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ON THE BIOCHEMISTRY AND PHYSIOLOGY OF VITAMIN B_1 AND VITAMIN B_6 BIOSYNTHESIS IN *ARABIDOPSIS THALIANA*.

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presented by

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“As we acquire more knowledge, things do not become

more comprehensible, but more mysterious.”

Albert Schweitzer

Dedication:

Obviously, I would not be sitting in front of my computer typing the last sentences of my thesis without having met Professor Meinhart H. Zenk, now at the Donald Danforth Plant Science Center in St. Louis, U.S.A.. He guided me during my first clumsy steps in science and never lost confidence in me. I thank him for his guidance and support throughout the past nine years, helping me to succeed and installing in me the confidence that I am capable of doing anything I put my mind to. I have always needed to work hard to achieve my scientific goals and he has always been there for me as an unwavering mentor.

I dedicate this work to you, to honour your belief, patience, advice, and support during all these years.

With my deepest gratitude,

Maja

This thesis is the account of three years of devoted work in the field of vitamin B_1 and vitamin B_6 research at the Institute of Plant Sciences, Group of Plant Biochemistry and Physiology, at the ETH Zurich, Switzerland, which would not have been possible without the help of many people.

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Table of contents:

Table of contents:

Table of contents:

0 Summary

The aim of the present study is to provide a more detailed insight into the *de novo* biosynthesis of vitamin B_1 (thiamin diphosphate, ThDP) and vitamin B_6 (pyridoxal 5'-phosphate, PLP) in plants. Both vitamins are essential compounds for growth and development, predominantly involved in carbohydrate and amino acid metabolism, respectively. Whereas bacteria, fungi and plants can synthesize both compounds *de novo*, humans and animals have to obtain them with their diet. Their *de novo* biosynthesis has been extensively studied in bacteria (*Escherichia coli*), whereas in plants a complete understanding of vitamin B_1 biosynthesis, in particular, is fragmentary.

In this study, *de novo* biosynthesis of the precursor molecule of the pyrimidine moiety of vitamin B_1, 2-methyl-4-amino-5-hydroxymethylpyrimidine phosphate (HMP-P) was addressed. In bacteria, HMP-P originates from 5-aminoimidazole ribonucleotide (AIR_t), catalyzed by ThiC. In yeast, however, HMP-P is derived from L-histidine and vitamin B_6, catalyzed by members of the THI5 or NMT1 family. In this study, comprehensive BLAST searches revealed the presence of a homolog of ThiC in *A. thaliana*, whereas no homolog of the THI5/NMT1 family could be identified. AtTHIC is indispensable for *de novo* synthesis of vitamin B_1 and is absolutely essential for plant development beyond the cotyledon stage. A knockdown of this gene leads to a clear reduction in the vitamin B_1 level, eventually leading to seedling lethality unless thiamin is provided as a nutrient. The THIC protein is localized in the chloroplasts. Furthermore, the *THIC* transcript level was found to be regulated by light and to be repressed when thiamin is present in the medium, indicative of the occurrence of a riboswitch-mediated transcriptional control of the gene. The heterologously expressed AtTHIC protein revealed the presence of an iron-sulfur cluster, which could also be shown with the bacterial ThiC homolog.

The second part of the study dealt with vitamin B_6. This compound has been shown, only recently, to be derived in plant systems from ribose 5-phosphate, glyceraldehyde 3-phosphate and glutamine. The biosynthesis is catalyzed by a heteromeric protein complex, consisting of PDX1 and PDX2, which acts as a glutamine amidotransferase giving directly rise to the formation of PLP. Whereas three *PDX1* homologs, *i.e. PDX1.1*, *PDX1.2*, *PDX1.3*, were identified in *A. thaliana*, only a single homolog of *PDX2* is present. Recently, vitamin B_6 has been found to also act as a potent quencher of reactive oxygen species. To investigate whether vitamin B_6 plays a comparable role in plant systems as well, *A. thaliana* lines were established

accumulating vitamin B_6 significantly above WT levels. Plants with increased vitamin B_6 levels show a delay in bolting and a reduced seed set in the siliques, but an overall increase in seed biomass yield. An increase in the major storage substances, *i.e.* lipid, carbohydrates and protein, contributes to the enlarged seed size. Experimental evidence is provided that vitamin B_6 may indeed act as a protectant in oxidative stress responses in *Arabidopsis*, using *flu* mutant lines, that are characterized by a burst of singlet oxygen upon the shift from the dark to the light, as well as by paraquat and rose bengal treatment.

Furthermore, PDX1.1 and PDX1.3 may have distinct roles in the regulation of vitamin B_6 biosynthesis in plants. In particular, PDX1.3 (as a housekeeping enzyme) may predominantly be involved in the maintenance of a certain level of vitamin B_6 throughout plant development, while PDX1.1 may be involved in ensuring vitamin B_6 homeostasis under certain environmental conditions, as demonstrated for a combination of high light intensity and cold. Vitamin B_6 levels increase upon treatment with paraquat and decrease in the presence of NaCl as well as rose bengal treatment (the latter in combination with sucrose) and with sucrose treatment alone. However, transcript levels of *PDX1* and *PDX2* were not clearly correlated with the vitamin B_6 content, implying regulatory mechanisms of vitamin B_6 biosynthesis beyond those of transcript synthesis.

In addition, previous studies had revealed that a knockout of both *PDX1.1* and *PDX1.3* is embryo lethal, thus excluding PDX1.2 from catalytic participation in *de novo* vitamin B_6 biosynthesis. Its actual function remains unclear to date. However, the response to certain stimuli was investigated in this study and revealed that the gene is up-regulated particularly in response to sucrose treatment and to the production of singlet oxygen. Constitutively enhanced protein levels of PDX1.2 increase the susceptibility to high-light exposure in *A. thaliana* and affect the transcript levels of other genes related to vitamin B_6 biosynthesis in the abovementioned *flu* mutant line.

0 Zusammenfassung

Die vorliegende Arbeit befasst sich mit der de novo Biosynthese von Vitamin B1 (Thiaminpyrophosphat, ThDP) und Vitamin B_6 (Pyridoxal 5'-Phosphat, PLP) in Pflanzen. Diese Vitamine sind essentiell für Wachstum und Entwicklung und insbesondere in Kohlenhydrat- und Aminosäuremetabolismus involviert. Während sie von Bakterien, Pilzen und Pflanzen synthetisiert werden, müssen Menschen und Tiere sie mit der Nahrung aufnehmen. Im Gegensatz zu Bakterien (*Escherichia coli*), in denen die *de novo* Biosynthese beider Substanzen detailliert untersucht wurde, ist das Wissen über die Vitamin B_1 Biosynthese in Pflanzen noch immer unvollständig.

In dieser Studie wurde die Biosynthese der Vorstufe der Pyrimidin-Untereinheit von Vitamin B_1, 2-Methyl-4-Amino-5-Hydroxymethylpyrimidinphosphat (HMP-P), betrachtet. In Bakterien wird HMP-P ausgehend von 5-Aminoimidazol Ribonukleotid (AIR_t), durch das Enzym ThiC, synthetisiert. Im Gegensatz dazu wird HMP-P in Hefen aus L-Histidin und Vitamin B_6 durch Mitglieder der Enzymfamilie THI5 bzw. NMT1 gebildet. Im Rahmen der vorliegenden Arbeit konnte ein Homolog von ThiC, jedoch nicht von THI5/NMT1, in *A. thaliana* identifiziert werden. AtTHIC ist für die *de novo* Biosynthese von Vitamin B_1 unabdingbar und ist damit für die Pflanze nach der Entwicklung der Keimblätter essentiell. Eine reduzierte Expression des Genes führt zu einer Reduktion des Vitamin B_1-Gehaltes und letztendlich zum Absterben des Sämlings, sofern kein Vitamin B_1 zugeführt wird. THIC ist ein chloroplastidäres Protein. Die THIC Transkription wird durch Licht stimuliert sowie durch exogenes Thiamin reprimiert. Letzteres weist auf die Kontrolle der Transkription durch einen sogenannten Riboswitch hin. In *E. coli* heterolog exprimiertes AtTHIC enthält, wie das bakterielle Homologe, ein Eisen-Schwefel-Cluster.

Der zweite Teil der Arbeit befasste sich mit Vitamin B_6. Die Biosynthese, ausgehend von Ribose 5-Phosphat, Glycerinaldehyd 3-Phosphat und Glutamin, wurde in Pflanzen gezeigt. Die Katalyse erfolgt durch einen heterogenen Proteinkomplex mit Glutaminamidotransferase-Aktivität bestehend aus den Proteinen PDX1 und PDX2. In *A. thaliana* wurden drei *PDX1* Gene, *PDX1.1*, *PDX1.2* und *PDX1.3*, identifiziert, hingegen nur ein *PDX2* Gen. Es wurde berichtet, dass Vitamin B_6, neben seiner Cofaktorfunktion, reaktive Sauerstoffspezies unschädlich macht. Um die Funktionen von Vitamin B_6 in Pflanzen weiter zu untersuchen, wurden *A. thaliana* Linien mit erhöhten Vitamin B_6 Gehalten erzeugt. Die Vitamin B_6 überproduzierenden Pflanzen zeigen einen verzögerten Beginn des Schossens und eine verringerte

Samenzahl pro Schote, jedoch ein höheres Samengewicht. In den signifikant grösseren Samen wurde eine Zunahme der drei Hauptspeicherstoffe Fette, Kohlenhydrate und Einweisse nachgewiesen. In Vitamin B_6 akkumulierenden *A. thaliana flu*-Mutanten Linien, in denen durch Belichtung Singulettsauerstoff freigesetzt wird, sowie bei Behandlung mit Paraquat und Rose Bengal, erhöht Vitamin B_6 die Toleranz gegen oxidativen Stress.

Des weiteren könnten PDX1.1 und PDX1.3 unterschiedliche Aufgaben in der Vitamin B_6-Biosynthese in Pflanzen übernehmen. PDX1.3 scheint vorrangig für die Sicherstellung des Vitamin B_6 Grundbedarfs verantwortlich zu sein, während PDX1.1 die Versorgung bei Stressreaktionen zu übernehmen scheint. Letzteres wurde mittels einer kombinierten Behandlung mit Starklicht und Kälte demonstriert. Der Gehalt an Vitamin B_6 nimmt bei Behandlung mit Paraquat zu, durch Behandlung mit NaCl oder Sacchrose, allein oder in Verbindung mit Rose Bengal, nimmt er ab. Es konnte aber keine Korrelation zwischen den Transkriptmengen von *PDX1* und *PDX2* sowie Vitamin B_6 gezeigt werden, was vermuten lässt, dass die Vitamin B_6 Biosynthese nicht ausschliesslich durch die Transkription von PDX1 und PDX2 reguliert wird.

Da der gleichzeitige Ausfall von *PDX1.1* und *PDX1.3* für die Pflanze letal ist, kann PDX1.2 nicht an der Katalyse der Biosynthese von Vitamin B_6 beteiligt sein. Eine spezifische Funktion konnte PDX1.2 bisher jedoch nicht zugeordnet werden. Daher wurde der Effekt verschiedener Stressfaktoren im Rahmen dieser Arbeit untersucht und es zeigte sich, dass insbesondere hohe Konzentrationen an Saccharose oder Singulettsauerstoff einen erhöhten *PDX1.2* Transkriptgehalt bewirken. Ein konstitutiv erhöhter Gehalt an PDX1.2 Protein vermindert die Toleranz gegenüber Starklicht und beeinflusst in der oben genannten *flu*-Mutanten Linie den Transkriptgehalt von Genen, die mit der Vitamin B_6 Biosynthese in Zusammenhang stehen.

1 Introduction

1.1 Vitamins and their involvement in general metabolism

The term “vitamin” (vital amine) was established by the Polish biochemist Casimir Funk in 1912. He elucidated that the deficiency disease Beriberi, these days endemic in many Asian countries, can be cured by a substance present in whole grains and particularly in rice bran, but not in polished rice [Funk, 1911]. He proposed that such essential compounds in the diet contain an amine group, hence the term vitamin.

Vitamins in general are defined as essential for life (the term “vitamin” is normally related to humans) in that they cannot be sufficiently synthesized in an organism. Thus, their immediate precursors or analogues have to be taken up with the diet, *e.g.* vitamin B_1 (unpolished rice, green leafy vegetables), vitamin B_6 (soybean, green leafy vegetables), vitamin C (citrus fruits), vitamin E (seeds, nuts), vitamin D (hen eggs, milk) [http://db.ancientfuture.net/vitamins.html]. To date, 13 compounds are classified as vitamins (table 1.1). In contrast to the three major nutrients (carbohydrates, proteins, lipids) as well as water, vitamins are needed in relatively small amounts by organisms, thus termed micronutrients [Lieberman and Brunig, 2003]. Other essential micronutrients include certain minerals, unsaturated fatty acids and certain amino acids.

The main contribution of vitamins is to maintain metabolism, in serving as coenzymes in numerous enzymatic reactions [Suckow *et al.,* 2006]. Furthermore, some of them play distinct roles, *e.g.* as antioxidants (vitamins C, E) [Cross *et al.*, 1994] or as a (pre-) hormone involved in calcium and phosphorus homeostasis in the blood stream (vitamin D). Vitamin A – retinol, retinal and many carotenoids (provitamin A) – is indispensable for the light-dependent synthesis of rhodopsin, a visual pigment in mammals [Noell *et al.*, 1971]. Vitamin B_{12}, a distinct group of cobalt-containing metabolites, is involved in the regulation of DNA synthesis, cell division, fatty acid biosynthesis and energy production in bacteria and animals [Wagner, 1995; Zingg and Jones, 1997; Frenkel *et al.*, 1973].

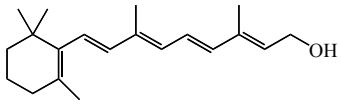
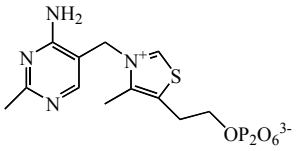
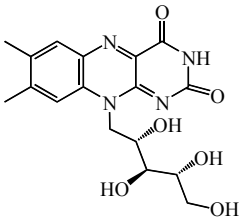
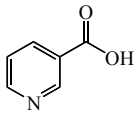
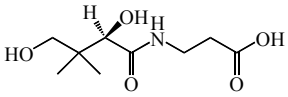
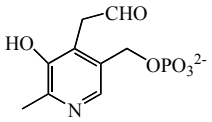
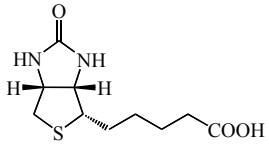
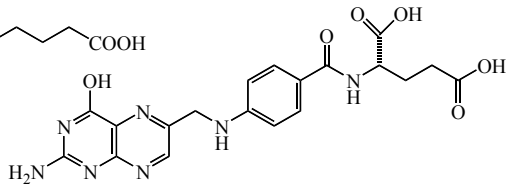
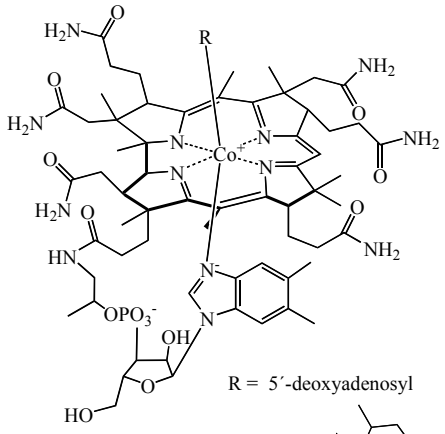
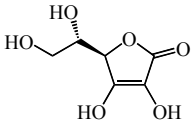
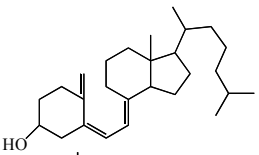
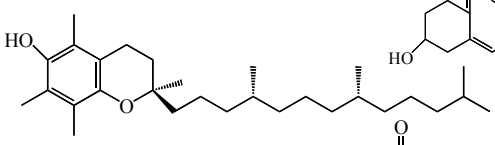
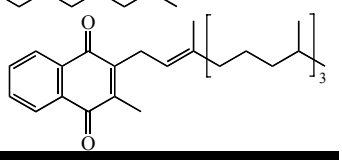
Vitamin	Chemical name (active form)	Solubility	Structure
A	Retinol	Lipophilic	
B_1	Thiamin diphosphate	Hydrophilic	
B_2	Riboflavin	Hydrophilic	
B_3	Niacin	Hydrophilic	
B_5	Pantothenic acid	Hydrophilic	
B_6	Pyridoxal 5'-phosphate	Hydrophilic	
B_7 (H)	Biotin	Hydrophilic	
B_9	Folic acid	Hydrophilic	
B_{12}	5'-Deoxyadenosylcobalamin	Hydrophilic	
C	L-Ascorbic acid	Hydrophilic	
D_3	Cholecalciferol	Lipophilic	
E	D-α-Tocopherol	Lipophilic	
K	Phylloquinone	Lipophilic	

Table 1.1: Metabolites classified as vitamins (for humans). The main active form is listed when various chemical compositions of a vitamin exist (based on http://de.wikipedia. org/wiki/Vitamine).

The majority of vitamins directly derive from intermediates of primary metabolism. Riboflavin and folic acid, vitamin B_2 and B_9, respectively, originate from purines or from intermediates of their biosynthesis [Young, 1986]. In many bacteria, fungi and plants, L-aspartate and α-ketovalerate are precursors for pantothenic acid – vitamin B_5 – whereas in the apicomplexan parasite *Toxoplasma gondii* L-aspartate and L-valine act as precursors [Müller and Kappes, 2007]. Pantothenic acid is a precursor for coenzyme A [Spry *et al.*, 2008]. L-Ascorbic acid – vitamin C – is derived from highly abundant sugars. In contrast to the other vitamins, ascorbic acid is present in high cellular abundance (10 to 100 mg / 100 g cell fresh weight). These levels are multiple orders of magnitudes above those of the other vitamins [Linster and van Schaftingen, 2006].

The fact that various organisms utilize different precursors, as exemplified above, is quite common in *de novo* vitamin biosynthesis. In the case of apicomplexan parasites, Wrenger *et al.* [2008] argued that this might be based on an evolutionary accommodation to their host cells as well as on the specificities of the particular organism's cell cycle. Whereas *T. gondii* possesses genes to synthesize pantothenic acid *de novo*, other representatives of the apicomplexan parasites like *Plasmodium falciparum*, *Cryptosporidium parvum* and *Cryptosporidium hominis* strictly depend on its supply. Furthermore, among these species, a rudimentary biosynthesis of vitamin B_1 dependent on the supply of 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) appears to be present exclusively in *P. falciparum* [Wrenger *et al.*, 2006]. The adaptation of vitamin requirements of a single species within closely related species amplifies the flexibility of evolution.

1.2 Vitamin B_1

1.2.1 The discovery of vitamin B_1

The discovery of vitamin B_1 is closely related to the deficiency disease Beriberi. In 1882, the Japanese scientist Kanehiro Takaki (1849-1929) demonstrated that a well-balanced nutrition (vegetables, barley, fish and meat) can, unlike polished rice, prevent Beriberi although these findings were barely recognized. Five years later, the Dutch scientist Christiaan Eijkman (1858-1930) proved that a component of rice bran that is absent in polished rice can prevent and cure the symptoms of this disease. For this finding, he and Frederick Gowland Hopkins (1861-1947) were awarded the Nobel Prize for Physiology or Medicine in 1929. In 1910, the Japanese scientist Umetaro Suzuki (1874-1943) received a patent for a compound entitled

"aberic acid" without having identified its chemical composition. The isolate from rice bran was active in curing patients of Beriberi. In 1912, Casimir Funk (1884-1967) was able to chemically identify the essential compound and suggested the name thiamin(e), due to its chemical composition. Isolation of the metabolite from rice bran in crystalline form was followed by its structural elucidation and chemical synthesis in 1936. In 1937, Lohmann and Schuster showed that the major active form is the diphosphate (ThDP) (figure 1.1) [Schellenberger, 1998].

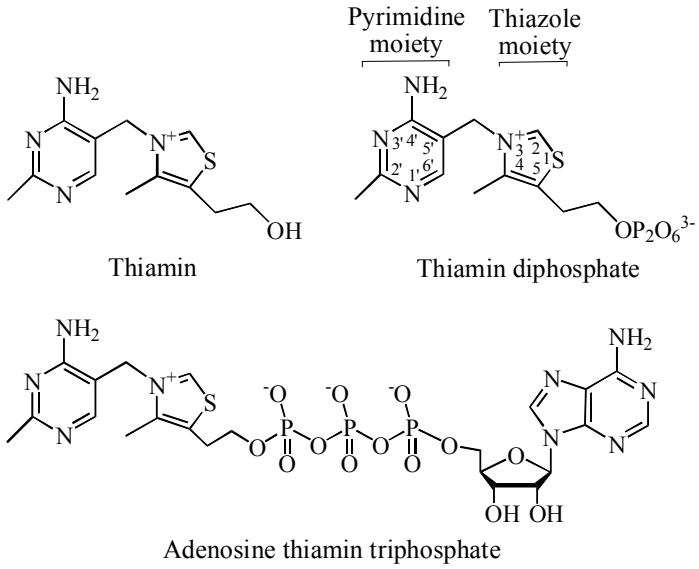


Figure 1.1: Chemical structures of thiamin, thiamin diphosphate and adenosine thiamin triphosphate. The basic molecule consists of the two moieties 2-methyl-4-amino-5-hydroxymethylpyrimidine (pyrimidine moiety) and 4-methyl-5(β-hydroxyethyl) thiazole (thiazole moiety) linked by a methylene bridge. Phosphorylation occurs at the hydroxyethyl side chain at C-5 of the thiazole moiety. The accepted numbering of the ring atoms and the chemical proportions are indicated for thiamin diphosphate, the major biologically active form. In addition, the mono- and triphosphate of thiamin occur naturally.

1.2.2 The biological relevance of thiamin diphosphate for metabolism – Examples of enzymes dependent on vitamin B_1

Generally, thiamin diphosphate (ThDP) is essential for several key enzyme reactions in anabolic and catabolic intermediary carbohydrate metabolism, like the pentose phosphate cycle and the Krebs cycle, thus making it indispensable for all living organisms [Godoi *et al.*, 2006]. Furthermore, it serves as a cofactor for enzymes of secondary metabolism. For example, it is indispensable for the initial reaction of the non-mevalonate isoprenoid biosynthetic pathway [Sprenger *et al.*, 1997; Lois *et al.*, 1998]. ThDP is also involved in the biosynthesis

of natural compounds like fosfomycin, fosmidomycin or bialaphos in various *Streptomyce*s strains that are of use as antibiotics or herbicides (figure 1.2, table 1.2).

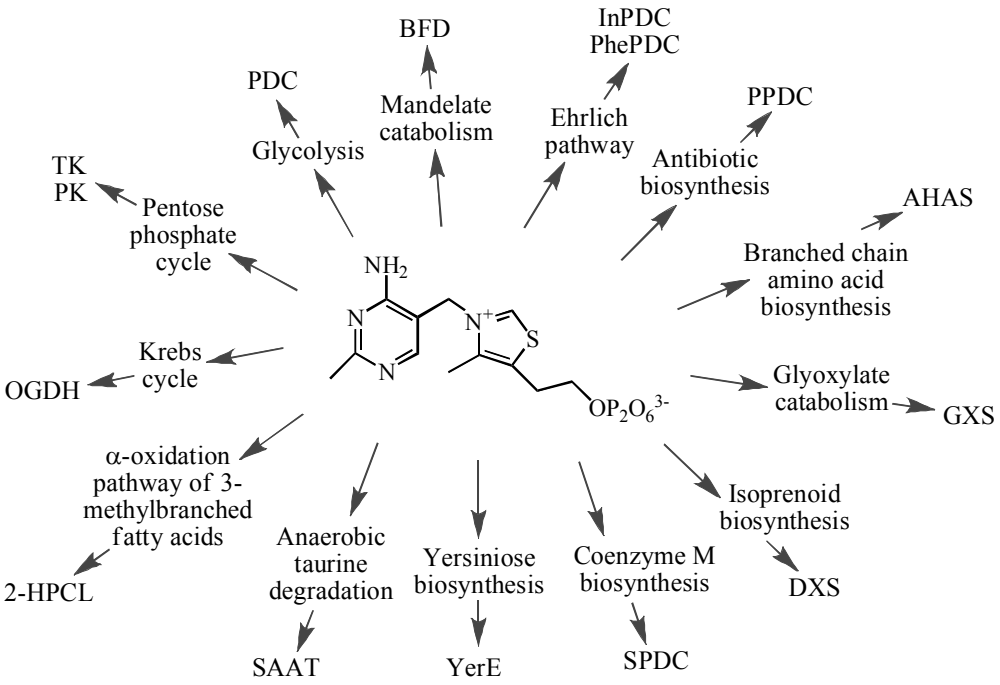


Figure 1.2: Overview of ThDP-dependent biosynthetic pathways and examples of the respective ThDP-dependent enzymes. Abbreviations used: AHAS: acetohydroxyacid synthase; BFD: benzoylformate decarboxylase; DXS: 1-deoxy-D-xylulose 5-phosphate synthase; GXS: glyoxylate carboligase; 2-HPCL: 2-hydroxyphytanoyl-CoA lyase; InPDC: indole 3-pyruvate decarboxylase; OGDH: 2-oxoglutarate dehydrogenase; PDC: pyruvate decarboxylase; PhePDC: phenylpyruvate decarboxylase; PK: phosphoketolase; PPDC: 3-phosphonopyruvate decarboxylase; SAAT: sulfoacetaldehyde transferase; SPDC: 3-sulfopyruvate decarboxylase; TK: transketolase. Further details are given in table 1.2 [based on Pohl *et al.*, 2004].

ThDP is involved in the formation and cleavage of bonds between a carbon atom and either a sulfur, oxygen, hydrogen or nitrogen atom or most noticeably to another carbon atom adjacent to a carbonyl group in various α-keto acid substrates [Frank *et al.*, 2007]. Among others, ThDP-dependent enzymes are known to form C–C-bonds in a stereo- and enantioselective manner, thus making them interesting for organic chemistry and the pharmaceutical industry [Demir *et al.*, 2007].

Experiments with isotopically labelled precursors revealed that the C-2 of the thiazole moiety rather than the *a priori* expected amino group attached at C-4' of the pyrimidine moiety is involved in catalysis [Holzer and Beaucamp, 1959]. The diphosphate chain at C-5 of the thiazole moiety acts as the essential linker group to the respective enzyme. Thiamin on its own

does not support catalysis in even high concentrations, whereas free pyrophosphate significantly competes for the active site of pyruvate decarboxylase (PDC) [Eppendorfer *et al.*, 1993; Schellenberger *et al.*, 1997]. It is believed that an indispensable bivalent metal ion (usually Mg^{2+} or Ca^{2+}) binds to specific amino acid residues that are conserved among all ThDP-dependent enzymes identified so far: $GDGX_{26}N(C)N$. Upon binding of the metal ion the diphosphate chain is bound to the enzyme [Jordan, 2003].

Due to various reaction types, Frank *et al.* [2007] suggest the classification of ThDP-dependent enzymes according to mechanistic similarities, *i.e.* either decarboxylating or non-decarboxylating reactions. The latter reactions include transferase activities as well. Another classification cited in the literature proposes the differentiation into oxidative and non-oxidative pathways [Jordan, 2003]. Examples of the latter include PDCs and benzoylformate decarboxylase (BFD). Oxidative reactions require a redox reagent, for example flavin (FAD; pyruvate oxidase (POX)) or lipoic acid (pyruvate dehydrogenase (PDH) multienzyme complex) [Jordan, 2003]. According to the International Union of Pure and Applied Biochemistry (IUPAC) and the International Union of Biochemistry (IUBMB), ThDP-dependent enzymes can be found in almost all enzyme classes, *e.g.* transferases (transketolase (TK), phosphoketolase (PK), sulfoacetaldehyde transferase (SAAT)), oxidoreductases (PDH, pyruvate ferredoxin-oxidoreductase (PFOR)), ATP-independent synthases/lyases (PDC, DXS), as well as ATP-dependent synthetases/ligases (glyoxylate carboligase (GXS)) (figure 1.2, table 1.2).

All ThDP-dependent enzymes share various similarities with regard to their catalytic mechanisms, amino acid sequences forming the binding fold for the essential metal ion Mg^{2+} or Ca^{2+}, and the domains identified with affinity to both the diphosphate chain and the pyrimidine subunit of ThDP [Costelloe *et al.*, 2008]. The overall amino acid sequences of ThDP-dependent enzymes generally show less than 20% sequence identity. The primary protein structures and the order of the domains can vary considerably, despite the fact that the tertiary structures identified so far are remarkably similar in more than 20 enzymes from eukaryotes, eubacteria and archaea [Frank *et al.*, 2007]. Additionally, the substrate specificities of those enzymes range from rather strict (PK, GXS) to more permissive (PDC, TK) [Schörken and Sprenger, 1998].

Enzyme	1st / 2nd substrate	1st / 2nd product	Metabolic pathway
PDC	2-Oxo acid / H^+	CO_2 / Acetaldehyde	Glycolysis (ethanolic fermentation)
OGDH	α-Ketoglutarate / CoA	CO_2 / Succinyl-CoA	Krebs cycle; NAD^+ dependent
E1 subunit of PDH	2-Oxo acid / Oxidized lipoyl group attached to PDH E2 subunit	CO_2 / Lipoyl domain (reductively acylated)	Krebs cycle
TK	Ketose / Aldose	Aldose / Ketose	Pentose phosphate cycle
PhePDC	Phenylpyruvate	CO_2 / Phenylacetaldehyde	Degradation of phenylalanine (Ehrlich pathway)
AHAS	Pyruvate / H^+	CO_2 / Acetolactate or 2-aceto-2-hydroxybutyrate	Branched-chain amino acid biosynthesis
DXS	Pyruvate / GAP	CO_2 / DXP	Isoprenoid biosynthesis
2-HPCL	2-Hydroxyphytanoyl-CoA	Formyl-CoA / Pristanal	α-Oxidation of 3-methyl-branched fatty acids
PPDC	3-Phosphonopyruvate	CO_2 / 2-Phosphono-acetaldehyde	Antibiotic biosynthesis
PFOR	Pyruvate / 2-Oxo acid	CO_2 / Acetyl-CoA	Linking the Wood-Ljungdahl pathway to glycolysis and cell carbon synthesis
GXS	Glyoxylate	CO_2 / Hydroxymalonic semi-aldehyde	Glyoxylate catabolism

Table 1.2: ThDP-dependent enzymes and their impact on metabolism. Abbreviations used: AHAS: acetohydroxyacid synthase; DXP: 1-deoxy-D-xylulose 5-phosphate; DXS: DXP synthase; GAP: D-glyceraldehyde 3-phosphate; GXS: glyoxylate carboligase; 2-HPCL: 2-hydroxyphytanoyl-CoA lyase; OGDH: 2-oxoglutarate dehydrogenase; PDC: pyruvate decarboxylase; PDH: pyruvate dehydrogenase; PFOR: pyruvate ferredoxin-oxidoreductase; PhePDC: phenylpyruvate decarboxylase; PPDC: 3-phosphonopyruvate decarboxylase; TK: transketolase [based on Frank *et al.*, 2007].

1.2.3 The biological relevance of other properties of vitamin B_1 for metabolism

While no physiological function could be assigned to thiamin and its monophosphate so far, recent studies suggest a physiological function not only for ThDP, but for the triphosphoester of thiamin (ThTP) and the adenylated ThTP (AThTP) as well (figure 1.1). Under normal conditions, the total cellular thiamin pool consists mainly of ThDP with minor quantities of the free form (Th), the monophosphate (ThMP) and, caused by a relatively high turnover rate, hardly detectable amounts of thiamin triphosphate (ThTP) and adenylated ThTP (AThTP) (figure 1.3*A*) [Makarchikov *et al.*, 2003; Bettendorff *et al.*, 2007]. Recently, it has been demonstrated that ThTP is able to activate high conductance anion channels in vertebrates and that this molecule is involved in the phosphorylation of various not yet identified proteins in eukaryotic organisms, *e.g.* in the brain of rodents [Nghiêm *et al.*, 2000; Bettendorff *et al.*, 1993].

It is proposed that this phosphorylation is triggered in unfavourable situations, evoking a stress response making the organism more resistant to apoptosis [Makarchikov *et al.*, 2003]. It has also been reported that *Escherichia coli* cultures grown in minimal medium without a carbon source have a significant increase in ThTP, whereas cells cultivated under amino acid starvation, but with a carbon source (*e.g.* glucose) show a transient increase in ThTP, which can represent up to 20% of the total cellular thiamin content [Bettendorff *et al.*, 2007; Lakaye *et al.*, 2004]. The molecular and enzymatic background of this phenomenon still needs to be deciphered. The ubiquitous occurrence of ThTP in bacteria to mammals has led to the proposal that ThTP could play a basic role in metabolism or might be involved in signalling [Makarchikov *et al.*, 2003].

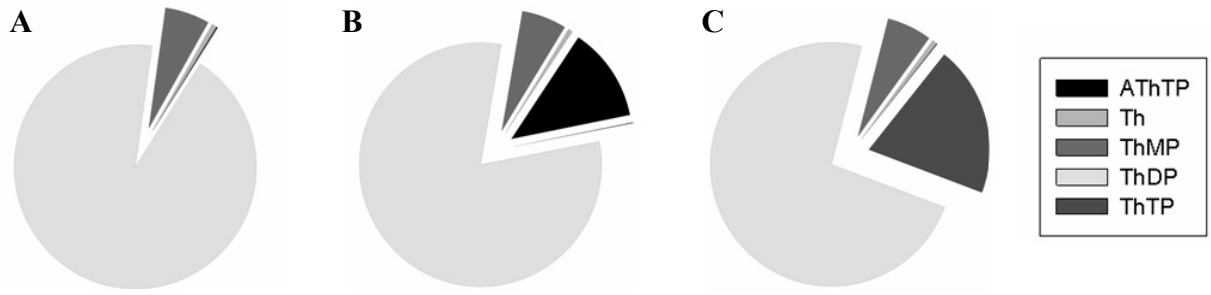


Figure 1.3: Composition of the total vitamin B_1 pool of *E. coli* cultivated under normal conditions ***(A)***, in minimal medium (M9) without ***(B)*** and with 10 mM D-glucose ***(C)***. AThTP and ThTP are hardly detectable under normal conditions ***(A)***, but transiently increase under starvation conditions for carbon (AThTP) ***(B)*** or amino acids (ThTP) ***(C)*** [based on Lakaye *et al.*, 2004; Bettendorff *et al.*, 2007].

1.2.4 Biosynthetic pathways of vitamin B_1

Vitamin B_1 is composed of a thiazole and a pyrimidine moiety (figure 1.1), which are synthesized separately. The pathways are described in the following sections.

1.2.4.1 *De novo* biosynthesis of the thiazole moiety

The biosynthesis of the thiazole moiety, 4-methyl-5(β-hydroxyethyl) thiazole monophosphate (HET-P) is carried out in a sequential and distinct organism-specific way (schemes 1.1, 1.2). In both facultative anaerobic and aerobic bacteria, the thiazole ring is the product of a condensation reaction (under release of a single molecule of carbon dioxide) between DXP and dehydroglycine (DHG). DXP provides the phosphorylated 4-methyl-5-hydroxyethyl group as well as the C-4 – methyl group, whereas the C-2 – N-3 unit derives from DHG [Godoi *et al.*, 2006; Begley *et al.*, 1999]. In aerobic bacteria like *B. subtilis*, DHG originates from the oxida-

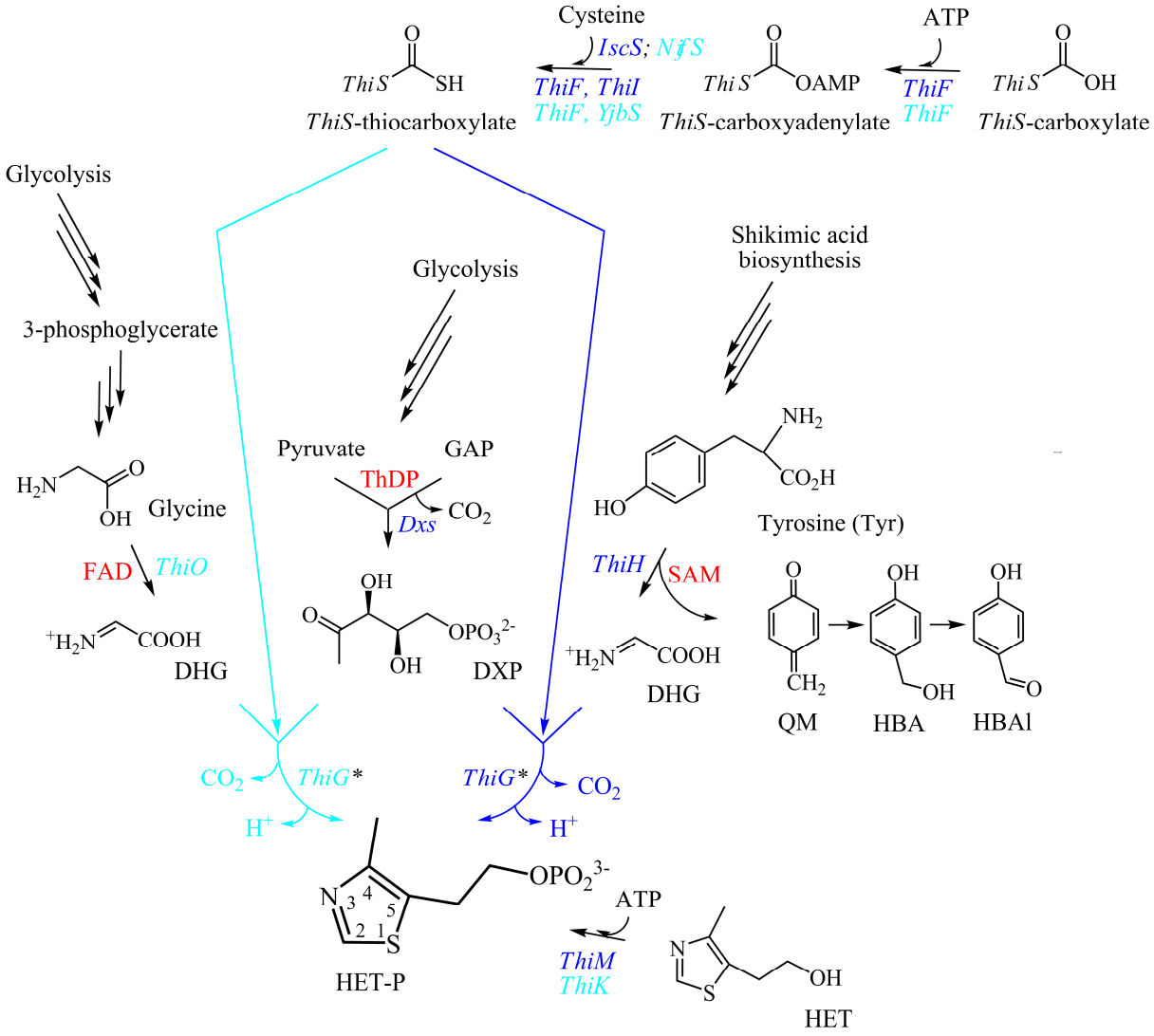
tion of glycine, which in turn is derived from 3-phosphoglycerate *via* serine, an intermediate in the glycolytic pathway. In facultative anaerobic bacteria like *E. coli*, *Salmonella typhimurium* or *Salmonella enterica*, DHG originates from the decomposition of tyrosine in which the benzyl side chain is released as quinone methide [Begley *et al.*, 1999; Kriek *et al.*, 2007]. Tyrosine is one of the end products of the shikimic acid biosynthetic pathway, which occurs exclusively in plants and microorganisms [Colombo *et al.*, 1996]. The sulfur atom at position 1 in the ring structure originates from cysteine [Tazuya *et al.*, 1987].

Most of the gene products involved in the biosynthesis of HET-P in bacteria have been identified. At least six genes are directly implied in the formation of the thiazole moiety (*E. coli* or *B. subtilis*): *IscS* or *NifS*, *ThiF-ThiS and ThiI*, *ThiG-ThiH* or *ThiO* and *ThiG*. It is a common feature that many of the mentioned genes occur in operons transcriptionally regulated by ThDP as a single unit (see section 1.2.5).

IscS (*E. coli*) and NifS (*B. subtilis*) are both PLP-dependent cysteine desulfurases degrading cysteine into alanine and elemental sulfur. The intermediate, a cysteine persulfide bound to the IscS active site, transfers the sulfur directly (*B. subtilis*) or by the action of ThiI (*E. coli*) to the ThiS-carboxyadenylate [Settembre *et al.*, 2003] modifying the C-terminus into a thiocarboxylate catalyzed by ThiF, which exclusively occurs as enzyme complex with ThiS [Xi *et al.*, 2003]. In *E. coli*, IscS activity is dependent on the cofactors ATP and PLP and the proteins ThiF and ThiI [Xi *et al.*, 2003]. In eukaryotes including yeast, no analogs of ThiF and ThiS have been identified [Nosaka *et al.*, 1980].

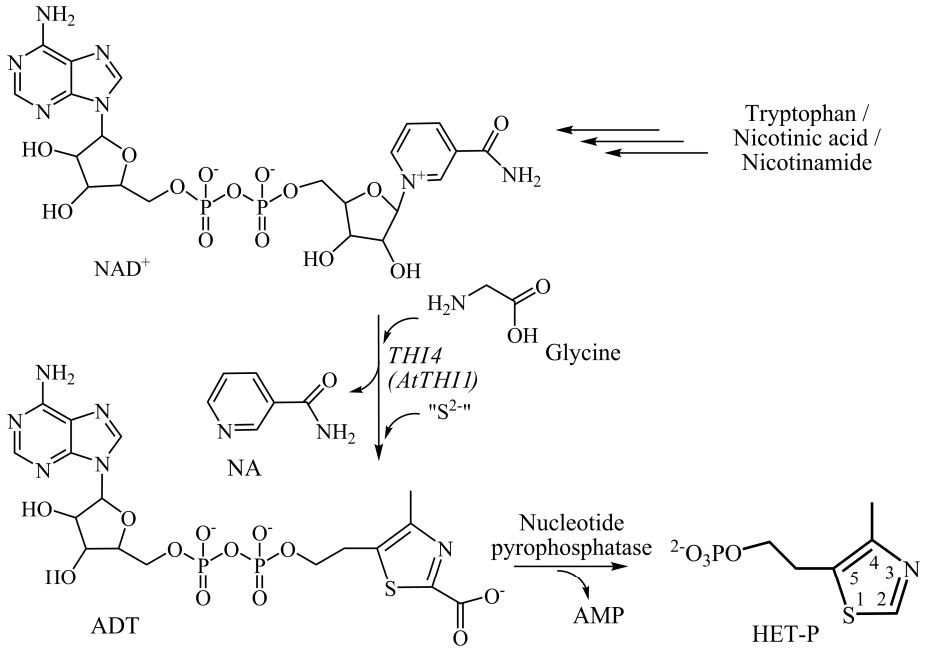
Homologous expression of ThiH and ThiG in *E. coli* revealed that they assemble as large (> 400kDa) complex. ThiH, on one side, shows several similarities to members of the "radical S-adenosyl-L-methionine (SAM)" super family, *i.e.* the conserved cysteine motif Cys-X-X-X-Cys-X-X-Cys that provides cysteine ligands for a labile [4Fe-4S] iron-sulfur cluster and the presence of an iron-sulfur cluster. Furthermore, the weakly defined SAM binding motif Lys-Lys-Val-Thr-Gly-Glu-His-Gln-Ala-Lys-Val has been identified [Leonardi *et al.*, 2003; Martinez-Gomez *et al.*, 2003]. Is has been predicted that SAM is reductively cleaved to the 5'-deoxyadenosyl radical by ThiH. The released radical is involved in the decomposition of tyrosine to release DHG which is subsequently used by ThiG [Kriek *et al.*, 2007]. *ThiG* (*E. coli*, *B. subtilis*) encodes a thiazole synthase which forms the entire HET-P from the immediate precursors DXP, DHG and ThiS-thiocarboxylate. DXP synthase, Dxs, is catalyzing the initial condensation reaction of the non-mevalonate biosynthesis pathway of isoprenoids yielding in DXP [Sprenger *et al.*, 1997; Lois *et al.*, 1998]. Interestingly, activity of Dxs is de-

pendent on ThDP, thus the metabolite is involved in its own biosynthesis [Sprenger *et al.*, 1997; Lois *et al.*, 1998]. ThiO from *B. subtilis* is homologous to D-amino acid oxidases. It is a FAD-dependent protein catalyzing the oxidation of glycine to DHG [Settembre *et al.*, 2003].



Scheme 1.1: Biosynthesis of the thiazole moiety of ThDP in bacteria (blue [facultative anaerobic; *E. coli*], turquoise [aerobic; *B. subtilis*]). Identified genes are indicated in italics, enzymes in capital letters, asterisks signify a possible gene replacement [Morett *et al.*, 2003]. Cofactor requirements are shown in red (FAD, SAM, ThDP). Abbreviations used: DHG: dehydroglycine; DXP: 1-deoxy-D-xylulose 5-phosphate; GAP: D-glyceraldehyde 3-phosphate; HBA: 4-hydroxybenzylalcohol; HBAl: 4-hydroxybenzaldehyde; HET: 4-methyl-5(β-hydroxyethyl) thiazole; HET-P: 4-methyl-5(β-hydroxyethyl) thiazole monophosphate; QM: quinone methide; SAM: S-adenosyl-L-methionine [based on literature cited in the text].

In *Saccharomyces cerevisiae*, a single gene has been related to the biosynthesis of the thiazole moiety so far, *THI4*. Homologs have been identified in archaea as well as in plants. In early studies it has been predicted that the overall reaction mechanism is comparable to that identified in bacteria and is the result of the condensation of DHG and an unidentified D-pentulose 5-phosphate (most probably D-ribulose 5-phosphate or D-xylulose 5-phosphate) and a sulfur atom deriving from a thiocarboxylate [White and Spenser, 1982]. This agrees with the prediction that THI4 (*S. cerevisiae*) and ThiG (*E. coli*) can replace each other [Morett *et al.*, 2003]. However, new evidence has appeared that this is not the case and a complete new route of HET-P biosynthesis has been hypothesized (scheme 1.2) [Chatterjee *et al.*, 2006/2007/2008]. According to this hypothesis, the substrates appear to be nicotinamide adenine dinucleotide (NAD^+), glycine and a yet unidentified sulfur donor. The initial reaction step may be the cleavage of the N-glycosyl bond in NAD^+ accompanied by the release of nicotinamide (NA). A non-oxidative rearrangement of ribose to ribulose is followed by the reaction with glycine. The incorporation of a sulfur atom would lead to the adenylated thiazole (ADT) which remains tightly bound to THI4. A nucleotide pyrophosphatase might catalyze the decomposition in the thiazole moiety, HET-P and AMP, respectively [Chatterjee *et al.*, 2006/2007/2008].



Scheme 1.2: Biosynthesis of the thiazole moiety of ThDP in fungi (*S. cerevisiae*). The identified gene (*THI4*) is indicated in italics. The homolog *THI1* from *A. thaliana* (*AtTHI1*) is given in brackets. Abbreviations used: ADT: adenylated thiazole; HET-P: 4-methyl-5(β-hydroxyethyl) thiazole monophosphate; NA: nicotinamide; NAD^+: nicotinamide adenine dinucleotide; "S^{2-}": unidentified sulfur donor [Chatterjee *et al.*, 2006/2007].

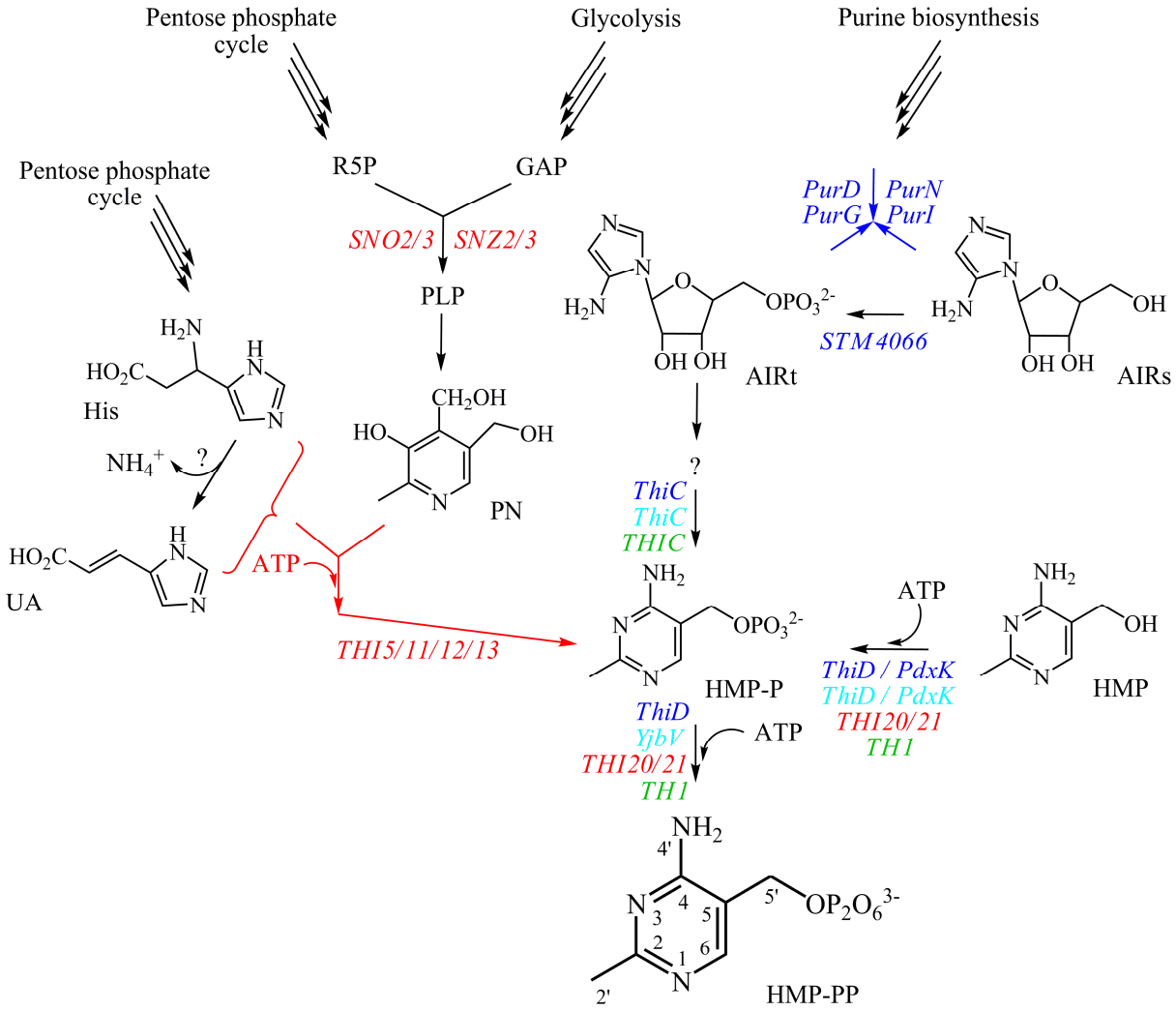
There is only a single report on a plant protein putatively involved in the formation of HET-P: THI1 (At5g54770) from *Arabidopsis thaliana* has been isolated and structurally characterized to be orthologous to THI4 from *S. cerevisiae* [Godoi *et al.*, 2006]. THI1 was initially postulated to be involved in DNA repair [Machado *et al.*, 1996]. Yet, it has not been experimentally shown that THI1 is active in the formation of HET-P.

1.2.4.2 *De novo* biosynthesis of the pyrimidine moiety

The formation of the second subunit, 2-methyl-4-amino-5-hydroxymethylpyrimidine diphosphate (HMP-PP) of ThDP in bacteria is distinct from the fungal pathway (scheme 1.3), but, neither are fully understood. Mechanistic proposals for the biosynthesis are generally based on incorporation experiments with isotopically labelled precursors. However, the final proof of intermediates and cofactors as well as the characterization of both reaction conditions and enzymes is often missing. In higher plants in particular, knowledge on this biosynthetic pathway is sparse.

In both aerobic and anaerobic bacteria, HMP-PP is derived from an intermediate of purine biosynthesis: 5-Aminoimidazole ribonucleotide (AIR_t). The next step, the conversion of AIR_t into 2-methyl-4-amino-5-hydroxymethylpyrimidine monophosphate (HMP-P) has not been elucidated, but results of precursor application experiments suggested that AIR_t cannot be directly metabolized by ThiC which led to the prediction of another intermediate and an additional catalytic activity [Begley *et al.*, 1999]. Precursor incorporation experiments suggested that the pyrimidine moiety arises in a most puzzling intramolecular rearrangement including both the sugar and imidazole moiety of AIR_t (table 1.3) [Estramareix and Therisod, 1984; Estramareix and David, 1990].

ThiD, a HMP-P kinase in *E. coli* (scheme 1.3), subsequently phosphorylates HMP-P to its diphosphate, HMP-PP. This enzyme also phosphorylates 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) to HMP-P and is thus essential in the salvage pathway of HMP-PP (scheme 1.4, see section 1.2.4.3) [Reddick *et al.*, 1998]. In addition to *ThiD*, *PdxK* has been identified in *E. coli*, a gene encoding a pyridoxine (PN) kinase with relatively low substrate specificity. PdxK phosphorylates various pyridine ring structures such as pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), but also HMP [Reddick *et al.*, 1998]. Thus, this enzyme might be involved in the salvage pathways of both ThDP and PLP [Begley *et al.*, 1999]. Recently, the occurrence of both HMP-P kinase (ThiD) and a PN kinase (PdxK) has been confirmed in *B. subtilis* [Park *et al.*, 2004; Newman *et al.*, 2006].



Scheme 1.3: Biosynthesis of the pyrimidine moiety of ThDP in bacteria (blue [facultative anaerobic; *E. coli*], turquoise [aerobic; *B. subtilis*]), fungi (red, *S. cerevisiae*) and plants (green, *A. thaliana*), respectively. The involvement of the plant genes is based on sequence homologies or complementation analyses. Identified genes are indicated in italics. Abbreviations used: AIR_s: 5-aminoimidazole ribonucleoside; AIR_t: 5-aminoimidazole ribonucleotide; DHAP: dihydroxyacetone phosphate; GAP: D-glyceraldehyde 3-phosphate; HMP-P: 2-methyl-4-amino-5-hydroxymethylpyrimidine monophosphate; HMP-PP: 2-methyl-4-amino-5-hydroxymethylpyrimidine diphosphate; R5P: ribose 5-phosphate; UA: urocanic acid [based on literature cited in the text].

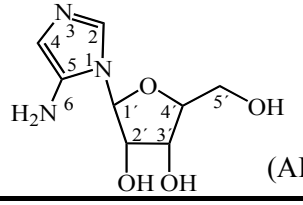
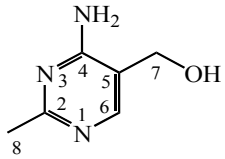
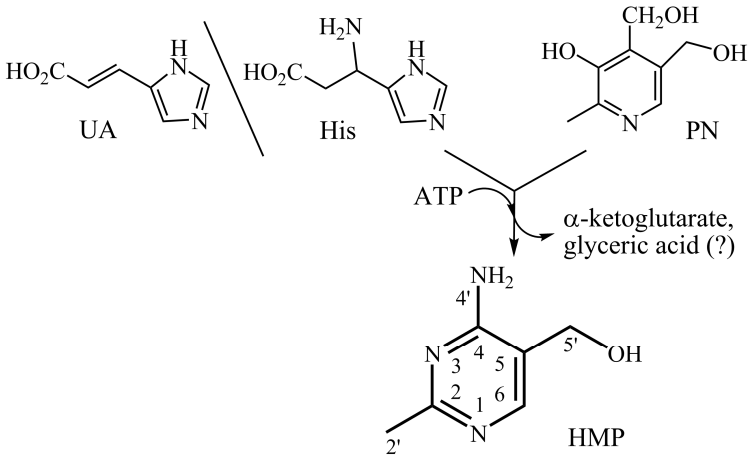
 (AIR$_t$)	 (HMP)
C-2 – N-3 – C-4	C-2 – N-1 – C-6
N-1 – C-5 – NH_2	N-3 – C-4 – NH_2
C-2'	C-8
C-4' – C-5' – OH	C-5 – C-7 – OH^-

Table 1.3: Origin of 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) from 5-aminoimidazole ribonucleotide (AIR$_t$) in bacteria. The Prediction is based on precursor application experiments in *S. typhimurium* and GC-MS analysis [based on Estramareix *et al.*, 1984/1990; Begley *et al.*, 1999].

In yeast, the biosynthesis of the pyrimidine subunit appears to be different from that in bacteria. It has been suggested that HMP-P is directly formed in an ATP-dependent reaction from L-histidine and pyridoxine (PN) [Tazuya *et al.*, 1989/1993/1995b]. However, Zeidler *et al.* [2003b] postulated, based on dilution studies with [^{13}C]formamide and urocanic acid in *S. cerevisiae* and subsequent NMR analyses, that urocanic acid, the degradation product of L-histidine, is the precursor instead. Although there are contradictory arguments regarding the utilization of L-histidine or urocanic acid, the origin and incorporation of the atoms in the pyrimidine moiety of thiamin are consistent throughout the experiments: The fragment C-2' – C-2 – N-1 – C-6 – C-5 – C-5' originates from C-2' – C-2 – N-1 – C-6 – C-5 – C-5' of PN whereas the other three atoms derive from N-3' – C-2' – N-1' of L-histidine/urocanic acid. It is believed that each fragment is transferred as an intact unit (scheme 1.4).



Scheme 1.4: Origin of 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) from L-Histidine (His) or urocanic acid (UA) in yeast. Predicted origin of the atoms based on precursor application experiments in *S. cerevisiae* [based on Tazuya *et al.*, 1989/1993/1995b; Zeidler *et al.*, 2003b].

Three distinct gene families have been related to the formation of the pyrimidine moiety in yeast: *SNO2/3* and *SNZ2/3* and *THI5/11/12/12*, respectively. Each member of the *SNO* and *SNZ* families has undergone preliminary characterization to elucidate its physiological function(s) [Rodríguez-Navarro *et al.*, 2002; Wightman and Meacock, 2003]. While *SNO2* and *SNO3* as well as *SNZ2* and *SNZ3* are almost identical, the third member, *SNO1* and *SNZ1* shares 72%, and 80% amino acid sequence identity, respectively, to the other two. Whereas SNO1 and SNZ1 are required in medium lacking pyridoxine and are thus more involved in maintaining the vitamin B_6 pool during growth (see section 1.3.6.2), apparently *SNO2/3* and *SNZ2/3* are regulated by ThDP [Rodríguez-Navarro *et al.*, 2002], most probably by the so-called *THI*-element, a common feature of genes involved in ThDP synthesis and uptake (see section 1.2.5.1) [Nosaka *et al.*, 2005]. Homologs of SNZ and SNO have been found in archaea, fungi, plants and most bacteria except representatives of the subgroup enterobacteria, *e.g. E. coli* and *S. typhimurium*. Evidence for SNO2/3 and SNZ2/3 being involved in the formation of the pyrimidine moiety of thiamin arose when yeast two hybrid screens indicated an interaction of SNZ2 and THI11. This led to the suggestion that the catalytic product of *SNZ2* (or *SNZ3*), might be efficiently transferred towards THI11 or another member of the THI5 family [Rodríguez-Navarro *et al.*, 2002]. This proposes a role for SNO2/3 and SNZ2/3 exclusively for the biosynthesis of the pyrimidine subunit of vitamin B_1 catalyzed by a multienzyme complex [Rodríguez-Navarro *et al.*, 2002; Nosaka *et al.*, 2006]. However, this has not yet been proven [Mittenhuber, 2001]. Members of the third gene family (*THI5*, *THI11*, *THI12*, *THI13*) share high sequence similarities in the promoter, open reading frames (ORF) and terminator regions. All genes are functionally redundant and exclusively found in *Saccharomyces sensu stricto* species [Wightman and Meacock, 2003]. These species, *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastoranus*, are either used in the fermentation industry or are of special scientific interest [Rainieri *et al.*, 2003]. *THI5*/*11*/*12*/*13* are homologous to *NMT1* and *NMT2* (no message in thiamin) of *Schizosaccharomyces pombe*. The latter are among the most highly expressed genes in this organism, but are completely repressed under thiamin supplementation [Maundrell, 1990; Manetti *et al.*, 1994].

Subsequently, HMP-P is phosphorylated to its diphosphate, the immediate precursor for ThDP, by the functionally redundant proteins THI20 and THI21 in *S. cerevisiae*. Both proteins occur as a fusion of two protein domains distinct in prokaryotes. The N-terminal part has HMP-P kinase activity that was identified and confirmed by complementation experiments and sequence comparisons with ThiD from *E. coli* and *S. typhimurium*. The C-terminus is

homologous to TenA (*ca.* 23% amino acid identity). The function of TenA has not yet been fully elucidated but it might be involved in the regeneration of HMP analogs (see section 1.2.4.3) [Haas Jenkins *et al.*, 2007].

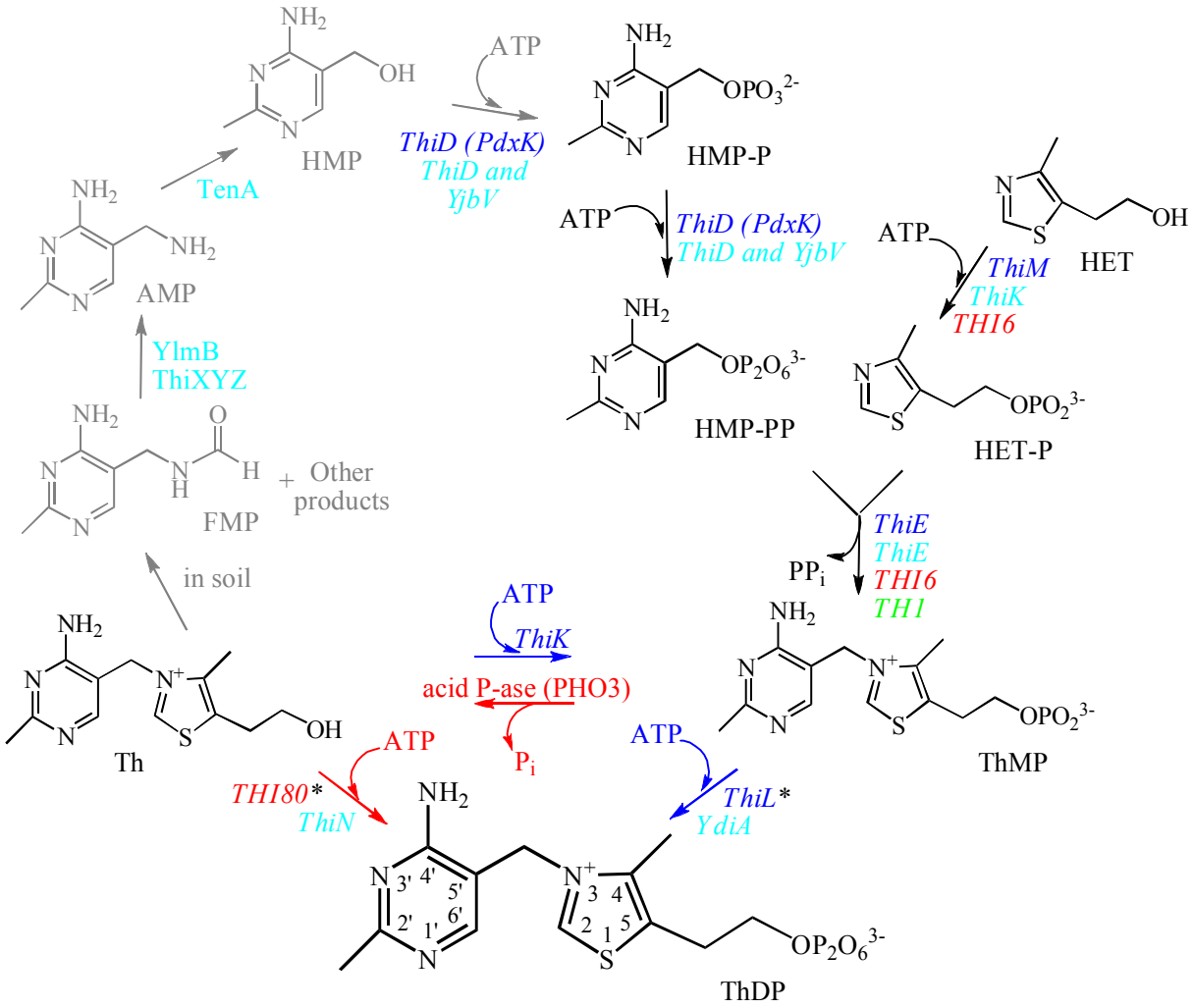
In higher plants, one gene has been related to *de novo* biosynthesis of the pyrimidine moiety of ThDP: *TH1* cDNAs were isolated from *Brassica napus* [Kim *et al.*, 1998] and *A. thaliana* [Ajjawi *et al.*, 2007]. Comparable to THI20/21 and THI6 of *S. cerevisiae*, TH1 occurs as a fusion of proteins which are discrete in prokaryotes (see section 1.2.4.3). In addition, *THIC* was identified in various higher plants, *e.g. A. thaliana* (At2g29630), *Poa secunda* and *Oryza sativa* based on sequence homology to *ThiC* of *E. coli* and *B. subtilis*, but remained to be characterized.

1.2.4.3 The formation of thiamin diphosphate and its distinct salvage pathways

The initial step in the direct formation of thiamin, *i.e.* the combining of HMP-PP and HET-P to yield thiamin monophosphate (ThMP) is ubiquitous among all organisms (scheme 1.5). This reaction is carried out by the thiamin monophosphate pyrophosphorylase (TMPP-ase) ThiE (*E. coli*, *B. subtilis*), THI6 (*S. cerevisiae*) and TH1 (*A. thaliana*, *B. napus*), respectively. A major difference between the prokaryotic and eukaryotic TMPP-ase is that the latter are bifunctional. Plant-originating TH1 carries a C-terminal TMPP-ase activity and a distinct HMP-P/HMP-PP kinase activity at the N-terminus [Ajjawi *et al.*, 2007; Kim *et al.*, 1998]. The yeast homolog THI6 is a fusion protein containing N-terminal TMPP-ase and C-terminal HET kinase domains [Nosaka *et al.*, 1994].

