Does Abiotic Stress Cause Functional B Vitamin Deficiency in Plants?[1][OPEN]

Andrew D. Hanson*, Guillaume A. Beaudoin, Donald R. McCarty, and Jesse F. Gregory III

Horticultural Sciences Department (A.D.H., G.A.B., D.R.M) and Food Science and Human Nutrition Department (J.F.G.), University of Florida, Gainesville, Florida 32611-0690

ORCID IDs: 0000-0003-2585-9340 (A.D.H.); 0000-0001-8694-5117 (D.R.M.).

B vitamins are the precursors of essential metabolic cofactors but are prone to destruction under stress conditions. It is therefore a priori reasonable that stressed plants suffer B vitamin deficiencies and that certain stress symptoms are metabolic knock-on effects of these deficiencies. Given the logic of these arguments, and the existence of data to support them, it is a shock to realize that the roles of B vitamins in plant abiotic stress have had minimal attention in the literature (100-fold less than hormones) and continue to be overlooked. In this article, we therefore aim to explain the connections among B vitamins, enzyme cofactors, and stress conditions in plants. We first outline the chemistry and biochemistry of B vitamins and explore the concept of vitamin deficiency with the help of information from mammals. We then summarize classical and recent evidence for stress-induced vitamin deficiencies and for plant responses that counter these deficiencies. Lastly, we consider potential implications for agriculture.

The great pioneers of modern plant physiology pointed out that B vitamins have much in common with hormones and even classified some vitamins as plant hormones (i.e. plant growth regulators in today's terms; Went et al., 1938; Bonner and Bonner, 1948; Thimann, 1963). In plants, as in animals, B vitamins and hormones are biologically active in minute amounts, are transported, and lead to similarly profound consequences when deficient (Bonner and Bonner, 1948). B vitamins and hormones might consequently be expected to have received comparable research attention. This has broadly been the case in biomedical research since vitamins and hormones were first discovered (Kohler, 1975). It has almost never been the case in any area of plant research, including abiotic stress. Thus, in the Arabidopsis (*Arabidopsis thaliana*) abiotic stress literature, articles involving hormones outnumber those involving B vitamins by a factor of 100 (Supplemental Table S1).

This stunning disparity suggests two things about past and present thinking in the abiotic stress field. First, it indicates a prevalent default assumption that the whole of B vitamin metabolism always and everywhere continues to work well in stressed plants and so can be safely ignored. Second, it signals a lack of attention to the possibility that stress-induced defects in B vitamin metabolism can be intermediate causes of system-wide plant stress responses. Neither the default assumption nor the inattention is reasonable a priori and they are not justified by the available evidence.

This review sets out to show that B vitamin deficiency is a simple, probable, but overlooked scenario in abiotic stress responses. We begin by providing key background information on the chemical and metabolic lability of B vitamins and the cofactors derived from them and on the

ADVANCES

- Recent research and the classical literature on "the chemical cure of climatic lesions" converge to indicate that stressed plants can develop functional B vitamin deficiencies.
- Fully appreciating the lability and metabolic centrality of B vitamins and cofactors makes clear that they are crucial stress-sensitive nodes in pathway networks that cells must seek to protect.
- Protection strategies include repairing or recycling damaged vitamins and cofactors, increasing biosynthesis by up-regulating biosynthesis gene expression, and probably mobilizing reserves.
- Thiamin is the most metabolically labile B vitamin and the only one whose synthesis depends on suicidal enzymes, implying that thiamin pools are doubly difficult to maintain in stress conditions.

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* Address correspondence to adha@ufl.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Andrew D. Hanson (adha@ufl.edu).

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concept of vitamin deficiency derived from work in animals. We then revisit classical studies that point clearly to roles for B vitamins in abiotic stress, but are no longer in the modern canon, and assess recent evidence for such roles. Next, we outline how plants combat stress-induced B vitamin deficiencies. Lastly, we consider how understanding stress-induced vitamin deficiency could inform crop breeding and management.

Two points should be made at the outset. First, there has been much research on the B vitamin contents of food plants and on B vitamin synthesis and metabolism in plants. However, the main, if not sole, driver for this valuable work has been plants as vitamin sources for humans rather than plants as vitamin sources for themselves (Fitzpatrick et al., 2012; Gerdes et al., 2012). This article takes the complementary position that "plants need their vitamins too" (Smith et al., 2007). Second, research on vitamin C (ascorbate) shows that plant stress biology does not invariably sideline vitamins (Foyer and Noctor, 2011). This article argues that B vitamins deserve as much attention as vitamin C.

CHEMICAL AND METABOLIC LABILITY: WHY BAD THINGS HAPPEN TO GOOD VITAMINS

B vitamins are indispensable because they are the metabolic precursors of essential cofactors. Figure 1 shows the seven B vitamins found in plants, along with the cofactors to which they are converted. In brief: Thiamin (vitamin B_1) is converted to thiamin diphosphate; riboflavin (vitamin B_2) to FMN and FAD; niacin (vitamin B_3) to NAD(P)$^+$; pantothenate (vitamin B_5) to coenzyme A and the prosthetic group of acyl carrier protein; pyridoxine (vitamin B_6) to pyridoxal 5′-phosphate; biotin (vitamin B_8) to the biotinyl side chain of enzymes; and tetrahydrofolate (historically termed vitamin B_9) to various one-carbon substituted folates and their polyglutamylated derivatives.

Cofactors function by participating in biochemical reactions, e.g. thiamin diphosphate forms covalent complexes with carbonyl substrates, FAD and NAD(P)$^+$ become reduced as substrates are oxidized, and coenzyme A forms thioesters with acyl groups. Cofactors and their vitamin precursors are thus chemically reactive by nature. Their chemically reactive groups are therefore prone to undergo spontaneous side-reactions such as oxidation, hydrolysis, racemization, or addition that damage or destroy the molecule, and stress conditions promote such side-reactions (Piedrafita et al., 2015). For example, most abiotic stresses cause accumulation of reactive oxygen species (You and Chan, 2015) that can inflict oxidative damage on every compound in Figure 1. Similarly, temperature extremes, high light levels, pH excursions, and stress-driven accumulations

