Doctoral Thesis

Metabolic engineering of Vitamin B1 and Vitamin B6 in staple crops

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METABOLIC ENGINEERING OF VITAMIN B1 AND VITAMIN B6 IN STAPLE CROPS

A thesis submitted to attain the degree of

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(Dr. sc. ETH Zurich)

presented by

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2014
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Abstract

Vitamin B1 and vitamin B6 are essential micronutrients for human health and plant growth. Both vitamins serve as cofactors in metabolic pathways, and are widely known as health-promoting compounds. Plants use \textit{THI1} and \textit{THIC} genes to produce thiazole and pyrimidine moieties separately, and subsequently couple these two moieties by a condensation to generate vitamin B1. The vitamin B6 \textit{de novo} biosynthesis pathway involves only two genes, \textit{PDX1} and \textit{PDX2}, in plants. Vitamin B1 and vitamin B6 deficiencies are associated with various chronic diseases including mental and physical disorders. The thesis aims at improving the vitamin B1 and vitamin B6 content in both cassava and rice via genetic engineering.

Strategies to increase vitamin B6 content in cassava and rice were based on the constitutive or edible organ-specific expression of Arabidopsis \textit{PDX1.1} and \textit{PDX2} genes. Transgenic cassava showed a maximal 10-fold increase in vitamin B6 content in leaves using the constitutive expression strategy and a maximal 12-fold increase in storage roots using root-specific expression strategy under field conditions. Transgenic rice showed up to 2-fold increase of vitamin B6 content in unpolished rice grains of both constitutive and endosperm-specific expression vectors. The results demonstrate the potential of vitamin B6 biofortification in cassava and rice.

Strategies to increase vitamin B1 content by constitutive expression of endogenous \textit{THI1} and/or \textit{THIC} genes have been conducted in cassava and rice. Transgenic cassava displayed no increase or even decreased levels of vitamin B1 content in leaves and storage roots. Moreover, adverse effects on plant growth (e.g. chlorotic leaf, die back of top) were observed in several transgenic cassava lines. Transgenic rice showed no consistent increase of vitamin B1 content. Some individual transgenic rice plants over-expressing \textit{THI1} or \textit{THIC} displayed higher vitamin B1 content at the seedling stage.

Additionally, transgenic plants based on constitutive or edible organ-specific expression of Arabidopsis \textit{THIC} were used to evaluate the impact of exogenous \textit{THIC} on cassava and rice. Transgenic cassava using both constitutive and root-specific expression strategies showed decreased levels of vitamin B1 in leaves and storage roots of several lines. Transgenic rice showed up to 1.4-fold increase at seedling stage using the constitutive expression strategy and up to 1.6-fold increase in polished seeds using endosperm-specific expression strategy.

The feasibility of vitamin B6 biofortification in cassava and rice was proven and is described in the thesis. It offers a sustainable and cost-effective approach to alleviate vitamin B6 deficiency in population having a diet mostly relying on cassava or rice consumption. Further characterization of transgenic plants expressing \textit{THI1} and \textit{THIC} will help to investigate the vitamin B1 biosynthesis in cassava and rice.
Zusammenfassung


Zusätzlich wurde THIC aus Arabidopsis konstitutiv und Speiseorgan-spezifisch in Maniok und Reis überexprimiert. Unabhängig von der Expressionsstrategie wurden in Blättern und Speicherwurzeln von transgenen Cassava verminderte Vitamin B1 Mengen gemessen. Transgene Reispflanzen zeigten unter konstitutiver Expression eine maximal 1,4-fache Steigerung des Vitamin B1 Gehaltes im Keimlingsstadium und bis zu 1,6-fache Erhöhung in polierten Körnern unter Endosperm-spezifischer Expression.

Die Machbarkeit von Vitamin B6 Biofortifikation in Maniok und Reis wurden in dieser Arbeit nachgewiesen und beschrieben und bietet einen nachhaltigen und kostengünstigen Ansatz zur Linderung von Vitamin B6 Mangel in einer Bevölkerung, die zur Ernährung hauptsächlich
auf Reis und Cassava angewiesen ist. Die weitere Charakterisierung von THI1 und/oder THIC exprimierenden Pflanzen wird zum Verständnis der Vitamin B1 Biosynthese in Cassava und Reis beitragen.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>35S promoter</td>
<td>CaMV 35S promoter</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA synthesized from RNA</td>
</tr>
<tr>
<td>cv.</td>
<td>Cultivar</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DW</td>
<td>Dry weight</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh weight</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<tr>
<td>hpt</td>
<td>Hygromycin phosphotransferase</td>
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<tr>
<td>nos</td>
<td>Nopaline synthase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PL</td>
<td>Pyridoxal</td>
</tr>
<tr>
<td>PLG</td>
<td>Pyridoxal 5’-glucoside</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal 5’-phosphate</td>
</tr>
<tr>
<td>PM</td>
<td>Pyridoxamine</td>
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<tr>
<td>PMG</td>
<td>Pyridoxamine 5’-glucoside</td>
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<td>PMP</td>
<td>Pyridoxamine 5’-phosphate</td>
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<td>PN</td>
<td>Pyridoxine</td>
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<tr>
<td>PNG</td>
<td>Pyridoxine 5’-glucoside</td>
</tr>
<tr>
<td>PNP</td>
<td>Pyridoxine 5’-phosphate</td>
</tr>
<tr>
<td>PP2A</td>
<td>Serine/Threonine protein phosphatase catalytic subunit 2A</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>QTLs</td>
<td>Quantitative trait locus</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SB</td>
<td>Southern blot</td>
</tr>
<tr>
<td>SSA</td>
<td>Sub-Saharan Africa</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>T-DNA</td>
<td>A. tumefaciens transfer DNA</td>
</tr>
<tr>
<td>TMP</td>
<td>Thiamin monophosphate</td>
</tr>
<tr>
<td>TPP</td>
<td>Thiamin pyrophosphate</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin-conjugating enzyme E2</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
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Chapter 1 Introduction

