Thiamin biofortification of crops

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Abstract

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 fivefold 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.

Introduction

Thiamin (vitamin B₁), 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 [10,11]. 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 [12,13], 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. The first step in the synthesis of the thiazole moiety involves 4-methyl-5- β -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 eightfold 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 *THII* 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. Similarly, *Arabidopsis* and rice plants expressing *THII* under the control of the *CaMV35S* promoter or the maize *UBI* promoter, respectively, and terminators which do not contain a riboswitch, did not accumulate thiamin in higher amounts than the control [10,11]. However, when *Arabidopsis* and rice plants that express *THIC* or *THII* transgenes alone were crossed to generate plants that overexpress both transgenes, total thiamin levels increased up to 3.4- and 2.5-fold in *Arabidopsis* and rice leaves, respectively, and up to 2.6- and 5-fold in *Arabidopsis* and rice unpolished seeds, respectively (Table 1). These results show that engineering of both pyrimidine and thiazole branches of the thiamin biosynthesis pathway is necessary in order to increase the thiamin content of leaves and seeds to higher amounts than those found in plants engineered for either branch.

Bottlenecks in thiamin biosynthesis

One potential limiting factor to further thiamin accumulation in plants engineered for both *THII* and *THIC* is a limiting supply of precursors. Three thiamin vitamers are typically present in plant leaves: thiamin, ThMP, and ThDP, ThDP making over 90% of the total thiamin content in wild type plants. In seeds, thiamin is stored in its unphosphorylated form that constitutes over 95% of the total thiamin content. In leaves of *Arabidopsis* and rice plants overexpressing both *THIC* and *THII*, thiamin levels increased over sixfold comprising up to 45% of the total thiamin pool, ThDP levels increased up to twofold comprising at least 50% of the total thiamin content, but ThMP pools remained very low, accounting for about 4% of the total thiamin pool [10,11]. This suggests that pools of some precursors of thiamin biosynthesis were exhausted. Therefore, combining overexpression of genes that are involved in precursors, *e.g.* *NADC* in NAD biosynthesis [32], and thiamin biosynthesis may be a strategy to further increase thiamin levels.

Limited activities of thiamin biosynthesis enzymes are also potential bottlenecks. Although *THIC* and *THII* transcript levels increased substantially in *Arabidopsis* and rice plants overexpressing *THIC* or *THII* [10,11], it is possible that increases in *THIC* and *THII* enzymatic activities were modest and not sufficient to allow higher increases in thiamin production. This could be due to limitations in factors which these enzymes rely upon. For instance, both *THIC* and *THII* activities may be dependent on reduction by thioredoxins as proteomics experiments have suggested [33,34]. Moreover, the supply of sulfur for *THIC*'s iron-sulfur cluster is

dependent on a cysteine desulfurase [14,35,36]. THI1 is a single turnover protein that loses its functionality after sacrificing a cysteine residue during reaction [24,36], which implies that a high demand of energy is necessary for THI1 protein synthesis to catalyze one single step of thiamin biosynthesis. In addition, ThMP synthase activity of TH1 could be inhibited by excess HMP-PP in *THIC*-overexpressing plants as was shown for recombinant TH1 from maize [25]. Protein quantification and enzymatic activity measurements will be necessary to confirm these hypotheses.

The specific issue of the endosperm and potential engineering strategies

Although engineered rice plants that overexpressed *THIC* and *THI1* under the control of the constitutive *UBI* promoter produced seeds with thiamin content up to fivefold greater than that of the control [11], thiamin content increased only up to 1.3-fold in the endosperm, suggesting that the endosperm has little ability to produce and/or store thiamin. While *THIC* and *THI1* are expressed in the early stages of endosperm development in maize, *THIC* expression decreases between 14 and 30 days post pollination [13], suggesting that the endosperm's ability to synthesize thiamin *de novo* is lost in the later stages of development (Figure 1). Therefore, the modest increase of the thiamin content in the endosperm of plants constitutively overexpressing *THIC* indicate that constituents of the endosperm which *THIC* relies upon for pyrimidine synthesis, *e.g.* precursors, are missing in the maturing endosperm. This is in accordance with the suggestion by Guan *et al.* [13] that part of the endosperm's thiamin content may be obtained by coupling *de novo* thiazole biosynthesis with pyrimidine salvage (Figure 1). Measurements of thiazole and pyrimidine in the endosperm of the overexpressor lines should help validate these hypotheses, but analytical methods are currently missing.

