International Journal on Recent Advancement in Biotechnology & Nanotechnology
Vol. 4, Issue 1 – 2021
ISSN: 2582-1571

Metabolic Engineering of Water-Soluble Vitamins in Plants for Enhanced Nutrition

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Abstract

Malnutrition or 'hidden hunger' is a prevalent and entrenched global socio-economic challenge that results due to the combined impact of poverty, poor access to food, inefficient food distribution infrastructure, and an over-reliance on subsistence mono-agriculture. Vitamin deficiencies are the major forms of micronutrient deficiencies, and are associated with severe physical and intellectual damages in humans. The dependence on the staple cereals, lacking major vitamins, without having a diverse diet, and nutrient losses during crop processing are the root of the problem in developing countries. Thus, providing a more balanced vitamin intake from high-quality food remains one of the formidable challenges for global human nutrition. Biofortification of crops through conventional breeding and/or genetic engineering plays a promising role in complementing in the battle against micronutrient deficiencies and combating vitamin deficiencies. The present review highlights the researches carried out so far in engineering of major metabolic pathways of water-soluble vitamins, viz., vitamins B and C to attain adequate levels of such vitamins in the dietary food items, so as to satisfy the Recommended Dietary Intake (RDI) of each vitamin, thereby offering a sustainable solution to populations with a suboptimal micronutrient intake.

Keywords: Malnutrition, water-soluble vitamins, metabolic engineering, biofortification, food security.

Introduction

Vitamins are the organic compounds, required in trace amounts for human health and considered as essential micronutrients. Vitamin deficiencies occur since the intake of vitamins is much lesser than the Recommended Dietary Intake (RDI) required in preventing several clinical deficiencies of 97-98% of the healthy individuals. Humans are unable to synthesize vitamins *de novo* and therefore the immediate precursors or analogs of vitamins must be provided through the diets. Vitamins are classified as either fat-soluble (A, D, E and K) or water-soluble (vitamin B complex: B1, B2, B3, B5, B6, B8, B9 and B12 and C).

Several life threatening diseases like night blindness, xerophthalmia and corneal scarring (due to vitamin A deficiency), neural tube defects in new-borns (due to folate deficiency), etc., occur due to insufficient intake of vitamins through food items (FAO/WHO 2001). Even small alterations in the levels of vitamins can have a disproportionately positive impact on the aspects of human health. Fruits usually provide several vitamins, but are poor sources of minerals or proteins. Therefore, a diversified well-balanced diet having proper concentration of nutrients is required to support human health. However, a major portion of low-income population in the developing countries cannot afford to consume diverse diet and consume only a single staple food crop which might lack several micronutrients (i.e., vitamins and minerals). Malnutrition is therefore more prevalent in the developing world which is due to lack of access to nutritious food, caused by intense poverty, leading to ill health and an inability to work (Jiang et al. 2017). Poverty, malnutrition and poor health therefore form a self-reinforcing cycle which is hard to escape by a vast section of a people. Micronutrient deficiency or ‘hidden hunger’ is therefore caused by the consumption of monotonous diets or micronutrient malabsorption. Even though staple crops provide a good source for the daily caloric intake, their micronutrient levels are usually low. In addition, food storage, processing and preparation cause substantial losses of the micronutrients. Rice milling is a widespread practice to prolong rice shelf life and avoid rancidification, but it lowers the nutritional value. Whole-grain rice, for example, is a good source of vitamin B1, which is depleted in the polished rice (Hurrell and Egli 2010).