Micronutrient malnutrition

More than half of people in the world especially in low and middle income countries suffer from a deficiency of essential micronutrients (Bouis et al., 2011; Mayer et al., 2008). Micronutrient malnutrition, the so-called “hidden hunger”, impairs mental and physical development in both infants and children, and reduce labor productivity of adults (Muthayya et al., 2013). Besides that, more than 50% of childhood deaths in the world are caused by micronutrient malnutrition, a significant threat to public health and economic development globally (Welch and Graham, 2004). Humans cannot synthesize essential micronutrients and obtain 13 vitamins and at least 22 minerals from their daily diet to maintain physiological processes (Fitzpatrick et al., 2012; White and Broadley, 2009). Vitamins are classified into two groups based on their solubility. There are four fat-soluble vitamins (A, D, E, and K) and nine water-soluble vitamins (B1, B2, B3, B5, B6, B8, B9, B12 and C). Seven mineral elements deficiencies including iron, zinc, copper, calcium, magnesium iodine and selenium remain prevalent and have a low content in edible organs of major staple crops (White and Broadley, 2009). On the contrary, plants can synthesize all vitamins de novo except for vitamin B12 and accumulate most minerals in tissues (Smith et al., 2007; White and Broadley, 2005). Plant-based foods are major sources of essential micronutrients for humans especially in resource-poor populations who rely on single staple as daily diet. Vitamin A, iron, zinc and iodine are the four predominant deficient micronutrients worldwide (Bouis and Welch, 2010; Mayer et al., 2008; Pfeiffer and McClafferty, 2007). There are other micronutrient deficiencies such as B-vitamins which are also common in both developed and developing countries (Muthayya et al., 2013).

The relationship between plant-based diets and human health has been well recognized and the benefits of increased nutrient uptake from foods includes an improvement in human health (Martin et al., 2011; Zhu et al., 2013). Besides its role in providing essential micronutrients, several vitamins (e.g. vitamin A and vitamin C) have been reported to increase absorption of minerals in the human gut (White and Broadley, 2005). The antioxidant properties of vitamins such as vitamin A, B1, B6, C and E lead to the elimination of reactive oxygen species (ROS), which can prevent chronic disease and mortality associated with these chronic diseases (Martin et al., 2013; Zhu et al., 2013). Other phytonutrients (e.g. anthocyanins, flavonoids) have also been demonstrated to improve human health especially when present in the food matrix than in their purified forms (Martin, 2013; Martin et al., 2011). For example, anthocyanins derived from foods can ameliorate the negative effects of high fat diet in mice, but the same beneficial impact was not observed with the
supplementation of purified anthocyanins (Guo et al., 2007; Prior et al., 2008; Titta et al., 2010).

Several strategies including dietary diversification, supplementation and food fortification have been suggested to tackle malnutrition problems (Newell-McGloughlin, 2008; White and Broadley, 2009; Zhu et al., 2007). However, due to technical and economic difficulties such as lack of extensive infrastructure and financial support especially in developing countries, these strategies cannot solve all malnutrition problems (Bhullar and Gruissem, 2013). Therefore, biofortification of crops through agronomic and/or breeding approaches can be a good strategy to combat micronutrient malnutrition. Biofortification is simply replacing ordinary crop varieties in existing food production systems without additional infrastructures and cost. This approach has been reported as one of the most effective and sustainable methods to tackle micronutrient malnutrition especially in developing regions such as Africa and south Asia where the most vulnerable groups, children and women subsist on single staple crop as the major diet (Bouis et al., 2011; Meenakshi et al., 2010). Most staple crops cannot provide all essential vitamins and minerals to meet human daily requirements (Fitzpatrick et al., 2012). Successful examples of orange sweet potato in African countries demonstrated the feasibility of implementing a biofortified staple crop to address vitamin A deficiency (Hotz et al., 2012; Low et al., 2007). More recently, a human study also demonstrated that consumption of biofortified cassava could potentially prevent vitamin A deficiency (La Frano et al., 2013).

**Strategies for biofortification**

Biofortification is used to increase the nutrients density of staple crops via agronomic practices or plant breeding techniques. Three approaches have been adopted to develop biofortified crops: 1) mineral fertilization; 2) conventional breeding; 3) transgenic approaches (Hirschi, 2009; Zhu et al., 2007). It has been reported that application of mineral fertilizer can significantly improve mineral elements content of crops in agricultural system (Rengel et al., 1999; White and Broadley, 2009). Unlike vitamins or other macronutrients, plants can take up minerals only from the external environment. Both foliar and soil applications of zinc fertilizers can increase levels of zinc in cereal grains such as wheat, rice and maize (Cakmak, 2008; Fang et al., 2008; Harris et al., 2007; Zou et al., 2012). The aforementioned strategies were also used to increase selenium content in wheat (Hawkesford and Zhao, 2007; Lyons et al., 2003). Irrigating with KI or KIO₃ solution in soils has been demonstrated to improve the uptake of iodine element from the soils to tomato fruits for biofortification purpose (Kiferle et al., 2013). However, the efficiency of this method depends on the soil types and crop species (Zhu et al., 2007). The phytoavailability of mineral element uptake by plants is based on the soil properties (e.g. soil pH, cation exchange capacity, microbes composition, soil structure) and its chemical forms (Frossard et
al., 2000; White and Broadley, 2009). Plants, for example, find it more difficult to take up iron and zinc when growing on calcareous or alkaline soils (White and Broadley, 2009). Legume seeds have a higher efficiency of accumulating iron and zinc than cereal grains (White and Broadley, 2005; White and Broadley, 2009). Foliar application of Zn-amino acid and ZnSO$_4$ increased levels of zinc content in polished rice grains than the application of Zn-EDTA and Zn-citrate (Wei et al., 2012). Moreover, the accumulation of minerals in different plant tissues is controlled by genetic factors, which are not able to target edible organs by mineral fertilization. In addition, the fertilization approach only works for mineral fortification and cannot be used for vitamin biofortification (Bhullar and Gruissem, 2013; Zhu et al., 2007). The over-accumulation of minerals in soil may also pollute the environment or be toxic to plants (Rengel et al., 1999; Zhao and McGrath, 2009). This drawback limits the application of mineral fertilization for crop biofortification.

