

REVIEW

Vitamin Deficiencies in Humans: Can Plant Science Help? ^W

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The term vitamin describes a small group of organic compounds that are absolutely required in the human diet. Although for the most part, dependency criteria are met in developed countries through balanced diets, this is not the case for the five billion people in developing countries who depend predominantly on a single staple crop for survival. Thus, providing a more balanced vitamin intake from high-quality food remains one of the grandest challenges for global human nutrition in the coming decade(s). Here, we describe the known importance of vitamins in human health and current knowledge on their metabolism in plants. Deficits in developing countries are a combined consequence of a paucity of specific vitamins in major food staple crops, losses during crop processing, and/or overreliance on a single species as a primary food source. We discuss the role that plant science can play in addressing this problem and review successful engineering of vitamin pathways. We conclude that while considerable advances have been made in understanding vitamin metabolic pathways in plants, more cross-disciplinary approaches must be adopted to provide adequate levels of all vitamins in the major staple crops to eradicate vitamin deficiencies from the global population.

INTRODUCTION

As plants are autotrophic, they have the ability to acquire the basic elements (minerals) and synthesize the full spectrum of organic molecules required to support their growth and propagation. While humans require the same basic elements as plants, they lack the ability to synthesize many organic molecules (i.e., so-called essential micronutrients [certain amino acids, and vitamins]), for which plants are the main dietary source. There-

fore, human nutritional health is dependent on plant food either directly or indirectly (through feeding on animals that feed on plants). In contrast with the three major nutrients (carbohydrates, proteins, and lipids), micronutrients by definition do not provide energy and are needed in relatively small amounts by organisms. We have known for well over a century that micronutrient deficiency is directly linked to human disease. Indeed, such observations instigated the discovery and categorization of various micronutrients, most notably the vitamins. The term "vitamine" was coined by the Polish biochemist Casimir Funk in 1912, when he isolated a substance (called beri-beri vitamine) that was present in rice bran, but not in polished rice (*Oryza*

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sativa) and could alleviate the deficiency disease beriberi, endemic in many Asian countries (Funk, 1912). At the time, he assumed (albeit wrongly) that all such essential compounds in the diet contain an amine group, hence, the term vitamin (vital-amine); the final “e” was later dropped to deemphasize the amine connection.

Micronutrients are essential for all life; however, the term “vitamin” is a medical definition pertaining to humans, emphasizing that they have lost the ability to synthesize these compounds *de novo*. Thus, the immediate precursors or analogs of vitamins must be obtained from the diet. To date, 13 compounds are classified as vitamins (Figure 1). They can be broadly classified into fat-soluble (A, D, E, and K) and water-soluble (vitamin B complex: B₁, B₂, B₃, B₅, B₆, B₈, B₉, and B₁₂, and vitamin C). Bacteria, fungi, and plants synthesize these compounds and their main function (both as micronutrients in these organisms and as vitamins in humans) is as cofactors or coenzymes in various enzymatic reactions. Furthermore, some of them play distinct roles, for example as antioxidants (vitamins C and E), in vision (β -carotene), or as a (pre-) hormone involved in calcium and phosphorus homeostasis in the blood stream (vitamin D). Being micronutrients in plants as well as animals, it follows that vitamin compounds are synthesized in tiny amounts. On the one hand, this makes it challenging to study the corresponding pathways and enzymes involved, but on the other hand, it means that even small alterations in the levels of these compounds can have a disproportionately positive impact on aspects of human health. It is only relatively recently, with the advent of genomic sequence information and the interest in manipulating the levels of these compounds in plants, that the metabolic pathways of these substances have begun to be deciphered.

That the concentration of many vitamins in the edible portions of the most abundantly grown plants used globally for human food is below minimal requirements (e.g., wheat [*Triticum aestivum*], rice, maize [*Zea mays*], potato [*Solanum tuberosum*], and cassava [*Manihot esculenta*]; Table 1) has profound implications for global human health. This deficit is exacerbated by the limited variety of foods that encompass the bulk of the average diet and a severe depletion of specific micronutrients in the five major crops as a result of postharvest processing. For example, whole-grain rice is a good source of vitamin B₁, but polished rice has been depleted of this vitamin (see Supplemental Table 1 online). Fruits usually provide several vitamins and carbohydrates but are poor sources of minerals or protein. Therefore, a diversified, balanced diet with the right concentration and combination of nutrients is required to support human health. Although the human requirements for the 13 vitamins are reasonably well defined (Figure 1, Table 2), at least at a population level, the vitamin status is far from being adequate in major sections of the global population. This is especially true in developing countries where billions of people still suffer from hunger and protein-energy malnutrition and are concomitantly deficient in numerous micronutrients (i.e., vitamins and minerals). In these countries, many people do not have the means to consume a diverse diet and rely on a single staple crop, which is almost invariably a poor source of several essential micronutrients (Table 1). For example, nearly one-half of the world’s population consumes rice as a staple food (typically produced by small farmers using highly labor-intensive techniques; Timmer, 2010),

while cassava is consumed by millions of people, mostly in tropical countries (see <http://www.fao.org/ag/agp/agpc/gcnds/>). In Western countries, most people have access to a broad variety of foods that provide all the required vitamins (see Supplemental Tables 2 and 3 online for the main vegetables and fruits, respectively) or are fortified during processing to achieve this; thus, vitamin deficiencies are scarce. Nevertheless, a significant portion of all populations do not have optimal intake in several vitamins for various reasons (Table 3). Current technology presents us with the opportunity to develop strategies to counteract these deficits and thereby improve the nutritional quality of unprocessed foodstuffs. The understanding of vitamin biosynthesis, transport, storage, and recycling in plants has progressed considerably in recent years. In addition, the accessibility of genomic tools, such as high marker density genetic maps, genome sequences, and genetic resources, is enabling the identification of vitamin-improved alleles and their introduction into elite varieties for many crop species (Giovannoni, 2006; Tester and Langridge, 2010).

In this article, we review the importance of each vitamin to human health and summarize efforts that have already been made to improve the vitamin content in various plants. We then provide a current and forward-looking perspective of how plant science can contribute to improving the vitamin content of various crop species via a combination of genetic modification, quantitative trait loci (QTL), and association mapping-based approaches.

VITAMIN BIOSYNTHESIS IN PLANTS, IMPORTANCE TO HUMAN HEALTH, AND CURRENT EFFORTS TO ENHANCE LEVELS

Given the enormity of research that has been performed in this area, we refer the reader to two recent volumes of *Advances in Botanical Research*, which provide a more detailed account of individual vitamins in plants (Rébeillé and Douce, 2011a, 2011b). Here, we provide a general overview of the extensive studies related to vitamin biosynthesis and their importance to human health.

Vitamin A (Retinoids)

Retinoic acid and retinal are the main molecules involved in the biological effects of vitamin A. This vitamin plays essential roles in vision, immune responses, and cellular growth, development, and reproduction. Its effects are conferred predominantly through the modulation of gene expression by retinoic acids (*all-trans* and *9-cis*) and by the ability of *11-cis* retinal to absorb photons in rhodopsin. Many health aspects can be impacted by deficiency in vitamin A, such as defects in immune responses and development. Extreme deficiency in this vitamin leads to xerophthalmia (dry eyes), corneal ulceration, blindness, and increased mortality, especially in children. In developing countries, particularly sub-Saharan Africa, vitamin A deficiency is a major health issue. It is estimated that one-third of the children under the age of five around the world suffer from vitamin A deficiency; indeed, 700,000 children die and 500,000 children

Vitamin	Chemical name	Structure
A	Retinol	
D ₃	Cholecalciferol	
E	d-α-tocopherol	
K	Phylloquinone	
B ₁	Thiamin diphosphate	
B ₂	Riboflavin	
B ₃	Niacin	
B ₅	Pantothenate	
B ₆	Pyridoxal 5'-phosphate	
B ₈	Biotin	
B ₉	Folate	
B ₁₂	5'-Deoxyadenosyl cobalamin	
C	L-ascorbate	

Figure 1. The 13 Vitamin Compounds Required in the Human Diet.

In the case of vitamin B₁₂, R represents 5'-deoxyadenosyl, Me, OH, or CN.