The conversion of ThMP to the diphosphate differs in the organisms investigated so far (scheme 1.5). In representatives of both the α- and γ-division of proteobacteria, *e.g. Rhizobium etli*, *E. coli* and *S. typhimurium*, ThMP is phosphorylated to its diphosphate in an ATP-dependent reaction by the thiamin monophosphate kinase (TMP-ase) ThiL [Miranda-Roís *et al.*, 1997; Kim *et al.*, 1998; Webb and Downs, 1997]. In *S. cerevisiae*, ThMP is dephosphorylated prior to an ATP- and Mg^{2+}-dependent diphosphorylation. The initial dephosphorylation is catalyzed either by an unspecific phosphatase or by the periplasmic ThMP-phosphatase PHO3. The latter is mainly involved in thiamin uptake. The subsequent phosphorylation is carried out by THI80, a thiamin diphosphokinase (TPK) [Nosaka *et al.*, 1993]. In *B. subtilis*, both alternatives may occur: ThMP can be phosphorylated by the TMP-ase encoded by *YdiA* (based on a 37% sequence similarity to *ThiL* of *E. coli* [Rodionov *et al.*, 2002]), and akin to

the fungal way, ThMP can be dephosphorylated by unspecific phosphatases or by Pho3 followed by the ThiN (*YloS*)-mediated diphosphorylation [Melnick *et al.*, 2004].



Scheme 1.5: *De novo* biosynthesis (black) and the proposed salvage pathway (grey) of ThDP and its precursors in bacteria (blue [facultative anaerobic; *E. coli*], turquoise [aerobic; *B. subtilis*]), fungi (red [*S. cerevisiae*]) and plants (green [*A. thaliana*]), respectively. Identified genes are indicated in italics, asterisks signify a possible gene replacement. Abbreviations used: acid P-ase: acid phosphatase; AMP: aminopyrimidine; FMP: Formyl aminopyrimidine; HET: 4-methyl-5(β-hydroxyethyl) thiazole; HET-P: 4-methyl-5(β-hydroxyethyl) thiazole monophosphate; HMP: 2-methyl-4-amino-5-hydroxymethylpyrimidine; HMP-P: 2-methyl-4-amino-5-hydroxymethylpyrimidine monophosphate; HMP-PP: 2-methyl-4-amino-5-hydroxymethylpyrimidine diphosphate; P_i: inorganic phosphate; PP_i: inorganic diphosphate [based on literature cited in the text].

Recently, a new salvage pathway acting in addition to the intracellular *E. coli*-type ThiM-E-D salvage pathway has been proposed: The extracellular recovery of HMP analogs. This pathway employs the hypothetical ABC transporter ThiX-Y-Z, the aminohydrolase YlmB and the

thiaminase II TenA [Haas Jenkins *et al.*, 2007]. The transporter ThiX-Y-Z occurs in the α- and γ-subdivision of proteobacteria and in some firmicutes including the *Bacilli*, *Clostridia* and *Mollicutes*. It is predicted to consist of a transmembrane domain (ThiX), a substrate-binding domain (ThiY) and an ATP-binding domain (ThiZ). ThiY has some amino acid sequence identity with THI5 (*S. cerevisiae*) and THI3 (*S. pombe*), respectively, involved in HMP biosynthesis [Rodionov *et al.*, 2002]. Most probably, this transporter is involved in the uptake of HMP rather than HET analogs to maintain the intracellular level of ThDP [Rodionov *et al.*, 2002]. TenA was previously described as thiaminase II, an enzyme catalyzing the decomposition of thiamin into its two subunits by using water as nucleophile [Toms *et al.*, 2005]. It was initially related to the regulation of extracellular enzymes, but TenA is not essential for cellular growth and extracellular enzyme production [Pang *et al.*, 1991]. Indeed, it often clusters with genes involved in the biosynthesis or transport of ThDP and its processing is strongly repressed by thiamin [Rodionov *et al.*, 2002]. Based on these findings and additional biochemical studies, Haas Jenkins *et al.* [2007] proposed that TenA is involved in the regeneration of HMP-analogs rather than in the degradation of thiamin (scheme 1.5). According to this prediction, thiamin is first decomposed in the basic and neutral environment of the soil. This reaction is catalyzed by a metal-ion-bound hydroxide on the clay surface yielding formyl aminopyrimidine (FMP), amongst other products. FMP could subsequently be transported into the cell by the putative ABC transporter ThiX-Y-Z and converted to aminopyrimidine (AMP) by YlmB. As the final step, AMP is deaminated at C-5' by TenA leading to HMP. The latter could add to *de novo* ThDP biosynthesis. This might be an energy- and metabolite-saving alternative to maintain the ThDP pool. The facts that YlmB is distributed among various species and that TenA has been identified in various organisms not restricted to growth in soil, might indicate that (1) the hydrolysis of FMP is catalyzed by a variety of unspecific enzymes, (2) this salvage pathway is widespread among organisms and (3) TenA might have additional catalytic activities [Haas Jenkins *et al.*, 2007].

Table 1.4: (page 19) Summary of enzymes related to the maintenance of the intracellular ThDP pool in various organisms. (*) The hypothesized participation of SNO2/3 and SNZ2/3 (grey) in the biosynthesis of HMP-PP in yeast has not yet been proven. Abbreviations used: acid P-ase: acid phosphatase; NR T: high-affinity nicotinamide riboside transporter with low thiamin affinity; TMP-ase: thiamin monophosphate kinase; TMPP-ase: thiamin monophosphate pyrophosphorylase; TPK: thiamin diphosphokinase [based on literature cited in the text].

Confirmed activity	*E. coli* (γ-Proteo-bacteria)	*R. etli* (α-Proteo-bacteria)	*B. subtilis* (Eubacte-ria)	*S. cere-visiae* (Fungus)	*A. thaliana* (Plant)	*Pyrococ-cus spp.* (Archaea)
HMP-PP biosynthesis						
	ThiC	ThiC	ThiC		THIC	ThiC
				SNO2/3(*)		
				SNZ2/3(*)		
				THI5/11/ 12/13		
HET-P biosynthesis						
	ThiG	ThiG	ThiG (YjbT)			
				THI4	THI1	
	ThiH	-	-			
	ThiI		YtbJ			
Adenyltransferase	ThiF		ThiF (YjbU)			
Sulfur transfer protein	ThiS (ThiG1)		ThiS (YjbS)			
Cysteine desulfurase	IscS	NifS	NifS/NifZ	NFS1 (YCL017)		
FAD oxidotransferase	-	ThiO	ThiO (YjbR)			
ThDP biosynthesis						
TMPP-ase	ThiE	ThiE	ThiC (ThiE)	THI6	TH1	ThiE
Thiamin kinase	ThiK					
TMP-ase	ThiL		ThiL (YdiA)			
TPK			ThiN (YloS)	THI80		
Salvage pathway						
HMP-P kinase	ThiD/PdxK	ThiD	ThiD (YjbV)	THI4	TH1	
HET kinase	ThiM	ThiM	ThiK (ThiM)	THI6		
Thiaminase II		TenA	TenA	PET18/ THI20/21/ 22		
Aminohydrolase			YlmB			
ABC transporter	ThiX-Y-Z	ThiX-Y-Z				
Transport, uptake						
ABC transporter	ThiB-P-Q	ThiB-P-Q				
Plasma membrane trans-porter			YuaJ	THI10 (7)/ 72		
Plasma membrane per-mease				THI73		
Mitochondrial transporter				TPC1		
NR transporter				NRT1		
Acid P-ase				PHO3		
Regulation						
Transcription factor				THI2 (PHO6)/ PDC2		
Regulatory gene				THI3		

1.2.5 Regulation of the vitamin B_1 biosynthetic pathways

Even though the discovery of vitamin B_1 dates back almost 100 years, the understanding of *de novo* ThDP biosynthetic pathway(s) with regard to the genetic, molecular and biochemical mechanisms is still incomplete. Furthermore, it is only more recently that the regulation of the biosynthesis of this vitamin has been explored. The currently proposed mechanisms of regulation are described below.

1.2.5.1 Riboswitch-mediated transcriptional control

A riboswitch-mediated regulation of ThDP biosynthesis was recently confirmed in prokaryotes and eukaryotes. Riboswitches are domains in the untranslated region of the mRNA and are characterized by an extremely high substrate specificity affording a tight gene regulation by sensing the concentration of a metabolite of low molecular weight, such as ThDP [Bocobza *et al.*, 2008]. In prokaryotes, these mRNA domains are restricted to the 5'-untranslated region, while in fungi and plants it is more randomly distributed among either the 5'- or 3'-untranslated region, respectively, or resides in introns that are absent in the genomes of most prokaryotes [Sudarsan *et al.*, 2003]. The ligand binding can force the formation of a transcription terminating hairpin (most Gram-positive bacteria) or a Shine-Dalgarno sequesting hairpin (most Gram-negative bacteria), thus preventing gene expression at the transcriptional or translational level [Rodionov *et al.*, 2002]. If the ligand binding domain is located in an intron, the formation of out-of-frame codons leading to unstable gene splicing variants of the primary transcript can be initiated.

In the case of vitamin B_1 it was thought that ThDP binds to the strictly conserved mRNA sequence GACCCGTTAACCTGATCCAGTTCATACTGGCGTAGGGA, proposed as “thi box” [Miranda-Roís *et al.*, 1997]. Based on recent results obtained in a comparative analysis of genes, operons and regulatory elements, the “thi box” has been extended towards a rather conserved *THI*-element [Rodionov *et al.*, 2002]. It consists of five helices and a single base stem, where only the first, fourth and fifth helices are conserved at sequence level. The latter two helices include the “thi box” sequence. The structural *THI*-element occurs in many eubacteria and was identified in some archaea of the species *Thermoplasma* as well. It is suggested, that ThDP binds directly to this site forming mRNA-effector complexes. The latter sequesters the ribosome-binding site thus reducing the translation of the mRNA (figure 1.4*A*) [Winkler *et al.*, 2002]. In both rokaryotes and eukaryotes, the “on”-state, as demonstrated for *ThiM* from

E. coli and THIC from *A. thaliana* the ThDP riboswitch promotes translation, whereas the "off"-state inhibits translation (figure 1.4*B*) [Thore *et al.*, 2006]. It is a common feature that many of the bacterial genes associated with the maintenance of the ThDP pool cluster in operons that are subject to regulatory control by ThDP as a single unit, *e.g. ThiC*, *ThiE-ThiF-ThiS-ThiG-ThiH* in *E. coli* [Van der Horn *et al.*, 1993; Leonardi *et al.*, 2003], *yjbR* (*ThiO*)-*ThiS-ThiG-ThiF-yjbV* (*ThiD*)-*TenA* in *B. subtilis* [Rodionov *et al.*, 2002] or *ThiC-ThiE-ThiF-ThiS-ThiG-ThiH*, *ThiM-ThiD* and the ABC transporter operon *ThiB-ThiP-ThiQ* in *S. typhimurium* [Petersen and Downs, 1997; Webb *et al.*, 1998]. Examples of genes that are unaffected by riboswitch-mediated control and do not cluster with any other gene involved in the maintenance of the vitamin B_1 pool are *IscS*, *Dxs* and *ThiI* [Rodionov *et al.*, 2002].

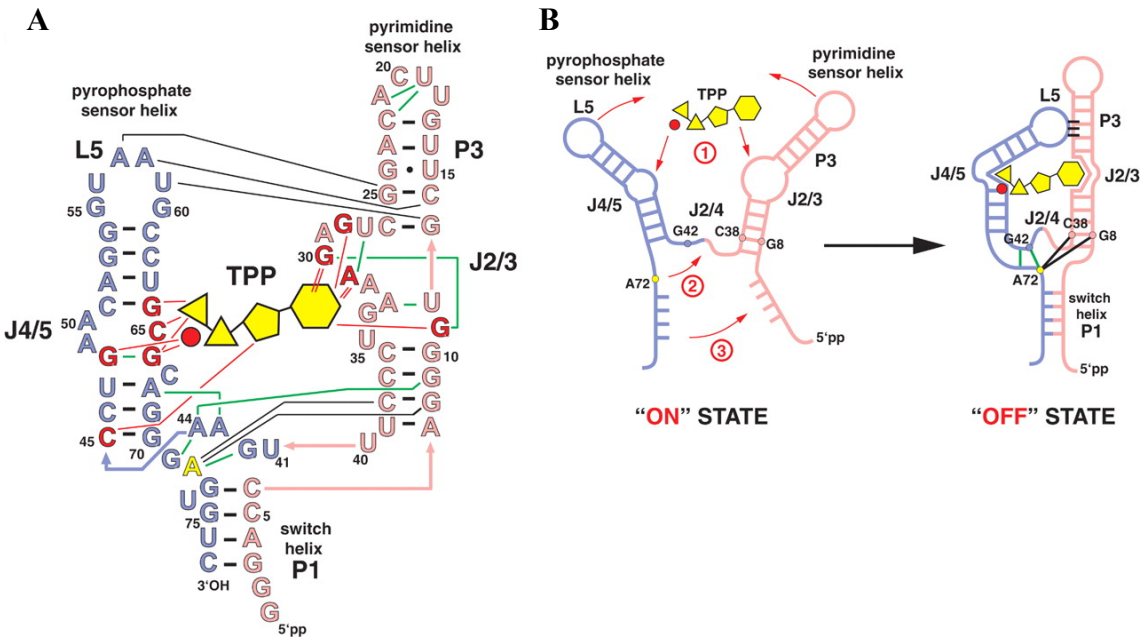


Figure 1.4: Model of the coupling between TPP binding and structural rearrangements in the TPP riboswitch in eukaryotes. *(A)* Summary of the contacts observed between the pyrophosphate (blue) and pyrimidine (pink) sensor helices, TPP, and the switch region. Watson-Crick base pairs are indicated in black, nonstandard base pairs are shown in green, and hydrogen bonds are indicated with thin black lines. TPP is schematically drawn in yellow: Phosphates, triangles; thiazole ring, pentagon; pyrimidine base, hexagon. The Mg^{2+} ion is shown as a red dot. Contacts between nucleotides and magnesium-bound TPP are highlighted in red. Red double-lines indicate stacking interactions with the ligand. Base A72, which forms the crucial assembly platform, is shown in yellow. ***(B)*** Upon TPP binding *(1)*, bulges J2/3 and J4/5 rearrange, thereby closing the sensor helix clamp and forming new hydrogen bonds at the tip of loop L5. The TPP-induced parallel positioning of the sensor helices stabilizes the three-way junction with A72 at the core of the interaction platform *(2)* and promotes the formation of the switch helix P1 *(3)*, which turns the riboswitch "off" [taken from Thore *et al.*, 2006].

Recent reports have deciphered riboswitches as a widespread phenomenon with the capacity to regulate more than 3% of all bacterial genes [Nudler, 2006; Sudarsan *et al.*, 2003]. Whereas in prokaryotes, riboswitches are used to recognize various metabolites like ThDP, flavin mononucleotide (FMN), cobalamin, purine or SAM [Tucker and Breaker, 2005], in eukaryotes only ThDP-dependent riboswitches have been confirmed so far [Thore *et al.*, 2006]. The first report on a riboswitch-mediated gene control in eukaryotes pertained to *THIC* of *P. secunda* [Sudarsan *et al.*, 2003]. Subsequently, its occurrence was confirmed for *THIC* of *A. thaliana* [Thore *et al.*, 2006], *THI4* and *NMT1* of *Neurospora crassa* [Cheah *et al.*, 2007], *THI10* (*THI7*) of *S. cerevisiae* [Enjo *et al.*, 1997] and *THIA* of *Aspergillus oryzae* [Kubodera *et al.*, 2003].

1.2.5.2 Transcriptional control of gene expression (by transcription factors)

To date, several mechanisms for a gene-mediated transcriptional control (by transcription factors) of the biosynthesis and transport of ThDP have been described, but only in yeast [Nosaka, 2006]. Transcription factors in general are characterized by the presence of the conserved N-terminal DNA binding domain G*AACY*TTAGA [Nosaka, 2006; Kowalska and Kozik, 2008]. Examples for transcription factors that positively regulate genes involved in the maintenance of the ThDP pool are THI2 (PHO6), the ThDP-binding protein and intracellular ThDP-sensor THI3 [Nosaka *et al.*, 2005; Hohmann and Meacock, 1998] and PDC2, an isoform of the ThDP-dependent pyruvate decarboxylase. In the absence of ThDP, the three proteins mentioned above form a ternary complex that binds to putative regulatory sequences upstream of *THI* genes, *e.g. THI4/6/20/21/22/80*. By that, the transcription of the genes is induced. At a sufficient cellular ThDP concentration, ThDP binds to THI3 preventing complex formation and impeding transcription of the *THI* genes [Nosaka, 2006]. Whether or not these genetic elements also affect ThDP biosynthesis in prokaryotes or in plants has not been elucidated so far [Begley *et al.*, 1999].

1.2.5.3 Control of the vitamin B_1 level based on uptake and transport

In addition, the level of ThDP is controlled by rates of uptake and intracellular transport. In yeast, the ThDP transporters TPC1 [Marobbio *et al.*, 2002], THI7 (*S. cerevisiae*) [Enjo *et al.*, 1997] and THI9 (*S. pombe*) [Vogl *et al.*, 2008] have been described. TPC1 is a mitochondrial transporter, whereas THI7 and THI9 are homologous and located in the plasma membrane. A

negative correlation between the extracellular thiamin level and the abundance of THI9 at both the mRNA and protein level was reported supporting the idea of ThDP acting as an intracellular signal in repressing genes associated to its synthesis and uptake [Vogl *et al.*, 2008]. PHO3, an acid phosphatase with a high affinity to bind thiamin and its phosphoesters is involved in the uptake of thiamin and its phosphates from the medium [Enjo *et al.*, 1997; Vogl *et al.*, 2008; Nosaka *et al.*, 1980]. In human retinal tissue, hTHTR-1 and hTHTR-2 are involved in thiamin uptake. These transporters are sensitive to the energy state, temperature and pH conditions, and the rate of uptake is inversely correlated to the concentration of the provided thiamin [Subramanian *et al.*, 2007].

In prokaryotes, as stated earlier, the uptake of thiamin and its phosphoesters is related to a class of ABC transporters which supports an ATP-dependent transport of various ions and small molecules into the cell. The protein complex consists of a periplasmic binding protein (PBP), a transmembrane permease and a cytosolic ATPase [Schmitt and Tampé, 2002]. In *E. coli* and *S. typhimurium*, the three genes encoding the components of the thiamin ATP transporter (*ThiB*, *ThiP*, *ThiQ*) are located within a single operon and encode the PBP domain, the transmembrane permease, and the cytosolic ATPase, respectively [Webb *et al.*, 1998].

1.3 Vitamin B_6

1.3.1 The discovery of vitamin B_6

The identification of vitamin B_6 is intimately connected with vitamin B_2 (riboflavin). Young rats fed with a synthetic diet containing only vitamins B_1 and B_2 developed deficiency symptoms like growth retardation and skin lesions (dermatitis acrodynia, pellagra-like dermatitis) especially in the paws, ears and snouts [Györgi, 1956/1971]. In 1934, Györgi identified a compound in a yeast concentrate missing vitamins B_1 and B_2, which was able to prevent and cure the pellagra-like dermatitis in rats [Györgi, 1934]. Four years later, Lepkovsky and others isolated vitamin B_6 in crystalline form [Lepkovsky, 1938]. Subsequently, the chemical structure was identified as 2-methyl-3-hydroxy-4,5-di-(hydroxymethyl)pyridine and its chemical synthesis was established soon after [Harris and Folkers, 1939]. The proposed name pyridoxine (PN, figure 1.5) for the free alcohol became generally accepted. Later on, two more compounds were identified having the same cofactor properties as pyridoxine (PN), 2-methyl-3-hydroxy-4-formyl-5-hydroxymethylpyridine (pyridoxal, PL) and 2-methyl-3-hydroxy-4-amino-methyl-5-hydroxymethylpyridine (pyridoxamine, PM) [Snell, 1944]. In 1944,

tyrosine decarboxylase from *Streptococcus faecalis* was characterized as the first vitamin B_6-dependent enzyme [Gunsalus and Bellamy, 1944].

1.3.2 Nomenclature and specificities of the different forms of vitamin B_6

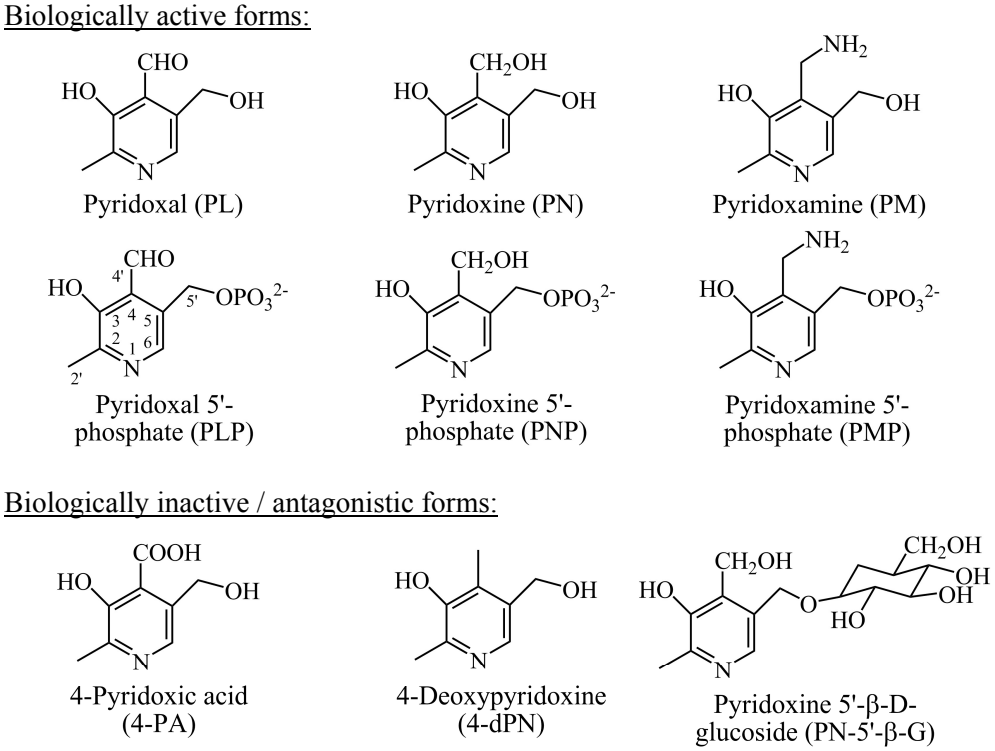


Figure 1.5: The predominant 3-hydroxy-2-methylpyridine derivatives. Pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated analogues PNP, PLP and PMP. 4-Pyridoxic acid (4-PN) is a degradation product of the free forms in bacteria, plants and animals. 4-Deoxypyridoxine (4-dPN) is a potent antagonist of the cofactor, whereas pyridoxine 5'-β-D-glucoside (PN-5'-β-G) is an example of glycosylated derivatives. The accepted numbering of the ring atoms is shown for PLP.

The basic structure of vitamin B_6 consists of a pyridine ring substituted with a methyl, hydroxyl and hydroxymethyl group at C-2, C-3 and C-5, respectively (figure 1.5). Depending on the substituent at C-4, the compound is characterized as pyridoxine (PN, R = CH_2OH), pyridoxal (PL, R = CHO) or pyridoxamine (PM, R = CH_2NH_2). The corresponding monophosphate esters at the hydroxymethyl side-chain at C-5 are classified as PNP, PLP and PMP, respectively (figure 1.5).

Another modification is a glycosidic linkage of one to several sugar molecules, predominantly glucose, at C-5' of PN leading for example to pyridoxine 5'-β-D-glucoside (PN-5'-β-G) or its stereoisomer PN-5'-α-D-G and at C-4' giving pyridoxine PN-4'-α-G. The latter two occur in

microorganisms, whereas the C-5'-glucoside is predominantly found in plants, where PN-5'-β-G can constitute 5 to 70% of the total vitamin B_6 pool [Gregory and Ink, 1987]. Because PN-5'-β-G is poorly metabolized by both rodents and humans, the bioavailability of plant-derived vitamin B_6 is reduced to *ca.* 25% and 58%, respectively. The rate-limiting step is not the intestinal absorption, but rather the enzymatic hydrolysis of the β-glycosidic linkage. Because of this, it is controversially discussed whether the glycosylated PN derivatives are forms of vitamin B_6 [Mahon *et al.*, 1997]. Interestingly, PN is not an exception among other vitamins to be conjugated by glycosylation. Other examples are riboflavin, pantothenic acid, cholecalciferol and niacin. The main contribution of glycosylated natural compounds to the organism might be to maintain the intracellular pool of a given compound through enhanced stability against heat, light and UV irradiation [Gregory, 1998; Nushimura *et al.*, 2008].

An inactive derivative of vitamin B_6 is 4-pyridoxic acid (4-PA, figure 1.5), which occurs in animals, bacteria and plants. It is the main degradation product upon oxidation of PN to PL or upon transamination of PM to PL (see section 1.3.6.3; scheme 1.11) [Burns and Conney, 1960; Stanulović *et al.*, 1976].

A potent antagonist of active B_6 vitamers was identified in animals as 4-deoxypyridoxine (4-dPN, figure 1.5). *In vitro* studies demonstrated that its phosphorylated form, 4-deoxypyridoxine 5'-phosphate, competes with the cofactor form PLP for the active site of vitamin B_6-dependent enzymes, such as tyrosine decarboxylase or aspartate transaminase [Scountzou *et al.*, 1989].

Another vitamin B_6 analog was identified as 4'-O-methoxypyridoxine ("Ginkgotoxin"), the C-4'-O-methylether of pyridoxine, which is present in seeds and leaves of *Ginkgo biloba*. This molecule has neurotoxic properties as it can induce epileptic seizures [Kaye *et al.*, 2002; Samuels *et al.*, 2008]. This finding has been related to an indirect inhibitory effect on glutamate decarboxylase, which is involved in the formation of γ-aminobutyric acid (GABA) caused by a reduced availability of PLP. This is triggered by the inhibition of enzymes involved in its formation, *i.e.* pyridoxal kinase [Kästner *et al.*, 2007].

1.3.3 The nutritional status of vitamin B_6 and its implication to human health

According to the *European Academy of Nutritional Science*s (EANS) a daily intake of 2.0 mg of vitamin B_6 is sufficient for 97-98% of healthy individuals in each stage of life and in both gender groups. As the value strongly depends on the protein taken up, a general model by *Die*

Deutsche Gesellschaft für Ernährung (DGE) suggests an uptake of 0.02 mg vitamin B_6 per gram of ingested protein [http://www.dge.de/pdf/ws/ReferenceValues.pdf]. Supplementation of vitamin B_6 in the human diet is rarely required, but may be essential under various physiological symptoms ranging from weakness, convulsive seizures, appetite and growth depression, anaemia, mental confusion to dermatitis caused by a long-term deficiency. Hypovitaminosis is related to a low-quality and unbalanced, protein-rich diet. In particular, it can occur in alcoholics, elderly people, epileptics, asthmatics and people dependent on medication against tuberculosis, Parkinson's disease or cancer [Merrill and Henderson, 1987]. Many diseases like epilepsy, diabetes, autism (in combination with magnesium) and Parkinson's disease have been positively affected by pharmacological doses of this vitamin. Symptoms of hypervitaminosis have been described following long-term daily intake of 200 mg or more of vitamin B_6 such as dermatitis, numbness in the extremities and neurological disorders [Lieberman and Bruning, 2003].

Next to physiological consequences, a variety of biochemical consequences that can occur after extensive intake of vitamin B_6 have been described as depression of antibody circulation and biosynthesis of nucleic acids and proteins. These effects are explainable by the central role of PLP predominantly in amino acid metabolism [Linkswiler, 1967]. Apart from this, elevated intracellular levels of vitamin B_6 in animal systems have a broad effect on systemic homeostasis. The repression of glucocorticoid hormone receptors [Tully *et al.*, 1994] and the inhibition of various genes, either related or completely unrelated to vitamin B_6, *e.g.* cytosolic aspartate aminotransferase, tyrosine aminotransferase and serum albumin, have been described. In particular, the expression of proteins carrying a binding fold for phosphorylated substrates or effectors, like both RNA and DNA polymerase or reverse transcriptase, is inhibited by PLP [Oka, 2001].

1.3.4 Nomenclature of enzymes dependent on vitamin B_6

According to the *Enzyme Commission (EC) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (NC-IUBMB, http://www.chem.qmul.ac.uk/iubmb/enzyme/), almost 1.5% of the genome in free-living prokaryotes encode vitamin B_6-dependent enzymes. Indeed, 145 catalytic reactions, representing *ca.* 4% of all enzymatic activities, have been classified so far.

1.3.4.1 An activity-based classification

Vitamin B_6-dependent enzymes exhibit a broad catalytic and structural diversity. Indeed, they represent the largest and most diverse group catalyzing cofactor-dependent reactions. They occur within five of the six general enzyme classes as described by the *Enzyme Commission* (EC) *of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (NC-IUBMB, http://www.chem.qmul.ac.uk/iubmb/enzyme/). Transaminases (EC 2.6.1) are the largest group among them (table 1.5). While a few exceptions are related to carbohydrate and lipid metabolism, the majority of enzymes dependent on vitamin B6 as a cofactor act in the intra- and extracellular metabolism of amino acids. The most predominant reactions that are catalyzed in dependence of vitamin B_6 are decarboxylation and transamination reactions. Other reactions like the interconversion of L- and D-amino acids by racemization and the removal or replacement of chemical groups located at C_β or C_γ of amino acid substrates can depend on PLP/PMP as well (see section 1.3.5) [Drewke and Leistner, 2001; Percudani and Peracchi, 2003].

Classification (EC)	Example	Total score
1. Oxidoreductases		**1**
1.4.4.2	Glycine dehydrogenase	
2. Transferases		**80**
2.4.1.1	Phosphorylase	
2.5.1.47	Cysteine synthase	
2.6.1.27	Tryptophan transaminase	
3. Hydrolases		**2**
3.5.99.7	1-Aminocyclopropane-1-carboxylate deaminase	
3.7.1.3	Kynureninase	
4. Lyases		**52**
4.1.1.15	Glutamate decarboxylase	
4.2.1.20	Tryptophan synthase	
4.2.3.1	Threonine synthase	
5. Isomerases		**13**
5.1.1.13	Aspartate racemase	
5.4.3.8	Glutamate-1-semialdehyde 2,1-aminomutase	
6. Ligases		**0**

Table 1.5: Activity-based classification of vitamin B_6-dependent enzymes (recommended by the *Enzyme Commission* (*EC*)). The four-digit number of each enzyme classifies the general type of reaction (1st digit), the enzyme family (2nd digit), its subfamily (3rd digit) and the enzyme itself (4th digit). The complete list can be found in [Percudani and Peracchi, 2003].

1.3.4.2 Classification according to sequence and structural similarities

In addition to a functional-related taxonomy, an evolutionary-based classification according to sequence and structural similarities is recently being preferred for the characterization of vitamin B_6-dependent enzymes [Grishin *et al.*, 1995; John, 1995; Christen and Mehta, 2001]. Sequence analyses of all enzymes identified so far requiring vitamin B_6 revealed that no overall sequence identity exists among them: Size, oligomeric state, catalytically active form and stability widely vary [Grishin *et al.*, 1995; John, 1995; Kern *et al.*, 1999; Christen and Mehta, 2001]. However, unique regions exist which are involved in the binding of the cofactor in particular, like a conserved lysine residue in the enzyme's PLP-binding site (it forms the Schiff base with the aldehyde group of PLP) and a glycine-rich loop with the general motif GXGXXG (*e.g.* GVGGGPGG [D-serine dehydratase from *E. coli*], GNGLLGNG [glycogen phosphorylase b from rabbit muscle], VGYGGGY [alanine racemase from *S. typhimurium*]) have been identified [Marceau *et al.*, 1990]. Amino acids functioning as hydrogen bond donors (*e.g.* Ser, Asp, Thr, Arg) interact with the phosphate group of PLP. Usually, aspartic acid (or more constrained a tyrosine or a methionine residue) can form a salt bridge to the N-1 of the cofactor to maintain the protonation of the cofactor and thus, its functionality (figure 1.6) [John, 1995; Eliot and Kirsch, 2004].

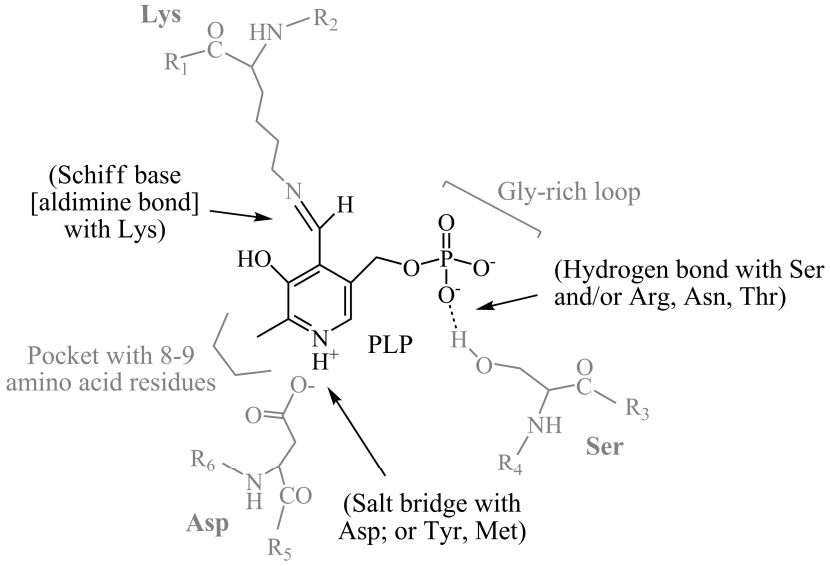


Figure 1.6: A scheme of conserved PLP-binding sites in enzymes dependent on vitamin B_6 as a cofactor. Despite a low level of overall sequence similarity, many amino acids interacting with the coenzyme are conserved and arranged in a similar pattern. The cofactor is presented in its imine form (black) bound to lysine. Residues indicated as R_1 to R_6 illustrate neighbouring amino acids in the enzyme (shown in grey) [based on literature cited in the text].

This alternative classification is mainly based on the relative orientation of the above mentioned motifs to each other and on folding characteristics of the protein. A polyphyletic origin has been proposed that leads to the well-characterized fold-types I to V, which include a variety of the common reaction types. Although the structures of several enzymes belonging to fold types VI and VII have been solved, they have not yet been conclusively specified [Eliot and Kirsch, 2004]. The structural features of each fold-type are summarized in table 1.6. Each fold-type includes a variety of common PLP-dependent reaction types and a single reaction type, based on structural features of the catalytic enzyme, can be related to more than one fold-type. For example, the bacterial alanine racemase belongs to fold-type III, whereas serine racemase and the fungal alanine racemase are classified in fold-types II and I, respectively [Eliot and Kirsch, 2004].

FT	Structural characteristics	Representatives (*e.g.*)
I	N-term --- Gly-rich domain --- hydrophobic β-strand --- Asp*[1] --- Lys ---C-term Planar β-sheets at N-term	**TA**: class I: aspartate transaminase (2.6.1.1) class II: glycine acetyltransferase (2.3.1.29) class III: β-alanine-pyruvate transaminase (2.6.1.18) class V: phosphoserine transaminase (2.6.1.52) **DC**: prokaryotic ornithine decarboxylase (4.1.1.17) **Lyases**: cystathionine γ-synthase (2.5.1.48)
II	N-term --- loop structure --- Lys --- Gly-rich domain --- Asp/Glu/Ser --- C-term Planar β-sheets at N-term Regulatory domain	**Synthases**: tryptophan synthase β subunit (4.2.1.20); threonine synthase (4.2.3.1) **Lyases**: D-serine ammonia-lyase (4.3.1.18)
III	N-term --- hydrophobic β-strand --- Lys --- Gly-rich domain --- Insertion*[2] --- C-term $(\beta/\alpha)_8$ barrel structure at N-term	**DC**: eukaryotic ornithine decarboxylase (4.1.1.17) **Racemases**: alanine racemase (5.1.1.1)
IV	N-term --- Lys --- Glu --- Asn --- C-term	**TA**: class IV: D-alanine transaminase (2.6.1.21)
V	Lactate dehydrogenase fold at C-term	**GTA**: Glycogen phosphorylase (2.4.1.1); starch phosphorylase (2.4.1.1)
VI	n.s.	**TA**: Succinyldiaminopimelate transaminase (2.6.1.17)
VII	n.s	**TA**: Valine-pyruvate transaminase (2.6.1.66)

Table 1.6: Classification of vitamin B_6-dependent enzymes into fold-types according to sequence and structural similarities. "---" indicate conserved residues, strands, helices, barrels and/or loops in the protein structure. The *EC*-numbers are given in parentheses. Abbreviations used: C-term: C-terminus; DC: decarboxylases; FT: fold type; GTA: glycosyltransferases; N-term: N-terminus; n.s. not specified; TA: transaminase. *[1]: Asp can be replaced by Ala, Ser, Tyr, Val, Ile or Met. *[2]: Insertion: a domain boundary between the N- and C-terminus is often encoding the catalytic active site [based on literature cited in the text].

1.3.5 The cofactor role of pyridoxal 5'-phosphate (and pyridoxamine 5'-phosphate)

As stated above, vitamin B_6 exhibits the largest versatility among cofactors involved in enzymatic reactions. The majority of all vitamin B_6-dependent enzymes, in particular those which are involved in amino acid metabolism, uses the aldehyde group of PLP to form a Schiff base with a conserved lysine residue in the enzyme's PLP-binding site. Dependent on the position where the amino acid substrate is enzymatically modified, different simple and combined reaction types are common (figure 1.7*A*, *B* and section below). In contrast, glucan phosphorylases, *i.e.* glycogen phosphorylase and starch phosphorylase, depend on the phosphate group at C-5' rather than on the aldehyde group at C-4' [Palm *et al.*, 1990]. These enzymes are found in all organisms and tissues from bacteria, higher plants and mammals where they play an essential role in the carbohydrate metabolism.

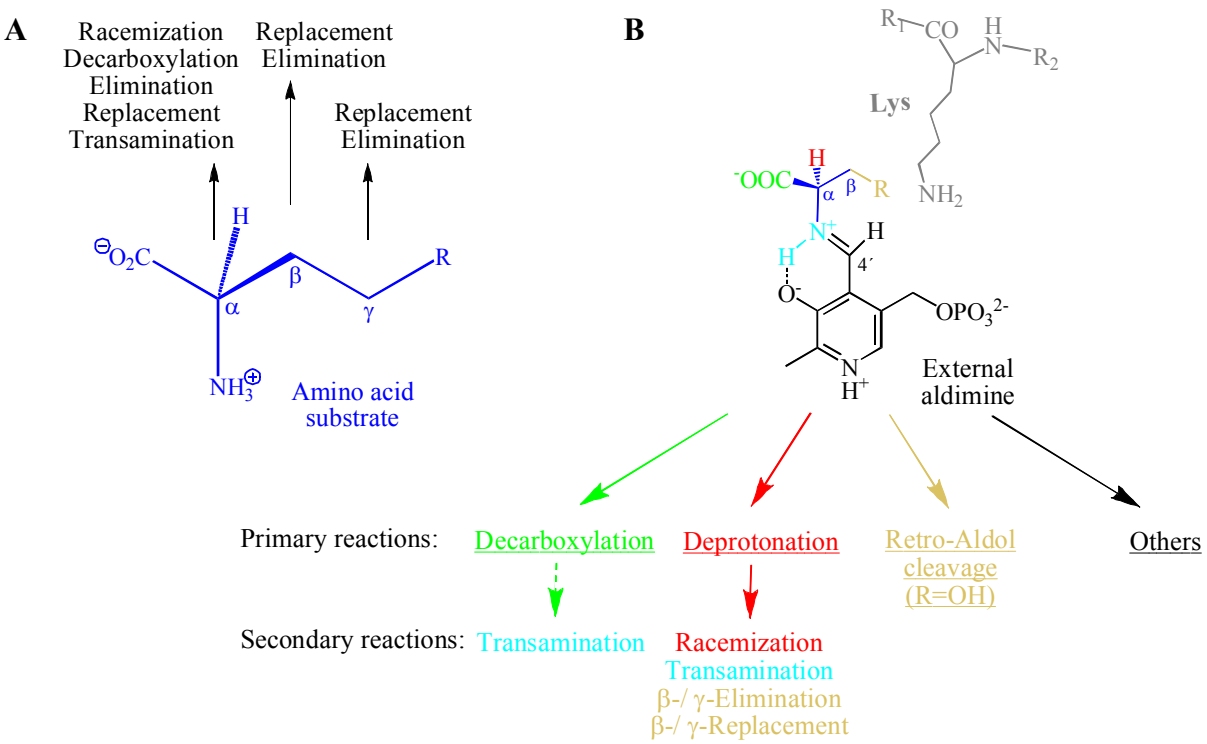
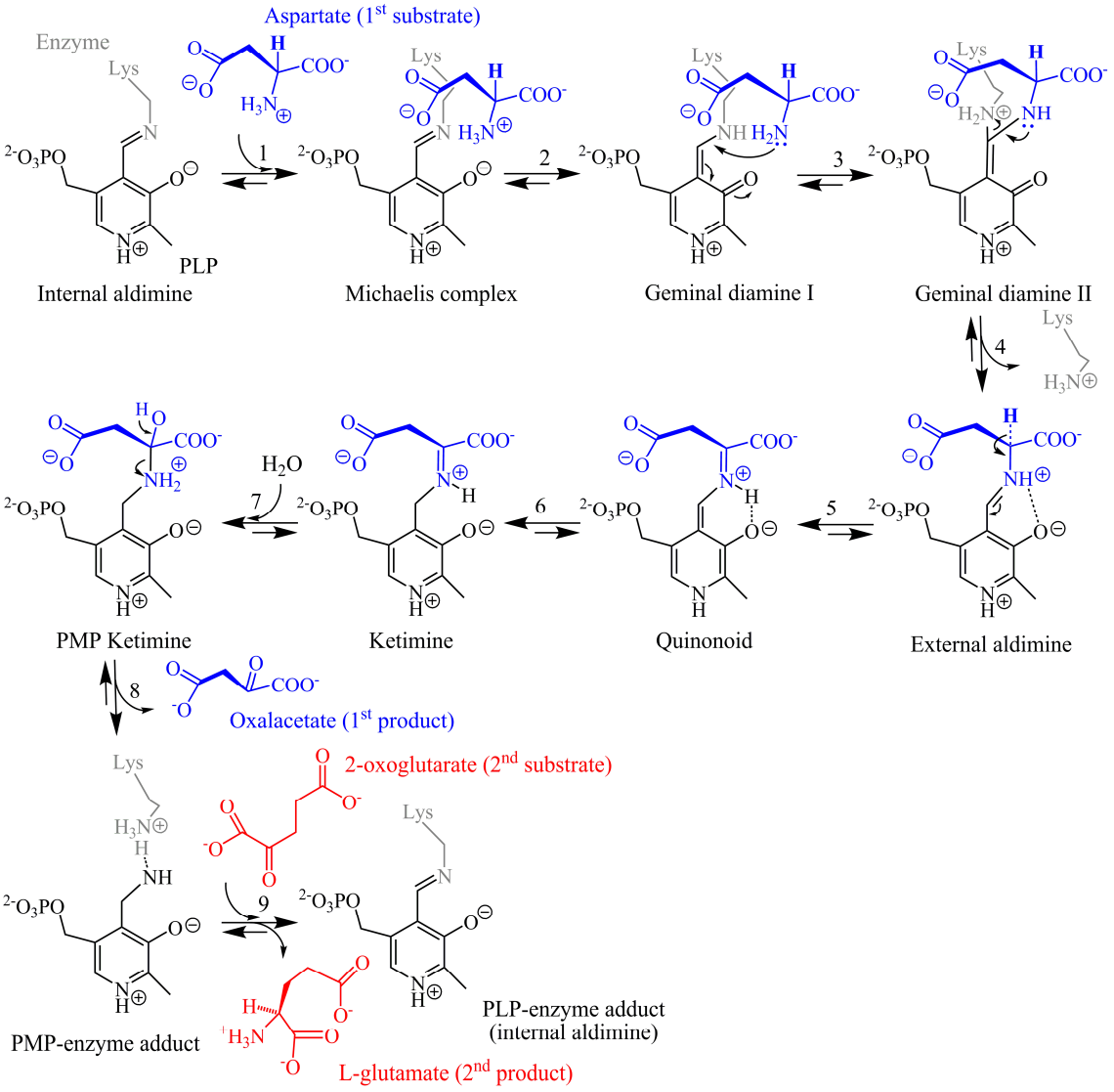


Figure 1.7: Basic reaction types facilitated by PLP (or PMP) on amino acid substrates. The reaction type depends on the relative orientation between PLP (black) and the substrate (blue) as hypothesized by Dunathan [Dunathan, 1966] *(A)*. The reaction diversity appears only after formation of the external aldimine. Depending on the relative orientation between substrate and cofactor either one of the bonds at C_α is cleaved (coloured arrows) initiating a primary reaction (underlined). Secondary reactions can occur (constrained: vertical solid arrow; not-constrained: vertical dashed arrow). The colour points out where the reaction is taking place in the substrate *(B)* [based on literature cited in the text].

Despite the fact that vitamin B_6-dependent reactions are diverse, they share a common principle in the first step of catalysis (scheme 1.6): PLP forms a Schiff base with the ε-amino group of a lysine residue which is conserved in the active site of the enzyme (internal aldimine). In the presence of the substrate, the internal aldimine is converted *via* the geminal diamine intermediates I and II to an external aldimine. The latter is characterized by a Schiff base between the aldehyde group of PLP and the amino group at C_α of the substrate. In the intermediate states, both the lysine and the substrate bind to C-4' of PLP. Subsequently, a proton transfer from the nitrogen atom of the substrate to the nitrogen atom of the lysine ensues. It has been discussed whether or not it would be more efficient to directly form the external aldimine between the aldehyde group of free PLP and the amino group at C_α of the substrate [Schonbeck *et al.*, 1975]. Non-enzymic model determinations revealed that no overall direct kinetic advantage results from the Schiff base between lysine and PLP. Indeed, the nucleophilic attack and subsequent transaldimation of the imine bond between lysine and PLP (to form the external aldimine) is two to four times slower compared to the use of free PLP. It has been speculated that the Schiff base formation between lysine and PLP is due to an increase of the association constant in a PLP-scarce environment [Schonbeck *et al.*, 1975]. Although with formation of the external aldimine the bond between the lysine residue and the cofactor is broken, the cofactor remains bound to the enzyme at various amino acid residues throughout catalysis (figure 1.6; scheme 1.6). Once the external aldimine is formed, subsequent reaction specificity is a function of the properties of the enzyme, *i.e.* its tertiary structure, coordinates the relative orientation and properties of the binding folds for both PLP and the substrate. The phenomenon is explained by Dunathan's hypothesis for reactions at C_α of an amino acid substrate (see section 1.3.5.1) [Dunathan, 1966]. After coordinating the substrate in the external aldimine it is subsequently modified by electron transfer. Here, the nitrogen atom (N-1) of the cofactor molecule acts as electron sink. Catalysis proceeds through delocalization of an electron from the substrate (or from an amino acid residue of the enzyme) *via* N-1 to the lysine residue. By that, the corresponding bond at C_α of the substrate is cleaved and the quinonoid intermediate is formed. The directed release of the charge from N-1, along with the transfer of protons or second substrates, results in the formation of new bonds, which leads to either a coenzyme-product-aldimine adduct as in the case of alanine racemases [Eliot and Kirsch, 2004] or to a ketimine intermediate as in the case of aspartate transaminases [Christen and Metzler, 1985]. After subsequent modifications, the latter yields the aldimine

adduct of the product with the coenzyme [Christen and Mehta, 2001]. Subsequently, the product is released and the cofactor in its PMP-form remains bound to the enzyme. In the second half of the reaction, as it has been demonstrated for transaminases, the cofactor is regenerated by donating the amino group to a 2-oxo acid, *e.g.* 2-oxoglutarate, yielding PLP and the corresponding amino acid, *e.g.* L-glutamate (scheme 1.6) [Eliot and Kirsch, 2004].



Scheme 1.6: PLP-dependent catalysis exemplified for aspartate transaminases. The binding of the substrate aspartate (blue) to the internal aldimine causes formation of the Michaelis complex *(1)*. A directed proton transfer from the substrate to the 3-phenolic oxygen of PLP (black) forces transaldimation, thus the enzyme (grey) is displaced by the substrate *(2-4)*. Dissociation of the C_α–H bond yields in the quinonoid intermediate *(5)*. Protonation at C-4' and subsequent hydrolysis gives the ketimine *(6)* and PMP-ketimine *(7)*. Release of the product causes an interaction between Lys and PMP *(8)*, which is recovered as PLP by converting 2-oxoglutarate to L-glutamate *(9)* [based on Christen and Metzler, 1985].

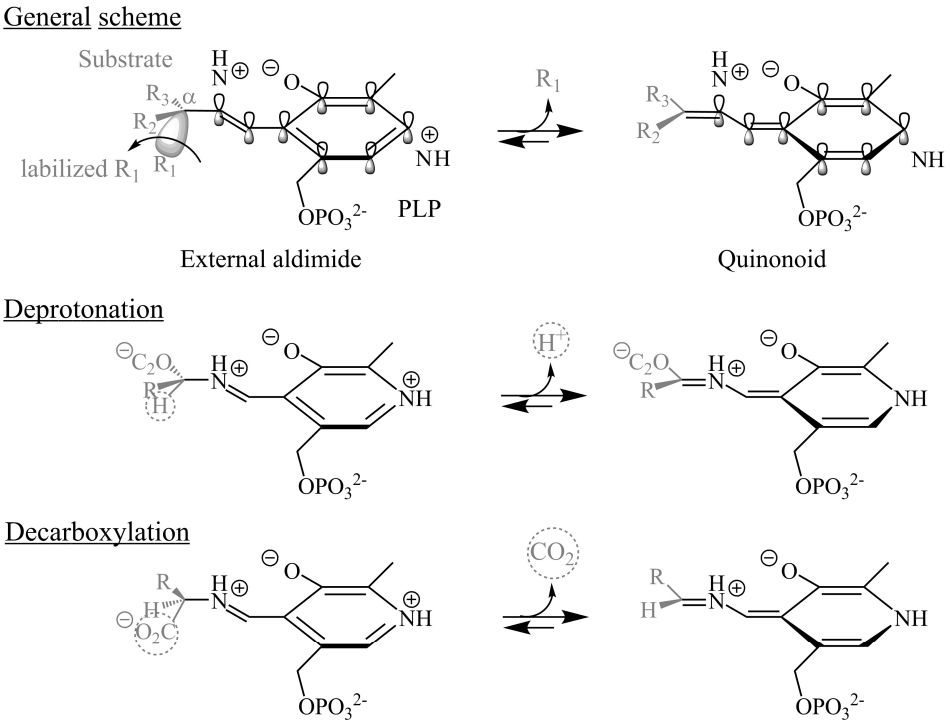
These reactions can occur, albeit very slowly, in the absence of an enzyme. This finding is based on the two essential properties of the cofactor, *i.e.* the ability to form a Schiff base with an amino group and the ability to act as an electron sink. But only the enzyme can provide a unique environment and thus a high substrate and reaction specificity [John, 1995; Christen and Mehta, 2001].

So far, only a single enzyme has been identified that uses PMP rather than PLP: CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-dehydrase (El). This enzyme catalyzes an unprecedented one electron redox reaction in the course of forming 3,6-dideoxy sugars, *e.g.* ascarylose, paratose, abequose and tyvelose. These sugars play an important role in bacterial pathogenicity [Rubenstein and Strominger, 1974]. In E1, the lysine residue conserved in the active site of PLP-dependent enzymes is replaced by histidine. Furthermore, an unusual [2Fe-2S] iron-sulfur cluster and the binding motif (C-X-C-X-C-X-C) have been identified. The reaction is initialized by the formation of a Schiff base between the amino group of PMP and the substrate. After isomerisation of the Schiff base, an electron is translocated to N-1 of PMP. Release of the hydroxyl group at C-3 yields an intermediate which is subsequently reduced by E3, a cytidine diphosphate (CDP)-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-reductase, in a NAD(P)H-dependent manner [Lei *et al.*, 1995; Smith *et al.*, 2008]. Interestingly, site-directed mutagenesis of four specific residues in the active site convert E1 to a PLP-dependent transaminase, which releases L-glutamate for the recovery of PLP in the second half of the reaction [Smith *et al.*, 2008].

1.3.5.1 Dunathan's hypothesis

The phenomenon of reaction specificity for PLP-dependent enzymes is explained by Dunathan's hypothesis for reactions at the C_α of the substrate [Dunathan, 1966]. Despite the fact that there is not much experimental evidence, this hypothesis is widely accepted [Toney, 2005]. The active site of a vitamin B_6-dependent enzyme coordinates the relative orientation of substrate and cofactor in such a way that the bond at C_α of the substrate that is to be cleaved lies parallel to the π-orbitals of the pyridine ring, thus achieving maximum overlap. This leads to a lowering of the transition state energy and to an increase in the reaction rate leading to the release of either H^+, CO_2 or the R-group, respectively (scheme 1.7) [Dunathan, 1966; Eliot and Kirsch, 2004; Toney, 2005]. The strict governing of several parameters, *e.g.* electron flow, proton transfer and the flux of water molecules, is assured by a conformational

change of the enzyme to favour, for example, transamination or racemization [Eliot and Kirsch, 2004; Toney, 2005].



Scheme 1.7: The Dunathan stereoelectric hypothesis. The reaction specificity of enzymes dependent on vitamin B_6 as a cofactor is explained by the relative orientation between the cofactor (black) and the substrate (grey). Exclusively the bond at C_α of the substrate that is to be cleaved is aligned with the π-orbitals of the cofactor exemplified for primary reaction types, *i.e.* deprotonation and decarboxylation. R, R_1, R_2, R_3 are unspecified side chains of the substrate [adapted from Eliot and Kirsch, 2004; Toney, 2005].

1.3.6 Biosynthetic pathways of vitamin B_6

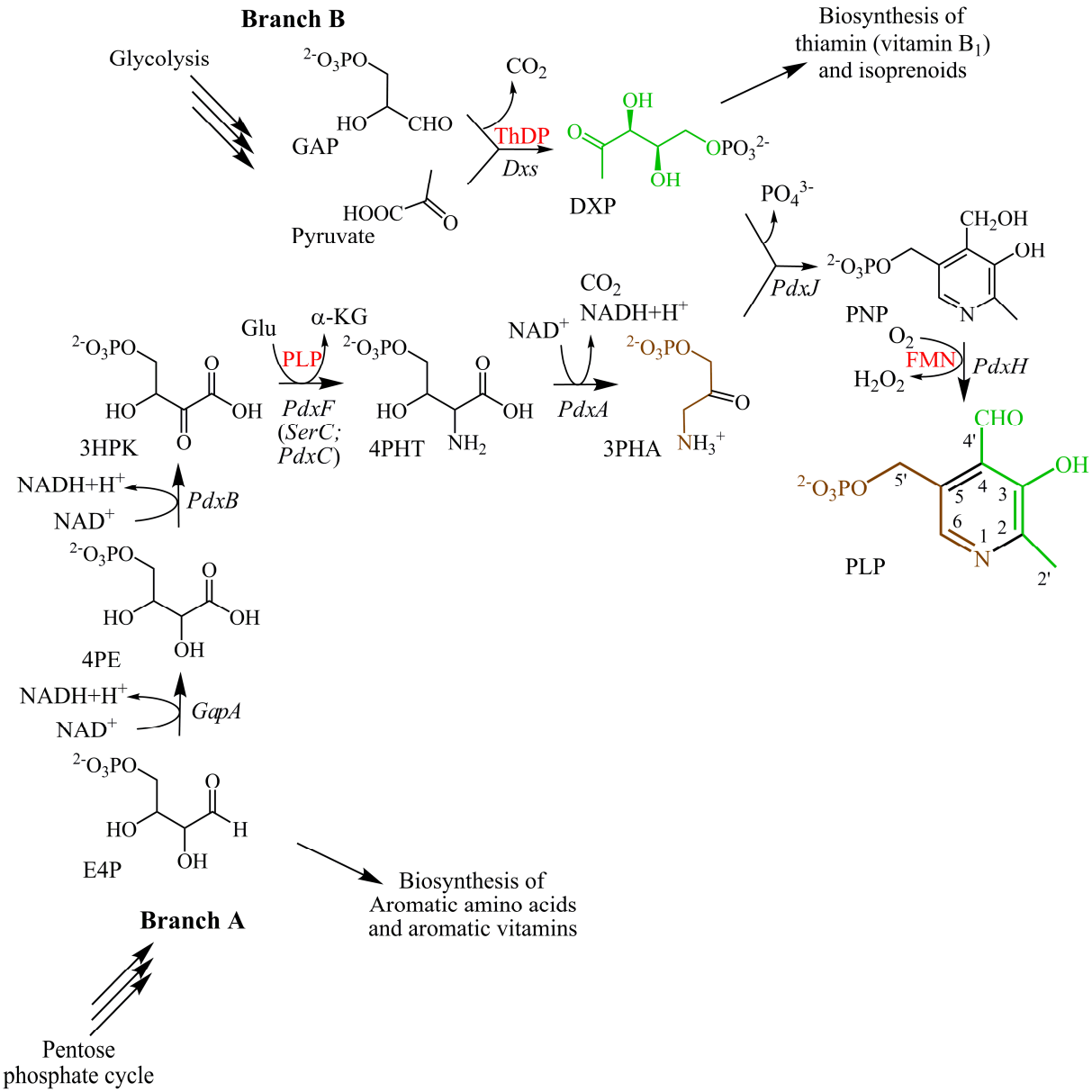
De novo biosynthesis of this vital compound was first explored in the model organism *E. coli* (see section 1.3.6.1) and was suggested to be ubiquitous in all organisms able to synthesize vitamin B_6 [Drewke and Leistner, 2001]. However, analyses of *C. nicotianae* mutated in the highly conserved gene *SOR1* led to PN auxotrophy. Notably, this gene showed no homology to any present in *E. coli* [Ehrenshaft *et al.*, 1998/1999b; Osmani *et al.*, 1999]. The presence of *Sor1* homologs in archaea, bacteria, fungi and plants led to the proposal of an alternative route of *de novo* vitamin B_6 biosynthesis (see section 1.3.6.2). Despite this, a salvage pathway has been identified in all living organisms (see section 1.3.6.3) [Garrido-Franco, 2003].

1.3.6.1 The 1-deoxy-D-xylulose 5-phosphate dependent pathway ("*E. coli*-like pathway")

De novo biosynthesis of PLP in γ-proteobacteria, *e.g. E. coli* and *S. typhimurium*, utilizes 4-phosphohydroxy-L-threonine (4HPT) and 1-deoxy-D-xylulose 5-phosphate (DXP), which are synthesized independent from each other (scheme 1.8). Incorporation experiments revealed that two single units build the entire C-skeleton of PLP: C-2' – C-2 – C-3 – C-4 – C-4' derives from DXP, whereas N-1 – C-6 – C-5 – C-5' originates from 4PHT (scheme 1.8) [Hill *et al.*, 1996]. 4PHT, a non-proteinogenic amino acid, derives from D-erythrose 4-phosphate (E4P), an intermediate in the pentose phosphate biosynthetic pathway. The central role of E4P in vitamin B_6 biosynthesis was confirmed in *E. coli* K12 mutant strains lacking the transketolases encoded by *TktA* and *TktB* that were dependent on supplementation with amino acids and PN [Zhao and Winkler, 1995]. E4P is also indispensable for the biosynthesis of aromatic compounds, *e.g.* L-phenylalanine, L-tyrosine, L-tryptophan and *p*-aminobenzoate, *p*-hydroxybenzoate and 2,3-dihydroxybenzoate, *via* the shikimate pathway in both bacteria and plants [Gibson and Pittard, 1968].

The formation of one of the two precursors, 4PHT, is initialized by a NAD^+-dependent oxidation of E4P to 4-phosphoerythronate (4PE) (branch A in scheme 1.8), catalyzed by the glyceraldehyde 3-phosphate dehydrogenase (GAPDHase, GapA) [Yang *et al.*, 1998b]. The second oxidation, *i.e.* the conversion of 4PE to 3-hydroxy-4-phosphohydroxy-α-ketobutyrate (3HPK) is catalyzed by PdxB, a NAD^+-dependent 2-hydroxy acid dehydrogenase. PdxB shares some amino acid sequence similarity with the serine biosynthesis enzyme SerA, *e.g.* conserved binding sites for both the cofactor NAD^+ and the substrate, which suggests a common evolutionary ancestor and a relationship between the two biosynthetic pathways in *E. coli* [Schoenlein *et al.*, 1989]. In the final transamination reaction, *i.e.* the conversion of 3HPK to 4PHT, L-glutamate acts as the nitrogen donor and α-ketoglutarate is released. This reaction is catalyzed by PdxF (SerC), a transaminase, which requires PLP as cofactor [Drewke *et al.*, 1996]. Thus, *de novo* biosynthesis of one of the two immediate precursors can only be completed in the presence of a certain intracellular concentration of PLP [Drewke *et al.*, 1996; Man *et al.*, 1997]. PdxF (SerC) is identical with PdxC, which is involved in serine biosynthesis. This enzyme has not only a catalytic function in both pathways, but a regulatory capacity as well. In serine biosynthesis, it controls the flux of intermediates through the pathway, whereas in vitamin B_6 biosynthesis it might play a crucial role in the rate of product re-

lease [Zhao *et al.*, 1995; Zhao and Winkler, 1996]. It is evident that vitamin B_6 biosynthesis evolved from serine biosynthesis by gene recruitment [Lam and Winkler, 1990].



Scheme 1.8: D*e novo* biosynthesis of PLP in the small subgroup of γ-proteobacteria, *e.g. E. coli*. The origin of the molecular skeleton of PLP is as shown (brown: 4PHT; green: DXP). Identified enzymes are indicated in italics and cofactor requirements (ThDP, PLP, FMN) are shown in red. Abbreviations used: DXP: 1-deoxy-D-xylulose 5-phosphate; E4P: D-erythrose 4-phosphate; GAP: D-glyceraldehyde 3-phosphate; 3HPK: 3-hydroxy-4-phosphohydroxy-α-ketobutyrate; α-KG: α-ketoglutarate; 4PE: 4-phosphoerythronate; 3PHA: 3-phosphohydroxy-1-aminoacetone; 4PHT: 4-phosphohydroxy-L-threonine [based on literature cited in the text].

The second branch (branch B in scheme 1.8) leads to the formation of DXP, which directly derives from central intermediates of glycolysis (pyruvate and D-glyceraldehyde 3-phosphate (GAP)). The reaction is catalyzed by DXP synthase (Dxs) (see section 1.2.4.1). This enzyme belongs to the transketolase-like enzyme family which is characterized by a high sequence similarity of its members [Sprenger *et al.*, 1997; Lois *et al.*, 1998].

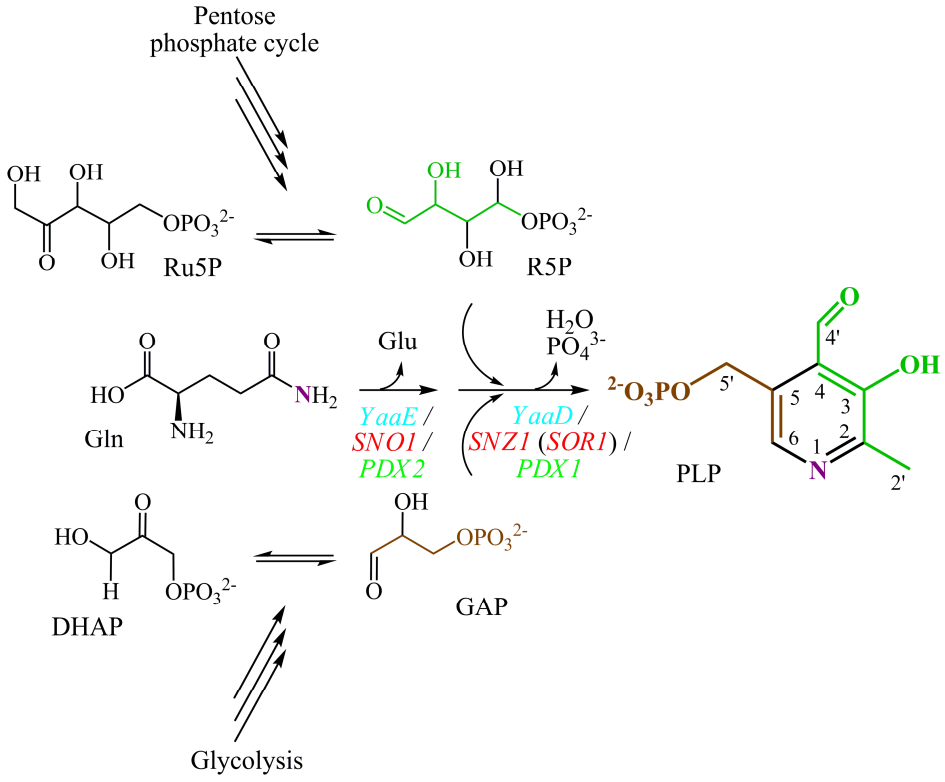
The condensation of 4PHT and DXP occurs as a coupled reaction employing the NAD^+-dependent dehydrogenase PdxA [Roa *et al.*, 1989; Cane *et al.*, 1998] and the PNP-synthase PdxJ [Lam *et al.*, 1992; Takiff *et al.*, 1992]. Initially, PdxA catalyzes the oxidation and decarboxylation of 4PHT. The product of this reaction was identified as 3-phosphohydroxy-1-aminoacetone [Cane *et al.*, 1998; Banks and Cane, 2004]. The latter is rather unstable and subsequently condenses with DXP yielding PNP accompanied by the release of inorganic phosphate. This reaction is catalyzed by PdxJ [Cane *et al.*, 1998/1999; Laber *et al.*, 1999]. PNP is then converted to the active cofactor PLP by the salvage pathway enzyme PdxH, a flavin mononucleotide (FMN) containing PNP oxidase (see section 1.3.6.3) [Lam and Winkler, 1992].