Conventional breeding is another approach to increase the nutritional value of staple crops. The genetic diversity in natural varieties can contribute to improving the levels of target nutrients in crops. A survey of micronutrients composition of major staple crops displays a high variation in different varieties (Hotz and McClafferty, 2007; Kandlakunta et al., 2008; Kennedy and Burlingame, 2003; White and Broadley, 2009). For instance, screening different cassava genotypes showed a 10- and 38-fold variation in vitamin A and iron content respectively in storage roots, and up to a 250-fold variation in vitamin C content was reported in cassava leaves (Chávez et al., 2000; Chávez et al., 2005). In tomato, concentration of vitamin C in fruits varies more than 50-fold, whereas in kiwi fruit, a 10-fold difference in vitamin C content was reported (Bulley et al., 2012; Galiana-Balaguer et al., 2006). The elucidation of molecular mechanism in vitamin A, C and E biosynthesis has been reported in several plant species. Single nucleotide polymorphism (SNP) in genes associated with vitamin A biosynthesis has been found in cassava (Welsch et al., 2010), maize (Harjes et al., 2008), and watermelon (Bang et al., 2007). For example, an allelic polymorphism of phytoene synthase (PSY) gene, a rate limiting factor in the vitamin A biosynthesis pathways, can lead to a 2-fold increase of carotenoid content in cassava storage roots (Welsch et al., 2010). Few nucleotide substitutions of gene sequences would dramatically increase the enzymatic activity to enhance vitamin A accumulation in plants. QTLs for vitamin C accumulation were reported in tomato, strawberry, and apple (Fitzpatrick et al., 2012). Furthermore, QTLs associated with vitamin E biosynthesis have been found in soybean, tomato, and Arabidopsis (Almeida et al., 2011; Gilliland et al., 2006; Li et al., 2010). The discovery of SNPs and QTLs for nutritional traits can be used for selecting varieties with high levels of various micronutrients. An understanding of biosynthesis pathways could be integrated into both conventional breeding techniques (e.g. marker-assisted selection, and QTL mapping) and genetic engineering to bio-fortify crops. However, propagated crops (e.g. banana and cassava) and the crops having a long vegetative period such as bamboo are not suitable for conventional breeding. Conventional breeding is also limited by existing traits. For example, it is difficult to increase vitamin A content in rice endosperm through
conventional breeding because the endosperm does not accumulate vitamin A (Ye et al., 2000).

Transgenic approaches provide a rapid, targeted and relatively faster solution to develop new crop varieties (Lemaux, 2008). Another advantage is that elite varieties with good agronomic traits can be improved for other traits directly without possibly altering the agronomic performance, thus, accelerating the breeding process. It was reported that the genetic diversity of vitamin A content in several staple crops is not sufficient to increase this vitamin via conventional breeding such as wheat, beans, cowpeas and pearl millet (Hotz and McClafferty, 2007). Transgenic approaches have been successfully applied to increase vitamin A content in polished rice grains, the so-called “Golden Rice”, by engineering transgenes from rice or the other plant species (Paine et al., 2005; Ye et al., 2000). Consumption of Golden Rice has been estimated to alleviate vitamin A deficiency significantly, which means thousands of children could be saved (Stein et al., 2006). Another example is the expression of endogenous dihydrodipicolinate synthase (DHDPS), the rate limiting enzyme in lysine biosynthesis pathway in plants, which is inhibited by the over-production of lysine (Ufaz and Galili, 2008). Although using the DHDPS gene from plants as a transgene could not significantly increase lysine content in transgenic plants because of the feedback regulation, transgenic tobacco and Arabidopsis transformed with lysine insensitive DHDPS gene from E. coli showed an increased level of lysine content (Shaul and Galili, 1992; Tzchori et al., 1996). This reveals that the technical feasibility of introduction of traits from microorganisms into plants. Tremendous progress of biofortification has been made in using transgenic approaches for biofortification in several important crops such as wheat, rice, maize, and cassava (Hirschi, 2009; Newell-McGloughlin, 2008; Sayre et al., 2011).

In this thesis, biofortification strategies for two essential micronutrients, vitamin B1 and vitamin B6, in cassava and rice will be tested. Both vitamin B1 and vitamin B6 are water-soluble and do not store for a long time in the human body. Therefore, increasing the levels of these two vitamins in staple crops could solve their deficiency in the daily diets of humans.

Target micronutrients: vitamin B6 and vitamin B1

Vitamin B6: Introduction

Vitamin B6 is an essential micronutrient for all forms of life and involved in protein, carbohydrate and lipid metabolism (Fitzpatrick et al., 2012). Mammals, including humans acquire vitamin B6 from their daily diet. In addition to taking it up from external environment, microorganisms and plants can also synthesize vitamin B6 de novo (Szydlowski et al., 2013). Vitamin B6 is composed of a mixture of so called vitamers pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and its phosphorylated derivatives pyridoxal 5’-phosphate (PLP), pyridoxine 5’-phosphate (PNP), pyridoxamine 5’-phosphate (PMP). PLP and
PMP serve as cofactors for several enzymes in more than 140 metabolic pathways to maintain physiological functions (di Salvo et al., 2012). PM and PL are abundant in animal tissues, whereas PN is the predominant form in plants. In addition to the six vitamers, pyridoxine-5'-β-glucoside (PNG) is the most predominant storage form of vitamin B6 in plants, accounting for 5-75% of total vitamin B6 pool in different plant-based foods (Gregory III, 1998). The free and phosphorylated forms of vitamin B6 are fully bioavailable, while the PNG form is only 50% bioavailable for human consumption (Gregory III, 2012).

Besides human nutrition and medical use, vitamin B6 and its derivatives are commonly used in feed additives, aquaculture, and cosmetic products. For instance, addition of PN to feed promoted growth of weanling pigs (Woodworth et al., 2000) while increased levels in dietary supplement of fish fingerlings can trigger immune response leading to improved disease resistance (Akhtar et al., 2012; Akhtar et al., 2010; Feng et al., 2010). Vitamin B6 and its derivatives are also widely used in cosmetic products (Baldessari et al., 1998; Snider and Dieteman, 1974).