Table 1. A Comparison of the Amount of Vitamins in the Five Major Crops as a Function of the RDA and the Fold Increase Required if Any One of These Crops Represents 80% of the Daily Intake of Calories

Vitamin	RDA							% RDA ^a					Fold Increase to Reach RDA				
	Adult Females ^b	Lactating Females ^b	Wheat/ 100 g ^c	Rice/ 100 g ^d	Corn/ 100 g ^e	Potatoes/ 100 g ^f	Cassava/ 100 g ^g	Wheat	Rice	Corn	Potatoes	Cassava	Wheat	Rice	Corn	Potatoes	Cassava
Calories (kcal)			361	130	59	87	160										
Vitamin A (μg/d) ^h	700	1300	2	0	3	0	2	4	0	6	0	4	25	>100	17	>100	50
Vitamin D (μg/d) ⁱ	15	15	0	0	0	0	0	0	0	0	0	0	>100	>100	>100	>100	>100
Vitamin E (mg/d) ^j	15	19	0.4	–	0.02	0.01	0.19	9	–	3	1	10	10.7	>100	35	>100	10
Vitamin K (mg/d)	90*	90*	0.3	–	0	2.1	1.9	1	–	0	43	21	68	>100	>100	2.3	4.7
Vitamin B ₁ (mg/d)	1.2	1.4	0.08	0.02	0.017	0.106	0.087	25	18	33	139	114	3.9	5.7	3.0	Suff.	Suff.
Vitamin B ₂ (mg/d)	1.1	1.6	0.06	0.016	0.006	0.02	0.048	17	12	10	23	30	6.0	8.1	9.8	4.3	3.3
Vitamin B ₃ (mg/d) ^k	14	17	1	0.4	0.175	1.439	0.854	26	29	28	156	92	3.8	3.5	3.6	Suff.	Suff.
Vitamin B ₅ (mg/d)	5*	7*	0.438	0.441	0.078	0.52	0.107	28	78	30	137	15	3.6	1.3	3.3	Suff.	6.5
Vitamin B ₆ (mg/d)	1.3	2.0	0.037	0.05	0.021	0.299	0.088	8	31	28	275	44	12.2	3.2	3.5	Suff.	2.3
Vitamin B ₈ (μg/d)	30*	35*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Vitamin B ₉ (μg/d)	400	500	33	2	1	10	27	29	5	5	37	54	3.4	20.3	18.4	2.7	1.9
Vitamin B ₁₂ (μg/d)	2.4	2.8	0	0	0	0	0	0	0	0	0	0	>100	>100	>100	>100	>100
Vitamin C (mg/d)	75	120	0	0	0	13	20.6	0	0	0	199	316	>100	>100	>100	Suff.	Suff.

The data were obtained from the USDA nutrient laboratory website (<http://www.nal.usda.gov/fnic/foodcomp/search>). The values for RDA are those sufficient to meet the requirements of nearly all (97 to 98%) healthy individuals in the indicated group, taken from <http://www.nap.edu>. The RDA is calculated from the estimated average requirement. If insufficient evidence is available to establish an estimated average requirement, and thus calculate an RDA, an adequate intake is used (denoted with an asterisk). Suff., when the nutrient is at sufficient levels and does not need to be increased to reach the RDA; –, denotes not reported. Note: Values used for wheat and cassava are from uncooked material (values for cooked material are not available). Of note also is that plants do not make vitamin B₁₂.

^aCalculated for lactating women, assuming 80% of daily calories (1600) comes from this crop.

^bValues for 19- to 30-year-old female adults or lactating female adults as they represent the groups with the highest requirements.

^cFlours and unenriched bread.

^dWhite, medium-grain, cooked, unenriched.

^eYellow, unenriched, and cooked with unsalted water.

^fBoiled or cooked in skin.

^gRaw.

^hAs retinol activity equivalents (RAEs); 1 RAE = 1 μg retinol, 12 μg β-carotene, 24 μg α-carotene, or 24 μg β-cryptoxanthin. The RAE for provitamin A carotenoids is twofold greater than retinol equivalents, whereas the RAE for preformed vitamin A is the same as retinol equivalent.

ⁱAs cholecalciferol; 1 μg cholecalciferol = 40 IU vitamin D. Values assume minimal sunlight.

^jAs α-tocopherol.

^kAs niacin equivalents; 1 mg niacin = 60 mg Trp.

become blind as a result of this disease each year (<http://www.who.int/nutrition/topics/vad/en/index.html>).

Structurally, vitamin A is a C₂₀ apocarotenoid derivative, its biosynthetic precursors being the provitamin A carotenoids β-carotene, α-carotene, and β-cryptoxanthin (see Supplemental Figure 1 online). β-Carotene, a C₄₀ carotenoid, is a natural pigment found in plants, algae, and some fungi and bacteria, but not in animals. Once absorbed into the body, β-carotene is centrally cleaved by a class of dioxygenase cleavage enzymes to yield vitamin A. The two unmodified β-ionone rings of β-carotene means that upon cleavage

two molecules of retinoic acid can be formed; this unique property among carotenoid molecules has led to β-carotene being the principal focus for alleviating provitamin A deficiency. Although vitamin A deficiency is not prevalent in Western societies, there is a wealth of scientific evidence to indicate that enhancing carotenoids in the diet, either provitamin A or non provitamin A (e.g., lycopene, lutein, and zeaxanthin), can contribute to the reduction of some chronic diseases, especially when consumed in fruits and vegetables (Van den Berg et al., 2000; Voutilainen et al., 2006; Tan et al., 2010). It should be noted that while two intervention studies using β-carotene

Table 2. A List of the Standard Blood Markers Used to Evaluate Vitamin Status for the General Human Population as well as the Thresholds Used to Estimate the Status

Vitamin	Plasma Marker	Deficiency Threshold	Suboptimal Status Threshold ^a	Toxic Threshold
Vitamin A	Retinol	<0.35 $\mu\text{mol/L}$	0.35–0.7 $\mu\text{mol/L}$	>4.50 $\mu\text{mol/L}$
Vitamin D	25-Hydroxycalciferol	<25 nmol/L	25–50 nmol/L	>125 nmol/L
Vitamin E	α -Tocopherol	<10 $\mu\text{mol/L}$	–	–
Vitamin K	Phytonadione	<0.30 nmol/L	–	–
Vitamin B ₁	Thiamin ^b	70–200 nmol/L ^c	–	–
Vitamin B ₂	Riboflavin ^d	<265 nmol/L	265–480 nmol/L	–
Vitamin B ₃	Nicotinic acid	<0.4 $\mu\text{mol/L}$	–	–
Vitamin B ₅	Pantotenic acid ^e	<4.5 pmol/L	–	–
Vitamin B ₆	Pyridoxal phosphate	<10 nmol/L	10–30 nmol/L	–
Vitamin B ₈	Biotin	<2 nmol/L ^f	–	–
Vitamin B ₉	Folates ^g	<370 nmol/L	370–460 nmol/L	–
Vitamin B ₁₂	Cobalamin	<120 pmol/L	–	–
Vitamin C	Ascorbic acid	<11 $\mu\text{mol/L}$	11–22 $\mu\text{mol/L}$	–

It should be noted that these markers are not always the most accurate but are commonly employed because of their ease of use. On the other hand, they are not used in several studies due to expense limits. The data have been adapted from Guillard et al. (2011). –, Concentration for thresholds is unknown.

^aSuboptimal intake can have long-term consequences on health (e.g., bone loss, macular degeneration, and diseases linked to increased oxidative stress, such as cardiovascular disease).

^bWhole blood total thiamin.

^cWindow of adequate status.

^dTotal riboflavin in red blood cells (i.e. includes FAD and FMN).

^eWhole blood total pantothenic acid.

^fLowest concentration observed in healthy well-nourished subjects in a clinical study (Velázquez et al., 1995).

^gIn red blood cells.

supplements (the ATBC and CARET studies) failed to confirm the beneficial role of this carotenoid on lung cancer (Rautalahti et al., 1997; Omenn, 2007), this does not call into question the beneficial role of this carotenoid when present in its natural matrix (i.e., fruits and vegetables). The only conclusion that came out of these studies is that a pharmacological amount of purified β -carotene, such as found in supplements that contain supranutritional amounts of β -carotene, should not be taken by smokers, former smokers, or people who were/are exposed to asbestos because in these cases β -carotene may increase the risk of this cancer. On the other hand, it is postulated that health-promoting properties of carotenoids are due to their potent antioxidant properties or the ability of carotenoid-derived metabolites to modulate the transcription of antioxidant response elements (Lindshield et al., 2007) or their ability to modulate the secretion of proinflammatory cytokines by adipose tissue. Consequently, there is interest in increasing carotenoid levels in plants, both for biofortification and for nutritional enhancement.

In most crop species examined, the first committed step in the pathway, catalyzed by phytoene synthase, has been found to be the most influential gateway step (Shewmaker et al., 1999; Fraser et al., 2007). Although modulation of phytoene synthase is now comparatively routine, the first attempts to alter this enzyme resulted in a number of pleiotropic effects, including phytohormone imbalances causing disproportionate pigmentation in different plant tissues and genetic effects related to cosuppression and gene silencing (Fray et al., 1995). The perturbations resulting from changes in phytoene synthesis or phytoene desaturation also revealed feed-forward (Ducreux et al., 2005) and feedback inhibition mechanisms (Römer et al., 2000). These detrimental

effects have been overcome in most cases through the use of tissue-specific promoters and optimized genes (e.g., altered codon usage and transcriptional and translational enhancers). It is also evident that each crop is different and the strategy must be optimized for the species or the specific tissue targeted within the plant. A classical example is rice endosperm, which, although devoid of carotenoids, expresses the majority of the enzymes in the pathway with the exception of the initial enzymes, phytoene synthase and desaturase. Therefore, the incorporation of these two enzymes is sufficient to induce β -carotene biosynthesis and is the basis of Golden Rice (Ye et al., 2000; Schaub et al., 2005).