1.3.6.2 The 1-deoxy-D-xylulose 5-phosphate independent pathway ("yeast-like pathway")

In the majority of organisms able to synthesize vitamin B_6 *de nov*o, *e.g. B. subtilis*, *S. cerevisiae* and *A. thaliana*, the active cofactor form PLP is the direct reaction product. This is different to the pathway described above, present only in the small subgroup of γ-proteobacteria in which PNP is initially synthesized (see section 1.3.6.1).

As early as in 1978, Pflug and Lingens proposed a hypothetical scheme for the biosynthesis of vitamin B_6 in *B. subtilis*, which was different from that in *E. coli*: Dihydroxyacetone phosphate (DHAP), or alternatively alanine or pyruvate, was proposed to act as the donor for the C-2' – C-2 – C-3 skeleton of the pyridine ring and glutamine was hypothesized to provide the nitrogen atom N-1 [Pflug and Lingens, 1978]. In *E. coli*, at that time only the C-2 – C-2' unit was predicted to derive from pyruvate, whereas C-3 – C-4 – C-4' was believed to be incorporated as an independent unit [Hill *et al.*, 1975]. Incorporation experiments using ^{13}C-labelled substrates in yeast revealed a ^{13}C-labelling in the positions C-2' – C-2 – C-3 – C-4 – C-4' in the PM molecule fitting with C-1 – C-2 – C-3 – C-4 – C-5 of the ribose component of cytidine. Thus, it has been predicted that both C_5 units derive from a common pentulose or pentose sugar, which is different from the findings in *E. coli* (see section 1.3.6.1) [Gupta *et*

al., 2001]. Zeidler *et al.* [2003a] proposed a scheme in which a pentulose and triose plus an additional nitrogen source generate the skeleton of PN. The authors predicted that the atoms C-6 – C-5 – C-5' of PM derive from a triose, hypothesized as D-glyceraldehyde [Zeidler *et al.*, 2003a]. Only in 2005, this alternative biosynthetic route was confirmed in *B. subtilis*. Here, the cofactor derives from a pentose or pentulose sugar, *i.e.* ribose 5-phosphate (R5P) or ribulose 5-phosphate (Ru5P), and from a triose, either GAP or DHAP (scheme 1.9). The substrates were confirmed by *in vivo* and *in vitro* studies in *A. thaliana* [Tambasco-Studart *et al.*, 2005] and in the apicomplexan protists *P. falciparum* [Wrenger *et al.*, 2005; Gengenbacher *et al.*, 2006] and *T. gondii* [Knöckel *et al.*, 2007]. From experiments with *S. cerevisiae* using ^{15}N-labelled substrates it was concluded, that N-1 derives from glutamine rather than from glutamate as in *E. coli* (see schemes 1.8 and 1.9) [Tazuya *et al.*, 1995a]. The earlier prediction of a pent(ul)ose isomerase activity for Pdx1 was rebutted of refining an utilization of R5P rather than Ru5P *in vitro* [Raschle *et al.*, 2007; Hanes *et al.*, 2008a].



Scheme 1.9: *De novo* biosynthesis of PLP in eubacteria (turquoise [*B. subtilis*]), fungi (red [*S. cerevisiae*]) and plants (green [*A. thaliana*]). The origin of the atoms is shown (brown: GAP; green: R5P; purple: Gln). Abbreviations used: DHAP: dihydroxyacetone phosphate; GAP: D-glyceraldehyde 3-phosphate; Gln: glutamine; R5P: ribose 5-phosphate; Ru5P: ribulose 5-phosphate [based on literature cited in the text].

Unlike *E. coli* which relies on seven enzymes to synthesize PLP (see section 1.3.6.1), in the majority of eubacteria as well archaea, fungi and plants, two enzymes are sufficient to sustain the intracellular vitamin B_6 pool [Fitzpatrick *et al.*, 2007]. During a screen for resistance against the photosensitizer cercosporin of *C. nicotianae*, *SOR1* was identified. Intriguingly, homologs are widespread in archaea, eubacteria, plants and fungi, and *SOR1* appears to be one of the most highly conserved genes in nature although photosensitizer resistance is uncommon [Ehrenshaft *et al.*, 1998]. Mutations in *SOR1* in *C. nicotianae* and in *Aspergillus flavus* (the homolog *PYROA*) led not only to sensitivity to photosensitizers such as cercosporin, but also to vitamin B_6 auxotrophy. Thus, these genes were renamed *Pdx1* (pyridoxine requiring) [Ehrenshaft *et al.*, 1998/1999b; Osmani *et al.*, 1999]. Homologs of the Pdx1 protein have been identified in various organisms, *e.g.* YaaD from *B. subtilis* [Sakai *et al.*, 2002; Burns *et al.*, 2005; Raschle *et al.*, 2005], SNZ from *S. cerevisiae* [Fuge *et al.*, 1994; Braun *et al.*, 1996], PDX1 from *Phaseolus vulgaris* [Graham *et al.*, 2004], *A. thaliana* [Tambasco-Studart *et al.*, 2005; Wagner *et al.*, 2006] and *G. biloba* [Leuendorf *et al.*, 2008]. The second related protein, Pdx2, was originally identified in 2001 in the filamentous fungi *C. nicotianae* [Ehrenshaft and Daub, 2001], and later in *B. subtilis* (YaaE) [Sakai *et al.*, 2002], *S. cerevisiae* (SNO1) [Dong *et al.*, 2004], *P. falciparum* [Wrenger *et al.*, 2005; Gengenbacher *et al.*, 2006] and in *A. thaliana* (PDX2) [Tambasco-Studart *et al.*, 2005]. Pdx2 does not show such a high conservation in its amino acid sequence compared to Pdx1. However, the two genes are distributed identically in the organisms and often in close physical proximity to each other suggesting a functional linkage [Galperin and Koonin, 1997]. It has been noted that the genome of organisms synthesizing vitamin B_6 along the DXP-dependent pathway (key enzymes PdxA/PdxJ) do not contain genes encoding enzymes active in the DXP-independent pathway (Pdx1/Pdx2) and *vica versa*, *i.e.* the two pathways are autoexclusive (table 1.7) [Ehrenshaft *et al.*, 1998/1999b; Osmani *et al.*, 1999; Mittenhuber, 2001].

Organism	Pdx1/Pdx2	PdxA/PdxJ	
Archaea			
Methanococcus jannaschii	+	−	
Pyrococcus horikoshii	+	−	
Eubacteria			
Bacillus subtilis	+	−	
Haemophilus influenzae	+	−	
Mycobacterium tuberculosis	+	−	
Clostridium acetobutylicum	+	−	
Streptococcus pneumoniae	+	−	
Escherichia coli	−	+	Representatives of the γ-division of proteobacteria
Salmonella typhimurium	−	+	
Yersinia pestis	−	+	
Pseudomonas aeruginosa	−	+	
Vibrio cholerae	−	+	
Dictyostelia			
Dictyostelium discoidum	+	−	
Fungi			
Saccharomyces cerevisiae	+	−	
Aspergillus nidulans	+	−	
Cercospora nicotianae	+	−	
Plant			
Arabidopsis thaliana	+	−	
Oryza sativa	+	−	
Brassica napus	+	−	
Animal			
Caenorhabditis elegans	−	−	

Table 1.7: Identification of PdxA/PdxJ or Pdx1/Pdx2 homologs among organisms representing either one of the *de novo* biosynthetic pathways for vitamin B_6. A more complete list can be found in [Ehrenshaft *et al.*, 1999b].

Interestingly, in various organisms more than one homolog of either Pdx1 or Pdx2 is present, *e.g.* in *S. cerevisiae*, SNZ1/2/3 (Pdx1) and SNO1/2/3 (Pdx2) have been identified. It has been proposed that SNZ2/3 and SNO2/3 are regulated by thiamin diphosphate (ThDP) and are perhaps involved in the synthesis of this compound (see section 1.2.4.2), whereas SNZ1 and SNO1 maintain the vitamin B_6 pool [Rodríguez-Navarro *et al.*, 2002; Dong *et al.*, 2004]. However, in the fission yeast *S. pombe* as well as in *N. crassa* and *C. nicotianae*, only a single homolog has been identified so far for both *Pdx1* and *Pdx2* [Bean *et al.*, 2001]. *Nicotiana tabacum* contains two homologs of *Pdx1* and a single copy of *Pdx2* [Denslow *et al.*, 2005]

whereas in *A. thaliana* three homologs of *Pdx1*, *i.e. PDX1.1* (At2g38230), *PDX1.2* (At3g16050), *PDX1.3* (At5g01410) and a single homolog of *PDX2* (At5g60540) exist. While AtPDX1.1 and AtPDX1.3 are functional in vitamin B_6 biosynthesis, the role of AtPDX1.2 is not yet completely clarified [Tambasco-Studart *et al.*, 2005; Titiz *et al.*, 2006]. Recent work has suggested that this homolog might function in response to oxidative stress (see section 1.3.8) [Denslow *et al.*, 2007].

Data derived from sequence comparison studies and *in vitro* studies using SNO1 from *S. cerevisiae*, in addition to the elucidation of the 3D structure of YaaE from *B. subtilis*, confirmed that Pdx2 is a member of the class I glutaminase family [Galperin and Koonin, 1997; Bauer *et al.*, 2004; Dong *et al.*, 2004] and that its catalytic activity is dependent on its counterpart Pdx1. Pdx1 and Pdx2 function in an enzyme complex as glutamine amidotransferase [Dong *et al.*, 2004; Wrenger *et al.*, 2005; Gengenbacher *et al.*, 2006; Tambasco-Studart *et al.*, 2007].

1.3.6.2.1 Glutamine amidotransferases

Glutamine amidotransferases (GATs) are essential for numerous metabolic pathways in which the δ-amide group of glutamine is the sole source of nitrogen for the synthesis of amino acids, purine and pyrimidine nucleotides, amino sugars, coenzymes and antibiotics [Zalkin and Smith, 1998]. In general, GATs consist of at least two globular domains or subunits which catalyze two distinct reactions: Glutamine hydrolysis and substrate amination. The first reaction, taking place at the active site of the glutaminase domain and being unique for all GATs, is a nucleophilic attack of the activated thiol group of a conserved cysteine residue in the glutaminase active site on the δ-amide of glutamine releasing nitrogen in the form of ammonia (glutaminase activity; *eq. 1*). Consequently, a negative charge is transiently formed at the carbonyl oxygen. The formation of a so called “oxyanion hole” comprising two amide nitrogens, stabilizes this negative charge in all GATs, although the mode of action can differ between them [Kim *et al.*, 1996; Larsen *et al.*, 1999; Chaudhuri *et al.*, 2003; Van den Heuvel *et al.*, 2004; Mouilleron *et al.*, 2006]. Commonly, as the glutaminase and synthase active sites are distinct from each other [Mouilleron and Golinelli-Pimpaneau, 2007] it is necessary to sequester nascent ammonia from the solvent to prevent its protonation and thus the formation of toxic ammonium ions [Zalkin and Smith, 1998]. Therefore, it is subsequently channelled to the active site of the synthase domain where it acts as a nucleophile. In the second reaction, ammonia reacts with the acceptor substrate S, which is specific for each GAT [Van der Heu-

vel *et al.*, 2004] and yields an aminated product (synthase activity; *eq. 2*). Both reactions can be summarized and represent the physiological catalysis occurring *in vivo* (*eq. 3*) [Zalkin, 1993].

$Gln + H_2O$	→	$Glu + NH_3$	(*eq. 1*)
$NH_3 + S$	→	$S\text{-}NH_2$	(*eq. 2*)
$Gln + S$	→	$S\text{-}NH_2 + Glu$	(*eq. 3*)

To ensure reaction efficiency, even though both reactions take place at distinct sites, substrate (ammonia) channelling and the synchronization of the two active centres are characteristics for the majority of GATs [Mouilleron and Golinelli-Pimpaneau, 2007]. Synchronization is often achieved by coupling the two enzyme activities. This, in a first instance, prevents futile hydrolysis of glutamine, *i.e.* ammonia is not produced until the acceptor substrate is bound to the active site of the synthase domain. However, this feature is not preserved in PLP synthase (see below). When a coupling of activities is observed, it is often achieved by conformational changes initiated mainly by acceptor-binding, referred to as interdomain signalling [Nagradova, 2003; Amaro *et al.*, 2007; Dossena *et al.*, 2007].

GATs can be categorized as class I (or triad GATs, *e.g.* PLP synthase, imidazole glycerol phosphate synthase), class II (or N-terminal nucleophilic (Ntn) GATs, *e.g.* glucosamine-6-phosphate synthase, glutamate synthase, glutamine phosphoribosylpyrophosphate amidotransferase) or a third, completely unrelated class of GATs including for example glutamine $tRNA^{Gln}$ GATs [Ollis *et al.*, 1992; Zalkin and Smith, 1998]. The third class belongs to the glutaminase superfamily although its members exhibit homologies to other enzyme families such as amidases, L-asparaginases or nitrilases [Zalkin and Smith, 1998; Mouilleron and Golinelli-Pimpaneau, 2007]. In contrast to the poorly characterized third class, triad and Ntn GATs have been widely studied and will only be considered here.

The classification of GATs is based on their structural and mechanistic features. No structural similarities have been found between the synthase domains of the different classes at the primary and tertiary structural level. Whereas members of the class I family typically share a common open $(\beta/\alpha)_8$ domain topology, those of Ntn GATs are mainly characterized by antiparallel β-sheets [Mouilleron and Golinelli-Pimpaneau, 2007]. In addition, the intramolecular tunnel formation for sequestering ammonia differs between them. For example, in carbamoyl phosphate synthetase (class I) [Thoden *et al.*, 1997] and asparagine synthetase (class II) [Lar-

sen *et al.*, 1999] the tunnel is constitutively formed. In contrast, in glutamine phosphoribosylpyrophosphate amidotransferase (class II) [Krahn *et al.*, 1997], its formation is initiated upon substrate binding to the synthase active site. For PLP synthase [Strohmeier *et al.*, 2007], anthranilate synthase [Knöchel *et al.*, 1999; Morollo and Eck, 2000; Spraggen *et al.*, 2001] and guanine monophosphate synthetase [Tesmer *et al.*, 1996] (all belong to class I GATs) the formation of an ammonia tunnel has been predicted, but not yet experimentally proven.

In addition to the structural features described above, the molecular mechanisms of glutaminase activation, control and the mode of regulation are different between the classes and can even differ within a single class [Van den Heuvel *et al.*, 2004]. In triad GATs, the active site cysteine residue is part of the catalytic triad Cys-His-Glu, in which histidine and glutamate are postulated to activate the thiol group of cysteine. In contrast, in Ntn GATs, the catalytically active cysteine residue is located at the N-terminus of the mature protein and the activation of the thiol group is assisted by the free amino-terminal group [Zalkin and Smith, 1998]. The structural changes taking place upon acceptor binding in the synthase active site, the first event in the reaction mechanism, distinguishes them further. In yeast imidazole glycerol phosphate synthase, a member of class I GATs, several loops surrounding the active site move slightly towards the acceptor substrate arranging the side chain of a catalytically active lysine residue such that it points towards, and can efficiently interact with the acceptor substrate [Douangamath *et al.*, 2002; Chaudhuri *et al.*, 2003]. Whereas for the imidazole glycerol phosphate synthase the largest conformational change appears to be a re-orientation of a partially ordered synthase site loop connecting strand β1 and helix α1 [Chaudhuri *et al.*, 2003], for representatives of class II GATs the induced conformational changes are more substantial. The C-terminus of the synthase domain becomes ordered forming a flexible loop which covers the active site to enhance affinity for the substrate [Krahn *et al.*, 1997; Muchmore *et al.*, 1998; Mouilleron *et al.*, 2008]. This flexible loop can also constitute (major) parts of the ammonia tunnel [Mouilleron and Golinelli-Pimpaneau, 2007]. Usually, the glutaminase activity is coupled with binding of the acceptor substrate to the synthase active site, although there are quantitative differences regarding the degree of regulation. Again, the level of regulation is not a feature of a particular class. Often, the catalytically active conformation of the glutaminase pocket is formed only upon stimulation by the synthase domain. Once the glutamine is bound to the glutaminase active site, it is often shielded by a flexible loop (Q-loop for Ntn GATs) [Van den Heuvel *et al.*, 2003] or by an ordered or re-organized loop of the glutaminase domain (some triad GATs) [Chaudhuri *et al.*, 2003; Goto *et al.*, 2004]. Glutaminase ac-

tivity is further regulated by positioning the catalytic residues in the glutaminase active site and the oxyanion hole in an optimal conformation [Mouilleron and Golinelli-Pimpaneau, 2007]. In conclusion, the main feature of most GATs, with the exception of PLP synthase or arginine synthase for instance, is the precise coordination of two reactions at distant sites. However, the level of regulation, the mode of inter-subunit crosstalk and the efficiency of coupling can widely differ between individual enzymes and cannot be generalized, not even within a given class of GATs.

Although several characteristics described for other class I family members cannot be attributed to PLP synthase [Tambasco-Studart *et al.*, 2007], a tight regulation of enzyme activity coordinated by an inter-subunit crosstalk between Pdx1 and Pdx2, the respective synthase and glutaminase domain, is expected [Raschle *et al.*, 2009]. The structure of the PLP synthase complex (figure 1.8), consisting of Pdx1 and Pdx2, has been solved from two organisms, *B. subtilis* [Strohmeier *et al.*, 2006] and *Thermotoga maritima* [Zein *et al.*, 2006], respectively. The complex occurs as a dodecamer arranged in two hexameric rings of Pdx1 to each of which one Pdx2 subunit is attached [Strohmeier *et al.*, 2006; Zein *et al.*, 2006]. This complex is a unique macromolecular assembly within the GAT family [Fitzpatrick *et al.*, 2007]. As for other GATs, the two active sites are localized remote from each other in PLP synthase [Strohmeier *et al.*, 2006; Zein *et al.*, 2006]. Ammonia, produced at the glutaminase active site, is channelled to the synthase active site through a putative tunnel that awaits confirmation [Strohmeier *et al.*, 2006; Tambasco-Studart *et al.*, 2007]. However, Zhu *et al.* [2005] originally predicted adjacent glutaminase and synthase active sites as they could not identify the ammonia tunnel in the structure they had obtained. The structure of autonomous Pdx1 homologs from *Geobacillus stearothermophilus* [Zhu *et al.*, 2005] and *B. subtilis* [Strohmeier *et al.*, 2006], PdxS and YaaD, respectively, have been elucidated by X-ray crystallography in their dodecameric forms, but in solution they appear in an equilibrium with the hexameric form that shifts towards the dodecamer when glutamine-bound Pdx2 is present. Structures of the autonomous homologs of Pdx2 from *B. subtilis* [Bauer *et al.*, 2004] and *P. falciparum* [Gengenbacher *et al.*, 2006], YaaE and Pdx2, respectively, have been elucidated by X-ray crystallography as well.

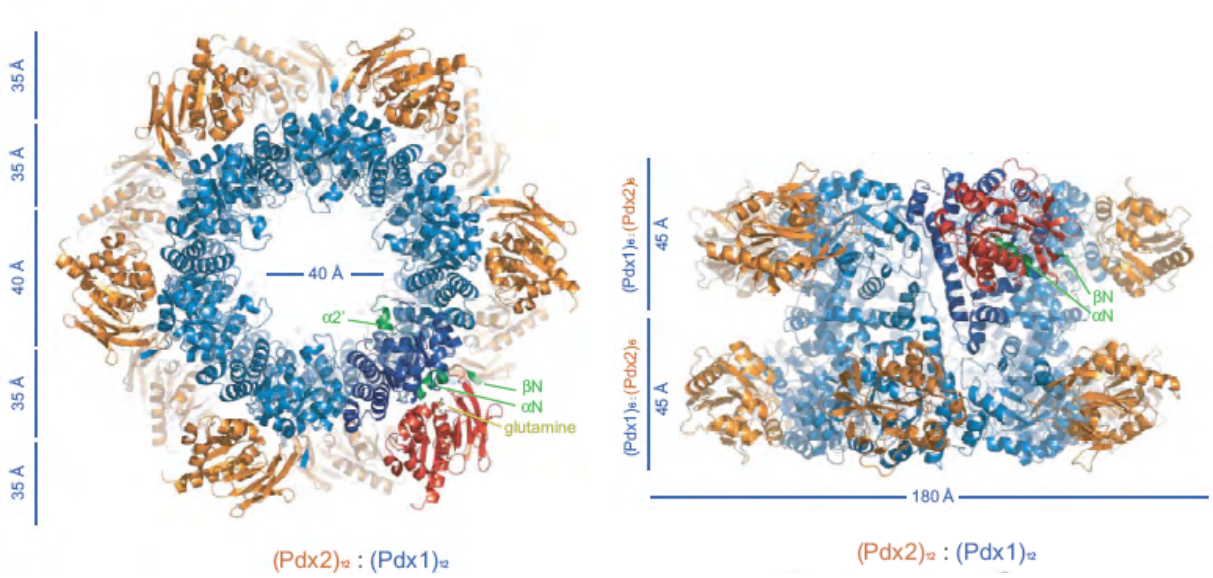


Figure 1.8: The Pdx1-Pdx2 enzyme complex. The Pdx1:Pdx2 H170N:glutamine ternary complex shown in two orientations turned by 90°. Twelve Pdx2 glutaminase subunits attach to a double-ring-like core formed by 12 Pdx1 subunits. The Pdx1 synthase subunits are colored blue and green, and the Pdx2 glutaminase subunits are in orange and red. A single heterodimer is highlighted in dark blue (Pdx1) and red (Pdx2), and the position of bound glutamine is shown in stick representation. The N-terminal β-strand, βN, and α-helix, αN, as well as helix α2' are highlighted in green. Approximate sizes are given in angstroms (10^{-10} m) [taken from Strohmeier *et al.*, 2006].

Upon formation of the Pdx1/Pdx2 complex, structural changes occur in Pdx1, namely the formation of an extra β-strand (βN), which is involved in β-completion with β7/β8 strands of Pdx2, and an ordering of the N-terminal α-helix, αN (residues 7-17), as well as helix α2' (residues 47-56) [Strohmeier *et al.*, 2006]. The N-terminal α-helix of Pdx1, a feature not observed in any of the other class I GAT enzyme structures identified to date [Myers *et al.*, 2005], is crucial for complex formation, glutaminase activation and PLP synthesis. Due to polar interactions with amino acid residues located in the catalytic site of Pdx2, αN in Pdx1 triggers the precise positioning, thus complex formation, and covers the glutaminase active site, thus sequestering the substrate glutamine from the surrounding solvent. In addition, upon comparing the structure of the autonomous protein with that of the protein in the ternary complex of Pdx1, Pdx2 and glutamine, αN triggers the formation of the oxyanion hole, a prerequisite for catalysis as it is required for both glutamine binding and hydrolysis [Strohmeier *et al.*, 2006]. The oxyanion hole is stabilized by slight re-orientations of residues surrounding the Pdx2 active site and by the interaction with residues originating from Pdx1 [Strohmeier *et al.*, 2006]. The importance of αN was further demonstrated by creating a "swap mutant" of

P. falciparum Pdx1, in which the complete αN-helix was replaced with that from the *B. subtilis* homolog. This mutant lost the ability to interact with Pdx2 from *P. falciparum* but showed cross-binding with Pdx2 from *B. subtilis* and exhibited partial glutaminase activity demonstrating that interface formation between Pdx1 and Pdx2 and the catalytic activation of the glutaminase are linked processes mediated by αN [Flicker *et al.*, 2007]. It also corroborates the recent hypothesis of species specificity in the formation of the catalytically active complex [Gengenbacher *et al.*, 2006]. Interestingly, binding of the pentose phosphate substrate (R5P) to the catalytically active lysine residue (Lys-81) in the synthase active site, one of the earliest steps in the reaction [Burns *et al.*, 2005; Raschle *et al.*, 2007], demonstrates positive cooperativity. Moreover, substrate affinity is enhanced by the presence of Pdx2 and glutamine in the PLP synthase complex [Raschle *et al.*, 2007/2009].

The C-terminus has very recently been shown to be indispensable for PLP synthase activity, but had eluded structural characterization, presumably due to an inherent flexibility [Raschle *et al.*, 2009]. It has been partly resolved in the structure of *T. maritima* PLP synthase in the presence of R5P [Zein *et al.*, 2006]. Cross-linking studies have revealed that the C-terminus of one Pdx1 monomer can act as a flexible lid that bridges and shields the active site of an adjacent protomer in Pdx1. It mediates an inter-subunit crosstalk within the enzyme complex [Raschle *et al.*, 2009]. A feature of other GATs examined so far, *i.e.* the tight coupling of glutaminase and synthase activity, has not been observed for PLP synthase. Although Pdx2 activity is only detectable in the presence of Pdx1 [Raschle *et al.*, 2005; Gengenbacher *et al.*, 2006; Tambasco-Studart *et al.*, 2007], an enhancement of glutaminase activity upon synthase substrate binding could not be demonstrated [Raschle *et al.*, 2005]. Furthermore, in many GATs the specific interdomain contact is most probably triggered by a salt bridge. This feature is missing in PLP synthase. Instead, it has been proposed that the interdomain contact is triggered by interdomain modifications in the Pdx1 protein that result in a higher hydrophobicity when glutamine is present. It has been demonstrated that the side chains of two methionine residues (Met-13, Met-16) form an ordered hydrophobic interface with the residue Ile-134 originating from Pdx2. These residues are disordered in the structure of autonomous Pdx1 [Strohmeier *et al.*, 2006]. This may significantly increase the affinity between Pdx1 and Pdx2 in *B. subtilis* [Flicker *et al.*, 2007]. Complex formation prior to enzyme activity is another factor that has not been completely elucidated. To date, the existence of mono-, hexa- and dodecameric Pdx1s and the knowledge that Pdx1 monomers are not able to interact with Pdx2, led to the proposal that complex assembly in *P. falciparum* follows a defined course: The

hexameric ring of Pdx1 molecules is formed prior to binding Pdx2, followed by the assembly of the dodecameric complex [Müller *et al.*, 2008]. Based on the observed stoichiometry and thermodynamics, Neuwirth *et al.* [2007] proposed that glutamine must bind first to Pdx2 prior to complex formation with Pdx1, rather than glutamine binding to the preformed Pdx1:Pdx2 complex ("encounter complex") in *B. subtilis*. It has been proposed that glutamine is a mediator for the formation of the Michaelis complex as the dissociation constant decreased in the presence of glutamine, leading to an increase in complex stability [Strohmeier *et al.*, 2006; Neuwirth *et al.*, 2007; Flicker *et al.*, 2007].

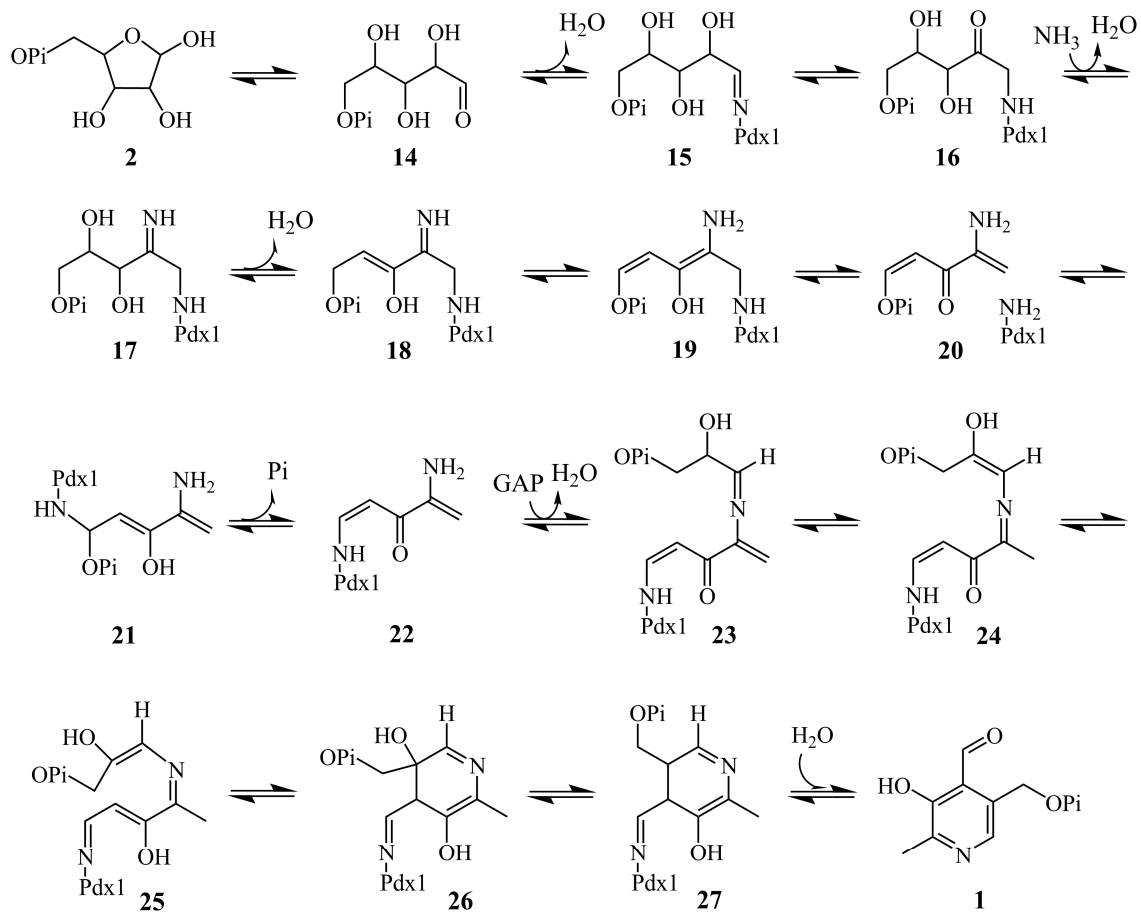
Interestingly, differences between PLP synthases from bacterial, apicomplexan and plant origin have been reported. A comparison of the amino acid sequences of Pdx1 from *B. subtilis* and *A. thaliana* revealed that the methionine residue M-13 in *B. subtilis* is replaced by a leucine in the *A. thaliana* homolog. This affords a much tighter coupling of glutaminase and synthase reactions and thus a higher catalytic efficiency for the use of ammonia by AtPDX1. Despite the prediction of an identical structure, the presumed ammonia tunnel and the entry mechanism must therefore differ between them [Tambasco-Studart *et al.*, 2007]. Substrate utilization is another feature in which the PLP synthase homologs differ from each other. In *A. thaliana* and *P. falciparum*, DHAP can only be efficiently used as a substrate in the presence of Pdx2 [Gengenbacher *et al.*, 2006; Tambasco-Studart *et al.*, 2007]. This has not been observed for PLP synthase from *B. subtilis* [Raschle *et al.*, 2005]. The entire protein complex is more stable in *P. falciparum* than in *B. subtilis* although the binding affinity to glutamine is similar for both [Flicker *et al.*, 2007]. However, whereas the "encounter complexes" differ between the organisms, they are similar when forming the catalytically active complex. Active complex formation and catalytic activation are linked through αN of Pdx1 in all homologs (see above). Although insight has been gained in the last years, many facets of this most interesting enzyme complex await further elucidation, for example, the mechanism behind the transfer of ammonia from Pdx2 to Pdx1 and the complete understanding of the biochemical mechanism behind PLP formation by Pdx1 and Pdx2 (see chapter 1.3.6.3).

1.3.6.2.2 Mechanistic aspects of PLP synthase

Substantial effort has been put into the elucidation of the mechanism behind the complex reaction catalyzed by PLP synthase [Raschle *et al.*, 2007; Hanes *et al.*, 2008a/b]. However, in spite of significant experimental support for hypotheses put forward, it is not yet fully under-

stood. A set of mechanistic proposals has been provided [Burns *et al.*, 2005; Raschle *et al.*, 2007; Hanes *et al.*, 2008a], the most recent one is given in scheme 1.10 and is discussed here [Hanes *et al.*, 2008a/b/c]. The initial step in PLP synthesis is assumed to be the opening of the furanose ring of R5P [Raschle *et al.*, 2007]. As mentioned above, R5P is the preferred C_5-substrate for Pdx1 from *B. subtilis* (see chapter 1.3.6.2.1) and its binding to Pdx1 does not require the presence of Pdx2 [Raschle *et al.*, 2007]. R5P (compound **14** in scheme 1.10) forms an imine, *i.e.* a covalent C=N bond (Schiff base), with a catalytically active lysine residue (Lys-81) of Pdx1 (**15**). The formation of the imine adduct **15** between Lys-81 of Pdx1 and the pent(ul)ose substrate is one of the earliest steps in the reaction scheme. The localisation of this imine was a matter of debate for some time. In 2005, Burns *et al.* [2005] localized the adduct to Lys-149, a residue that is absolutely conserved between the Pdx1 homologs. This hypothesis was rejected later on, and the lysine residue involved was demonstrated to be Lys-81 based on tandem mass spectrometry [Raschle *et al.*, 2007] and the structural elucidation of R5P bound to Pdx1 from *T. maritima* [Zein *et al.*, 2006]. However, Lys-149 is known to be required in PLP synthesis, as site-directed mutagenesis studies clearly showed. It has been suggested to act as an acid/base catalyst [Raschle *et al.*, 2007]. The imine **15** subsequently isomerizes to compound **16**. It was originally thought that the bond was formed at C-2 of Ru5P, based on the mechanism of imidazole glycerol phosphate synthase, and it was assigned as such in the X-ray structure of Pdx1 with Ru5P bound [Zein *et al.*, 2006]. However, a recent series of NMR experiments suggest that the imine is formed by C-1 of R5P (Hanes *et al.*, 2008). Ammonia derived from glutamine hydrolysis catalyzed by Pdx2 is incorporated into **16** to form **17**. Elimination of water (**18**) is followed by deprotonation of C-5 yielding **19**. This deprotonation has been demonstrated to be stereospecific for the pro-*R* hydrogen atom at C-5 of R5P and is partially rate-limiting. Hanes *et al.* [2008c] suggested that the next step is elimination of Lys-81 from C-1 to form the intermediate **20** the C-5 of which now reacts with Lys-81 to form **21**. While this step remains to be proven, this C-1 to C-5 migration of the lysine residue could greatly enhance the catalytic versatility of the Pdx1 active site by shifting the intermediate into a new environment. Future work will have to show if this is mechanistically feasible and also, whether the migration actually involves the same lysine residue of the active site. Phosphate is subsequently eliminated from **21** yielding a chromophoric intermediate (**22**). The identification of the chromophoric intermediate marked a turning point in the history of Pdx1 mechanistic proposals, as it had previously been overlooked [Raschle *et al.*, 2007]. In the same study, a chromophoric adduct with an extinction coefficient of approxi-

mately 16,200 M^{-1} cm^{-1} at an absorbance maximum of 315 nm has been characterized. The authors demonstrated that the rate of its formation is dependent on the concentration of R5P and ammonia. Although the catalytic competence of the compound was proven by addition of GAP, reconstitution of the chromophore and subsequent ESI-MS analysis indicated the absence of nitrogen from the structure. As addition of GAP alone led to the formation of PLP [Raschle *et al.*, 2007; Hanes *et al.*, 2008a], it was suggested that the nitrogen atom of PLP must be part of the chromophoric intermediate. Therefore, Hanes *et al.* [2008a] proposed an alternative structure for the chromophoric intermediate (**22**) even though the presence of nitrogen could not be confirmed by ESI-FTMS analysis. Hanes *et al.* [2008b] recently used a trapping reaction, and NMR analysis of the trapped product revealed that the nitrogen atom is covalently incorporated into the chromophoric intermediate supporting structure **22**. All experimental data indicate that the chromophoric intermediate is covalently attached to the enzyme *via* Lys-81 and occurs subsequent to imine formation in the presence of both Pdx2 and glutamine. Alternatively, ammonium in high concentration is sufficient for its formation *in vitro* [Hanes *et al.*, 2008c]. This highly conjugated system [Raschle *et al.*, 2007; Hanes *et al.*, 2008c], is characterized by the presence of all five C-atoms from R5P, the loss of phosphate, water and a proton (pro-*R* hydrogen atom; see above) [Raschle *et al.*, 2007; Hanes *et al.*, 2008a]. The rate of phosphate loss was shown to be identical to that of the formation of the kinetically competent chromophoric intermediate [Raschle *et al.*, 2007; Hanes *et al.*, 2008c]. The remaining steps in the reaction mechanism to yield PLP are hypothetical, but in order to be consistent with the mechanism discussed, Hanes *et al.* suggest that an imine bond is subsequently formed between **22** and the C_3-substrate (GAP) to give rise to **23**, which, after two tautomerization reactions, followed by electrocyclic ring closure and aromatization forms **27**. The compound thus formed is PLP bound to Pdx1 *via* an imine bond to C-4 [Hanes *et al.*, 2008c]. The final hydrolysis of the imine yields the free reaction product PLP (**1**) [Hanes *et al.*, 2008a]. An earlier demonstration that whole cells of *S. cerevisiae* convert 2'-hydroxypyridoxol to PM [Zeidler *et al.*, 2002] led to a hypothetical, alternative scheme for PLP formation [Raschle *et al.*, 2007]. However, the intermediacy of 2'-hydroxypyridoxol 5'-phosphate could not be confirmed for *B. subtilis* [Raschle *et al.*, 2007; Hanes *et al.*, 2008a].



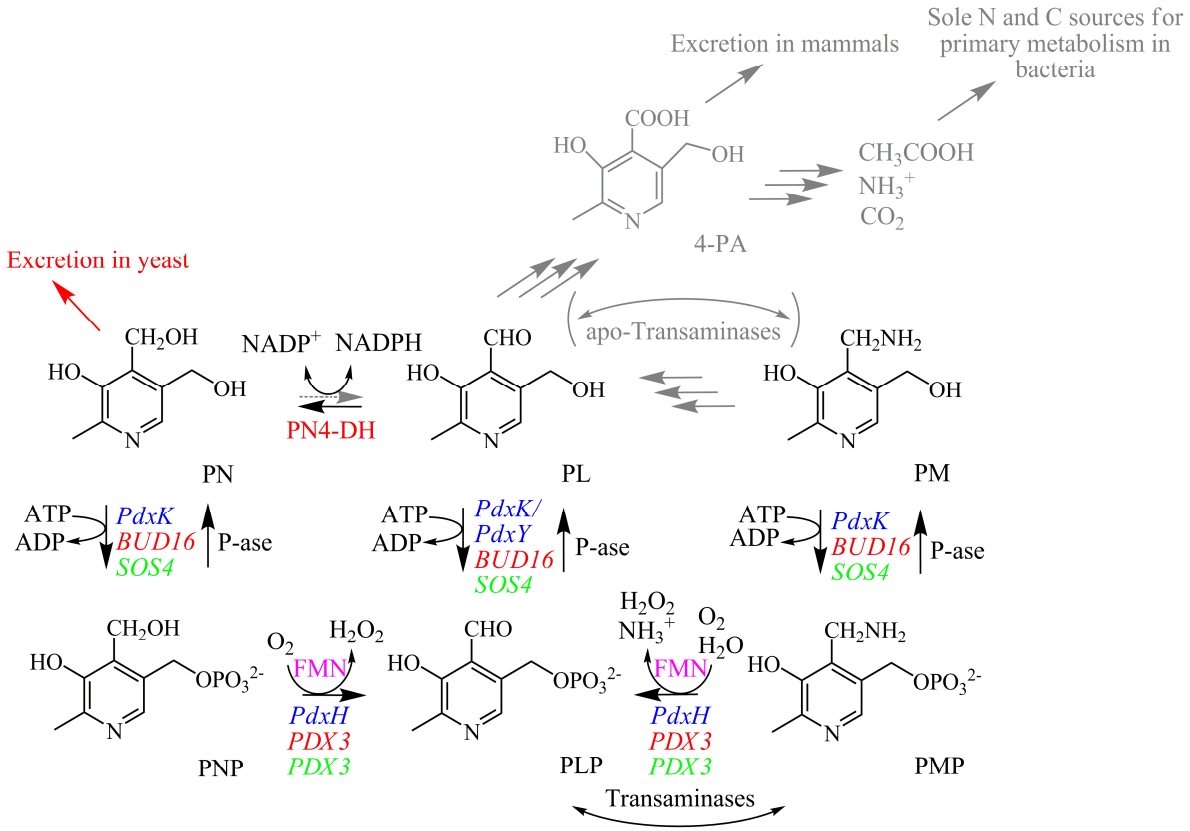
Scheme 1.10: Mechanistic proposal for the formation of PLP by PLP synthase. See text for details [taken from Hanes *et al.*, 2008a].

It will be interesting to explore the generality of the mechanism established for *B. subtilis* PLP synthase for other organisms that use the DXP-independent biosynthetic pathway for PLP formation. Finally, the later steps, *i.e.* conversion of the chromophoric intermediate to PLP, need to be defined [Raschle *et al.*, 2007; Hanes *et al.*, 2008a/c].

1.3.6.3 Salvage pathway(s) and degradation of vitamin B_6

PN, PL, PM and their respective 5'-phosphoesters are embraced by the term "vitamin B_6" (figure 1.5). Unlike *de novo* biosynthesis of vitamin B_6, the salvage pathway is active in all organisms identified so far (scheme 1.11). In this pathway, the different vitamers are inter-converted to salvage vitamin B_6 [Di Salvo *et al.*, 2004]. The free forms can be phosphorylated by an ATP-dependent PN/PL/PM-kinase that has been identified as PdxK in *E. coli* [Yang *et al.*, 1996], BUD16 in *S. cerevisiae* [Tanaka *et al.*, 2005], SOS4 in *A. thaliana* [Shi *et al.*, 2002] as well as in numerous other organisms like *Klebsiella pneumoniae*, *P. falciparum*,

Candida albicans, *Drosophila melanogaster* and *Homo sapiens* [Mittenhuber, 2001]. In *E. coli*, PdxY, a specific PL-kinase, has been identified in addition to PdxK. PdxY exhibits rather strict substrate specifi-city towards PL and is exclusively active in the salvage pathway of vitamin B_6, whereas PdxK is predicted to be involved in vitamin B_1 biosynthesis as well (see section 1.2.4.3; scheme 1.5) [Yang *et al.*, 1998a]. The dephosphorylation of PNP, PLP and PMP in order to restore the free forms is apparently catalyzed by unspecific phosphatases [Mittenhuber, 2001]. The conversion of both PNP and PMP to PLP is catalyzed by a PNP/PMP oxidase, encoded by *PdxH*. PdxH, extensively studied and characterized in mammalian systems in the early 1980s, is a flavin (FMN) containing protein that utilizes PNP and PMP to the same extent in mammals [Choi *et al.*, 1983], but exhibits significant selectivity for PNP in *E. coli* [Zhao and Winkler, 1995]. Molecular oxygen is used as an electron acceptor and hydrogen peroxide is released during catalysis [McCormick and Chen, 1999]. Interestingly, the activities of *E. coli* and mammalian PdxH are strongly inhibited by PLP in a competitive manner suggesting a regulatory role for PdxH in the biosynthesis of the cofactor [Choi *et al.*, 1987; Zhao and Winkler, 1995]. Homologs of *PdxH* have been verified in the genomes of all organisms sequenced so far, *e.g. PDX3* in *S. cerevisiae* [Loubbardi *et al.*, 1995] and *A. thaliana* [Sang *et al.*, 2007]. PLP can also derive from PMP by the activity of unspecific transaminases in a so-called shuttle mechanism of coenzyme action (see section 1.3.5; scheme 1.6) [Jenkins and Sizer, 1957]. Interestingly, the apoenzyme of aspartate transaminases (apo-transaminase) interconverts, although rather inefficiently, PM and PL as the first step in the degradation pathway of PM (see below; scheme 1.11) [Wada and Snell, 1962a/b]. Additionally, in yeast a $NADP^+$-dependent pyridoxine 4-dehydrogenase (PN-4DH, PL reductase) has been identified that is able to interconvert PN and PL, with a clear preference toward the reduction of PL [Morino and Sakamoto, 1960; Holzer and Schneider, 1961; Guirard and Snell, 1988]. The reduction of PLP to PNP is catalyzed by this enzyme as well [Holzer and Schneider, 1961]. Recent studies in *S. cerevisiae* suggest that PN-4DH, encoded by *PLR1*, is predominantly involved in the secretion of PN rather than in the salvage pathway [Morita *et al.*, 2004].



Scheme 1.11: Salvage and degradation pathways of the different forms of vitamin B_6. The salvage pathway (black) involves ATP-dependent kinase, oxidase, transaminase and unspecific phosphatase activities. In yeast, PL and PN can be interconverted in a NADP(H+H^+)-dependent manner with a preference for the reduction of PL to PN. The cofactor requirement (FMN) is shown in purple. Identified genes (given in italics) are presented in blue (bacteria [*E. coli*]), red (fungi [*S. cerevisiae*]) and green (plants [*A. thaliana*]). The free forms of the vitamin can be degraded *via* PL to 4-PA in mammals and even further serving as a sole source of nitrogen and carbon in certain bacteria (grey). Abbreviations used: P-ase: unspecific phosphatase; 4-PA: 4-pyridoxic acid; PN4-DH: pyridoxine 4-dehydrogenase (PL reductase) [based on literature cited in the text].

The oxidative degradation of vitamin B_6 predominantly occurs at the level of the free forms (scheme 1.11), in particular PL. Both PN and PM are converted to PL by oxidation and transamination, respectively. The latter is converted to 4-pyridoxic acid (4-PA) in subsequent reactions. 4-PA is not converted further and is excreted in the urine of mammals [Burns and Conney, 1960; Stanulović *et al.*, 1976]. In bacteria, *e.g. Pseudomonas* MA-1 (ATCC 33286) and *Arthrobacter sp.*, 4-PA can either be first isomerised to 5-pyridoxic acid (isopyridoxine) or directly degraded to ammonia, carbon dioxide and acetic acid [Nelson and Snell, 1986]. Thus, several bacteria can use vitamin B_6 as the sole source of carbon and nitrogen [Burg *et al.*, 1960; Yagi *et al.*, 1983].

1.3.7 Regulation of vitamin B_6 biosynthetic pathways

The majority of information on the regulation of vitamin B_6 biosynthesis refers to *E. coli*, but it is not yet fully understood. Only preliminary data is available on the recently established DXP-independent pathway.

Many of the genes involved in the biosynthesis and salvage of vitamin B_6 in *E. coli* are clustered within multifunctional operons that include genes involved in other biosynthetic pathways (table 1.8). Roa *et al.* [1989] pointed out that such an arrangement in transcriptional units might provide a genetic linkage with other metabolic pathways and processes. For example, *PdxJ* forms an operon with *Acps* (*dpj*) encoding an acyl carrier protein synthase which is involved in the biosynthesis of fatty acids and lipids [Lam *et al.*, 1992]. *PdxA*, which catalyzes the terminal reaction in branch A of vitamin B_6 biosynthesis (scheme 1.8), clusters in a complex operon with *SurA*, *KsgA*, *ApaH* and *ApaG* [Roa *et al.*, 1989]. S*urA* encodes a peptidyl prolyl isomerase involved in folding of outer membrane proteins [Lazar and Kolter, 1996]. *KsgA*, a putative 16S rRNA methyltransferase is followed by *ApaG*, a gene of unknown function, and by *ApaH*, a diadenosine tetraphosphatase. The latter is involved in the synthesis of unusual nucleotides, such as an adenosine molecule linked by a tri- or tetraphosphatechain to another adenosine or guanosine molecule. These molecules are thought to affect the priming reaction of replication [Grummt, 1983] and/or to act as "alarmones" to signal the onset of cellular oxidative stress [Bochner *et al.*, 1984]. Moreover, *PdxF* forms a regulatory operon with *AroA*, an enzyme involved in aromatic amino acid biosynthesis in *E. coli* [Man *et al.*, 1997]. It has been speculated that the linkage between the three pathways, *i.e.* PLP, serine and aromatic amino acid biosynthesis, at the transcriptional level ensures sufficient amounts of both serine and PLP for the terminal step of tryptophan biosynthesis which is carried out by the PLP-dependent tryptophan synthetase [Dempsey, 1987]. *PdxH* forms a multifunctional operon with *TyrS* encoding an aminoacyl-$tRNA^{Tyr}$ synthetase [Bosshard *et al.*, 1975]. This is also evident in *B. subtilis* where the physically linked and co-regulated *YaaD* and *YaaE* form a cluster with *SerS*, encoding an aminoacyl-$tRNA^{Ser}$ synthetase [Sakai *et al.*, 2002].

Upstream of *PdxJ*, *PdxB* and *PdxA*, the consensus motif ACGT(G/T)AAAATCC has been identified and designated the "PDX-box" [Roa *et al.*, 1989; Schoenlein *et al.*, 1989]. It has been proposed that this region might be involved in the regulation of transcription by feedback inhibition, or possibly a repression-derepression mechanism, by the catalytic product [Lam *et al.*, 1992]. However, the presence of the "PDX-box" motif is not solely responsible

for the regulation of vitamin B_6 biosynthesis as the activity of PdxH is competitively inhibited by PLP, but its coding sequence is not preceded by a "PDX-box" [Choi *et al.*, 1987; Zhao and Winkler, 1995].

On the other hand, in many organisms, *e.g. Methanococcus mannaschii*, *S. cerevisiae* and *A. thaliana*, *Pdx1* and *Pdx2* are localized in the genome distant from each other. However, *Pdx1* and *Pdx2* may be co-regulated under certain conditions, as was reported for *S. cerevisiae SNZ1* and *SNO1* under nutrient, in particular amino acid, starvation [Padilla *et al.*, 1998; Ehrenshaft and Daub, 2001; Bean *et al.*, 2001]. This observation has been related to the presence of a regulatory motif (*cis* element) identified in the promoter region of both *SNZ1* and *SNO1*, which is a consensus target for *GCN/cpcA*-like transcriptional activators mediating amino acid biosynthesis [Hoffman *et al.*, 2001].

Genetic linkage of *Pdx* genes	Regulation	Operon	Organism	Reference
PdxJ *– Dpj (AcpS)* Fatty acid & lipid biosynthesis	"PDX box"	Multi	*E. coli*	Lam *et al.*, 1992
PdxB *– HisT* tRNA modification	"PDX box"	Multi	*E. coli*	Arps and Winkler, 1987
SurA – ***PdxA*** *– KsgA – ApaG/H* 16S ribosomal RNA modification & chaperone	"PDX box"	Multi	*E. coli*	Roa *et al.*, 1989
PdxH*-TyrS* tRNA synthetase	n.i.	Multi	*E. coli*	Lam and Winkler, 1992
PdxF **(*SerC*)** *– AroA* Shikimate pathway	n.i.	Multi	*E. coli*	Man *et al.*, 1997
YaaD *(Pdx1)* **–** ***YaaE*** *(Pdx2)*	Co	Related	*B. subtilis*	Galperin and Koonin, 1997
SNZ1 *(Pdx1)* and ***SNO1*** *(Pdx2)*	Co	No	*S. cerevisiae*	Padilla *et al.*, 1998

Table 1.8: Enzymes involved in vitamin B_6 biosynthesis (in bold) are often clustered within multifunctional or functionally related operons. Abbreviations used: Co: co-regulation; multi: multifunctional; n.i.: not identified [based on literature cited in the text].

In plants, in particular *A. thaliana*, the regulation of genes involved in *de novo* vitamin B_6 biosynthesis, *i.e. PDX1.1*, *PDX1.3* and *PDX2*, as well as *PDX1.2*, has been investigated at the transcriptional and posttranscriptional level in response to various abiotic stresses [Denslow *et al.*, 2005/2007; Titiz *et al.*, 2006]. There is evidence that the transcription of all four genes in is enhanced in response to environmental stresses that are accompanied by a release of reac-

tive oxygen species (ROS) such as high light, chilling and drought [Denslow *et al.*, 2005/2007], wounding [Graham *et al.*, 2004] as well as the intracellular concentration of hormones such as ethylene, gibberellins (GA) [Graham *et al.*, 2004], salicylic acid (SA) [Sivasubramaniam *et al.*, 1995] and jasmonic acid (JA) [Titiz, 2008]. Ethylene is produced throughout the life cycle of a plant, *e.g.* germination, fruit ripening and senescence and upon environmental stimuli such as mechanical wounding, hypoxia, ozone, chilling and freezing [Wang *et al.*, 2002]. Apart from a putative relation to stress, the biochemical link between *de novo* vitamin B_6 and ethylene biosynthesis may lie in the PLP-dependent 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which catalyzes the conversion of S-adenosyl-L-methionine (SAM) to ACC, the final intermediate in ethylene synthesis in plants [Capitani *et al.*, 1999]. Jasmonic acid (JA) is involved in developmental processes such as fruit ripening, senescence and pollen development, as well as in defence responses against biotic and abiotic stresses such as wounding, water deficit and ozone [Balbi and Devoto, 2007]. Furthermore, an early report revealed that the PDX1 homolog from *Hevea brasiliensis* (HEVER) is stress-induced upon treatment with salicylic acid (SA) and ethephon, an ethylene-releasing agent. In addition, a short-lived stimulated response of *P. vulgaris* PDX1 in leaf discs upon treatment with ACC and GA_3 has been reported [Graham *et al.*, 2004]. To further specify the regulation of vitamin B_6 biosynthesis in plants and to elucidate whether a regulatory difference between the two homologs PDX1.1 and PDX1.3 in *A. thaliana* exists, the promoter regions were analyzed for the occurrence of regulatory motifs (*cis* elements; table 1.9) [Denslow, 2005; Titiz, 2008]. Based on the differential motif pattern of the promoter regions, PDX1.1 and PDX1.3 may be unequally regulated upon external stimuli in particular upon ozone exposure [Denslow *et al.*, 2007], ethylene production, salicylic acid and methyl jasmonate treatment [Titiz, 2008]. As *PDX2* carries most of the regulatory elements identified for *PDX1.1* and/or *PDX1.3*, one may suspect a co-regulation of these genes at the transcriptional level as has been suggested for the yeast homologs *SNZ1* and *SNO1*, respectively [Padilla *et al.*, 1998]. Interestingly, a G-Box motif (CACGTG) has been identified exclusively in the promoter region of *PDX1.2* from *A. thaliana*. This motif is specific for G-box binding transcription factors (GBF) involved in the phosphorylation-dependent regulation of light-responsive promoters and putatively in seed maturation [for review see Jakoby *et al.*, 2002]. In fact, the light regulation of *PDX1.2* has been studied in etiolated seedlings, but only a slight increase in the transcript abundance 15 min post transfer was observed [Titiz *et al.*, 2006].

Gene / Element	*PDX1.1* (At2g38230)	*PDX1.2* (At3g16050)	*PDX1.3* (At5g01410)	*PDX2* (At5g60540)
Cold	MYB (4) MYC (3)	C/DRE (1) MYB (8) MYC (9)	C/DRE (1) MYB (4) MYC (4)	MYB (5) MYC (1)
Drought	ABRE (3) MYB (4) MYC (3)	ABRE (3) C/DRE (1) MYB (8) MYC (9)	C/DRE (1) MYB (4) MYC (4)	MYB (5) MYC (1)
High light		G-Box (2)		
Heat shock	C/EBP (6)	C/EBP (1)	C/EBP (2)	C/EBP (3)
Osmotic stress	ABRE (3)	ABRE (3) C/DRE (1)	C/DRE (1)	
Ozone			ERE (2)	ERE (1)
Pathogen attack	TGA (1) WRKY (2)	TGA (4) WRKY (2)	ERE (2) WRKY (2)	ERE (1) WRKY (5)
Wounding	WRKY (2)	WRKY (2)	WRKY (2)	WRKY (5)
Abscisic acid	ABRE (3) MYB (4) MYC (3)	ABRE (3) G-Box (2) MYB (8) MYC (9)	MYB (4) MYB (4)	MYB (5) MYB (1)
Ethylene		G-Box (2)	ERE (2)	ERE (1)
Jasmonic acid		G-Box (2)	ERE (2)	ERE (1)
Salicylic acid	TGA (1) WRKY (2)	TGA (4) WRKY (2)	WRKY (2)	WRKY (5)

Table 1.9: Potential stress-responsive regulatory motifs (*cis* elements) in the promoter region (1.4 kb upstream) of the *PDX* genes present in *A. thaliana*. The abundance of the motifs is given in parentheses [adopted from Denslow, 2005].

1.3.8 The involvement of vitamin B_6 in oxidative stress

All living organisms have to cope with numerous environmental or anthropogenic stressors. Thus, they require mechanisms to avoid and/or adapt to stress situations [Apel and Hirt, 2004]. Many of the biotic and abiotic stresses that occur in plants cause the formation of reactive oxygen species (ROS), *i.e.* the superoxide radical ($O_2^{\cdot -}$), hydroperoxyl radical ($\cdot O_2H$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) or the hydroxyl radical ($\cdot OH$) [Laloi *et al.*, 2007]. ROS are continuously produced during aerobic metabolic reactions, *i.e.* energy transfer ($^1O_2$) and electron transfer reactions (H_2O_2, $O_2^{\cdot -}$, $\cdot O_2H$) [Asada, 2006]. To prevent oxidative damage, a low cellular level of ROS is maintained by enzymatic and non-enzymatic scavenging systems [Mittler, 2002]. Recently, a "ROS gene network" consisting of more than 150

genes involved in ROS-production, perception and scavenging as well as in signal sensing and transmission has been proposed in *A. thaliana* [Mittler *et al.*, 2004]. Therefore, ROS may have a new role in plants: Altering gene expression and affecting signal transduction pathways by acting as a cellular signal in response to intra- and extracellular stimuli (figure 1.9) [Wojtaszek, 1997; Mittler, 2002; Laloi *et al.*, 2004/2007], apart from their damaging effects on a large variety of biomolecules, such as proteins, lipids and DNA [Kim *et al.*, 2008]. This duality of function can only by facilitated by a delicate balance between the production and scavenging of ROS [Mittler *et al.*, 2004]. The authors argue that cellular events such as photosynthesis, pathogen attack and recognition, drought, salt, high-light, temperature stress and hormonal perception can result in an enhanced production of ROS ("oxidative burst"). The subsequent perception of ROS by sensor molecules leads to the adaptation of photosynthesis, activation of specific stress response mechanisms, *e.g.* through modulating gene expression by transcription factors and zinc finger proteins, programmed cell death and hormonal responses. In a parallel event, ROS are enzymatically scavenged (see below). The intensity, duration and localization of ROS (signals) depend on the interplay between production and scavenging pathways and may determine the mode of cellular response [Mittler *et al.*, 2004]. Although the signalling chain has not yet been fully elucidated and all ROS can cause a similar cytotoxic damage of membranes and cells, their signalling specificities may differ, and an intimate cross-talk between them has been predicted [Laloi *et al.*, 2007].

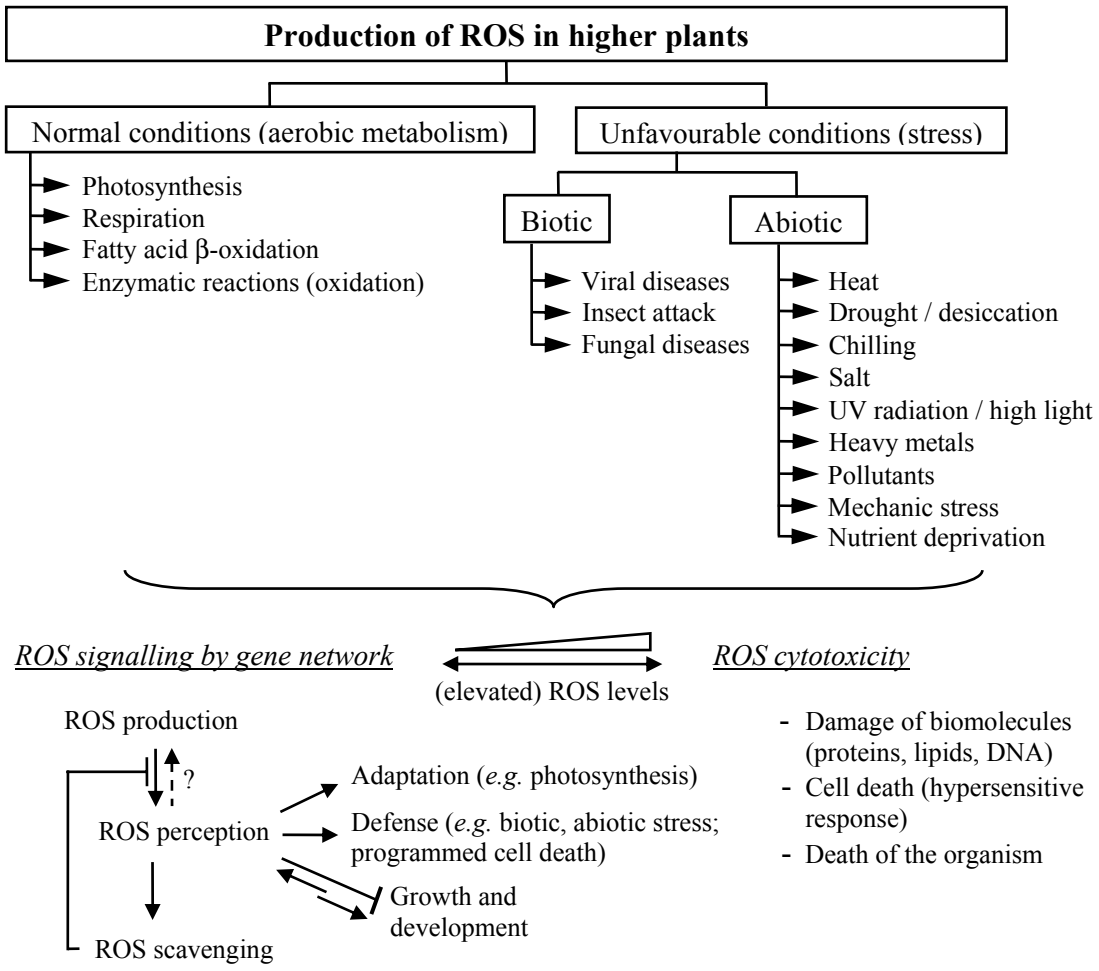


Figure 1.9: Occurrence of reactive oxygen species (ROS) in higher plants under normal and unfavourable environmental conditions, which can cause an "oxidative burst" (upper part) [based on Lichtenthaler, 1998; Mittler, 2002] and the predicted model of ROS signalling in plants in view of their cytotoxic nature (lower part) [based on Mittler *et al.*, 2004].

Biotic stress, such as pathogen attack, primarily leads to the formation of superoxide and H_2O_2 at the site of the attempted invasion [Apel and Hirt, 2004]. The formation of the two ROS in response to biotic stress has primarily been related to the activity of the complex plasma membrane-bound NADPH oxidase found in a variety of cells [Babior, 1999]. It uses NADPH as an electron donor in the one-electron reduction of oxygen to form the superoxide radical ($O_2^{\cdot -}$). The latter is rather inactive against many macromolecular compounds, but can subsequently form reactive oxidants such as oxidized halogens, free radicals and singlet oxygen, which are used by phagocytes to kill the invading pathogen but the surrounding host cells as well [Apel and Hirt, 2004]. Under physiological conditions at neutral or slightly acidic pH, the superoxide radical can disproportionate either spontaneously or catalyzed by superoxide

dismutase to H_2O_2 and O_2 both intra- and extracellularly [Wojtaszek, 1997]. H_2O_2, in addition to arising from the disproportionation of the superoxide radical, can be formed by apoplastic peroxidases in the presence of NADPH in a chain reaction [Chen and Schopfer, 1999]. H_2O_2 can, in the presence of transition metals such as Fe^{2+} or Cu^{+}, be intracellularly reduced to the highly reactive hydroxyl radical (·OH) *via* the Fenton reaction catalyzed by peroxidases. As no detoxification mechanism for the highly reactive and toxic hydroxyl radical has evolved, its generation must be avoided by a tight regulation of the cellular levels of superoxide and H_2O_2 [Apel and Hirt, 2004].

Apart from being released in response to biotic stresses, ROS are by-products of aerobic metabolism. For example, superoxide and H_2O_2 are released during photosynthesis in photosystem I. H_2O_2 is a product of CAT-peroxidase catalysis in the peroxisomes and fatty acid β-oxidation in mitochondria and peroxisomes, respectively. The homeostasis between production and destruction of ROS is tightly balanced both enzymatically and non-enzymatically, but may be disturbed by abiotic factors such as high light, drought, low temperature, chilling and salt [Lichtenthaler, 1998; Mittler, 2002].

Enzymatic scavenging systems include the catalytic activities of superoxide dismutase (SOD), ascorbate peroxidases (APX), catalase (CAT) and glutathione peroxidases (GPX). SOD decomposes superoxide and the hydroperoxyl radical (·O_2H) to hydrogen peroxide, the immediate substrate of APX, CAT and GPX, respectively. In contrast to CAT, both APX and GPX require an ascorbate and/or glutathione regeneration system for their activity including monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (figure 1.10) [Apel and Hirt, 2004]. The homeostasis between the different scavenging enzymes appears to be crucial for the efficient suppression of elevated levels of superoxide and H_2O_2 to prevent from the formation of hydroxyl radicals [Rizhsky *et al.*, 2002].

A *Superoxide (and hydroperoxyl radical) disproportionation*:

(1) $2\ O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ (SOD)

(2) $2\ {}^{\cdot}O_2H + O_2^{\cdot -} + H^+ \rightarrow H_2O_2 + O_2$ (SOD)

(3) $2\ {}^{\cdot}O_2H \leftrightarrow H_2O_2 + O_2$ (SOD)

B *Catalase activity*:

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ (CAT)

C *Ascorbate-glutathione cycle (Halliwell-Asada pathway)*:

(1) H_2O_2 + ascorbate → H_2O +MDA (APX)

(2) MDA + NAD(P)H → ascorbate + $NAD(P)^+$ (*e.g.* MDAR)

(3) DHA + GSH → ascorbate + GSSG (*e.g.* DHAR)

(4) GSSG + NAD(P)H → GSH + $NAD(P)^+$ (GR)

D *Glutathione-peroxidase cycle*:

(1) H_2O_2 + GSH → H_2O + GSSG (GPX)

(2) GSSG + NAD(P)H → GSH + $NAD(P)^+$ (GR)

Figure 1.10: Principal modes of enzymatic ROS scavenging systems by superoxide dismutase (SOD) ***(A)***, catalase (CAT) ***(B)***, ascorbate peroxidases (APX) ***(C (1))***, glutathione reductase (GPX) ***(D (1))*** and the respective regeneration cycles of APX and GPX, ascorbate-glutathione cycle ***(C (2-4))*** and glutathione peroxidases cycle ***(D (2))***. The enzymes are given in parentheses. Abbreviations used: DHA: dehydroascorbate; DHAR: DHA reductase; GR: glutathione reductase; MDA: monodehydroascorbate; MDAR: MDA reductase [based on Wojtaszek, 1997; Apel and Hirt, 2004].