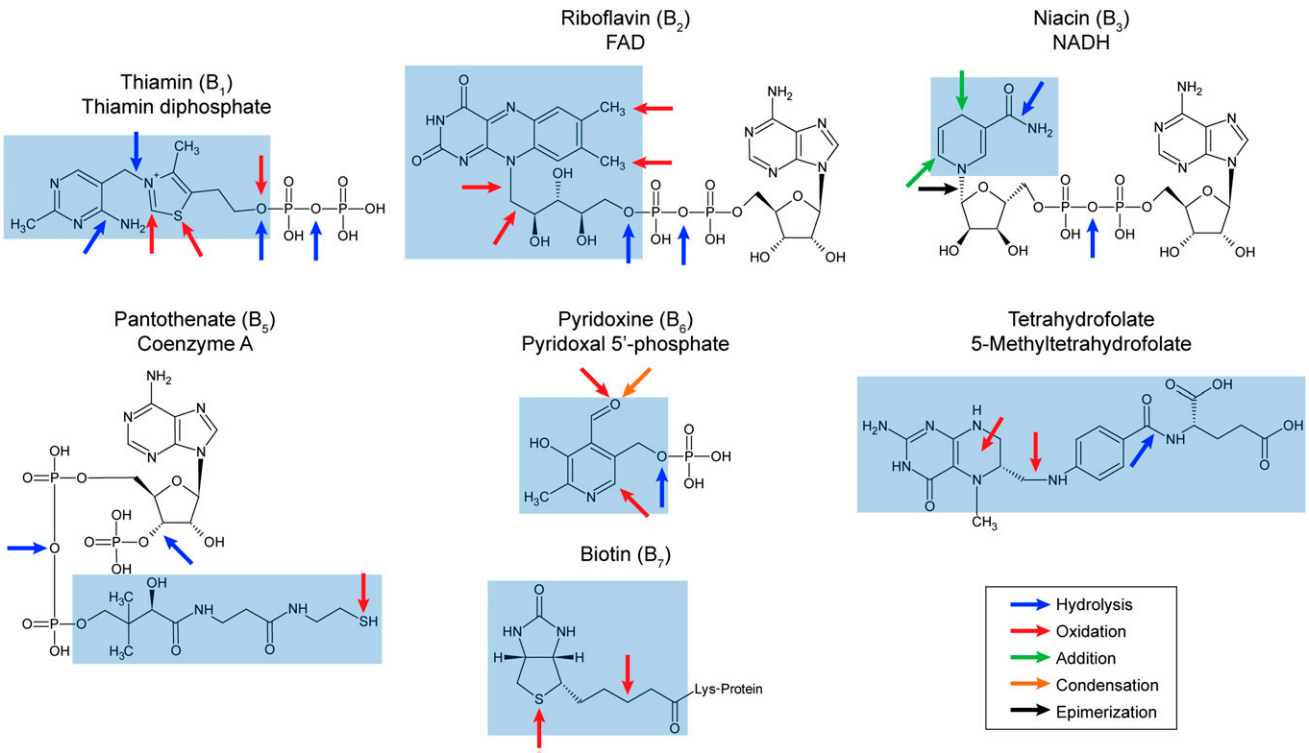


Figure 1. Structures and damage reactions of the seven B vitamins found in plants and of representative cofactors derived from them. The vitamin moieties are highlighted in blue. Note that folates generally have a short γ-linked poly-Glu chain attached to the glutamyl moiety. Color-coded arrows show the site and nature of spontaneous chemical or enzymatic damage reactions that each vitamin/cofactor can undergo in vivo. The damage reactions involved are documented in Table I.

of metabolites with which cofactors react all directly accelerate diverse types of spontaneous cofactor damage (Treadwell and Metzler, 1972; Baggott, 2000; Mills et al., 2006; Marbaix et al., 2011). Abiotic stresses can also promote cofactor damage indirectly by altering compartmentation (Akhtar et al., 2010; Mohammadi et al., 2012) by inducing enzymes that break down cofactors (Rapala-Kozik et al., 2008; Higa et al., 2012) and by creating harsh cellular conditions in which enzymes that normally act on other substrates become more promiscuous (Piedrafita et al., 2015) and mistakenly attack cofactors.

Figure 1 uses color-coded arrows to show the site and nature of damage reactions that vitamins and cofactors can undergo in physiological conditions, and Table I catalogs the reactions corresponding to the arrows. Note that Figure 1 is bristling with arrows, which helps explain why most of the B vitamins/cofactors made it into a "Top 30" list of damage-prone metabolites (Lerma-Ortiz et al., 2016) and why all organisms have dedicated systems to deal with vitamin/cofactor damage (Linster et al., 2013). "Bad things happen" to B vitamins and cofactors because these compounds are basically chemical and metabolic "accidents waiting to happen." Also, unlike many other reactive metabolites, cofactors are end-products, not short-lived intermediates that are quickly converted to something else. The longer an end-product lives, the higher the probability of its getting damaged at some point.

Thiamin diphosphate deserves special mention for metabolic lability because, unlike other cofactors, it can be damaged during the catalytic cycle (catalysis-induced inactivation; McCourt et al., 2006). Oxygen-dependent side-reactions in enzyme active sites convert the thiamin diphosphate cofactor to an inactive thiazolone derivative (Sümegi and Alkonyi, 1983; Bunik et al., 2007). Such use-dependent damage explains why thiamin is the only B vitamin whose dietary requirement in animals is proportional to nonfat energy intake (McCourt et al., 2006). More generally, the damage reactions undergone by all B vitamins account for their dietary requirements; if vitamins were not continuously damaged, they would last forever and not need to be constantly resupplied. In this context, it is noteworthy that the whole-body half-life of thiamin (and its phosphates) in humans is one to two orders of magnitude shorter than those of other B vitamins for which estimates are available, as follows: thiamin, 9.5 to 18.5 d (Ariaey-Nejad et al., 1970); folates, 60 to 150 d (Gregory and Quinlivan, 2002); vitamin B_6, ~500 d (Coburn, 1990); vitamin B_{12} (absent from plants), 480 to 1284 d (Hall, 1964).

Table I. *Chemical and enzymatic damage reactions of B vitamins and the corresponding cofactors*

Vitamin/Cofactor	Reactions	C/E[a]	References[b]
Thiamin (vitamin B_1)/thiamin diphosphate	Pyrimidine ring deamination	C	Windheuser and Higuchi (1962)
	Thiazole ring oxidation	C,E	Bunik et al. (2007); Dwivedi and Arnold (1973)
	Thiazole ring breakdown	C	Jenkins et al. (2007)
	Oxidation to thiamin acetate	E	Dalvi et al. (1974)
	Hydrolysis to thiazole + pyrimidine	E	Jurgenson et al. (2009)
	Dephosphorylation	E	Rapala-Kozik et al. (2009)
	Thiamin triphosphate formation	E	Linster et al. (2013)
Riboflavin (vitamin B_2)/FMN, FAD	(Photo)oxidative flavin ring loss	C	Choe et al. (2005)
	(Di)phosphate bond hydrolysis	E	Ogawa et al. (2008); Rawat et al. (2011)
	7α- and 8α-hydroxylation	E	Ohkawa et al. (1983)
	Cyclic FMN formation from FAD	C,E	Pinto et al. (1999); Sánchez-Moreno et al. (2009)
Niacin (vitamin B_3)/NAD(P)(H)	Hydration of nicotinamide ring	C,E	Marbaix et al., (2011)
	Epimerization of β- to α-NAD(P)H	C	Oppenheimer and Kaplan (1975)
	Hydrolytic loss of nicotinamide ring	E	Everse et al. (1975)
	Diphosphate bond hydrolysis	E	Ogawa et al. (2008)
	Nicotinamide ring addition reactions	C	Everse et al. (1971)
Pantothenate (vitamin B_5)/ coenzyme A, ACP[c]	Oxidations of the thiol group	C	Huang et al. (2016)
	Diphosphate bond hydrolysis	E	Ogawa et al. (2008)
	Hydrolysis of the 3′ phosphate group	E	Paizs et al. (2008)
Pyridoxine (vitamin B_6)/pyridoxal 5′-phosphate	Aldehyde group oxidation	C/E	Gerdes et al. (2012)
	Aldehyde group condensations	C	Dalling et al. (1976)
	Dephosphorylation	E	Gerdes et al. (2012)
	6-Hydroxylation	C	Tadera et al. (1986)
Biotin (vitamin B_8)/biotinylated enzymes	Oxidation to biotin sulfoxide	C	Melville (1954)
	Side chain β-oxidation	E	Izumi et al. (1973)
Tetrahydrofolate/C1-substituted folates	Oxidative cleavage of C_9-N_{10} bond	C	Gregory (1989)
	Pteridine ring oxidation	C/E	Noiriel et al. (2007)
	5-Formyltetrahydrofolate formation	C/E	Baggott (2000); Goyer et al. (2005)
	γ-Glutamyl bond hydrolysis	E	Orsomando et al. (2005); Bozzo et al. (2008)

[a]C, chemical (i.e. nonenzymatic, spontaneous) reaction; E, enzymatic reaction or side-reaction. [b]Certain reactions have so far been reported only from animals or microbes. [c]ACP, acyl carrier protein, which has a bound 4′-phosphopantetheinyl prosthetic group derived from coenzyme A.

WHAT ACTUALLY CONSTITUTES B VITAMIN DEFICIENCY?

The concept of vitamin deficiency comes from human and animal nutrition; it refers to the consequences of a shortfall in the dietary supply of a specific vitamin. Because plants make their own B vitamins, and indeed are the ultimate source of most of the B vitamins consumed by animals, the concept of "B vitamin deficiency" might seem inapplicable to plants, and hence irrelevant. But, as we will show, this concept is actually very relevant to plants.