Potato is the only crop out of the five most important staples (wheat, rice, maize and cassava) with potentially high vitamin B6 content sufficient to meet the recommended dietary allowance of 1.3 mg/day (Fitzpatrick et al., 2012). The human populations particularly in developing countries where people rely on these staple crops as the major diet usually suffer from vitamin B6 deficiency. Elucidation of vitamin B6 biosynthesis in plants has been mostly conducted in the model plant Arabidopsis. The accumulated high levels of vitamin B6 in transgenic Arabidopsis plants opens up the possibility of addressing vitamin B6 deficiency issue in staple crops via genetic engineering (Chen and Xiong, 2009; Raschke et al., 2011). The current knowledge of vitamin B6 in plant genetics, physiology, metabolism and molecular biology can be exploited of the development of new strategies for vitamin B6 biofortification through genetic engineering.

**Vitamin B6: Benefits for humans**

Vitamin B6 deficiency in humans is associated with various diseases and disease processes such as diabetes, neurological disease, cardiovascular disease, tumor progression, inflammation, pellagra and mental disorders (Galluzzi et al., 2013; Hellmann and Mooney, 2010; Larsson et al., 2010; Morris et al., 2010). Besides its role as a cofactor, several studies suggest that vitamin B6 could function as a protectant against reactive oxygen species (Jain and Lim, 2001) and prevent chronic diseases and cancers independently or in combination with other micronutrients (Fitzpatrick et al., 2012; Galluzzi et al., 2013). Epidemiological evidence showed increased levels of vitamin B6 supplements were associated with the improved survival rate of HIV patients (Tang et al., 1996). Of particular interest is PM, which has pharmaceutical functions preventing and ameliorating diabetic complications and aging-related diseases (Ahmad et al., 2013; Lewis et al., 2012; Metz et al., 2003; Voziyan and
Hudson, 2005). More recently, vitamin B6 deficiency has been postulated to be one of the factors contributing to an epidemic disease referred to as nodding syndrome in South Sudan and northern Uganda, and medical studies have revealed the prevalence of vitamin B6 deficiencies in developing countries (di Salvo et al., 2012; Foltz et al., 2013). Remarkably, suboptimal vitamin B6 status is also prevalent in the USA (Morris et al., 2008). Women, elderly people and populations with limited diet diversity appear to be particularly at risk of vitamin B6 deficiency.

**Vitamin B6: Benefits for plants**

The benefit of vitamin B6 for plants has been reviewed (Vanderschuren et al., 2013). The active cofactor form of vitamin B6, PLP, was estimated to interact with 177 enzymes in Arabidopsis (Percudani and Peracchi, 2009). These PLP-dependent enzymes participate in various metabolic pathways including starch, auxin and ethylene metabolism in plants (Mooney and Hellmann, 2010). It has been reported that PN can act as a protectant to scavenge reactive oxygen species (ROS) as well as vitamin C and vitamin E (Jain and Lim, 2001; Kannan and Jain, 2004). Analysis of Arabidopsis loss-of-function mutants for genes involved in vitamin B6 biosynthesis confirmed the importance of this vitamin in plant development and abiotic stress responses (Chen and Xiong, 2005; Titiz et al., 2006). Transgenic Arabidopsis accumulating high levels of vitamin B6 showed increased biomass as well as improved oxidative stress tolerance (Raschke et al., 2011). The importance of vitamin B6 in plant responses to stress is also illustrated by an increased vitamin B6 accumulation in tobacco subjected to abiotic stresses such as chilling, UV radiation, drought, osmotic pressure and high light intensity (Huang et al., 2013). When exposed to UV-B light, Arabidopsis also showed vitamin B6 accumulation (Ristilä et al., 2011). The genes associated with the vitamin B6 de novo biosynthesis pathway (AtPDX1.1, AtPDX1.3 and AtPDX2) were up-regulated in Arabidopsis when the plant was under abiotic stress pressure (e.g. high light, chilling, ozone) (Denslow et al., 2007). In addition, exogenous infiltration of PN can trigger an hypersensitive response to alleviate the bacterial infection in tobacco leaves (Denslow et al., 2005). From these observations, it has been postulated that enhanced levels of vitamin B6 may improve biotic and abiotic stress tolerance in plants. The antioxidant properties of vitamin B6 can provide new directions of improving traits in crops.

**Vitamin B1: Introduction**

Vitamin B1 was the first vitamin that was characterized by Casimir Funk, who isolated an essential compound from rice bran in 1912 (Funk, 1912), which later was identified as vitamin B1 (Eggersdorfer et al., 2012). The de novo synthesis of vitamin B1 occurs in only microorganisms and plants with mammals acquiring vitamin B1 from their daily diet (Goyer,
Vitamin B1 plays a central role in the citric acid cycle, pentose phosphate pathway, branched-chain amino acid biosynthesis, isoprenoid biosynthesis as well as the Calvin cycle in photosynthetic plants (Goyer, 2010; Moulin et al., 2013). Three predominant B1 vitamers exist: thiamin, thiamin monophosphate (TMP) and thiamin pyrophosphate (TPP). These three B1 vitamers have equivalent bioavailability for human consumption (Gregory III, 2012). TPP is the active form which serves as a cofactor for several enzymes of carbohydrate and amino acid metabolism.

Potato and cassava contain high levels of vitamin B1, which is assumed to be sufficient for human daily requirements (Fitzpatrick et al., 2012). However, the vitamin B1 content in cassava was calculated based on the raw storage roots. The actual value of vitamin B1 in cooked cassava needs further determination. The vitamin B1 content in the important cereal crops is below the recommended dietary allowance (1.2 mg/day) in a standard portion of daily consumption (e.g. wheat, rice and maize) (Fitzpatrick et al., 2012). The aforementioned study estimated that a 3.9-, 5.7- and 3-fold increase in vitamin B1 in wheat, rice and maize can meet human daily requirements, respectively.