Non-enzymatic scavengers such as the major cellular redox buffers ascorbate and glutathione as well as tocopherols, flavonoids, alkaloids and carotenoids have a major impact on disposing ROS. Ascorbate is directly and indirectly involved in scavenging hydrogen peroxide and superoxide by ascorbate peroxidases [for review see Smirnoff, 2001]. Glutathione, the most abundant low-molecular weight thiol, can reach a concentration between 1 to 5 mM. Its predominant task is to keep the intracellular environment in a reduced state. Ninety per cent of glutathione present in a cell occur in its reduced form, GSH. This reduced compound can directly be oxidized to GSSG by hydrogen peroxide. Furthermore, essential electrons for the catalytic activity of glutathione peroxidases for instance are provided by GSH as well [for review see Rouhier *et al.*, 2008]. Carotenoids predominantly occur in the thylakoid membranes of chloroplasts where they act as accessory light-harvesting pigments thereby extending the range of light that can be absorbed during photosynthesis. Additionally, these molecules play a photoprotective role by quenching triplet-state chlorophyll [for review see Young, 1991]. α-Tocopherol is involved in the maintenance of photosynthetic membranes and in photo- and

antioxidative protection. It scavenges singlet oxygen and lipid hydroperoxy radicals, thus preventing lipids and other membrane components from photooxidative damage and from the subsequent lipid peroxidation of the membrane lipid layer [for review see Munné-Bosch, 2007]. Recently, a new candidate has been postulated to efficiently quench ROS comparable to ascorbic acid and α-tocopherol: Vitamin B_6. Vitamin B_6 is rapidly degraded in the presence of singlet oxygen both *in vitro* [Bilski *et al.*, 2000] and *in vivo* [Ehrenshaft *et al.*, 1998/1999a/1999b; Osmani *et al.*, 1999]. Theoretical studies suggest that singlet oxygen and, to some extent, hydroxyl radicals can attack vitamin B_6, in particular PN, PL and to a lesser extent PLP, forcing the subtraction of a hydrogen atom from either C-5' or C-4' [Matxain *et al.*, 2006/2007]. Whereas there is no theoretical evidence that PN is attacked by superoxide radicals [Matxain *et al.*, 2006], this reaction was found to occur *in vitro* [Ehrenshaft *et al.*, 1999b; Denslow *et al.*, 2005]. In mammals, the supporting effects of vitamin B_6 in the therapy of diseases which involve the release or the accumulation of ROS have been demonstrated (see section 1.3.3).

1.4 Objectives of the present work

The elucidation of the alternative pathway of vitamin B_6 biosynthesis in the majority of organisms capable of producing this vitamin opens a rather new field of scientific interest. The identification of the enzymes PDX1 and PDX2 and the characterization of the respective knockout lines in *A. thaliana*, the elucidation of the complex reaction mechanism in both *B. subtilis* and *A. thaliana*, including precursor and product release studies, are of interest and had been established before the onset of this thesis. Within this project, one objective was the *de novo* biosynthesis of the pyrimidine moiety of vitamin B_1 (see chapter 2). Another objective was the over-expression of the endogenous *PDX* genes in *A. thaliana* with the aim to increase the vitamin B_6 content (see chapter 3). Lastly, the regulation of vitamin B_6 biosynthesis, and in this context, an initial characterization of PDX1.2, is addressed in chapter 4.

1.5 References

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2 Vitamin B_1 biosynthesis in plants requires the essential iron-sulfur cluster protein, THIC

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2.2 Abstract

Vitamin B_1 (thiamin) is an essential compound in all organisms acting as a cofactor in key metabolic reactions and has furthermore been implicated in responses to DNA damage and pathogen attack in plants. Despite the fact that it was discovered almost a century ago and deficiency is a widespread health problem, much remains to be deciphered about its biosynthesis. The vitamin is composed of a thiazole and pyrimidine heterocycle, which can be synthesized by prokaryotes, fungi, and plants. Plants are the major source of the vitamin in the human diet, yet little is known about the biosynthesis of the compound therein. In particular, it has never been verified whether the pyrimidine heterocycle is derived from purine biosynthesis through the action of the THIC protein as in bacteria, rather than vitamin B_6 and histidine as demonstrated for fungi. Here, we identify a homolog of THIC in *Arabidopsis* and demonstrate its essentiality not only for vitamin B_1 biosynthesis, but also plant viability. This step takes place in the chloroplast and appears to be regulated at several levels, including through the presence of a riboswitch in the 3'-untranslated region of THIC. Strong evidence is provided for the involvement of an iron-sulfur cluster in the remarkable chemical rearrangement

reaction catalyzed by the THIC protein for which there is no chemical precedent. The results suggest that vitamin B_1 biosynthesis in plants is in fact more similar to prokaryotic counterparts and that the THIC protein is likely to be the key regulatory protein in the pathway.

2.3 Introduction

Vitamin B_1, in the form of thiamin diphosphate (TPP), acts as a cofactor for several enzymes in key cellular metabolic pathways such as glycolysis, the pentose phosphate pathway and the citric acid cycle (TCA), in addition to amino acid and nonmevalonate isoprenoid biosynthesis, respectively [Pohl *et al.*, 2004]. More recently, it has been implicated in tolerance to DNA damage [Machado *et al.*, 1996] and as an activator of disease resistance in plants [Ahn *et al.*, 2005/2007]. It is an essential compound in all living systems, but *de novo* biosynthesis is only found in prokaryotes, fungi, and plants; therefore, animals must acquire it from dietary sources. Deficiency of the vitamin is a widespread health problem particularly in countries where rice is a major constituent of the diet, because grain polishing removes most of the thiamin in the bran. Therefore, it is of major interest to define the pathways of biosynthesis, which may assist in the overproduction of the vitamin for beneficial purposes.

Thiamin biosynthesis occurs through the separate formation of the pyrimidine and thiazole heterocycle, which are then coupled to form the cofactor (supplementary scheme 2.1). Although the kinases involved in the phosphorylation of the precursor molecules have been identified in many organisms, the *de novo* formation of the heterocycles is poorly understood. In bacteria, the mechanism of thiazole formation is reasonably well documented and involves at least five enzymes that transform 1-deoxy-D-xylulose 5-phosphate (DXP), cysteine and either glycine or tyrosine (*Bacillus subtilis* or *Escherichia coli*, respectively), in a complex oxidative condensation [Settembre *et al.*, 2003; Begley *et al.*, 1999]. Much less is known about the formation of the pyrimidine moiety, which is thought to be derived from 5-aminoimidazole ribonucleotide (AIR_t) an intermediate in the purine biosynthetic pathway (supplementary scheme 2.1) [Himmeldirk *et al.*, 1998; Estramareix and Therisaud, 1984]. AIR_t is then converted into 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P), or its alcohol, through the action of THIC. The mechanism by which THIC performs this remarkable rearrangement reaction is of particular interest, as there appears to be no chemical precedent, but little is known of its requirements [Lawhorn *et al.*, 2004].

In eukaryotes, neither the biosynthesis of the thiazole nor of the pyrimidine moiety has been

fully elucidated. A study in spinach has implicated DXP, cysteine and tyrosine as precursors for thiazole synthesis [Julliard and Douce, 1991]. Yet, to date, only a single thiazole biosynthetic enzyme has been identified, called THI4 in *Saccharomyces cerevisiae* [Praekelt *et al.*, 1994] and THI1 in *Arabidopsis* [Machado *et al.*, 1996], but shows no homology to the bacterial enzymes. Although the involvement of this protein in thiazole biosynthesis has been deduced from genetic studies, its biochemical function has not been clarified. Interestingly, recent studies based on structural and mechanistic data of the *S. cerevisiae* protein have led to the intriguing implication of the redox cofactor NADH as a precursor in this organism [Chatterjee *et al.*, 2007]. In contrast to bacteria, in eukaryotes the pyrimidine moiety appears to be derived from vitamin B_6 and histidine [Tazuya *et al.*, 1993/1994/1995; Zeidler *et al.*, 2003]. Again, only a single protein involved in this transformation is known, THI5 or NMT1 for "no message in thiamin1" in *S. cerevisiae* and *Schizosaccharomyces pombe*, respectively [Maundrell, 1990; Schweingruber *et al.*, 1991]. Thus, although much remains to be deciphered, it is assumed that the pathways of thiamin biosynthesis are distinct from each other in prokaryotes and eukaryotes [Hohmann and Meacock, 1998].

Here, we have focused on the synthesis of the pyrimidine moiety in plants employing *Arabidopsis*. It could be envisaged that the pyrimidine heterocycle is derived either from AIR_t as in bacteria, or vitamin B_6 and histidine as in fungi, or both (supplementary scheme 2.1). In this study, we demonstrate that the predominant and perhaps sole source of the pyrimidine heterocycle in *Arabidopsis* is *via* a bacterial type pathway. A single *THIC* homolog is present and is essential for plant viability. However, the *thiC* knockdown mutant can be rescued with thiamin. Metabolic profiling allows us to demonstrate the essentiality of the vitamin for primary metabolic function in plants. Localization studies show the protein to be partitioned to chloroplasts. Expression analysis reveals positive regulation of the expression of the gene by light and severe negative regulation by thiamin itself. We provide strong evidence that the THIC protein contains an iron-sulfur (Fe-S) cluster that is required for functionality, and we suggest a further level of regulation of the protein by the chloroplast thioredoxin-ferredoxin system.

2.4 Material and Methods

2.4.1 Plant material and growth conditions

Unless indicated otherwise, *Arabidopsis* (wild-type (WT, ecotype Col-0), SAIL_793_H10 [Sessions *et al.*, 2002] and CpNIFS RNAi-inducible lines [Van Hoewyk *et al.*, 2007]) was

either grown in soil or sterile culture under long-day conditions (16 hrs light at a light intensity of 100 µmol of photons m^{-2} s^{-1}) at 22°C/19°C day/night, respectively, and in the presence or absence of thiamin as indicated. In the case of the light induction analysis, plates were exposed to 100 µmol of photons m^{-2} s^{-1} for one hr after vernalisation and then transferred to continuous dark at 20°C for six days. The etiolated seedlings were then transferred to light (as above) and whole plantlets were collected after various times of exposure to light or a 24-hr light-dark cycle (*i.e.*, 16-hr light, 8-hr dark). In all cases, samples of plant material collected were immediately frozen in liquid nitrogen and maintained at -80°C until analysis.

For the tissue expression analysis, roots, stems, flowers, siliques, rosette leaves, and cauline leaves were collected from at least ten plants. Cotyledons were collected from seedlings grown under the same conditions at two weeks. When grown in sterile culture, *Arabidopsis* seeds were first surface-sterilized, plated on Murashige and Skoog (MS) medium [Murashige and Skoog, 1962] containing 1% (w/v) sucrose and 0.9% (w/v) agar and kept for four days in the dark at 4°C, after which the plates were transferred to the same conditions as described for soil-grown plants. Samples for the developmental analysis were collected from seedlings grown in culture 2, 5, 7, 9, and 12 days after germination in the absence of thiamin. Complementation experiments were performed by growing seedlings on MS plates under the same light and temperature conditions, either in the absence or presence of thiamin (0.5 µM) or after transfer to soil, by watering with 1.5 µM or 100 µM thiamin, respectively, as indicated.

2.4.2 Analysis of mutants by PCR

Genomic DNA of the *Arabidopsis* SAIL_793_H10 line was extracted and screened for the T-DNA at the *THIC* locus. Forward and reverse primers from the sequence of *THIC* were designed for PCR screening with a combination of a T-DNA left border-specific primer [Sessions *et al.*, 2002]. Flanking sequences of insertion loci were confirmed by sequencing. Sequences of primers used are as follows: THIC-F, 5'-CGCTAAGCAAGGGATCATAACTG-3'; THIC-R, 5'-CCGCTCGAGTTTCTGAGCAGCTTTGACATAGC-3'; and SAIL T-DNA LB3 primer, 5'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3'. PCR conditions were as follows: Denaturation (2 min at 94°C), 35 cycles of: 45 s at 94°C, 45 s at 57°C, 1 min at 72°C, followed by 5 min at 72°C.

2.4.3 Isolation of RNA, RT-PCR and immunochemical analyses

Total RNA was extracted using the RNeasy mini kit (Qiagen) and treated with RNase-free DNase (Qiagen) to remove traces of DNA, according to the manufacturer's instructions. First strand cDNA synthesis was performed at 42°C for 60 min in a volume of 20 µl, containing one or two µg of total RNA, 20 pmol of oligo(dT) primers, and 200 units of reverse transcriptase from the Advantage RT-for-PCR kit (Takara BioEurope/CLONTECH). For transcript quantification, the equivalent of 50 ng of total RNA was used. The PCR conditions were as follows: Denaturation at 95°C for 2 min, followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The primers used were THIC N-term F, 5'-AAAAAGCAGGCTACATGGCTGCTTCAGTACACTG-3', and THIC N-term R, 5'-AGAAAAGCTGGGTCTTCTTCAAAAGATGGAATTG-3'; and, as a control, actin-2 F, 5'-ATTCTTGCTTCCCTCAGCAC-3', and actin-2 R 5'-CCCCAGCTTTTTAAGCCTTT-3'. Western blot analysis was performed essentially as described recently [Titiz *et al.*, 2006], except that immunodetection was carried out employing an antibody raised in rabbit against the *Bacillus subtilis* ThiC protein, which had been affinity-purified before use against *Arabidopsis* DN71-THIC (1:1,000 dilution).

2.4.4 Expression of recombinant protein and purification

A truncated form of *Arabidopsis* THIC missing 71 amino acids from the N terminus was constructed by amplifying the DNA fragment from a full-length cDNA obtained from the *Arabidopsis* Biological Resource Center (stock U16941) [Yamada *et al.*, 2003]. The amplified product was cloned into the *Nde*I/*Xho*I sites of pET21a (Novagen) to allow expression of the protein with a C-terminal hexa-histidine affinity tag in *E. coli* BL21(DE3) cells. The resulting clone was named pETDN71AtTHIC. Expression was induced by addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside, followed by growth for 8 h at 24°C. The protein was purified by Ni-NTA chromatography (Qiagen) using the non-denaturing protocol described by the manufacturer and was judged to be >90% homogeneous from an SDS/PAGE analysis.

2.4.5 Vitamin B_1 determination

Total vitamin B_1 was measured by a microbiological assay employing *S. cerevisiae* strain *thi4* [Winzeler *et al.*, 1999] auxotrophic for vitamin B_1 grown in thiamin deficient medium (For-Medium). Vitamin B_1 was extracted from plant tissue as described for vitamin B_6 [Tambasco-Studart *et al.*, 2005], with the exception that the extract was treated with alkaline phosphatase

before quantification. The vitamin B_1 content of plant extracts (either mature dry seeds or seedlings as indicated) was extrapolated from a standard curve of growth of cultures supplemented with known amounts of thiamin (0-9 ng).

2.4.6 Sub-cellular localization

The N-terminal 90 amino acids of THIC were amplified from a full-length cDNA clone obtained from the *Arabidopsis* Biological Resource Center (stock U16941) and fused to both the N- and C-terminus of YFP. Transient expression was carried out in *Arabidopsis* protoplasts essentially as described [Tambasco-Studart *et al.*, 2005].

The N-terminal 90 amino acids of THIC were amplified from a full-length cDNA clone obtained from the *Arabidopsis* Biological Resource Center (stock U16941) (44), supplied in the Gateway entry vector pENTR/SD/D-TOPO. The cloned fragments were fused to either the N or C terminus of YFP by employing the binary Gateway-compatible destination vectors pB7WGY2.0 and pB7YWG2.0, respectively [Karimi *et al.*, 2005]. The primers used were THIC N-term F and R as described for RT-PCR for the N1-90AtTHIC-YFP fusion, and THIC target N-term F and THIC target+stop R: 5'-AGAAAAGCTGGGTCTTATTCTTCAAAAGA TGGAATTG-3' for the YFP-N1-90AtTHIC fusion. Transient expression was carried out in *Arabidopsis* protoplasts essentially as described in [Jin *et al.*, 2001], using three-week old seedlings maintained on MS plates, but with some modifications: incubation in enzyme solution was performed overnight, in the dark, without agitation. Cells were allowed to recover 48 h before analysis by confocal laser scanning microscopy (Leica Microsystems) equipped with an ArKr laser at 488 nm. YFP fluorescence was recorded between 500 and 550 nm.

2.4.7 CpNIFS silencing

Silencing of the chloroplast localized cysteine desulfurase (CpNIFS) was carried out essentially as described recently [Van Hoewyk *et al.*, 2007]. Briefly, RNA interference was induced in 14-day old *CpnifS* RNAi inducible plants grown in soil by spraying every four days with a 2% (v/v) ethanol solution. Samples were taken for analysis 19 days after induction.

2.4.8 Metabolite analysis and chlorophyll determination

Cotyledons of 11-day old seedlings (100 mg fresh weight) grown in the presence or absence of thiamin supplementation (0.5 µM) as indicated were used for the metabolite analysis. The

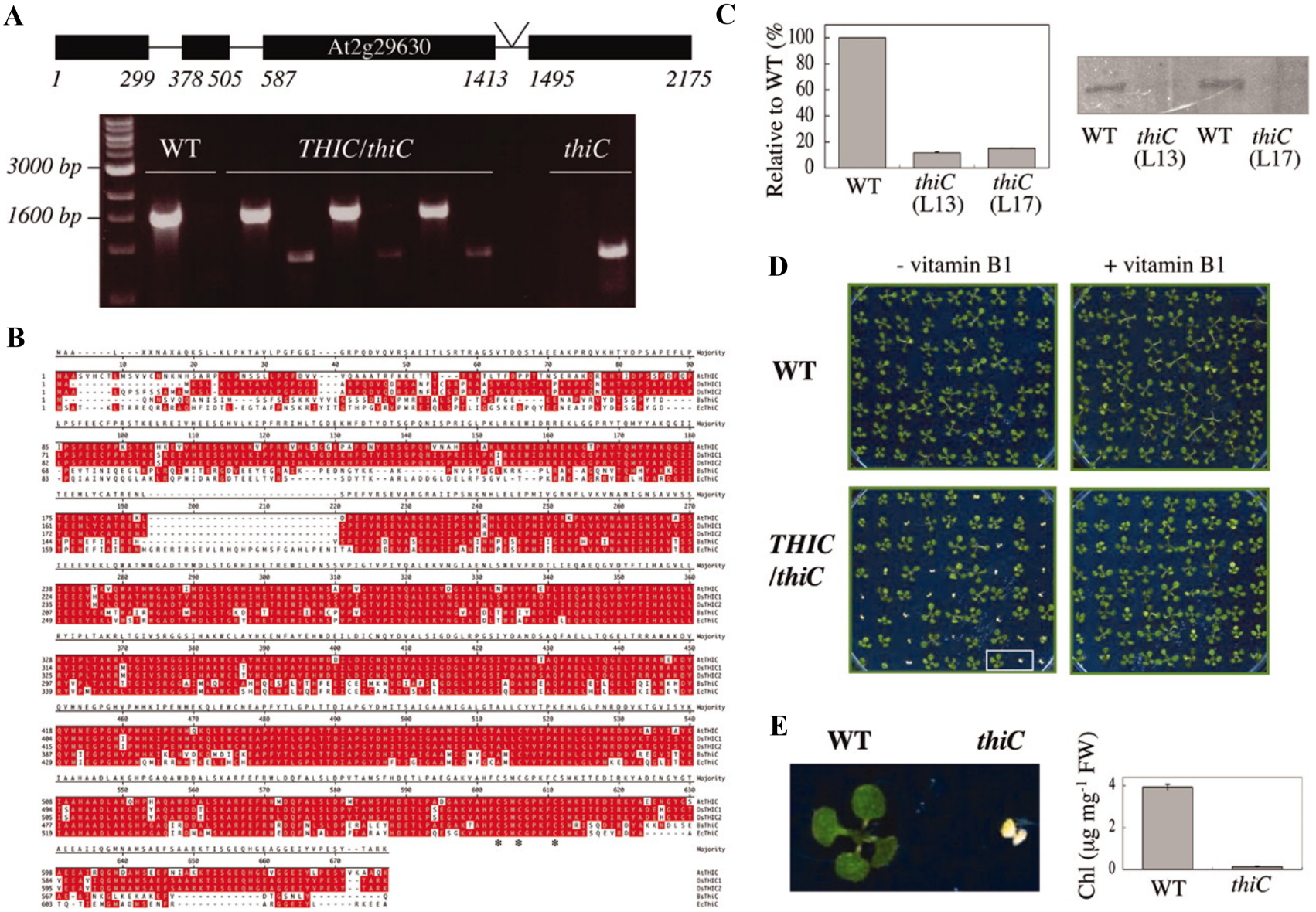
analysis was performed employing gas chromatography mass spectrometry essentially as described in [Lisec *et al.*, 2006]. Total chlorophyll was determined according to [Porra *et al.*, 1989].

2.5 Results and Discussion

2.5.1 *De novo* biosynthesis of the pyrimidine moiety of vitamin B_1 *in planta via* THIC

As either the bacterial or fungal route of pyrimidine heterocycle biosynthesis, or both, could be in operation *in planta*, we first searched for homologs of known genes of either route in *Arabidopsis*, *i.e.*, *THIC* or *NMT1*, respectively. Whereas one homolog of *THIC* was identified (At2g29630), no homolog of *NMT* could be found. The *THIC* gene contains three introns and four exons (figure 2.1*A*). The predicted protein sequence is 644 amino acids with a calculated molecular mass of 71,994 Da. Homologs of *THIC* were identified in prokaryotes and plants, but not in fungi or apicomplexa. An amino acid sequence alignment (figure 2.1*B*) demonstrates that the *Arabidopsis* protein is highly homologous to THIC from *B. subtilis* and *E. coli* (58% and 53% identity, respectively) and is *ca.* 80% identical to the two homologs identified in rice.

Figure 2.1: (page 83) *Arabidopsis* THIC characteristics. ***(A)*** (*Upper*) Exon-intron structure of *Arabidopsis THIC* indicating the location of the SAIL_793_H10 insertion. (*Lower*) PCR analysis indicating plant genotypes employing primer pairs specific for either WT or the T-DNA insertion in *thiC*, respectively. From left to right: molecular marker, WT, three lines heterozygous for *thiC*, and a homozygous line (L13). ***(B)*** Amino acid sequence alignment of THIC homologs identified in *A. thaliana* (At), *O. sativa* (Os), *B. subtilis* (Bs), and *E. coli* (Ec). Amino acids identical in at least two of the sequences are shaded in red. The asterisk denotes the identified $C(X)_2C(X)_4C$ motif. ***(C)*** (*Left*) Quantitative RT-PCR of *THIC* transcript in WT or *thiC* plants. The values indicated are the percentage mRNA levels relative to WT (=100%). Error bars indicate the standard deviation of three independent experiments. (*Right*) Western blot analysis of the same samples probed with a THIC antibody. L13 and L17 refer to lines 13 and 17, respectively. ***(D)*** *thiC* mutant phenotype. (*Left*) WT and progeny of selfed *THIC*/*thiC* plants grown in sterile culture lacking vitamin B_1. *Ca.* one quarter of the population shows a pale green phenotype and does not develop beyond the cotyledon stage. (*Right*) Supplementation with vitamin B_1 (0.5 µM) restores growth of *thiC* to that of WT. Ten-day old seedlings grown under long day conditions. ***(E)*** (*Left*) Enlarged picture of two seedlings from *D*. (*Right*) Total chlorophyll content (Chl) of WT and *thiC* (L13) under the same conditions. Error bars indicate the standard deviation of three independent experiments.


A
At2g29630
1
299
378
505
587
1413
1495
2175
3000 bp
1600 bp
WT
THIC/thiC
thiC
B
C
Relative to WT (%
100
80
60
40
20
0
WT
thiC (L13)
thiC (L17)
WT
thiC (L13)
WT
thiC (L17)
D
- vitamin B1
+ vitamin B1
WT
THIC /thiC
E
WT
thiC
Chl (μg mg-1 FW)
4
2
0
WT
thiC

To address the question of whether THIC is involved in the biosynthesis of the pyrimidine moiety of thiamin, loss of function studies were performed employing a putative insertion mutant of *THIC* identified in the SAIL collection (SAIL_793_H10) [Sessions *et al.*, 2002]. SAIL_793_H10 is a T-DNA insertion line that carries the *BAR* gene conferring resistance to phosphinothricin (Basta). PCR analysis with primers designed to hybridize close to either end of the inserted element and the flanking gene sequence revealed bands of the expected size, thus confirming the respective insertion(s) in *THIC* (figure 2.1*A*). Sequencing of the amplified fragments revealed that the T-DNA insertion is located at base pair 1,464 relative to the start codon, *i.e.*, within the third intron of *THIC* (figure 2.1*A*). A segregation analysis among the progeny of selfed *THIC*/*thiC* plants revealed that Basta-resistant and -sensitive seedlings, respectively, segregated in a ratio of 3:1 (supplementary table 2.1), indicating that the insertion is at a single locus. A quantitative RT-PCR analysis revealed that the *THIC* transcript is severely reduced (*ca.* ten-fold) in the *thiC* mutant plants (figure 2.1*C*). Furthermore, Western blot analysis using a THIC antibody detected the ascribed protein band at substantially lower abundance in the mutant as compared with WT (figure 2.1*C*). Thus, we conclude that this mutant represents a strong knockdown version of THIC in *Arabidopsis*.

To assess for a *thiC* phenotype, seedlings heterozygous for the T-DNA insertion were grown on thiamin-deficient medium. One quarter of the progeny exhibited a chlorotic phenotype and did not develop beyond the cotyledon stage (figure 2.1*D*, *E*). PCR analysis confirmed that the segregating phenotype were *thiC* seedlings (data not shown). This phenotype was rescued by supplementation with thiamin (figure 2.2*D*). Moreover, *thiC* lines grown in thiamin-free sterile culture for ten days and then transferred to soil could be maintained by continuously supplying them with a thiamin solution (figure 2.2*A*). If the *thiC* plants were not supplemented with thiamin, they eventually died. A concentration of thiamin as low as 1.5 µM was sufficient to allow growth of the seedlings, but these were chlorotic; at 100 µM thiamin, seedlings had normal coloration, but were smaller than WT seedlings (figure 2.2*A Left*). To further corroborate that the seedling lethal phenotype of *thiC* is due to impairment in thiamin biosynthesis, a quantitative microbiological assay employing a strain of *S. cerevisiae* auxotrophic for thiamin was established. Ten-day old *thiC* seedlings grown in the absence of thiamin had only 30% total vitamin B_1 content compared with WT seedlings (table 2.1).

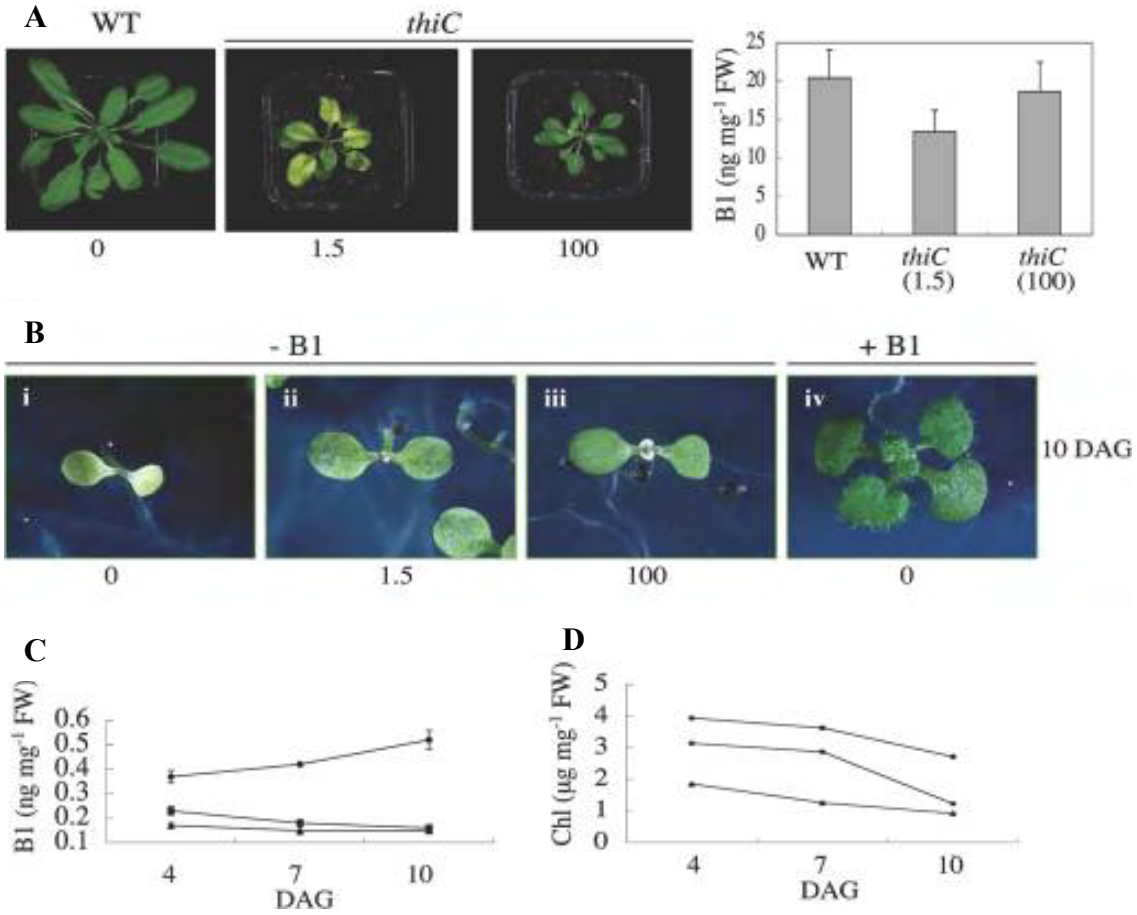


Figure 2.2: Rescue of *Arabidopsis thiC* mutants. ***(A)*** (*Left*) Rescue of *thiC* by supplementation with thiamin after transfer to soil. Ten-day old segregating *thiC* seedlings grown on medium lacking vitamin B_1 (figure 1*D*) were transferred to soil and watered with the indicated concentrations of thiamin (µM). Plants are 30 days after transfer to soil. (*Right*) Total vitamin B_1 content of seeds of rescued plants. ***(B)*** Progeny of rescued *thiC* lines as described in ***(A)*** compared with segregating non-supplemented *thiC* from a heterozygous population. The numbers indicate the concentration of thiamin (µM) supplied to the mother plants. Seedlings (ten-day old) were grown in the absence of thiamin. As a control, a segregating *thiC* seedling from a heterozygous mother grown in the presence of vitamin B_1 is shown. ***(C)*** Total vitamin B_1 content of WT (circles), *thiC* progeny rescued with 100 µM (squares) and 1.5 µM (triangles) thiamin, respectively, grown in the absence of thiamin over the time period indicated. ***(D)*** Total chlorophyll content (Chl) of the same seedlings as in ***(C)***. Error bars indicate the standard deviation of three independent experiments. DAG, days after germination.

Interestingly, we observed that the total vitamin B_1 level in seeds of rescued *thiC* is 66% and 91% of WT when supplemented with 1.5 and 100 µM thiamin, respectively (figure 2.2*A Right*). This was reflected in a noticeable developmental advance of the progeny even when grown in the absence of vitamin B_1, compared with segregating *thiC* seedlings from a non-supplemented heterozygous population (figure 2.2*B*). The level of vitamin B_1 in the *thiC*

progeny was seen to decrease over time, whereas that of the WT increased over the same period (figure 2.2*C*). Most likely, stored vitamin B_1 gets used up in the *thiC* mutant, whereas thiamin biosynthesis is induced in WT plants when the compound becomes limiting (see expression analysis below). Indeed, the increase in thiamin levels observed over time implies that a certain threshold level is required for plant viability at this stage. Even when *thiC* seedlings have enhanced thiamin content, they are not able to proceed to primary leaf development in the absence of supplementation, thus emphasizing the need for a certain level of the vitamin for survival. Furthermore, we observed that seedlings diminished in thiamin content were paler due to a decrease in total chlorophyll (figure 2.2*C*, *D*) and were approaching albino in appearance 12 days after germination. This can be explained by the fact that the biosynthesis of the phytol moiety of chlorophyll depends on the action of 1-deoxy-D-xylulose 5-phosphate synthase, a key enzyme of the nonmevalonate pathway of isoprenoid biosynthesis, which in turn is critically dependent on TPP as a cofactor [Sprenger *et al.*, 1997]. As 1-deoxy-D-xylulose 5-phosphate synthase is the initial enzyme of this pathway, it is likely that all isoprenoids biosynthesized *via* this route are reduced.

Sample	Vitamin B_1 (ng/mg FW)	% of WT
WT	0.47 ± 0.04	100
thiC	0.14 ± 0.05	30

Table 2.1: Vitamin B_1 content of WT and *thiC* mutant plants. Total vitamin B_1 content was quantified in whole seedlings employing a microbiological assay. Seedlings were grown in sterile culture in medium lacking vitamin B_1 and analyzed ten days after germination. The results shown are the average of four independent experiments. In the case of the *thiC* mutant, segregating homozygous seedlings from a heterozygous population of line 17 were used.

2.5.2 Metabolite profiling reveals the importance of THIC in primary metabolism

As vitamin B_1 in the form of TPP is a necessary cofactor for key enzymes of primary metabolism, we compared the relative level of certain metabolites in *thiC* and WT. Whole seedlings were analyzed 11 days after germination, reflecting the state of more severe depletion in total thiamin content (figure 2.2). The most notable alteration in metabolites of *thiC* plants, when they were grown in the absence of thiamin supplementation, appears in the dramatic relative increase in the level of certain amino acids, in particular alanine, asparagine, methionine, phenylalanine, and tryptophan (table 2.2). In addition, certain constituents of the TCA cycle

were seen to increase, *i.e.* malate, whereas there was a significant decrease in the level of fumarate. It is possible to explain these changes based on the requirement of certain primary metabolic enzymes on TPP as a cofactor. Notably, impairment of pyruvate dehydrogenase would lead to a breakdown of the TCA cycle. In this context, fumarate is thought to represent a major fraction of fixed carbon in *Arabidopsis* [Chia *et al.*, 2000], thus the *thiC* seedlings can maintain the cycle by using these reserves, which ultimately leads to the observed depletion of this metabolite at day 11. An impairment of pyruvate dehydrogenase could also lead to an increase in glycolytic intermediates, *e.g.*, pyruvate or phosphoenolpyruvate (PEP), which in turn may explain the increases in alanine, phenylalanine, and tryptophan. An increase in the accumulation of either pyruvate or PEP could also elevate the oxaloacetate pool through the action of either pyruvate carboxykinase or PEP-carboxylase. This then may explain not only the observed increase in the pool sizes of methionine and asparagine, but also of malate through the reverse reaction of enzymes of the TCA cycle. It should also be pointed out that transketolase, a participant in both the Calvin and non-oxidative pentose phosphate cycle, whose products and substrates in turn are starting points of several other chloroplast pathways, is a TPP-dependent enzyme. A decrease in transketolase activity is expected to be reflected not only in the Calvin cycle, where the enzyme seems to be rate limiting, but also in carbohydrate, amino acid and natural product metabolism [Henkes *et al.*, 2001]). Although a partial reversion of *thiC* metabolites to WT levels was observed when grown in the presence of thiamin, notably some metabolites remained at elevated levels, in particular certain amino acids (table 2.2, supplementary table 2.2). The reason for this is unknown at present.

Metabolite (nmol/g FW)	WT -B_1	*thiC* -B_1	*thiC* +B_1
Amino acids			
Alanine	1.000±0.053	**9.659±0.913**	1.448±0.053
Arginine	1.000±0.080	1.283±0.110	2.458±0.144
Asparagine	1.000±0.070	**6.316±0.534**	**5.037±0.248**
Aspartate	1.000±0.042	0.964±0.025	1.098±0.006
β-Alanine	1.000±0.027	**3.545±0.155**	1.396±0.019
Cysteine	1.000±0.043	**3.513±0.123**	**4.329±0.135**
Glutamate	1.000±0.050	**3.018±0.128**	1.416±0.054
Glycine	1.000±0.255	*0.131±0.009*	*0.051±0.003*
Lysine	1.000±0.013	**4.563±0.243**	**4.392±0.101**
Methionine	1.000±0.027	**8.292±0.598**	**8.103±0.290**
Phenylalanine	1.000±0.071	**23.397±1.129**	**16.529±0.514**
Proline	1.000±0.046	1.216±0.029	**2.119±0.088**
Serine	1.000±0.022	1.285±0.014	0.539±0.005
Threonine	1.000±0.008	1.290±0.023	0.575±0.005
Tryptophan	1.000±0.142	**44.130±4.096**	**42.691±1.915**
Tyramine	1.000±0.038	1.129±0.0.23	1.016±0.028
Valine	1.000±0.038	**2.840±0.159**	1.980±0.040
Organic acids			
Butyrate	1.000±0.057	**6.111±0.347**	**2.479±0.045**
Citrate	1.000±0.144	1.211±0.172	0.835±0.122
Fumarate	1.000±0.054	*0.231±0.016*	*0.220±0.005*
Glycerate	1.000±0.107	1.309±0.094	0.526±0.033
Glycerol	1.000±0.064	0.693±0.031	0.690±0.089
Isocitrate	1.000±0.078	1.769±0.134	1.704±0.040
Malate	1.000±0.058	**2.509±0.271**	0.938±0.033
Sugars			
Fructose	1.000±0.078	1.443±0.066	1.412±0.121
Gentiobiose	1.000±0.085	*0.196±0.031*	*0.435±0.006*
Glucose	1.000±0.019	1.681±0.128	1.801±0.201
Raffinose	1.000±0.154	*0.453±0.041*	*0.382±0.016*
Ribose	1.000±0.022	*0.293±0.012*	*0.373±0.017*
Sucrose	1.000±0.023	0.789±0.036	0.782±0.046
Trehalose	1.000±0.023	1.031±0.047	0.781±0.011

Table 2.2: Relative metabolite levels between WT and *thiC*. Samples were grown in the absence or presence of vitamin B_1 (0.5 µM) as indicated and cotyledons were harvested 11 days after germination. Substantial increases are shown in bold (*ca.* two-fold), whereas decreases (*ca.* 0.5-fold) are italicized. Standard errors are the average of six experimental repetitions. Quantitative data are provided in supplementary table 2.2.

2.5.3 Sub-cellular localization of THIC

So far, proteins involved in thiamin biosynthesis in plants have been reported to have differential subcellular localizations. For example, THI1 involved in the synthesis of the thiazole moiety, is targeted to both mitochondria and plastids [Chabregas *et al.*, 2001], whereas the recently described thiamin pyrophosphokinase is reported to be cytosolic [Ajjawi *et al.*, 2007]. To assemble the site(s) of synthesis of the thiamin molecule, it is thus of interest to know the subcellular localization of THIC. An *in silico* analysis indicates amino acid sequence alignment does not reveal an obvious N-terminal presequence in the *Arabidopsis* protein in comparison with bacterial counterparts (figure 2.1*B*). Moreover, the algorithms differ with respect to the length of the expected target sequence, *e.g.*, TargetP [Emanuelsson *et al.*, 2000] and PSORT [Bannai *et al.*, 2002] predict 37 and 48 amino acids, respectively. We investigated the subcellular location of THIC by fusing the N-terminal 90 amino acids to YFP at either the N or C terminus (N90THIC-YFP and YFP-N90THIC, respectively). The constructs were transformed into *Arabidopsis* mesophyll protoplasts and the intracellular compartmentation was observed by confocal microscopy (figure 2.3).

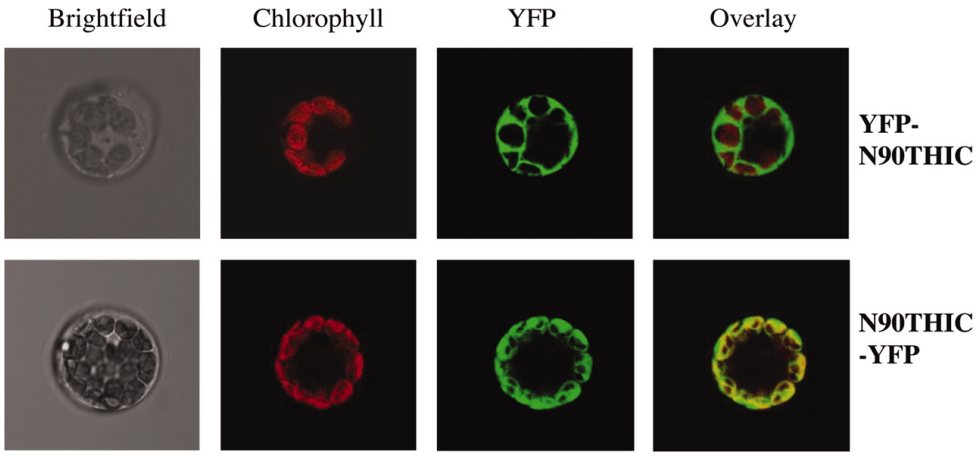


Figure 2.3:. Sub-cellular localization of THIC in *Arabidopsis*. The N-terminal 90 amino acids of THIC were fused to either the N or C terminus of YFP to give N90THIC-YFP and YFP-N90THIC, respectively, and transiently expressed in isolated *A. thaliana* mesophyll protoplasts. Confocal laser scanning microscopy was used to monitor fluorescence.

In the case of the N90THIC-YFP fusion protein, the overlay with chlorophyll autofluorescence clearly demonstrates that the protein is exclusively found in the chloroplasts. Moreover, a stromal localization within the chloroplast is indicated by the fluorescence pattern. This statement is corroborated by a recent study of the stromal proteome of *Arabidopsis*, in which

THIC was identified as a component [Peltier *et al.*, 2006]. The YFP-N90THIC fusion protein, on the other hand, is observed throughout the cytosol, as is the case for YFP alone (data not shown). This demonstrates that THIC is localized to the plastid and that moreover the targeting sequence must be contained within the N-terminal 90 amino acids.

2.5.4 Regulatory mechanisms revealed by expression analysis

To further characterize *THIC*, its transcript abundance was analyzed by using RT-PCR. Consistent with a chloroplastic localization, a tissue expression analysis revealed that the transcript was predominantly found in green tissue, with barely detectable expression in roots or stems (figure 2.4*A*). This is also consistent with the involvement of thiamin diphosphate primarily in photosynthesis and carbohydrate metabolism. As the *thiC* mutant has a seedling lethal phenotype, we also analyzed the expression of *THIC* during early developmental stages. The *THIC* transcript was not detectable two days after germination, but was readily detectable at day five (figure 2.4*B*). This suggests that there may be no net thiamin biosynthesis in *Arabidopsis* during the first days of germination, which agrees with an earlier report on maize [Belanger *et al.*, 1995]. Therefore, we assume that thiamin stored in the seed (most likely translocated from other parts of the plant) is used during the first stages of seedling development, after which expression of *THIC* is induced and homeostasis is restored. Indeed, the variable thiamin levels described above in the differentially supplemented *thiC* progeny corroborate this statement.

As THIC is localized to the chloroplast, regulation of expression by light was examined. *THIC* transcript was detected in both dark and light grown seedlings (figure 2.4*C*). After transfer of etiolated seedlings to light, the level of the *THIC* transcript increases gradually over a 24-hr period. The transcript level in seedlings grown in 24 hrs of light is substantially higher than that in seedlings exposed to a single long day cycle (16 hrs light, 8 hrs dark). This is even more pronounced in six-day old seedlings subjected to the same conditions (figure 2.4*C*). The THIC protein could also be detected by Western blot analysis in extracts from the seedlings grown for 24 hrs or six days in the light (data not shown), *i.e.*, when transcript abundance was high, indicating that protein and transcript levels may correlate. In an attempt to further investigate the regulation of *THIC*, we probed a rather exciting phenomenon recently discovered: the concept of gene regulation by conserved regions of mRNA that bind specific metabolites, known as riboswitches [Winkler *et al.*, 2002; Mironov *et al.*, 2002]. Discovered in 2002, riboswitches were originally thought to occur only in the 5′ UTR of genes

and moreover to be restricted to prokaryotes. This notion is no longer tenable with the very recent discovery of a thiamin pyrophosphate binding riboswitch in the 3´ UTR of the *THIC* gene of *Arabidopsis* [Sudarsan *et al.*, 2003; Thore *et al.*, 2006]. As the gene had not been studied in any detail, it was not known at the time if it is actually involved in thiamin biosynthesis *in planta*. Clearly, with this study, this latter question has now been answered. Moreover, we observed that expression of the *THIC* gene is negatively regulated by thiamin itself (figure 2.4*D*). It is thought that this occurs due to instability of the mRNA conferred by thiamin diphosphate binding. As the thiamin riboswitch only responds to thiamin diphosphate but not thiamin [Thore *et al.*, 2006], the down-regulation of *THIC* mRNA observed here implies that the externally provided thiamin is converted to thiamin diphosphate inside the cell leading to the conformational change inducing mRNA instability [Thore *et al.*, 2006].

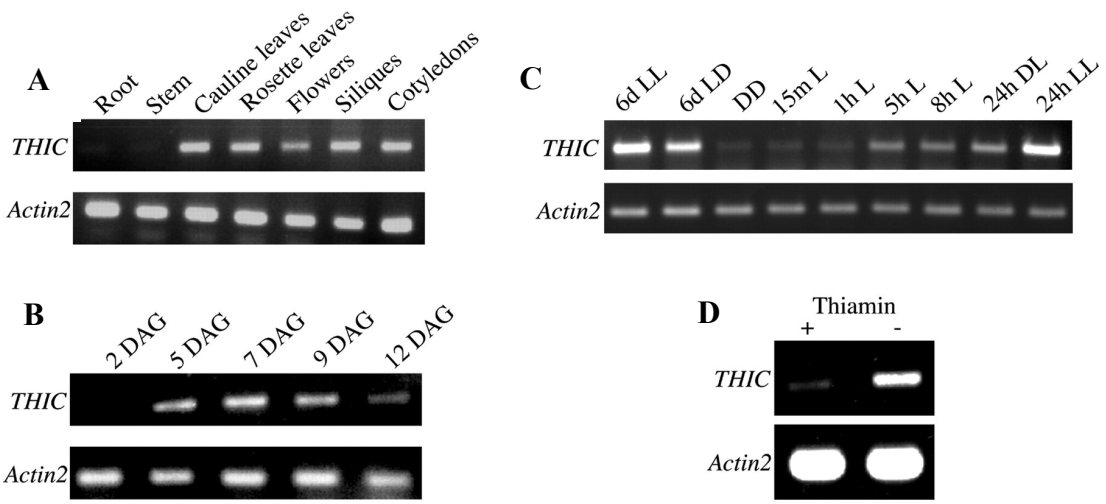


Figure 2.4: Expression analysis of *THIC* as assessed by RT-PCR. Transcript abundance in the indicated tissues ***(A)*** and days after germination (DAG) in the absence of thiamin ***(B)*** grown under long day conditions (LD, 100 µmol photons m^{-2} s^{-1} photons for 16 hrs, 8 hrs dark). ***(C)*** Samples of whole seedlings were analyzed six days after growing in either continuous light (LL) or LD, or continuous dark (DD). In addition, samples of six-day old etiolated seedlings were analyzed at the indicated times after transfer to light, as well as after a single 24 hrs LD cycle. ***(D)*** Seedlings grown in the presence and absence of 0.5 µM thiamin under LD. In all cases, actin2 mRNA serves as the control.

2.5.5 THIC is a Fe-S cluster protein

THIC is thought to convert AIR_t into HMP-P (supplementary scheme 2.1). This fascinating reaction has been described as the most complex unresolved rearrangement in primary metabolism [Lawhorn *et al.*, 2004] and has no chemical precedent. Remarkably, the reaction has been reconstituted *in vitro* employing the *Escherichia coli* protein [Lawhorn *et al.*, 2004].

However, reconstitution necessitated the addition of a crude extract from the bacterium rendering it difficult to dissect factors necessary for functionality. We observed that all THIC homologs harbour the consensus motif $C(X)_2C(X)_4C$ at the C terminus (figure 2.1*B*). However, the motif is not exclusive to THIC as illustrated by the fact that the same motif is displayed, in the *E. coli* genome, by at least 27 additional enzymes. This motif is similar to the $C(X)_3C(X)_2C$ motif normally found at the N terminus of enzymes dependent on the 5'-deoxyadenosyl radical generated from S-adenosyl-L-methionine (SAM) and a Fe-S cluster [Sofia *et al.*, 2001], albeit that of THIC is at the other end of the protein and has altered the order and number of residues between the cysteines. In this context, it is noteworthy that the capacity to reconstitute the THIC reaction as described by [Lawhorn *et al.*, 2006] was reduced if SAM was omitted. As "radical SAM"-dependent enzymes require a Fe-S cluster for functionality we were prompted to check for evidence of such a cofactor in THIC.

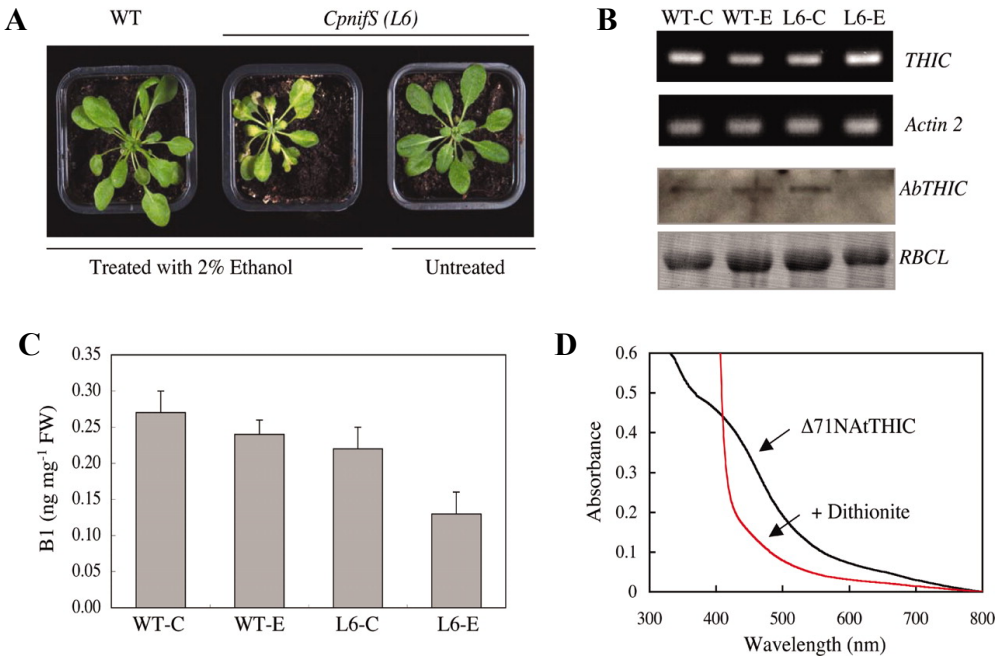


Figure 2.5: THIC is an iron-sulfur cluster protein. ***(A)*** RNA interference of *CpnifS* as evidenced by the chlorotic phenotype. Plants were grown on soil for 14 days, after which silencing was induced by spraying with 2% (v/v) ethanol every four days. The plants shown are 19 days after induction of *CpnifS* silencing. ***(B)*** (*Upper*) Transcript abundance of *THIC* in WT or *CpnifS* plants (as shown in ***(A)***). WT-C, WT-E, L6-C, and L6-E refer to wild-type or *CpnifS* line 6 control or ethanol-treated plants, respectively. Actin2 mRNA serves as the control. (*Lower*) Western blot of the same samples probed with a THIC antibody in addition to the Ponceau S-stained membrane where RBCL indicates the large subunit of Rubisco and serves as a protein loading control. ***(C)*** Total thiamin content of the same samples described in ***(B)***. ***(D)*** UV-visible spectrum of *Arabidopsis* THIC as isolated aerobically (black line), and one min after addition of 0.3 M sodium dithionite (red line).

In plants, cysteine desulfurase (NIFS) supplies the sulfur for Fe-S clusters. In *Arabidopsis*, two distinct NIFS proteins exist, localized to the chloroplast and mitochondria (CpNIFS and MtNIFS, respectively). Recently, a study employing a *CpnifS* mutant has shown that the protein is essential for the maturation of plastidic Fe-S proteins [Van Hoewyk *et al.*, 2007]. As THIC is localized to the chloroplast, we examined both THIC abundance and thiamin content in the *CpnifS* mutant. Silencing of the gene is achieved by using an ethanol-inducible RNA interference (RNAi) approach and results in a chlorotic phenotype [Van Hoewyk *et al.*, 2007] (figure 2.5*A*). Two independent transgenic lines (L6 and L9, kindly provided by Elizabeth A. H. Pilon-Smits, Colorado State University, Ft. Collins) were used for analysis. Although L6 showed a clear phenotype after ethanol treatment (figure 2.5*A*), induction of CpNIFS silencing could not be consistently observed in L9 in our hands; thus, only the results for L6 are shown. Concomitant with silencing of CpNIFS, mutant plants exhibiting the phenotype have a clear reduction in THIC accumulation, whereas when silencing of CpNIFS does not occur, the levels of THIC are similar to WT (figure 2.5*B Lower*). In contrast, a reduction of *THIC* mRNA was not observed in CpNIFS silenced plants (figure 2.5*B Upper*), suggesting that the stability of the protein depends on the Fe-S cluster, and that apoTHIC does not accumulate. Moreover, the total thiamin content of *CpnifS* plants is reduced (figure 2.5*C*).

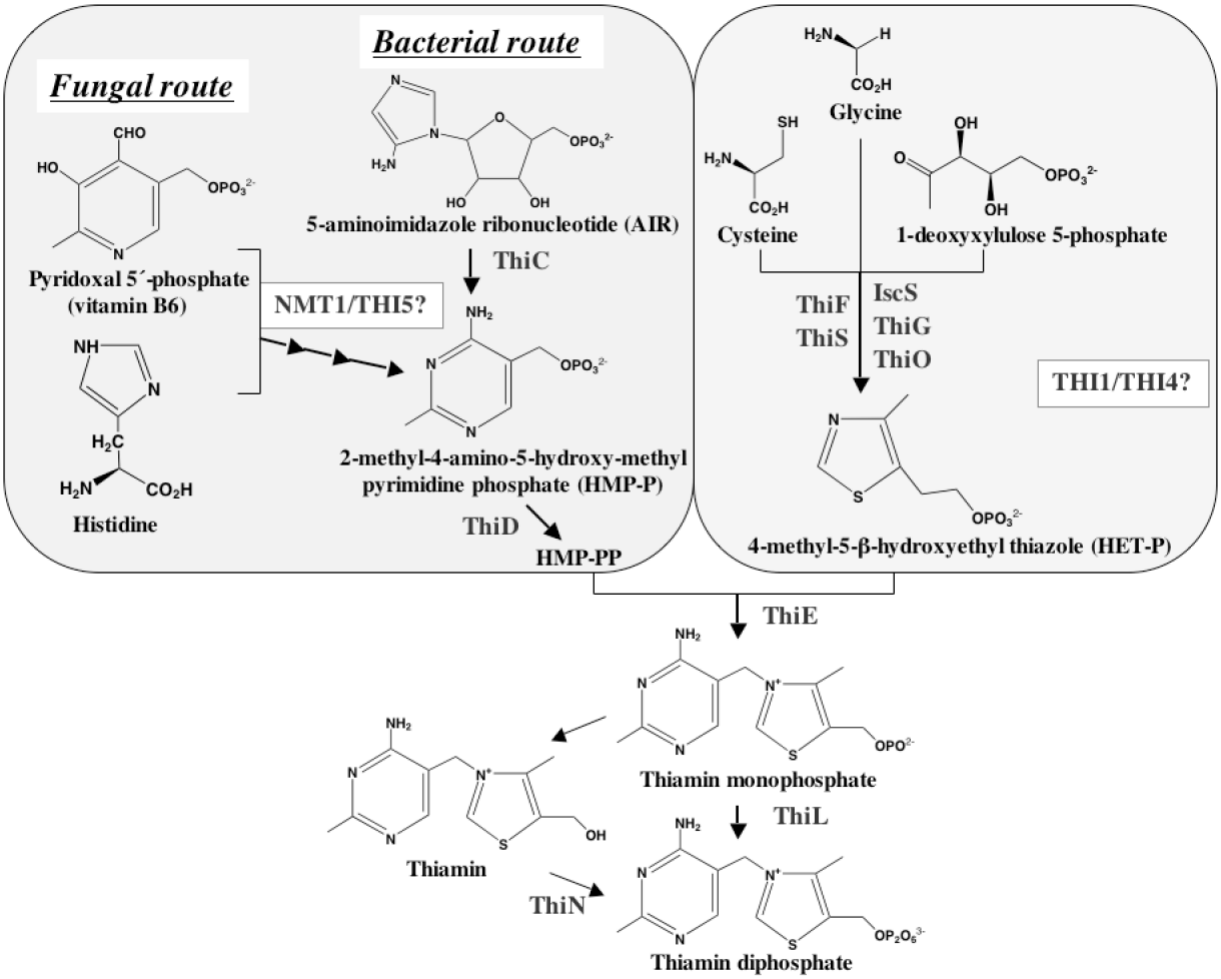
To corroborate the above result, the *Arabidopsis* THIC protein was produced recombinantly in *E. coli* with a C-terminal hexa-histidine tag. Whereas the full-length protein was completely insoluble, N-terminally truncated version lacking the first 71 amino acids was soluble (data not shown). Using Ni-NTA chromatography, the protein was purified to homogeneity in a very high yield (42 mg per litre of bacterial culture). Although the protein was isolated under aerobic conditions, it was intensely brown in colour with an absorption maximum at 410 nm and a shoulder at 320 nm that strongly decreased after reduction with sodium dithionite (figure 2.5*D*). These features are clear indicators of a Fe-S cluster [Külzer *et al.*, 1998]. A more detailed characterization of the Fe-S cluster will appear in a future report, and demonstration of THIC activity will await the availability of substrates.

2.6 Concluding remarks

Vitamin B_1 was discovered in 1932, its structure was elucidated in 1936, and it was the first such compound to be recognized as an essential metabolic cofactor. Yet, surprisingly many facets of its metabolism still remain unresolved. Nowhere is this more apparent than in plants. In this study, we show that THIC plays an essential role in the synthesis of vitamin B_1 and

moreover is necessary for plant viability. The presence of a Fe-S cluster in THIC suggests that it must be coupled with a reductant to enable catalytic activity. In bacteria, this can be accomplished by the flavodoxin/flavodoxin reductase/NADPH system as for the non-mevalonate isoprenoid biosynthesis proteins, IspG and IspH [Rohdich *et al.*, 2003]. However, flavodoxin is absent from the plastids of phototrophic organisms, but reduction could be achieved by ferredoxin, which can supply the electrons either in the presence of light or in the dark *via* ferredoxin-$NADP^+$ reductase and NADPH [Seemann *et al.*, 2006]. In plants, many chloroplastic enzymes are also activated and deactivated through oxidation/reduction reactions *via* the thioredoxin system. THIC has been found as a potential thioredoxin target protein in chloroplasts [Balmer *et al.*, 2003] and could influence the protein at several levels, including activity, oxidative regulation, and assembly or folding [Buchanan and Balmer, 2005]. Thus, the regulation of thiamin biosynthesis in plants may occur at several levels, *i.e.*, riboswitch and redox. We attempted to complement an *E. coli thiC* mutant with the full-length *Arabidopsis* THIC and various N-terminally truncated versions; however, with the exception of the *E. coli* protein itself, none of these could restore thiamin prototrophy to the *E. coli* mutant, suggesting that a specific "plant/chloroplast factor" is necessary for functionality of the plant protein. Given the central role that both *THIC* and its riboswitch likely serve in modulating the concentration of thiamin, this protein or its mRNA may serve as a new target for drug discovery. Furthermore, previous efforts to engineer RNAs that perform as ligand-dependent molecular switches have proven that RNA has an enormous potential for molecular sensing.

2.7 Supplementary information



Supplementary scheme 2.1: Thiamin biosynthesis in bacteria and fungi. The blue panel illustrates current knowledge on the biosynthesis of the pyrimidine moiety, which in bacteria is derived from AIR_t and in fungi is derived from vitamin B_6 and histidine. The THIC reaction is shown to result in the phosphorylated form of HMP, but no equivocal evidence for this is yet available. The green panel illustrates the biosynthesis of the thiazole moiety; for simplicity, the pathway deciphered from *Bacillus subtilis* is shown. THI4/THI1 are known to be involved in biosynthesis of the thiazole moiety in fungi and plants, respectively, but their exact biochemical function is not known. Note that although the phosphorylation of thiamin monophosphate (TMP) by ThiL is known to occur in *E. coli*, in yeast and plants TMP is first dephosphorylated and then pyrophosphorylated by thiamin pyrophosphate kinase (ThiN).

Line	Basta™-resistant	Basta™-sensitive	Total	% Sensitive
13	295	102	397	25.7
16	344	87	431	20.2
17	385	123	508	24.2

Supplementary table 2.1: Segregation ratio of the *BAR* gene conferring phosphinothricin herbicide resistance to SAIL_793_H10 (*thiC*). Progeny of selfed heterozygous line SAIL_793_H10 were analyzed for resistance and sensitivity to Basta™.

Metabolite (nmol/g FW)	WT-B_1	At*thiC* KO -B_1	At*thiC* KO +B_1
Amino acids			
Alanine	1659.32±214.93	**16026.86±3712.54**	2402.78±214.83
Arginine	1581.89±309.04	2030.18±427.94	**3888.56±558.68**
Asparagine	13482.14±2387.63	**86217.40±19301.98**	**67971.35±8343.32**
Aspartate	7352.95±752.17	7089.73±445.70	8073.12±113.20
β-Alanine	736.45±48.27	**2610.44±279.74**	1027.77±34.12
Cysteine	1.87±0.17	**6.83±0.68**	8.95±0.55
Glutamate	1619.25±206.45	**4123.96±274.74**	2244.80±199.96
Glycine	3550.87±2215.50	*464.03±80.87*	*179.72±22.54*
Lysine	110.74±4.70	**571.08±70.56**	**555.22±30.51**
Methionine	20.14±1.34	**167.02±29.48**	**163.21±14.28**
Phenylalanine	148.88±25.91	**3483.38±411.69**	**2460.96±187.36**
Proline	3803.58±426.11	4626.14±273.41	**8058.20±818.25**
Serine	6508.78±350.53	8361.67±229.09	3509.06±79.55
Threonine	3540.00±72.35	4566.50±197.11	2036.17±42.78
Tryptophan	271.82±94.45	**11995.54±2727.50**	**11604.36±1275.24**
Tyramine	70.65±6.58	79.74±4.00	74.94±4.82
Valine	427.56±50.37	**1214.24±167.00**	846.54±42.21
Organic acids			
Butyrate	1321.15±184.33	**8073.63±1121.52**	**3275.12±146.53**
Citrate	346.98±122.07	420.19±145.76	289.86±103.60
Fumarate	4730.97±620.11	*1092.53±188.12*	*289.86±103.60*
Glycerate	2.04±0.83	2.69±1.10	0.35±0.14
Glycerol	n.d.	n.d.	n.d.
Isocitrate	1566.49±301.14	2770.63±514.87	2668.75±154.82
Malate	2706.74±382.09	**6792.31±1798.61**	2538.61±1590.66
Sugars			
Fructose	5374.28±1029.98	7755.64±863.73	7590.55±1590.66
Gentiobiose	n.d.	n.d.	n.d.
Glucose	13072.23±953.39	18219.74±1473.54	18134.25±2172.60
Raffinose	65.29±24.62	*29.55±6.50*	*24.92±2.61*
Ribose	79.76±4.39	*23.34±2.33*	*29.72±3.37*
Sucrose	28462.30±1636.23	22251.03±3202.63	22455.11±2537.12
Trehalose	483.55±27.80	498.42±56.26	347.26±13.39

Supplementary table 2.2: Absolute metabolite contents of *Arabidopsis* WT and *thiC*. Substantial increases are shown in bold (*ca.* two-fold), whereas decreases (*ca.* 0.5-fold) are italicized. Standard errors are the average of six experimental repetitions. n.d.: not determined.

2.8 Acknowledgements

We dedicate this study to Prof. Meinhart H. Zenk (Donald Danforth Plant Science Center, St. Louis, MO) on the occasion of his 75th birthday. We thank Prof. Elizabeth A. H. Pilon-Smits for her kind gift of the *CpNifS* RNAi lines, Fabian Ramseyer and Sandro Steiner (ETH, Zurich) for assistance in the preliminary stages of this project, Dr. Hironori Niki at the National Institute of Genetics in Japan for supplying the *E. coli thiC* knockout mutant and the pCA24N*Ec*ThiC construct that were used in the mentioned complementation experiments, Dr. Dieter Rubli for photographic work, and Prof. Julia Fritz-Steuber and Po-Chi Lin at the University of Zurich, and Prof. Sam Zeeman at the ETH Zurich, for useful discussions. This work was supported by Swiss National Science Foundation Grant 3100A0-107975/1 (to N.A. and T.B.F.). D.A. acknowledges support from Novartis International AG. The *Arabidopsis* Biological Resource Center is greatly acknowledged for supplying line 793_H10 and stock U16941 (*THIC* cDNA).

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2.10 Appendix to chapter 2: Recent investigations of ThiC

Whereas *de nov*o synthesis of the thiazole moiety of vitamin B_1 has been elucidated in much detail in bacteria, the biosynthesis of the pyrimidine moiety, *i.e.* 2-methyl-4-amino-5-hydroxymethylpyrimidine diphosphate (HMP-PP) is less well understood. In *E. coli*, the HMP-P synthase ThiC has so far been identified as the only enzyme committed to *de novo* biosynthesis of HMP-PP [Van der Horn *et al.*, 1993]. It is involved in the conversion of 5-aminoimidazole ribonucleotide (AIR_t) to HMP-P. Homologs of ThiC are present in archaea, bacteria, cyanobacteria and higher plants, but not in fungi. In the latter, members of the THI5/11/12/13 family, identified in several ascomycota (*e.g. Saccharomyces cerevisiae*) as well as their close homologs NMT1 (no message in thiamin 1) and THI3, found in other fungi (*e.g. Schizosaccharomyces pombe*), have been predicted to be involved in an alternative pathway of ATP-dependent HMP-P biosynthesis [Schweingruber *et al.*, 1991] using pyridoxine and L-histidine/urocanic acid as precursors [Tazuya *et al.*, 1989/1993/1995; Zeidler *et al.*, 2003]. Before the study presented here in chapter 2, the synthesis of the pyrimidine moiety had not been studied in plants.