In tissues naturally possessing a low level of carotenoids or even devoid of carotenoids, minipathways have been successfully engineered (Diretto et al., 2007; Fujisawa et al., 2009). More recently, the manipulation of organelle parameters has been used successfully as a tool to enhance carotenoid contents in both source and sink plant organs. In tomato (*Solanum lycopersicum*), the *hp-1* and *-2* genes that are negative regulators of light perception have been used (Davuluri et al., 2005). Initially, the mode of action attributed to these gene products for increasing pigment content was an elevation in plastid area per cell. However, more integrative approaches now suggest the effect is due to the coordinated expression of core metabolic components (Enfissi et al., 2010). The cauliflower (*Brassica oleracea*) *Or* gene, which regulates chromoplast formation, is another example where plastid-related parameters have been altered to increase carotenoid content (Lu et al., 2006). These examples highlight several targets for metabolite engineering through QTL. As a result of a combined association-based mapping and linkage analyses in maize, Harjes et al. (2008) recently showed

Table 3. A List of Factors That Increase the Risk of Having Vitamin Deficiencies for the General Human Population

Factor	Vitamin												
	A	D	E	K	B1	B2	B3	B5	B6	B8	B9	B12	C
Age or Physiological State													
Pregnancy or nursing mothers	-	+	-	-	+	-	-	-	+	-	+++	-	++
Premature babies	+	+++	+++	+++	-	-	-	-	+	-	++	-	+
Infants or children	-	++	-	+	-	-	-	-	-	-	-	-	-
Teenagers	-	++	-	-	-	-	-	-	-	-	-	-	-
Elderly people	-	+++	+	-	++	-	-	-	-	-	+++	+++	+
Diseases and Behavior													
Undernutrition	++	+	+	+	+	+	+	+	+	+	++	+	+
Malabsorption	++	++	++	++	+	+	+	-	+	-	++	++	+
Aids	+	++	+	+	++	++	+	-	+	-	++	++	+
Alcoholism	+	+	-	++	+++	+	+	-	+	-	++	+	-
Liver cirrhosis	+	++	+	++	+	+	+	-	+	-	++	+	+
Smoking	+	-	+	-	-	-	-	-	+	-	-	-	+
Obesity	-	+	-	-	++	-	-	-	-	-	-	-	-
Dialysis	-	-	-	-	+	+	+	-	+	-	+	+	+
Drug therapy	-	++	-	+	+	-	-	-	+	++	++	-	-
Dietary Habits													
Veganism	-	++	-	-	-	-	-	-	-	+ ^a	-	+++	-
Low-calorie diet	+	+	+	+	+	+	+	-	+	-	+	+	+
Low-residue diet	+	+	-	-	-	-	-	-	-	-	++	-	++

The number of plus signs indicates the following: +, can have a negative effect; ++, has a significant negative effect; +++, has a strong negative effect; -, concentration for thresholds is unknown. The data have been adapted from Guillard et al. (2011).

^aRaw egg white-rich diet.

that variation at the lycopene epsilon cyclase (*lcyE*) and β -carotene hydroxylase 1 (*CrtRB1*) loci are key targets for provitamin A biofortification. This work selected favorable *lcyE* and *CrtRB1* alleles and developed inexpensive molecular markers that are now being deployed by breeders in developing countries to produce maize grain with higher provitamin A levels (Harjes et al., 2008; Yan et al., 2010). Interestingly, these alleles conferred higher β -carotene by preventing its metabolism, unlike many of the genetic engineering approaches that have focused on the enhancement of biosynthetic capacity. Through the use of this approach a target of $15 \mu\text{g}\cdot\text{g}^{-1}$ provitamin A was reached, which is predicted to alleviate vitamin A deficiency in most developing areas.

The Vitamin B Complex

The B vitamin complex is a group of eight compounds that all act as essential enzymatic cofactors in metabolism (B₁, B₂, B₃, B₅, B₆, B₈, B₉, and B₁₂). With the exception of vitamin B₁₂, plants can synthesize all B vitamins; therefore, we will restrict our discussion to these.

Vitamin B₁ (Thiamin)

The first vitamin to be characterized was vitamin B₁ (Funk, 1912). Vitamin B₁ acts, in the form of thiamin diphosphate, as an essential cofactor for crucial metabolic pathways, such as the conversion of pyruvate to acetyl-CoA at the mitochondrial mem-

brane, 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 manifests itself as the well-known disease beriberi, which can cause detrimental neurological effects and/or affect the cardiovascular system with a fatal outcome if not cured by thiamin administration. Less severe deficiency can result in weight loss, irritability, confusion, and impairment of short-term memory (Wernicke's encephalopathy). Deficiency is surprisingly common, particularly in developing countries where the main food sources are low in thiamin, such as communities where polished rice forms a major part of the diet. Cereal grains are an important source of thiamin; however, it is found mostly in the outer layers of the grain and in the germ, which are removed during the refining process. For example, 100 g of whole-wheat flour contains 0.55 mg of thiamin, whereas 100 g of white flour contains only 0.06 mg. In the US, processed flour is enriched with chemically synthesized thiamin mononitrate (along with niacin, ferrous iron, riboflavin, and folic acid) to replace that lost in processing.

Structurally, thiamin consists of a thiazole and a pyrimidine moiety (Figure 1); the two heterocyclic compounds are synthesized separately in plants (by thiazole synthase, THI1 [Machado et al., 1996], and 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate synthase, THIC [Raschke et al., 2007], respectively) and then linked together by an oxidative condensation reaction catalyzed by thiamin monophosphate synthase (TH1) to form thiamin monophosphate (Ajajawi et al., 2007b) (see Supplemental Figure 2 online). The latter is then dephosphorylated and the

resulting thiamin is subsequently pyrophosphorylated by a pyrophosphokinase (annotated TPK) to form thiamine diphosphate (Ajawi et al., 2007a). A major step in negative regulation of vitamin B₁ biosynthesis was elucidated through the discovery of a riboswitch associated with the *THIC* gene (Sudarsan et al., 2003). 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 (Breaker, 2011). The *THIC* riboswitch is the only confirmed example in plants to date. Upon binding thiamine diphosphate, an alternative splicing event occurs that is thought to interfere with polyadenylation leading to an unstable transcript (Bocobza et al., 2007; Wachter et al., 2007). This feature could be exploited to manipulate the overproduction of thiamin in plants. To date, there have been no reports on the isolation of plants with enhanced thiamin levels either through genetic engineering or improved alleles.

Vitamin B₂ (Riboflavin)

Riboflavin is the precursor of the cellular cofactors flavin adenine mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Numerous enzymes require these flavin cofactors, including those involved in mitochondrial electron transport, photosynthesis, and fatty acid oxidation. Humans cannot synthesize riboflavin but have the ability to synthesize FMN and FAD from dietary riboflavin. In humans, deficiency in vitamin B₂ has been linked to cancer, cardiovascular disease, anemia, and various neurological and developmental disorders (Powers, 2003).

In addition to acting as precursors for FMN and FAD, in plants, flavins are important components of photoreceptors (cryptochromes and phototropins) and certain signaling molecules in plants (e.g., FKF1, a F-box protein involved in regulating the circadian clock). De novo biosynthesis of riboflavin in plants (reviewed in Bacher et al., 2000; Roje, 2007) begins with the conversion of guanosine triphosphate into 5-amino-6-ribitylamino-2,4-pyrimidinedione involving four enzymes and the conversion of ribulose 5-phosphate to 3,4-dihydroxy-2-butanone 4-phosphate involving one enzyme (see Supplemental Figure 3 online). Dephosphorylation of the latter and condensation with 5-amino-6-ribitylamino-2,4-pyrimidinedione results in the formation of 6,7-dimethyl-8-ribityllumazine, which is then converted into riboflavin by the correspondingly named synthase. Phosphorylation and adenylation of riboflavin yield FMN and FAD, respectively. The entire pathway has been elucidated in bacteria and is assumed to be the same in plants, although homologs for all of the genes have not yet been identified. There are several studies on the overproduction of riboflavin or isolation of natural riboflavin-overproducing strains in bacteria and fungi, but we are not aware of any such studies to date in plants.

Vitamin B₃ (Niacin)

Niacin, initially described by Weidel (1873), comprises two derivatives of pyridine that carry either a carboxyl or carboxamide group at the pyridine's 3'-position, leading to nicotinic acid or nicotinamide, respectively. Niacin and nicotinamide serve as dietary precursors for the enzymatic cofactors NAD⁺ and NADP⁺

that are required in many redox reactions and are involved in fatty acid and carbohydrate metabolism (Wahlberg et al., 2000). NAD⁺ is also a critical metabolite in cellular signaling reactions as it serves as a substrate for ADP-ribosyltransferases and ADP-ribosyl cyclases to generate ADP-ribosylated substrates and cyclic ADP-ribose, respectively (Lepiniec et al., 1995; Doucet-Chabeaud et al., 2001; Virág and Szabó, 2002; Hassa et al., 2006; Pollak et al., 2007; Lamb et al., 2011). ADP-ribosylation plays a critical role in various cellular processes, such as DNA repair (Doucet-Chabeaud et al., 2001), stress responses (Monks et al., 2006; Adams-Phillips et al., 2008), cell cycle control (Shiotani et al., 2006), and chromatin structure (Laroche et al., 1980; Houben et al., 2007), and cyclic ADP-ribose is important in the control of the cellular calcium flux (Higashida et al., 2001; Pollak et al., 2007; Morgan and Galione, 2008).