One approach to tackle malnutrition is artificial supplementation of food sources with micronutrients, just like iodine-fortified salt. However, this is not practical on a broader term, since it is dependent on robust distribution infrastructure, government co-ordination and consumer compliance. The broad term biofortification (i.e., manipulation of metabolic pathway through transgenic technology) has been applied to enhance the level of additional micronutrients, including vitamins in seeds and tubers of crops as well as in leafy vegetables and fruits, as a target to mitigate micronutrient deficiency. This approach has several advantages over conventional breeding approaches, where several generations would be required to introgress nutritional traits into elite germplasms. First-generation genetically-engineered crops have already shown their potential to enhance agricultural productivity and reduce poverty in the developing countries (Zhu et al. 2007). The second generation of genetically-engineered crops is currently under development that can fulfill nutritional requirements directly by contributing to multipoint intervention strategies. The nutritional traits should be targeted sometimes to specific tissues (e.g., seeds for cereals) and gene pyramiding need to be undergone to engineer the entire pathway. The understanding of vitamin biosynthesis, transport, storage and recycling in plants has progressed considerably in recent years. Recent advancements in genome sequence information, accessibility to genetic tools like high marker density genetic maps and genetic resources, quantitative trait loci (QTL) and association mapping-based approaches as well as technologies related to the manipulation of the levels of micronutrients have created urge to decipher the metabolic pathways of vitamins and enhance their level in edible crops through genetic engineering (Simkin 2019). This is because vitamin levels in edible parts of most plant species is far below the minimal requirements. Several alleles involved in vitamin improvement have been identified and their introduction into elite varieties of crop species is already underway. The aim of this review is to highlight the progresses achieved so far in the manipulation of vitamin B and C biosynthetic pathways in plants to develop vitamin-enriched crops.

Vitamin B1

The first vitamin to be characterized was vitamin B1. This vitamin, as thiamine diphosphate, acts as essential cofactor for crucial metabolic pathways, such as the conversion of pyruvate to acetyl-CoA at the mitochondrial membrane in the citric acid cycle, and the branched chain amino acid biosynthesis pathway, as well as the cytosolic nonoxidative stage of the pentose phosphate pathway. Deficiency in vitamin B1 causes the well-known disease, beriberi, chiefly exerting the neurological defects. Other symptoms like loss in weight, irritability and short-term memory impairment may also be caused due to less-severe deficiency. The RDA of Vitamin B1 is 1.3 mg/day (Fitzpatrick et al. 2012). Cereal grains are important sources of thiamin; however, it is found mostly in the outer layers of the grain and in the germ cells, which are removed during the refining process. Hence, polished rice lacks sufficient levels of thiamine. A riboswitch associated with *THIC* gene is responsible for negative regulation of Vitamin B1 synthesis. Riboswitches are structured noncoding RNA domains that bind specific metabolites and control gene expression at the transcriptional or translational level or through splicing events in eukaryotes. Alternative splicing event occurring after thiamine diphosphate binding interfere with polyadenylation, leading to an unstable transcript. This mode of regulation is mediated through the direct binding of the product, thiamine pyrophosphate (TPP) to the pre-mRNA of particular thiamin biosynthetic genes without the need for intermediary proteins. The binding causes a change in RNA secondary structure that interferes with gene expression. A TPP riboswitch sequence has been found in the 3’-UTR of the *THIC* gene of species across the plant kingdom. Thiamin is formed by the condensation of two separately biosynthesized moieties, hydroxyethylthiazole phosphate (HET-P) and hydroxymethylpyrimidine pyrophosphate (HMP-PP) to form TMP. The phosphomethyl-pyrimidine synthase (THIC) converts amino imidazole ribonucleotide (AIR) to hydroxymethyl pyrimidine phosphate (HMP-P). The latter is then phosphorylated to HMP-PP by a bifunctional protein characterized in maize as THI3 and in *Arabidopsis* as TH1 (Rapala-Kozik et al. 2009).The same protein catalyzes the condensation step between HET-P and HMP-PP to produce TMP. All of these steps have been shown to occur in the chloroplast. Supplementation with TPP (1 µM) or thiamin (100 µM) indicated that under such conditions, the pre-mRNA of *THIC* undergoes a conformational change, exposing a splice site in the 3’-UTR of this gene. Thus, there is splicing of intron 2 within the 3’-UTR of *THIC*, eliminating the consensus polyadenylation signal and resulting in an unstable transcript. On the other hand, in the absence of supplementation, there is strong accumulation of an intron-retained variant (as the splice site is not exposed). The latter transcript has a single, more stable consensus polyadenylation signal. It is therefore assumed that when TPP reaches sufficiently high levels, *THIC* expression occurs through this alternative splicing mechanism, i.e., high TPP levels lead to an unstable mRNA, whereas the gene is stably expressed when TPP levels are low (Pourcel et al. 2013). Such a riboswitch mechanism offers an efficient strategy for manipulation of Vitamin B1 levels through genetic engineering.