During the course of this study, it was established that a major pathway of *de novo* biosynthesis of the pyrimidine moiety of thiamin occurs in *A. thaliana* comparable to that present in *E. coli* and other prokaryotes. A knockdown of *THIC* led to a chlorotic phenotype and an abortion in early development, unless thiamin was supplemented as a nutrient. By that, the phenotype is distinct from the characteristic thiamin-deficient phenotype [Papini-Terzi *et al.*, 2003], *i.e.* the development of green cotyledons and white true leaves before the developmental abortion. Therefore, THIC is essential for viability, growth and development of *A. thaliana* at an early state of development.

A sequence analysis of the *A. thaliana* THIC revealed that three of the cysteine residues reside in the motif **C**(S/T)M**C**XXXX**C** which is present near the C-terminus and was identified in all THIC homologs. This motif is vaguely reminiscent of the motif **C**XXX**C**XX**C** which is present at the N-terminus of the majority of enzymes depending on S-adenosyl-L-methionine (SAM) as a cofactor, *i.e.* the SAM-radical enzyme family, although the motif is mirrored and contains an altered number of residues between the cysteines. The cysteine residues form the binding sites for the iron atoms of the oxygen-labile iron-sulfur cluster present in the majority of these enzymes [Marsh *et al.*, 2004]. In general, iron-sulfur clusters assist as a prosthetic group in enzymatic reactions such as electron transfer, substrate binding/activation, iron/sul-

fur storage, regulation of gene expression or enzyme activation as a one electron reductant [Johnson *et al.*, 2005]. Cysteine desulfurases (NifS) are present in all kingdoms of life and required for the mobilization of the sulfur atom from cysteine and thus for the protein's iron-sulfur cluster biogenesis in a complex biosynthetic machinery [Johnson *et al.*, 2005]. In plants, two cysteine desulfurase-like proteins have been identified in chloroplasts and mitochondria, respectively [Van Hoewyk *et al.*, 2007]. Silencing of the chloroplastidic NIFS protein leads to a reduced THIC level and overall declined vitamin B_1 content in *A. thaliana* [Raschke *et al.*, 2007]. This indicates that the mature enzyme contains an iron sulfur cluster which, in addition, was demonstrated for several homologs by reducing the recombinantly expressed protein with dithionite [Raschke *et al.*, 2007; Martinez-Gomez and Downs, 2008; Chatterjee *et al.*, 2008]. The presence of the iron sulfur cluster is a prerequisite for activity of the enzyme [Martinez-Gomez and Downs, 2008]. EPR and Mössbauer spectroscopy demonstrated that ThiC from *Caulobacter crescentus* contains an iron sulfur cluster most probable of the [4Fe4S]-type [Chatterjee *et al.*, 2008]. This type of iron-sulfur cluster is characteristic for members of the SAM-radical enzyme family [Marsh *et al.*, 2004]. Such an iron-sulfur cluster is particularly sensitive to molecular oxygen and completely decomposes under mild oxidizing conditions. Unlike in other cluster forms, such as [2Fe2S], [3Fe4S] or [8Fe7S] clusters in which a cysteine residue completes the tetrahedral coordination of the sulfur atom at each iron site, in the above-mentioned [4Fe4S]-cluster only three iron atoms are ligated each by one cysteine residue, whereas the fourth iron atom presumably interacts with the cofactor SAM through its amino and carboxylate group, respectively [Marsh *et al.*, 2004]. A single structure of a ThiC homolog has been partially solved from *Caulobacter crescentus* (figure A2.1) [Chatterjee *et al.*, 2008]. Unfortunately, this study did not reveal the presence of the iron-sulfur cluster at all, as the C-terminus was unresolved. However, the authors modelled the iron-sulfur cluster using biotin synthase as a guide. Biotin synthase is one out of four SAM-radical enzymes the structures of which have been solved [Berkovitch *et al.*, 2004]. Using the backbone of biotin synthase, the iron sulfur cluster was modelled to ThiC in such a way that the four cysteine residues were in an orientation to provide ligands for the iron atoms of the iron-sulfur cluster. Furthermore, a glycine rich motif **G**IVSR**GG**(S/A) [Martinez-Gomez and Downs, 2008], a further characteristic of SAM-radical enzymes, is present in the centre of all ThiC amino acid sequences and is absolutely conserved. The glycine residues shown in bold are considered essential for binding the cofactor SAM [Sofia *et al.*, 2001].

Based on the many common features between the SAM-radical enzymes and ThiC it is assumed that ThiC is a member of the SAM radical superfamily [Raschke *et al.*, 2007; Martinez-Gomez and Downs, 2008]. However, the molecular mechanism of the remarkable and complex chemical re-arrangement reaction catalyzed by ThiC has not yet been unravelled. In addition, an appropriate reducing system, *e.g.* NADH, NADPH or dithionite as well as a yet unknown cellular component is necessary for efficient *in vitro* reconstitution of catalytically active ThiC producing HMP-P and 5'-deoxyadenosine [Lawhorn *et al.*, 2004; Martinez-Gomez and Downs, 2008]. The *in vivo* regeneration of the reduced state of the ThiC iron sulfur cluster may for example be triggered by the ferredoxin/thioredoxin system, comprised of reduced ferredoxin and the ferredoxin-thioredoxin reductase or the glutathione/glutaredoxin system [for review see Buchanan and Balmer, 2005].

The solving of the structure of the ThiC homolog from *Caulobacter crescentus* in 2.8 Å and 2.0 Å resolution, respectively (figure A2.1) [Chatterjee *et al.*, 2008], reveals its assembly as a homodimer in which each protomer consists of three domains. The N-terminal domain, a novel fold consisting of six α-helices and five β-strands, forms a thin-blanket-like structure which covers parts of the other two domains. The second domain is a $(\beta/\alpha)_8$ barrel fold consisting of β-strands (6-13) and α-helices (6-18), whereas domain three is characterized by an anti-parallel three α-helix bundle which is followed by a loop and 66 disordered, thus unresolved, residues which include the three cysteines forming the binding sites of the iron atoms from the iron-sulfur cluster. This structural feature is anchored to the top of the $(\beta/\alpha)_8$ barrel of the adjacent protomer. During dimer formation, the $(\beta/\alpha)_8$ barrel of one protomer is arranged approximately anti-parallel to the $(\beta/\alpha)_8$ barrel of the second protomer. The interface, carrying hydrophobic characteristics with 72.7% of the residues being non-polar, composed of the last three α-helices of the $(\beta/\alpha)_8$ barrel and the three α-helix bundles of domain three and two, respectively. The active site is located at the C-terminal end of the $(\beta/\alpha)_8$ barrel in the third domain. In addition, a transition metal ion, most likely Zn^{2+}, is coordinated within the active site. The HMP-P site is located in the $(\beta/\alpha)_8$ barrel at the N-terminus of helix α_{12} and the C-terminus of the adjacent protomer (figure A2.1).

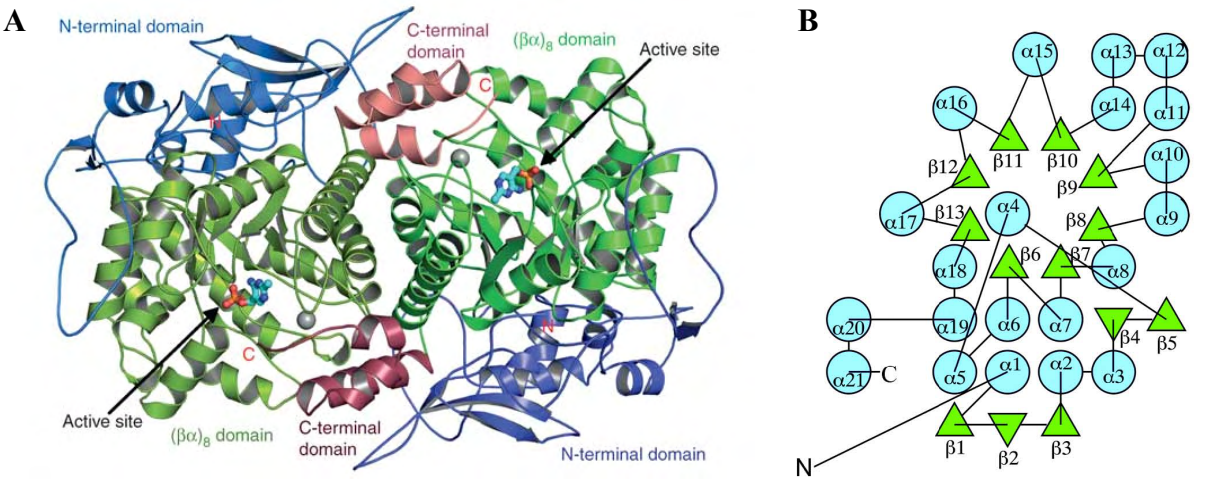

Figure A2.1: Structure of *C. crescentus* ThiC protein. ***(A)*** The HMP-P synthase homodimer. Each protomer consists of three domains. The N-terminal domains are colored in shades of blue, the $(\beta/\alpha)_8$ core domains in green, and the C-terminal domains in red, respectively. HMP-P is shown as a ball-and-stick model in the active sites. ***(B)*** A secondary structure topology diagram. The N-terminal domain (domain 1) comprises α-strands 1-5 and β-helices 1-5. The $(\beta/\alpha)_8$ core domain (domain 2) comprises α-strands 6-13 and β-helices 6-18. The C-terminal domain (domain 3) comprises β-helices 19-21 plus 66 disordered residues [taken from Chatterjee *et al.*, 2008].

The observation of an early developmental arrest of the *A. thaliana thiC* mutant line (SAIL_793_H10 [Raschke *et al.*, 2007]) limits the likelihood of the presence of an alternative pathway for the biosynthesis of the pyrimidine moiety of vitamin B_1 akin to that in fungi. Such a pathway, if present at all, may act concomitant and/or additional to the THIC-route throughout a plant's development. Nevertheless, an attempt was made in this study to investigate whether the pyrimidine moiety of vitamin B_1 can be synthesized by a route other than the THIC-route. The occurrence of the fungal-like pathway was examined, in which pyridoxine acts as a precursor. As a starting point, the protein sequence of THI13 from *S. cerevisia*e, a member of the THI5/11/12/13 gene family [Wightman and Meacock, 2003] was used as reference sequence and blasted against a protein database (UniProt Knowledgebase at EMBL [Altschul *et al.*, 1997]) aligned at the European Bioinformatics Institute (KALIGN; http://www.ebi.ac.uk/Tools/kalign/index.html) and processed using the online tool Jalview (http://www.jalview.org/ [Clamp *et al.*, 2004]) (figures A2.2, A2.3). THI13 homologs present in other fungi share at least 57% amino acid identity to the reference protein. In *A. thaliana*, no homologous protein appears to be present; a putative mitochondrial proline oxidase has the highest score in identity (35%). In *Oryza sativa* as well, the second plant with a fully sequenced genome, no THI13 homolog seems to be present. Unequivocally, both *Populus jackii*

and *Zea mays* contain a protein highly homologous to THI13 from *S. cerevisiae* (63% and 69% amino acid sequence identity, respectively). Both proteins, *i.e.* A9PK65 (*P. jackii*) [Ralph *et al.*, 2008] and B4FBP6 (*Z. mays*) are comparable to other fungal homologs, but appear not to have been further characterized (table A2.1). Both homologs have been added only recently to the protein database and were identified from a full-length cDNA library and by subsequent BLAST analysis. However, a contamination of the cDNA libraries with fungal sequences can not be excluded at this stage which would also explain the rather high amino acid sequence homology with *S. cerevisiae* THI13 (figures A2.2) and the clustering of the sequences from *A. niger* and *Z. mays* (figure A2.3).

Protein	Organism	Identical amino acids (%)	Gaps (%)	Activity
Fungi				
THI13	*Saccharomyces cerevisiae*	100	-	Subtelomeric protein family involved in the synthesis of HMP
THI11	*Saccharomyces cerevisiae*	100	-	
THI5	*Saccharomyces cerevisiae*	99	-	
THI12	*Saccharomyces cerevisiae*	99	-	
NMT1	*Aspergillus niger*	69	-	Synthesis of HMP
NMT1	*Neurospora crassa*	66	-	Synthesis of HMP
Q2GQH3	*Chaetomium globosum*	65	-	Hypothetical protein
THI3	*Schizosaccharomyces pombe*	62	-	Synthesis of HMP
NMT1	*Ustilago maydis*	57	-	Synthesis of HMP
Bacteria				
NMT1	*Legionella pneumophila*	47	3	Thiamin biosynthesis (HMP)
Q1AU98	*Rubrobacter xylanophilus*	27	6	HMP-binding protein
Plants				
B4FBP6	*Zea mays*	69	-	Uncharacterized
A9PK65	*Populus jackii*	63	-	Uncharacterized
At5g38710	*Arabidopsis thaliana*	35	7	Mitochondrial proline oxidase/dehydrogenase; putatively responsive to osmotic stress; similar to AtERD5
At4g32790	*Arabidopsis thaliana*	25	14	Exostosin family protein; similar to At5g25820
A8IGH4	*Chlamydomonas reinhardtii*	22	19	Probable lipoxygenase

Table A2.1: Best hits of a BLAST search using the protein sequence of *S. cerevisiae* THI13 as a reference against a protein database (UniProt Knowledgebase at EMBL) [Altschul *et al.*, 1997]. Abbreviations used: ERD: early responsive to dehydration; HMP: 2-methyl-4-amino-hydroxymethyl-pyrimidine.

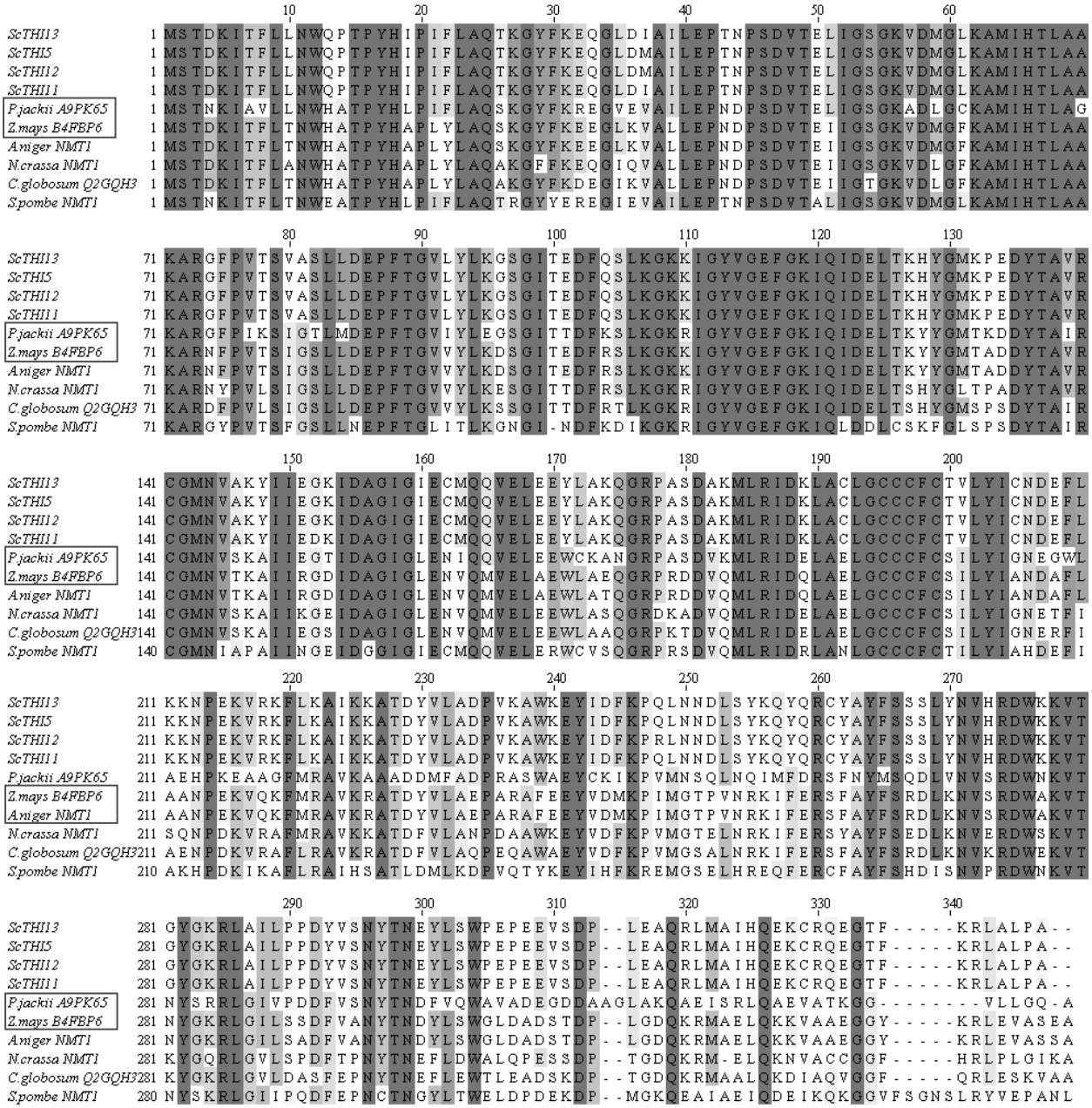

Figure A2.2: Alignment (European Bioinformatics Institute (KALIGN; http://www.ebi.ac.uk/Tools/kalign/index.html)) of homologs of the *S. cerevisiae* THI13 identified by a BLAST search against a protein database (UniProt Knowledgebase at EMBL [Altschul *et al.*, 1997]). Other homologs listed in table A2.1 have been omitted for reasons of clarity. The two plant sequences are highlighted by a grey box. The organisms are as follows: *A. nige*r: *Aspergillus niger*; *C. globosum*: *Chaetomium globosum*; *N. crassa*: *Neurospora crassa*; *P. jackii*: *Populus jackii*; *Sc*: *Saccharomyces cerevisiae*; *S. pombe*: *Schizosaccharomyces pombe*; *Z. mays*: *Zea mays*. Further details can be found in table A2.1 and figure A2.3, respectively. Alignment analysis was performed using the online tool Jalview (http://www.jalview.org/ [Clamp *et al.*, 2004]).

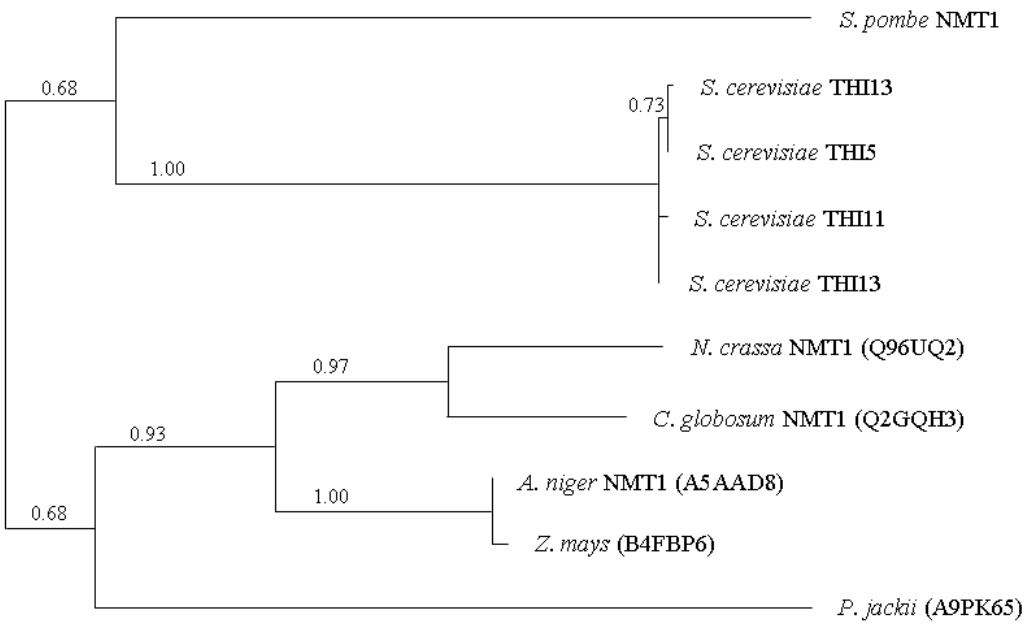

Figure A2.3: Average distance tree of THI13 homologs using % identity as a basis. Protein sequences used for the calculation are as given in figure A2.2. The two plant homologs of THI13 are underlined. The distance tree was created using Jalview based on the protein sequences (http://www. jalview.org/ [Clamp *et al.*, 2004]). Branch support values are as indicated. Organisms names are the same as indicated in figure A2.2.

As no homolog of *S. cerevisiae* THI13 was identified in *A. thaliana*, *Spinacia oleracea* was used as an alternative model organism to find out whether the fungal-like route of HMP-P biosynthesis may occur in plants as well. *S. oleracea* is, next to *Pisum sativum*, a well-established plant system to study chloroplastidic biosynthetic pathways. This system was chosen because of an early study which proposed thiamin biosynthesis to occur in the chloroplasts of higher plants [Julliard and Douce, 1991]. Initial biochemical studies were performed in which [5,5',6-$^{13}C_3$]-pyridoxine was synthesized and applied to isolated intact chloroplasts as well as to the isolated stroma fraction of *S. oleracea*. Subsequent mass spectrometric analysis of the purified plant extract gave preliminary evidence of the incorporation of ^{13}C-atoms into the pyrimidine, but not into the thiazole moiety of vitamin B_1 in intact chloroplasts but not in the stroma fraction. However, a repetition of these experiments, by using [5,5',6-$^{13}C_3$]- and [2,2',3,4,4'-$^{13}C_5$]-pyridoxine as substrates that were applied to intact chloroplasts, separated stroma and thylakoid fractions as well as intact leaves, failed. While these results need to be clarified and substantiated they may suggest that the fungal-like route may be present in at least some plants as well (unpublished data; not shown). However, studies with the *A. tha-*

liana thiC knockdown mutant [Raschke *et al.*, 2007; Kong *et al.*, 2008] suggest that the fungal-like route represents either a minor biosynthetic pathway or is not sufficiently present throughout plant development to complement the knockdown of THIC, at least in *A. thaliana*.

One characteristic of *E. coli ThiC* is the presence of a riboswitch in the 5'-untranslated region of the gene [Winkler *et al.*, 2002]. Such a motif was confirmed in the 3'-untranslated region of *THIC* from *A. thaliana* [Sudarsan *et al*., 2003] and from *Chlamydomonas reinhardtii* [Croft *et al.*, 2007] as well. Supplementation studies revealed that increased levels of exogenous thiamin repress the expression of THIC supporting the functionality of the riboswitch [Wachter *et al.*, 2007; Raschke *et al.*, 2007; Croft *et al.*, 2007; Kong *et al.*, 2008]. Over-expression of THIC under control of the 35S CaMV promoter in *A. thaliana* wild-type background caused a slight accumulation of vitamin B_1 above WT (1.3-fold) [Kong *et al.*, 2008], indicating that the riboswitch alone may be a sufficient regulatory element. The presence of an alternative route for the synthesis of the pyrimidine moiety could bypass the riboswitch-mediated control of *ThiC* expression and hence, the observed regulation of the intracellular vitamin B_1 level. However, alternative biosynthetic routes of essential compounds may be of advantage, in particular under unfavourable conditions, *i.e.* when a fast change of the respective metabolite is necessary to sustain growth and development. The occurrence of coexisting metabolic pathways has been reported for isoprenoid biosynthesis in higher plants [for review see Kuzuyama, 2002] and fatty acid biosynthesis in yeast [Veen and Lang, 2005]. Very recently, a link between thiamin metabolism and responses to abiotic stress was proposed based on the finding that the total vitamin B_1 content increases upon osmotic and oxidative stress in maize seedlings [Rapala-Kozik *et al.*, 2008]. Unfortunately, the focus of this study was not on the early enzymes, *i.e.* THI1 and THIC, providing the two subunits of the thiamin molecule, but rather on the late enzymes thiamin monophosphate synthase (TMPS) and thiamin pyrophosphokinase (TPK). However, THI1 has been related to abiotic stress responses, DNA damage and pathogen attack [Machado *et al.*, 1996/1997; Ribeiro *et al.*, 2005; Wang *et al.*, 2006]. Interestingly, in transgenic *A. thaliana* lines over-expressing PDX1.1 and over-accumulating vitamin B_6 (AtPDX1.1 OE; see chapter 3), the vitamin B_1 levels are elevated above WT (*ca.* two-fold) as well (figure A2.4). This correlation may be explained by an interaction of the biosynthetic pathways of the two vitamins. Equally well, the hypothesis that the active cofactor form of vitamin B_6 stimulates general metabolism, thus affording higher enzyme activities leading to an increased requirement not only of vitamin B_1, but for other cofactors as well, is

plausible in this context. This might be accompanied by an, in comparison to WT plants, altered metabolic profile. It is therefore suggested to cross AtPDX1.1 OE with the *A. thaliana thiC* mutant line to investigate whether the increased vitamin B_1-levels quantified in shoot material of AtPDX1.1 OE have any positive impact on the phenotype reported for the *A. thaliana thiC* mutant line.

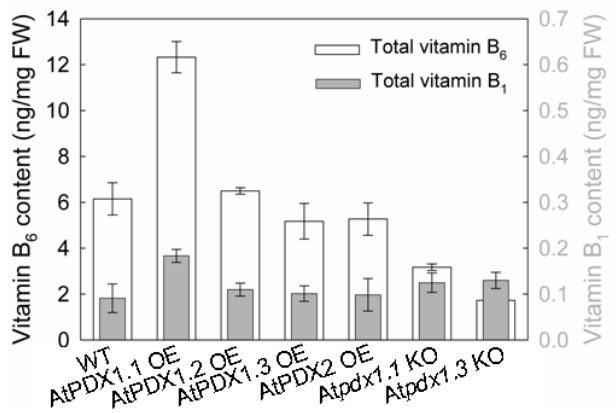

Figure A2.4: *A. thaliana* lines over-accumulating vitamin B_6 have increased levels of vitamin B_1. Seeds were sown on medium lacking both pyridoxine and thiamin and cultivated for ten days at a light intensity of 100 µmol photons m^{-2} s^{-1} (continuous light). Shoot material was harvested and total vitamin B_1 and vitamin B_6 contents were quantified by yeast bioassays after treatment of the extracts with acid phosphatase (vitamin B_1) or acid phosphatase and β-glucosidase (vitamin B_6), respectively.

2.11 References

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3. Increasing seed size and stress tolerance in Arabidopsis with enhanced levels of vitamin B_6

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3.1 Abstract

Vitamin B_6 is an essential nutrient in the human diet, being derived primarily from plant sources. Currently, many people are afflicted by deficiency in vitamin B_6, which is associated with various dysfunctions of the nervous, hormone and immune systems, as well as atherosclerosis and cardiovascular disease. More recently, an antioxidant function for vitamin B_6 has been uncovered. Here we report on overproduction of the vitamin in Arabidopsis and the effect(s) this process has on plant development. *De novo* vitamin B_6 biosynthesis in plants was recently shown to be catalyzed by the two proteins, PDX1 and PDX2. We demonstrate that over-expression of the endogenous PDX proteins in Arabidopsis leads to an increase in the vitamin B_6 content, manifested by interplay between both the *de novo* and salvage pathways of biosynthesis. The highest accumulation of the vitamin occurs in seeds and leads to their substantial enlargement. Moreover, the bigger seeds are enriched in protein, lipid and carbohydrate content, and there is an overall increase in seed yield. In addition, plants that over-accumulate the vitamin have a considerably increased tolerance to oxidative stress.

3.2 Introduction

Vitamin B_6 is an essential metabolite in all organisms in that it acts as a cofactor for numerous metabolic enzymes. It is an essential nutrient in the human diet, being derived primarily from plant sources. Currently, the vitamin is recognized as deficient in many people including those of developed countries and is associated with a number of disorders, for example nervous and immune system dysfunctions, impairments in red blood cell formation (heme biosynthesis), hormone dysfunction, as well as atherosclerosis and cardiovascular disease [Fitzpatrick *et al.*, 2007; Maggini *et al.*, 2007]. While the benefits of vitamin B_6 as an enzyme cofactor are well established, a completely unprecedented function of vitamin B_6 as an antioxidant with a potency equivalent to that of vitamins C and E, was uncovered only recently [Ehrenshaft *et al.*, 1999; Bilski *et al.*, 2000]. It is well established that many human diseases are correlated with

the release and accumulation of reactive oxygen species. Interestingly, clinical trials have provided evidence that vitamin B_6 can assist in chronic kidney disease (Swaminathan and Shah, 2008), in complicated forms of diabetes [Stitt *et al.*, 2002; Metz *et al.*, 2003] and in the reduction of epileptic seizures [Grillo *et al.*, 2001]. Furthermore, there is growing evidence that the vitamin may also have a novel role as an anti-tumour agent [Komatsu *et al.*, 2003; Kanellis *et al.*, 2007]. Thus, there is an interest to overproduce the vitamin for nutrient and other beneficial purposes. Much of the vitamin B_6 used for supplementation purposes is currently produced chemically. Indeed 1,600 tons of the vitamin are produced in this way every year [Vandamme, 1992]. However, bottlenecks imposed by low yields, poor and inconsistent product quality and difficulties with downstream processing pose a challenge to this field. Crop plants are a source of essential dietary components and a renewable resource that, in comparison to microbial production, is often favourable with respect to environment and economy. Therefore, plants are now becoming accepted as a platform for production of biochemicals either for extraction to be used in industry or directly in crop plants to increase their nutritional value. There has been much effort to increase the abundance of vitamins in plants that are related to human health, *e.g.* vitamin E [Shintani and DellaPenna, 1998], provitamin A [Ye *et al.*, 2000; Beyer *et al.*, 2002] and vitamin B_9 [Hossain *et al.*, 2004]. The majority of these studies focused on the rate-limiting step of enzymatic synthesis or the translocation of the complete metabolic pathway in question to a certain plant tissue.

In order to increase the content of a particular metabolite, the biosynthetic pathway of the compound in question needs to be known. It was tacitly assumed that the vitamin B_6 biosynthesis pathway in plants is the same as that in the Gram-negative bacterium *Escherichia coli,* the workhorse of molecular biology. Very recently, it has been demonstrated that this is not the case and a completely novel pathway has since been unravelled [Ehrenshaft *et al.*, 1999; Osmani *et al.*, 1999; Burns *et al.*, 2005; Raschle *et al.*, 2005; Tambasco-Studart *et al.*, 2005; Raschle *et al.*, 2007]. This pathway is made up of only the two proteins PDX1 and PDX2 [Ehrenshaft *et al.*, 1999; Osmani *et al.*, 1999; Ehrenshaft and Daub, 2001; Tambasco-Studart *et al.*, 2005], which, in a complex, can directly synthesize the cofactor form of the vitamin, pyridoxal 5'-phosphate (PLP), from ribose 5-phosphate, glyceraldehyde 3-phosphate and glutamine [Burns *et al.*, 2005; Raschle *et al.*, 2005]. Over-production of these two proteins would thus be expected to enhance the level of vitamin B_6 in a plant tissue.

On the other hand, there is presently much interest to generate crop plants with increased tolerance to environmental stresses such as oxidative stress, drought, salinity, and deviant tem-

peratures. In plants, the response to these adverse conditions is largely mediated through an imbalance in the production and scavenging of reactive oxygen species, such that the latter when present in excessive amounts can lead to cell death and necrosis. Thus, plants with an enhanced scavenging system would be expected to be less sensitive to such stresses. In addition, in the current climate of limited food production there is great interest in the generation of crops with increased yield. This phenomenon pertains to the seed component in particular, as they are a basic source of food, an important source of pharmaceuticals and a source of raw materials for industrial use.

Here we report on the overproduction and accumulation of vitamin B_6 in Arabidopsis by over-expressing the endogenous proteins of the biosynthetic pathway. Significantly, the accumulation of the vitamin is in both green tissue and seeds and leads to an increased oxidative stress tolerance. Furthermore, an unprecedented consequence of overproducing the vitamin is that the seeds of the modified plants are substantially enlarged. This feature is manifested by enriched protein, lipid and carbohydrate content, most likely due to a general increase in primary metabolism. Moreover, even though seed set is decreased in plants with enhanced vitamin B_6 levels, total seed yield by weight is significantly increased. Taken together, an approach to increase the abundance of vitamin B_6 in plants appears to have great potential for agronomic, nutritional and pharmaceutical purposes.

3.3 Materials and methods

3.3.1 Construction of expression vectors

AtPDX1.1 (At2g38230) and *AtPDX1.3* (At5g01410) were amplified by PCR using the primer pairs 5'-GGGGTACCATGGCAGGAACCGGAGTTGTGG-3', 5'-GCTCTAGATTACTCAGAACGACTAGCGAACC-3' and 5'-GGGGTACCATGGAAGGAACCGGCGTTGTGG-3', 5' -GCTCTAGATCACTCGGAGCGATTAGCGAACC-3', respectively; the underlined region in each primer pair represents the introduced restriction sites, KpnI and XbaI, respectively. *AtPDX2* (At5g60540) was amplified using the primers 5'- GGTACCATGACCGTCGGAGTTTTAGCTTTG-3' and 5'-GAATTCTTATTGAAATATAGGAAGATCAGGC-3' with KpnI and EcoRI restriction sites introduced, respectively. In each case, isolated cDNA was used as the template. The purified PCR products were subcloned into the commercially available PCR2.1 TOPO® vector (Topo TA Cloning Kit, Invitrogen, California, U.S.A.) according to the manufacturer's recommendations and subsequently cloned into the plant binary vectors

pCAMBIA1300.1 (*AtPDX1.1* and *AtPDX1.3*) and pSH9 (*AtPDX2*), respectively, using the restriction sites indicated above, and confirmed by sequencing. In the case of *AtPDX2*, the pSH9 cassette containing the CaMV 35S promoter and *AtPDX2* was cloned into pBIN19 as the final destination vector using the HindIII restriction sites.

3.3.2 Transgenic *A. thaliana*

The expression vectors (pCAM-*AtPDX1.1*, pCAM-*AtPDX1.3* or pBIN-*AtPDX2*) were transfected into *Agrobacterium tumefaciens* C58 to be used for plant transformation. Transformation of *Arabidopsis thaliana* wild-type (WT, ecotype Col-0) was performed using the floral dip method [Clough and Bent, 1998]. Surface sterilized seeds of the transformed plants (T0) were selected for resistance to either hygromycin (50 mg l^{-1}) (*AtPDX1.1* and *AtPDX1.3*) or kanamycin (35 mg l^{-1}) (*AtPDX2*) on MS medium [Murashige and Skoog, 1962] including vitamins (Duchefa, Haarlem, The Netherlands). Segregation analyses were performed with a population of at least 100 seeds from at least six independent transformants on selective medium and statistically analyzed (χ^2 test with one degree of freedom). Lines homozygous for each over-expressed gene and carrying a single insertion of the gene cassette were obtained in the fourth generation (T4) and utilized. Lines co-over-expressing both AtPDX1.1 and AtPDX2 were established by crossing the above described T4 homozygous plants of AtPDX1.1 OE and AtPDX2 OE. Seeds of the crossed lines were selected for both hygromycin and kanamycin resistance on MS medium as described above. Crossing into the *flu* background (ecotype Col-0 [Lee *et al.*, 2007] was performed with chosen lines from the above PDX over-expressors and the respective progeny was termed *flu* AtPDX1.1 OE, *flu* AtPDX2 OE and *flu* AtPDX1.1-2 OE. Lines were selected based on resistance to either hygromycin or kanamycin or both and display of the *flu* phenotype, *i.e.* chlorosis and cell death following a shift from the dark to the light [Meskauskiene *et al.*, 2001].

3.3.3 Cultivation conditions

For phenotype determinations, plants were simultaneously cultivated in soil under long-day conditions at a light intensity of 120 µmol photons m^{-2} s^{-1}. To perform experiments under defined conditions, seeds were sown on solidified MS medium [Murashige and Skoog, 1962] supplemented with 1% (w/v) sucrose and 0, 1.2, 2.4, 4.9, 12.4, 26.4, 49.2 or 492 µM pyridoxine, respectively, and cultivated for 15 days under long-day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1}. Leaves were harvested and immediately frozen in liquid nitrogen

prior to vitamin B_6 quantification. For the sterile cultivation of plants for the purpose of seed harvesting, individual seeds were sown in a Magenta vessel (Sigma-Aldrich, St. Louis, U.S.A.) on 200 ml solidified MS medium supplemented with 1% (w/v) sucrose and containing either none or 100 µM of pyridoxine. Plants were sterile cultivated under long-day conditions at a light intensity of 130 µmol photons m^{-2} s^{-1}. To allow seed maturation, the pots were left ajar after development of the siliques. The seeds of each plant were harvested separately after reaching maturity.

3.3.4 RNA and protein methods

At least four independent lines each of AtPDX1.1 OE, AtPDX1.3 OE, AtPDX2 OE or of crossed progeny were tested for their potential to over-express either PDX1.1, PDX1.3, PDX2, or both PDX1.1 and PDX2 at the RNA as well as the protein level. Total RNA was extracted from 10-day old plant shoot material using Trizol® (Invitrogen Life Technologies, California, U.S.A.) following the protocol provided by the company. A total of 2 µg of RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase and oligo dT(20) primers (Invitrogen Life Technologies) according to the manufacturer's recommendations and analyzed by quantitative real-time RT-PCR using the primers and methods described in Titiz *et al* [Titiz *et al.*, 2006] and Tambasco-Studart *et al.* [Tambasco-Studart *et al.*, 2007]. For Western blot analysis, soluble protein was extracted from shoot material in 50 mM sodium phosphate, pH 7.0, containing 5 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride and 1% (v/v) complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, U.S.A.). Cell debris was removed by centrifugation at 16,000 *g* for 10 min at 4°C. Subsequent SDS-PAGE (15%) and Western blot analyses were performed as described in Titiz *et al* [2006] and Tambasco-Studart *et al.* [2007] with the exception that PDX1.1 and PDX1.3 were detected with specific antibodies (diluted 1:1,000) raised against the peptides GEGAMTETKQKSP and CEGNGAITEAKKSP, respectively, in rabbit (Eurogentec, Seraing, Belgium). The material used in each case was from 10-day old seedlings grown on MS medium lacking pyridoxine under long day conditions (16 hr photoperiod at a light intensity of 100 µmol photons m^{-2} s^{-1}). Shoot and root material was harvested separately, immediately frozen in liquid nitrogen and kept at -80°C until analysis.

3.3.5 Vitamin B_6 analysis

Total vitamin B_6 was quantified using a yeast microbiological assay as described [Tambasco-Studart *et al.*, 2005]. In brief, the vitamin was extracted from up to 2 mg of plant material by autoclaving for 60 min in 1 ml of 0.028 M sulphuric acid. Free and modified forms, *i.e*. phosphorylated and glycosylated ones, were determined by comparison of samples with and without pre-treatment with acid phosphatase and/or β-glucosidase (Sigma Aldrich, St. Louis, U.S.A.) for 12-15 hrs at 37°C. In addition, the different vitamers were quantified by a HPLC method based on that described by González *et al.* [2007], employing a Beckman System Gold apparatus (Beckman Coulter Inc., California, U.S.A.) and a programmable Spectroflow 980 fluorescence detector (Kratos Analytical Ltd, Manchester, U.K.). Pyridoxine, pyridoxal, pyridoxamine, pyridoxal 5'-phosphate, 4-deoxypyridoxine and 4-pyridoxic acid were obtained from Sigma-Aldrich (St. Louis, U.S.A.). Pyridoxine 5'-phosphate and pyridoxamine 5'-phosphate were synthesized from pyridoxine and pyridoxamine, respectively in a total volume of 500 µl containing 100 mM Tris-HCl, pH 8.0, 1.5 mM ATP, 10 mM MgCl2, each 1 mM PN or PM and 80 µg of purified recombinant PdxK from *Bacillus subtilis* [Park *et al.*, 2004] expressed in *E. coli*. The reaction mixture was incubated for 16 hrs at 37°C in the dark. After incubation at 70°C for 5 min the reaction mixture was centrifuged at 16,000 *g* for 10 min at 10°C and the supernatant was transferred to light-impermeable tubes and kept at -20°C until use. The standards were separated on a 250 x 4 mm LiChrospher 100 RP-18e (5 µm) column (Merck, Darmstadt, Germany) during 30 min of isocratic flow (1 ml min^{-1}) of aqueous 0.05 M phosphoric acid enriched with 0.5% (v/v) acetonitrile, pH 3.2 (adjusted with potassium hydroxide; solvent A). Samples were detected by fluorescence emission at 360 nm following excitation at 288 nm. Sodium bisulfite (0.1% (w/v)) was used as a post-column reagent to enhance B_6 vitamer fluorescence by pumping it into the postcolumn eluate at 0.1 ml min^{-1} [Sampson *et al.*, 1995]. Green plant material (2-5 g fresh weight) from ten-day old seedlings grown in MS medium lacking pyridoxine under long-day conditions (16 hr photoperiod at a light intensity of 100 µmol photons m^{-2} s^{-1}), was frozen in liquid nitrogen and lyophilized in light-impermeable tubes for 24 hrs. The lyophilized green plant material as well as mature desiccated seeds (*ca.* 100 mg fresh weight) were homogenized in liquid nitrogen and extracted in 25 ml of 5% (w/v) trichloroacetic acid. The extracts were transferred to light-impermeable tubes, frozen in liquid nitrogen and lyophilized to complete dryness. The residue was taken up in a small volume of solvent A (see above) and transferred to 2 ml tubes. The light-impermeable tube was washed with small volume of solvent A and the content trans-

ferred to the same 2 ml tube. Normally, the total volume of extract was 1.5-1.8 ml. The extract was subsequently centrifuged at 16,000 *g* for 40 min at 12°C. Typically, extract equivalent to *ca.* 150 mg (fresh weight) of shoot material and 10 mg (fresh weight) of desiccated seeds was used, diluted to a total volume of 100 µl solvent A and injected into the HPLC. For mapping the plant extract profile with the B_6 vitamers, the plant extract was spiked with the above mentioned standards and separated by HPLC as described above.

3.3.6 Total protein, lipid and carbohydrate analysis of seeds

Total protein was extracted according to Focks and Benning [1998]. Seeds (*ca.* 10 mg) were homogenized in 1 ml of acetone followed by incubation for 20 min at 4°C. The extract was centrifuged for 5 min at 16,000 *g* at 4°C. The supernatant was discarded and the pellet was evaporated to dryness followed by resuspension in 200 µl of 50 mM Tris-HCl, pH 7.5, containing 250 mM sodium chloride, 1 mM EDTA and 1% (w/v) SDS. The suspension was incubated for 2 hrs at 25°C followed by centrifugation at 16,000 *g* for 5 min. Total protein of the supernatant was determined as described recently [Bradford, 1976]. Soluble carbohydrates were extracted according to Siloto *et al.* [2006]. Seeds (*ca.* 10 mg) were homogenized in 1 ml of 80% (v/v) ethanol and incubated for 90 min at 70°C with slight shaking. The extract was centrifuged at 16,000 *g* for 10 min and the pellet was washed three times with 0.3 ml of 80% (v/v) ethanol. All supernatants were combined followed by evaporation to dryness and resuspension in 100 µl of water. The pellet was resuspended in 200 µl of 0.2 M sodium hydroxide and incubated at 95°C for 60 min by slight shaking. After neutralization (35 µl of 1 M acetic acid) the suspension was centrifuged at 16,000 *g* for 5 min. The supernatant (insoluble carbohydrates) was heated to 90°C for 15 min before analysis. Quantification of carbohydrates was performed according to Smith and Zeeman [2006]. Lipids were extracted according to Siloto *et al.* [2006]. Briefly, seeds (*ca.* 10 mg) were homogenized and lipids extracted in 1 ml 2-propanol. After evaporation, the residue was first dissolved in 1 ml of methanol:chloroform:water (2:2:1.8 (v/v)). The soluble fraction was decanted and the remaining residue repeatedly extracted with 1 ml of methanol:chloroform:water (1:2:0.8 (v/v)). All supernatants were combined and evaporated to dryness followed by an additional desiccation step for 24 hrs. Total lipids were determined gravimetrically and related to seed fresh weight.

3.3.7 Determination of PDX enzyme activity in freshly isolated plant extract

Approximately 1 g of leaf material from soil-grown plants was harvested and immediately frozen in liquid nitrogen. The plant material was homogenized in 2 ml of pre-cooled extraction buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 x complete prote-ase inhibitor cocktail (Sigma-Aldrich, St. Louis, U.S.A.) in an ice-cold 2 ml glass tissue hand homogenizer (Sartorius, Goettingen, Germany). Total protein content was determined as described by Bradford [1976]. The crude material was used to monitor the formation of pyridoxal 5'-phosphate at 30°C in a double beam spectrophotometer (Uvikon XL, Secomann, ALES Cetex, France) at 414 nm using the same extract without substrates as a reference in a total volume of 300 µl containing 50 mM Tris-HCl, pH 8.0, 0.5 mM ribose 5-phosphate, 1 mM D,L-glyceraldehyde 3-phosphate and plant extract equivalent to 0.1 mg total protein. The reaction was initiated upon addition of L-glutamine to a final concentration of 10 mM.

3.3.8 Stress treatments and cell death measurements

Surface sterilized seeds were sown on MS medium containing sucrose (1% w/v), but lacking pyridoxine and in the presence or absence of 1 µM paraquat. Seedlings cultivated under long day conditions (16 hr photoperiod at a light intensity of 100 µmol photons $m^{-2}s^{-1}$) were analyzed 12 days after imbibition. For the electrolyte leakage experiments, plants grown for 21 days in soil in continuous light (100 µmol photons m^{-2} s^{-1}) were placed in the dark for 8 hrs. Electrolytes were determined according to Laloi *et al.* [2007] in a time-dependent manner employing a conductivity meter (TetraCon-325, Universal Pocket Multiline P4; WTW, Weilheim, Germany) directly after placing the plants back to light. Cell death was also quantified by staining detached leaves with Evan's Blue solution [Ochsenbein *et al.*, 2006]. In brief, four leaves were cut at the petiole to minimize tissue injury, weighed and incubated in 4 ml of 0.1% (w/v) Evan's Blue for 24 hrs at room temperature in the dark with slight shaking. Leaves were washed with 0.05% (w/v) sodium chloride until the washing solution was colourless. The dye was extracted by incubating the leaves in 4 ml 50% (v/v) methanol containing 1% (w/v) SDS. The extract was quantified against the extraction buffer (50% (v/v) methanol, 1% (w/v) SDS) in a spectrophotometer at 600 nm and related to leaf fresh weight. Measurements of maximum quantum efficiency of photosystem II photochemistry (Fv/Fm) were determined as described in Titiz *et al.* [2006].

3.3.9 Determination of seed fresh weight (FW) and seed dry weight (DW)

Seeds from four independent sets of plants and three biological replicates were analyzed. In each case, approximately 100 seeds were counted, cleaned from dried plant debris, and either the weight was determined directly (fresh weight) or after drying in an oven for 24 hrs at 100°C followed by cooling in a desiccator (dry weight).

3.3.10 Determination of seed size

The length and width of at least 55 seeds of WT, AtPDX1.1 OE (L2), AtPDX2 OE (L4) or AtPDX1.1-2 OE (L4), respectively, from three biological replicates, were determined under a microscope. The respective volume was calculated according to Riefler *et al.* [2006] employing the mathematical formula for a spheroid ($^4/_3$ π*length*width*depth) making the assumption that width and depth are equal.

3.3.11 Pollen viability assay

Pollen was harvested on microscope slides from freshly opened flowers from the primary stem of soil-grown plants cultivated under long-day conditions at a light intensity of 120 µmol photons m^{-2} s^{-1} and stained with aqueous Alexander's stain (16.4% (v/v) ethanol, 1.64% (v/v) of a 10.8 mM malachite green solution (in ethanol), 32.7% (v/v) glycerol, 8.2% (v/v) of an aqueous 17.3 mM acid fuchsine solution containing 1 g phenol and 1.64% (v/v) lactic acid) [Alexander, 1969]. After removing excessive staining solution the pollen was analysed using a bright-field microscope and viable (dark purple) and non-viable pollen (pale green) were counted with a lab counter.

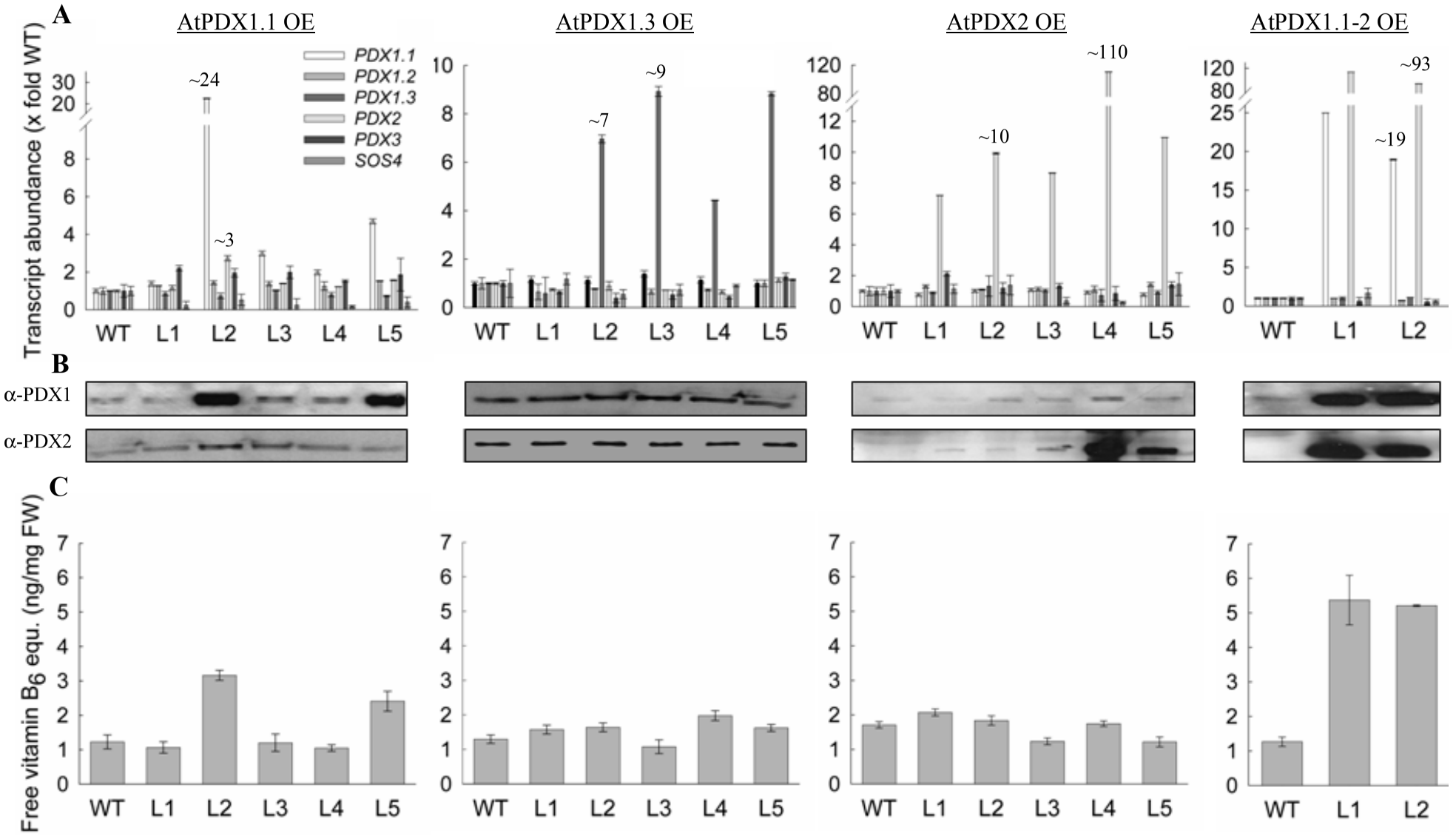
3.3.12 Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: β-*ACTIN2*, At3g18780; PDX1.1, At2g38230; PDX1.3, At5g01410; PDX2, At5g60540; FLU, At3g14110. Germplasm information for the, *pdx1.3* T-DNA insertion line (SALK_086418) used in this study can be found in the Nottingham Arabidopsis Stock Center under accession number N586418.

3.4 Results

3.4.1 Generation of Arabidopsis plants with increased levels of vitamin B_6

Agrobacterium-mediated transformation was used to introduce constructs directing the over-expression of the endogenous *PDX1* and *PDX2* genes in *Arabidopsis thaliana*. In Arabidopsis, there are two functional homologs of PDX1 (AtPDX1.1 and AtPDX1.3) and a single homolog of PDX2 [Tambasco-Studart *et al.*, 2005]. The constructs pCAM-At*PDX1.1*, pCAM-At*PDX1.3* and pBIN-At*PDX2* include the sequences encoding AtPDX1.1 (At2g38230), AtPDX1.3 (At5g01410) and AtPDX2 (At5g60540), respectively, which are under the control of the constitutive cauliflower mosaic virus 35S promoter. Seedlings of the respective transformed plants were selected for resistance to either hygromycin (pCAM-At*PDX1.1*, pCAM-At*PDX1.3*) or kanamycin (pBIN-At*PDX2*). In each case, lines homozygous for the insertion cassettes were identified from segregation analyses, and progeny of the fourth generation (T4) carrying a single insertion were analyzed. An expression analysis was carried out on shoot material from five independent lines of each transformation. Real-time RT-PCR was used to assess transcript levels relative to wild-type (WT) with actin 2 serving as a control. A substantial increase in transcript accumulation could be observed in certain lines, *e.g.* line 2 for At*PDX1.1* (*ca.* 24-fold increase), while the highest accumulation of the At*PDX2* transcript was in line 4 (*ca.* 110-fold increase) (figure 3.1*A*, supplementary table 3.1). A Western blot analysis of the same samples with specific antibodies against the respective proteins confirmed their substantial over-expression in these lines (figure 3.1*B*). It is noteworthy that while an increase was observed for the At*PDX1.3* transcript in several lines (up to nine-fold), no corresponding protein accumulation was detected and thus, these lines were not pursued further in this context (figure 3.1*A*, *B*).


A
AtPDX1.1 OE
AtPDX1.3 OE
AtPDX2 OE
AtPDX1.1-2 OE
Transcript abundance (x fold WT)
PDX1.1
PDX1.2
PDX1.3
PDX2
PDX3
SOS4
~24
~3
~7
~9
~10
~110
~19
~93
WT
L1
L2
L3
L4
L5
B
α-PDX1
α-PDX2
C
Free vitamin B6 equ. (ng/mg FW)

Employing a microbiological assay [Tambasco-Studart *et al.*, 2005], the amount of free vitamin B_6 was found to be increased about two-fold in shoot tissue from the AtPDX1.1 over-expressing lines 2 and 5, respectively (AtPDX1.1 OE), while no significant increase was observed in lines over-expressing AtPDX2 (AtPDX2 OE) (figure 3.1*C*). This latter observation can be explained by the fact that AtPDX2 expression was also increased in the AtPDX1.1 OE lines (figure 3.1*B*), and it suggested that the vitamin content could be further increased in lines over-expressing both proteins. Therefore, reciprocal crosses of AtPDX1.1 OE lines and AtPDX2 OE lines were performed and the progeny were selected for resistance to both hygromycin and kanamycin on MS medium as described above. The reciprocal hybrids from crossing the strongest over-expressors, *i.e.* AtPDX1.1 OE line 2 and AtPDX2 OE line 4 were chosen for detailed analysis. While the hybrids (lines 1 and 2) did not differ in their phenotype, that from female parent AtPDX1.1 OE and male parent AtPDX2 OE (line 2) was designated AtPDX1.1-2 OE and subjected to detailed investigation unless specified otherwise. The free vitamin B_6 content of shoot material from the double over-expressors was increased up to four-fold over WT (figure 3.1*C*). Moreover, the increase in vitamin B_6 could be correlated with an increase in the activity of PLP synthase (figure 3.2*A*). It is noteworthy that supplementation of WT plants with vitamin B_6 did not result in an increase in the tissue-level of this vitamin (figure 3.2*B*). Only upon supplementation with non-physiological levels of the vitamin (*ca.* 500 μM) was an accumulation observed.

Figure 3.1: (page 122) Analysis of the expression of genes involved in vitamin B_6 biosynthesis upon over-expression of the *PDX* genes in *Arabidopsis thaliana* and quantification of free vitamin B_6 content. ***(A)*** Quantitative real-time RT-PCR analysis of WT and five independent lines (L1-L5) in which either AtPDX1.1 (AtPDX1.1 OE), AtPDX1.3 (AtPDX1.3 OE) or AtPDX2 (AtPDX2 OE), in addition to two independent lines (L1 and L2) in which both AtPDX1.1 and AtPDX2 (AtPDX1.1-2 OE) are under control of the CaMV 35S promoter. The relative transcript level (see supplementary table 3.1 for details) of At*PDX1.1* (white), At*PDX1.2* (light *grey*), At*PDX1.3* (medium *grey*), At*PDX2* (light blue *grey*), At*PDX3* (black), At*SOS4* (dark blue) is shown compared to WT (set to 1). Shoot material of ten-day old seedlings grown under a 16 hr photoperiod (100 μmol photons m^{-2} s^{-1}) on MS medium lacking vitamin B_6 was used. ***(B)*** Western blot analysis of total protein extracted from the same samples as described for ***(A)*** using antibodies specific to AtPDX1.1, AtPDX1.3 or AtPDX2 as indicated. Total protein loaded per lane was 40 μg for detection of AtPDX1.1 or AtPDX1.3 in all lines and for detection of AtPDX2 in WT, AtPDX1.1 OE and AtPDX1.3 OE, whereas 10 μg was used for detection of AtPDX2 in AtPDX2 OE and AtPDX1.1-2 OE. ***(C)*** Free vitamin B_6 content of the same samples as in ***(A)***.

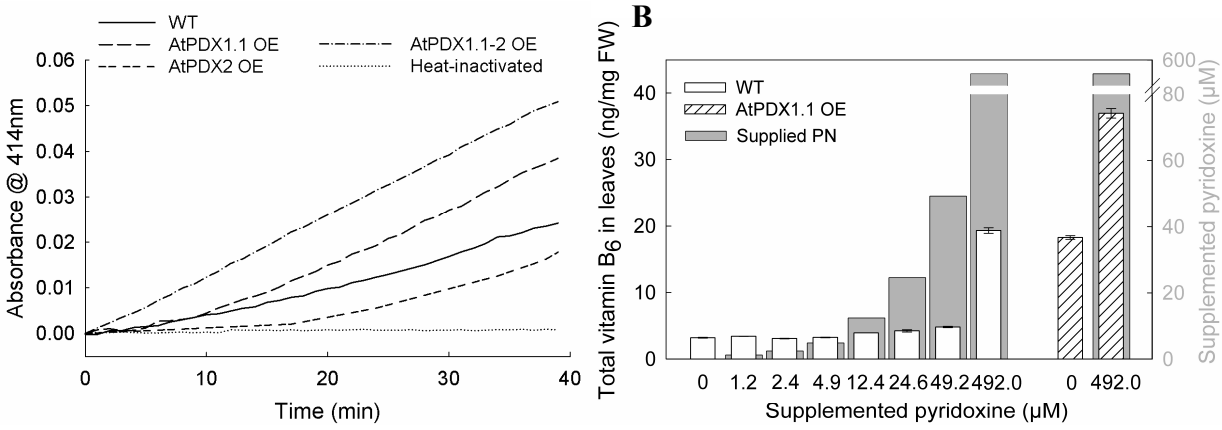


Figure 3.2: Pyridoxal 5'-phosphate synthase (PLPS) activity and effect of vitamin B_6 supplementation on Arabidopsis. *(A) In vitro* PLPS activity test with freshly isolated total protein (0.1 mg) from leaf material of 21-day old plants cultivated under a 16 hr photoperiod at 120 µmol photons m^{-2} s^{-1} in soil. In all cases, the background absorbance at λ = 414 nm was subtracted. Heat-inactivated plant extracts did not exhibit PLPS activity. This is a representative activity test for in total four independent analyses. *(B)* Effect of supplementation on total vitamin B_6 content of Arabidopsis plants. Seeds were sown on MS medium containing 1% (w/v) sucrose supplemented with up to 492 µM pyridoxine (PN). Plants were cultivated under a 16 hr photoperiod at a light intensity of 100 µmol photons m^{-2} s^{-1} for 15 days. Total vitamin B_6 content was quantified after treatment with acid phosphatase and β-glucosidase (Sigma-Aldrich) from leaf material. The values given are the average of three biological replicates with at least two technical repeats. A statistical analysis (Student's T-test) was performed for $P < 0.05$ and related to seedlings cultivated without PN supplementation. The *p*-values are as follows: 1.2 µM, *p*=0.039; 2.43 µM, *p*=0.1651; 4.86 µM, *p*=0.6292; 12.4 µM, *p*=0.0023; 24.6 µM, *p*=0.0135; 49.2 µM, *p*=0.0028; 492.0 µM, *p*=0.0002.

Vitamin B_6 can occur as free (pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM)) and as phosphorylated (pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP)) derivatives, respectively. The yeast strain *Saccharomyces carlsbergensis* employed for the quantification of vitamin B_6 takes up the free forms only. Therefore, plant extracts were treated with acid phosphatase before analysis. As a result, in addition to the free also the phosphorylated forms increased (figure 3.2*B*), suggesting a coordination between *de novo* and salvage pathway. The salvage pathway is responsible for the interconversion of the free and phosphorylated forms. So far, two enzymes of the salvage pathway have been identified in Arabidopsis: PDX3 [Sang *et al.*, 2007] and SOS4 [Shi *et al.*, 2002]. PDX3 is a FMN-containing enzyme that exhibits PNP/PMP-oxidase activities, thus forming PLP. At the transcript level, this gene appears to be up-regulated in all AtPDX1.1 OE lines (*ca.* two-fold above WT) only in L2 of the AtPDX2 OE lines and in neither of the

AtPDX1.1-2 OE lines (figure 3.1*A*). The increased levels of PDX3 transcripts in the AtPDX1.1 OE and AtPDX2 OE lines may contribute to the PLP pool (table 3.1*A*). The reason this is not observed in the double over-expressing lines is not readily apparent. To explain the higher transcript level, the protein abundance as well as enzyme activity of PDX3 should be monitored. The second enzyme, SOS4, catalyzes the conversion of the free forms of vitamin B_6 to their phosphorylated counterparts in an ATP-dependent manner, thus exhibiting PN/PL/PM-kinase activity. Interestingly, expression of SOS4 appeared to be opposite of that of PDX3 in most of the lines analysed. This down-regulation is most pronounced in the AtPDX1.1 OE and AtPDX1.1-2 OE (figure 3.1*A*). However, the level of transcript change did not correlate with the level of vitamin B_6 alteration (figure 3.1*A*, *C*). It could be speculated that the different pools of vitamin B_6 are well-balanced implying a higher phosphatase activity, which is consistent with the increased free vitamin B_6 levels determined (figure 3.1*C*). A higher level of free vitamin B_6 may be sensed triggering the down-regulation of SOS4 as it has been demonstrated for PdxK, the SOS4 homolog from *E. coli*, which is inhibited by PL *in vitro* [Yang *et al.*, 1996]. These inhibitory mechanisms of PDX3 and SOS4 activities may be important regulatory paths for the intracellular vitamin B_6 homeostasis.

3.4.2 Bioavailable forms of vitamin B_6 are predominantly formed

It must be taken into account that vitamin B_6 can occur in its free form as either pyridoxine (PN), pyridoxal (PL) or pyridoxamine (PM), and in addition their phosphorylated derivatives (PNP, PLP, PMP, respectively). All six forms can be interconverted through the salvage pathway of vitamin B_6 biosynthesis (supplementary figure 3.1). Two of the salvage pathway enzymes have been identified in Arabidopsis, namely PDX3 (a PNP/PMP oxidase) and SOS4 (a PN/PL/PM kinase). Furthermore in plants a substantial amount (up to 70%) of this vitamin can be made up of glycosidic derivatives, in particular, pyridoxine 5'-β-D-glucoside (PNG), which has a reduced bioavailability to humans due to the lack of appropriate intestinal hydrolytic enzymes [Gregory III, 1998]. Therefore, in order to determine the total vitamin B_6 content and to distinguish different pools of vitamers, the vitamin was extracted from plants and treated with acid phosphatase and β-glucosidase prior to quantification. The former enzyme hydrolyzes the phosphorylated vitamers and the latter enzyme hydrolyses pyridoxine glycosides present in plant-based foods. In shoot material, the total increase in vitamin B_6 content

had contributions from free, phosphorylated and glycosylated forms, with the bioavailable free and phosphorylated forms being the strongest contributors (figure 3.3*A*).

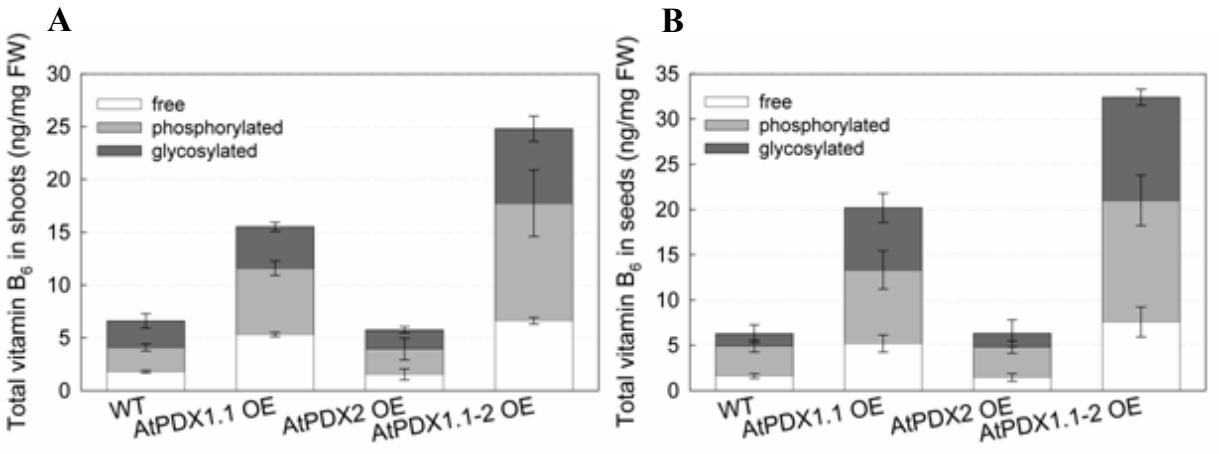


Figure 3.3: Analysis of free, phosphorylated and glycosylated forms of vitamin B_6. ***(A)*** Characterization of free (white), phosphorylated (light *grey*) and glycosylated (dark *grey*) forms of vitamin B_6 in shoot material of ten-day old plants cultivated on MS medium lacking vitamin B_6 under long day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1}. ***(B)*** As for ***(A)*** but employing seeds of identically cultivated soil-grown plants (long-day conditions at a light intensity of 150 µmol photons m^{-2} s^{-1}). In both cases, the experiment was performed in triplicate, *i.e.* three biological replicates with each two technical repeats.

