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# Thiamin biofortification of crops

## Aymeric Goyer<sup>1,2</sup>

Thiamin is essential for human health. While plants are the ultimate source of thiamin in most human diets, staple foods like white rice have low thiamin content. Therefore, populations whose diets are mainly based on low-thiamin staple crops suffer from thiamin deficiency. Biofortification of rice grain by engineering the thiamin biosynthesis pathway has recently been attempted, with up to 5-fold increase in thiamin content in unpolished seeds. However, polished seeds that retain only the starchy endosperm had similar thiamin content than that of non-engineered plants. Various factors such as limited supply of precursors, limited activity of thiamin biosynthetic enzymes, dependence on maternal tissues to supply thiamin, or lack of thiamin stabilizing proteins may have hindered thiamin increase in the endosperm.

### Addresses

<sup>1</sup> Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97330, United States

<sup>2</sup> Hermiston Agricultural Research and Extension Center, Oregon State University, Hermiston, OR 97838, United States

Corresponding author: Goyer, Aymeric  
([aymeric.goyer@oregonstate.edu](mailto:aymeric.goyer@oregonstate.edu))

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## Introduction

Thiamin (vitamin B<sub>1</sub>), in its diphosphate form (ThDP), functions as a cofactor for key enzymes of carbohydrates and amino acid metabolism in all living organisms [1,2]. While plants, fungi and bacteria can synthesize thiamin *de novo*, humans cannot and must obtain it from food. Although plant foods are a major source of thiamin, some staple crops like rice contain relatively low amounts of thiamin. Industrialized milling of the rice grain that removes the outer layers of the seed, i.e. the pericarp, testa, nucellus, and aleurone layer, as well as the embryo (Figure 1), further depletes the grain's thiamin content [3]. The remaining white starchy endosperm that is widely consumed worldwide contains very small amounts of thiamin. Populations whose diets are mostly based on

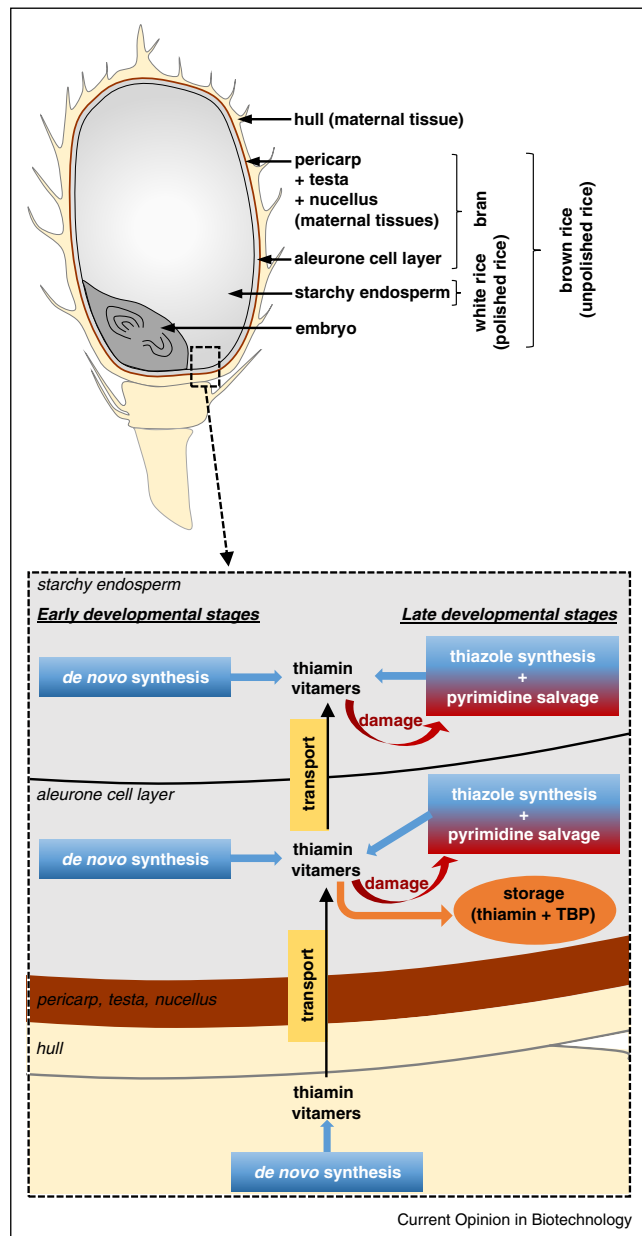
low thiamin and high carbohydrates foods like white rice commonly suffer from thiamin deficiency [4,5]. Symptoms of severe thiamin deficiency include degeneration in the nerves and heart, a disease known as beriberi, which can be fatal. Severe cases of thiamin deficiency are rare in countries like the United States where programs to fortify food with thiamin have been implemented [6]. However, marginal thiamin deficiencies are still common and often remain undiagnosed because of the lack of obvious clinical symptoms [7,8].