In humans, severe deficiencies were first described in the context of Pellagra, a disease where patients suffer from dementia, dermatitis, and diarrhea (Kohn, 1938). Niacin deficiency is also associated with mental disorders, which become apparent as amnesia, poor concentration, apathy, or depression (Lanska, 2010). A severe dearth in niacin nutrition is often present in areas where humans rely mainly on a maize-based diet and where the seed does not undergo nixtamalization before consumption. Nixtamalization is a process in which maize is soaked and prepared in an alkaline solution (such as lime), which improves the extraction of proteins and vitamins from the grain.

While in mammals vitamin B₃ can be synthesized in the liver (and in some species in the kidney) from Trp via the intermediates kynurenine and quinolinate (Kumasov et al., 2003), a proposed pathway in plants is located in the chloroplasts and proceeds from Asp through α -aminosuccinate to enter the quinolinate pathway (Katoh et al., 2006) (see Supplemental Figure 4 online). Quinolinate is further metabolized in several steps to NAD⁺, nicotinamide, and niacin via the pyridine nucleotide cycle (Roje, 2007). Currently, little is known about the impact of genetically engineering enzyme activities of the niacin biosynthetic pathway on plant development and vitamin B₃ content. However, the few reports that exist suggest that plants are highly sensitive to changes in this biosynthetic pathway. For example, it was recently shown that overexpression of NAD⁺ synthetase in *Arabidopsis thaliana* efficiently increased niacin and nicotinamide, although not NAD⁺, resulting in early senescence and flowering and negatively affecting seed development (Shinnosuke et al., 2010). Likewise, loss-of-function mutants affected in quinolinate synthase, which catalyzes the step from α -aminosuccinate to quinolinate, results in reduced NAD⁺ levels and early aging (Schippers et al., 2008). While it is conceivable that increasing niacin content is of benefit for human nutrition, the negative impacts on plant growth and development using forward and reverse genetic approaches indicate that it may be technically demanding to generate plants with elevated vitamin B₃ levels without significantly affecting other processes, and marker-assisted genetic approaches have not yet been reported for this compound.

Vitamin B₅ (Pantothenate)

Pantothenate provides the 4'-phosphopantetheine moiety in CoA and is an acyl carrier involved in lipid biosynthesis and catabolism as well as in pyruvate decarboxylase activity (Webb

et al., 2004). No known disease has been associated with a lack of this compound, as it is found in small amounts in many foods and its content is particularly high in human foods such as whole-grain cereals, legumes, eggs, meat, and avocado (Table 3). The biosynthesis of pantothenate proceeds via three enzyme catalyzed reactions in plants (ketopantoate hydroxymethyl transferase, pantothenate synthase, and an as yet to be identified ketopantotate reductase) (Smith et al., 2007; see Supplemental Figure 5 online). However, because no deficiency diseases are attributed to this vitamin, in the context of this article we will not discuss it further.

Vitamin B₆ (Pyridoxine)

Vitamin B₆ comprises three chemically distinct molecules: pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL). They have a pyridine ring in common but carry different substitutions at the 4'-position: a hydroxyl, an amino, or an aldehyde group, respectively. Vitamin B₆ is involved in diverse enzymatic reactions, including transaminations, α -decarboxylations, racemizations, β - and γ -eliminations, and aldol cleavages (Drewke and Leistner, 2001). To function as an enzymatic cofactor, the different B₆ vitamers require additional phosphorylation at their 5'-position leading to PNP, PMP, or PLP. Although vitamin B₆ is primarily required for amino acid biosynthesis and catabolism, it is also involved in additional processes, such as sugar and fatty acid metabolism (Mittenhuber, 2001; Fitzpatrick et al., 2007). Furthermore, the biosynthesis of other B vitamins, such as B₈ (biotin), B₉ (folate), and B₁₂ (cyanocobalamin), requires the activities of PLP-dependent enzymes (Battersby and Leeper, 1998; Basset et al., 2004; Pinon et al., 2005). Besides its role as an enzymatic cofactor, vitamin B₆ is also known as a potent antioxidant (Ehrenshaft et al., 1998; Bilski et al., 2000).

Due to its pleiotropic effects, vitamin B₆ has major impacts on cellular biology and has been implicated in various health concerns, including cardiovascular disease, blood pressure problems, diabetes, neurological disorders, carpal tunnel syndrome, premenstrual syndrome, and pellagra skin disease (Mooney and Hellmann, 2010). As a result of its antioxidant capacities, it may also serve as an important protectant against overproduction of reactive oxygen species in the body (Jain and Lim, 2001; Han et al., 2010). Notably, a recent clinical study in the US showed a general deficiency of vitamin B₆ in the population (Morris et al., 2008); as a consequence, the authors proposed raising the current recommended dietary intake from 2 mg to 4.9 mg.

Biosynthesis of vitamin B₆ has only recently been elucidated in plants (Tambasco-Studart et al., 2005; Wagner et al., 2006; Tambasco-Studart et al., 2007) (see Supplemental Figure 6 online). It requires the concerted activities of the PDX1 and PDX2 proteins, which use either ribose 5-phosphate or ribulose 5-phosphate, in addition to either glyceraldehyde 3-phosphate or dihydroxyacetone phosphate as well as Gln to synthesize PLP (Burns et al., 2005; Raschke et al., 2005; Tambasco-Studart et al., 2005). Besides a de novo pathway, organisms that synthesize vitamin B₆ (bacteria and fungi in addition to plants) also have a salvage pathway. In plants, two enzymes of this pathway have been characterized: a kinase that can phosphorylate PN, PL, and PM (to PNP, PLP, and PMP, respectively) and an oxidase that

converts either PMP or PNP to the active cofactor form, PLP. In plants, while activity of the de novo pathway and the kinase appear to be cytosolic (although additional localizations have been visualized) (Shi et al., 2002; Chen and Xiong, 2005; Tambasco-Studart et al., 2005), the oxidase has been shown to be restricted to plastids (Sang et al., 2011). A few recent studies in *Arabidopsis* have independently demonstrated that overexpressing the biosynthetic enzymes (Chen and Xiong, 2009; Leuendorf et al., 2010; Raschke et al., 2011) leads to increased accumulation of vitamin B₆. The most recent of these studies, which demonstrated the highest boost in B₆ content, showed that such an increase can lead to larger organ size and, thus, potentially higher yield rates (Raschke et al., 2011). Vitamin B₆ has also been associated with photosynthetic efficiency (Havaux et al., 2009; Triantaphylidès and Havaux, 2009). As a consequence of its potent antioxidant activity, plants affected in B₆ metabolism are often highly sensitive to abiotic stress conditions (Shi et al., 2002; Chen and Xiong, 2005; Titiz et al., 2006; González et al., 2007). All of these findings suggest there is great potential to generate crop plants with modified vitamin B₆ content that may have increased productivity, greater stress tolerance, and a generally improved vitamin B₆ value from which human consumers may benefit.

Vitamin B₈ (Biotin)

Biotin acts as a cofactor for a few enzymes that are involved in carboxylation, decarboxylation, and transcarboxylation reactions in carbohydrate and fatty acid metabolism. There are four biotin-dependent carboxylases in humans: those of propionyl-CoA, β -methylcrotonyl-CoA, pyruvate, and acetyl-CoA. In recent years, biotin has also been implicated in cell signaling and histone biotinylation via biotinidase (Kobza et al., 2005). Similar to pantothenate, deficiency of this vitamin is rare in humans, with the exception of individuals with inborn errors of biotin metabolism and in some women during pregnancy (Said, 2002).

De novo biosynthesis of biotin in plants is thought to be similar to the pathway in bacteria, beginning with L-Ala and pimelic acid and proceeding through the intermediates pimeloyl-CoA, 7-keto-8-aminopelargonic acid, 7,8-diaminopelargonic acid, and D-thiobiotin, involving five enzymes (Alban et al., 2000) (see Supplemental Figure 7 online). The only procedure on increasing biotin production in plants that we are aware of is the claim of a U.S. patent (US5869719), which states it is possible to overproduce biotin in plants through heterologous expression of either diaminopimelate aminotransferase or biotin synthase from *Escherichia coli*, or through overexpression of the corresponding endogenous genes, in the *bio1 Arabidopsis* mutant (Patton et al., 1996).