Key questions about B vitamin nutrition in animals are: What dietary intake of each vitamin is needed for optimal growth and health? Also, what happens when the supply of a vitamin falls below the optimal level? Both questions are often answered by measuring weight gain in young animals given various vitamin doses in the diet and by tracking levels of the vitamin and its corresponding cofactor in tissues and organs. Figure 2 (left half) summarizes data from such studies, in which various levels of thiamin, pyridoxine, or folate were supplied to rats whose growth and liver cofactor levels were monitored. Two points that emerge from these data are as follows: (1) There is a continuum rather than a sharp divide between vitamin sufficiency and deficiency, and (2) the cofactor level in liver does not have to fall much before growth is substantially impacted (as are development, metabolic functions, and behavior; data not shown), and declines of 30 to 60% from optimal are devastating. Animals therefore operate their vitamin and cofactor systems with rather narrow margins of safety.

The same appears to be true of plants. Nutritional trials analogous to those above have not yet been conducted using totally vitamin-deficient mutants. However, mutant plants partially deficient in thiamin or vitamin B_6 have been studied (Woodward et al., 2010; Rueschhoff et al., 2013), as have cultured cells depleted in folate using a reasonably specific antifolate drug (Loizeau et al., 2008; Fig. 2, right half). Although these experiments produced a single deficient state, not a range as in rats, the data clearly suggest that a 25 to 40% loss of cofactor leads to severe consequences. To summarize: The red zone on the B vitamin fuel gauge seems to be in roughly the same place in plants and animals and is quite close to the full mark.

A subsidiary concept within vitamin deficiency is "functional vitamin deficiency," wherein vitamin and cofactor measurements need not show marked depletion but strong metabolic disturbances nevertheless ensue. A prime example is vitamin B_{12} (absent from plants), whose deficiency in humans is better diagnosed by its metabolic consequences (elevated levels of methylmalonic acid and homo-Cys) than by measuring B_{12} itself (Stabler et al., 1996). Another example is folate, for which, in contrast to B_{12}, deficiency causes elevation only of homo-Cys and not of methylmalonic acid (Green, 2008). Functional deficiencies of other B vitamins in humans generally affect enzymes having the weakest affinity for their cofactor relative to the cofactor's intracellular concentration, as is the case for vitamin B_6 (Ueland et al., 2015). However, the hierarchy of biochemical reactions most susceptible to deficiency of B vitamins has not been fully clarified.

Functional vitamin deficiencies also can arise when the deficiency occurs in a particular (inaccessible) tissue or compartment but does not affect the whole organism, or at least the part convenient for assessing vitamin or cofactor status. Such a situation could develop in roots for thiamin and niacin because roots of certain species import these vitamins from shoots (Bonner and Bonner, 1948). Intracellular deficiencies are also possible given that B vitamins and cofactors are synthesized and used in different compartments. For example, thiamin diphosphate is made in the cytosol but used mainly in plastids and mitochondria (Rapala-Kozik et al., 2012), and vitamin B_6 is made in the cytosol but used throughout the cell (Fitzpatrick, 2011). In this connection, note that the plastids of the vitamin B_6 deficient Arabidopsis mutant in Figure 2 were far more severely deficient in B_6 than the leaf as a whole (Rueschhoff et al., 2013) and that a thiamin-requiring tobacco (*Nicotiana sylvestris*) mutant appeared to suffer primarily from thiamin deficiency in chloroplasts (McHale et al., 1988).

EVIDENCE FOR STRESS-INDUCED B VITAMIN DEFICIENCY

The previous sections explained that B vitamins and cofactors are chemically and metabolically unstable, that stresses potentially make them even more unstable, and that modest falls in cofactor levels slow growth. We might therefore predict that (1) abiotic stresses cause vitamin and cofactor deficiencies, (2) the deficiencies degrade plant performance, and (3) supplementing stressed plants with the deficient vitamin(s) improves performance. Evidence from both classical and modern work indicates that all these things happen.

Classical Evidence

The classical evidence was considered in a short 1957 article with the evocative title "The Chemical Cure of Climatic Lesions" (Bonner, 1957). The idea captured in the title was that negative effects of "climatic lesions" (i.e. physicochemical environmental stresses) could be "cured" by applying vitamins or other essential metabolites, singly or in mixtures. "Chemical cures" by B vitamins were reported for various plants and various abiotic stresses during the 1940s to 1960s; typical data are shown in Figure 3. In each case, applying a vitamin or vitamin mixture promoted growth in unfavorable conditions but not in favorable ones. These results are de facto confirmation that stress can lead to functional

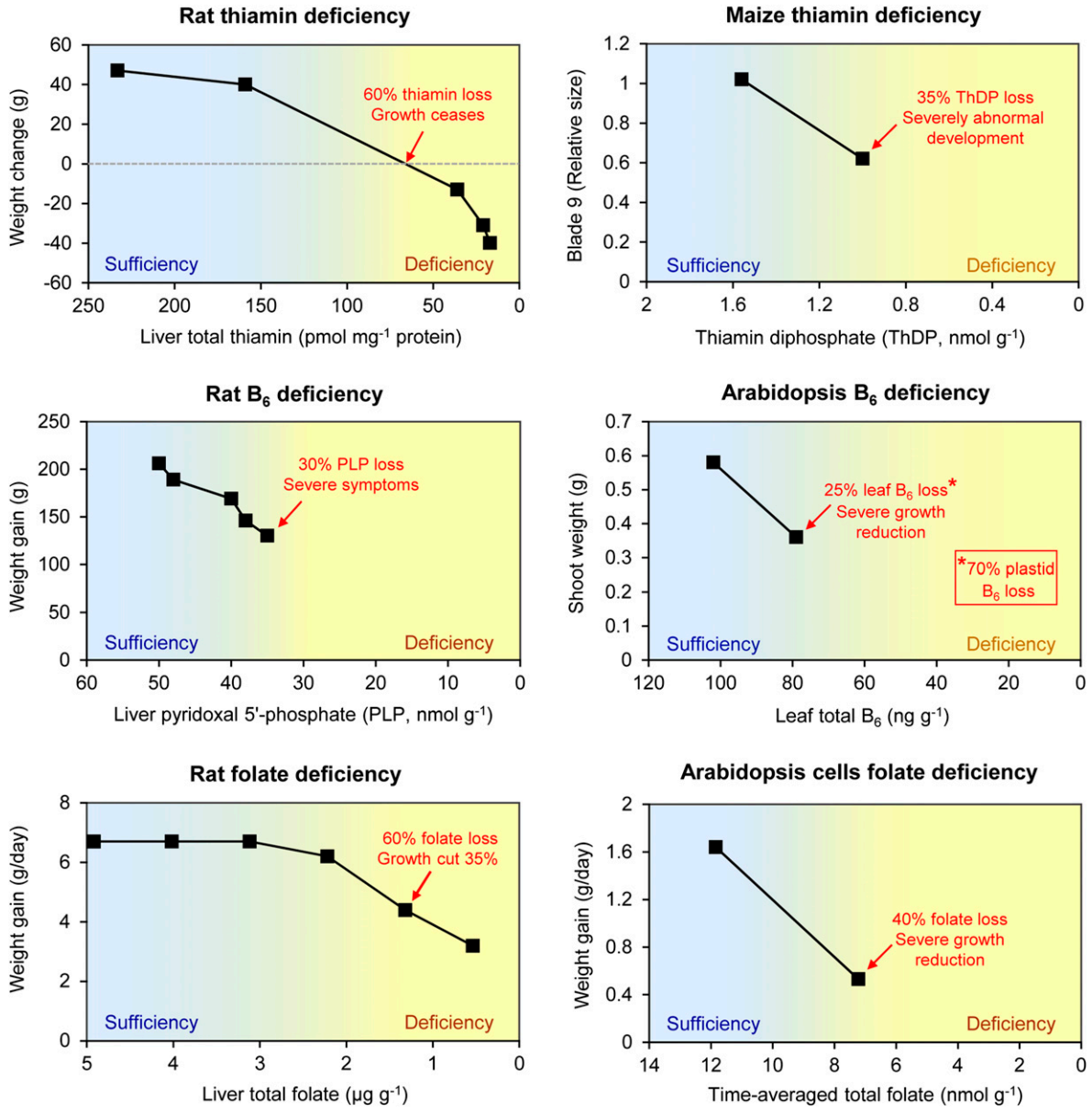


Figure 2. Comparing growth responses to deficiencies of thiamin, B_6, or folate in rats versus plants. The decline from vitamin sufficiency to deficiency is approximated by a color gradient. For rats, deficiencies were obtained by varying dietary vitamin content; liver was used to assess vitamin status. For plants, the experiments involved vitamin deficient mutants (*blk-1* for thiamin in maize; *pdx1.3* for B_6 in Arabidopsis) or cell cultures treated with an antifolate drug for folate in Arabidopsis. Whole plants, leaves, or cells were used to assess vitamin status. Data sources: rat thiamin (Rains et al., 1997), rat B_6 (Mackey et al., 2003; Scheer et al., 2005), rat folate (Clifford et al., 1993), maize thiamin (Woodward et al., 2010), Arabidopsis B_6 (Rueschhoff et al., 2013), and Arabidopsis folate (Loizeau et al., 2008).