3.4.3 Interplay between *de novo* and salvage pathways

The observations above suggest substantial interaction between *de novo* and salvage pathway of vitamin B_6 biosynthesis, respectively. Thus, a HPLC assay was established which allowed separation of the various vitamer forms (supplementary figure 3.2). In this way, it could be demonstrated that the cofactor vitamer PLP is one of the major contributors to the overall enhanced vitamin B_6 level (table 3.1*A*, *B;* supplementary table 3.2). There is also a substantial increase in the levels of PN and to a lesser extent, of PM. As PLP is formed directly by the PDX1/PDX2 complex [Tambasco-Studart *et al.*, 2005], it must be assumed that PN and PM are generated through the subsequent action of phosphatases, a PL reductase or transaminases that are part of the salvage pathway, but have not yet been identified in Arabidopsis. Upon examination of the transcripts of the known salvage pathway genes in Arabidopsis, *i.e. SOS4* and *PDX3*, a substantial decrease in the level of *SOS4* transcripts in vitamin B_6 accumulating lines was observed (figure 3.1*A*). In this context, it is interesting to note that a recent study has demonstrated that the Arabidopsis *sos4* mutant substantially accumulates PLP and PN [Gon-

zález *et al.*, 2007]. While this again argues for interaction of the different vitamer biosynthetic pathways, in our study one of the vitamers with an increased level, *i.e.* PLP, PN or PM presumably triggers the down-regulation of *SOS4*. Thus, it must be concluded that the increase in total vitamin B_6 observed in this report, is not only due to over-expression of AtPDX1 and AtPDX2, but must also include modulation of the salvage pathway, *i.e. SOS4*.

A

Seedlings	PN	PM	PNP	PMP	4-PA	4-dPN	PL	PLP
WT	100 ± 47	100 ± 16	100 ± 29	100 ± 32	100 ± 40	100 ± 41	100 ± 21	100 ± 56
AtPDX1.1 OE	**340 ± 33**	164 ± 32	114 ± 25	106 ± 47	117 ± 40	148 ± 23	107 ± 39	**354 ± 30**
AtPDX2 OE	144 ± 61	129 ± 78	87 ± 29	99 ± 12	79 ± 45	121 ± 49	119 ± 50	68 ± 16
AtPDX1.1-2 OE	**447 ± 181**	**306 ± 35**	206 ± 86	126 ± 25	174 ± 72	210 ± 35	171 ± 39	**419 ± 42**

B

Seeds	PN	PM	PNP	PMP	4-PA	4-dPN	PL + PLP
WT	100 ± 56	100 ± 43	100 ± 30	100 ± 36	100 ± 66	100 ± 30	100 ± 33
AtPDX1.1 OE	**333 ± 105**	120 ± 35	116 ± 36	152 ± 49	187 ± 122	137 ± 12	177 ± 35
AtPDX2 OE	94 ± 26	94 ± 13	120 ± 57	110 ± 16	106 ± 21	93 ± 32	94 ± 25
AtPDX1.1-2 OE	**484 ± 88**	156 ± 42	128 ± 42	224 ± 78	134 ± 25	150 ± 74	286 ± 47

Table 3.1: Relative contributions of vitamers to the total vitamin B_6 pool in ***(A)*** 10-day old seedlings grown in sterile culture medium lacking vitamin B_6 and ***(B)*** mature desiccated seeds. The data were acquired by HPLC analysis and are the average of three biological replicates performed in duplicate and are expressed in percent relative to WT. The approximate values of the B_6 vitamers in WT (in ng / mg FW) are as follows: Seedlings: PN, 0.13±0.06, PM, 0.02±0.002; 4-PA, 0.002±0.001; 4-dPN, 0.002±0.001; PL, 0.26±0.054; PLP, 0.99±0.552. Seeds: PN, 0.14±0.08; PM, 0.51±0.22; 4-PA, 0.01±0.007; 4-dPN, 0.01±0.033. Under the conditions used, PL and PLP could not be distinguished in seed extracts. The approximate numbers in bold represent substantial increases over WT. See supplementary table 3.2 for details regarding the statistical analysis. Abbreviations used: PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; PNP, pyridoxine 5'-phosphate; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; 4-PA, 4-pyridoxic acid; 4-dPN, 4-deoxypyridoxine.

3.4.4 Plants with enhanced vitamin B_6 levels have enlarged seeds

In the first instance, it was observed that the overall increase in vitamin B_6 was predominantly found in shoots rather than in roots (figure 3.4*A*). Given that the expression is under control of the CaMV 35S promoter, an explanation for this is not readily apparent, but this phenomenon may point to a tight regulation in root tissue. The enhanced vitamin B_6 level did not affect de-

velopment of the plants until bolting, when a significant delay was observed, an effect which appeared to be correlated with the free vitamin B_6 content (figure 3.4*B*) but to be not related to the rosette leaf number before bolting (supplementary table 3.3). At maturity however, plants were indistinguishable from WT (figure 3.4*C*). Interestingly, a substantial accumulation of the vitamin was observed in the seeds of plants (Figure 3.4*A*). Indeed, the seed represents the part of the plant with the highest accumulation of the vitamin. As for shoot tissue, the overall increase in vitamin B_6 appears to be contributed by free, phosphorylated and glycosylated equivalents (figure 3.3*B*).

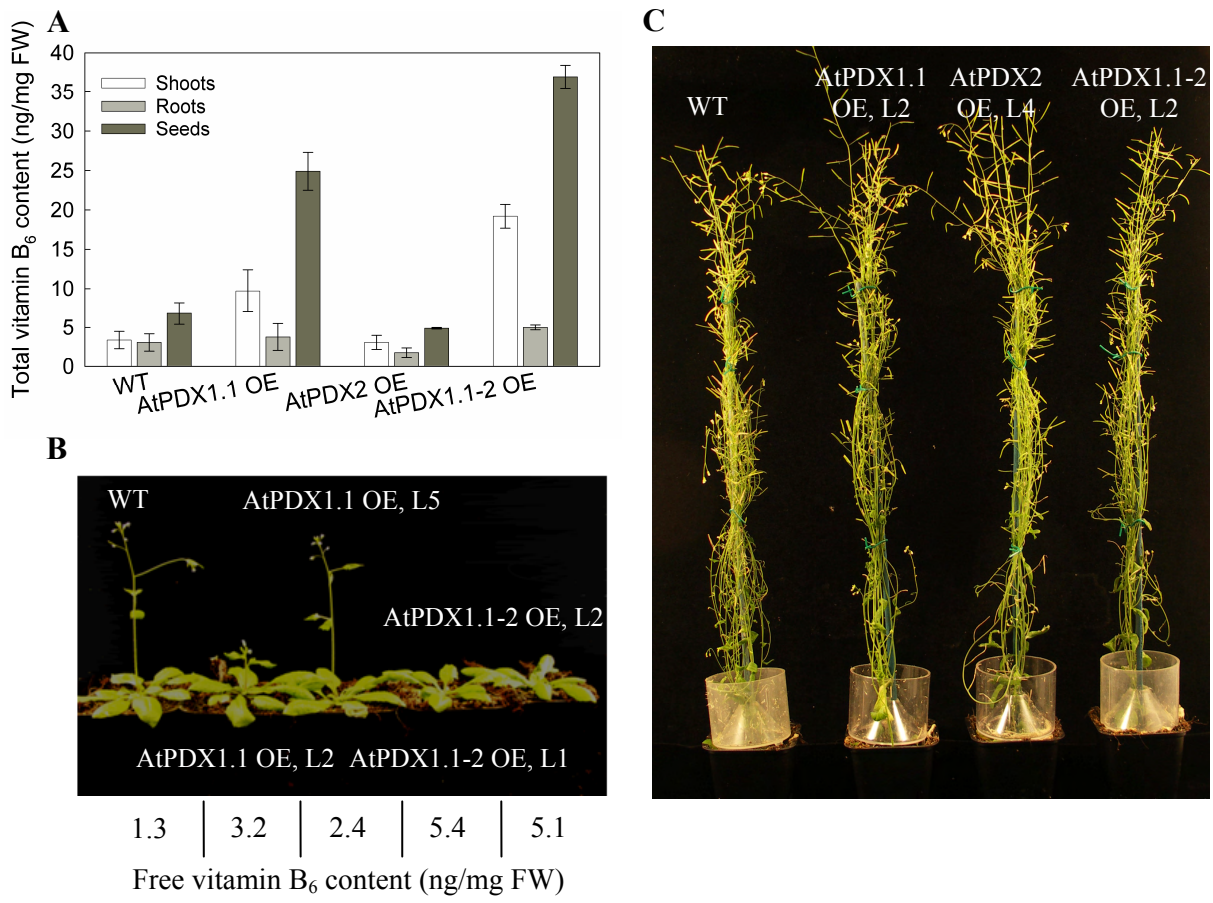


Figure 3.4: Characteristics of *Arabidopsis* plants with enhanced vitamin B_6 levels. ***(A)*** Total vitamin B_6 content in shoots (white), roots (light *grey*) and seeds (dark *grey*). Contents of shoot and root were determined in ten-day old seedlings. ***(B)*** Plants with elevated vitamin B_6 show a delay in bolting. The respective lines, 35 days after germination, were simultaneously cultivated under the conditions described for ***(A)*** and have free vitamin B_6 contents as shown underneath. ***(C)*** As for ***(B)***, but the lines shown are 63 days after germination.

Furthermore, the seeds of such plants are substantially enlarged (figure 3.5*A*). The average length and width of seeds were increased up to 1.4-fold over WT (figure 3.5*B*) and the calculated seed volume (employing a spheroid formula) showed an up to 2.2-fold increase over WT (figure 3.5*C*). Moreover, vitamin B_6 content and seed size can be correlated in the different lines (figure 3.5*D*). The increase in seed size is related to a general increase in total protein, lipid and carbohydrate content (table 3.2). In particular, protein and lipid appear to be the largest contributors to the increase in mass. However, the increase in seed size appears to be offset by an incomplete seed set, *i.e.* AtPDX1.1 OE and AtPDX1.1-2 OE only produced 82% and 72% of WT seed set, respectively (figure 3.5*A*, table 3.3). The latter is not caused by a reduced set of ovules (table 3.3*A*) or by a reduction of pollen viability (table 3.3*B*).

Line	Seed FW (µg)	Seed DW (µg)	Total proteins (µg/FW)	Total lipids (µg/FW)	Total soluble carbohydrates (ng/FW)	Total insoluble carbohydrates (ng/FW)
WT	20.6 ± 2.8	12.9 ± 0.3	7.5 ± 0.7	8.1 ± 2.2	506.2 ± 15.6	100.6 ± 14.3
AtPDX1.1 OE	**32.4 ± 1.7**	**19.3 ± 1.7**	**12.1 ± 1.0**	11.6 ± 2.3	**887.6 ± 60.4**	158.3 ± 21.9
AtPDX1.1-2 OE	**35.7 ± 2.2**	**19.8 ± 1.0**	13.8 ± 3.1	13.7 ± 1.5	**1,055.9 ± 90.5**	**165.3 ± 25.1**

Table 3.2: Nutrient content of seeds of plants over-expressing the PDX proteins involved in vitamin B_6 biosynthesis. The numbers represent the average data of at least four biological replicates cultivated simultaneously under identical conditions (16 hr photoperiod, 120 µmol photons m^{-2} s^{-1}). Each analysis was performed in triplicate analysis. Numbers in bold represent substantial increases over WT. A statistical analysis (Student's T-test) was performed for $P < 0.05$ and related to WT. The *p*-values are as follows: AtPDX1.1 OE: seed FW, p=0.0008; seed DW, p=0.0248; protein, p=0.0395; lipids, p=0.31; sol. CH, p=0.01; insol. CH, p=0.1005. AtPDX1.1-2 OE: seed FW, p=0.0001; seed DW, p=0.0089; protein, p=0.0821; lipids, p=0.0111; soluble carbohydrates, p=0.0097; insoluble carbohydrates, p=0.0326. Abbreviations used: FW: fresh weight, DW: dry weight.

A

	WT	AtPDX1.1 OE	AtPDX2 OE	AtPDX1.1-2 OE
Silique length (mm)	10.8 ± 0.5	**12.8 ± 0.2**	10.9 ± 0.5	**12.9 ± 0.2**
Total seeds	50 ± 1.8	**41 ± 0.5**	50 ± 1.2	**36 ± 0.4**
Total ovules	56 ± 0.6	**57 ± 1.5**	56 ± 0.7	**58 ± 1.1**

B

Line	Pollen (total)	Viable (total)	Non-viable (total)	Non-viable (%)
WT	2,651	2,534	117	4.4 ± 0.3
AtPDX1.1 OE	3,492	3,267	225	**6.4 ± 0.2**
AtPDX2 OE	4,260	4,040	220	**5.2 ± 0.3**
AtPDX1.1-2 OE	4,375	4,099	276	**6.3 ± 0.7**

Table 3.3: *(A)* Comparative analysis of the silique length and the average ovule and seed number, respectively, in siliques from the primary stem of five-week old plants as indicated. At least 30 individual primary stem siliques from a minimal set of six plants per line and two independently cultivated sets of plants (16 hr photoperiod, 120 µmol photons m^{-2} s^{-1}) were analyzed. The significance of the results was confirmed by a double-sided Student's T-test for $P < 0.05$ (silique length: AtPDX1.1 OE, $p = 0$; AtPDX2 OE, $p = 0.0586$; AtPDX1.1-2 OE, $p = 0$; total seeds: AtPDX1.1 OE L2, $p = 0$; AtPDX2 OE L4, $p = 1$; AtPDX1.1-2 OE L2, $p = 0$; total ovules: AtPDX1.1 OE, $p = 0.0017$; AtPDX2 OE, $p = 1$; AtPDX1.1-2 OE, $p = 0$). Significant changes are indicated in bold. ***(B)*** Pollen viability assays. Pollen was taken from freshly opened flowers on the primary stem from at least five plants per line, cultivated simultaneously under long-day conditions (16 hr photoperiod, 120 µmol photons m^{-2} s^{-1}) and stained with Alexander's stain [Alexander, 1969]. The significance of the results was confirmed by a double-sided Student's T-Test for $P < 0.05$ (AtPDX1.1 OE: p=0.0005; PDX2 OE: p=0.0283; AtPDX1.1-2 OE: p=0.0015).

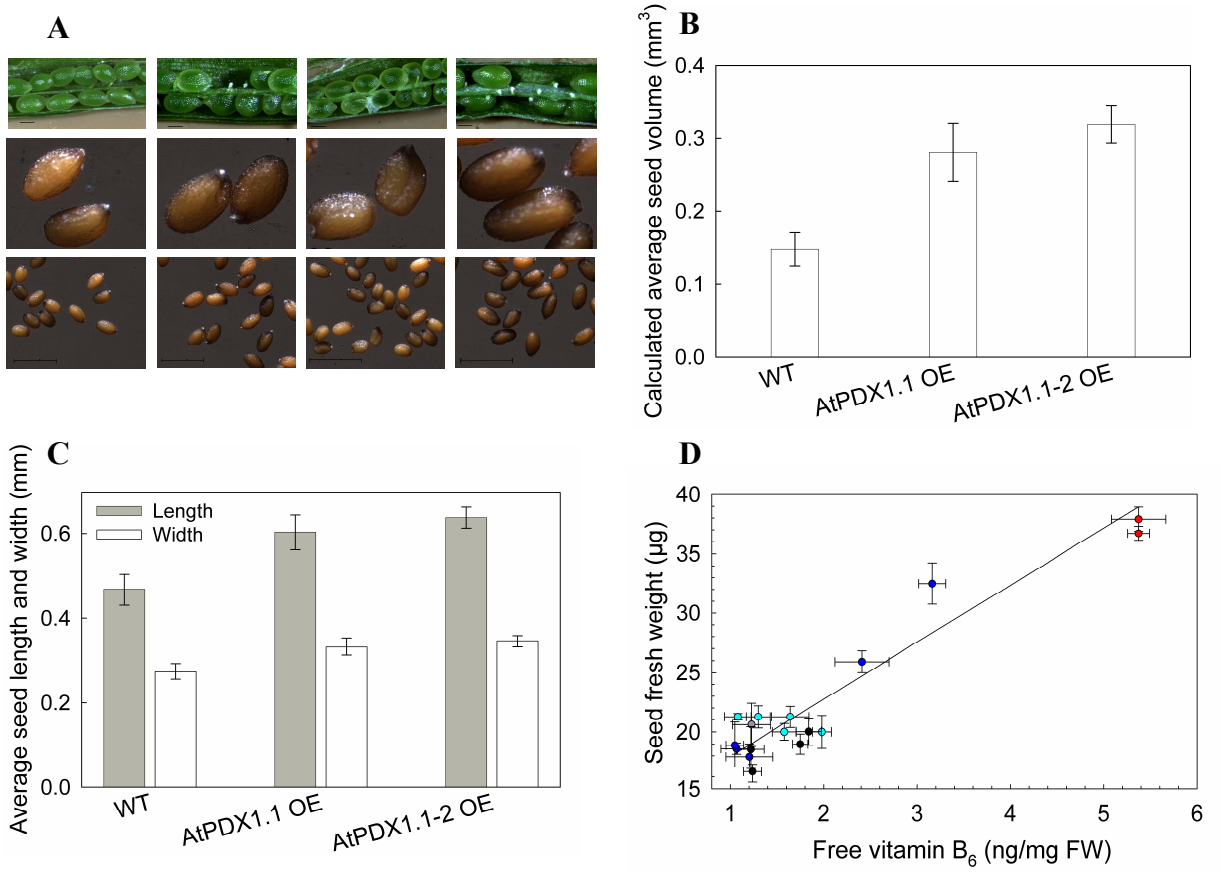


Figure 3.5: Plants with enhanced vitamin B_6 levels have enlarged seeds. ***(A)*** Upper panel: Dissected immature siliques; centre panel and lower panel: Representative sets of mature seeds of *Arabidopsis* as indicated. The scale bar on the lower panel represents 1 mm. The centre panel is a four-fold magnification of seeds from the lower panel. ***(B)*** The length and width of seeds. At least 55 seeds from three biological replicates were employed. ***(C)*** Seed volume. The seed volume was calculated according to Riefler *et al.* [2006] employing the mathematical formula for a spheroid ($^4/_3$ π*length*width*depth) making the assumption that the width and depth are equal. ***(D)*** Correlation between vitamin B_6 and seed size in independent lines of WT (grey), AtPDX1.1 OE (blue), AtPDX1.3 OE (black), AtPDX2 OE (cyan) and AtPDX1.1-2 OE (red), equated by non-linear regression ($y = 4.7577x + 13.0247$ ($R = 0.9233$, $t = 12.0887$, $P < 0.0001$). All lines used were simultaneously cultivated under a 16-hr photoperiod (120 µmol photons m^{-2} s^{-1}). WT, AtPDX1.1 OE, AtPDX1.3 OE, AtPDX2 OE and AtPDX1.1-2 OE refer to wild-type or lines either over-expressing AtPDX1.1, AtPDX1.3, AtPDX2 or both AtPDX1.1 and AtPDX2, respectively.

However, the overall yield of seeds from mature plants in each case significantly exceeds that of WT (23.1 % and 17.5 % increase in the case of AtPDX1.1 OE and AtPDX1.1-2 OE, respectively; figure 3.6*A*). In order to address the possibility of altered carbon partitioning in the transgenic lines that may account for the increase in seed size, we removed 20-30% of the developing siliques from WT plants. This mimics the reduction in seed set observed in the

AtPDX1.1 OE lines and AtPDX1.1-2 OE lines, respectively. However, while we observed a slight increase in seed weight in WT plants (1.1-fold), it was not significant (p=0.0632 for $P < 0.05$) compared to the increase observed for AtPDX1.1 OE lines and AtPDX1.1-2 OE lines (1.6-fold and 1.7-fold, respectively). Therefore, the increase in seed size cannot be correlated with the reduced seed set. In addition we noted that a comparable proportion of the plants total dry weight is partitioned to the seed in all lines (figure 3.6*B*).

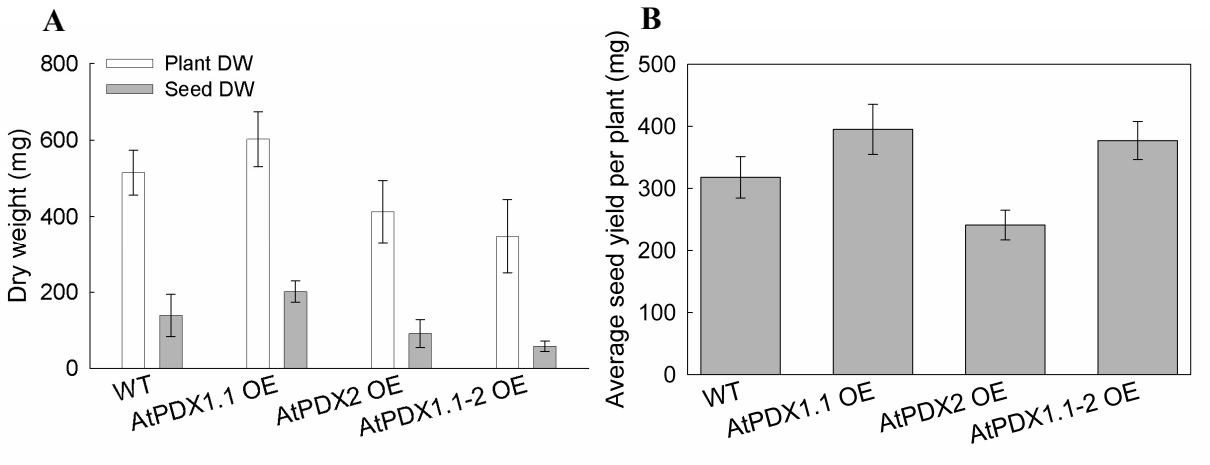


Figure 3.6: Seed yield and partitioning of dry weight to seeds. ***(A)*** Seed yield. The values given are the average weight of seeds harvested separately from at least seven plants. The significance of the results compared to WT was confirmed with a Student's T-test for $P < 0.05$ (AtPDX1.1 OE, p=0.0013; AtPDX2 OE, p=0.0006; AtPDX1.1-2 OE, p=0.0043). Both WT and AtPDX2 OE were harvested 97 days after germination, whereas AtPDX1.1 OE and AtPDX1.1-2 OE were harvested 125 and 141 days after germination, respectively. ***(B)*** Allocation of plant dry weight to the seeds. Seeds were sown in soil and cultivated under a 16 hr photoperiod at a light intensity of 150 µmol photons m^{-2} s^{-1}. After bolting and stem elongation, the aboveground parts of the plants were covered by an air-permeable bag and cultivated further until maturity. The completely dried aboveground plant material was harvested and the weight was determined from the whole material (white bars) and from seeds only (grey bars), respectively. The standard deviation was calculated from at least seven plants per line. A Student's T-test was performed for $P < 0.05$ and related to WT (shoot dry weight: AtPDX1.1 OE: p=0.0169, AtPDX2 OE: p=0.0183, AtPDX1.1-2 OE: p=0.006; dry weight compartmented to seeds: AtPDX1.1 OE: p=0.196, AtPDX2 OE: p=0.1036, AtPDX1.1-2 OE: p=0.0074).

3.4.5 Plants with enhanced vitamin B_6 levels are more resistant to abiotic stress

As vitamin B_6 is now considered an antioxidant with a particular potency against the reactive oxygen species superoxide ($O_2^{\cdot-}$) and singlet oxygen ($^1O_2$) [Bilski *et al.*, 2000; Jain and Lim, 2001], we tested if plants with an enhanced vitamin B_6 content are less susceptible to oxida-

tive damage. In the first instance, growing seedlings on media containing paraquat (an $O_2^{\cdot-}$-inducer), demonstrated that lines enhanced in vitamin B_6 are more resistant to this compound than WT (figure 3.7*A*).

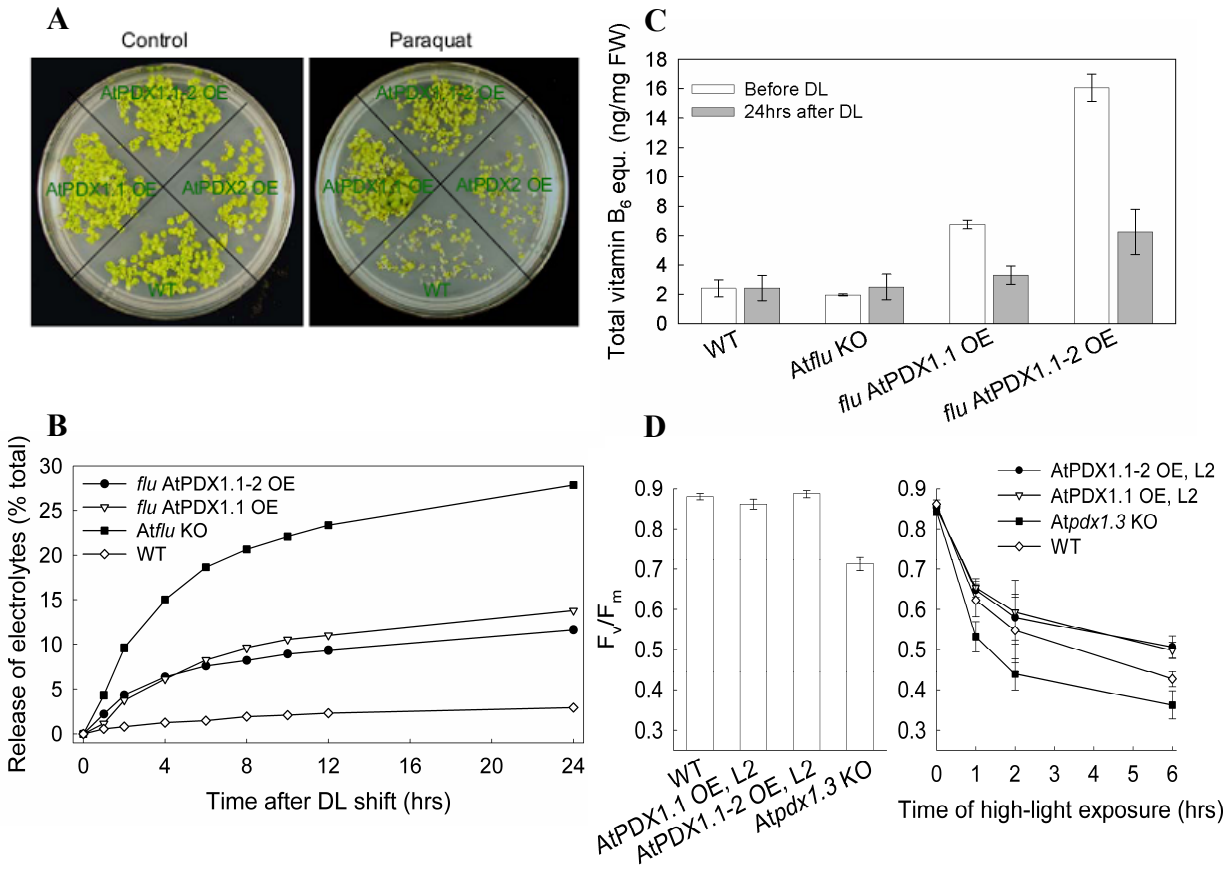


Figure 3.7: Plants with enhanced vitamin B_6 levels are more tolerant to oxidative stress. ***(A)*** Seeds obtained from identically cultivated plants were surface-sterilized and plated onto MS medium lacking vitamin B_6 and including paraquat (1 µM). Seedlings were cultivated under a 16 hr photoperiod (100 µmol photons m^{-2} s^{-1}). Pictures were captured 12 days after imbibition. ***(B)*** Electrolyte leakage in lines as indicated after a shift from the dark (8 hrs) to the light (DL = dark/light shift). The values given are related to total electrolytes determined after boiling (=100%) and are the average of eight determinations. The significance of the results related to the *flu* mutant (At*flu* KO) was confirmed with a Student's T-test for $P < 0.05$ (*flu* AtPDX1.1 OE, p=0.0459; *flu* AtPDX1.1-2 OE, p=0.0431). ***(C)*** Total vitamin B_6 content in leaf material of the same lines as shown in ***(B)*** before and 24 hr after DL. ***(D)*** Response to photoinhibitory treatment. Maximum quantum efficiency of photosystem II photochemistry of the lines indicated grown under either 100 µmol photons m^{-2} s^{-1} (control, left panel) or 10 µmol photons m^{-2} s^{-1} for seven days and then exposed to 1,100 µmol photons m^{-2} s^{-1} for the times indicated (right panel). The significance of the results was analyzed with a Fisher's F distribution test for $P < 0.05$ (AtPDX1.1 OE, L2, p=0.0.519; AtPDX1.1-2 OE, L2, p=0.0558; At*pdx1.3* KO, p=0.0761).

The response to $^1O_2$ could be demonstrated through the use of the Arabidopsis conditional *flu* mutant (At*flu* KO) [Meskauskiene *et al.*, 2001]. The *flu* mutant is characterized by a rapid burst of $^1O_2$ in the chloroplast following a shift from a dark period to the light, due to accumulation in the dark of the photosensitizer molecule protochlorophyllide, a precursor to chlorophyll. Under such conditions, cell death ensues rapidly following transfer to light [Op den Camp *et al.*, 2003]. For the purpose of this study, AtPDX1.1 OE and AtPDX1.1-2 OE lines were crossed into the *flu* background and triple homozygote plants of the third generation were analyzed. The extent of cell death in leaves was determined by measuring electrolyte leakage in leaves of 21-day old plants grown initially under continuous light before transfer to the dark. Plants harbouring the *flu* mutation showed the expected rapid onset of cell death compared to WT (figure 3.7*B*). However, plants over-expressing the AtPDX proteins in the *flu* background and having an enhanced vitamin B_6 level (figure 3.7*C*), showed a dramatic decrease in the extent of cell death (figure 3.7*B*). Cell death was additionally monitored in leaves from the same lines 24 hrs after the dark-light shift by Evan's Blue dye, which specifically stains dead cells. The results corroborate those from the electrolyte leakage experiments (supplementary figure 3.3). This provides evidence, albeit indirect, for scavenging of reactive oxygen species by the excess vitamin B_6. This conclusion is supported by the observed decrease in the total vitamin B_6 content of these lines 24 hrs after the transfer from dark to light (figure 3.7*C*).

The response to oxidative stress was further corroborated by experiments in which WT and the AtPDX over-expressors in WT background were subjected to high light stress, which leads to a dramatic increase in the production of reactive oxygen species and subsequent photoinhibition. We compared the extent of damage to photosystem II under such conditions by determining the maximum quantum efficiency of photosystem II photochemistry (F_v/F_m). Plants over-expressing the AtPDX proteins with an increased vitamin B_6 content had F_v/F_m values significantly higher than WT, indicating lower susceptibility to photosystem II photoinhibition (figure 3.7*D*) and emphasizing resistance of such plants to abiotic stress. As an additional control, a knockout line of AtPDX1.3 was included that shows considerably increased photoinhibition compared to WT under these conditions as previously described [Titiz *et al.*, 2006]. This then substantiates our previous postulate that vitamin B_6 plays a photoprotective role in plants [Titiz *et al.*, 2006]. In the absence of high light stress, no difference in F_v/F_m was observed between WT and the AtPDX over-expressors (figure 3.7*D*).

3.5 Discussion

Engineering of plants for enhanced vitamin levels is thought to hold great promise for the improvement of the nutritional content of foods or even for plants as bioreactors to produce such compounds. This study provides an impetus to realize this goal with fervour in the case of vitamin B_6. A recent study suggested that the vitamin B_6 content of plants could not be significantly increased, via the expression of heterologous *PDX* genes, purportedly due to strict regulation of the pathway [Herrero and Daub, 2007]. It must be emphasized, therefore, that the increase in vitamin B_6 levels reported here was achieved through the over-expression of the *endogenous* proteins that produce the vitamin. On the biochemical level this point is important because it is now known that not only must PDX1 and PDX2 interact in order to be enzymatically active, but also the interaction interface is apparently species specific and there may even be species specific regulatory mechanisms [Tambasco-Studart *et al.*, 2007].

The increase in seed size and yield were unanticipated, but this trait is highly significant. While, this study demonstrates that vitamin B_6 content correlates with the increase in seed size, the precise secondary physiological mechanisms behind this increase will be the basis of future studies. As seeds are a major resource for both human consumption and animal feed, there are many ongoing projects worldwide that aspire to increasing seed biomass/yield. The features of increased biomass observed here could be exploited to achieve exactly this in plant species of economical interest by introducing the *PDX* genes into a crop plant in the appropriate way. Plant seeds are especially attractive as production units. In addition to a high production capacity, they offer several important advantages over other plant parts. Seeds can be stored for a long time without losing the nutrients' effectiveness, so that a reserve can always be kept on hand. This means that the nutrients can be isolated from the seeds at the moment they are actually needed. With leaves or tubers, such lengthy storage is not possible: the nutrient must be isolated shortly after production. So, production in plant seeds provides the clear advantage of timely processing. A recent study has demonstrated that species and individual plants with larger seeds respond more positively to elevated CO_2 [Jones and Reekie, 2007]. This feature is of particular significance in the context of the potential effects of rising atmospheric CO_2 concentrations, and moreover in understanding the factors affecting plant performance at current and elevated atmospheric CO_2 levels.

Furthermore, the plants described here have the potential to sustain high yield under environmentally stressful conditions. In this context, it is well established that many environmental

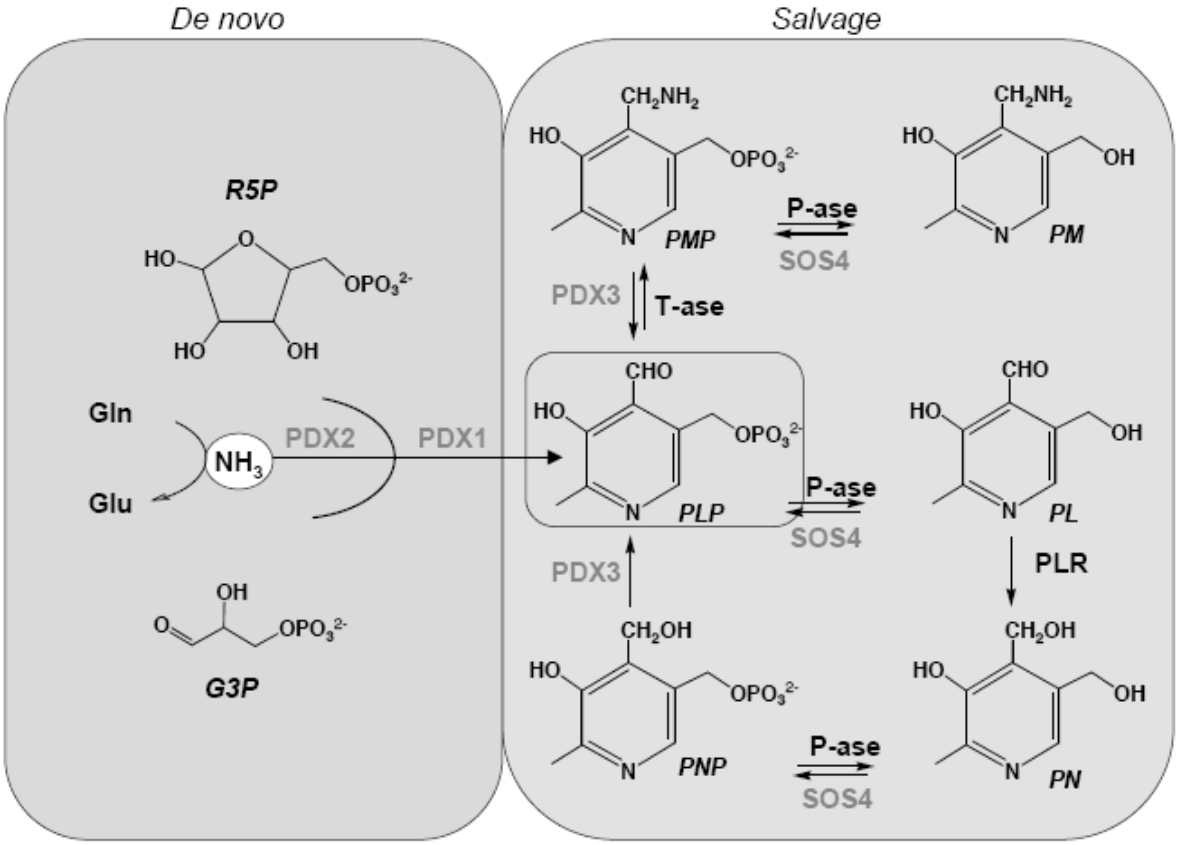
stressors, *e.g.* drought, salinity, temperature, heavy metals, cause a rapid and excessive accumulation of reactive oxygen species in plants [Sunkar *et al.*, 2003; Apel and Hirt, 2004]. Future work will encompass a comprehensive study of the response of these plants to extensive combination stress programs as suggested by Mittler [2006]. Indeed, preliminary experiments indicate that PDX over-expressors have significant tolerance to drought. In any case, the findings reported herein substantiate the previous postulation that vitamin B_6 can play a protective role in plants under oxidative stress, as has been demonstrated both *in vitro* [Bilski *et al.*, 2000] and *in vivo* by genetic knockout studies in *Arabidopsis* [Shi *et al.*, 2002; Denslow *et al.*, 2005; Titiz *et al.*, 2006]. The generation of lines over-accumulating vitamin B_6, will help to address the molecular details behind these phenomena, in particular where the primary source of its protective action is to be found. Indeed, one may doubt that the positive effect of elevated vitamin B_6 levels on oxidative stress is actually due to a direct quenching of reactive oxygen species. Thus, in the case of the *flu* mutant which produces an excess of singlet oxygen that is characterized by a very short half-life and diffusion distance [Asada, 2006], vitamin B_6 would need to be localized in the reaction centre or antenna subunit complex of photosystem II, the primary source of singlet oxygen production. As *de novo* vitamin B_6 biosynthesis occurs in the cytosol [Tambasco-Studart *et al.*, 2005], this would implicate a mechanism for transport into the chloroplast. However, we cannot rule out that vitamin B_6 through its primary function as a cofactor for amino acid, carbohydrate and lipid metabolism provides an as yet unknown factor, which is responsible for the protective effects observed.

The mechanism behind the developmental delay observed in plants that overproduce vitamin B_6 is beyond the scope of this study. However, it should be noted that the active cofactor form, *i.e.* PLP, could interrupt the transcriptional responses of receptor proteins in signalling cascades. In particular, a negative effect on specific signal cascades has been reported for glucocorticoid, progesterone and estrogen hormone receptors in mammalian cell lines, such that elevated endogenous PLP levels decrease the transcriptional response and thus the activation of the nuclear transcription factor NF1, which leads to depression of the activation of responsive genes [Tully *et al.*, 1994]. Recently, studies in mammalian cell lines revealed that PLP in particular, could modify a nuclear receptor interacting protein (RIP140), thereby enhancing the interaction with histone deacetylases subsequently resulting in repression of DNA transcription [Huq *et al.*, 2007]. Such mechanisms have not been studied in plants to date, but could suggest novel physiological functions for PLP. An alternative explanation for the de-

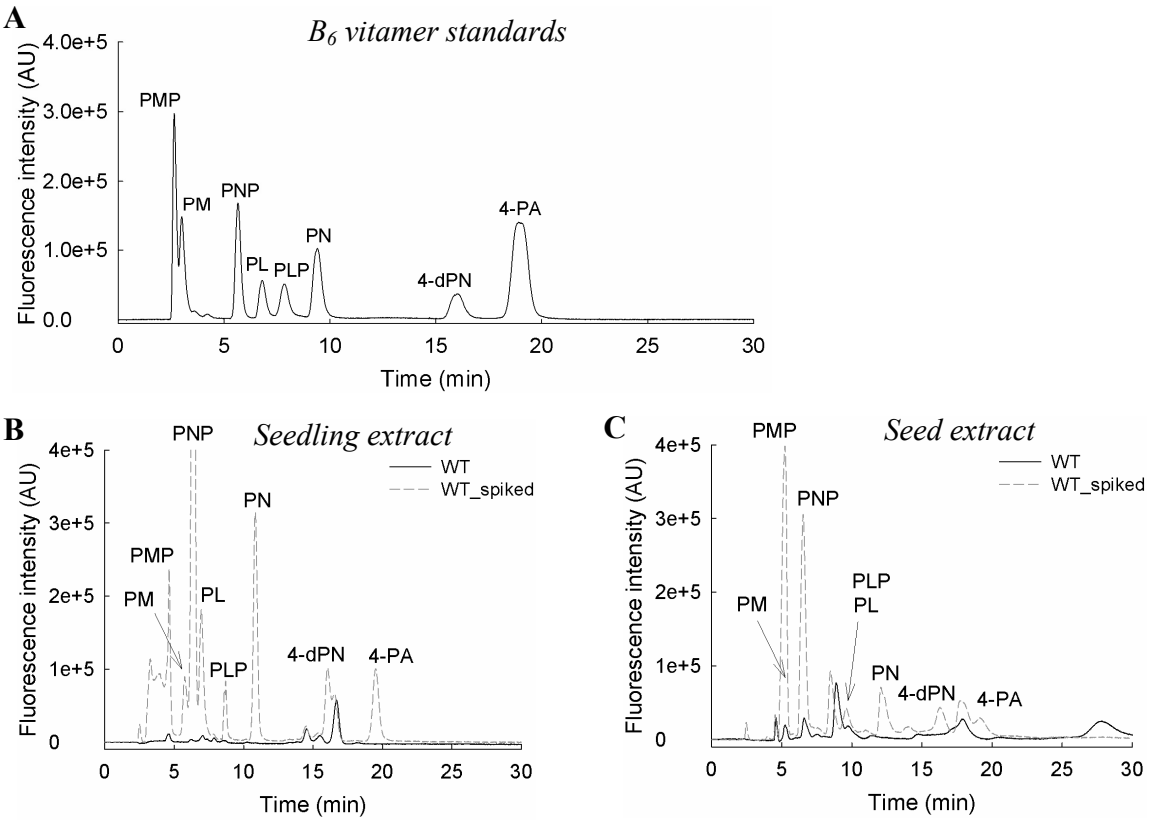
velopmental delay could possibly be related to polyamines, a group of compounds that have been associated with a variety of developmental processes [Evans and Malmberg, 1989]. *De novo* biosynthesis of polyamines is dependent on PLP as a cofactor, in particular the arginine and ornithine decarboxylases, respectively, both of which catalyze initial reactions of polyamine biosynthesis. Polyamines have been related to a delay in senescence as well as to stress protection, radical scavenging and membrane stabilization. If, through the overproduction of vitamin B_6, the levels of polyamines are increased, this may account for the stress-protective effects observed in this study. On the other hand, it has been reported that under long-day conditions, inhibition of polyamine biosynthesis results in a reduction of both bolting and flowering in Arabidopsis [Applewhite *et al.*, 2000]. In rats, PLP can complex the polyamine, spermine, thereby reducing its toxicity under high cellular abundance [Keniston *et al.*, 1987]. If such a phenomenon occurs in plants, it could be argued that increased levels of vitamin B_6 may at least partially prevent the accumulation and/or translocation of polyamines and may account for the observed delay in development.

One should note again that supplementation with vitamin B_6 does not greatly affect total vitamin B_6 levels in plants, which implies that both uptake and transport of the B_6 vitamers are controlled. In particular, it is likely that the pool of the vitamin present in the seeds is predominantly synthesized *in situ* during seed development rather than being imported from the parent plant. The finding that the embryo lethal *pdx2* knockout lines can only be rescued by watering with highly excessive amounts of the vitamin, and that yet the seeds of such rescued plants still abort at the globular stage, supports this. Thus, the supplied vitamin does not appear to be transported to the developing seeds [Tambasco-Studart *et al.*, 2007]. Indeed, promoter-*GUS* and quantitative real-time RT-PCR analyses, respectively, have revealed that the *PDX* genes are highly expressed in seeds [Wagner *et al.*, 2006; Tambasco-Studart *et al.*, 2007].

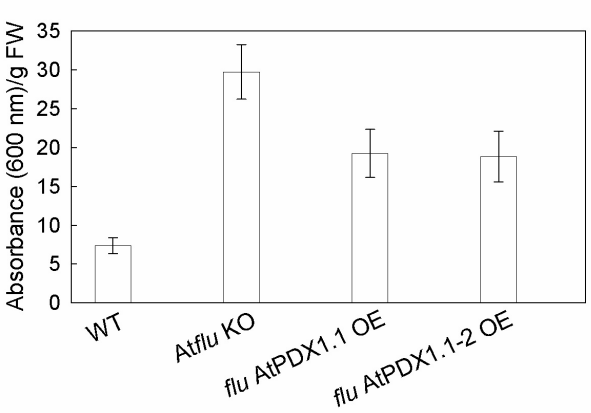
3.6 Supplementary information



Supplementary figure 3.1: Biosynthesis of vitamin B_6. Pyridoxal 5'-phosphate is produced *de novo* from ribose 5-phosphate (R5P), glyceraldehyde 3-phosphate (G3P) and glutamine (Gln) through the action of PDX1 and PDX2. Enzymes of the salvage pathway can interconvert the vitameric forms, *i.e.* PN (pyridoxine), PL (pyridoxal), PM (pyridoxamine) or their phosphorylated derivatives (PNP, PLP, PMP, respectively) as depicted. Only the PN/PM oxidase (PDX3) and PN/PL/PM kinase (SOS4) have been identified in *Arabidopsis*. T-ase, P-ase and PLR refer to transaminase, phosphatase and PL reductase, respectively.



Supplementary figure 3.2: Separation of standard B_6 vitamers and plant and seed extracts of *A. thaliana* WT by HPLC. 4-dPN: 4-deoxy-pyridoxine; 4-PA: 4-pyridoxic acid; PL; pyridoxal; PLP: pyridoxal 5'-phosphate; PM: pyridoxamine; PMP: pyridoxamine 5'-phosphate; PN: pyridoxine; PNP: pyridoxine 5'-phosphate. ***(B**, **C)*** Analysis of *A. thaliana* WT plant extracts from shoot material of ten-day old seedlings cultivated on MS medium lacking pyridoxine under long-day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1} ***(B)*** and desiccated seeds from simultaneously cultivated *A. thaliana* WT plants obtained after cultivation on soil under long-day conditions at a light intensity of 130 µmol photons m^{-2} s^{-1} ***(C)***. The dashed line in ***(B**, **C)*** represents the same extract but spiked with the standards as given in ***(A)***.



Supplementary figure 3.3: Monitoring of cell death in leaf material of the lines indicated with Evan's blue dye upon a shift from the dark (eight hrs) to the light.

	PDX1.1 (At2g38230)	*PDX1.2 (At3g16050)*	*PDX1.3 (At5g01410)*	*PDX2 (At5g60540)*	*PDX3 (At5g49970)*	*SOS4 (At5g37850)*
AtPDX1.1 OE, L1	1.39±0.097 (*p*=0.082)	1.28±0.014 (*p*=0.1187)	0.86±0.112 (*p*=0.1774)	1.17±0.123 (*p*=0.308)	**2.21±0.141 (*p*=0.038)**	0.24±0.203 (*p*=0.1281)
AtPDX1.1 OE, L2	**22.56±0.129 (*p*=0)**	**1.44±0.104 (*p*=0.0378)**	0.74±0.140 (*p*=0.0846)	**2.74±0.136 (*p*=0.0029)**	1.96±0.215 (*p*=0.0857)	0.52±0.301 (*p*=0.2588)
AtPDX1.1 OE, L3	**2.99 ±0.147 (*p*=0.0051)**	1.39±0.116 (*p*=0.1378)	1.03±0.019 (*p*=0.297)	**1.39±0.02 (*p*=0.0201)**	1.99±0.332 (*p*=0.1231)	0.25±0.334 (*p*=0.1411)
AtPDX1.1 OE, L4	1.98±0.54 (*p*=0.0985)	1.26±0.226 (*p*=0.3779)	0.81±0.116 (*p*=0.1098)	1.22±0.01 (*p*=0.0553)	1.53±0.061 (*p*=0.123)	**0.17±0.06 (*p*=0.031)**
AtPDX1.1 OE, L5	**4.69 ±0.129 (*p*=0.0013)**	**1.53±0.019 (*p*=0.038)**	**0.73±0.033 (*p*=0.001)**	**1.57±0.022 (*p*=0.0041)**	1.86±0.867 (*p*=0.2931)	0.40±0.279 (*p*=0.1802)
AtPDX1.3 OE, L1	1.16±0.161 (*p*=0.2008)	0.65±0.320 (*p*=0.3726)	0.59±0.662 (*p*=0.3942)	**0.75±0.040 (*p*=0.0263)**	0.65±0.068 (*p*=0.0699)	1.18±0.233 (*p*=0.7109)
AtPDX1.3 OE, L2	1.15±0.125 (*p*=0.3082)	0.77±0.021 (*p*=0.2372)	**6.69±0.163 (*p*=0.0003)**	0.90±0.176 (*p*=0.4345)	0.39±0.190 (*p*=0.0658)	0.55±0.187 (*p*=0.3649)
AtPDX1.3 OE, L3	**1.40±0.048 (*p*=0.0275)**	0.66±0.103 (*p*=0.1877)	**8.92±0.201 (*p*=0.0002)**	**0.72±0.004 (*p*=0.0049)**	0.53±0.184 (*p*=0.1018)	0.74±0.221 (*p*=0.5809)
AtPDX1.3 OE, L4	**1.15±0.024 (*p*=0.0147)**	0.73±0.040 (*p*=0.1943)	**4.42±0.013 (*p*=0)**	**0.65±0.079 (*p*=0.0256)**	**0.44±0.070 (*p*=0.0297)**	0.90±0.054 (*p*=0.8033)
AtPDX1.3 OE, L5	1.01±0.008 (*p*=0.8086)	1.00±0.136 (*p*=0.9964)	**8.83±0.085 (*p*=0)**	1.14±0.089 (*p*=0.1582)	1.28±0.131 (*p*=0.1807)	1.14±0.031 (*p*=0.7088)
AtPDX2 OE, L1	0.75±0.161 (*p*=0.0919)	1.31±0.094 (*p*=0.2515)	0.89±0.049 (*p*=0.533)	**7.20±0.003 (*p*=0)**	2.13±0.151 (*p*=0.0576)	1.14±0.293 (*p*=0.5424)
AtPDX2 OE, L2	1.01±0.044 (*p*=0.874)	1.09±0.037 (*p*=0.6382)	1.32±0.662 (*p*=0.5529)	**9.92±0.051 (*p*=0)**	1.19±0.341 (*p*=0.5658)	1.38±0.633 (*p*=0.4148)
AtPDX2 OE, L3	1.07±0.048 (*p*=0.8213)	1.13±0.113 (*p*=0.5948)	1.04±0.095 (*p*=0.8498)	**8.65±0.014 (*p*=0)**	1.34±0.154 (*p*=0.3526)	0.38±0.211 (*p*=0.0557)
AtPDX2 OE, L4	0.91±0.101 (*p*=0.4739)	1.13±0.198 (*p*=0.6766)	0.72±0.393 (*p*=0.4999)	**110.35±0.098 (*p*=0)**	0.85±0.447 (*p*=0.7837)	**0.06±0.063 (*p*=0.013)**
AtPDX2 OE, L5	**0.77±0.008 (*p*=0.0349)**	1.42±0.128 (*p*=0.1891)	0.92±0.111 (*p*=0.6237)	**10.95±0.005 (*p*=0)**	1.42±0.178 (*p*=0.2948)	1.45±0.732 (*p*=0.4082)
AtPDX1.1-2 OE, L1	**24.10±0.008 (*p*=0)**	1.00±0.016 (*p*=0.9884)	1.02±0.16 (*p*=0.9112)	**113.19±0.001 (*p*=0)**	0.66±0.454 (*p*=0.3885)	1.68±0.634 (*p*=0.2213)
AtPDX1.1-2 OE, L2	**18.95±0.068 (*p*=0)**	**0.71±0.037 (*p*=0.0079)**	1.13±0.004 (*p*=0.0752)	**92.81±0.141 (*p*=0)**	0.55±0.382 (*p*=0.2528)	0.62±0.204 (*p*=0.162)

Supplementary table 3.1: Relative transcript levels of the genes related to vitamin B_6 biosynthesis in the PDX over-expressing lines as given in figure 3.1 and statistical analysis of the results. The significance of the results (related to WT) was confirmed (significant changes are indicated in bold) with a double-sided Student's T-test for $P < 0.05$. The calculated *p*-values are given in brackets.

A

Seedlings	PN	PM	PNP	PMP	4-PA	4-dPN	PL	PLP
AtPDX1.1 OE	***p*=0.0326**	*p*=0.0521	*p*=0.6952	*p*=0.9031	*p*=0.6302	*p*=0.1826	*p*=0.8043	***p*=0.0064**
AtPDX2 OE	*p*=0.5452	*p*=0.6062	*p*=0.6122	*p*=0.9683	*p*=0.709	*p*=0.7223	*p*=0.6527	*p*=0.4705
AtPDX1.1-2 OE	***p*=0.0945**	***p*=0.0024**	*p*=0.209	*p*=0.5045	*p*=0.2249	*p*=0.1271	*p*=0.0711	***p*=0.0285**

B

Seeds	PN	PM	PNP	PMP	4-PA	4-dPN	PL + PLP
AtPDX1.1 OE	***p*=0.0437**	*p*=0.6963	*p*=0.7123	*p*=0.3884	*p*=0.3621	*p*=0.2255	*p*=0.1885
AtPDX2 OE	*p*=0.8733	*p*=0.8512	*p*=0.6296	*p*=0.7442	*p*=0.9036	*p*=0.8628	*p*=0.8711
AtPDX1.1-2 OE	***p*=0.0393**	*p*=0.372	*p*=0.5572	*p*=0.0817	*p*=0.5378	*p*=0.4448	*p*=0.0525

Supplementary table 3.2: Statistical analysis of the changes in different B_6 vitamers in the PDX over-expressing lines compared to WT (see table 3.1) in seedlings ***(A)*** and seed ***(B)***, respectively. The significance of the results was confirmed (significant changes are indicated in bold) with a double-sided Student's T-test for $P < 0.05$. Under the conditions used, PL and PLP could not be distinguished in seed extracts. Abbreviations used: n.d.: not distinguishable; n.q.: not quantified.

Line	Leaf number	*p*-value for $P < 0.05$
WT	26 ± 2.82	-
AtPDX1.1 OE, L2	25 ± 2.00	0.430
AtPDX1.1-2OE, L2	18 ± 5.84	0.006

Supplementary table 3.3: Quantification of the rosette leaf number before bolting in at least eight plants per line. A statistical analysis (Student's T-test) was determined for $P < 0.05$ and related to WT, the *p*-values are given.

3.7 Acknowledgements

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4 Regulation of vitamin B_6 biosynthesis under abiotic stress – a comparison of *Arabidopsis thaliana* lines modified in *PDX* genes

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Dr. Marina Tambasco-Studart, ETH Zurich (construction and transformation of the pCAMBIA1302-*AtPDX1.2* construct into wild-type (WT, ecotype Col-0, designated AtPDX1.2 OE), and in the *flu*-mutant background (ecotype Col-0, designated At*flu* PDX1.2 OE)). Dr. Rasa Meskauskiene, Dr. Christophe Laloi and Prof. Klaus Apel, ETH Zurich (providing both conditions and equipment for the high-light – cold and high-light treatment. Kind gift of seeds of the *A. thaliana flu* knockout line (designated At*flu* KO)). Edgar Herren (construction of the pCAMBIA 2301-*AtPDX1.2-RNAi* and pCAMBIA1302-*AtPDX1.2-RNAi* constructs).

4.2 Abstract

In *Arabidopsis thaliana*, *de novo* vitamin B_6 biosynthesis involves the two proteins PDX1 and PDX2. There are two functional homologs of PDX1, PDX1.1 (At2g38230) and PDX1.3 (At5g01410), which, in a complex with PDX2 (At5g60540), directly form pyridoxal 5'-phosphate, the active cofactor form of vitamin B_6. In this report, we concentrate on the regulation of vitamin B_6 biosynthesis by analyzing transcript as well as protein abundance corresponding to genes involved in both *de novo* and salvage pathways. The quantification of total vitamin B_6 levels upon stress treatments suggests that their regulation is not only restricted to the transcriptional and/or translational level, but may include post-translational modifications as well. We provide evidence that PDX1.1 and PDX1.3 are indeed distinguishable in their response to various abiotic stress situations, thus acting in distinct mode(s). Further evidence for these findings comes from the *pdx1.1* and *pdx1.3* knockout lines of *A. thaliana*, as well as from attempts to over-express both PDX1 proteins in *A. thaliana*. The latter approach revealed a strict regulation of PDX1.3 at the translational or post-translational level. Apart from paraquat treatment, the differences in the vitamin B_6 content under conditions of oxidative stress are minor, indicating an efficient regulation. Although definitive proof for vitamin B_6 acting as a quencher of reactive oxygen species is lacking, the results emphasize that this indeed might be the case and is most pronounced in vitamin B_6 over-accumulating lines.

AtPDX1.2 (At3g16050), the third PDX1 homolog in *A. thaliana*, is not actively involved in vitamin B_6 catalysis, but is rather suggested to have a regulatory function.

4.3 Introduction

The versatility of vitamin B_6 in its active cofactor forms pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP) to act in enzymatic reactions has been known for a long time. The majority of enzymes which depend on its chemical properties are related to the intra- and extracellular utilization and transformation of amino acids, but are also involved in carbohydrate and lipid metabolism [Drewke and Leistner, 2001]. PLP is a most versatile cofactor involved in reactions like transamination, decarboxylation and racemization [Drewke and Leistner, 2001; Percudani and Peracchi, 2003]. It is required by all organisms for metabolic homeostasis. Recently, two mutually exclusive pathways of *de novo* vitamin B_6 biosynthesis have been elucidated. *E. coli* and other members of the γ-division of proteobacteria possess the PdxA/PdxJ route, whereas archaea, the majority of eubacteria, fungi as well as plants depend on the activity of the Pdx1/Pdx2 proteins (supplementary scheme 4.1) [Ehrenshaft *et al.*, 1999; Osmani *et al.*, 1999; Mittenhuber, 2001; Tanaka *et al.*, 2005]. In *E. coli*, pyridoxine 5'-phosphate (PNP) is synthesized from the precursors 4-phosphohydroxy-L-threonine (4PHT) and 1-deoxy-D-xylulose 5-phosphate (DXP) through the activity of the two proteins PdxA and PdxJ [Drewke *et al.*, 1996; Laber *et al.*, 1999; Cane *et al.*, 1999]. Then, PNP is converted to the active cofactor form PLP, by PdxH, a FMN-containing PNP/pyridoxamine 5'-phosphate (PMP) oxidase [Lam and Winkler, 1992]. The second pathway, completely novel and widely distributed among four kingdoms of life, has since been unravelled [Osmani *et al.*, 1999; Burns *et al.*, 2005; Raschle *et al.*, 2005/2007; Tambasco-Studart *et al.*, 2005]. This pathway is made up of only the two proteins Pdx1 and Pdx2, which act in a complex and are dependent on each other *in vivo* [Ehrenshaft and Daub, 2001]. Pdx1 is a dodecameric entity arranged in two hexameric rings to which 12 individual Pdx2 subunits are attached [Strohmeier *et al.*, 2006]. The assembled complex exhibits glutamine amidotransferase activity in which Pdx2 acts as the glutaminase and Pdx1 as the synthase domain [Burns *et al.*, 2005; Raschle *et al.*, 2005/2007, Strohmeier *et al.*, 2006]. In a complicated reaction series, ribose 5-phosphate, glyceraldehyde 3-phosphate and glutamine are converted to the active cofactor form PLP [Burns *et al.*, 2005; Raschle *et al.*, 2005/2007; Tambasco-Studart *et al.*, 2005]. Unlike *de novo* biosynthesis, a salvage pathway which interconverts the different forms of vitamin B_6, *i.e.*

pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their phosphorylated derivatives PNP, PLP and PMP, respectively, is present in all organisms that, upon vitamer uptake, can maintain vitamin B_6 homeostasis (supplementary scheme 4.1) [Di Salvo *et al.*, 2004]. In particular, a PN/PL/PM-kinase, PdxK, converts the non-phosphorylated forms to their phosphorylated analogs PNP/PLP/PMP. In *E. coli* PdxY, a specific PL-kinase, has been identified in addition to PdxK [Yang *et al.*, 1998]. The conversion of both PNP and PMP to PLP is catalyzed by a PNP/PMP-oxidase, PdxH [Lam and Winkler, 1992]. It is worth mentioning that the oxidation of PMP can alternatively be accomplished by PLP-dependent transaminases [Mittenhuber, 2001]. Unspecific phosphatases maintain the pool of the free vitamers [McCormick and Chen, 1999]. In addition, a PL reductase (PN-4-dehydrogenase, PN-4DH) that reduces PL to PN has been related to the vitamin B_6 salvage pathway in yeast [Guirard and Snell, 1988; Nakano *et al.*, 1999]. Among all enzymes involved in the salvage pathway, PdxH has been assigned a regulatory role in *E. coli* and mammals, as this enzyme is strongly and competitively inhibited by its catalytic product PLP [Choi *et al.*, 1987; Zhao and Winkler, 1995].

In *Arabidopsis thaliana*, three homologs of Pdx1, referred to as PDX1.1 (At2g38230), PDX1.2 (At3g16050), PDX1.3 (At5g01410), and one homolog of PDX2 (At5g60540) exist. Only PDX1.1, PDX1.3 and PDX2 have been demonstrated to be involved in *de novo* biosynthesis of vitamin B_6 [Tambasco-Studart *et al.*, 2005]. The third PDX1 homolog, PDX1.2, appears to be divergent from the other two. Apart from a different expression pattern [Titiz *et al.*, 2006; Tambasco-Studart *et al.*, 2007] and abundance [Tambasco-Studart *et al.*, 2007; Denslow *et al.*, 2007], it slightly differs in the amino acid sequence as well. Interestingly, it is predominantly the catalytic active residues that are absent in PDX1.2, but present in PDX1.1 and PDX1.3 [Tambasco-Studart *et al.*, 2005/2007]. In addition, a partial copy of PDX1.1 (At2g38210), which is identical to the first 240 nucleotides, is located 4kb upstream from *PDX1.1* itself. Its function is unclear to date [Denslow *et al.*, 2007]. Thus far, two salvage pathway enzymes, namely SOS4, exhibiting PN/PL/PM-kinase activity [Shi and Zhu, 2002], and PDX3, a PNP/PMP-oxidase [Sang *et al.*, 2007], have been identified in Arabidopsis.

An unprecedented function of vitamin B_6 as an antioxidant with a potential to quench reactive oxygen species (ROS) equivalent to that of the vitamins C and E, was uncovered recently in fungi [Ehrenshaft *et al.*, 1999; Bilski *et al.*, 2000; Chumnantana *et al.*, 2005]. From a medical point of view, it has been demonstrated that pharmaceutical doses of pyridoxamine, *e.g.* 50 mg/day can assist in treating complicated forms of diabetes [Williams *et al.*, 2007]. A daily dose of 0.5 mg/day can control epileptic seizures [Grillo *et al.*, 2001]. This is particularly in-

teresting as both release and accumulation of ROS is a frequent phenomenon concomitant with these and other diseases. Furthermore, knockout studies in *Saccharomyces cerevisiae* revealed that SNZ1 and SNO1, the respective Pdx1 and Pdx2 homolog, increase at both transcript and protein levels upon entry into the stationary phase which is characterized by a burst of ROS [Braun *et al.*, 1996; Padilla *et al.*, 1998]. In addition, proteins involved in PLP-homeostasis were up-regulated at the transcript level upon H_2O_2 treatment in *Schizosaccharomyces pombe* [Chen *et al.*, 2003]. Recent knockout studies, predominantly performed in the model plant *A. thaliana*, gave evidence that the vitamin may indeed alleviate various forms of stress, in particular oxidative, osmotic and ionic treatments [Chen and Xiong, 2005; Titiz *et al.*, 2006]. A knockout of either *PDX1.1* or *PDX1.3* reduces the level of free vitamin B_6 not significantly or by *ca*. 20%, respectively. This is even more pronounced in plants harbouring only a single copy of either *PDX1.1* or *PDX1.3*. The complete knockout of both genes leads to embryo lethality [Titiz *et al.*, 2006]. On the other hand and somewhat unexpectedly, a mutation in the *SOS4* gene significantly increases the total B_6 content with PLP being the highest contributor to the overall increase, whereas a knockdown of PDX3, the second confirmed salvage pathway enzyme in *A. thaliana*, does not show any major effect on the total B_6 content and on the distribution of the different vitamers [González *et al.*, 2007]. It has been demonstrated that an *A. thaliana PDX1.3* knockout mutant, but not the respective *PDX1.1* knockout line, is sensitive to elevated NaCl concentrations [Chen and Xiong, 2005; Titiz *et al.*, 2006]. However, a mutant carrying a single point mutation in *PDX1.3* (G162A), leading to the amino acid exchange G54S, does not resemble the above-mentioned salt sensitive phenotype of At*pdx1.3* KO [Wagner *et al.*, 2006]. In addition, UV-treatment, affects the *PDX1.3* mutant line more severely than WT, which is evident from an increased membrane lipid peroxidation [Chen and Xiong, 2005]. High-light treatment led to an increased rate of photoinhibition in case of the At*pdx1.3* KO, whereas At*pdx1.1* KO is only slightly affected in the photosystem II efficiency [Titiz *et al.*, 2006]. Plants lacking *SOS4* are hypersensitive to salt as well [Chen and Xiong, 2005; González *et al.*, 2007] and, although they are strongly affected by sucrose, they appear to be less susceptible to osmotic stress induced by mannitol [González *et al.*, 2007]. All these mutants are altered in their vitamin B_6 composition, and because of their altered responses to abiotic stress a function of this vitamin beyond that of a co-factor in plant systems has been implied.