Vitamin B₉ (Folate)

Folate is a generic term for a group of compounds that comprise the cofactor 5,6,7,8-tetrahydrofolate and its respective one-carbon derivatives. Folates play critical roles in one-carbon transfer systems and as such are required in the biosynthesis of DNA (purines and thymidylate) and also for the production of vitamin B₅, Met, or Met formyl-Met-tRNA (Basset et al., 2004; Goyer et al., 2004; Roje, 2007). Folate deficiency is a common

worldwide phenomenon in human populations and may cause serious disorders, such as Pellagra, birth defects, cardiovascular problems, megaloblastic anemia (a type of anemia caused by impaired DNA synthesis), aggravation of iron deficiency anemia, and cell cycle defects that affect red blood cell production (Wickramasinghe, 2006). Folate deficiency may also cause neural tube defects, such as anencephaly and spina bifida (Pitkin, 2007). Malnutrition in vitamin B₉ is likely to be caused by the fact that over half of the world's population relies on a cereal-based diet, a food resource that is generally poor in folate content. For example, in China, where many people depend on a rice-based diet, ~20% of the population is considered to be folate deficient (De Steur et al., 2010).

In plants, folates are synthesized from *para*-aminobenzoic acid (*p*ABA) and pterin precursors (Goyer et al., 2004; Roje, 2007) (see Supplemental Figure 8 online). *p*ABA 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 tetrahydrofolate. Metabolic engineering of cereal crop plants to increase production of folate may serve as a valuable tool to fortify folate nutrition. Recent work has demonstrated that transgenic rice plants cooverexpressing key enzymes in folate biosynthesis from *Arabidopsis* (aminodeoxychorismate synthase and GTP cyclohydrolase I; (Storozhenko et al., 2007) or wheat (6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase and 7,8-dihydropterolate synthase; Gillies et al., 2008) increase folate content. In transgenic grains, up to a 100-fold elevation in comparison to wild-type plants has been measured without any reported defects on plant development (Storozhenko et al., 2007). Similarly, transgenic tomato plants overexpressing human GTP cyclohydrolase I and aminodeoxychorismate synthase from *Arabidopsis* resulted in a 25-fold increase of folate in fruits (Díaz de la Garza et al., 2007). Moreover, first reports on the identification of QTL for folate content in crop species are emerging (Khanal et al., 2010). In the latter study, Khanal et al. identified a set of five QTL markers based on the analysis of two *Phaseolus vulgaris* varieties, Othello and Redhawk, and could prove that genetic variation among the germplasms cause up to 11% change in folate content. Hence, generating or breeding and farming such plants may help fortification of improved phytonutrient nourishment and better health in many countries worldwide.

Vitamin C (Ascorbate)

The major source of vitamin C in the human diet is from plant-derived ascorbic acid (AsA), mostly found in fruit and vegetables.

Despite numerous epidemiological studies establishing a positive link between vitamin C content in food and/or plasma and health benefits (e.g., the prevention of cardiovascular disease, cancer, and other diseases) (Blot et al., 1993; Steinmetz and Potter, 1996) or the increase in iron bioavailability (López and Martos, 2004), the only clinical benefit of AsA supplementation, with a proven mechanism of action, is in the prevention of scurvy, which is caused by vitamin C deficiency (Lykkesfeldt and Poulsen, 2010). However, there are indications that vitamin C consumed from plant products might be more efficient for the prevention of atherosclerosis than vitamin C delivered as a supplement (Inoue et al., 2008). In humans, biochemical evidence links AsA to molecular events associated with oxygen sensing, redox homeostasis, and carcinogenesis (Valko et al., 2006). Surprisingly, it was not until 1998 that the first vitamin C biosynthetic pathway was described in plants (Wheeler et al., 1998) (see Supplemental Figure 9 online). In this pathway, D-glucose is converted to AsA through the following intermediates: GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, and L-galactono-1,4-lactone. This pathway is known as the L-galactose pathway. A second pathway initiating from D-galacturonate was proposed after the identification of a D-galacturonate reductase gene in strawberry (*Fragaria* spp; Agius et al., 2003). Additionally, L-gulose (Wolucka and Van Montagu, 2003) and *myo*-inositol (Lorence et al., 2004) have been proposed as primary metabolites in the origin of AsA biosynthesis pathways. Though the main pathway is the L-galactose pathway described by Wheeler et al. (1998), the prevalence of this pathway is dependent on the plant species, tissues, and the developmental stage. In plants, as in humans, AsA plays a critical role in many processes and is essential for normal development (Pavet et al., 2005). This requires tight control of its biosynthesis, catabolism, and reductive recovery from the oxidized form, dehydroascorbic acid.

Most of the dietary sources of vitamin C, such as fruits (mango [*Mangifera indica*], kiwifruit [*Actinidia deliciosa*], *Citrus*, strawberry, and tomato), flowers (broccoli [*Brassica oleracea*]), or tubers (potato), are sink organs in which ascorbate can accumulate to high levels. However, the natural vitamin C content of fruit varies widely across plant species (ranging from <10 mg/100 g of fresh weight in apple to >2.5 g/100 g of fresh weight in the Amazonian fruit *Camu camu*), as well as across cultural and environmental conditions (e.g., light and postharvest losses during storage and processing; Table 4). Additional elements when considering food nutritional value are which plant tissue is edible (vitamin C is mostly accumulated in the epidermis or rind in

Table 4. Sensitivity of Vitamins to Different Agents

Sensitivity	Heat	Light	Oxidants	Reducers	Moisture	Acids	Bases
Very Sensitive	B ₁	A, D, K, B ₂	A, D, B ₉ , C	B ₉ , B ₁₂	–	B ₅ , B ₁₂	K, B ₁ , B ₂ , B ₅ , B ₁₂ , C
Sensitive	A, D, E, C	E, B ₁ , B ₆ , B ₈ , B ₉ , B ₁₂ , C	E, K, B ₁₂	B ₁ , B ₂ , B ₃	B ₁ , B ₃ , B ₅ , B ₁₂ , C	A, D, B ₆ , B ₈ , B ₉ , C	D, E, B ₆ , B ₈ , B ₉
Stable	K, B ₂ , B ₃ , B ₆ , B ₈ , B ₉ , B ₁₂	B ₃ , B ₅	B ₁ , B ₂ , B ₃ , B ₅ , B ₆ , B ₈ , B ₁₂	A, D, E, K, B ₅ , B ₆ , B ₈ , C	A, D, E, K, B ₂ , B ₆ , B ₈ , B ₉	E, K, B ₁ , B ₂ , B ₃	A, B ₃

The data have been adapted from Guillard et al. (2011). –, Symbolizes that no reports of very sensitive have been recorded.

many fruit species, which is rarely consumed) and whether the product is consumed raw or processed (vitamin C is heat labile; Table 4).

There is a large potential for improving vitamin C content in food products by means of both genetic engineering and breeding. At the genetic engineering level, success has been achieved by the expression of plant or animal vitamin C biosynthetic genes from various pathways in the model plant *Arabidopsis* and in crop species (lettuce [*Lactuca sativa*], potato, and maize) (Ishikawa et al., 2006). Mean increases in vitamin C content were usually two- to threefold, except for the sevenfold rise observed in lettuce following ectopic expression of rat L-gulonolactone oxidase and a similar magnitude in potato plants ectopically expressing a bacterial pyrophosphorylase or a yeast invertase (Farré et al., 2008). However, the most promising results were obtained more recently with the newly discovered GDP-L-galactose phosphorylase (VTC2) enzyme that catalyzes the first committed step in the L-galactose ascorbate biosynthetic pathway and is highly regulated by developmental and environmental cues (Dowdle et al., 2007; Laing et al., 2007; Linster et al., 2007). Ectopic expression of kiwifruit VTC2 alone in *Arabidopsis* augmented vitamin C content almost fourfold (from 60 to 216 mg/100 gram fresh weight), while transient coexpression of both VTC2 and GDP-mannose epimerase, another key enzyme of the L-galactose pathway, increased tobacco (*Nicotiana tabacum*) leaf ascorbate content by up to 12-fold (from 37 to 453 mg/100 gram fresh weight) (Bulley et al., 2009). Recently, the stable overexpression of VTC2 in tomato, strawberry, and potato succeeded in increasing fruits and tuber vitamin C by two- to sixfold (i.e., 111, 131, or 1.83 mg/100 gram fresh weight in ripe fruits of tomato, strawberry, or potato tubers, respectively) (Bulley et al., 2011). Increasing the vitamin C content by breeding is well advanced (Davey et al., 2006; Stevens et al., 2007; Zorrilla-Fontanesi et al., 2011) and will therefore be discussed below to illustrate how this strategy can be applied as an alternative to genetic engineering with regard to the other vitamins.