Vitamin B6

Vitamin B6 refers to a group of six water-soluble vitamins, among which pyridoxal-5-phosphate (PLP) is of central importance, since it is required as a cofactor for over 140 chemical reactions within the cell, including transaminations, α-decarboxylations, racemizations, β- and γ-eliminations and aldol cleavages. This vitamin is primarily required for amino acid biosynthesis and catabolism, and sugar and fatty acid metabolism, as well as it regulates the biosynthesis of other B vitamins, such as B8 (biotin), B9 (folate) and B12 (cyanocobalamin). It is a powerful antioxidant that protects the cells from reactive oxygen species. Deficiency may lead to blood pressure problems, cardiovascular diseases, neurological disorders, diabetes, and pellagra skin disease. The RDI for vitamin B6 is 1.3 mg/ day for adults (Fitzpatrick et al. 2012). Biosynthesis of vitamin B6 requires the concerted activities of the PDX1 and PDX2 proteins, which use either ribose 5-phosphate or ribulose 5-phosphate, in addition to glyceraldehyde 3-phosphate or dihydroxyacetone phosphate as well as Gln to synthesize PLP. The *Arabidopsis*, rice and cassava genomes have three homologues of *PDX1* (*PDX1.1*, *PDX1.2*, and *PDX1.3*) and one *PDX2* gene. Only PDX1.1 and PDX1.3 are considered to be important in vitamin B6 biosynthesis. In *Arabidopsis*, constitutive over expression of AtPDX1.3 and AtPDX2 raised the vitamin B6 content in seeds by 20%, whereas seed-specific expression resulted in a threefold increase in seeds without affecting the plant performance (Chen and Xiong 2009; Leuendorf et al. 2010; Raschke et al. 2011). Simultaneous overexpression of Arabidopsis pyridoxal phosphate synthase (*PDX1.1*) and pyridoxal phosphate glutaminase (*PDX2*) genes by CaMV35S promoter in cassava allowed a 9.0-fold increase of the vitamin in the leaves to 54.74 mg g^{-1}, and a 15.4-fold increase in the roots to 16.21 mg g^{-1} by root-enhanced patatin promoter respectively (Li et al. 2015). The correct choice of PDX homologues is therefore important for maximum vitamin accumulation.

Folates

Folates (vitamin B9) including tetrahydrofolate (THF) and its derivatives are essential water-soluble B-vitamins, acting as one-carbon donors and acceptors in all the organisms. They are tripartite molecules, combining pterin, p-aminobenzoate (p-ABA) and one or more glutamate moieties. Folate deficiency may cause several disorders like Pellagra, megaloblastic anemia, cardiovascular problems, neural tube defects, iron-deficiency anemia and birth defects (Fitzpatrick et al. 2012). In plants, folates are synthesized from para-aminobenzoic acid (p-

ABA) and pterin precursors. Folate biosynthesis is compartmentalized among cytosol, mitochondria and chloroplasts. p-aminobenzoate is generated from chorismate in the chloroplasts, while pterin branches are synthesized in the cytosol from GTP. Both p-ABA and pterins are imported to mitochondria where they are condensed to form dihydropteroate, which is then glutamylated to form folates. The folate biosynthesis pathway in plants is diagrammatically shown in Fig. 1