B vitamin deficiency. Importantly, the Arabidopsis work in Figure 3 gave stress-induced vitamin deficiency a genetic basis by defining ecotypic differences attributable to one or a few genes (Langridge and Griffing, 1959). This is the pattern expected if the enzymes that use a particular cofactor bind to that cofactor with different strengths. When the vitamin starts to run out and cofactor levels fall, the weakest binder loses activity first, and small allelic differences in the affinity of this enzyme for the cofactor become critical determinants of performance of the organism as a whole (Guenther et al., 1999).

Modern Evidence

Although the exogenous application approach ("spray and pray") has fallen out of fashion, it can be perfectly valid, and beneficial effects of applying B vitamins, particularly thiamin, to stressed crop species continue to be reported, e.g. for salinized wheat

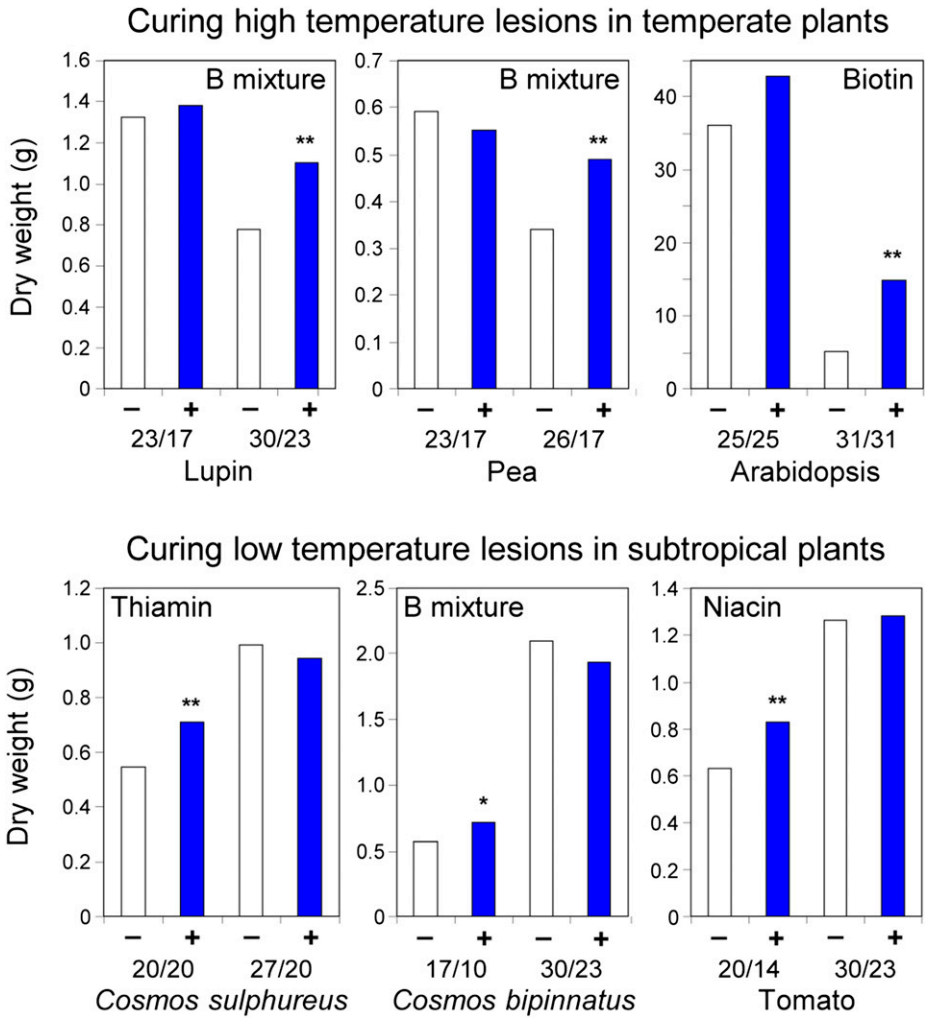


Figure 3. Classical data on the chemical cure of high- or low-temperature growth lesions. Day/night temperatures are in °C. **+**, Vitamin(s) added; **–**, no vitamin; differences significant at $*P < 0.05$ and $**P < 0.01$. The data are for species of the ornamental plant *Cosmos* (Bonner, 1943), for Arabidopsis (Langridge and Griffing, 1959), and for other species (Ketellapper, 1963).

(*Triticum aestivum*), sunflower (*Helianthus annuus*), and maize (*Zea mays*; Al-Hakimi and Hamada, 2001; Sayed and Gadallah, 2002; Tuna et al., 2013; Kaya et al., 2015). Also, a rigorous Arabidopsis study showed that thiamin enhances tolerance to oxidative stress imposed by paraquat (Tunc-Ozdemir et al., 2009). Such protective effects of thiamin on stressed plants are generally interpreted in terms of the antioxidant properties of thiamin and its diphosphate (Lukienko et al., 2000), but the in vivo relevance of these properties has been questioned (Lesgards et al., 2005), and it is not clear that the observed protection is due to direct antioxidant effects (Asensi-Fabado and Munné-Bosch, 2010). The experimental data are equally compatible with the protection being due to relief of a functional deficiency of thiamin diphosphate and the consequent restoration of normal metabolic fluxes. Although bias from prior positive reports may be at work, it is interesting that thiamin features so prominently in the literature on vitamin application, given that thiamin and thiamin diphosphate are particularly labile in vivo (see above). It is also interesting in the light of the extremely high energetic cost and inefficiency of thiamin biosynthesis (Box 1), to which we will return later.

Further modern support for stress-induced deficiency of four different B vitamins has come from molecular genetics, as follows. (1) Pyridoxine: The Arabidopsis *SOS4* (*SALT OVERLY SENSITIVE4*) gene for pyridoxal kinase was cloned via the salt-sensitive phenotype of *sos4* mutants, which was reverted by pyridoxine (Shi et al., 2002), a textbook "cure" of a stress lesion. Arabidopsis mutants in the *PDX1.3* gene for pyridoxal 5′-phosphate synthase were found to be hypersensitive to salt, osmotic, and oxidative stress (Chen and Xiong, 2005; Titiz et al., 2006). (2) Pantothenate/coenzyme A: The Arabidopsis *HAL3A* (*HALOTOLERANCE DETERMINANT 3A*) gene, first identified for its relation to stress tolerance (Espinosa-Ruiz et al., 1999), was shown to code for a key enzyme in pantothenate conversion to coenzyme A (Kupke et al., 2001), and *HAL3A* overexpression increased salt tolerance (Espinosa-Ruiz et al., 1999; Yonamine et al., 2004). (3) Folate: Ablation of the Arabidopsis gene encoding a cytosolic folate synthesis enzyme (HPPK/DHPS) specifically impacted salt and oxidative stress