Vitamin D (Calciferol)

Vitamin D is a fat soluble prohormone that has emerged as a key biological predictor of increased rates of cardiovascular disease risk factors (hypertension, obesity, diabetes mellitus, and metabolic syndrome) and progression factors (e.g., inflammation and fibrosis) (Artaza et al., 2011). In humans, the optimal plasma level of vitamin D is commonly said to be 30 ng/mL or above, while levels of 21 to 29 ng/mL are generally considered insufficient and levels below 20 ng/mL considered deficient (Holick, 2007). Vitamin D deficiency is highly prevalent and has been associated with a diverse range of chronic medical conditions, such as rickets, osteomalacia, osteoporosis, myopathy, lung disease, increased inflammation, and reduced immunity (Janssens et al., 2009; Kumar et al., 2011). The term vitamin D includes the vitamins D₂ (ergocalciferol) and D₃ (cholecalciferol) that are produced by photolysis from naturally occurring sterol precursors under UV-B light (280 to 320 nm) by both plants and animals. Therefore, production of vitamin D from its precursors takes place in sun-exposed tissues: the skin in animals and the leaves in plants.

In the past, it was believed that plants synthesize only vitamin D₂ and that animals synthesize only vitamin D₃ (see Supplemental Figure 10 online), although more recently, vitamin D₃ has also been found in a variety of plants. While vitamin D₂ is produced from ergosterol in fungi, the precursor is not known in plants. On the other hand, vitamin D₃ is produced from 7-dehydrocholesterol, found in some plants and in the skin of animals. Both vitamin D₂ and D₃ are further metabolized to the active forms 25-hydroxyvitamin D (25[OH]D) and 1,25-dihydroxyvitamin D (1,25[OH]₂D), which is a strong modulator of gene expression. In animals, the 25-hydroxylation takes place in the liver (Cheng et al., 2004), while the second 1-hydroxylation occurs primarily in the kidney, although other tissues have this enzyme. These steps are also thought to occur in the leaves of plants (Napoli et al., 1977; Boland et al., 2003). Species that have been identified as containing 25[OH]D and 1,25[OH]₂D are the Solanaceae, Cucurbitaceae, Fabaceae (Leguminosae), and Poaceae (Gramineae) (Boland, 1986; Boland et al., 2003), with the leaves of the plants, not surprisingly, containing the highest concentration. Despite synthesis occurring most commonly in light-exposed tissues, a biosynthetic route for vitamin D₃ that is independent of light has been reported in *Solanum glaucophyllum* cultured in vitro (Curino et al., 1998). Not much is known about the function of vitamin D₃ in plants. It has been proposed that 1,25[OH]₂D, in particular, is involved in plant defense (Piel et al., 1997; Burlini et al., 2002). There is evidence that vitamin D₃ at concentrations of 10⁻¹⁰ to 10⁻⁹ M stimulates root growth (Vega et al., 1988), which is mediated by an activation of the Ca²⁺ messenger system (Boland et al., 2003), similar to other classical plant growth regulators like auxins, cytokinins, and gibberellins. Nevertheless, the physiological function of the vitamin D₃ pathway in plants is still obscure, and metabolic engineering strategies aimed at enhancing its content have as yet not been reported.

Vitamin E (Tocopherol)

Tocopherols and tocotrienols are members of a group of compounds synthesized exclusively by photosynthetic organisms and known collectively as tocochromanols. Tocopherols and tocotrienols can occur as four differentially methylated species (α , β , γ , and δ), and while all are excellent lipid soluble antioxidants, they differ dramatically in their vitamin E activity in vivo, with α -tocopherol having the highest activity on a molar basis. Since their first isolation from wheat germ oil (Evans et al., 1936), tocochromanols have been recognized as an important component of the human body's defense against oxidative damage through their quenching of polyunsaturated free radicals, thus breaking the chain reaction of lipid peroxidation. Novel functions of vitamin E have also been reported, such as influences on enzyme activities, signaling cascades, and gene regulation, although the molecular mechanism(s) in vivo has not been elucidated (Azzi, 2007; Traber and Atkinson, 2007; Brigelius-Flohé, 2009). Like all essential dietary nutrients, humans cannot synthesize vitamin E and must obtain a sufficient quantity from their diet, primarily from plant-derived oils, which can vary by orders of magnitude in total tocochromanol levels, composition, and activity (Grusak and DellaPenna, 1999). Vitamin E deficiency can lead to hemolytic anemia in premature babies and to

neurological and ophthalmological disorders as well as myopathy in children. People in developing countries suffer from vitamin E deficiency (Dror and Allen, 2011), while in developed countries, vitamin E deficiency is rare and is observed only in specific conditions: premature babies, digestive pathology, and genetic pathology.

The first biosynthetic enzyme in the tocochromanol pathway was cloned from *Arabidopsis* in 1998, and during the intervening years, the entire pathway was elucidated, primarily by approaches integrating the model organisms *Arabidopsis* and *Synechocystis* sp PCC6803 (reviewed in DellaPenna and Pogson, 2006). The cloned vitamin E biosynthetic genes allowed individual and multiple pathway steps to be manipulated in *Arabidopsis* to define their functions and demonstrate their potential, individually and in combination, for modifying the content and composition of tocochromanols in leaf and seed tissues (DellaPenna and Pogson, 2006). The vitamin E biosynthetic pathway starts with the conversion of hydroxyphenylpyruvate to homogentisate, which is then condensed with phytyl pyrophosphate by homogentisate phytyl transferase (VTE2) (see Supplemental Figure 11 online). The 2-methyl-6-phytylquinol produced is then subject to methylation and/or cyclization by dimethyl-phytylquinol methyl transferase (VTE3) and tocopherol cyclase (VTE1), respectively, and/or methylation by γ -tocopherol methyl transferase (VTE4). The different combinations and numbers of these reactions result in formation of α , β , γ , and δ -tocopherols. A phytol kinase (VTE5) converts phytol to phytol phosphate (Valentin et al., 2006) and a yet to be cloned second kinase activity converts phytol phosphate to phytol diphosphate (Ischebeck et al., 2006).

The information obtained from *Arabidopsis* has been readily transferable to other plants and because orthologs of tocopherol biosynthetic genes can be identified in the genome sequences of crop plants, this has enabled substantial progress in transgenic manipulation of the pathway and end product accumulation in several crop species, including maize, soybean (*Glycine max*), canola (*Brassica napus*), and others (Shukla and Mattoo, 2009). Since the first demonstration that vitamin E composition can be shifted in favor of α -tocopherol (Shintani and DellaPenna, 1998), results from subsequent studies suggested that VTE3 and VTE4 represent key points controlling the ratios between the different tocopherol species (Collakova and DellaPenna, 2003a; Van Eenennaam et al., 2003; Quadrana et al., 2011), while other studies demonstrated that homogentisic acid geranylgeranyl transferase and VTE2 are limiting activities for tocotrienol and tocopherol syntheses, respectively (Cahoon et al., 2003; Collakova and DellaPenna, 2003b; Sattler et al., 2004). Soybean perhaps represents the most comprehensively engineered crop example to date. It has been shown that tocopherols present in seed can be converted nearly completely to α -tocopherol by engineering increased expression of the two pathway methyl transferases, VTE3 and VTE4 (Van Eenennaam et al., 2003), and, in another study, manipulation of four VTE genes, singly and in combination, was shown to impact both tocopherol composition and total content (Karunanandaa et al., 2005). Several groups have identified QTL explaining the variance of seed vitamin E composition and content in biallelic populations of *Arabidopsis* (Gilliland et al., 2006), sunflower (*Helianthus annuus*; Hass et al., 2006), maize (Chander et al., 2008), and the fruit of tomato (Schauer et al.,

2006; Almeida et al., 2011). For many of these studies, vitamin E biosynthetic genes colocalized to some of the QTL intervals, but the size of the QTL intervals had limited definitive demonstration of the molecular basis of the QTL and, thus, whether they are due to modifications of biosynthetic gene function or expression. In the last decade, several studies of transcription factors involved in the tomato fruit-ripening program have reported alterations in tocopherol levels (Adato et al., 2009; Itkin et al., 2009; Karlova et al., 2011). However, until mechanistic explanations can be provided for these observations, they should be considered cautiously, as such alterations in tocopherol levels could be pleiotropic effects of broad alterations in transcription networks leading to increased stress, altered photosynthetic electron transport and oxidation, or to increased phytol levels from chlorophyll degradation. This latter explanation is in line with results showing that overexpression of a *phytoene synthase-1* (Fraser et al., 2007) and a bacterial *phytoene desaturase* (Römer et al., 2000), core biosynthetic enzymes in the synthesis of tomato carotenoids, also result in higher tocopherol content in fruit. In only one tomato transcription study (alteration in *det1* expression; Enfissi et al., 2010) has it been shown that the substantial increases in tocopherol content (twofold to 10-fold) are directly correlated with upregulation of geranylgeranyl diphosphate reductase and VTE4 expression, providing a mechanistic explanation for light having a role in regulation of tocopherol synthesis and accumulation in tomato fruit.