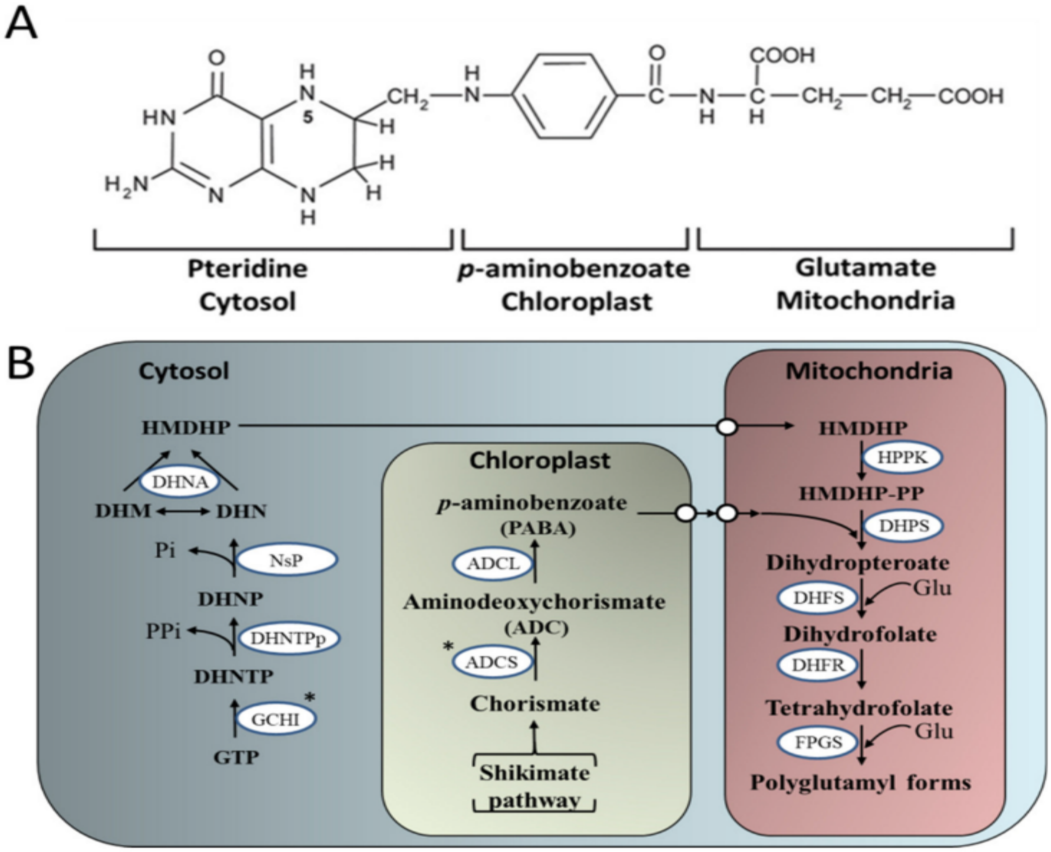

Fig.1. (**A**) The chemical structure of monoglutamyl tetrahydrofolate (**B**) The pteridine pathway leading to hydroxymethyldihydropterin (HMDHP) in the cytosol; the pathway leading to p-aminobenzoate in the plastid, and condensation steps localized in the mitochondria are shown. DHN, dihydroneopterin; -P, monophosphate; -PP, pyrophosphate; -PPP, triphosphate; DHM, dihydromonapterin; Glu, glutamate; ADCS, aminodeoxychorismate synthase; ADCL, aminodeoxychorismate lyase; GCHI, GTP cyclohydrolase I; DHNTPp, dihydroneopterin triphosphate pyrophosphatase; NsP, non-specific phosphatase; DHNA, dihydroneopterin aldolase; HPPK, hydroxymethyldihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase. White

circles represent points of substrate transport. * targeted enzymes for over-expression (Figure extracted from Simkin 2019).