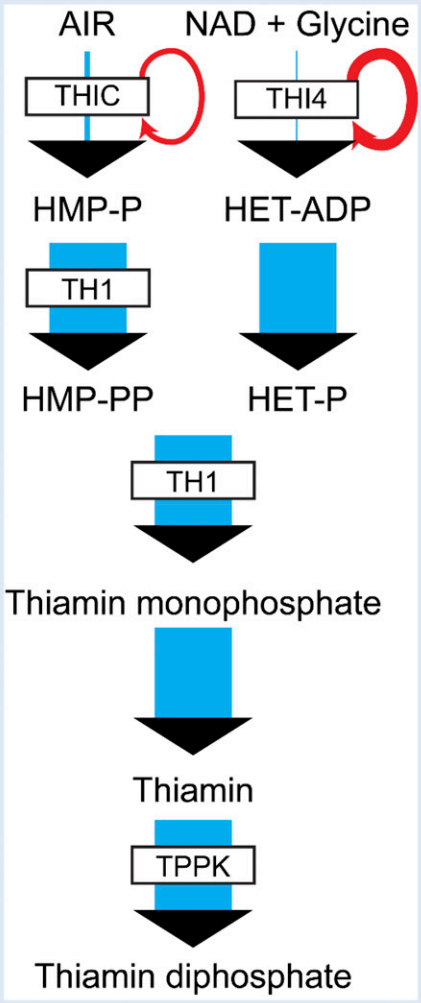
Box 1. Thiamin Biosynthesis: A High-Cost, High-Risk Operation

Enzymes all have finite life spans, but some have much shorter life spans than others. These include so-called "suicide enzymes" in which a residue is irreversibly altered after just one turnover, as well as enzymes employing reactive radicals as intermediates, e.g. radical *S*-adenosyl-Met enzymes, which typically catalyze fewer than a dozen turnovers in vitro (Broderick et al., 2014). Enzymes with short life spans are energetically costly because they have to be replaced so often.

Thiamin is much more energetically costly to produce than other vitamins because its first biosynthetic enzymes, THI4 and THIC, have extremely short life spans. THI4 (also called THI1) synthesizes the thiazole moiety of thiamin (see fig.). The sulfur atom in the thiazole ring comes from a conserved Cys in THI4 whose loss permanently inactivates the protein, making THI4 a suicide enzyme (Chatterjee et al., 2013). THIC synthesizes the pyrimidine moiety of thiamin. THIC is a radical *S*-adenosyl-Met enzyme that may achieve no more than five catalytic turnovers in vitro (Palmer and Downs, 2013). Thus, THI4 and THIC mediate orders of magnitude fewer catalytic events during their lives than most other enzymes.

This catalytic inefficiency is manifested in vivo. In proteomic analyses of barley leaves, the THI4 and THIC proteins had the first and fifth highest turnover rates, respectively, of more than 500 proteins studied (Nelson et al., 2014). The half-lives of THI4 and THIC were just a few hours compared to days for most proteins.

Another consequence may be high vulnerability of the thiamin biosynthetic pathway to stress. Global responses to abiotic stress include shutting down protein synthesis in general (Dhindsa and Cleland, 1975; Good and Zaplachinski, 1994) and specifically inducing proteins involved in protein folding and processing (Liu and Howell, 2010). Such reprioritization could adversely impact the replacement of THI4 and THIC, without which thiamin synthesis cannot proceed.



Box 1 Figure. Catalytic longevity and protein turnover in thiamin synthesis. The number of catalytic turnovers that each pathway enzyme mediates during its lifetime (represented by the width of the blue reaction arrows) varies from thousands to <10 for THIC and one for THI4. The consequential rapid turnover of the THIC and THI4 proteins is schematized by red arrows. AIR, Aminoimidazole ribotide; HET, hydroxyethylthiazole; HMP, hydroxymethylpyrimidine; PP, diphosphate.

resistance at germination (Storozhenko et al., 2007; Navarrete et al., 2012). (4) Riboflavin: The Arabidopsis *phs1* (*PHOTOSENSITIVE1*) mutant, first identified by its sensitivity to high-light stress (Ouyang et al., 2010), was found to lack a functional domain of the riboflavin biosynthesis enzyme PyrR (Hasnain et al., 2013; Frelin et al., 2015). Note the pattern here: Either a gene identified via a stress-sensitive phenotype turned out to be a B vitamin or cofactor synthesis enzyme or ablating a known B vitamin/cofactor enzyme caused stress sensitivity.

COUNTERING STRESS-INDUCED B VITAMIN DEFICIENCY

Conceptually, resource deficiencies can be countered by (1) maintaining and then mobilizing reserves, (2) obtaining more of the resource, (3) repairing or recycling it, or (4) using less of it. Plants certainly deploy strategies 2 and 3 to confront B vitamin deficiency and potentially deploy the others.

Strategy 1: Storing and Mobilizing B Vitamins and Precursors

Storage forms of B vitamins have had little attention in the plant literature, in sharp contrast to storage forms of hormones (Ludwig-Müller, 2011; Piotrowska and Bajguz, 2011). Storage forms of vitamins and vitamin precursors, mainly glucosides and Glc esters, nevertheless exist and some can reach higher concentrations than the corresponding free forms (Gregory, 1998). The only B vitamin for which no storage forms have been reported is thiamin. Storage forms have not been analyzed in abiotic stress studies, perhaps because they are not commercially available as standards. Unfortunately, this means that the contribution of storage forms to B vitamin homeostasis during stress remains unresolved; however, this contribution could be large. The main storage forms are shown in Figure 4 and described below, starting with the two vitamins (B_6 and B_3) whose storage forms are reported to be more abundant than the free forms in certain species and tissues.

Pyridoxine (B_6)

The 5′-β-D-glucoside of pyridoxine, and other glycosides linked via the 4- or 5-hydroxymethyl groups, can contribute up to 75% of the total vitamin B_6 in plant tissues (Gregory, 1998), i.e. they can be the largest item in the B_6 budget. A glucosyltransferase responsible for pyridoxine-5′-β-D-glucoside synthesis has been detected in pea (*Pisum sativum*; Tadera et al., 1982); enzymatic hydrolysis of the glucoside has been shown in vitro (Yasumoto et al., 1976), so it can presumably be mobilized in vivo.

Niacin (B_3)

Nicotinic acid-*N*-β-D-glucoside and *N*-methylnicotinate (trigonelline) are widespread in plants and can be major metabolites of nicotinamide and nicotinate (Matsui et al.,