So far, the regulation of *de novo* vitamin B_6 biosynthesis has been addressed at the transcriptional level for both biotic and abiotic stresses, but overall these studies revealed only minor

modifications in the expression of the *PDX* genes. In *A. thaliana*, *PDX1.1*, *PDX1.3* and *PDX2* are up-regulated upon high-light treatment, drought and chilling stress [Denslow *et al.*, 2007]. Unfortunately, NaCl treatment was focused on *PDX1.3* only, which was down-regulated under these conditions [Chen and Xiong, 2005]. In contrast, ozone, UV-B and paraquat did not significantly change the *PDX1.1*, *PDX1.3* and *PDX2* transcript levels [Denslow *et al.*, 2007]. In *Phaseolus vulgaris*, both H_2O_2 and rose bengal treatment or wounding did not significantly affect the transcript levels of *PDX1* [Graham *et al.*, 2004]. While H_2O_2 and rose bengal were reported to cause an increase in ethylene production in *P. vulgaris*, without stimulating *PDX1* transcript levels, treatment with 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, led to a transient enrichment of *PDX1* transcripts [Graham *et al.*, 2004]. A similar finding has been reported for *Hevea brasiliensis* (rubber tree) in which a direct treatment with ethephon, a growth regulator that is metabolized to ethylene (Rhône-Poulenc; http://extoxnet.orst.edu/pips/ethephon.htm), indeed induced HEVER, a *PDX1* homolog [Sivasubramaniam *et al.*, 1995]. In summary, the majority of these reports provide strong evidence that vitamin B_6 plays an essential role in the plant's tolerance against salt, osmotic and oxidative stress, which are often accompanied by the release of ROS. This report presents initial results of experiments designed to reveal the regulation of *de novo* vitamin B_6 biosynthesis in *A. thaliana* based on the actual vitamin B_6 content, furthermore on transcript analysis of both *de novo* and known salvage pathway enzymes as well as the protein abundance of enzymes involved in *de novo* vitamin B_6 biosynthesis during various forms of abiotic stress. Consistent with recent knockout studies in *A. thaliana* [Chen and Xiong, 2005; Titiz *et al.*, 2006; Wagner *et al.*, 2006], it is concluded that PDX1.1 and PDX1.3 are not redundant. While they share a primary function in *de novo* vitamin B_6 biosynthesis and are both predicted to localize to the cytosol in *A. thaliana* [Tambasco-Studart *et al.*, 2005], they respond differentially at the transcript level upon various stress treatments, at least in seedlings. The data further suggest that the regulation of *de novo* vitamin B_6 biosynthesis cannot exclusively be related to changes in transcript or protein abundance of *de novo* biosynthetic genes, but may in addition be triggered by activation/inactivation of enzymes at the post-translational level. Furthermore, the present report delves deeper into the role of PDX1.2 by examining its response under the above-mentioned conditions.

4.4 Materials and methods

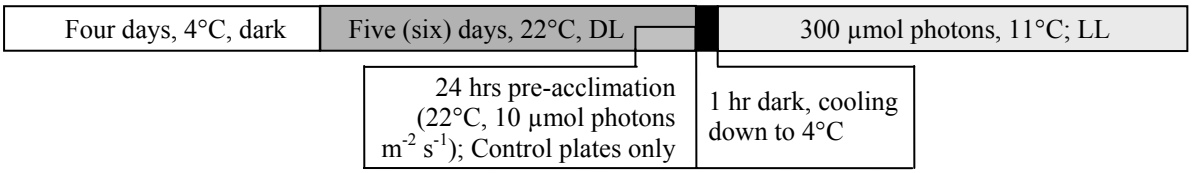
4.4.1 Generation of recombinant *Arabidopsis thaliana* lines

The establishment of lines over-expressing (OE) PDX1.1 (AtPDX1.1 OE, At*flu* PDX1.1 OE), PDX1.3 (AtPDX1.3 OE, At*flu* PDX1.3 OE), PDX2 (AtPDX2 OE, At*flu* PDX2 OE) alone or both PDX1.1 and PDX2 (AtPDX1.1-2 OE, At*flu* PDX1.1-2 OE) in both *A. thaliana* wild-type (WT) and the At*flu* knockout (KO) [Meskauskiene *et al.*, 2001] background, respectively, have been described in chapter 3. Lines over-expressing AtPDX1.2 in both the WT and At*flu* KO background were established by Dr. M. Tambasco-Studart ([Tambasco-Studart, 2007]; the protocol can be found in chapter 4.8.1). In supplementary table 4.1, the respective antibiotic and additional screening procedures used for the different transgenic lines are described.

4.4.2 Growth conditions and stress treatments

Plants, unless specified further, were routinely cultivated under 100 µmol photons $m^{-2}s^{-1}$ under long-day conditions except lines carrying a mutation in the *FLU* gene, which were cultivated under continuous light at the same light intensity in sterile culture and at a light intensity of 120-150 µmol photons m^{-2} s^{-1} in soil, respectively. Stress experiments were performed under sterile conditions with homozygous plants on MS medium [Murashige and Skoog, 1962] lacking PN, supplied with 1% (w/v) sucrose, as indicated, and/or 25-50 µM rose bengal, 0.5-1 µM paraquat, 1 mM H_2O_2, 100 mM NaCl or 3.3% (w/v) sucrose. Germination experiments were performed with surface sterilized seeds, which were directly spread on MS medium lacking or containing one of the above-mentioned compounds at the given concentration. Stress-induction experiments were initiated with seven-day old seedlings cultivated on MS medium lacking pyridoxine that were subsequently transferred to MS medium containing one of the stress-inducing compounds mentioned above and cultivated for another one or ten days, respectively, under long-day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1}. Five-day old sterile-cultivated seedlings were subjected to high-light – cold treatment, *i.e.* a combination of elevated light intensity (300 µmol photons m^{-2} s^{-1}) and low temperature (11°C). Seeds were sown on MS medium without pyridoxine and supplemented with 1% (w/v) sucrose as indicated, and prior to treatment grown under long-day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1} at 22°C. After five days, control plates were pre-acclimated for 24 hrs under low-light intensities (10 µmol photons $m^{-2}s^{-1}$, 22°C; these plates are indicated as "*pre*"; pre-acclimated plants exhibit an increased tolerance against the subse-

quent stress conditions). All plates, with or without pre-treatment, were subsequently subjected to a 24 hrs dark period in which the temperature was reduced to 11°C, followed by a four-day continuous high-light (300 µmol photons m^{-2} s^{-1} (designated LL)) – cold period (scheme 4.1). [The conditions were optimized and the experiment was conducted by Dr. Rasa Meskauskiene, unpublished]. The experiments with the *A. thaliana flu* mutant lines were conducted as described in chapter 3.



Scheme 4.1: Experimental setup of the high-light – cold treatment. See text for details.

4.4.3 Isolation of RNA, RT-PCR and immunochemical analyses

The isolation of RNA, reverse transcription into cDNA and quantitative real-time RT-PCR as well as Western blot analysis was performed as described by Titiz *et al.* [2006] and Tambasco-Studart *et al.* [2007] employing a slightly modified protein extraction buffer (see chapter 3) and specific antibodies for the detection of PDX1.1 and PDX1.3. These were raised in rabbit against the peptides GEGAMTETKQKSP (PDX1.1) and CEGNGAITEAKKSP (PDX1.3), respectively (Eurogentec, Seraing, Belgium) and used in a 1:1,000 dilution.

4.4.4 Pigment analysis

The method for the quantification of anthocyanins was adapted from Lange *et al.* [1971]. In brief, 200-500 mg leaf material was harvested and transferred to 1 ml of extraction buffer (H_2O:1-propanol:HCl (81:18:1 (v/v)) in the dark. After boiling for 1.5 min, extraction was continued in the dark for 24 hrs at room temperature with slight shaking. The absorbance of the supernatant obtained after centrifugation at 16.000 g was measured at OD_{535} and OD_{650}. The anthocyanin content was calculated according to Rayleigh's formula $OD_{535\ (corr.)} = OD_{535} - 2.2*OD_{650}$ as given in Lange *et al.* [1971] and related to the extracted leaf fresh weight.

4.4.5 Vitamin B_6 determination

The quantification of free, phosphorylated, glycosylated as well as total vitamin B_6 content was performed directly or after treatment with acid phosphatase and/or β-glucosidase, respectively, as described in chapter 3, employing the *Saccharomyces carlsbergensis* strain

(ATCC9080) auxotrophic for vitamin B_6 as described [Tambasco-Studart *et al.*, 2005; Titiz *et al.,* 2006].

4.4.6 Cell death measurements

Electrolyte leakage experiments were performed as described recently [Laloi *et al.*, 2007]. The extent of cell death was also assessed by staining with Evan's Blue solution [Wright *et al.*, 2000; Ochsenbein *et al.*, 2006]. In brief, leaves were cut at the petiole to minimize tissue injury and incubated in 4 ml 0.1% (w/v) Evan's blue for 24 hrs at room temperature with slight shaking. Leaves were washed with 0.05% (w/v) saline until the washing solution was colourless. The incorporated dye was extracted from leaf material by incubating the leaves in 50% (v/v) methanol containing 1% (w/v) SDS. The staining was quantified against extraction buffer by measuring the OD at 600 nm and related to leaf fresh weight.

4.5 Results

4.5.1 Indications for distinct roles of PDX1.1 and PDX1.3 in *A. thaliana* – an experimentally-based comparison

So far, there is one report in the literature that attempted to over-produce vitamin B_6 in plant systems [Herrero and Daub, 2007]. In this study, the genes coding for PDX1 and PDX2 from the phytopathogenic fungus *Cercospora nicotianae* were transfected into *Nicotiana tabacum*. Accumulation of the *CnPDX1* transcript was at the expense of the endogenous *NtPDX1*, accompanied by a 1.2-fold accumulation of the vitamin above wild-type (WT) [Herrero and Daub, 2007]. However, in our experiments *A. thaliana* transformed with the endogenous *PDX1.1*, either alone or in combination with the endogenous *PDX2*, *PDX1.1*/*PDX2* transcripts as well as the respective proteins over-accumulated. This was accompanied by a significant increase in the vitamin B_6 content in several lines (see chapter 3). When using the same approach for PDX1.3, the transcript level of *PDX1.3* could be raised up to nine-fold above WT. But, no significant accumulation of the PDX1.3 protein (figure 4.1*A*, *B*) or vitamin B_6 above WT could be observed (figure 4.1*C*). Moreover, the different pools of vitamin B_6 in the latter lines, *i.e.* free (PN, PL, (PM), phosphorylated (PNP, PLP, PMP) as well as the glycosylated PN derivative pyridoxine 5'-β-D-glucoside (PNG), in both green tissue and seeds, were not significantly different from WT. This is in contrast to the *PDX1.1*-transgenic line AtPDX1.1 OE (see chapter 3; figure 4.1*D*, *E*).

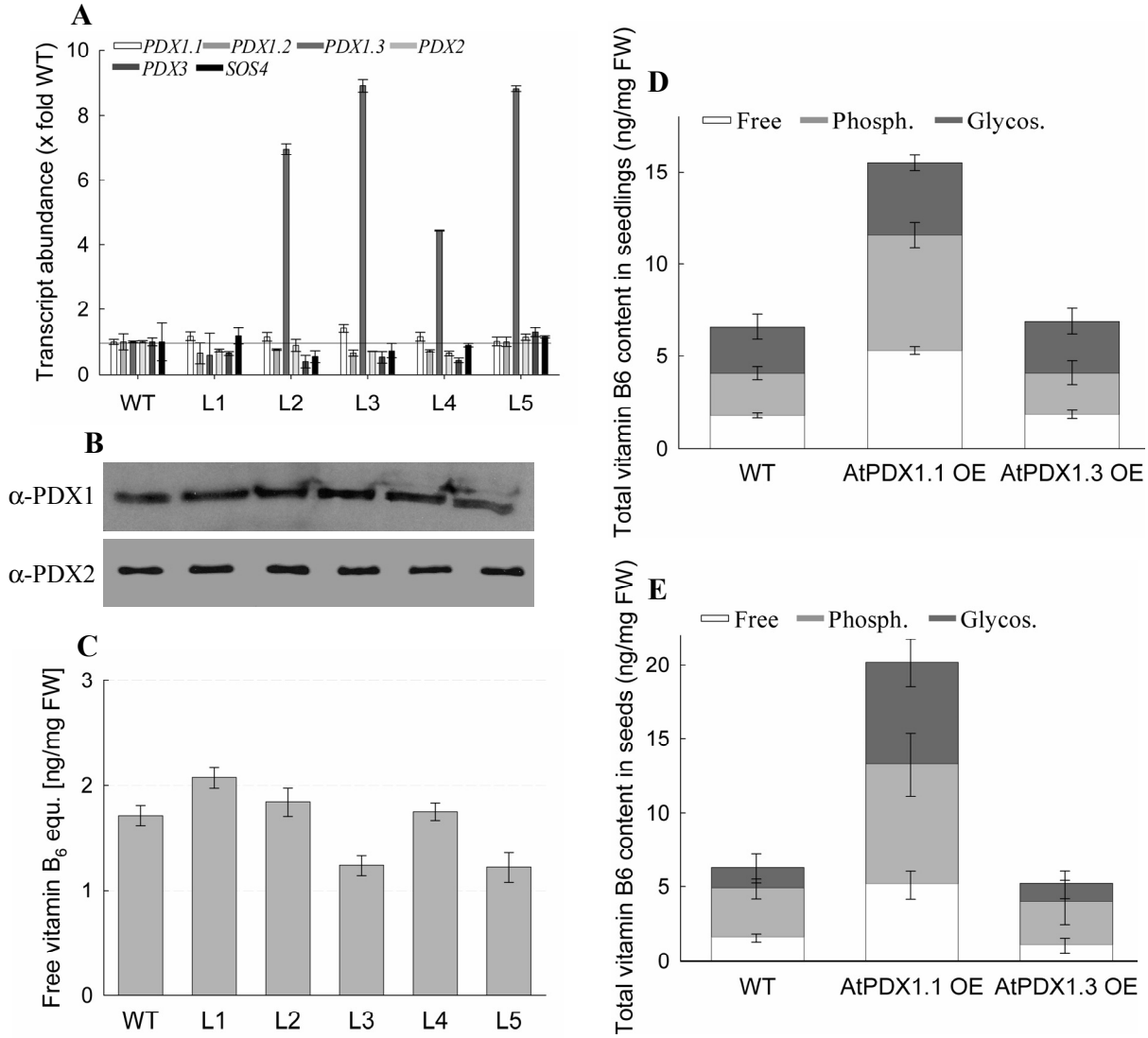


Figure 4.1: Comparative analysis of *PDX1.3*-transgenic lines. ***(A)*** Quantitative RT-PCR analysis of WT and five independent lines (L1-L5) of T4 homozygous seedlings carrying a single copy of the gene cassette. The relative transcript level of *PDX1.1* (white), *PDX1.2* (light grey), *PDX1.3* (medium grey), *PDX2* (light blue grey), *PDX3* (dark blue grey), *SOS4* (black) compared to WT (set to 1) in shoot material of ten-day old seedlings grown under a 16 hr photoperiod (100 µmol photons m^{-2} s^{-1}) on MS medium lacking PN. ***(B)*** Western blot analysis of total protein extracted from the same samples as in ***(A)*** using antibodies specific to either PDX1.3 or PDX2 as indicated. Total protein loaded per lane was 40 µg. ***(C)*** Free vitamin B_6 content from the same samples as in ***(A)***. ***(D***, ***E)*** Comparison of the free (white), phosphorylated (light grey) and glycosylated (dark grey) vitamin B_6 equivalents in WT, AtPDX1.1 OE (L2) and AtPDX1.3 OE (L2) seedlings cultivated as above ***(D)*** and in desiccated seeds from simultaneously cultivated plants under a 16 hr photoperiod (150 µmol photons $m^{-2}s^{-1}$) ***(E)***. Data given for ***(A*** to ***E)*** are the average of three biological replicates with at least two technical repeats.

4.5.2 Divergent responses to oxidative and osmotic stress

PDX1.1 and *PDX1.3* have a high level of sequence identity (89%) with an open-reading frame composed of a single exon. This feature is characteristic for genes encoding stress-related proteins to avoid the process of splicing, as this can be affected by stress leading to alternate splice variants. The proper processing of a basic pool of genes to the respective gene products is thought to be essential to maintain metabolic functionality under various conditions, including abiotic and biotic stress situations [Iida *et al.*, 2004]. Promoter studies revealed stress-responsive motifs some of which differ between PDX1.1 and PDX1.3 [Denslow, 2005; Titiz, 2008]. Under high-light, chilling and drought treatment *PDX1.1* and *PDX1.3* transcript abundances changed in parallel (table 4.1) suggesting a possible redundancy of the two [Denslow *et al.*, 2007]. However, a deletion of *PDX1.3* has more severe effects under normal as well as several conditions of stress such as oxidative, osmotic or salt stress [Shi *et al.*, 2002; Titiz *et al.*, 2006]. It was therefore suggested that PDX1.3 is more essential than PDX1.1, but the mode of regulation of the two genes remains to be explored.

Treatment	Time interval (days)	*PDX1.1*	*PDX1.2*	*PDX1.3*
Control	As below	Set to 1	Set to 1	Set to 1
High-light	1 / 4 / 7	4 / 0.9 / 2	1.5 / 0.5 / 1.2	3.3 / 0.9 / 1.9
Chilling	1 / 4 / 7	7.8 / 3.6 / 2	1.8 / 1.5 / 0.9	5.8 / 3 / 1
Drought	7 / 14	1.1 / 1.8	1.3 / 1.1	1.3 / 1.7
Ozone	1 / 2	0.5 / 0.7	3 / 2.5	1.2 / 0.9

Table 4.1: Determination of the transcript abundance (x-fold control) of *A. thaliana PDX1.1*, *PDX1.2* and *PDX1.3*, at the time-points indicated after stress treatments. Grey boxes indicate significant changes in the transcript abundance compared to the control [extracted from Denslow *et al.*, 2007].

To monitor the response of *A. thaliana* to oxidative, osmotic and salt stress in more detail, studies were performed with PDX over-expressing as well as PDX knockout lines. The response of different *A. thaliana* lines to the respective stress-inducing compounds was assessed in both seven-day old seedlings (figures 4.4, 4.5) and germinating seeds of WT and AtPDX1.1 OE L2 (figure 4.6), respectively. The focus was on phenotype observations, quantification of vitamin B_6 and the transcript as well as protein abundance related to the genes *PDX1.1*, *PDX1.2*, *PDX1.3* and *PDX2*. In the germination experiments, *PDX3* and *SOS4* were included as well. It is important to note that sucrose added to MS medium at a concentration of 1% (w/v) (as is usual in culture conditions) already induced a stress response, which in-

creased the tolerance to subsequent treatments (*i.e.* a pre-acclimation). This was first observed upon rose bengal (50 µM) treatment (figure 4.2*A*; table 4.2*A*) or high-light – cold stress treatment (see below). In contrast, sucrose was essential to induce a stress response in developing seedlings to paraquat (figure 4.2*B*; table 4.2*B*).

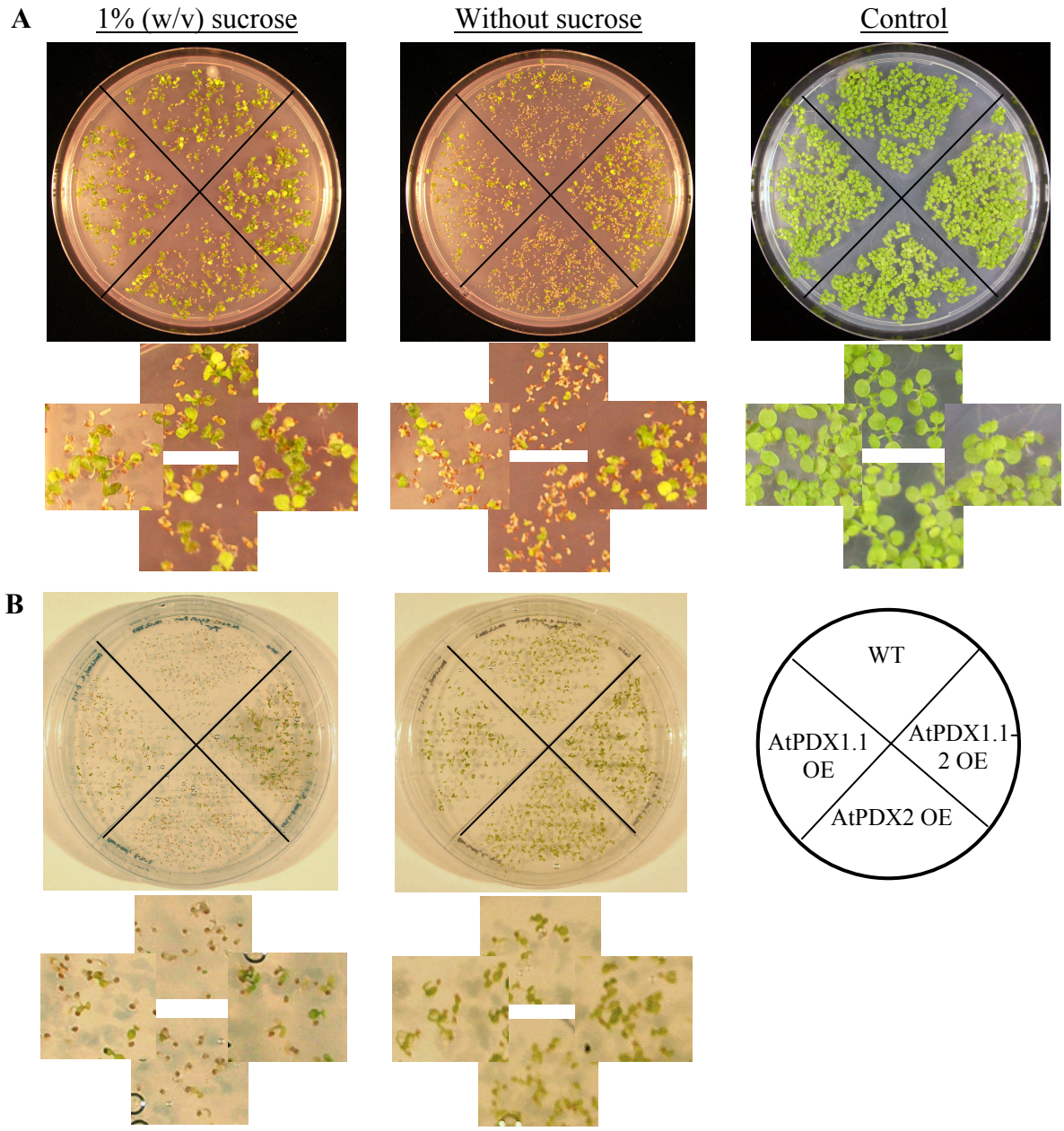


Figure 4.2: Effect of sucrose on stress responses. Seeds were sown on MS medium lacking PN containing sucrose (1% (w/v), left panel) or not (central panel), 50 µM rose bengal ***(A)*** or 1 µM paraquat ***(B)***. Plates were cultivated under long-day conditions (100 µmol photons m^{-2} s^{-1}) for ten ***(A)*** and six ***(B)*** days, respectively. The control is presented in the right panel under ***(A)*** and the distribution of the lines is given in ***(B)***. The lower panel in ***(A)*** and ***(B)*** are a magnified picture of the above. See table 4.2 for details.

A

Line (rose bengal treatment)	Seeds sown	Germinated seeds	Developing seedlings	Survival rate [%]
WT, Control*	95	95	94	98.9
WT, RB, w/o sucrose#	349	342	81	23.7
WT, RB, 1% (w/v) sucrose#	341	339	204	60.2
AtPDX1.1 OE, Control*	115	113	111	98.2
AtPDX1.1 OE, RB, w/o sucrose#	402	400	173	43.3
AtPDX1.1 OE, RB, 1% (w/v) sucrose#	347	344	191	55.2
AtPDX1.2 OE, Control*	83	81	81	100.0
AtPDX1.2 OE, RB, w/o sucrose#	499	499	98	19.6
AtPDX1.2 OE, RB, 1% (w/v) sucrose	n.d.	n.d.	n.d.	n.d.
AtPDX1.3 OE, Control*	125	123	120	97.6
AtPDX1.3 OE, RB, w/o sucrose#	389	380	78	20.5
AtPDX1.3 OE, RB, 1% (w/v) sucrose	n.d.	n.d.	n.d.	n.d.
At*pdx1.1* KO, Control*	124	117	116	99.1
At*pdx1.1* KO, RB, w/o sucrose#	243	236	43	18.2
At*pdx1.1* KO, RB, 1% (w/v) sucrose	n.d.	n.d.	n.d.	n.d.
At*pdx1.3* KO, Control*	117	113	109	96.5
At*pdx1.3* KO,RB, w/o sucrose*	153	150	41	27.3
At*pdx1.3* KO, RB, 1% (w/v) sucrose	n.d.	n.d.	n.d.	n.d.

B

Line (paraquat treatment)	Seeds sown	Germinated seeds	Developing seedlings	Survival rate [%]
WT, P, w/o sucrose*	133	133	96	72.2
WT, P, 1% (w/v) sucrose*	183	181	70	38.7
AtPDX1.1 OE, P, w/o sucrose*	90	90	83	92.2
AtPDX1.1 OE, P, 1% (w/v) sucrose*	170	170	145	85.3
AtPDX1.2 OE, P, w/o sucrose	n.d.	n.d.	n.d.	n.d.
AtPDX1.2 OE, P, 1% (w/v) sucrose*	82	81	29	35.8
AtPDX1.3 OE, P, w/o sucrose	n.d.	n.d.	n.d.	n.d.
AtPDX1.3 OE, P, 1% (w/v) sucrose*	92	92	24	26.1
At*pdx1.1* KO, P, w/o sucrose	n.d.	n.d.	n.d.	n.d.
At*pdx1.1* KO, P, 1% (w/v) sucrose*	129	128	76	59.4
At*pdx1.3* KO, P, w/o sucrose	n.d.	n.d.	n.d.	n.d.
At*pdx1.3* KO, P, 1% (w/v) sucrose*	148	146	43	29.5

Table 4.2: Germination rate of WT and *PDX* transgenic lines as indicated in the presence of 50 µM rose bengal (RB) ***(A)*** or 1 µM paraquat (P) ***(B)***, respectively. Seeds were sown on MS medium lacking pyridoxine, supplemented with or without (w/o) sucrose (1% (w/v)) as indicated. Plates were cultivated for ten days under long-day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1}. The developing seedlings were counted and related to the total number of germinated seeds. Grey boxes indicate significant differences to WT plants. Controls in ***(B)*** are the same as in ***(A)***. The data was acquired from one (*) or three (#) independent analyses. Abbreviation used: n.d.: not determined.

In contrast to the germination experiments mentioned above, the transfer of seedlings to plates containing either rose bengal (inducer of singlet oxygen), paraquat (inducer of superoxide), H_2O_2, high sucrose (inducer of osmotic stress) or NaCl (inducer of salt stress), did not reveal any difference in the phenotype whether sucrose was present or not (data not shown). Furthermore, the total vitamin B_6 contents one day after the transfer were not affected by sucrose supplementation (figure 4.3).

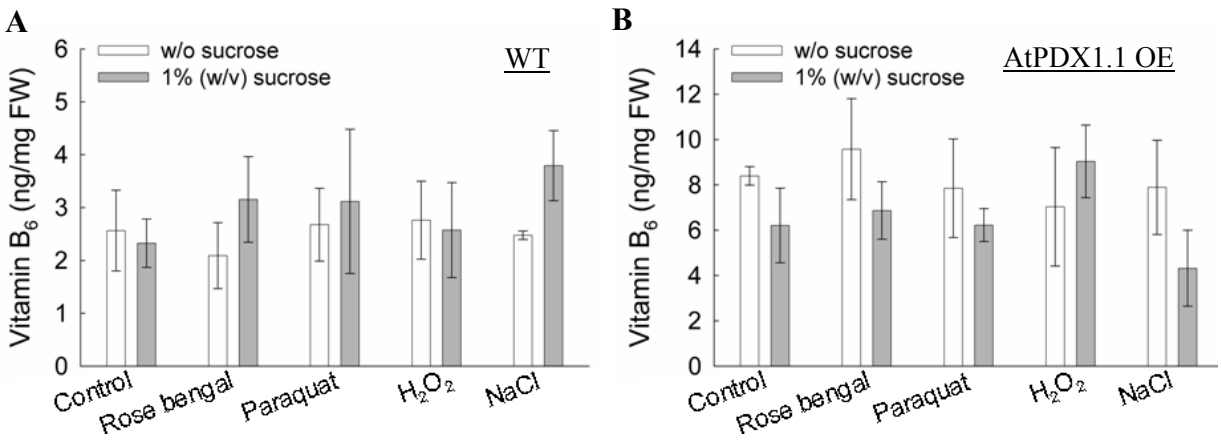


Figure 4.3: Effect of sucrose on the total vitamin B_6 content in seedlings under various stress treatments. Seven-day old seedlings cultivated on MS medium lacking PN and supplemented with (1% (w/v) or without sucrose, cultivated under long-day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1} were transferred to MS plates lacking PN containing 50 µM rose bengal, 1 µM paraquat, 1 mM H_2O_2 or 100 mM NaCl without (white bars) or with (1% (w/v); grey bars) sucrose. Seedlings were cultivated for another day before quantification of the total vitamin B_6 content in shoot material of WT ***(A)*** and AtPDX1.1 OE ***(B)***, respectively.

In a first instance, the effect was addressed by quantifying the vitamin B_6 content in seedlings after transferring them at an age of seven days to MS plates containing either stress-inducing compound. For that purpose, WT, AtPDX1.1 OE, AtPDX1.2 OE, AtPDX1.3 OE, At*pdx1.1* KO as well as At*pdx1.3* KO lines were cultivated for one and ten days, respectively, after the transfer (figure 4.4). A comparison of the different lines is possible as long they were cultivated on medium lacking sucrose. This, in particular, refers to the *PDX1.3* knockout mutant At*pdx1.3* KO, whose short-root phenotype, distinct in pyridoxine-deficient MS medium, is present upon sucrose supplementation only [noted by González *et al.*, 2007 and data not shown]. After one day, especially sucrose increased the vitamin B_6 content compared to the controls in several of the lines tested. The strongest effect was observed for At*pdx1.3* KO which showed a *ca.* three-fold increase (p=0.0208 for $P < 0.05$ in a double-sided Student's T-test; figure 4.4*F*). Interestingly, the increase in the At*pdx1.1* KO was less than in the At*pdx1.3*

KO indicating that PDX1.1 rather than PDX1.3 may respond to sucrose, which has been confirmed at the transcript level in WT seedlings (figure 4.5A). Thus, it is predominantly PDX1.1 that accounts for the increase in the vitamin B_6 content (see below). After ten days of sucrose treatment, the total vitamin B_6 content in WT seedlings was slightly increased (*ca.* 1.3-fold) above WT control seedlings, but this difference was, according to a double-sided Student's T-test for $P < 0.05$, not significant (p=0.3533) (figure 4.4*A*, grey bars). On the other hand, when seeds germinated on MS medium containing high sucrose, the total vitamin B_6 content was reduced by *ca.* 20% and 70% in WT and in AtPDX1.1 OE, respectively (figure 4.6*A*, *B*). Sucrose treatment reduced the free forms (PN, PL, PM) in WT by *ca.* 50% (p=0.0255) (figure 4.6*A*). In contrast, the pools of the free and phosphorylated B_6 vitamers as well as PNG were equally reduced by *ca*. 60% in AtPDX1.1 OE (figure 4.6*B*). Interestingly, *PDX1.1* transcripts were reduced in germinating WT seedlings, whereas *PDX1.2* transcripts were significantly up-regulated (figures 4.5*A*, 4.6*C*; see chapter 4.6.3).

Rose bengal and paraquat treatment yielded in moderate but statistically not significant changes (Student's T-test for $P < 0.05$: p=0.0667 (rose bengal) and p=0.0586 (paraquat)) in the total vitamin B_6 content of WT seedlings after cultivation for ten days reaching levels of *ca.* 1.4- and 1.6-fold, respectively, compared to the control. Notably, the effect of both compounds was most pronounced in WT seedlings followed by AtPDX1.3 OE, AtPDX1.2 OE and AtPDX1.1 OE, which were showing less of an enhancement in the overall vitamin B_6 levels after ten days of cultivation (figure 4.4 grey bars).

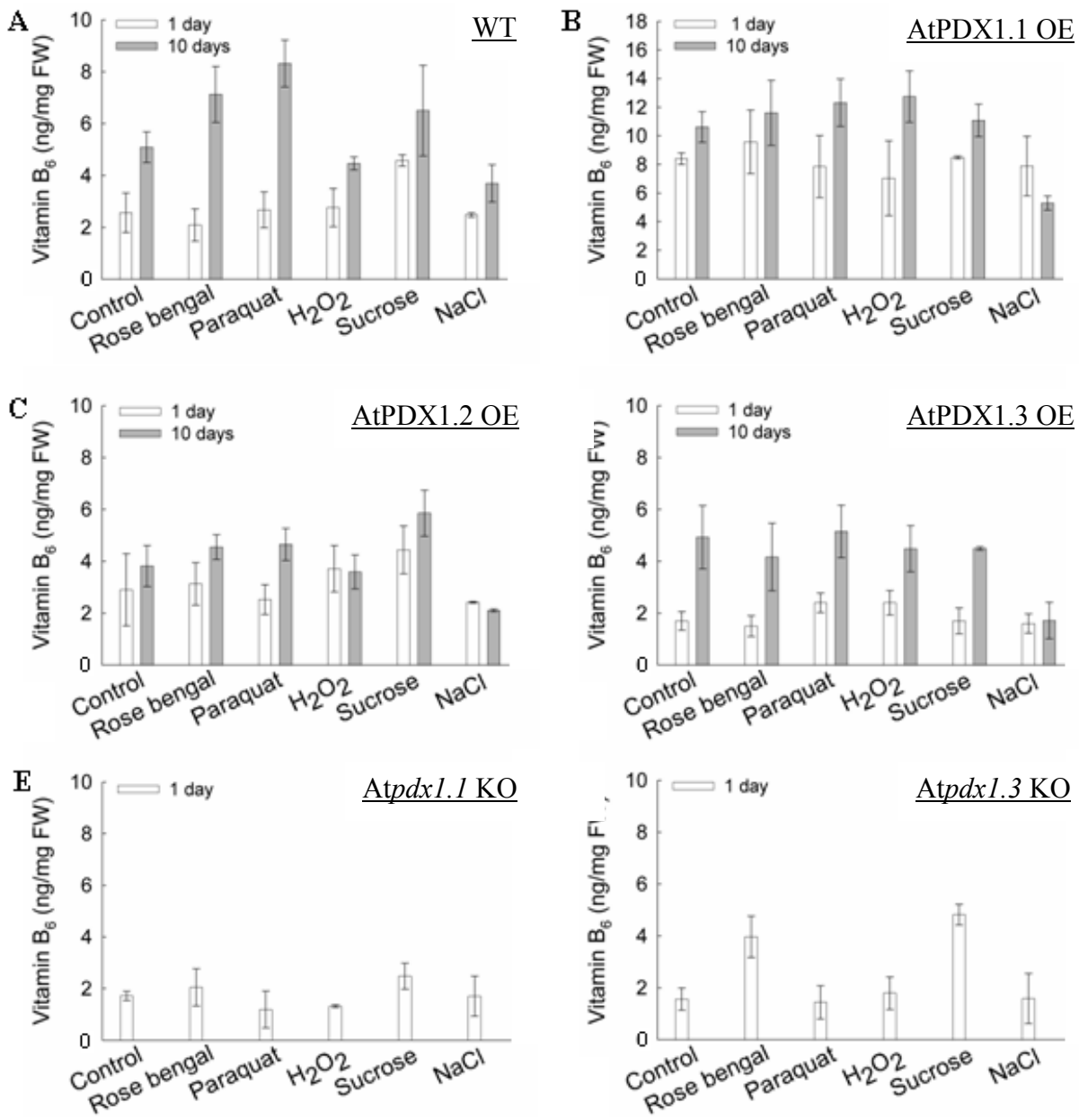


Figure 4.4: Effect of stress treatments on the vitamin B_6 content in seedlings. Seven-day old seedlings were transferred to MS plates lacking PN containing 25 µM rose bengal, 0.5 µM paraquat, 1 mM H_2O_2, 100 mM NaCl or 3.3% (w/v) sucrose. Seedlings were further cultivated for one (white bars) and ten days (grey bars), respectively. Cultivation in each case was under long-day conditions at a light intensity of 100 µmol photons m^{-2} s^{-1}. The total vitamin B_6 content was quantified in shoot material of WT ***(A)***, AtPDX1.1 OE ***(B)***, AtPDX1.2 OE ***(C)***, AtPDX1.3 OE ***(D)***, At*pdx1.1* KO ***(E)*** and At*pdx1.3* KO ***(F)***. The data presented are the average of three independent experiments with at least two technical repeats.

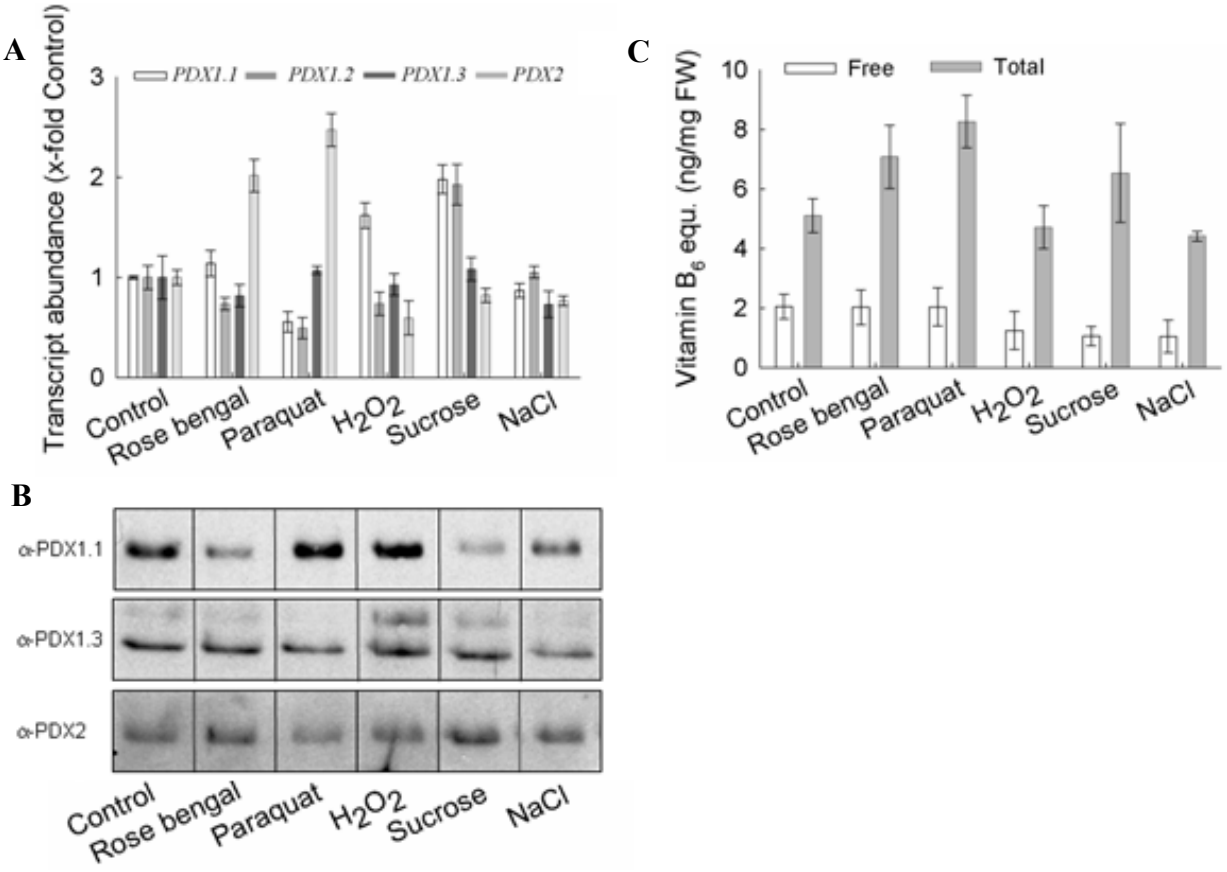
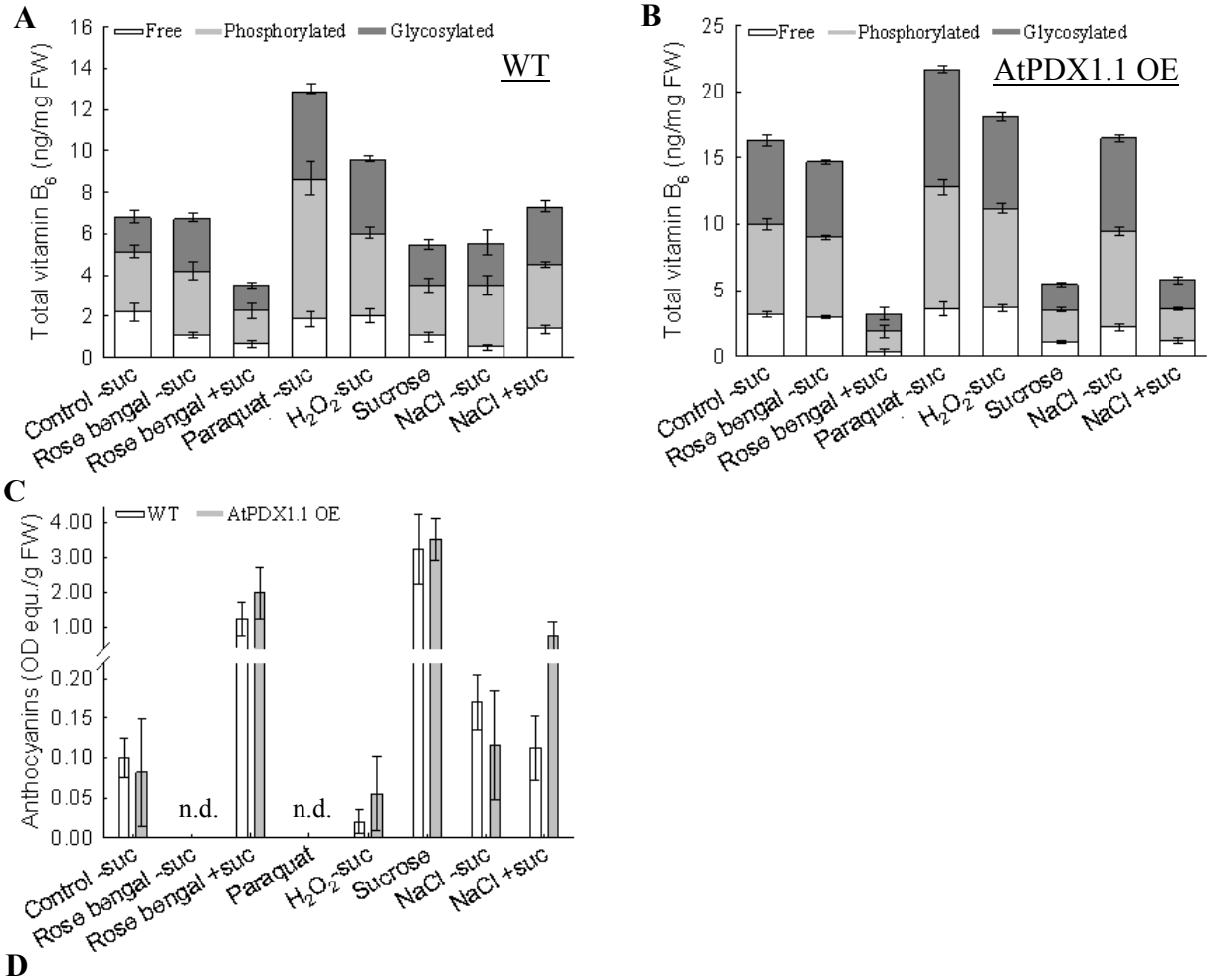


Figure 4.5: Analysis of transcript *(A)* and protein *(B)* abundance as well as vitamin B_6 content *(C)* in *A. thaliana* WT. Seven-day old seedlings cultivated on MS medium lacking pyridoxine under long-day conditions (100 µmol photons m^{-2} s^{-1}) were transferred to fresh MS plates lacking pyridoxine, containing either 25 µM rose bengal, 0.5 µM paraquat, 100 mM NaCl, 1 mM H_2O_2 or 3.3% (w/v) sucrose, and further cultivated for ten days. The transcript abundance was quantified by real time RT-PCR and related to control seedlings (set to 1) *(A)*. Protein extract (30 µg) was subjected to 11.5% SDS-PAGE prior to Western blot analysis using specific antibodies against PDX1.1 (upper panel), PDX1.3 (middle panel) and PDX2 (lower panel), respectively *(B)*. Vitamin B_6 was quantified directly (free) or after treatment with acid phosphatase and β-glucosidase (total) by the yeast assay *(C)*.



D

WT	*PDX1.1* (At2g38230)	*PDX1.2* (At3g16050)	*PDX1.3* (At5g01410)	*PDX2* (At5g60540)	*PDX3* (At5g49970)	*SOS4* (At5g37850)
Control	1.0 ± 0.03	1.0 ± 0.01	1.0 ± 0.12	1.0 ± 0.02	1.0 ± 0.45	1.0 ± 0.17
Rose bengal -suc	2.3 ± 0.01	1.1 ± 0.03	1.1 ± 0.04	0.7 ± 0.10	2.3 ± 0.51	1.6 ± 0.13
Rose bengal +suc	0.8 ± 0.02	2.6 ± 0.03	2.1 ± 0.03	1.9 ± 0.04	0.8 ± 0.22	0.4 ± 0.10
Paraquat	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
H_2O_2	1.1 ± 0.07	0.6 ± 0.03	0.7 ± 0.06	0.6 ± 0.03	0.6 ± 0.24	0.4 ± 0.10
Sucrose	0.4 ± 0.03	2.6 ± 0.01	1.4 ± 0.03	1.5 ± 0.18	0.9 ± 0.16	0.5 ± 0.14
NaCl -suc	1.0 ± 0.02	1.0 ± 0.06	1.3 ± 0.06	1.0 ± 0.03	0.8 ± 0.15	5.0 ± 0.38
NaCl +suc	0.7 ± 0.04	1.3 ± 0.02	1.0 ± 0.01	0.7 ± 0.03	0.8 ± 0.17	0.6 ± 0.15

Figure 4.6: Detailed analysis of *A. thaliana* Col-0 (WT) and AtPDX1.1 OE (see chapter 3) after germination on MS medium lacking PN, containing 50 µM rose bengal, 1 µM paraquat, 100 mM NaCl or 1 mM H_2O_2, with or without sucrose as indicated (1% (w/v)), and 3.3% (w/v) sucrose, respectively. Germinating seeds were cultivated for ten days under long-day conditions (120 µmol photons m^{-2} s^{-1}) and the vitamin B_6 content was determined in shoot material ***(A, B)***. ***(C)*** Anthocyanins (AC) were extracted from shoot material and quantified as described in materials and methods. ***(D)*** Transcript abundance was quantified by quantitative real-time RT-PCR and related to control plants (set at 1). Grey boxes indicate significant changes in the transcript abundance. Abbreviations used: n.a.: not analyzed; n.d.: not detectable.

In contrast to the transfer experiments mentioned above, the germination experiments using WT and AtPDX1.1 OE revealed variations in the vitamer distribution compared to the respective controls (figure 4.6A). Rose bengal predominantly reduced the free vitamer levels by *ca.* 50% (p=0.0576). The reduction was accompanied by slightly increased levels of the phosphorylated vitamers (*ca.* 1.1-fold; p=0.7159) as well as the glycosylated forms (*ca.* 1.5-fold; p=0.0783), thus, in the end, the overall vitamin B_6 content did not change. In contrast, when sucrose was added in addition to rose bengal, it strongly enhances the effect such that the vitamin B_6 content was reduced to *ca.* 46% and 20%, respectively (figure 4.6*A*, *B*). This was accompanied by a significant increase in the tolerance of WT against rose bengal (figure 4.2*A*, table 4.2*A*). The effect of sucrose was further manifested in the transcript abundance of the *PDX* and *SOS4* genes. In WT seedlings grown on sucrose-deficient MS medium containing rose bengal (50 µM) *PDX1.1*, *PDX3* and *SOS4* were up-regulated *ca.* 2.3-, 2.3- and 1.6-fold, respectively, while *PDX1.2*, *PDX1.3* and *PDX2* remained unchanged. In contrast, when sucrose was additionally applied, *PDX1.1*, *PDX3* and *SOS4* remained unchanged whereas *PDX1.2*, *PDX1.3* and *PDX2* increased in abundance *ca.* 2.6-, 2.1- and 1.9-fold, respectively, (figure 4.6*D*). Furthermore, treatment with paraquat, which acts as a terminal oxidant of photosystem I and results in the reduction of oxygen to the superoxide radical that in turn can be converted to hydrogen peroxide [Mehler, 1951], led to a reduction of the free vitamer level (*ca.* 0.8-fold; p=0.5251) accompanied by a significant increase in the phosphorylated (*ca.* 2.3-fold; p=0.0193) and in the glycosylated (*ca.* 2.6-fold; p=0.0436) forms, respectively.

In contrast to oxidative and osmotic stress, which tended to increase the total vitamin B_6 level, salt stress reduced the vitamin B_6 content in both seedlings and germinating seeds (figures 4.5*C*, 4.6*A*). Notably, in AtPDX1.3 OE seedlings, cultivated for ten days on NaCl-containing medium, this effect was most pronounced and appeared to be significant (*ca.* 35%; p=0.0312), followed by the vitamin B_6-accumulating line AtPDX1.1 OE (*ca.* 50%; p=0.0218) and AtPDX1.2 OE (*ca.* 55%; p=0.0653). In WT seedlings, vitamin B_6 levels were least effected, being *ca.* 73% of the control (p=0.2007) (figure 4.4*A*-*D*, grey bars). This finding is consistent with the vitamin B_6 levels quantified in WT when germinated on NaCl-containing medium, namely *ca.* 80% (p=0.111) of the controls (figure 4.6*A*). A closer look at the vitamer distribution revealed that mainly the free forms are reduced by NaCl (p=0.0268) accompanied by a slightly increased pool of the phosphorylated analogs (figure 4.6*A*). Interestingly, in AtPDX1.1 OE, but not in WT, sucrose in combination with NaCl reduced the total vitamin B_6 content by *ca.* 70%. Thus, sucrose, in combination with NaCl, has the same effect on the vi-

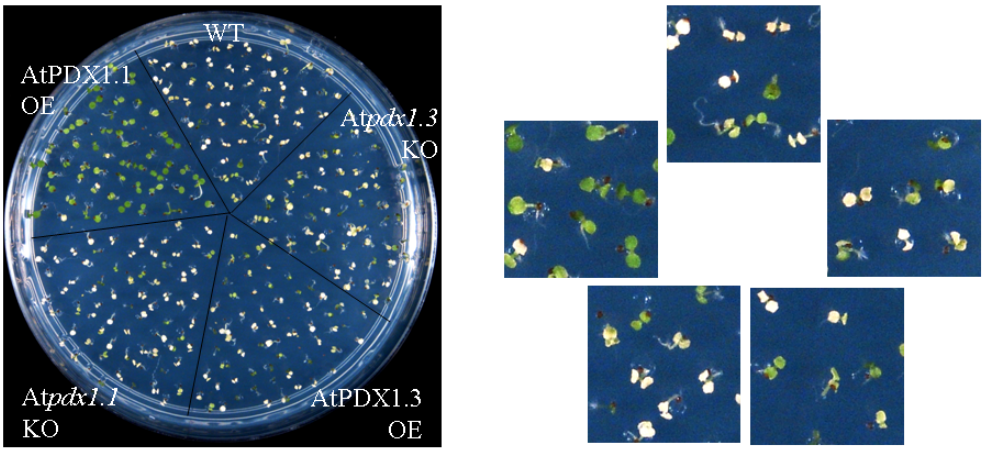
tamer distribution as it has in combination with rose bengal (figure 4.6*B*). However, the transcript abundance of *PDX1.1*, *PDX1.2*, *PDX1.3*, and *PDX2* did not significantly change in the presence of salt, independent of whether sucrose was present in the medium or not (figure 4.6*D*). In contrast, *SOS4* was up-regulated *ca.* five-fold in a sucrose-dependent manner (figure 4.6*D*). Correspondingly, it had previously been reported that *A. thaliana sos4* lines are more susceptible to salt stress [Shi *et al.*, 2002]. The authors hypothesized that SOS4 is essential for salt tolerance as it produces PNP, PMP and PLP, which are thought to regulate the intracellular Na^+ and K^+ homeostasis by modulating ion influx by ATP-gated transporters in plants [Shi *et al.*, 2002], comparable to the P_2X-receptor ion channels identified in animals [Ralevic and Burnstock, 1998].

Changes in anthocyanins are a frequent plant stress response although their role is not yet completely understood. Their antioxidant properties in the context of a general stress response may explain the health-promoting qualities in animals [Winkel-Shirley, 2002]. Neither H_2O_2 nor NaCl significantly induced anthocyanin accumulation in the shoots of WT or AtPDX1.1 OE when seeds were germinated on MS medium lacking sucrose. Most significantly, sucrose alone (3.3% (w/v)) or in a reduced concentration of 1% (w/v), in combination with rose bengal or NaCl (for AtPDX1.1 OE) triggered an increase in the anthocyanin content (figure 4.6*C*).

The response of the genes involved in *de novo* vitamin B_6 biosynthesis was also studied in seedlings exposed to high light, to cold temperature and to an oxidative burst in the chloroplasts (see chapter 4.6.3). A combination of high light intensities and low temperature is known to result in a systemic response which mainly affects photosystem (PS) II photochemistry. Increased irradiation can result in photoinhibition caused by an over-excitation and over-reduction of PS II which results in singlet oxygen production and destruction of the D1 protein of PS II [Sundby *et al.*, 1993]. Cold temperature does not affect the photochemistry, but causes a reduced rate of protein synthesis, which in turn attenuates the D1 protein repair process for the functional PS II machinery. Furthermore, the dissipation of excess light energy through alternative pathways is impaired [for review see Huner *et al.*, 1993]. A light intensity of 300 µmol photons m^{-2} s^{-1} in combination with low temperature (11 °C) is detrimental to seedlings of *A. thaliana* (Dr. R. Meskauskiene, ETH Zurich; personal communication). Arabidopsis with enhanced vitamin B_6 content is less susceptible to such conditions compared to WT while the *pdx1* KO mutants show divergent responses (figure 4.7). The A*tpdx1.1* KO mutant is more susceptible than the At*pdx1.3* KO mutant (first observed by Dr. R. Meskaus-

kiene, personal communication; confirmed by own studies). This is surprising as the At*pdx1.3* KO mutant is characterized by an overall reduced vitamin B_6 level and reduced chlorophyll content, compared to At*pdx1.1* KO mutant and WT [Titiz *et al.*, 2006]. Yet, these plants appear to be more tolerant to these conditions tested (figure 4.7). One could argue that under high-light – low-temperature conditions, it is primarily PDX1.1 that is involved in directing *de novo* synthesis of vitamin B_6, which then could alleviate the stress by a yet unknown mechanism.

A



B

Line	Total germinated seeds	Total developing seedlings	Survival rate (%)
WT	58	30	51.7
WT, *pre*	52	46	88.5
AtPDX1.1 OE	56	45	80.4
AtPDX1.1, OE, *pre*	58	58	100.0
At*pdx1.1* KO	59	18	30.5
At*pdx1.1* KO, *pre*	58	56	96.6
AtPDX1.3 OE	56	31	55.4
AtPDX1.3 OE, *pre*	55	33	60.0
At*pdx1.3* KO	51	41	80.4
At*pdx1.3* KO, *pre*	56	53	94.6

Figure 4.7: Susceptibility of seedlings to high-light – cold treatment. Seeds of the lines indicated were sown on MS medium lacking PN without (***A***) or with sucrose (1% (w/v)) (***B***) and cultivated for five days under moderate conditions (22°C, 100 µmol photons m^{-2} s^{-1}). One half of the plates was pre-acclimated for 24 hrs (22°C, 10 µmol photons m^{-2} s^{-1}; indicated as "*pre*" in (***B***)) before a dark period of 24 hrs in which the system was cooled down to 11°C, followed by a high-light period (300 µmol photons m^{-2} s^{-1}) at 11°C. The picture was captured four days after starting the experiment. The right panel is a magnification of the picture on the left ***(A)***. The survival rate of the lines was determined at day 11 (***B***). Grey boxes indicate significant changes in the survival rate compared to WT.

4.5.3 Distinct role(s) for PDX1.2 in *A. thaliana*

Based on *in vitro* activity studies, *in vivo* complementation as well as sequence and mutant analysis [Titiz *et al.*, 2006], PDX1.2 is not functional in *de novo* vitamin B_6 biosynthesis [Tambasco-Studart *et al.*, 2005/2007]. To date, no function has been assigned to this protein, although it is expressed in all parts of Arabidopsis, except in the embryo and the primary root, albeit in relatively low abundance, preferentially in roots, flowers and siliques. Its expression level is only about 0.5% of either PDX1.1 or PDX1.3 [Denslow *et al.*, 2007]. A previous study suggested that PDX1.2 may be involved in cell death responses [Tambasco-Studart, 2007]. In that study, *A. thaliana* lines over-expressing the endogenous PDX1.2 under control of the CaMV 35S promoter (AtPDX1.2 OE) as well as in the *flu*-mutant (At*flu* KO) background (*flu* AtPDX1.2 OE) had been established (see chapter 4.8.1) [Tambasco-Studart, 2007]. The response of the *flu* AtPDX1.2 OE to a chloroplast-specific burst of singlet oxygen was examined. In the constitutive *flu* mutant, the regulatory mechanism in the Mg^{2+} branch of tetrapyrrole biosynthesis, leading to chlorophyll, is disrupted leading to an accumulation of protochlorophyllide in the dark [Meskauskiene *et al.*, 2001]. Upon illumination, protochlorophyllide is rapidly (within minutes after irradiation) converted to chlorophyllide, which is accompanied by the release of singlet oxygen ($^1O_2$), a non-radical reactive oxygen species. Singlet oxygen is perceived as a signal which induces cell death by apoptosis indicated by the occurrence of chlorotic lesions and an immediate retardation of growth [Op den Camp *et al.*, 2003]. An adequate method for quantifying the level of damage is to determine the rate of electrolyte release, which is indicative of the integrity of the cell membranes. Staining leaves with Evan's blue can also be used to determine the integrity of cell membranes as this dye exclusively enters damaged cells and thus allows the quantification of the damage [Ochsenbein *et al.*, 2006]. Initial studies with *flu* AtPDX1.2 OE gave evidence, although not consistently, that this line might be more susceptible to the oxidative burst than At*flu* KO [Tambasco-Studart, 2007]. This work was extended her by comparing the response of At*flu* KO and *flu* AtPDX1.2 OE lines, with both *flu* AtPDX1.1 OE and *flu* AtPDX1.3 OE serving as additional controls. In addition to the determination of the level of cell death, the vitamin B_6 content and the transcript abundance of the three *PDX1* homologs, *PDX2*, *PDX3* and *SOS4* was elucidated. The plants were cultivated 21 days under continuous light before subjecting them to an eight-hr dark period followed by a re-exposure to light (DL shift). The release of electrolytes was monitored in detached leaves starting immediately after the DL shift (figure 4.8*A*). In our

hands, over-expression of PDX1.2 in the *A. thaliana flu* mutant did not affect the release of electrolytes and cell death, whereas over-expression of PDX1.1 led to a significantly reduced susceptibility to the oxidative burst (see chapter 3; figure 4.8*A*). Evan's blue staining of detached leaves corroborated the above findings (figure 4.8*B*). Furthermore, apart from the *flu* AtPDX1.1 OE line, in which a significant decline in the total vitamin B_6 content was observed, the DL-shift did not significantly affect the vitamin levels in the other lines (figure 4.8*C*). One should note that the total vitamin B_6 content is already elevated above WT levels in this particular line.

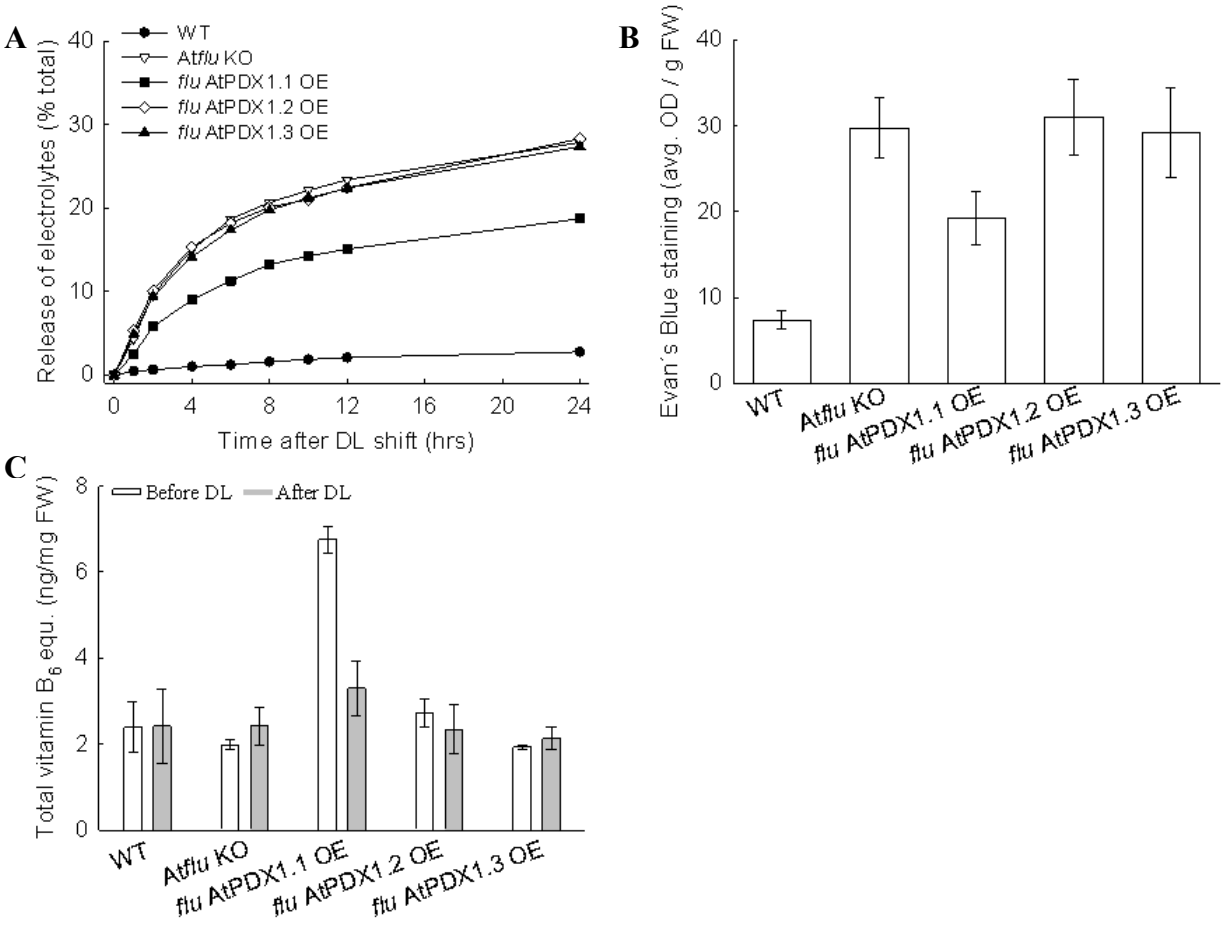


Figure 4.8: Response against an oxidative burst in different *A. thaliana flu* mutant lines. Plants were cultivated under continuous light at 120 µmol photons m^{-2} s^{-1} in soil. 21-day old plants were transferred to an eight-hr dark period before returning them to light (referred to as DL shift). ***(A)*** Electrolyte leakage measurements immediately before (= 0) and for the times indicated. The values given are related to total electrolytes determined after boiling (= 100%) and are the average of eight determinations. ***(B)*** Rosette leaves from a set of five plants per line were harvested 24 hrs after the DL shift and stained with Evan's Blue to assess the extent of cell death. ***(C)*** Total vitamin B_6 content was quantified from rosette leaves before and 24 hrs after the DL-shift. Samples taken after the DL shift were from the leaf tip of chlorotic leaves. Three plants were analyzed in duplicate per time point and line.

These results are corroborated by experiments in which protoplasts of both At*flu* KO and *flu* AtPDX1.2 OE were isolated and kept in the dark for eight hrs. No difference in the viability was observed after the DL-shift (Dr. Tambasco-Studart, ETH Zurich; unpublished). Furthermore, a comparison of WT and AtPDX1.2 OE lines for their ability to germinate and to develop on MS medium containing rose bengal or paraquat did not reveal any difference (table 4.2). However, *PDX1.2* transcripts increased in abundance (at least two-fold) in WT seedlings cultivated on high sucrose but not when treated with rose bengal, paraquat, H_2O_2 or high salt, respectively (figures 4.5*A*, 4.6*C*). In summary, constitutively elevated PDX1.2 levels do not evoke an obvious phenotype and have no effect on the vitamin B_6 content under moderate growth conditions (figures 4.4*C*, 4.8*C*, control bars). However, the stress response differed from WT in several cases (figure 4.4*A*, *C*). Interestingly, a dense cultivation of WT increased the transcript abundance of *PDX1.2* up to two-fold (data not shown), but not of the other *PDX* transcripts. In addition, Genevestigator data indicates a transient up-regulation of *PDX1.2* after six hrs of UV-B irradiation or 24 hrs of paraquat treatment (https://www.genevestigator.ethz.ch/ [Zimmermann *et al.*, 2004]). The relative expression level of *PDX1.2* at different developmental stages, in different tissues and in response to stress were extracted from the Genevestigator database and correlated to the expression of the other two *PDX1* genes, indicating a differential mode of regulation [Titiz *et al.*, 2006]. Denslow *et al.* demonstrated that *PDX1.2* transcript levels are unaffected by high-light, chilling or drought one, four and seven days, respectively, after the treatment, but they are so one and two days after treatment with ozone (table 4.1) [Denslow *et al.*, 2007].