Vitamin K (Phylloquinone)

Vitamin K (2-methyl-3-phytyl-1,4-naphthoquinone) is a terpenoid-quinone conjugate made up of a redox active naphthalenoid ring attached to a liposoluble phytyl side chain. Vertebrates require vitamin K as a cofactor for the γ -carboxylation of specific Glu residues, thus increasing their chelating properties, on certain proteins that must bind calcium to be biologically active. These include blood coagulation factors (prothrombin, factors VII, IX, and X), proteins involved in bone metabolism and homeostasis (osteocalcin and Matrix Gla Protein), and the cell signaling factor Gas6 (Vermeer et al., 2004). There is also experimental evidence that vitamin K acts as a transcriptional regulator in mammals (Ichikawa et al., 2006). For humans, phylloquinone from green leafy vegetables and vegetable oils is the main contributor of dietary vitamin K (Booth and Suttie, 1998). Symptoms of vitamin K deficiency include bruising, hemorrhages, and increased risk of bone fracture (Suttie, 1995; Szulc et al., 1996; Booth et al., 2000). In neonates in particular, the lack of vitamin K is the leading cause of a life-threatening condition known as vitamin K deficiency bleeding of the newborn (American Academy of Pediatrics Committee on Fetus and Newborn, 2003). Vitamin K prophylaxis at birth is therefore a standard practice in many countries (American Academy of Pediatrics Committee on Fetus and Newborn, 2003). The link between vitamin K intake and bone mineral density, on the other hand, is still debated (Booth et al., 2000; Rejnmark et al., 2006). Phylloquinone is synthesized exclusively by plants, green algae, and some species of cyanobacteria that use it as an electron carrier in photosystem I and for protein disulfide bond formation (Brettel et al., 1986; Furt et al., 2010). Red algae,

diatoms, the cyanobacterium *Gloeobacter violaceus*, as well as many bacterial and archaeal species synthesize a related form called vitamin K₂ or menaquinone [2-methyl-3-(all-*trans*-polyprenyl)-1,4-naphthoquinone], the side chain of which is fully saturated and can vary in length between species.

The vitamin K biosynthetic pathway consists of two separated metabolic branches: one for the polyprenyl moiety and the other for the naphthoquinone ring (see Supplemental Figure 12 online). In plants, the phytol diphosphate precursor originates from the methylerythritol-phosphate pathway and is common to the biosyntheses of tocopherol and chlorophyll. The immediate precursor of the naphthoquinone ring is chorismate. It is first converted to isochorismate to serve as a substrate for a multifunctional enzyme, termed PHYLLLO, that catalyzes sequential steps of addition, elimination, and aromatization (Gross et al., 2006; Strawn et al., 2007; Garcion et al., 2008). PHYLLLO's product, *o*-succinylbenzoate (OSB), is then activated by ligation with CoA (Kim et al., 2008) and cyclized, yielding the CoA thioester of 1,4-dihydroxy-2-naphthoate (DHNA). DHNA-CoA is subsequently hydrolyzed and the freed DHNA is prenylated and methylated (Shimada et al., 2005; Lohmann et al., 2006; Kim et al., 2008). Two of these steps have not yet been characterized in plants: the cyclization of OSB-CoA catalyzed by DHNA-CoA synthase (OSB-CoA → DHNA-CoA) and the hydrolysis of DHNA-CoA catalyzed by DHNA-CoA thioesterase (DHNA-CoA → DHNA). Homology searches indicate that plants possess orthologs of prokaryotic DHNA-CoA synthase but that DHNA-CoA thioesterase is missing outside the red algal and diatom lineages (Widhalm et al., 2009). All the plant vitamin K₁ biosynthetic enzymes characterized so far have been shown to occur in plastids (Shimada et al., 2005; Gross et al., 2006; Lohmann et al., 2006; Strawn et al., 2007; Garcion et al., 2008; Kim et al., 2008). However, the apparent plastid-exclusive localization of the vitamin K₁ biosynthetic pathway was recently questioned by proteomic and green fluorescent protein fusion studies that demonstrated that OSB-CoA ligase and homologs of DHNA-CoA synthase occur in peroxisomes (Babujee et al., 2010). To date, there have not been any engineering or breeding attempts *sensu stricto* to modify vitamin K₁ status in plants. However, while engineering salicylate biosynthesis in tobacco, it has been observed that overexpression of a bacterial isochorismate synthase targeted to plastids led to a fourfold increase in vitamin K₁ content (Verberne et al., 2007). Functional complementation experiments in *Arabidopsis* showed that overexpression of demethylphyloquinone methyltransferase or OSB-CoA ligase did not significantly modify vitamin K₁ levels (Lohmann et al., 2006; Kim et al., 2008).

CURRENT AND FUTURE STRATEGIES TO ENHANCE VITAMIN CONTENT

The complete elucidation of the biosynthesis and regulation of vitamin pathways will be important for the manipulation of the nutritional quality of foodstuffs. While engineering vitamin pathways or vitamin-related metabolism is one avenue that is worthwhile to pursue, the exploitation of natural variation is another feasible approach for the development of crops containing

adequate, balanced levels of vitamins. For example, in the case of β -carotene, the visual change observed with increased content means that natural alleles can be observed easily among diverse accessions. To date, QTLs for β -carotene accumulation have been found in maize (Harjes et al., 2008), cassava (Welsch et al., 2010), and tomato (Lippman et al., 2007). These are important tools that contribute to enhanced provitamin A levels in crop plants. Interestingly, no transcription factors like those discovered and used for elevating flavonoids and anthocyanins (Butelli et al., 2008) have yet been found for carotenoids or their precursors. However, there is considerable variation in vitamin content in many crop species. For example, fruit vitamin C content of wild tomato species accessions examined ranges from ~10 to >500 mg per 100 g of fresh weight (Galiana-Balaguer et al., 2006), while in kiwifruit and related species, vitamin C ranges from 80 to 800 mg per 100 g of fresh weight (Bulley et al., 2009).

Strategies to exploit natural genetic variability of a given trait using molecular markers are now well established in plants and have been applied to some vitamins (A, C, and E) in a variety of species. In the case of vitamin C, QTL have been located on genetic maps using introgression line, advanced backcrossing, and recombinant inbred line populations, derived from crosses between a cultivated variety and other cultivars or wild related species in tomato (Rousseaux et al., 2005; Stevens et al., 2007), strawberry (Zorrilla-Fontanesi et al., 2011), and apple (*Malus domestica*; Davey et al., 2006). This approach can be applied to most species for which parental lines with differential vitamin content can be identified. A candidate gene approach or positional cloning can then be used to identify the polymorphic locus responsible for the vitamin variation. In tomato, one allelic form of monodehydroascorbate reductase, a gene involved in the ascorbate recycling pathway, was shown after fine mapping to cosegregate with a major vitamin C QTL and to explain >80% of the variation in vitamin C levels following storage (Stevens et al., 2007; Stevens et al., 2008). Once vitamin QTL have been located, their stability in various genetic backgrounds and environmental conditions can be assessed and molecular markers can be used to help their transfer to elite varieties.

As an alternative and complement to conventional QTL mapping, the association mapping approach that scans the genome for significant association between genetic markers and the trait studied (reviewed in Myles et al., 2009) might be used to discover new alleles controlling vitamin content. This requires a population of known structure, displaying wide natural genetic variation in the particular vitamin, as well as high-density molecular marker maps and well-annotated genomes, which are or will be soon available in several species. The introgression of useful traits from wild species into cultivated varieties may, however, pose specific problems, such as strong unbreakable linkages with many other undesirable traits.

Mutant collections, in which a very large genetic variability beyond the natural variation found in domesticated species can be induced in a cultivated variety (Menda et al., 2004), could provide a useful source of genotypes with enhanced vitamin content, such as in the case of vitamin C (Stevens et al., 2006). These collections can be further exploited by TILLING (Minoia et al., 2010). However, since most mutagenesis methods

produce loss-of-function mutants, the TILLING approach may be mostly adapted to known candidate genes that negatively regulate vitamin biosynthesis (e.g., the *AMR1* gene and vitamin C; Zhang et al., 2009). Map-based cloning approaches can also be applied after high-throughput phenotyping of mutant collections for vitamin content. Alternatively, in crop species with available mutant genetic resources, the development of next-generation mapping of unknown mutations, which is based on whole-genome sequencing and has been already developed in model species (Austin et al., 2011), becomes increasingly attractive for the discovery of new genes and allelic series enhancing vitamin content of food products. When plant fitness is not affected by the mutation, this approach is one of the most promising for the rapid generation of high-quality elite varieties with enhanced vitamin content. Indeed, in all cases, the consequences of manipulating the production of these compounds on the plant itself will need to be taken into account, and a compromise between a nutritionally rich and environmentally robust plant will need to be found. For example, the current status of vitamin E metabolism points to a fine equilibrium between effective control mechanisms within cells to prevent oxidative damage and the synthesis of molecules involved in the process that are also beneficial in terms of human nutrition. This could mean that approaches to improve plant resistance to oxidative stress may involve manipulating the levels of compounds that have a beneficial side effect of also improving the nutritional quality of crop plant products. In this context, the recent demonstration that tocochromanols are essential for maintaining the viability of *Arabidopsis* seeds (Mène-Saffrané et al., 2010) adds a new perspective to the importance of these molecules. Although further investigation is needed, plants engineered for optimal tocochromanol contents for seed storage, for example, would not only add nutritional value to foodstuffs but could also have a tremendous economic impact on crop production. As discussed elsewhere for other phytonutrients (Martin et al., 2011), availability of near-isogenic lines differing only by the content of a particular vitamin should help establish these properties. However, to achieve biofortification of multiple vitamins particularly in a local elite variety, it is likely that a combination of breeding and genetic engineering will be required given that breeding does not appear to be an adequate solution on its own (Naqvi et al., 2009). Engineering in plants will benefit from the development of multi-gene transfer and transformation strategies, such as large capacity T-DNA-based vectors, artificial chromosomes, or unlinked cotransformation approaches (Naqvi et al., 2010).