**Vitamin B1: Benefits for human**

Vitamin B1 deficiency causes a well-known neurological disease, the so-called “beriberi”, which is common in populations with limited diet diversity and mostly rely on polished rice as a staple food (Fitzpatrick et al., 2012). In African countries, where people consume improperly processed bitter cassava as a major staple, another neurological disease, konzo, is also associated with vitamin B1 deficiency (Adamolekun, 2010). In addition, vitamin B1 deficiency is associated with mental disorders, congestive heart failure, alcoholism and HIV infection (Hanninen et al., 2006; Luong and Nguyẽn, 2013; Sriram et al., 2012). Epidemiological studies have demonstrated that supplementation of dietary intake with vitamin B1 can slow HIV progression and increase the survival rate of HIV patients (Tang et al., 1993; Tang et al., 1996). Prevalence of vitamin B1 deficiency remains high in tropical countries especially in south east Asian countries (e.g. Laos) where people consume single staple foods with low vitamin B1 content (Coats et al., 2013; Kauffman et al., 2011; Khounnorath et al., 2011; Muthayya et al., 2013). According to a national survey in 2002, nearly 80% of adults in China do not take adequate vitamin B1 (below the recommended dietary allowance 1.3 mg/day) (Zhang et al., 2013). Elderly, infants and refugees are at high risk of vitamin B1 deficiency (Khounnorath et al., 2011).
Vitamin B1: Benefits for plants

Besides its role as an essential micronutrient, vitamin B1 is also a key factor in plant responses to both abiotic and biotic stresses. Arabidopsis and maize accumulate vitamin B1 when exposed to abiotic stresses such as salt, drought, and oxidative damage (Rapala-Kozik et al., 2008; Rapala-Kozik et al., 2012). The accumulation of vitamin B1 is potentially due to an up-regulation of genes associated with vitamin B1 biosynthesis by stress stimuli. Also, under dehydrating conditions, genes involved in vitamin B1 metabolism were up-regulated in a desiccation-tolerant plant (C. plantagineum) (Rodriguez et al., 2010). These results infer the important role of vitamin B1 in plant stress response or adaptation. Another interesting approach to prime plants against both biotic and abiotic stresses has been to soak seeds in thiamin solution or spray plants with thiamin. This approach resulted in improved salt tolerance in sunflower and wheat (Al-Hakimi and Hamada, 2001; Sayed and Gadallah, 2002), and induced plant defense systems in Arabidopsis, cucumber, grapevine and rice (Ahn et al., 2005; Ahn et al., 2007; Bahuguna et al., 2012; Boubakri et al., 2012). Furthermore, thiamin has been reported to induce systematic acquired resistance through priming to combat viral, fungal and bacterial infections (Ahn et al., 2005; Ahn et al., 2007). Treatments with thiamin can also reduce the nematode population on eggplant (El-Zawahry and Hamada, 1994; Hamada et al., 2001). More recently, exogenous application experiments in pea and barley showed that thiamin can reduce aphid population growth and aphid acceptance (Hamada and Jonsson, 2013). In the aphid infestation study, thiamin was demonstrated to induce the expression of defense-related genes in barley. However, the comprehensive mechanism of thiamin-induced defense system is still elusive. Nevertheless, these observations suggest the potential use of thiamin as an alternative to chemical insecticides and fungicides for the control of pests and pathogens in crop production. Spraying or pretreatment of crop plants with thiamin can be a novel and environmentally friendly approach to pathogen and pests control in agricultural systems. With the exception of transgenic Arabidopsis which was reported to show a 1.5-fold increase in vitamin B1 under short day conditions (Bocobza et al., 2013), increasing endogenous thiamin content has not yet been achieved in any crop plant through genetic engineering. Biofortification of vitamin B1 in staple crops still remains a potential and innovative approach to tackle micronutrient malnutrition and improve tolerance to different biotic and abiotic stresses.

Target crops: cassava and rice

Importance of cassava

Cassava (Manihot esculenta Crantz) is one of the most important staple crops in tropical and subtropical countries, where more than 800 million people rely on its storage roots as a major calorie source (Burns et al., 2010). Cassava ranks amongst the top six staple crops in terms of global production (http://faostat.fao.org/), and is the most important staple in Sub-
Saharan Africa. Several factors including, tolerance to drought stress, simple propagation by stem cutting, flexible harvesting date as well as relatively good agronomic performance under suboptimal environment with low input account for its worldwide cultivation by smallholder farmers in developing countries (Burns et al., 2010; Cock, 1982; El-Sharkawy, 2004). The flexible harvesting times of cassava, from six months up to three years, makes it a good food security crop especially in times of famine (Burns et al., 2010). Recently, it has been reported that cassava can have an increased yield potential under elevated CO₂ conditions (Rosenthal et al., 2012). This highlighted the role of cassava for food production under changing climatic conditions. In addition to smallholder farmer plantation as a staple food, cassava is also an economic crop in large-scale cultivation for biofuel and industrial starch production because of its high yield potential (Anggraini et al., 2009; Jansson et al., 2009).

Besides its storage roots, cassava leaves are important source of several micronutrients and proteins including vitamin B1, vitamin B2 and vitamin C for human nutrition and animal feed in several African and Asian countries (Montagnac et al., 2009). The leaves contain high levels of protein which range from 14-40% on dry weight basis (Eggum, 1970). In contrast, the storage roots, which are the main part of the plant that is consumed, are rich in carbohydrates, accounting for nearly 90% on dry weight basis. However, the protein content is low in storage roots which ranges from 1-3% on a dry weight basis and 0.4-1.5% on a fresh weight basis in different varieties (Montagnac et al., 2009). The sulfur-containing essential amino acids including methionine and cysteine are also low in cassava storage roots (Stupak et al., 2006). The B vitamins content is also low in storage roots and lost partially due to cooking processes (Montagnac et al., 2009). Consumption of cassava storage roots does not provide all required elements due to low protein content and low levels of certain essential micronutrients (Montagnac et al., 2009; Sayre et al., 2011). It provokes major nutritional deficiencies for populations whose diets are predominantly cassava-based. Moreover, the populations who rely on cassava storage roots as a staple are usually suffering from the Protein-Energy-Malnutrition in African countries (Stupak et al., 2006). In addition, consuming poorly processed bitter cassava exposes consumers to cyanide toxicity and may cause a neurological disease, konzo, due to the high content in cyanide (Adamolekun, 2010).