To further elucidate the role of PDX1.2, the abundance of *PDX1.1*, *PDX1.2*, *PDX1.3*, *PDX2*, *PDX3* and *SOS4* transcripts, respectively, was compared in At*flu* KO and *flu* AtPDX1.2 OE before and after a DL-shift (see above; figure 4.9). In our hands, the peak in the abundance of *PDX1.2* transcripts (*ca.* 2.5-fold) was reached one hour after the DL-shift in At*flu* KO. After six hrs, *PDX1.2* was again on the basal level at which it remained. *PDX3*, *PDX1.3* and *PDX1.1* transcripts also accumulate, but with a significant delay compared to *PDX1*.2. In *flu* AtPDX1.2 OE, a comparable response of *PDX1.2* was not observed, most likely because *PDX1.2* is already elevated in this line (*ca*. 50-fold). Surprisingly, the time-courses of the responses of *PDX3* and *PDX1.3* differ between *flu* AtPDX1.2 OE and At*flu* KO (figure 4.9*B*). The maximal abundance for both transcripts was reached at the six-hrs time-point (*flu* AtPDX1.2 OE) and after 24 hrs (At*flu* KO). Whereas the response of *SOS*4 in both lines was indistinguishable, *PDX1.1* did respond in At*flu* KO (the maximum was after 24 hrs) but

not in *flu* AtPDX1.2 OE (figure 4.9*A*, *B*). A comparison of the transcript abundances in both lines revealed that, with exception of *PDX3* (which is enriched in *flu* AtPDX1.2 OE) and *SOS4* (which responds the same way in both lines), in *flu* AtPDX1.2 OE the levels are below those in the At*flu* KO. This direct relation between the transcript abundances in both lines substantiates the different responses at the transcript level (figure 4.9*C*, *D*).

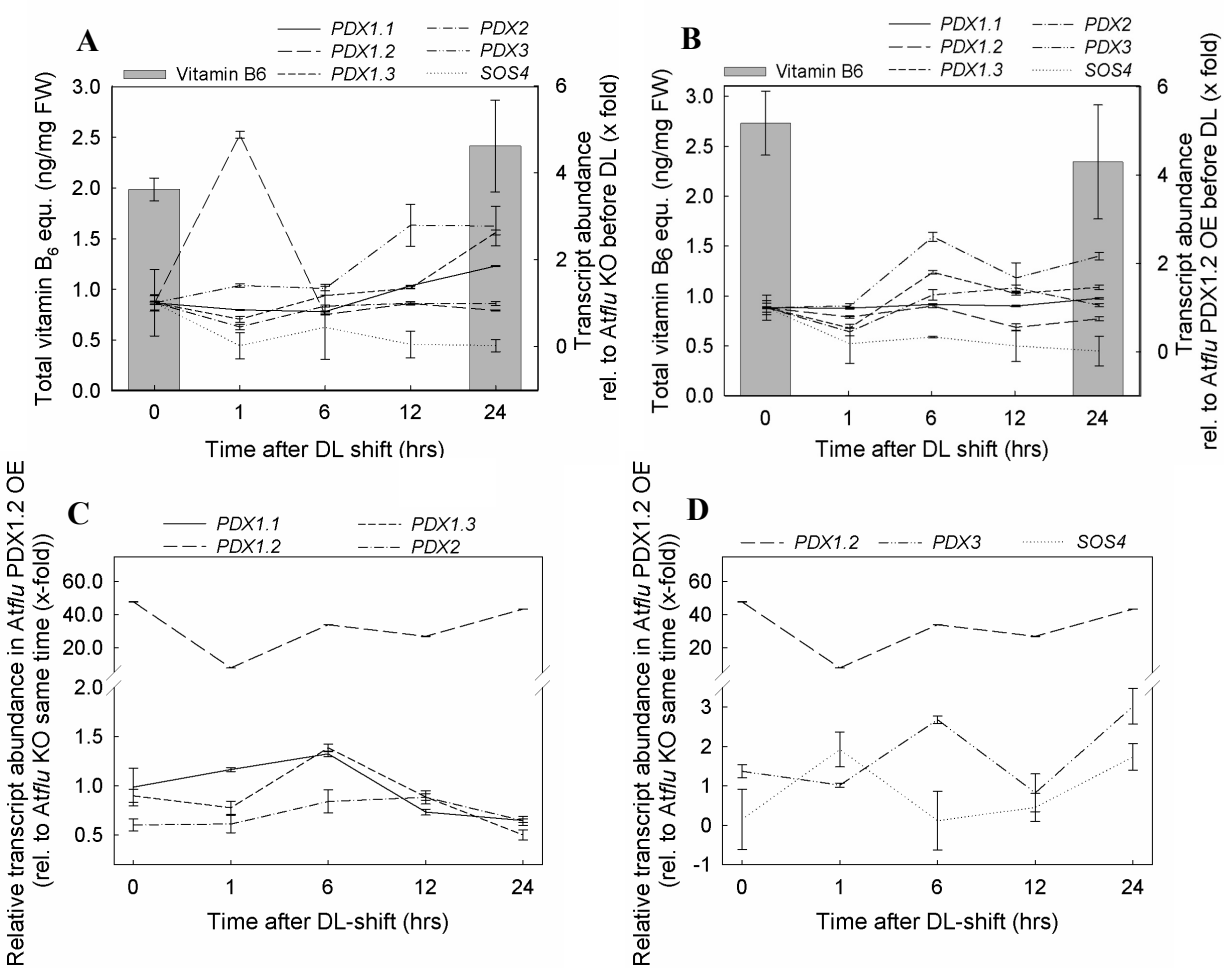


Figure 4.9: Comparative analysis of At*flu* KO ***(A)*** and *flu* AtPDX1.2 OE ***(B)*** in response to an oxidative burst. The plants were cultivated under continuous light (120 µmol photons m^{-2} s^{-1}). 21-day old plants were transferred to an eight-hr dark period before placing them back to light (referred to as DL-shift). Total vitamin B_6 content was determined before and 24 hrs after the DL-shift. The transcript abundance of *de novo* ***(C)*** and salvage pathway ***(D)*** enzymes in At*flu* PDX1.2 OE relative to At*flu* KO. Transcript abundance was quantified by quantitative real time RT-PCR and related to the time-point "0" of the same line (set to 1) ***(A, B)*** and to the same time-point in At*flu* KO ***(C, D)***, respectively. Values in ***(C, D)*** are as follows: x = 1: equal abundance in At*flu* KO and *flu* AtPDX1.2OE; x < 1: higher abundance in At*flu* KO; x > 1: higher abundance in At*flu* PDX1.2 OE. The values given are the average of two (transcript analysis) and three (vitamin B_6 quantification), respectively, independent determinations performed in duplicates.

High-light treatments were performed to further monitor the response of PDX1.2. Such a treatment can induce the production of singlet oxygen and superoxide/hydrogen peroxide/hydroxyl radicals [Fryer *et al.*, 2002]. Recently, it was reported that high-light treatment has no effect on the *PDX1.2* transcript levels [Denslow *et al.*, 2007]. In this particular study, the transcript abundance was not determined until one day after the treatment but, according to the results presented above, this may not be sufficient (figure 4.9). Therefore, the response to high-light intensities was monitored in WT and AtPDX1.2 OE for a period of six hrs only, with AtPDX1.1 OE and At*pdx1.3* KO serving as additional controls. For the latter two lines, it has recently been demonstrated that they are less and more susceptible, respectively, to photodamage [see chapter 3; Titiz *et al.*, 2006]. Seven-day old seedlings, cultivated under low light intensity (10 µmol photons m^{-2} s^{-1}) on MS medium lacking pyridoxine, were transferred to high light (1,100 µmol photons m^{-2} s^{-1}) and the maximum quantum efficiency of PS II photochemistry in dark-adapted plant material was determined. The efficiency level of PS II photochemistry in the AtPDX1.2 OE line was lower than that of WT but slightly higher than that of the At*pdx1.3* KO [Titiz *et al.*, 2006] after six hrs of high-light exposure. It is interesting to not, that the overall pattern of response differed between AtPDX1.2 OE and At*pdx1.3* KO (figure 4.10*A*). The deviation between WT and AtPDX1.2 OE became more evident only during the later part of the time course of the experiment (figure 4.10*A*). Under moderate light conditions (100 µmol photons m^{-2} s^{-1}) no difference between seven-day old seedlings from WT and AtPDX1.2 OE was found (figure 4.10*B*). Whereas the response of both AtPDX1.1 OE and At*pdx1.3* KO has been related to their elevated and reduced vitamin B_6 levels, respectively, this statement can not be extended to AtPDX1.2 OE as the level of the vitamin is similar to that of WT (figure 4.8*C*).

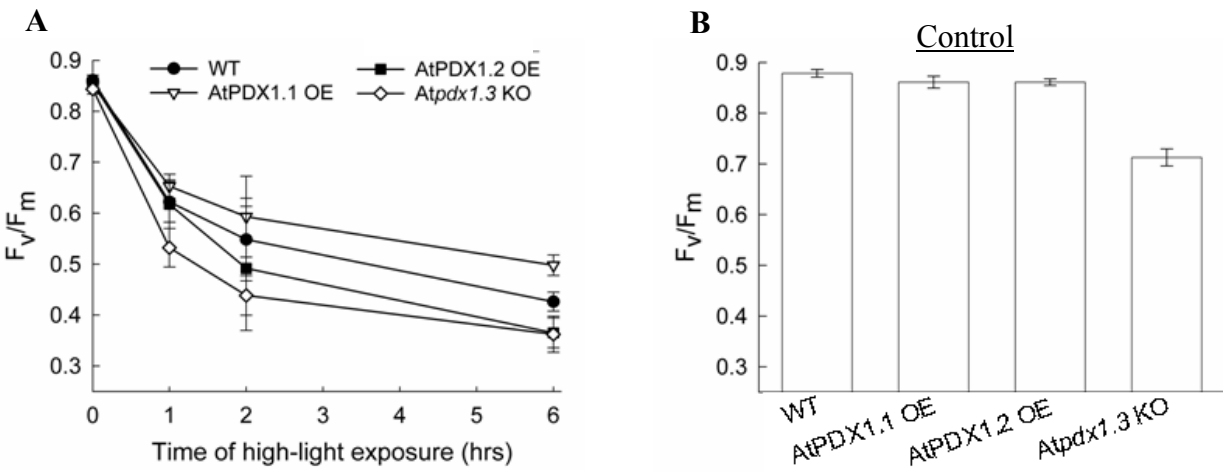


Figure 4.10: Maximum quantum efficiency of PS II photochemistry (F_v/F_m) for the lines indicated. Seeds were sown on MS medium lacking pyridoxine at a light intensity of 10 µmol photons m^{-2} s^{-1} for seven days (time point 0) and subsequently exposed to 1,100 µmol photons m^{-2} s^{-1} (***A***). Controls were cultivated at a light intensity of 100 µmol photons m^{-2} s^{-1} for seven days (Control, ***B***). The significance of the results (six hr time-point) was determined by a Student's T-test for $P < 0.1$ (AtPDX1.1 OE: p=0.0871; AtPDX1.2 OE: p=0.1095; At*pdx1.3* KO: p=0.1278)

4.6 Discussion

4.6.1 Regulation of PDX1.1 and PDX1.3 by abiotic stress

Vitamin B_6 has been related to tolerance against the photosensitizer compound cercosporin, in the fungus *Cercospora nicotianae* [Ehrenshaft *et al.,* 1999]. Moreover, PM, PN, PL and PLP are rapidly oxidized by singlet oxygen *in vitro* [Bilski *et al.*, 2000]. In plants, the effect of physiological stimuli (stress or hormones) on vitamin B_6 biosynthesis has just started to be addressed. In particular, stress-related motifs have been identified in the promoter regions of the *PDX* genes in *A. thaliana* [Denslow *et al.*, 2005; Titiz, 2008] and varying abundances at the transcript and/or protein level have been demonstrated [Sivasubramaniam *et al.*, 1995; Graham *et al.*, 2004; Chen and Xiong, 2005; Denslow *et al.*, 2007]. On the one hand, regulation of the catalytic activity can occur *via* enzyme concentration, for example, by protein *de novo* synthesis and degradation [Aragón and Sols, 1991]. Interestingly, the two catalytically active PDX1, *i.e.* PDX1.1 and PDX1.3, differ in their predicted half-life time being ca. 220 hrs and 5-31 hrs, respectively (supplementary table 4.2; http://www.isv.cnrsgif.fr/terminator2/test.php) [Meinnel *et al.*, 2005]. This may give an explanation as to why PDX1.1, but not PDX1.3 can be over-expressed in *A. thaliana* lines (figure 4.1 compare with figure 3.1 in chapter 3). The sub-cellular localization is another factor that can modulate enzyme activities

either through the binding of molecules (ions, substrates, inhibitors, cofactors) or the physiological conditions of the respective compartment. This may also pertain to PDX1 and PDX2, as recent reports varied in the assignment of their localization, either cytosolic [Tambasco-Studart *et al.*, 2005] or attached to the endomembrane system [Chen and Xiong, 2005; Denslow *et al.*, 2007], respectively. Furthermore, post-translational amino acid modifications can affect enzyme's activity as well. The predominant one of such modifications is phosphorylation/dephosphorylation, which is carried out by protein kinases and protein phosphatases [Swarup, 1998]. These modulating enzymes are often regulated by external (physiological) stimuli to direct molecular as well as cellular functions of other proteins independent from *de novo* biosynthesis [Barford *et al.*, 1998]. The amino acid sequences of PDX1.1, PDX1.2, PDX1.3 and PDX2 contain several potential phosphorylation sites (supplementary table 4.3; http://www.cbs.dtu.dk/services/NetPhos/ and http://www.cbs.dtu.dk/services/NetPhosK/) [Blom *et al.*, 1999]. Among the predicted kinases, $MAPK^{P38}$ kinase is specifically activated upon biotic and abiotic stresses and, in particular, by singlet oxygen, as demonstrated in human skin fibroblasts [Klotz *et al.*, 1999]. In all three PDX1 protein sequences regions similar to the classic MAPK-docking motif, (**R**/**K**)XXXX#X# (where # stands for a hydrophobic amino acid), have been identified preceding the predicted $MAPK^{P38}$-phosphorylating site, *i.e.* PDX1.1: AA 19-28 (KQ**K**SPFS**V**K**V**) and AA 85-95 (KEI**K**NAVT**I**P**V**); PDX1.2: AA 79-87 (R**R**MPDP**V**L**I**), AA 88-98 (KEV**K**RAVS**V**P**V**) and AA 91-98 (**K**RAVS**V**P**V**); PDX1.3: AA 19-27 (K**K**SPFS**V**K**V**) and AA 84-94 (KEI**K**QAVT**I**P**V**) (http://elm.eu.org/) [Puntervoll *et al.*, 2003] (supplementary table 4.3). Another phosphorylation site that is conserved in all three PDX1 proteins has been related to casein kinase II (CK II). This kinase has been implicated in the regulation of central cellular functions, *e.g.* cell division, growth and DNA replication. In particular, CK II positively affects the binding of the G-box binding factor I (GBF I), a transcription factor required for the expression of genes that are induced, for example, by light, oxygen deprivation, ethylene, abscisic acid and methyl jasmonate [Menkens *et al.*, 1995]. These features have recently been described for PDX1.1 and/or PDX1.3 in *A. thaliana* as well [Denslow, 2005; Titiz, 2008]. Interestingly, the predicted phosphorylation motifs appear to differ between PDX1.1 and PDX1.3 (supplementary table 4.2*A*, *C*). Furthermore, the rather low correlation between transcript and protein levels for both PDX1.1 and PDX1.3 [Titiz *et al.*, 2006] suggest several levels of regulation, *i.e.* at transcriptional, translational and/or post-translational level. However, this topic was beyond the scope of this study but may be worthwhile addressing in the future, *e.g.* by enzyme activ-

ity tests, gel shift assays and the identification of the B_6 vitamer profile of stressed and unstressed seedlings.

While NaCl may inhibit the activity of the PDX1-PDX2 complex (figure 4.4), it does not significantly affect the transcription of the genes related to *de novo* vitamin B_6 biosynthesis (figures 4.5*A*, *B*; 4.6*D*). Interestingly, *SOS4* was found to be up-regulated *ca.* five-fold in WT, but only in the absence of sucrose. This finding is not without precedent as a mutation in SOS4 renders *A. thaliana* more susceptible to salt stress [Shi *et al.*, 2002]. It was hypothesized that SOS4 is essential for salt tolerance as it triggers the formation of PLP (from PL), which in turn may regulate the intracellular Na^+ and K^+ homeostasis by modulating influx activities of ATP-gated ion transporters in plants. This has been demonstrated for the P_2X-receptor ion channels identified in animals [Ralevic and Burnstock, 1998]. PLP may antagonize the cofactor ATP in the channel, thereby preventing opening and thus the flow of monovalent cations across the membrane according to their electrochemical gradient [Ralevic and Burnstock, 1998]. In accordance with this, the vitamin B_6-accumulating lines AtPDX1.1 OE and AtPDX1.1-2 OE are less sensitive to salt treatment *in vivo* (see chapter 3 and supplementary figure 3.3). However, vitamin B_6 quantification in *A. thaliana sos4* knockout mutants revealed not only increased levels of the free vitamer forms such as PN and PM, but an unexpected *ca.* nine-fold enhancement of the PLP content [González *et al.*, 2007]. Thus, the response to salt stress cannot be solely based on the PLP content. Therefore the overall predicted preventive mode of vitamin B_6 against salt stress needs to be taken with caution. One possible way to explain the discrepancy is as follows. At*sos4* KO mutants have elevated PLP levels and are hypersensitive to salt treatment. Thus, enhanced levels of vitamin B_6 by themselves, PLP in particular, may not sufficiently prevent Na^+-influx into the cell by the mechanisms proposed for P_2X ion channels in animals, meaning that an adequate efflux-system for Na^+ ions is crucial for intracellular Na^+ homeostasis. SOS1, a Na^+/H^+-antiporter located in the plasma membrane, is an interesting candidate for the extrusion of Na^+ [Zhu, 2002]. It has been speculated that SOS4 may regulate SOS1, in addition to the SOS2-SOS3 regulatory mechanism [for review see Mahajan *et al.*, 2008]. In such a scenario, SOS4 may provide PLP as a ligand to SOS1 thereby enhancing its efflux activities. Support for this comes from the identification of a putative PLP-binding sequence in SOS1 [Zhu, 2002].

Unlike NaCl, both rose bengal and paraquat tended to increase total vitamin B_6 levels. Paraquat treatment, in particular, led to a further increase in the vitamin B_6 content AtPDX1.1

OE (see chapter 3; figures 4.4*A*, 4.6*A*, *B*), implying that the actual concentration of the vitamin is not recognized/sensed under these conditions. Interestingly, the transcription of *PDX1.1* was found to be reduced whereas *PDX1.3* was rather unaffected (figure 4.5*A*). Unlike treatments with rose bengal in combination with sucrose, as well as the *A. thaliana flu* mutant experiments (see chapter 3; figure 4.8*C*), paraquat treatments did not provide evidence that vitamin B_6 is directly involved in quenching the superoxide radical, as for this a reduction of the vitamin B_6 level would have been anticipated. However, based on the available data it can not be dismissed that the steady-state level of vitamin B_6 may increase upon paraquat treatment, thus eliciting an increased *de novo* biosynthesis and degradation of the vitamin. Activity tests may shed light on that. Another explanation may be that vitamin B_6 is related to an overall stabilization of proteins involved in a response against paraquat, either as a compound ensuring stability or indeed as a cofactor. As mentioned earlier, in animal systems it has been demonstrated that vitamin B_6 can compete with ATP in P_2X ion channels and by that mechanism it may block the uptake of paraquat [Ravelic and Burnstock, 1998]. In addition, superoxide stimulates the generation of ethylene in the presence of PLP [Elstner *et al.*, 1978]. In ethylene biosynthesis, PLP is involved as a cofactor for the conversion of S-adenosyl-L-methionine (SAM) to ACC catalyzed by ACC synthase [Adams and Yang, 1979]. On the contrary, an increased demand for ACC may additionally stimulate *de novo* PLP biosynthesis.

An interesting observation, worth addressing in the future, is the effect a combination of sucrose with other stress-inducing compounds has on the total vitamin B_6 content and on the general stress tolerance. It has been reported that carbohydrate-accumulating plants exhibit an enhanced tolerance against drought and salt [for review see Gupta and Kaur, 2005] and that soluble sugars accumulate upon pathogen attack, drought, salt, high light, low temperature or abscisic acid treatment, implying an adaptive response to these stress conditions [Roitsch, 1999]. Indeed, the presence of sucrose significantly increased the survival rate of WT seedlings when germinated on medium containing rose bengal (figure 4.2*A*, table 4.2*A*). Furthermore, the sensitivity significantly decreased when sucrose-grown seedlings were treated with a combination of low temperature and high light (supplementary figure 4.1). This sucrose-induced tolerance is comparable to a pre-acclimation, for example by low-light intensities (figure 4.7*B*). Sucrose specifically led to an up-regulation of *PDX1.2*, *PDX1.3* and *PDX2* transcription accompanied by a down-regulation of *SOS4*, a situation even more pronounced in the presence of rose bengal. Furthermore, the increase in *SOS4* transcript upon NaCl treatment is significantly repressed by sucrose (figure 4.6*D*) and the susceptibility against paraquat

is dependent on the presence of sucrose (table 4.2*B*). Taking all findings together, extracellular sucrose may mediate a signal that activates alternative stress response pathways, thus providing an accelerated tolerance for the plant. However, this awaits further elucidation.

4.6.2 Distinct role(s) for PDX1.2?

PDX1.2 does not contain the complete PdxS/SNZ family signature [L/V]-P-[V/I]-[V/T/P/I]-[N/Q/L/H/T]-[F/L]-[A/T/V/S]-[A/S]-G-G-[L/I/V]-[A/T]-T-P-[A/Q/S]-D-[A/G/V/S]-[A/S]-[L/M] and can not compensate for the loss of the other two PDX1 proteins in *A. thaliana* [Titiz *et al.*, 2006]. Furthermore, parsimony analysis revealed that PDX1.2 is distinct from other PDX1 homologs identified to date [Denslow *et al.*, 2007; Titiz *et al.*, 2006]. Two other plant species have been noted to contain a PDX1.2 homolog: *Populus trichocarpa* (LG_I_903) [Tambasco-Studart, 2007] and *Vitis vinifera* (A7P7B7). However, no function has been assigned to this low-abundant protein so far. Recent studies suggested that it may be involved in early stress responses as it is transiently up-regulated in the At*flu* KO mutant one to two hrs after the DL-shift and was shown to elevate cell death responses under these conditions [Tambasco-Studart, 2007]. In addition, PDX1.2 was recently identified by a microarray analysis to be induced by glucose [Li *et al.*, 2006]. Own studies revealed that the transcript level is increased by sucrose treatment (figures 4.5*A*, 4.6*D*) as well as under light- and nutrient-limiting conditions in densely cultivated plants (data not shown).

An experiment to address the question whether PDX1.2 is involved in stress responses was performed by subjecting both WT and the PDX1.2-accumulating AtPDX1.2 OE lines to high-light treatment. It revealed striking differences between the two lines (figure 4.10). High-light treatment predominantly affects the efficiency of PS II, which can be quantified by the F_v/F_m value. A reduction in this value indicates photoinhibition [Aro *et al.*, 1993]. High-light treatment can lead to an acceptor side-induced photoinhibition (ASP) and a donor side-induced photoinhibition (DSP) of PS II. ASP is characterized by the formation of excess amounts of chlorophyll in the triplet state and the subsequent formation of singlet oxygen. In contrast, DSP is caused by an impairment of the electron donation from water, which occurs when the reduction of chlorophyll and tyrosine cation radicals in the PS II photosynthetic centre is retarded. Unlike ASP, DSP does not depend on oxygen and does not trigger the release of singlet oxygen [Aro *et al.*, 1993]. Both mechanisms have predominantly been investigated *in vitro*, but there is also evidence for their occurrence *in vivo* [for review see Barber and Andersson, 1992; Hideg *et al.*, 1998]. PDX1.2 may either be directly or indirectly involved in

the PS II-mediated high-light stress responses *via* DSP rather than impairing ASP, as the latter most probably would have led to increased cell death and thus to an increased release of electrolytes in the *flu* AtPDX1.2 OE line comparable to At*flu* KO upon the DL-shift (figure 4.8*A*, *B*). In contrast, lines harbouring modified vitamin B_6 levels such as AtPDX1.1 OE and At*pdx1.3* KO might primarily respond to ASP, which would be congruent with the finding that vitamin B_6 can quench singlet oxygen, among other ROS, *in vitro* [Bilski *et al.*, 2000], and thus protect from cell death responses as observed in fungi [Ehrenshaft *et al.*, 1999; Chumnantana *et al.*, 2005]. This statement is in agreement with the decline in the total vitamin B_6 content observed in *flu* AtPDX1.1 OE 24 hrs after the DL-shift (figure 4.8*B*). Furthermore, the D1 protein of PS II is characterized by a rapid turnover upon oxidative stress, thus it plays a crucial role in the repair cycle of PS II [Aro *et al.*, 1993]. Therefore, the overall reduced level of D1 protein in At*pdx1.3* KO mutants, a line carrying reduced vitamin B_6 levels, points towards increased concentrations of singlet oxygen suggesting that vitamin B_6 plays a photo-protective role in controlling $^1O_2$ detoxification and hence protein D1 turnover [Titiz *et al.*, 2006]. Interestingly, an *in vitro* reconstitution of ASP and DSP revealed different fragmentation patterns of the D1 protein [Barbato *et al.*, 1991/1992; Salter *et al.*, 1992]. This might be of relevance for investigating the specificity of PDX1.2 as well as for the involvement of vitamin B_6 in plant stress tolerance. Although final *in vivo* data is lacking, both ASP and DSP might be relevant protective mechanisms in PS II. Thus, the transient up-regulation of PDX1.2 in At*flu* KO upon the DL-shift (figure 4.9*A*) could be explained in a way that most probably both DSP and ASP occur *in vivo*. However, ASP may dominate because of the burst of singlet oxygen in the chloroplasts. To date, it is believed, that, based on the short half-life and interaction distance, singlet oxygen cannot move across the chloroplast envelope to directly induce nuclear gene expression. Recently, a triple knockout mutant of *A. thaliana* has been reported to suppress the $^1O_2$-mediated signal transduction from the chloroplasts to the nucleus. In this line, in which the genes *FLU*, *EXECUTER1* and *EXECUTER2* are knocked out, nuclear-encoded $^1O_2$-responsive genes are not up-regulated in the face of high plastidic $^1O_2$-concentrations [Lee *et al.*, 2007]. It would be interesting to determine the time-course of *PDX1.2* transcription in this line after DL-shift. The singlet oxygen-mediated signalling network is currently under intense investigation in plants. The involvement of calcium ion channels [Pei *et al.*, 2000], different MAP kinase cascades [Samuel *et al.*, 2000; Yuasa *et al.*, 2001], ethylene responsive factors, WRKY transcription factors, zinc-finger and DNA-binding proteins, protein kinases, calcium- and calmodulin-binding proteins as well as further

proteins putatively involved in signal-transduction pathways and calcium regulation is being discussed in this context [for review see Kim *et al.*, 2008].

In summary, the experiments performed cannot assign a definitive function to PDX1.2 in *A. thaliana*. Constitutively enhanced levels of PDX1.2 (AtPDX1.2 OE) did not change the overall vitamin B_6 content and did not lead to a phenotype when cultivated under standard growth conditions (data not shown). Twenty-one and 39-day old AtPDX1.2 OE lines cultivated in soil (long-day conditions, at a light intensity of 120 µmol photons m^{-2} s^{-1}) and subsequently exposed to drought or salinity behaved similar to WT (data not shown). Other stress treatments, such as oxidative stress (rose bengal, paraquat) caused lethality comparable to WT (table 4.1). *Flu* mutant experiments revealed that several of the genes involved in vitamin B_6 *de novo* and salvage biosynthetic pathways are affected in PDX1.2 accumulating plants (figure 4.9). This implies that a certain interaction between these proteins may occur. High-light treatments support this hypothesis, as the efficiency of the PS II of AtPDX1.2 OE was more severely affected than that of WT (figure 4.10*A*). Taking these findings and the rather low transcript and protein abundance of PDX1.2 into consideration, it could be that *PDX1.2* is a so-called pseudogene. Pseudogenes have been originally defined as non-functional, in the sense of not producing a functional protein. They are sequences of genomic DNA that originate from other genes often sharing a high degree of sequence similarity with them [Woodmorappe, 2003]. However, many of the pseudogene features such as absent or disabled promoters, premature stop codons, frame-shift-causing insertions or deletions [Balakirev and Ayala, 2003] cannot be related to *PDX1.2*. A hypothesized feature of pseudogenes is the ability to be transcribed and to modulate the expression of paralogous genes [McCarrey and Riggs, 1986]. Thus, a possible interaction may come from the formation of short antisense RNA chains of *PDX1.2* that hybridize with the sense RNA of *PDX1.1* and/or *PDX1.3*, thus repressing the expression of them. The high sequence homology of PDX1.2 with PDX1.1 (61.4%) and PDX1.3 (63.4%), respectively, may account for that. In addition, the results of the *flu* mutant experiments, when the transcript abundances in the At*flu* KO and *flu* AtPDX1.2 OE lines have been compared, may contribute to this (figure 4.9). Another feature of pseudogenes that may apply to PDX1.2 is a putative interaction with its paralogous protein(s). This hypothesis is based on recent findings for the nitric oxidase synthase (NOS). This gene can be regulated at the level of transcriptional repression (by sense-antisense hybridization) and post-translational modifications. By that, NOS can form a catalytically inactive heterodimer with antiNOS-2, a short, catalytically inactive version of NOS. This interaction leads to

a strong suppression of NOS activity *in vivo* [Korneev and O'Shea, 2002]. For PDX1.2, yeast two-hybrid and pull-down experiments revealed that PDX1.2 can interact with PDX1.1 and PDX1.3, but not with PDX2 [Wagner *et al.*, 2006]. In addition, the use of a hormone-treated seedling prey library in a yeast two-hybrid screen led to the finding that PDX1.1 can interact with PDX1.2 [Dr. L. Bürkle, ETH Zurich; personal communication]. The specificity of the latter interaction may indicate that the interaction depends on the developmental stage and external stimuli. This finding is supported by an early observation that over-expression of PDX1.2 or PDX1.1 in *A. thaliana* leads to an increased appearance of a protein(s) of *ca*. 66 kD in size. The presence of this complex, that is particularly stable upon treatment with denaturing agents such as SDS or DTT [Dr. M. Tambasco-Studart, ETH Zurich; personal communication], is more pronounced under certain conditions, such as treatment with low concentrations of rose bengal and appears to be light-stimulated as well (supplementary figure 4.2). Based on preliminary Western blot analysis, PDX1.1 and PDX1.2, but not PDX1.3 and PDX2 may be part of this complex. Thus, it may be speculated that PDX1.2, under certain conditions, affects the rate of vitamin B_6 *de novo* biosynthesis by reducing the freely available PDX1.1 in the cell and thus limiting the formation of active PDX1.1-PDX2 complexes. Although the results of the *flu* mutant experiments indicate that the expression of PDX1.1 at the level of transcription, the precise mechanism behind this awaits further elucidation. However, this, indeed, may be part of the regulation of the vitamin B_6 content in plants.

However, it is worth mentioning that it is not necessarily PDX1.2 alone which may affect particularly the transcription and/or translation of PDX1.1. The specific down-regulation of PDX1.1 under ozone (table 4.1) [Denslow *et al.*, 2007], paraquat (figure 4.5*A*) and sucrose (figure 4.6*D*) at the transcript level and upon treatment with rose bengal or sucrose at the protein level (figure 4.5*C*) could also be attributed to a protein which is identical to the first 240 amino acid residues of PDX1.1. This protein is encoded by a partial copy of *PDX1.1* (At2g38210) which is located four kb upstream from *PDX1.1* itself [Denslow *et al.*, 2007] and should be considered in future studies.

4.7 Supplementary information

4.7.1 Supplementary protocols for established *A. thaliana* lines and lines generated but not yet studied

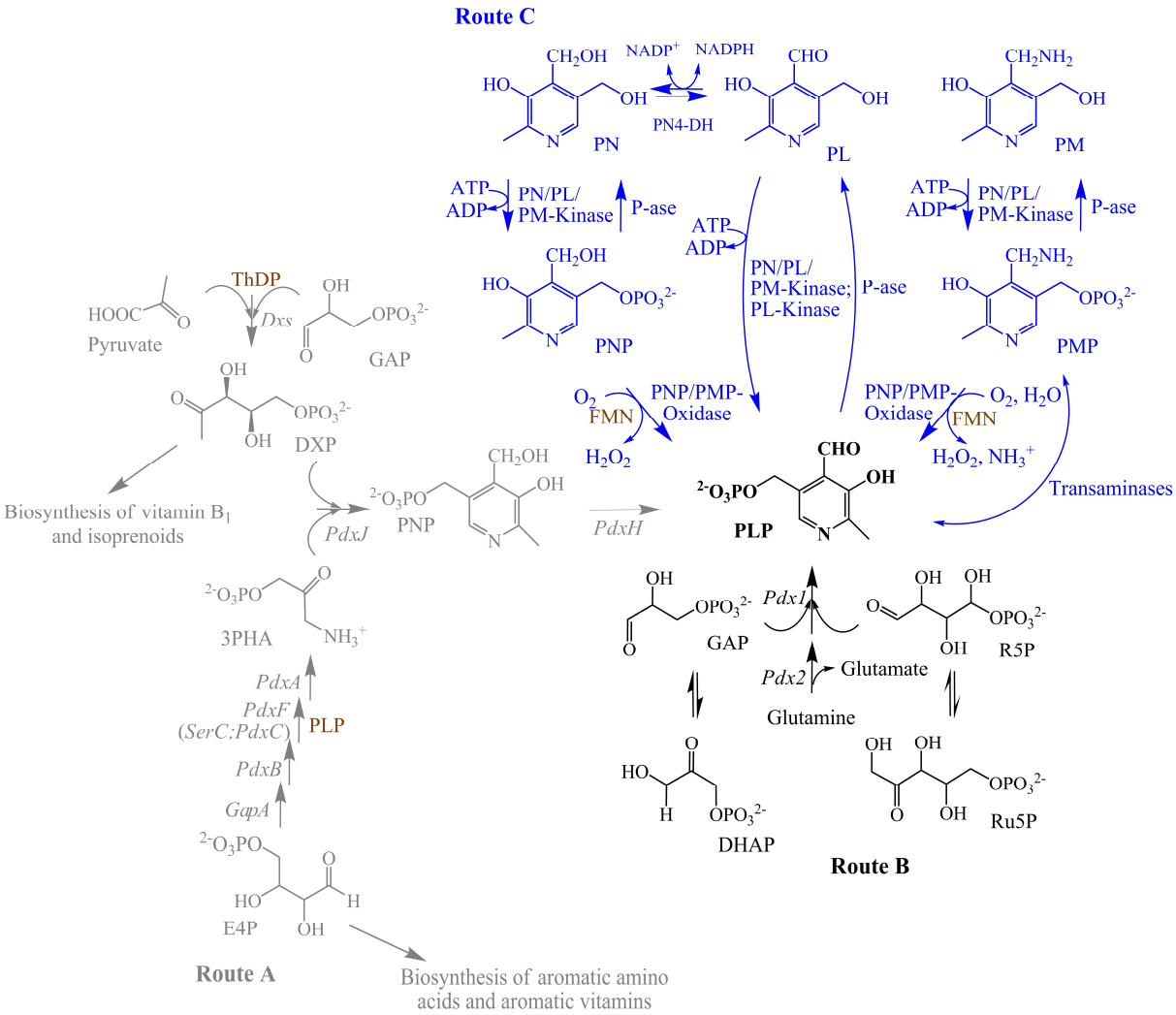
A. thaliana lines over-expressing the endogenous *PDX1.2* in WT, and the At*flu* KO background, designated AtPDX1.2 OE and *flu* AtPDX1.2 OE, respectively, were established by Dr. M. Tambasco-Studart (unpublished) by transforming *A. thaliana* WT and the At*flu* KO (both ecotype Col-0) with the *pCAMBIA1302-AtPDX1.2* construct under control of the constitutive 35S CaMV promoter. Endogenous *PDX1.2* was amplified using the primer pair: 5'-CATGCCATGGCGGATCAAGCTATGACG-GAT-3' (fw) and 5'-GGACTAGTTCAAA-CACTGCCTTGGCCAAAGTC-3' (rv) and cDNA as a template. The underlined sequences represent the incorporated restriction sites, NcoI, and SpeI, respectively. The purified PCR products were sub-cloned into the commercially available PCR2.1 TOPO® vector (Topo TA Cloning Kit, Invitrogen, California, U.S.A.) according to the manufacturer's recommendations and subsequently cloned into the plant binary vector pCAMBIA 1302. *A. thaliana* WT and At*flu* KO (both ecotype Col-0) were stably transformed employing *Agrobacterium tumefaciens*-mediated transformation [Clough and Bent, 1998]. Homozygous lines of the fourth generation (T4) carrying a single insertion of the gene cassette of the pCAM-*AtPDX1.2* construct were selected according to their resistance against hygromycin (50 mg l^{-1}) on MS medium including vitamins [Murashige and Skoog, 1962] supplemented with 1%(w/v) sucrose. In the case of *A. thaliana* lines harbouring the *flu* mutation, the plants were additionally selected for a chlorotic phenotype upon a shift from continuous light to an eight-hr dark period and back to the light. Homozygous lines in either background were used in the experiments.

Homozygous *A. thaliana* lines carrying the pCAM-*AtPDX1.2* construct were crossed into At*pdx1.1* KO and At*pdx1.3* KO background [Titiz *et al.*, 2006] and designated At*pdx1.1* PDX1.2 OE, and At*pdx1.3* PDX1.2 OE, respectively. The presence of a single insertion of the gene cassette of the pCAM-*AtPDX1.2* construct and the homozygous insertion in the respective knockout lines was confirmed by selection/segregation analysis on MS plates containing hygromycin (50 mg l^{-1}) and by Western blot analysis. After PDX1.2 over-expressing plants were selected these plants were analyzed (by PCR) for a homozygous knockout of *PDX1.1* or *PDX1.3*, respectively, as described recently [Titiz *et al.*, 2006]. Remark: These lines were established and the homozygous state for both the inserted *PDX1.2* gene cassette and the knockout of *PDX1.1* and *PDX1.3* has been confirmed.

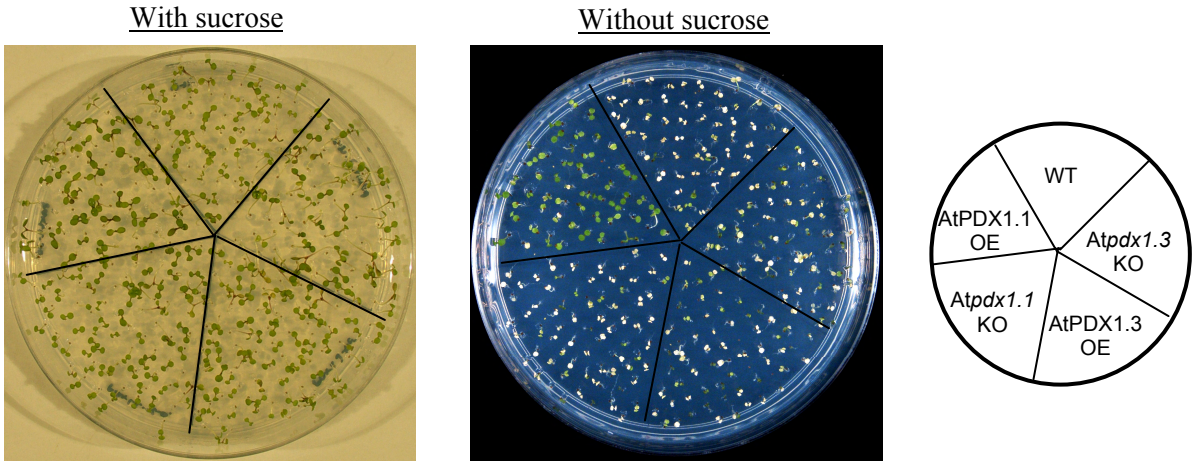
A. thaliana lines over-expressing *PDX1.2* and *PDX1.1* or *PDX1.2* and *PDX2* were established by crossing the respective homozygous lines with the highest expression level. The double over-expressors were selected against hygromycin (50 mg l^{-1}) (AtPDX1.2-1.1 OE) or both hygromycin (50 mg l^{-1}) and kanamycin (35 mg l^{-1}), respectively (AtPDX1.2-2 OE). The higher abundance of the proteins compared to WT level was confirmed by quantitative real-time RT-PCR, Western blot analysis and by the occurrence of the large-seed phenotype related to over-expression of PDX1.1 in addition to the increased vitamin B_6 content. These lines are designated AtPX1.2-1.1 OE and AtPDX1.2-2 OE, respectively.

As no true *A. thaliana* knockout line for *PDX1.2* was available, a sense-antisense PDX1.2-RNAi line was initiated. As sense fragment, bp 3-460, and as antisense fragment, bp 3-303, of the endogenous *PDX1.2* was amplified from cDNA using the primer pairs 5'-CATGCCATGGGGCGGATCAAGCTATGACG-3', 5'-GCACTAGTCTCCTGTATCACG-GCAACC-3' and 5'-GCACTAGTCGTGCCATCACAGGTACAG-3', 5'-CAGGGTAACCGGCGGATCAAGCTATGACG-3', for the sense and antisense fragment, respectively. The underlined sequences represent the incorporated restriction sites, NcoI, SpeI (sense) and SpeI, BstEII (antisense), respectively. The sense and antisense fragments are coupled by the linker sequence ACTAGT. The purified PCR products were subsequently cloned into the binary plant vector pCAMBIA1302 (carrying a hygromycin resistance cassette) and pCAMBIA2301 (carrying a kanamycin resistance cassette) using the restriction sites mentioned above. The pCAMBIA2301-*AtPDX1.2 RNAi* construct was introduced by *A. tumefaciens*-mediated transformation [Clough and Bent, 1998] into *A. thaliana* WT, AtPDX1.1 OE, *PDX1.3*-transgenic line (designated AtPDX1.3 OE) and At*flu* KO [Meskauskiene *et al.*, 2001], and the construct pCAM-*AtPDX1.2 RNAi* was transfected into AtPDX2 OE and AtPDX1.1-2 OE, respectively. Transformed plants carrying a single cassette of each construct were selected by resistance to both hygromycin (50 mg l^{-1}) and kanamycin (35 mg l^{-1}), respectively. Remark: These lines were selected and homozygous PDX1.2 RNAi lines have been obtained. However, it needs to be confirmed whether they are true RNAi lines.

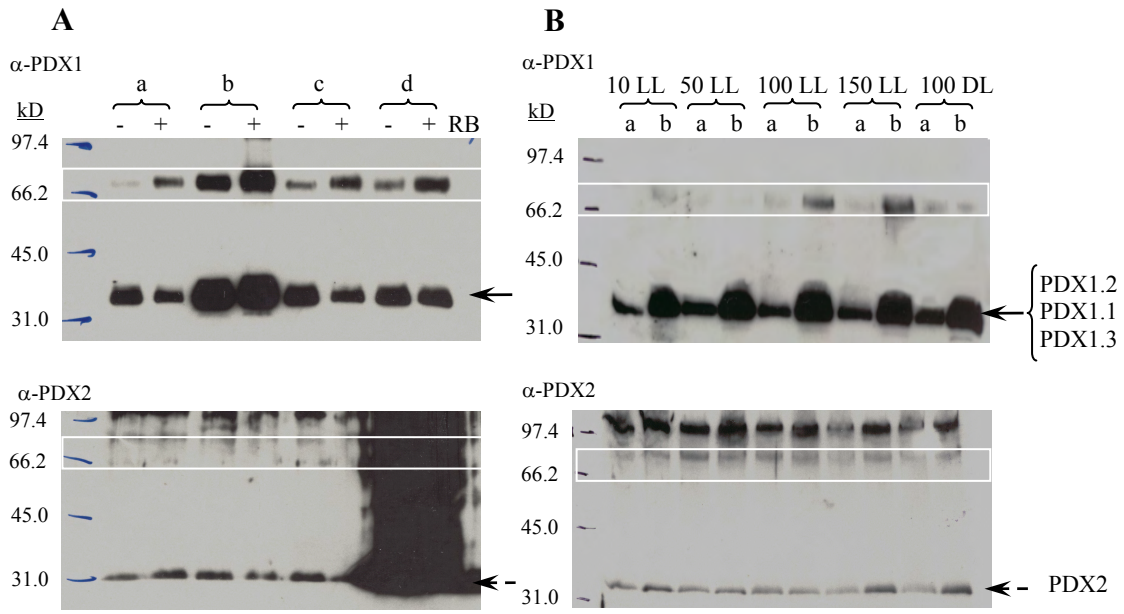
4.7.2 Supplementary figures and tables



Supplementary scheme 4.1: *De novo* and salvage pathways of vitamin B_6 biosynthesis. 1-Deoxy-D-xylulose 5-phosphate (DXP) dependent (*route A*; dark grey) and independent (*route B*; black) *de novo* biosynthesis of vitamin B_6 and the salvage pathway for the inter-conversion of the different B_6 vitamers (*route C*; blue) identified in a small subgroup of γ-proteobacteria (*route A*), in the majority of bacteria, archaea, fungi as well as plants (*route B*) and in all living organisms (*route C*), respectively. The identified enzymes are indicated in italics. PN/PL/PM-kinase: *PdxK* (*E. coli*), *SOS4* (*A. thaliana*), *BUD16* (*S. cerevisiae*); PL-kinase: *PdxY* (*E. coli)* and predicted in *A. thaliana* (At5g58730) [González *et al.*, 2007]; PNP/PMP-oxidase: *PdxH* (*E. coli*), *PDX*3 (*A. thaliana*, *S. cerevisiae*) have been identified (see text for details) to be involved in the salvage pathway. Cofactor requirements are given in brown colour (PLP, ThDP, FMN). Abbreviations used: DHAP: dihydroxyacetone phosphate; E4P: erythrose 4-phosphate; FMN: flavin mononucleotide; GAP: D-glyceraldehyde 3-phosphate; 3PHA: 3-phosphohydroxy-1-aminoacetone; PLP: pyridoxal 5'-phosphate; PNP: pyridoxine 5'-phosphate; R5P: ribose 5-phosphate; Ru5P: Ribulose 5-phosphate; ThDP: thiamin diphosphate.



Supplementary figure 4.1: Effect of sucrose on stress responses under a high-light – cold regime. Seeds were sown on MS medium lacking pyridoxine, with ((1% (w/v), left panel) and without (middle panel) sucrose and cultivated under the conditions described for figure 4.7*A*, *B*. The right panel indicates the orientation of the lines in the left and middle panel.



Supplementary figure 4.2: Inducible formation of a protein complex of about 66 kD containing PDX1 but not PDX2. Western blot analysis of protein extracts isolated from *A. thaliana* WT (a), AtPDX1.1 OE (b), AtPDX1.3 OE (c), AtPDX2 OE (d) cultivated with or without 10 µM rose bengal ***(A)*** or under different light intensities (10, 50, 100 and 150 µmol photons m^{-2} s^{-1} continuous light (LL) or long-day conditions (DL)) as indicated ***(B)*** for 10 and 15 days, respectively. Total protein (40 µg) was loaded on a 12.5% SDS page and probed with PDX1 (upper panel) and PDX2 (lower panel) antibody, respectively. The complex is highlighted by a white box. PDX1 and PDX2 proteins are indicated by solid and dashed arrows, respectively. The approximate protein mass is given in kD. Abbreviations used: AB: antibody; RB: rose bengal.

Line	Background (Col-0)	Resistance	Additional selection
AtPDX1.1 OE	WT/At*flu* KO	Hygromycin	DL-shift (At*flu* KO)
AtPDX1.2 OE	WT/At*flu* KO/ At*pdx1.1* KO/ At*pdx1.3* KO	Hygromycin	DL-shift (At*flu* KO), PCR (At*pdx1.1/1.3* KO)
AtPDX1.3 OE	WT/At*flu* KO	Hygromycin	DL-shift (At*flu* KO)
AtPDX2 OE	WT/At*flu* KO	Kanamycin	DL-shift (At*flu* KO)
AtPDX1.1-2 OE	WT/At*flu* KO	Hygromycin, kanamycin	DL-shift (At*flu* KO)
AtPDX1.2-2 OE	WT	Hygromycin, kanamycin	
AtPDX1.2-1.1 OE	WT	Hygromycin	
AtPDX1.2 RNAi	WT/AtPDX1.1 OE/ AtPDX1.3 OE	Hygromycin, kanamycin	
AtPDX1.2 RNAi	AtPDX2 OE/ AtPDX1.1-2 OE	Hygromycin, kanamycin	

Supplementary table 4.1: A summary of the transgenic lines generated and their respective antibiotic resistance used for selection. In addition to the antibiotic selection for the incorporated transgene, homozygosity for the *FLU* mutation was confirmed by monitoring the chlorotic phenotype after an eight-hour dark period followed by 24 hrs of re-illumination (DL-shift) [Meskauskiene *et al.*, 2001]. The homozygous knockout of *pdx1.1* and *pdx1*.3, respectively, was confirmed by PCR [Titiz *et al.*, 2006].

Protein	N-terminus	Flanking sequence	Likelihood (%)	Half-life (hrs)
AtPDX1.1	Ac-Ala (2)	(M)**A**GTGVVAV	83	220
AtPDX1.2	Ac-Ala (2)	(M)**A**DQAMTDQ	83	220
AtPDX1.3	Ac-Met (1)	**M**EGTGVVAV	100	5-31
AtPDX2	T(2)	(M)TVGVLALQ	75	65
AtPDX3	T(2)	(M)TFTFLLQS	75	65
AtSOS4	P(2)	(M)PFSFPTTT	100	?

Supplementary table 4.2: Predicted N-terminus and its putative co-translational modification site (in bold) for acetylation (indicated as Ac-), the likelihood for its occurrence (%) and the putative half life (hrs) of the PDX proteins involved in *de novo* and salvage vitamin B_6 biosynthesis. When the methionine residue M(1) is given in brackets, it is most probably removed during maturation. (http://www.isv. cnrs-gif.fr/terminator2/test.php [Meinnel *et al.*, 2005]).

A

	AtPDX1.1	Site	Sequence	Probability	Predicted kinase
*		S22	TKQK**S**PFSV	0.962	CDK5; $MAPK^{P38}$
*		S25	KSPF**S**VKVG	0.990	PKC
*		S79	VARM**S**DPEM	0.992	CKII; PKA
		S121	YVDE**S**EVLT	0.612	
		S193	RLLR**S**MDDD	0.995	CKII
		S306	ERFA**S**RSE-	0.962	PKA
		T4	-MAG**T**GVVA	0.792	
*		T125	SEVL**T**LADE	0.600	CKII
*		T165	AMIR**T**KGEA	0.743	PKG
*		T234	GGVA**T**PADA	0.932	$MAPK^{P38}$
		Y117	IGVD**Y**VDES	0.783	

B

	AtPDX1.2	Site	Sequence	Probability	Predicted kinase
		S32	NHPF**S**VKVG	0.994	PKC
		S51	VEVS**S**VNQA	0.789	PKC
		S74	DPVR**S**RGGV	0.683	
		S95	KRAV**S**VPVM	0.789	PKA
*		S128	SEII**S**VADD	0.982	CKII
		S271	KKLR**S**IVQA	0.738	PKA
		T152	GCRD**T**GEAL	0.685	
		T181	NIAE**T**VKNV	0.794	PKC
*		T238	GGIT**T**PADA	0.978	$MAPK^{P38}$

C

	AtPDX1.3	Site	Sequence	Probability	Predicted kinase
*		S21	EAKK**S**PFSV	0.979	$MAPK^{P38}$
*		S24	KSPF**S**VKVG	0.996	PKC
*		S78	VARM**S**DPQM	0.856	PKA
*		T124	SEVL**T**LADE	0.600	CKII
*		T164	AMIR**T**KGEA	0.743	PKG
*		T233	GGVA**T**PADA	0.932	$MAPK^{P38}$
		Y274	AVTH**Y**SDPE	0.665	

D

AtPDX2	Site	Sequence	Probability	Predicted kinase
	S48	PGGE**S**TTMA	0.902	
	S129	PQLT**S**QEGG	0.866	DNAPK; ATM
	S227	EQGA**S**SSSS	0.939	CDC2
	S228	QGAS**S**SSSK	0.877	PKC; CDC2
	S230	ASSS**S**SKTI	0.975	
	S236	KTIV**S**VGET	0.992	PKC
	S241	VGET**S**AGPE	0.768	
	T128	VPQL**T**SQEG	0.724	
	T136	GGPE**T**YRGV	0.939	PKC
	Y169	NKVL**Y**SSST	0.799	

Supplementary table 4.3: (page 183) Predicted phosphorylation sites (in bold) for AtPDX1.1 (***A***), AtPDX1.2 (***B***), AtPDX1.3 (***C***) and AtPDX2 ***(D)*** and the protein kinase(s) putatively involved. The kinases PKA/PKG/PKC (given in grey) have not been identified in plants but respective homologous proteins, *e.g.* PVKP1, are known [Stone and Walker, 1995]. Conserved kinase recognition sites for the PDX1 proteins are indicated by an asterisk. The significance threshold was set to 60%. Abbreviations used: ATM: ataxia telangiectasia mutated nuclear protein kinase; CDC2: cell division control protein kinase 2; CDK5: cyclin-dependent kinase; CKII: casein kinase II; DNAPK: DNA-activated protein kinase; $MAPK^{P38}$: mitogen-activated protein kinase P38; PVPK1: (*Phaseolus vulgaris*) protein kinase 1; PKA/PKG/PKC: protein kinase A/C/G. The data was obtained from http://www.cbs.dtu.dk /services/NetPhos/ and http://www.cbs.dtu.dk/services/NetPhosK/ [Blom *et al.*, 1999].

4.8 Acknowledgements

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5 Outlook

5.1 Vitamin B_1 biosynthesis

In the present study we have demonstrated that a major pathway of *de novo* synthesis of the pyrimidine moiety of vitamin B_1 in *A. thaliana* is akin to that of bacteria, and that it depends on the THIC protein. A biochemical investigation revealed the presence of an iron-sulfur cluster in this protein. However, structural insight is still lacking, even though a recent publication reported on the structure of ThiC from *Caulobacter crescentus*, but unfortunately the C-terminus was missing where the iron sulfur cluster is predicted to be [Chatterjee *et al.*, 2008]. The aerobic isolation of ThiC from *Salmonella enterica* resulted in a colourless protein solution without any spectroscopic feature [Martinez-Gomez and Downs, 2008]. *A. thaliana* THIC recombinantly expressed and aerobically isolated from *E. coli* was intensely brown in colour, and the reduction with dithionite indicated the presence of an iron-sulfur cluster. Crystallization studies with THIC from *A. thaliana* may be a promising to shed new light on this domain.

In addition, preliminary results from the application of ^{13}C-labeled pyridoxine to chloroplasts from *Spinacia oleracea* and subsequent mass spectrometric analysis gave evidence that, in addition to the bacterial ThiC-route, the pyrimidine moiety of vitamin B_1 might be synthesized like in yeast. Confirmation of these results would provide evidence that an alternative, albeit minor, pathway for this biosynthetic route exists *in planta*. In addition, the experimental setup may be expanded, *e.g.* by applying both [5,5',6-$^{13}C_3$]- and [2,2',3,4,4'-$^{13}C_5$]-pyridoxine as well as ^{13}C-labeled L-histidine, the second postulated precursor, as a substrate.

To date it remains unknown how the rearrangement reaction of AIR_t to HMP-P occurs exactly and furthermore, whether ThiC is the sole enzyme involved in this catalysis. No enzymatic studies have been performed with the plant enzyme. Setting up biochemical studies using AIR_t as a substrate would help to address these questions. Although it has not been in the focus of this study, many questions remain to be answered concerning vitamin B_1 biosynthesis in plants. This for example pertains to THI1 (At5g54770) from *A. thaliana*, an enzyme that has been isolated and structurally characterized to be orthologous to the THI4 protein from *S. cerevisiae* [Godoi *et al.*, 2006] and thus involved in the formation of HET-P, the second constituent, beside HMP-P, of vitamin B_1. However, the reaction catalyzed by this enzyme has not been elucidated.

In vitamin B_6-accumulating lines, the vitamin B_1 content is increased *ca.* two-fold above WT levels. It would be interesting to investigate how the two vitamins are linked. The vitamin B_6-accumulating line AtPDX1.1 OE has already been crossed with the vitamin B_1-deficient *THIC* mutant At*thiC* KO, and the progeny of the cross could be analyzed, to see whether the increase in the vitamin B_1 content observed in AtPDX1.1 OE may revert the vitamin B_1-deficient phenotype of At*thiC* KO to WT.

5.2 Vitamin B_6 biosynthesis: PDX1.1 and PDX1.3

Analysis of vitamin B_6-accumulating plants

A genetic approach demonstrated that it is possible to enhance the vitamin B_6 content in *A. thaliana* by constitutive over-expression of the endogenous *PDX1.1* alone or in combination with *PDX2*. Such plants have enlarged seeds. This could be related to an increase of storage compounds such as lipids, carbohydrates and proteins in the seeds. In addition, a comparison of cotyledons of WT and vitamin B_6-accumulating plants indicated that the cotyledons of the latter are increased in size, which would be indicative of larger embryos. To support these findings, embryos should be analyzed at the level of both cell number and cell size. In addition, it may be of interest to determine whether the increase in endosperm is based on increased cell number or cell volume or both.

The delay in development observed upon entry to bolting appears to be dependent on the actual vitamin B_6 content. The PDX1.1 over-expressing line L5 that accumulates vitamin B_6 *ca.* two-fold above WT is very interesting in this context, as in this line the developmental delay is not as pronounced as in AtPDX1.1 OE L2 and the progeny of the cross AtPDX1.1-2 OE L6, which contain *ca.* three and five times, respectively, of the vitamin B_6 content of WT. Therefore, this line should be considered in subsequent experiments with regard to seed yield and stress tolerance in particular.

Metabolite profiling of vitamin B_6-accumulating and -deficient plants, will reveal whether primary metabolism in these plants is generally altered or whether there is an enhanced translocation of metabolites into the seeds. This is of particular interest to reveal how vitamin B_6 assists in abiotic stress tolerance.

Whereas it was possible to over-express PDX1.1 in *A. thaliana*, such an approach failed for PDX1.3. A reason for the difference between the two proteins could be due to the reduced protein stability of PDX1.3, or on the other hand transcriptional gene silencing [Baulcombe,

2004]. The latter aspect may be addressed by transforming *A. thaliana* with *PDX1.3* under the control of a weaker promoter, *e.g.* the nopaline synthase promoter (pNOS). In this context, it is necessary to keep in mind that with the use of a relatively weak promoter the site of integration within the genome of *A. thaliana* may affect the level of gene expression to a greater extent than is the case with a stronger promoter such as the CaMV 35S [Lechtenberg, 2002].

Analysis of stress responses – Addressing the difference(s) between PDX1.1 and PDX1.3

A burst of singlet oxygen in chloroplasts of *A. thaliana flu* mutant lines induced by a dark-light-shift causes chlorotic lesions on the leaves accompanied by the release of electrolytes, indicative of cell death. In *A. thaliana flu* mutant lines over-accumulating vitamin B_6, the release of electrolytes was reduced and accompanied by a significant reduction of the total vitamin B_6 content. This might be explained by an active degradation of the vitamin supporting the notion that vitamin B_6 is a potent quencher of reactive oxygen species *in vitro* and *in vivo* [Bilski *et al.*, 2000; Ehrenshaft *et al.*, 1998/1999a/b; Osmani *et al.*, 1999]. To prove this interpretation, plant extracts could be analyzed by mass spectrometry for the presence of vitamin B_6 degradation products. Furthermore, as singlet oxygen is characterized by a very short half life, thus disabling the compound from crossing the chloroplast envelope membrane, vitamin B_6 should be in close proximity to the site of singlet oxygen evolution, *i.e.* in the chloroplasts near the photosystem II reaction centre. Therefore, it would be worth checking for the presence of vitamin B_6 in the membrane systems and/or chloroplasts of the plant. This may be addressed for example by non-aqueous fractionation studies.

The regulation of vitamin B_6 biosynthesis was investigated by determining the relation between vitamin B_6 *de novo* synthesizing enzymes (at the transcript level) and the total vitamin B_6 content upon stress treatment. A discrepancy between transcript (or protein) abundance and the vitamin B_6 level was observed frequently, which may be explained by the presence of additional mechanisms regulating enzyme activity, *e.g.* inhibition and post-translational modifications. In a first instance, protein abundance should be investigated and related to the actual enzyme activity, thereby providing information beyond that obtained from the steady-state level of vitamin B_6. This approach will also help to elucidate whether inhibition of enzyme activities or degradation of the vitamin are responsible for the reduction of the vitamin B_6 levels. Phosphorylation/dephosphorylation is a well-known mechanism to alter enzyme activity post-translationally, thus circumventing costly *de novo* protein biosynthesis [Swarup, 1998]. Putative phosphorylation sites in the amino acid sequence have been identified (see chapter 4,

supplementary table 4.3). Western blot analysis of proteins from stressed plants with and without phosphatase treatment, as well as mass spectrometry, will allow the determination of whether such a modification is valid for the PDX proteins as well. The enzymes involved in the salvage pathway (PDX3 and SOS4) as well as the predicted specific PL-kinase [González *et al.*, 2007] have not been in the focus of this study. To fully elucidate the putative link between vitamin B_6 and stress, it will be essential to investigate them at both the transcript and protein levels.

The transcript analysis of WT seedlings revealed that particularly for rose bengal, sucrose and NaCl, responses at the transcript level differ between *PDX1.1* and *PDX1.3*. Furthermore, plants lacking *PDX1.1* are more severely affected by a combination of high light intensity and low temperature as well as by a rose bengal treatment, but interestingly, are more tolerant against paraquat treatment than plants lacking *PDX1.3*, whereas under normal growth conditions *PDX1.3* is more prevalent than its close homolog [Titiz *et al.*, 2006]. To address this in more detail, the stress treatments should be extended towards both *A. thaliana PDX1* knockout lines, *i.e.* At*pdx1.1* KO and At*pdx1.3* KO.

Salt treatments of various plants evoked more questions than could be answered. The *A. thaliana sos4* mutant has been reported to be hypersensitive to NaCl treatment [Shi *et al.*, 2002]. This line is characterized by enhanced PLP levels [González *et al.*, 2007] questioning the prediction that vitamin B_6 can assist in NaCl tolerance. Further doubt came from the observation that the vitamin B_6-deficient At*pdx1.3* KO mutant line is more tolerant to NaCl treatment [González *et al.*, 2007]. Our own studies showed the root growth of the vitamin B_6-accumulating plants AtPDX1.1 OE and AtPDX1.1-2 OE did not differ from WT which may also be attributed to the fact that vitamin B_6 does not accumulate in the roots, whereas soil-grown bolting plants appeared to be more tolerant to a single dose of NaCl. A possible explanation would be that the enhanced level of vitamin B_6 alone is not sufficient to provide salt tolerance. Thus, vitamin B_6 may not be involved in preventing the influx of Na^+ as was proposed from animal systems, but may positively stimulate the efflux of the cation. Based on the details mentioned above, this may depend on the activity of SOS4. Therefore, interaction studies using SOS4 should be performed, and the influx-efflux of Na^+ should be determined in the various lines in the presence, as well as in the absence, of pyridoxine. Furthermore, our study showed that the simultaneous application of NaCl and sucrose prevented the up-

regulation of SOS4. Thus, it would be interesting to address the salt tolerance of the plants in the presence and absence of sucrose as well.

5.3 Regulation of vitamin B_6 biosynthesis: PDX1.2

It has been hypothesized that PDX1.2 may be a negative regulator of *de novo* vitamin B_6 biosynthesis [Tambasco-Studart, 2007]. The data presented in this study provide evidence that a regulatory role for PDX1.2 under specific circumstances can be visualized. However, for a complete understanding, several additional experiments need to be performed, *e.g.*:

Putative complex formation between PDX1.1 and PDX1.2

Initial results presented in this study suggest that PDX1.1 and PDX1.2 can form a complex of an appr. mass of app. 66 kD *in vivo*. This finding is based on preliminary Western blot analysis using specific antibodies as well as on the fact that the complex was first observed by Western blot analysis in protein extracts of lines over-expressing PDX1.2 [Tambasco-Studart, 2007] or PDX1.1, *i.e.* AtPDX1.2 OE and AtPDX1.1 OE, respectively. However, the final proof that PDX1.1 and PDX1.2 are indeed part of the complex is missing. Furthermore, the vitamin B_6 content should be related to the presence of the complex. A comparable analysis of lines carrying constitutively enhanced levels of both PDX1.1 and PDX1.2 (AtPDX1.1-1.2 OE) as well as lines over-expressing PDX1.2, but lacking either PDX1.1 or PDX1.3 (At*pdx1.1* PDX1.2 OE; At*pdx1.3* PDX1.2 OE) should be carried out in this context. These lines have already been established and are available for characterization.

Elucidation of the function of PDX1.2 during abiotic stress responses

The above-mentioned lines represent a suitable material to investigate the role of PDX1.2 during stress, in particular at the level of vitamin B_6. Attention should be paid particularly to progeny of the cross between AtPDX1.2 OE and At*pdx1.1* KO or At*pdx1.3* KO (in both WT and At*flu* KO background). PDX1.2 RNAi lines in *A. thaliana* WT, AtPDX1.1 OE, At*flu* KO background will help to further back up the results (the PDX1.2 RNAi lines have been established, but the level of PDX1.2 protein remains to be quantified).

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