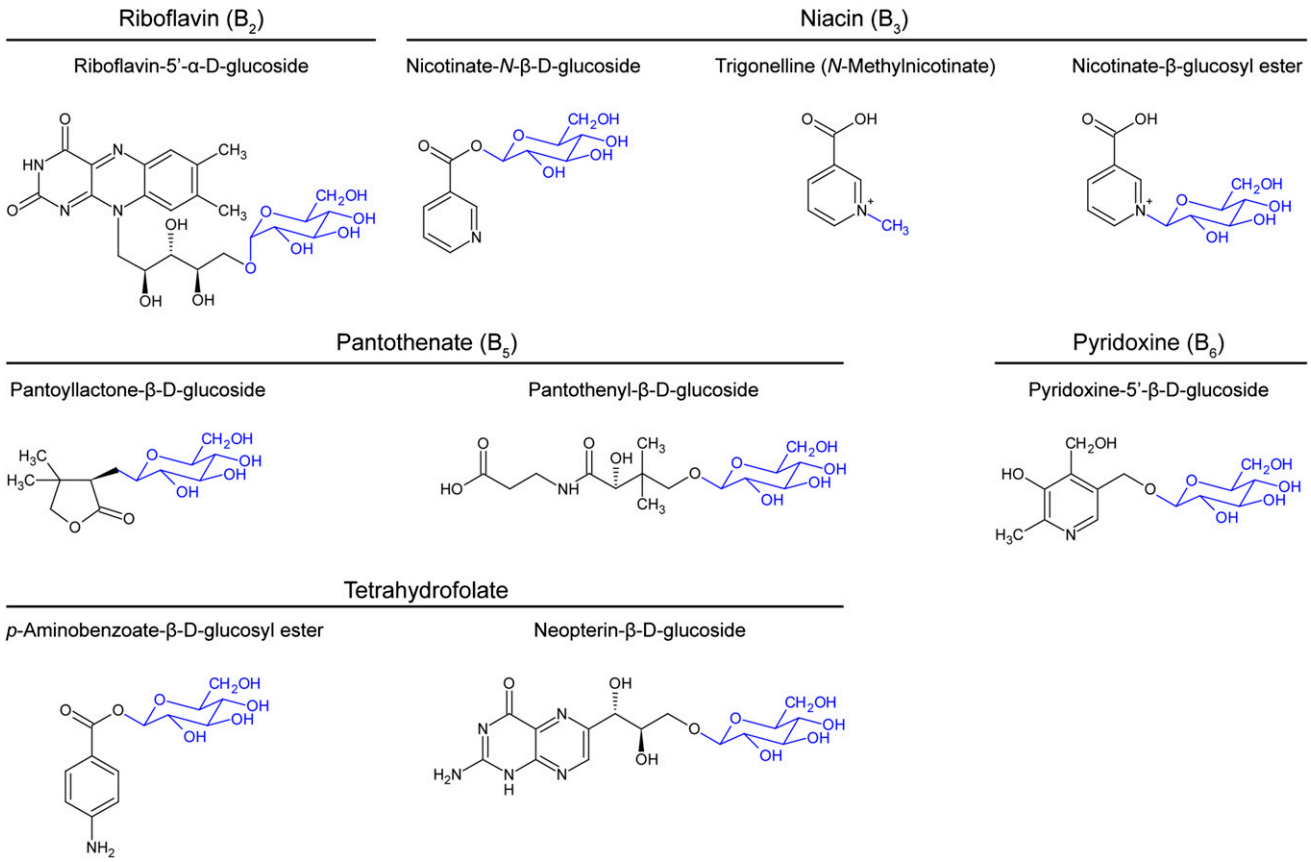


Figure 4. Structures of conjugated forms of B vitamins and their precursors that occur in plants. The molecules to which the vitamins and precursors are conjugated are colored blue.

2007; Ashihara et al., 2010); more complex glycosides also occur (Gregory, 1998). Enzymes that would allow the *N*-glucoside and trigonelline to act as mobilizable storage forms have been characterized (Upmeier et al., 1988; Shimizu and Mazzafera, 2000; Mizuno et al., 2014). Brassicaceae contain the β-D-glucosyl ester of nicotinic acid, which almost certainly acts as a mobilizable storage form (Li et al., 2015).

Pantothenate (B_5)

Pantothenate 4′-*O*-β-D-glucoside has been isolated from tomato fruit (*Solanum lycopersicum*; Amachi et al., 1971) and is most likely present in a wide range of species and tissues (Yoshizumi and Amachi, 1969). Glycosides of the lactone form of pantoate, the immediate precursor of pantothenate (pantoyllactone-β-D-glucopyranoside and pantoyllactone primeveroside) occur in rice (*Oryza sativa*) seedlings, the levels in coleoptile tissue being in the millimolar range (Menegus et al., 2002). These pantoate derivatives seem not to have been sought in any plant besides rice; they could conceivably be widespread.

Tetrahydrofolate Precursors

The precursor *p*-aminobenzoate was converted to its β-D-glucosyl ester by all tissues and species tested and was found to be the major endogenous form of *p*-aminobenzoate (Quinlivan et al., 2003). The esterification reaction is reversible and the ester is stored in vacuoles (Eudes et al., 2008). Glycosides (probably β-D-glucosides) of the tetrahydrofolate precursors neopterin and monapterin were found in tomato fruit engineered to overproduce pterins (Díaz de la Garza et al., 2004); it is not known whether such glycosides occur naturally or whether they can be mobilized.

Riboflavin (B_2)

The alpha and beta forms of riboflavin-5′-D-glucoside have been found in germinating barley (*Hordeum vulgare*) seedlings supplied with riboflavin (Suzuki and Uchida, 1983). As with the pterin glycosides above, it is it is not known whether riboflavin glucosides occur naturally or whether they can be mobilized.

Biotin (B_7)

While no small-molecule conjugates of biotin have been reported, a biotinyl protein that represents >90% of the total protein-bound biotin has been characterized and cloned from pea seeds (Dehaye et al., 1997) and is conserved among higher plants (Gerdes et al., 2012).

Strategy 2: Increasing B Vitamin Supply

Many analyses of gene and protein expression have indicated that abiotic stresses up-regulate flux through some but not all B vitamin biosynthesis pathways and studies of the effects of stress on the levels of vitamins and cofactors tend to support this inference. As is usual in stress research, differences in experimental design (the species and tissue used, the timing, duration, and severity of stress, and the analysis methods applied, i.e. blots/microarrays/RNA-seq, etc.) preclude meta-analysis of all the data. Below, we therefore focus first on a high-throughput data set, the AtGenExpress global stress expression data set (available at http://jsp.weigelworld.org/expviz/expviz.jsp), supplemented with broadly comparable data from several independent Arabidopsis stress studies (Supplemental Table S2). We then highlight illustrative articles on pyridoxine and thiamin.

Note that even when a vitamin biosynthetic pathway is clearly upregulated in response to stress, stress-induced vitamin or cofactor deficiency may still occur for several reasons. First, activating biosynthetic gene expression may fail to increase vitamin production due to downstream constraints, e.g. the energy cost of making THI4 protein for thiamin biosynthesis (see below and Box 1). Second, as noted above, a high vitamin or cofactor status, as measured in whole organs, tissues, or cells, can obscure functional deficiencies within subcompartments. Lastly, conversion of vitamins to cofactors may become limiting, so that increases in vitamin levels do not always result in higher cofactor levels. Cofactor levels can consequently fall even when free vitamin levels rise, and cofactor levels, not vitamin levels, determine metabolic outcomes.

Arabidopsis Gene Expression

The stresses used for the AtGenExpress global stress expression data set (heat, cold, drought, salt, high osmolarity, UV-B light, and wounding) were essentially single-regime shock treatments, lasting 24 h or less, given to Arabidopsis seedlings (Kilian et al., 2007); the protocols in the other studies listed in Supplemental Table S2 were quite similar. The transcriptional responses observed in these experiments therefore necessarily only included part of the plant's full repertoire. These responses nonetheless follow the coherent pattern seen in Figure 5, a bird's-eye schematic of B vitamin synthesis and salvage pathways in which enzymes are represented by rectangles. The color density of each enzyme is proportional to the number of different stresses that induce the corresponding gene by 2-fold or more. The pathways are arranged in order of prevalence of gene induction. At one extreme (biotin, pantothenate/CoA, and tetrahydrofolate pathways), just one or two biosynthetic genes are induced by a single stress, whereas at the other extreme (vitamin B_6 and thiamin pathways), all the known biosynthetic genes are induced by at least three stresses. Except for B_6, the prevalence of induction of salvage genes basically tracks that of biosynthetic genes. This overview of gene-level data thus indicates that certain B vitamin biosynthesis pathways, particularly those for B_6 and thiamin,

