine thiamine triphosphate, in *E. coli* grown under carbon starvation, and suggested that it also may act as a signal rather than a cofactor. Small amounts of adenosine thiamine triphosphate were also detected in roots of Arabidopsis and parsley (14 and 33 pmol/g fresh weight, respectively). Although it was present in much lower amounts relative to TDP (**10**) and free thiamine (**9**) in parsley roots (1340 and 540 pmol/g FW of TDP (**10**) and free thiamine (**9**), respectively), it was present in relatively similar amounts compared to TDP (**10**) and free thiamine (**9**) in Arabidopsis roots (83 and 7 pmol/g FW of TDP and free thiamine (**9**), respectively) (Makarchikov et al., 2003). This suggests that adenosine thiamine triphosphate may play an important function in roots. Its physiological role remains unknown, but it has been suggested to serve as chemically and enzymatically more stable storage form for TDP (**10**) and TTP (Jordan, 2007).

4. Conclusions

The data reviewed here shows the tremendous progress made in recent years toward resolving thiamine metabolism and functions in plants. Functional genomics and reverse genetics have enabled depiction of most of the thiamine (**9**) biosynthetic pathway and discovery of riboswitch regulation of gene expression which allows fine-tuning of its biosynthesis. Functions of thiamine (**9**) that extend beyond its role as a cofactor have started to emerge. It may act as a signaling molecule in response to biotic and abiotic stress, and modulation of its metabolism is important for plant responses to changing environments. This review also illustrates the complexity of thiamine (**9**) metabolism and shows that many aspects of its biochemistry remain unclear. Future efforts are needed to completely unravel the roles that thiamine (**9**) plays in plants, to depict its catabolism and salvage pathways, to characterize transporters, and to better understand the regulation and coordination of these pathways. From a crop improvement perspective, engineering thiamine (**9**) metabolism may be an innovative way to improve plant performance as well as to increase the nutritional value of staple foods like rice. It will be interesting to see the outcomes of such endeavors.

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