A large proportion of the endosperm's thiamin pool may actually be supplied from maternal tissues. *In vitro* radiolabeling feeding experiments showed that thiamin can be transported from maternal tissues (cob) into the endosperm of maize seed [37], and over 90% of the total thiamin content may come from maternal tissues in developing *Arabidopsis* seeds [1,14]. Therefore, increasing transport of thiamin from maternal tissues (*e.g.* leaves, hull, pericarp) into the endosperm is a potential strategy to increase the endosperm's thiamin content. A thiamin transporter that is involved in long-distance transport of thiamin from leaves that biosynthesize thiamin to roots that do not biosynthesize thiamin was recently characterized [38]. Similar transporters that supply thiamin from source tissues to the endosperm will need to be identified in order to implement such strategies.

Another limitation to further increasing thiamin in the endosperm (and other tissues or organs) may be the lack of stability of thiamin. Thiamin is extremely prone to chemical or enzymatic damage [12,39-41]. Preemption of damage and salvage mechanisms in thiamin metabolism have recently been described [42-44], but it remains unclear how much these contribute to the homeostasis of thiamin pools in the endosperm. In addition, seeds of many plant species [2,45] synthesize thiamin-binding proteins (TBPs) that may protect thiamin from

damage. Several studies have reported a close relationship between the increase in thiamin content and the increase in TBP activity during seed maturation [46-49]. Most TBP activity was found in rice bran and little activity was detected in milled rice [45,50,51]. Immunolocalization experiments have shown that TBPs are localized in the aleurone layer of wheat seeds [49]. These data indicate that the endosperm lacks the capability of retaining thiamin during seed maturation and storage, and suggests that expressing TBP in the endosperm may increase the endosperm's thiamin content.

Conclusions

Recent progress in our understanding of thiamin metabolism in plants has led to the first attempts in the thiamin biofortification of staple crops in the past two years. Overexpression of two genes that are involved in thiamin biosynthesis increased thiamin content up to fivefold in unpolished rice seeds. However, there was very little increase in thiamin content in the starchy endosperm, the tissue remaining after removal of the grain's outer layers during milling to produce white rice. Factors limiting thiamin increase in the endosperm of engineered plants may be a limited supply of thiamin precursors and/or a lack of increased activity of thiamin biosynthetic enzymes. In addition, the endosperm may be a sink tissue that mostly relies on import of thiamin from source tissues. Finally, the endosperm seems to lack thiamin binding proteins that may be essential for thiamin stability. Future research should investigate the divisions of labor in thiamin metabolism in seed tissues and develop tissue-specific metabolic models [52] in order to design rational engineering strategies for thiamin biofortification of the rice endosperm, one of the main food source for billions of people worldwide.

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Figure captions

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 *THII*, suggesting that thiamin is synthesized *de novo* in these tissues. In the late stages of development, the expression of *THIC* decreases while that of *THII* 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).

Figure 2. The *de novo* thiamin biosynthetic pathway in plants. Thiazole and pyrimidine synthesis branches are highlighted in green and gold, respectively. Thiamin biosynthetic enzymes and transporters are indicated in white font in grey boxes. ThDP-dependent enzymes are indicated in white font in blue boxes. The red line illustrates the negative regulation of HMP-PP on ThMP synthase activity of TH1. TK, transketolase; DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; PDH, pyruvate dehydrogenase; AHAS, acetohydroxyacid synthase; PDC, pyruvate decarboxylase; KGDH, α -ketoglutarate dehydrogenase; BCKDH, branched-chain α -ketoacid dehydrogenase.

Figure1

Figure2

Table 1. Maximum increase in total thiamin obtained by engineering the thiamin biosynthesis pathway in *Arabidopsis* and rice leaves and seeds. Fold increases are relative to non-engineered plants. n.m., not measured.

Organism	Organ	Thi1	ThiC	Thi1 x ThiC	Reference
<i>Arabidopsis</i>	Leaves	0	1.6	3.4	[8,28]
	Seeds	0	1.7	2.6	
Rice	Leaves	0	0	2.5	[9]
	Unpolished seeds	0	0	5	
	Polished seeds	n.m.	n.m.	1.3