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Determination of the genetic, molecular, and biochemical basis of the *Arabidopsis thaliana* thiamin auxotroph *th*1

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Abstract

2-Methyl-4-amino-5-hydroxymethylpyrimidine phosphate kinase/thiamin monophosphate pyrophosphorylase (HMPPK/TMPPase) is a key enzyme involved in thiamin biosynthesis. A candidate HMPPK/TMPPase gene identified in the *Arabidopsis* genome complemented the thiamin auxotrophy of the *th*1 mutant, thus proving that the *th*1 locus corresponds to the structural gene for the HMPPK/TMPPase. Sequence comparisons between the wild-type HMPPK/TMPPase gene and the *th*1–201 mutant allele identified a single point mutation that caused the substitution of a phenylalanine for a conserved serine residue in the HMPPK domain. Functional analyses of the mutant HMPPK/TMPPase in *Escherichia coli* revealed that the amino acid substitution in the HMPPK domain of mutant enzyme resulted in a conformational change that severely compromised both activities of the bifunctional enzyme. Studies were also performed to identify the chloroplast as the specific subcellular locale of the *Arabidopsis* HMPPK/TMPPase.

Keywords: Thiamin; 2-Methyl-4-amino-5-hydroxymethylpyrimidine phosphate kinase; Thiamin monophosphate pyrophosphorylase; Vitamin B1; Genetic complementation; *Arabidopsis*

Thiamin pyrophosphate (TPP)[3] is an enzyme cofactor that is required for the catalytic transfer of activated aldehyde groups to specific keto acid acceptors. A number of key metabolic enzymes such as pyruvate dehydrogenase, α-ketogluturate dehydrogenase, transketolase, allolactate synthase, and deoxyxylulose phosphate synthase, require TPP as a cofactor. Due to its central role in intermediary metabolism, TPP is required by all biological systems. Since animals and humans cannot synthesize TPP, they must obtain this cofactor as free (unphosphorylated) thiamin (i.e. Vitamin B1) from plant sources.

Although plants are a primary source of dietary thiamin, very little is understood about how plants synthesize this compound. What is known is that thiamin is synthesized from two major intermediates, 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (HET-P) and 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP). These two compounds are condensed to form thiamin-monophosphate (TMP) in a reaction catalyzed by the enzyme TMP pyrophosphorylase (TMPPase) (Fig. 1).

As with many other biosynthetic pathways, auxotrophic mutants have played a pivotal role in elucidating the different enzymatic steps in thiamin biosynthesis. Thiamin auxotrophs, which are conditionally lethal and require thiamin supplementation for growth, have been identified from a number of bacteria [2], fungi [12], algae [10], and higher plants [10,19,21]. The nutritional requirements of individual mutants have been used to assign where specific mutants disrupt the thiamin biosynthetic pathway.

In *Arabidopsis*, five thiamin auxotrophs, *th*1, *th*2, *th*3, *py*, and *tz*, have been identified and mapped to the genome

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[3] *Abbreviations used:* TPP, thiamin pyrophosphate; HET-P, 4-methyl-5-(β-hydroxyethyl) thiazole phosphate; HMP-PP, 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate; TMPPase, TMP pyrophosphorylase.

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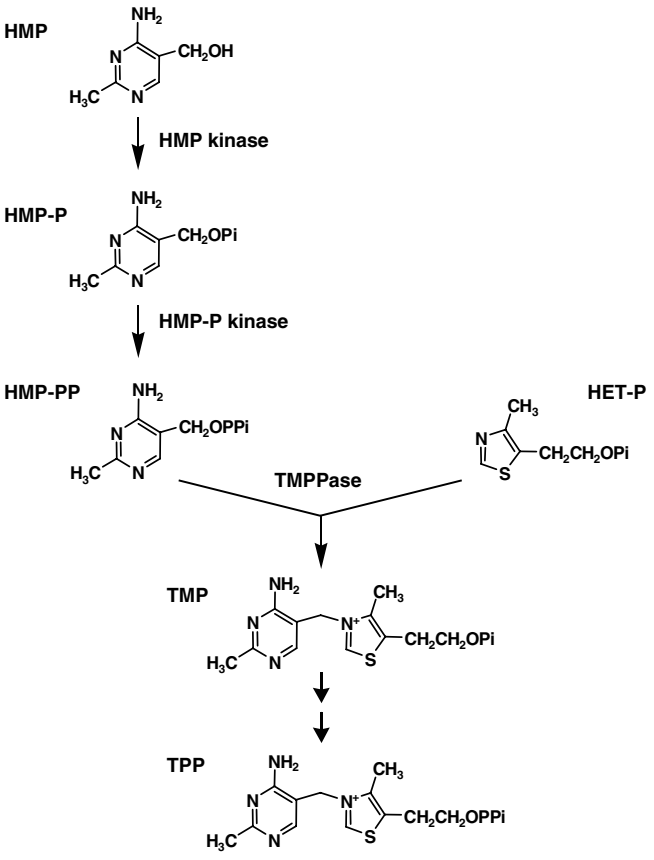