Thiamin biofortification of staple crops that have low thiamin content is an attractive strategy to avert thiamin deficiency [9]. In the past two years, the first efforts to biofortify food crops with thiamin have been reported [10<sup>\*\*</sup>,11<sup>\*\*</sup>]. These attempts were feasible because of the tremendous progress in our understanding of thiamin metabolism in plants in recent years. The availability of complete plant genome sequences has enabled the identification and cloning of several genes involved in thiamin biosynthesis, transport, and salvage [12<sup>\*\*</sup>,13<sup>\*\*</sup>], and the biochemical characterization of the corresponding proteins. Regulatory elements have also been discovered. This has provided the foundation for the first thiamin engineering strategies. Yet, many aspects of thiamin metabolism remain poorly understood, which hinders the implementation of rational thiamin engineering strategies. In this review, I will first summarize the current knowledge about thiamin biosynthesis in plants. Next, I will highlight efforts that have been made in the last two years to increase thiamin content in plants by genetic engineering. Finally, I will address the specific issue of the seed and discuss future potential strategies to further increase thiamin content in major food grains.

## The *de novo* thiamin biosynthetic pathway in plants

Thiamin is made of a pyrimidine and thiazole moiety that are synthesized in the chloroplast (Figure 2). Today, most genes that are involved in *de novo* thiamin biosynthesis have been identified and some regulatory features of the pathway have been characterized. The first step in the synthesis of the pyrimidine moiety involves 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P) synthase (THIC) that utilizes 5-aminoimidazole ribonucleotide (AIR) and *S*-adenosylmethionine (SAM) as substrates to form HMP-P [14,15] (Figure 2). An important element of *THIC* regulation is the presence of a riboswitch in the 3' untranslated region (UTR) of *THIC* mRNA precursor (Figure 2) [16–20]. This riboswitch is a sensor of ThDP concentration in the cell that controls *THIC* mRNA stability and thereby ThDP production.

Figure 1



Rice grain anatomy and model of thiamin synthesis, salvage, transport and storage in the rice grain. In the early stages of development, the endosperm, *i.e.* the starchy endosperm and the aleurone cell layer, expresses the thiamin biosynthetic genes *THIC* and *THI1*, suggesting that thiamin is synthesized *de novo* in these tissues. In the late stages of development, the expression of *THIC* decreases while that of *THI1* increases in the endosperm, as shown in maize [11\*\*], suggesting that the endosperm loses its capability to synthesize thiamin *de novo* but may still be able to produce thiamin by coupling *de novo* thiazole biosynthesis and pyrimidine salvage. Thiamin vitamers transported from maternal tissues to the endosperm may be a major source of thiamin for the endosperm, especially during the late stages of development. Thiamin in its free form can be stored in the aleurone cell layer by interaction with thiamin binding proteins (TBPs).

The first step in the synthesis of the thiazole moiety involves 4-methyl-5- $\beta$ -hydroxyethylthiazole phosphate (HET-P) synthase (*THI1*) [21] that utilizes nicotinamide adenine dinucleotide (NAD) and glycine as substrates and supplies a sulfide from a conserved cysteine residue to form an adenylated thiazole intermediate (ADT) [22–24]. ADT is then hydrolyzed to HET-P but the NUDIX hydrolase responsible for this step has not been characterized. A bifunctional HMP-P kinase/thiamin monophosphate (ThMP) pyrophosphorylase (*TH1*) phosphorylates HMP-P to HMP-PP and then condenses HMP-PP to HET-P to form ThMP [25,26]. ThMP is then dephosphorylated to thiamin by a specific haloacid dehalogenase (HAD) family phosphatase (*TH2*) that is directed to the cytosol or mitochondria (AD Hanson, personal communication); other HAD phosphatases may also contribute to ThMP dephosphorylation [27]. Thiamin is then pyrophosphorylated to ThDP by ThDP kinases (TDPKs) that are located in the cytosol in *Arabidopsis* [28]. ThDP serves as a cofactor in enzymatic reactions in the cytosol, chloroplast, and mitochondria. One transporter has so far been characterized for ThDP transport to organelles [29].

### Engineering of the thiamin biosynthesis pathway

Bypassing the control exerted by the *THIC* riboswitch is a rational strategy to increase thiamin content. *Arabidopsis* plants expressing *THIC* with a deficient riboswitch in a *THIC* knockdown background had up to 1.6 and 1.7-fold increase in total leaf and seed thiamin content, respectively [30]. Similar increases in total thiamin content of leaves were reported when *THIC* was under the control of the *Arabidopsis* *UBIQUITIN(UBI)-1* promoter or the *CAULIFLOWER MOSAIC VIRUS (CaMV) 35S* promoter and terminators which do not contain a riboswitch [15,30]. Although this approach produced increases in thiamin content, these were relatively modest, suggesting that the thiazole moiety of thiamin may be limiting in plants overexpressing *THIC* and that both branches of the thiamin biosynthetic pathway should be engineered to obtain larger increase in total thiamin pools. The first validation of this hypothesis came from *in vitro* feeding studies in *Arabidopsis* [31]. Wild-type *Arabidopsis* seedlings that were grown on basal salt medium supplemented with both HMP and HET had thiamin levels 8-fold higher than that of the control while thiamin levels in seedlings supplemented with HMP or HET alone remained unchanged.

In the last two years, biofortification *per se* has been attempted in *Arabidopsis* and rice by overexpressing *THIC* and *THI1* alone or together under the control of strong constitutive promoters and terminators with no riboswitch [10\*\*,11\*\*]. Rice plants expressing *THIC* under the control of the maize *UBI* promoter and the *NOPALINE SYNTHASE* terminator did not contain higher amounts of any thiamin forms than those of the control.

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