Limited information exists on the correlation between genetics and agronomic traits in cassava compared to the other important staple crops such as rice, wheat, and maize (Okogbenin and Fregene, 2003). Also, because of its high heterozygosity, allopolyploid and non-synergistic flowering time, it is difficult to develop new cassava varieties through conventional breeding (Ceballos et al., 2004). Thus, the development of cassava varieties with improved traits such as nutritional content, increased virus resistance, prolonged shelf-life and decreased cyanogen content through genetic engineering is a valuable option to address some of these drawbacks in this crop (Sayre et al., 2011). This approach has been given support with the launch of the BioCassava Plus program aimed at developing
biofortified cassava to solve some health problems in Sub-Saharan Africa (SAA) through genetic engineering (Sayre et al., 2011). Progress has been reported for iron, protein and β-carotene biofortification (Ihemere et al., 2012; Narayanan et al., 2011; Welsch et al., 2010).

**Importance of rice**

*Rice (Oryza sativa)* ranks amongst the top three staple crops in terms of global production (http://faostat.fao.org/). Over 50% of the world’s population, mostly in Asian countries, consumes rice as a daily staple. Rice is grown worldwide in Asia, America, Africa, the Caribbean and the Pacific as a major crop. The whole rice grain contains high levels of vitamins and minerals which are mostly accumulated in the embryo and aleurone layer. Consumption of whole rice grains has been reported to prevent chronic diseases such as cardiovascular diseases and cancers (Dipti et al., 2012). Nevertheless, to avoid rancidity during storage, rice grains are often polished to remove the embryo and aleurone layer and most micronutrients are removed together. The polished rice grains are the most common type for consumption in most countries.

However, consumption of polished grains does not provide the complete nutrients for human daily requirements. For example, all essential vitamins in standard portions of polished rice grains are below the recommended dietary allowance, e.g. vitamin B1 (18% RDA), vitamin B2 (12% RDA), vitamin B3 (29% RDA), vitamin B5 (78% RDA), vitamin B6 (31% RDA) and vitamin B9 (29% RDA) (Fitzpatrick et al., 2012). Polished rice grains do not contain vitamins A, C, D and B12. The levels of vitamin E and K in rice grains have not yet been reported. These micronutrient deficiencies are prevalent in populations who mainly rely on rice as a daily calories source.

Because of its worldwide importance, rice is considered to be a strategic target crop for biofortification to combat malnutrition and prevent chronic disease (Dipti et al., 2012). Increasing the levels of essential micronutrients and health-promoting compounds in polished rice grains can potentially address both malnutrition and chronic disease issues together. The genetic diversity of several nutrients has been documented (e.g. iron, zinc, vitamin B1) (Kennedy and Burlingame, 2003) and this knowledge of natural variation can be integrated into breeding programs. This enhances the feasibility of improving the nutritional value of rice by conventional breeding. A more targeted and promising strategy to develop new rice varieties is through the use of genetic transformation (Bashir et al., 2013; Bhullar and Gruissem, 2013). This strategy has been successfully conducted targeting diverse nutrients and health-promoting compounds including vitamin A, iron, zinc, folate, flavonoids and certain essential amino acids (Johnson et al., 2011; Lee et al., 2001; Ogo et al., 2013; Storozhenko et al., 2007; Wakasa et al., 2006; Wirth et al., 2009; Ye et al., 2000).
**Aim of this thesis**

The thesis aims at improving the nutritional quality of both cassava and rice via genetic engineering. It has two major components: 1) increasing vitamin B6 content in cassava and rice; 2) improvement of the vitamin B1 content in cassava and rice.

To examine the feasibility of vitamin B6 biofortification in staple crops, transgenic cassava and rice over-expressing two Arabidopsis genes, *AtPDX1.1* and *AtPDX2*, essential for vitamin B6 *de novo* biosynthesis have been generated. Both *PDX* transgenes were expressed under the control of the 35S promoter for constitutive expression. To investigate vitamin B6 accumulation in edible organs, a second vector has been generated in which *PDX* transgenes are under the control of root-specific *patatin* promoter to restrict the expression of transgenes to cassava roots. In another vector for rice transformation, *PDX* transgenes have been placed under the control of the endosperm-specific *globulin* promoter to restrict the expression of transgenes to rice endosperm.

Secondly, strategies to enhance vitamin B1 were explored in cassava and rice via genetic engineering. To avoid the issue of protein specificity in different plant species, endogenous *THI1* and *THIC* genes were cloned from cassava and rice for transformation. Transgenic plants over-expressing endogenous *THI1* or *THIC* have been generated to examine the impact of single moiety accumulation on vitamin B1 biosynthesis. Together, the transgenic plants over-expressing *THI1* combined with *THIC* were generated to increase vitamin B1 content. Both endogenous *THI1* and *THIC* were expressed under the control of the 35S promoter for constitutive expression. In addition, transgenic plants expressing *AtTHIC* were generated to investigate the impact of constitutive and edible organ-specific expression of exogenous *THIC* in cassava and rice.

Transgenic cassava and rice plants were generated using *Agrobacterium*-mediated transformation and analyzed for vitamin accumulation. Together, the vitamin extraction protocols for cassava and rice was established as well as quantification methods using yeast microbiological assays and HPLC analysis. The interaction between endogenous genes and transgenes in transgenic plants was further investigated.
Chapter 2 Vitamin B6 biofortification of cassava by metabolic engineering

Abstract

Vitamin B6 is an essential micronutrient involved in amino acid biosynthesis and catabolism as well as key metabolic processes. It is also widely known as a health-promoting compound as vitamin B6 deficiencies have been associated with various chronic diseases including cardiovascular disease and diabetes. Recent investigations of vitamin B6 status revealed the prevalence of vitamin B6 deficiency in certain African populations. We report genetic engineering of cassava, one of the most important staple crops in Africa, expressing two Arabidopsis genes, AtPDX1.1 and AtPDX2, for de novo biosynthesis of vitamin B6. Transgenic cassava displayed a maximal 10- and 12-fold increase of total vitamin B6 content in leaves and storage roots, respectively, under field conditions. High vitamin B6 cassava has the potential to meet the human daily requirements as well as to serve as renewable alternative feedstock for industrial synthesis.