CONSIDERATIONS FOR ENGINEERING VITAMIN-BIOFORTIFIED CROPS

Arguably the most viable strategy for producing vitamin-biofortified crop foods is to target staple crops that are already widely produced and consumed by the target population and to target the organ or tissue that constitutes the edible portion of the plant (e.g., the endosperm of cereal grains, such as rice, wheat, and maize). A major consideration is then the targeted level of biofortification to be achieved for the vitamin(s) or micronutrient(s) in question, which depends on three main variables: the requirement of the targeted population for the target vitamin, the retention of the vitamin

following storage and processing or cooking, and the bioavailability of the vitamin (e.g., for human uptake and metabolism). The requirement of the targeted population for the target vitamin depends on how far below the recommended dietary allowance (RDA) or recommended dietary intake it lies and the upper level of safe intake (Table 1), which in turn relies on whether the biofortified food will be the primary source of the vitamin or secondary to other sources already prevalent in the local diet. Retention of the vitamin following storage and processing (e.g., cooking) of the biofortified plant is a critical consideration that varies widely depending on the vitamin and crop plant in question (Table 4). For example, retention of β -carotene in a variety of orange-fleshed sweet potato (*Ipomoea batatas*) averages 80% after cooking (Nestel et al., 2006). The third acknowledged variable is the bioavailability of the vitamin in the food source of the biofortified plant, which also varies widely depending on the vitamin and the form in which it is delivered, as discussed above. Fortunately, several *in vitro* models are available, such as the *in vitro* digestion model (Garrett et al., 1999; Reboul et al., 2006b) and the Caco-2 cell line (Reboul et al., 2007). The choice of the animal model depends on the studied vitamin because not all laboratory animals have a vitamin metabolism similar to humans. For example, ferrets are considered better than rats to extrapolate results on carotenoid absorption in humans (White et al., 1993). Finally, because vitamin bioavailability can be affected by food processing, bioavailability must be monitored on food products prepared in the same way as the target population's culinary habits.

Another challenge will be for nutritionists to determine the impact of a vitamin-biofortified plant on the dietary intake of the vitamin in the targeted population(s). There are several factors that can impact the vitamin intake from plants or plant-derived products. The first is the stability of the vitamin in the plant product. Indeed, numerous factors can affect vitamin shelf life (Table 4). The second factor is the bioavailability of the vitamin from the plant product. For example, some vitamins in plant-derived foods are less efficient than when provided in supplements (Castenmiller et al., 1999) or found in foods from animal origin (Chung et al., 2004). This is mainly due to the effect of the plant matrix, which can impair the release of some vitamins in the gut and thus diminish their absorption (Castenmiller et al., 1999; Borel, 2003). It should also be considered that some vitamins compete for absorption in the human body, for example, vitamin E and lutein (Reboul et al., 2006a), and vitamin E and vitamin D (Reboul et al., 2011).

Two recent clinical studies have shown that β -carotene-biofortified rice (Golden Rice), as well as β -carotene-biofortified maize, are effective sources of vitamin A (Tang et al., 2009; Li et al., 2010). However, the benefits to be derived from biofortification in a particular location will depend on (1) the amount of staple food consumed locally, (2) the prevalence of existing vitamin deficiencies, (3) the vitamin requirement as affected by daily losses of vitamin from the body (e.g., antioxidant vitamins such as vitamin C and E in people subjected to oxidative stress) and particular developmental stages, such as growth, pregnancy, and lactation (Table 3), as well as (4) genetic variations of individuals (Borel, 2011). The prevalence of infection and other micronutrient deficiencies in some developing countries may affect the ability of biofortified plants to improve the vitamin status.

It is also critical to assess whether or not the vitamin biofortified plant food is indeed able to improve the vitamin status (e.g., blood vitamin concentrations) and health (e.g., resistance to diseases) in targeted populations. Some attempts have been made to evaluate the health impact of some vitamin-biofortified plants. For example, folate-biofortified rice was evaluated by applying the disability adjusted life years (DALYs) method (i.e., the number of DALYs lost). This number equals the sum of the “years lived with disability” and “years of life lost.” According to low- and high-impact scenarios, it was calculated that folate-biofortified rice in China would save between 116,090 and 257,345 DALYs per year (De Steur et al., 2010). Similarly, it was estimated that the use of Golden Rice in India might save 1.38 million DALYs per year, under the highest impact scenario (Qaim, 2010). Indeed, the cost-effectiveness of Golden Rice intervention was compared with other vitamin A interventions. It was calculated that the cost per DALY saved was (U.S.\$) 3.1, 134, and 84 through Golden Rice, supplementation (pills), and industrial fortification (food fortificants), respectively (Qaim, 2010).

A final important issue is to what extent the target population will accept the food source(s) of the biofortified plant. Acceptance of a new food depends on a range of factors, including the cost compared to similar food sources, the taste, and the shelf life. For example, the acceptance of biofortified plants with visible traits (e.g., yellow maize rich in β -carotene) requires that consumers accept the color change. This is not obvious, for example, in some areas of Africa where there is a well-established strong cultural consumer preference for white maize for human consumption (Nestel et al., 2006). However, carotenoids are an exception in this regard as most other vitamins are colorless and flavorless at the levels needed in the diet to provide nutritional sufficiency, and their engineering/breeding for adequate levels in staple crops should not impact taste or consumer preferences. From an economic perspective, the use of biofortified plants has been estimated to be more cost-effective than other strategies (i.e., supplementation and industrial fortification) (see example for vitamin A above; Qaim, 2010). Indeed, even though the initial investments are high to produce them and to test them, for example, on the bioavailability of the vitamins, the recurrent costs are assumed to be low because biofortified plants will spread through existing formal and informal distribution channels and can be produced by farmers themselves. Conversely other strategies (e.g., vitamin pills and food fortificants) will need to reach the target population in remote rural areas, which require large investments and monitoring on a regular basis. The success of the HarvestPlus initiative (<http://www.harvestplus.org>), whose vision is to reduce micronutrient deficiency through biofortification of staple crops, is proving the sustainability and cost-effectiveness of this targeted approach.

CONCLUSIONS

We believe that there is an obvious and important role for plant science in addressing nutritional vitamin deficiencies on a global scale, especially in at risk populations in developing countries. The potential for plant sciences to make a meaningful difference in the daily lives of billions of individuals on the planet is now within our grasp. For certain vitamins, namely A, B, and E, viable strategies to enhance the overall levels of these metabolites in

plants have been presented; furthermore, results from forward genetic screens for at least a subset of these vitamins look promising. Another reason for optimism is the fact that, unlike a decade ago, our understanding of the plant biosynthetic pathways for all vitamins is relatively complete. It is our hope that the coming decade will continue to see advancements in the basic science of vitamin biosynthesis in plants but that this also be married to a global effort to move and deploy this knowledge into major crop plants used as primary staples in developing countries. In moving forward, it is imperative that the problem of dietary vitamin deficiency be tackled in a cross-disciplinary manner, including collaboration between plant scientists, nutritionists, politicians, national and international funding agencies, and educators.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Biosynthetic Pathway of Vitamin A.

Supplemental Figure 2. Biosynthetic Pathway of Vitamin B₁.

Supplemental Figure 3. Biosynthetic Pathway of Vitamin B₂.

Supplemental Figure 4. Biosynthetic Pathway of Vitamin B₃.

Supplemental Figure 5. Biosynthetic Pathway of Vitamin B₅.

Supplemental Figure 6. Biosynthetic Pathway of Vitamin B₆.

Supplemental Figure 7. Biosynthetic Pathway of Vitamin B₈.

Supplemental Figure 8. Biosynthetic Pathway of Vitamin B₉.

Supplemental Figure 9. Biosynthetic Pathway of Vitamin C.

Supplemental Figure 10. Biosynthetic Pathway of Vitamin D.

Supplemental Figure 11. Biosynthetic Pathway of Vitamin E.

Supplemental Figure 12. Biosynthetic Pathway of Vitamin K.

Supplemental Table 1. A Comparison of the Amounts of Vitamins in White versus Brown Rice.

Supplemental Table 2. A Comparison of the Amounts of Vitamins in Four Main Vegetable Sources.

Supplemental Table 3. A Comparison of the Amounts of Vitamins in Four Main Fruit Sources.

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