Though abundant in leafy vegetables, their concentration is poor in rice, wheat, potato, tomato, etc., so that there is a dietary deficit of folate in humans, which is less than the RDI of 400 mg per day in adults and 600 mg daily for pregnant women. Currently, flour and cereal-grain products are fortified with folic acid to overcome the issues of folate deficiency within the population; however, this does not address the issues of folate deficiency in poorer communities. Overexpression of the GTP cyclohydrolase I (GCHI) in Arabidopsis resulted in a >1000-fold increase in pterins and only a 2.0- to 4.0-fold increase in folates. Feeding p-ABA in tomato fruit though fruit stalk could increase folate by up to 10.0-fold. Transgenic tomato overexpressing human GTP cyclohydrolase I (the first step in the pterin branch) and aminodeoxychorismate synthase (the first step in the p-ABA branch) from Arabidopsis was shown to enhance (25.0- fold) folate content. In the individual transgenics, the maximum enhancement was double the normal level of folate. However, combining the two transgenes in a single line through crossing released the bottlenecks in both the branches with a simultaneous 25.0-fold increase in the folate levels (Storozhenko et al. 2007; Dıaz de la Garza et al. 2007). However, such approach failed to give desirable results in potato tubers, establishing a major bottleneck in folate synthesis. A triple-gene approach with the bifunctional enzyme hydroxymethyl dihydropterin pyrophosphokinase/dihydropteroate synthase (HPPK/DHPS) was suggested. Transgenic rice expressing *Arabidopsis thaliana* genes of the pterin (Glb-1) and p-ABA (GluB1) branches of the folate biosynthetic pathway have shown almost 100.0-fold increase in folate content. Folate content in rice was also increased when aminodeoxychorismate synthase and GTP cyclohydrolase I from Arabidopsis and 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase from wheat was overexpressed in rice. In case of corn, the rate limiting enzyme, dihydrofolate synthetase was overexpressed to achieve 2.0-fold increase in endosperm (1.94 mg g^{-1}). Another enzyme that catalyzes the first committed step in the cytosol, when overexpressed in tomato, increased the folate content by 2.0-fold up to 2.99 nmol g^{-1} and by 8.5 folds in lettuce up to 1.85 mg g^{-1}. Combination of folylpolyglutamate synthetase (FPGS), GTPCHI, and ADCS from Arabidopsis with mammalian folate binding proteins (FBP) has been successful in improving folate accumulation (up to 25.3 mg g^{-1}) and stability in rice. However, the folate pathway needs to be better understood to design an engineering strategy for several staple food crops. Khanal et al. (2010) identified five quantitative trait loci (QTL) markers in two varieties of *Phaseolus vulgaris* (Perez-Massot et al. 2013; Blancquaert et al. 2017). Folate-biofortified crops could be invaluable in addressing the incidence of the folate deficiency in whole populations.