Introduction

Genes associated with vitamin B6 de novo and salvage biosynthesis in plants were identified in the model plant Arabidopsis (Figure 1) (González et al., 2007; Herrero et al., 2011; Sang et al., 2007; Shi and Zhu, 2002; Tambasco-Studart et al., 2007; Tambasco-Studart et al., 2005). The salvage and de novo pathways co-exist in plants and regulate the interconversion of B6 vitamers and the de novo synthesis of pyridoxal 5'-phosphate (PLP) respectively. All organisms use the salvage pathway to interconvert the six different B6 vitamers including pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), pyridoxal 5'-phosphate (PLP), pyridoxine 5'-phosphate (PNP), and pyridoxamine 5'-phosphate (PMP) (Fitzpatrick et al., 2007). PDX3 is a PN oxidase, PLR is a PL reductase, and SOS4 is PN/PL/PM kinase in the salvage pathway in Arabidopsis (González et al., 2007; Herrero et al., 2011; Lum et al., 2002; Sang et al., 2007; Shi and Zhu, 2002). Besides these three characterized enzymes in Arabidopsis, non-specific phosphatases may exist to dephosphorylate the phosphorylated derivatives. More recently, it was shown that only two genes, namely PDX1 and PDX2, are involved in de novo biosynthesis of vitamin B6 in Arabidopsis (Tambasco-Studart et al., 2007; Tambasco-Studart et al., 2005). Vitamin B6 de novo biosynthesis occurs in the cytosol (Tambasco-Studart et al., 2005). Three homologs of PDX1 and one homolog of PDX2 have been identified in Arabidopsis. However, only two of the three PDX1 homologs in Arabidopsis are functional
(AtPDX1.1 and AtPDX1.3) (Tambasco-Studart et al., 2005). A strategy to enhance vitamin B6 through genetic engineering has been successful in Arabidopsis by over-expressing AtPDX1 together with AtPDX2 (Chen and Xiong, 2009; Raschke et al., 2011). It has been demonstrated that over-expression of AtPDX1.1 and AtPDX2 is the best combination to enhance vitamin B6 content in Arabidopsis (Raschke et al., 2011).

In this present study, we genetically engineered cassava expressing AtPDX1.1 and AtPDX2 to increase vitamin B6 content in storage roots and leaves. Our results demonstrate that vitamin B6 content is significantly increased in both cassava storage roots and leaves using a combination of Arabidopsis PDX1.1 and PDX2 genes.

**Figure 1. The vitamin B6 biosynthesis pathways in plants.** Two enzymes, PDX1 and PDX2, of the *de novo* pathway catalyze ribose 5-phosphate (R5P), glyceraldehyde 3-phosphate (G3P) and glutamine (Gln) to produce pyridoxal 5'-phosphate (PLP). Enzymes of the salvage pathway (i.e. PDX3, PN/PM oxidase; SOS4, PN/PL/PM kinase; T-ase, transaminase; P-ase, phosphatase; PLR, PL reductase) interconvert the six B6 vitamers: PN (pyridoxine); PL (pyridoxal); PM (pyridoxamine) and its respective phosphorylated derivatives (PNP, PLP, PMP). Glu, glutamate. (Figure is adapted from Raschke et al., 2011.)

**Results**

**Generation of transgenic cassava expressing AtPDX1.1 and AtPDX2**

In order to modulate the vitamin B6 content in cassava, we selected the AtPDX1.1 and AtPDX2 genes for genetic engineering; the combination of genes demonstrated to confer the highest vitamin B6 accumulation in transgenic Arabidopsis (Raschke et al., 2011). The Arabidopsis PDX transgenes were placed under the control of the 35S promoter for constitutive expression and later referred to as 35S-PDX1-35S-PDX2 vector (Figure 2a). In a second vector, the PDX transgenes were placed on the control of the *patatin* promoter (Kim et al., 1994) and later referred to as PAT-PDX1-PAT-PDX2 vector. The PAT-PDX1-PAT-PDX2 vector was generated to drive expression of the Arabidopsis PDX transgenes in cassava roots.
(Figure 2b). Fifteen and 14 putative independent transgenic lines resulting from the transformation with 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 vectors, respectively, were selected for molecular characterization. Southern blot analysis confirmed the single and multiple integration events in all putative transgenic lines initially selected (Supplementary Figure 1a,b). Four independent transgenic lines with a single T-DNA insertion (35S-1, 35S-2, 35S-3, 35S-5) and one transgenic line (35S-7) with two T-DNA insertions of 35S-PDX1-35S-PDX2 vector were selected for further analysis. Additionally, four independent transgenic lines (Pat-2, Pat-3, Pat-7, Pat-12) with a single T-DNA insertion of PAT-PDX1-PAT-PDX2 vector were selected for further analysis. Integration of the AtPDX2 gene in the selected transgenic lines of both transformation vectors was validated by PCR (Supplementary Figure 1c,d). It further confirmed that the full T-DNA region was integrated into the cassava genome. Selected transgenic cassava lines were transferred to a greenhouse with control plants to assess their performance. All transgenic lines did not differ phenotypically from the wild-type control. Evaluation of their below ground (Supplementary Figure 2a) and above ground (Supplementary Figure 2b) fresh weight (FW) biomass revealed equal performance between transgenic and wild-type controls under greenhouse conditions.

Transgenic cassava lines accumulate high levels of vitamin B6 in leaves and storage roots under greenhouse conditions

We initially quantified the total vitamin B6 content of fresh leaves and storage roots in transgenic lines under greenhouse conditions using a microbiological assay. Transgenic and wild-type cassava plants were harvested after 6 months of growth. The yeast microbiological assay of vitamin B6 offers a simple and economical way to estimate total vitamin B6 content. Cassava extracts were pretreated with phosphatase and β-glucosidase in order to hydrolyze phosphorylated and glycosylated derivatives into free vitamers.

Total vitamin B6 content was consistently increased 10-fold above wild-type levels in leaves of four out of five transgenic 35S-PDX1-35S-PDX2 lines (Supplementary Figure 3a). The transgenic line 35S-7 displayed a low level of vitamin B6 enrichment in leaves. As expected, vitamin B6 enrichment was limited in leaves of transgenic PAT-PDX1-PAT-PDX2 lines.
Transgenic strategies using constitutive and root-specific expression of the transgenes provided a three to four-fold increase of vitamin B6 content in storage roots (Supplementary Figure 3b). The transgenic line 35S-7 consistently displayed vitamin B6 levels similar to wild-type in storage roots.