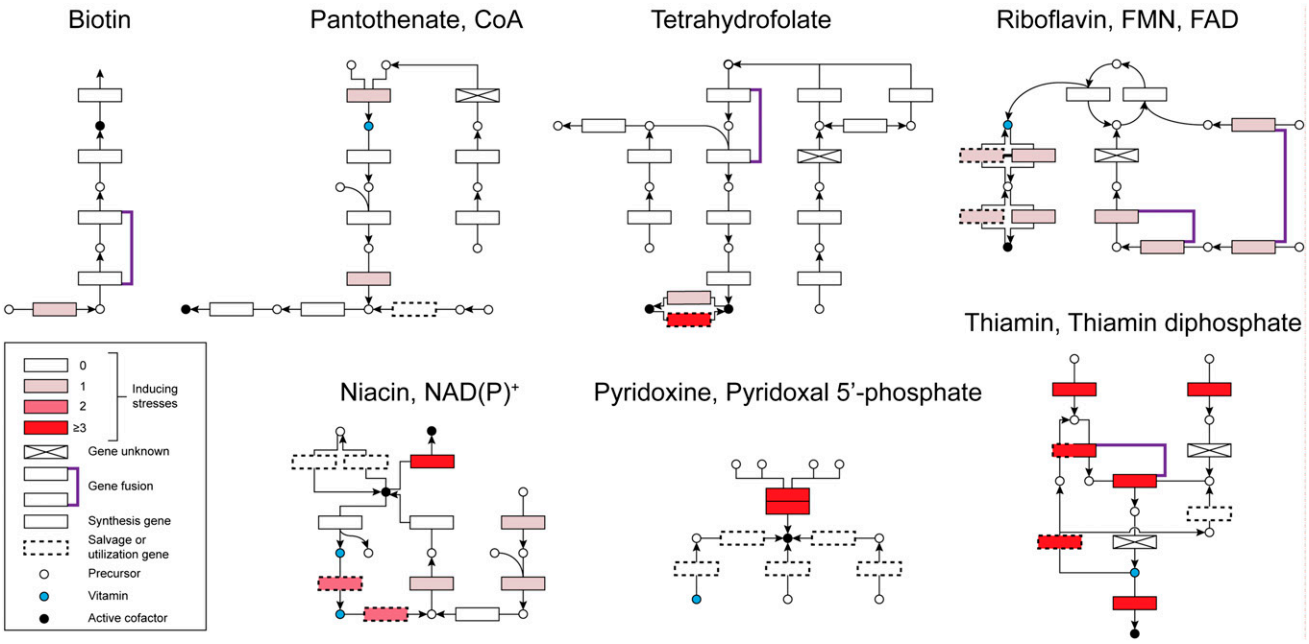


Figure 5. Stress induction of B vitamin and cofactor synthesis and salvage genes in Arabidopsis. Synthesis and salvage pathways for each vitamin are schematized as by Gerdes et al. (2012) except for the omission of compartmentation and the addition of two thiamin salvage reactions (Yazdani et al., 2013; Zallot et al., 2014). Circles are metabolites, arrows are reactions, and rectangles are enzymes. The full pathway schemes, which include enzyme and metabolite names, are available at http://pubseed.theseed.org/seedviewer.cgi?page=PlantGateway. Enzymes whose genes are induced at least 2-fold by 0, 1, 2, or ≥3 different abiotic stresses (in shoots or roots) are color-coded white and three shades of red, respectively. Gene expression data were taken from AtGenExpress and literature summarized in Supplemental Table S2.

are upregulated by several abiotic stresses and that others, such as the tetrahydrofolate pathway, are largely not. Vitamin/cofactor analysis data and protein-level data for vitamin B_6 and thiamin are consistent with this picture, as discussed next.

Vitamin B_6

Pyridoxal 5′-phosphate is made by the pyridoxal synthase complex, which consists of two proteins, PDX1 and PDX2 (the two red-colored boxes in the pyridoxal 5′-phosphate section of Fig. 5). Arabidopsis has three PDX1 homologs, of which PDX1.1 and PDX1.3 are active enzymes and PDX1.2 is a positive regulator (Titiz et al., 2006; Moccand et al., 2014). Paralleling the stress induction of Arabidopsis *PDX* genes (Fig. 5; Supplemental Table S2), PDX1.3 protein accumulated and the total B_6 level increased by 60% in response to UV-B exposure (Ristilä et al., 2011); total B_6 also increased by 60% in response to heat stress (Moccand et al., 2014). Levels of pyridoxal 5′-phosphate and free pyridoxal or pyridoxine likewise increased by about 2-fold in tobacco exposed to chilling, UV radiation, osmotic stress, oxidative stress, or drought (Huang et al., 2013).

Thiamin

Thiamin is synthesized by a pathway that is very energetically expensive because the thiazole moiety is made by a suicide enzyme (THI4), and the pyrimidine moiety is made by a radical SAM (*S*-adenosyl-Met) enzyme (THIC) that probably lasts for only a few catalytic cycles (Box 1). Paralleling the stress induction of Arabidopsis THI4, THIC, and other thiamin synthesis genes (Fig. 5; Supplemental Table S2), the level of total thiamin and of its components (thiamin mono- and diphosphates and free thiamin) increased by up to 2-fold in Arabidopsis seedlings subjected to cold, osmotic, salinity, or oxidative stress (Tunc-Ozdemir et al., 2009). Similarly, total thiamin increased by up to 60% in Arabidopsis seedlings exposed to salt, osmotic, or oxidative stress, with free thiamin contributing as much or more than thiamin diphosphate to the increase (Rapala-Kozik et al., 2012). Also similarly, in maize seedlings subjected to water, salt, or oxidative stress, total thiamin increased by 70 to 150%, due to increases in free thiamin and thiamin monophosphate (Rapala-Kozik et al., 2008). However, in this case levels of the cofactor thiamin diphosphate stayed constant or fell by up to 30%, which is quite enough to cause severe deficiency symptoms (Fig. 2) and illustrates the point made above about cofactor levels falling even though free vitamin levels rise.

Given the suicide nature of the THI4 enzyme, it is striking that *THI4* was the most highly or second most highly induced gene in cotton (*Gossypium hirsutum*) seedlings exposed to cold, drought, salt, or high pH

stress; the induction of *THI4* relative to unstressed controls ranged from 14- to 44-fold (Zhu et al., 2013). It is equally striking that the THI4 protein showed, among all proteins analyzed, the second largest increase in expression (3.2-fold) in heat-stressed poplar leaves (Ferreira et al., 2006) and the third largest increase in expression (3.7-fold) in heat-stressed wild rice leaves (Scafaro et al., 2010).

Strategy 3: Repairing or Recycling Damaged B Vitamins and Cofactors

Like other organisms, plants have enzyme systems that deal with some of the chemical and enzymatic damage reactions shown in Figure 1 (Linster et al., 2013). These enzyme systems may repair damaged vitamins and cofactors, i.e. directly restore them to the original state, or salvage parts of damaged molecules for reuse in biosynthesis. As vitamin and cofactor repair (Hanson et al., 2016) and recycling (Gerdes et al., 2012) were recently reviewed, we focus here on three cases where the repair or recycling-related activity appears to be upregulated by stress and not simply constitutive.

Thiamin Salvage Hydrolase TenA_E

TenA_E mediates two successive steps in the recovery of the pyrimidine moiety from thiamin damaged in the thiazole moiety (Zallot et al., 2014). The Arabidopsis gene encoding TenA_E is upregulated by several abiotic stresses, in common with thiamin synthesis genes (Fig. 5; Supplemental Table S2). The Arabidopsis gene specifying the thiazole salvage enzyme ThiM (Yazdani et al., 2013) is not upregulated (Fig. 5), which fits with the thiazole moiety of thiamin being more prone to irreversible damage than the pyrimidine moiety, and hence not as recyclable (Zallot et al., 2014).

Thiamin Diphosphate-Related Nudix Hydrolases

These enzymes (NUDT20 and NUDT24 in Arabidopsis) are diphosphatases whose preferred substrates are damaged, and toxic, forms of thiamin diphosphate, including the previously mentioned thiazolone form generated by oxygen-dependent side-reactions (Goyer et al., 2013). The diphosphatase reaction is both a detoxification step and the first step in salvage of the pyrimidine moiety. The Arabidopsis genes (which are not distinguished by microarrays) are induced between 2- and 5-fold by salt, drought, and osmotic stress, and probably also oxidative stress (Kilian et al., 2007; Goyer et al., 2013).

NAD(P)$^+$ Salvage Module

Two enzymes mediating consecutive steps in NAD(P)$^+$ salvage (nicotinamidase and nicotinate phosphoribosyltransferase) are each induced by two stresses in Arabidopsis (Fig. 5), as is a transporter (NiaP) for nicotinate, the intermediate that these enzymes share (Kilian et al., 2007). Arabidopsis NiaP also transports trigonelline (Fig. 4) (Jeanguenin et al., 2012). NiaP is probably located in the plasma membrane (Jeanguenin et al., 2012), suggesting that nicotinate salvaged in one location could be reused for NAD(P)$^+$ synthesis in another. This would be consistent with the classical evidence for inter-organ exchange of nicotinate (Bonner and Bonner, 1948).