Vitamin C

Ascorbate (vitamin C) is found in citrus fruits (oranges and lemons), grapefruit, mango, kiwifruit, broccoli, Brussels sprouts, tomatoes and a variety of berry fruits (strawberries, raspberries, blueberries, and cranberries). It acts as a cofactor of several enzymes, including those required for the synthesis of collagen, carnitine, cholesterol and certain amino acid hormones and the enzymatic production of some neurotransmitters. It is very well-known for its role in boosting the immune system, especially against allergies due to its antioxidant properties. It is also involved in energy metabolism and can aid in the treatment of Alzheimer’s, Huntington’s and Parkinson’s disease. Vitamin C deficiency causes the ulceration disease, scurvy, reflecting the breakdown of the connective tissues, together with joint pains, bone and connective tissue disorders, poor healing, and a weak immune system (Fitzpatrick et al. 2012). Humans and some other primates have lost the ability to synthesize and store Vitamin C and depend on the dietary provision to cover the RDI for vitamin C, which is 75-90 mg per day for adult. Potato and tomato contain one fourth to one fifth of the RDI for vitamin C per 100 g weight. Vitamin C is quite low and usually undetectable in the grains of cereal crops, including barley, corn, millet, rice and wheat. There are several biosynthetic pathways that generate ascorbate in plants, and when ascorbate is oxidized, it can be recycled through an additional pathway with reduced glutathione as the reductant. The amount of ascorbate accumulating in plants can therefore be enhanced not only by increasing its biosynthesis, but also the rate at which the molecule is recycled. In the first approach, the overexpression of the rat L-Gulono-1, 4-γ-lactone oxidase (GuLO) in lettuce resulted in the accumulation of up to 580 nmol/g fresh weight of ascorbate, a 7.0-fold improvement, which is the largest increase, observed up to date. A 2.0-fold increase was achieved by expressing the same gene in potato tubers. Multivitamin maize, expressing the rice *DHAR* gene from the ascorbate recycling pathway under the control of the barley *D-hordein* promoter, accumulated 6.0-times the normal level of ascorbate. Unlike overexpression, knockdown of the genes participating in recycling, such as *monodehydroascorbate reductase* (*MDHAR*) and *dehydroascorbate reductase* (*DHAR*), led to lesser increase in tomato, maize and potato. *GDP-l-galactose phosphorylase* (*GGP* or *VTC2*) gene overexpression under CaMV35S promoter led to enhanced ascorbate in tomato (3.0-6.0- fold), strawberry (2.0-fold) and potato (3.0-fold in one line only). In plants, several biosynthesis pathways for Vitamin C have been characterized (Locato et al. 2013). The Smirnoff-Wheeler pathway is the primary pathway in plants, along with three ‘alternative’ pathways: myo-inositol pathway, L-gulose pathway and D-galacturonate pathway. Overexpression of GDP-Man-3’, 5’-epimerase enzyme involved in Smirnoff-Wheeler pathway and L-gulose pathways, was shown to result in a 1.2- to 1.6-fold increase in ascorbate in tomato fruit (Simkin 2019). Furthermore, over-expression of GDP-L-Gal phosphorylase enzyme, also active in both the Smirnoff-Wheeler and L-gulose pathways, was shown to result in a 2.0- to 6.0-fold increase in ascorbate levels in strawberry and tomato fruit, respectively, and up to 3.0-fold increase in ascorbate in potato tubers. Overexpression of GDP-D-Man pyrophosphorylase also facilitated an increase in ascorbate levels. Co-expression of GDP-D-Man pyrophosphorylase and GDP-L-Gal phosphorylase resulted in higher ascorbate levels, compared to each enzyme alone (Jain et al. 2000; Radzio et al. 2003). Furthermore, co-overexpression of GDP-D-Man pyrophosphorylase, GDP-Man-3’, 5’-epimerase and GDP-L-Gal phosphorylase significantly increased ascorbate contents, compared to the dual expression of GDP-D-Man pyrophosphorylase and GDP-L-Gal phosphorylase. Overexpression of the enzymes involved in the three ‘alternative’ pathways has also showed desirable results, viz., 2.0- to 7.0-fold increase with L-gulonolactone oxidase, 2.0-3.0-fold increase with D-galacturonate reductase or myo-inositol oxygenase (Suekawa et al. 2019).

Conclusion

Food security is one of the pillars of proper human health that can maintain well-being in the society, since food supplies energy as well as provide nutrients that build up the immune system, maintaining the body in a sound state of repair, correlated with lower morbidity and mortality from infectious and non-infectious diseases. The poverty-malnutrition-disease cycle needs to be broken by multipoint interventions that provide direct, effective and sustainable approaches to increase the economic welfare of the world’s poorest people, including the provision of drugs and vaccines that tackle poor health, with adequate access to nutritious food (Hoddinott et al. 2008). Even in the developed countries, intakes of several vitamins are below the recommended levels in a significant part of the population. Moreover, there is a gap between vitamin intake and requirements for a significant proportion of the population, despite the availability of diverse foodstuffs. Several reports in maize, tomato and lettuce have demonstrated the potential of biofortification in alleviating vitamin deficiencies in human, by means of ensuring an adequate uptake of nutrients (such as folate) from biofortified foods without processing and cooking. Natural folates from biofortified tomato or rice have the potential to improve folate status in humans. For better implementation of genetic engineering, it is essential to understand the genetic networks and biochemical and molecular processes controlling the accumulation of the target vitamins in crops by employing novel technologies, such as isotope labeling-based metabolomics, vitamin-macromolecule and vitamin-micromolecule interactions (Bhullar and Gruissem 2013). One of the major concerns is that very few biofortified transgenic crops, including those with enriched vitamin, have been commercialized for general cultivation. To benefit from this technology, the regulators and policy makers have to revisit different protocols, so that all unnecessary regulations based on ‘extra precautionary’ principles could be removed. Such an approach will greatly benefit the society at large and will be instrumental in achieving food security.

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