Fig. 1. *De novo* thiamin biosynthetic pathway. HMPPK/TMPPase has three distinct activities, HMP kinase, HMP-P kinase and TMPPase. HMP kinase is responsible for the phosphorylation of 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) into 2-methyl-4-amino-5-hydroxymethylpyrimidine phosphate (HMP-P). HMP-P kinase phosphorylates HMP-P into 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP). TMPPase catalyzes the condensation of HMP-PP and 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (HET-P) into thiamin monophosphate (TMP). TMP is eventually converted to the active cofactor thiamin pyrophosphate (TPP) by other enzymes depicted by two successive arrows.

[18,21]. Each of the mutants exhibits a seedling lethal phenotype that can be complemented by supplementation with exogenous thiamin. While *th*1, *th*2, and *th*3 have an absolute requirement for thiamin, the *py* and *tz* mutants can also survive when provided with exogenous HMP and HET, respectively. These observations indicate that while *th*1, *th*2, and *th*3 genes must encode proteins involved in the terminal steps of TPP biosynthesis [18], the *py* and *tz* genes likely encode enzymes involved in HMP and HET biosynthesis, respectively [21].

Komeda et al. [17] showed that leaf extracts from the *th*1 mutant were unable to form thiamin monophosphate when provided with HMP and HET, suggesting that the thiamin auxotrophy was due to a lesion in the TMPPase gene. Unfortunately at the time this work was done, the gene encoding the *Arabidopsis* TMPPase was not yet identified and genetic complementation experiments could not be performed to confirm the identity of the gene responsible for the *th*1 phenotype.

Recently, the gene encoding TMPPase was cloned and the corresponding enzyme was characterized from several microbial species including *Escherichia coli* [17,32], *Bacillus subtilis* [20], and *Saccharomyces cerevisiae* [27]. The first plant TMPPase gene sequence was identified from *Brassica napus* through a mutant complementation screen of a cDNA expression library using the *E. coli* TMPPase mutant *thi*E [15]. One of the major differences between the prokaryotic and eukaryotic enzyme is that the yeast and plant enzymes are bifunctional. However, while the yeast TMPPase is fused to a 4-methyl-5-(β-hydroxyethyl) thiazole (HET) kinase domain, the plant TMPPase is fused to a 2-methyl-4-amino-5-hydroxymethylpyrimidine phosphate (HMP-P) kinase domain (HMPPK) [15]. Subsequently, it was demonstrated that the *E. coli* HMPPK possessed two distinct activities corresponding to HMP kinase and HMP-P kinase [24]. HMPPK therefore catalyzes the sequential phosphorylation of HMP to HMP-P and HMP-P to HMP-PP, respectively (Fig. 1). Sequence similarity alignments suggest that plant HMPPK domains are highly likely to also possess HMP kinase activity [15].

While the *B. napus* HMPPK/TMPPase was used to identify a putative *Arabidopsis* ortholog [15], the authors were unable to confirm that this gene corresponded to the *th*1 mutant locus. To examine this possibility, we complemented the *th*1 mutant with a wild-type copy of the *Arabidopsis* HMPPK/TMPPase gene. Our results showed that constitutive expression of the HMPPK/TMPPase gene complements the *th*1 mutation. We further determined that the *th*1–201 allele encoded a protein containing a point mutation, which results in the substitution of the amino acid phenylalanine for the highly conserved serine at position 63 of the HMPPK domain of the enzyme. These results demonstrate that the *Arabidopsis th*1 mutation is a mutation in the HMPPK/TMPPase structural gene. Furthermore, the *Arabidopsis* HMPPK/TMPPase was shown to be localized within the chloroplast compartment.

Materials and methods

Plant material

Wild-type *Arabidopsis thaliana* ecotype Landsberg erecta (Ler) and the *Arabidopsis th*1–201 seeds were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Seeds were germinated on a thiamin free medium consisting of Murashige and Skoog (MS) basal medium supplemented with 2% sucrose, 100 mg/L inositol, 0.5 mg/L nicotinic acid, and 0.5 mg/L pyridoxine [25], and allowed to grow for 14 days at 25 °C under constant light of 150 $\mu E\, m^{-2}\, s^{-1}$.

Plant transformation and antibiotic selection for transformed plants

A BLAST [1] search of the TIGR *Arabidopsis* Gene Index database with the *B. napus* HMPPK/TMPPase (GenBank Accession No. AF015310) revealed an *Arabidopsis* cDNA, EST # 96C21T7 (GenBank Accession No. T21651), which corresponded to the At1g22940 gene locus. The cDNA was completely sequenced and found to have the first four nucleotides including the first ATG codon missing. Therefore the four missing base pairs and a Kozak consensus translational start sequence [29] were engineered through PCR onto the 5′ end of the truncated cDNA.