Strategy 4: Decreasing Demand for B Vitamins

Metabolism can sometimes be rerouted to bypass certain cofactor-dependent steps, as in the phosphate starvation response, in which alternative bypass pathways of cytosolic glycolysis are upregulated to spare scarce adenylates and phosphate (Plaxton and Tran, 2011). There are as yet no cases of full-blown rerouting in response to vitamin deficiency, although two studies provide indirect evidence for redirection of fluxes that may be, at least in part, active responses. In the first study, tobacco leaves overexpressing transketolase, a thiamin diphosphate-dependent enzyme, became moderately thiamin diphosphate-deficient and showed reduced isoprenoid synthesis via the thiamin diphosphate-dependent enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS); this reduction was associated with lower *DXS* transcript levels (Khozaei et al., 2015). In the second study, a drastic, rapid-onset tetrahydrofolate deficiency in Arabidopsis cells treated with antifolate drugs led to initial depletion of Met and *S*-adenosyl-Met pools, reflecting reduction in the tetrahydrofolate-dependent flux of one-carbon units to Met as one-carbon fluxes were adaptively reprioritized in favor of nucleotides (Loizeau et al., 2008). Surprisingly, the Met and *S*-adenosyl-Met pools, and by inference the associated fluxes, subsequently returned to normal, in part via a conserved adaptive mechanism, triggered by tetrahydrofolate deficiency, that involves proteolytic removal of the N-terminal regulatory region of the Met synthesis enzyme cystathionine γ-synthase (Loizeau et al., 2008).

AGRICULTURAL IMPLICATIONS

As Abraham Blum has emphasized (Blum, 2014), humility and caution are needed when trying to predict agricultural benefits by extrapolating from the responses of seedlings of Arabidopsis or other species to short, sharp stresses in growth chambers (which describes most of the experiments covered above). With this warning in mind, what might stress-induced B vitamin and cofactor deficiency imply for crop breeding and management? The primary implication is that breeding for enhanced vitamin content (biofortification), by

transgenic or other approaches, could pay off in terms of stress resistance as well as human nutrition (Fitzpatrick et al., 2012). If stress indeed predisposes to deficiency, a clear prescription for stress resistance breeding would be to "keep your cofactors safe."

The two B vitamins most connected with stress responses, vitamin B_6 and thiamin (Fig. 5), are targets of transgenic biofortification efforts in Arabidopsis (Raschke et al., 2011; Pourcel et al., 2013; Vanderschuren et al., 2013; Dong et al., 2015), and stress tests have been run on the engineered plants, albeit mainly with plantlets cultured on agar medium containing Suc (i.e. not photosynthesizing normally). The total B_6 content of leaf tissue was boosted up to 4-fold and included large increases in the cofactor pyridoxal 5′-phosphate and pyridoxine-5′-β-D-glucoside (Raschke et al., 2011). The total thiamin content of leaf tissue was boosted up to 3.4-fold, contributed by up to 2-fold increases in the cofactor thiamin diphosphate and up to 6-fold increases in thiamin (Dong et al., 2015). The B_6-biofortified plants were larger than controls and more resistant to paraquat-imposed oxidative stress; no other stress data were reported (Raschke et al., 2011). The thiamin-fortified plants showed no change in resistance to salt, cold, osmotic, drought, or oxidative stress (Dong et al., 2015). To summarize: These first biofortification results for B_6 and thiamin are inconclusive and merit follow-up.

Stress-induced B vitamin deficiency also has implications for crop management (Plaut et al., 2013). Reported benefits of vitamin applications are cited above; such applications are most likely to be useful in practice if they can be given as seed-priming treatments (Al-Hakimi and Hamada, 2001), i.e. applied to seed before sowing. Such seed treatments are cost-efficient (Tanou et al., 2012).

FUTURE PERSPECTIVES

The preceding overview of work relating B vitamins to abiotic stress raises five issues that are listed in the "Outstanding Questions." Each issue is developed briefly below.

Itemizing Stress-Relevant Enzyme Reactions

Although various enzymes and genes for repair or recycling of damaged vitamins and cofactors are known, this is still not the case for about half of the 30 damage reactions in Figure 1. While the products of some of these reactions may have no possible fate besides complete degradation, comparative biochemistry suggests that others may well be reclaimed in plants; examples include biotin sulfoxide and chain-shortened biotin (Linster et al., 2013), pyridoxine and nicotinamide *N*-oxides (Sakuragi and Kummerow, 1960; Shibata et al., 1991), and riboflavin-4′,5′-phosphate (cyclic FMN; Fraiz et al., 1998). We also don't know most of the enzymes or genes for the synthesis and mobilization of the vitamin conjugates in Figure 4, and it is not even clear that we have fully inventoried the conjugates that plants can make. Notably, conjugated forms of thiamin and its pyrimidine and thiazole precursors have not been reported from plants, but they seem not to have been sought.

Graduating to Crops and Realistic Stress

As noted previously, most of the modern data linking abiotic stress with B vitamins and cofactors comes from young Arabidopsis plants and unrealistic stress protocols. This is a generic weakness of molecular-level stress research, and the resulting disconnect from agriculture goes a long way toward explaining why molecular work has had a very limited impact on breeding for stress environments (Blum, 2014). It is consequently important to extend stress/vitamin work from Arabidopsis to good genetic model crops such as tomato, canola (*Brassica napus*), maize, and rice and to adopt stress protocols that mimic field stresses to plants beyond the seedling stage.

Diagnosing Functional Deficiencies

Vitamin deficiencies, particularly functional ones affecting only certain cells or compartments, may be better diagnosed from characteristic changes in metabolite profiles than by directly measuring vitamin levels, as discussed above for vitamin B_{12} deficiency in humans (Stabler et al., 1996). Given the possibility that direct vitamin measurements can fail to detect subtle but physiologically critical functional deficiencies, and the high cost and low-throughput nature of many vitamin assays, indirect assessment of plant vitamin and cofactor status via metabolomics is appealing. Confirming or disconfirming the validity of this approach in plants, as in humans (Stabler et al., 2013), will require prior metabolic profiling studies of vitamin-deficient mutants. An alternative, indirect, high-throughput way to detect functional vitamin deficiencies may be via characteristic transcriptome changes, analogous to the induction of specific genes by mineral nutrient deficiencies (Nikiforova et al., 2003; Zheng et al., 2009). Furthermore, transcriptome analysis could uncover mechanisms that reroute metabolism to mitigate the effects of vitamin deficiency (see above). Again, evaluation of these possibilities will require studies of vitamin-deficient mutants.

Genotypic Variation

Although there is natural genotypic variation in vitamin content (Hanson and Gregory, 2011; Fitzpatrick et al., 2012), it would be impractical to use vitamin

OUTSTANDING QUESTIONS

- Have we documented all the relevant metabolic reactions of B vitamins and cofactors?
- Do the results from model stresses in Arabidopsis apply to realistic stresses in crops?
- Can functional B vitamin deficiency be diagnosed from metabolome/transcriptome changes?
- How far can genotypic variation in vitamins or cofactors explain variation in stress resistance?
- Is thiamin really the most stress-sensitive B vitamin?

content as a selection criterion to develop experimental populations due to the issues of assay cost and efficacy just outlined. On the other hand, were high-throughput indirect methods to assess deficiency available (see above), it would be feasible to assess vitamin/cofactor status in populations subjected to realistic stresses and then to work back via genome-wide association studies to genes governing vitamin/cofactor status. Once identified, such genes, which would presumably include known and novel genes with repair/recycling, synthesis, storage, or bypass functions, could be used to explore a "keep your cofactors safe" breeding strategy for abiotic stress resistance.

Is Thiamin an Achilles' Heel in Stress Metabolism?

A thread running through the whole of this review is that thiamin and thiamin diphosphate are not like the other B vitamins and cofactors. Thiamin diphosphate may turn over exceptionally fast, at least in part because of its unusual feature of catalytic inactivation. Thiamin is energetically costly to make due to the suicidal nature of one of its biosynthetic enzymes and the suicidal tendency of another. Because thiamin synthesis depends absolutely on concurrent protein synthesis, it is vulnerable to stress in a way that other B vitamins are not. Unlike most other B vitamins, thiamin and its precursors lack known storage forms. Thiamin synthesis and salvage genes are particularly highly stress-regulated in Arabidopsis seedlings. Lastly, thiamin is more prominent than other B vitamins in the "spray and pray" literature. Taken together, these features suggest that thiamin could be an Achilles' heel, a crucial weak point, in plant stress metabolism.

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. Analysis of Arabidopsis stress literature based on PubMed search terms.

Supplemental Table S2. Stress-induced B vitamin/cofactor synthesis and salvage genes in Arabidopsis.

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