Expression of PDX transgenes correlate with vitamin B6 accumulation in transgenic cassava under greenhouse conditions

An expression analysis of the PDX transgenes was subsequently performed in selected transgenic lines. Expression of AtPDX1.1 could be achieved in cassava leaves and storage roots of the two selected transgenic 35S-PDX1-35S-PDX2 lines, accumulating high levels of vitamin B6 (Supplementary Figure 4a). Expression analysis of AtPDX2 (Supplementary Figure 4b) revealed that transcripts of the AtPDX2 transgene accumulate at lower levels compared to AtPDX1.1. Noticeably, line 35S-2 accumulated significantly lower levels of both AtPDX1.1 and AtPDX2 transcripts compared to line 35S-1 in storage roots. The latter observation was consistent with the limited vitamin B6 enrichment initially observed in storage roots of line 35S-2 (Supplementary Figure 3b). Consistent with the vitamin B6 quantification data, expression of AtPDX1.1 and AtPDX2 was barely detectable in leaves and roots of line 35S-7 (Supplementary Figure 4a,b). Expression of the AtPDX1.1 and AtPDX2 transgenes under the patatin promoter in storage roots was significantly higher than their expression in leaves for the selected lines transformed with the PAT-PDX1-PAT-PDX2 vector (Supplementary Figure 4a,b). The RT-qPCR analysis revealed that the root-specific patatin promoter retained expression in leaves of the selected transgenic lines. However, expression of transgenes under patatin promoters was significantly lower than the ones under a 35S promoter in cassava leaves (Supplementary Figure 4a,b). Expression levels of PDX transgenes were overall consistent with total vitamin B6 content in transgenic plants.

Expression of PDX transgenes does not alter accumulation of endogenous PDX transcripts in transgenic cassava

Previous reports have shown that heterologous expression of exogenous PDX1 and PDX2 genes in planta can alter accumulation of endogenous PDX transcripts (Herrero and Daub, 2007). To investigate whether the expression of endogenous PDX genes in transgenic cassava were affected by PDX transgenes, we identified three PDX1 homologs and one PDX2 homolog in the recently released cassava genome (Prochnik et al., 2012), which we later referred to as MePDX1.2, MePDX1.3a, MePDX1.3b (Supplementary Figure 5a) and MePDX2 (Supplementary Figure 5b). We designed primers to quantify transcript levels of these four endogenous PDX genes in cassava (Supplementary Table 1). In cassava leaves, MePDX1.3a transcript accumulation was in the range of the internal control (PP2A) and it had a
significantly higher expression (around 10-fold) than *MePDX1.3b*. On the contrary, *MePDX1.3b* transcripts accumulated to higher levels (around 3-fold) than *MePDX1.3a* in cassava storage roots. In our analysis, *MePDX1.2* transcripts were barely detectable in leaves and storage roots of greenhouse plants. Expression of *MePDX2* was in the range of *MePDX1.3b* in leaves and of *MePDX1.3a* in storage roots (Supplementary Figure 6a,b).

Expression levels of the *MePDX1* and *MePDX2* genes were analyzed in leaves and storage roots of the selected transgenic cassava lines (Supplementary Figure 6a,b). Expression analysis also demonstrated that expression of *Arabidopsis PDX* transgenes did not significantly alter expression patterns of *MePDX1* and *MePDX2* genes in selected transgenic lines. These results suggest that high vitamin B6 accumulation in transgenic cassava is mediated by transgenes expression and does not interfere with endogenous *de novo* vitamin B6 biosynthesis.

Transgenic cassava lines accumulate high levels of vitamin B6 in leaves and storage roots under field conditions

In order to confirm the vitamin B6 trait under field conditions, three transgenic 35S-PDX1-35S-PDX2 lines (35S-3, 35S-4, 35S-5), three transgenic PAT-PDX1-PAT-PDX2 lines (Pat-2, Pat-7, Pat-12) and wild-type controls were grown under field conditions. Field-grown cassava plants were harvested 6 months after planting. Transgenic cassava was phenotypically indistinguishable from wild-type control plants. However, the limited number of plants did not allow a robust agronomic performance evaluation. We first measured the total vitamin B6 content of field-grown cassava using a microbiological assay. All 6 transgenic lines of both 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 vectors displayed high vitamin B6 accumulation in fresh leaves (Supplementary Figure 7a) and storage roots (Supplementary Figure 7b) under field conditions.

Distribution of B6 vitamers is altered in transgenic cassava and the vitamin B6 increase is largely due to PNG under field conditions

In order to evaluate the contribution of each B6 vitamer to the overall vitamin B6 enrichment in transgenic cassava, an HPLC assay was established to measure B6 vitamers in cassava extracts. Chromatograms of wild-type and transgenic cassava extracts revealed significant differences in the elution peaks of B6 vitamers for both leaves (Figure 3a) and storage roots (Figure 3b). Glycosylated derivatives of vitamin B6, in particular pyridoxine-5’-β-D-glucoside (PNG), can serve as a predominant storage form of vitamin B6 in plants (Gregory III, 1998). Glycosylated forms were also estimated in cassava extracts by performing an hydrolysis of glycosylated derivatives using a β-glucosidase treatment,
measuring total free vitamers and subsequently extrapolating the amount of glycosylated derivatives.

![HPLC chromatogram of vitamin B6 extract from cassava leaves.](image-a)

![HPLC chromatogram of vitamin B6 extract from cassava storage roots.](image-b)

![Free (white), phosphorylated (black), glycosylated (gray) forms of vitamin B6 in cassava leaves.](image-c)

![Free (white), phosphorylated (black), glycosylated (gray) forms of vitamin B6 in cassava storage roots.](image-d)

Figure 3. Analysis of B6 vitamers and glycosylated derivatives in transgenic cassava expressing Arabidopsis PDX1.1 and PDX2 under field conditions.

(a) HPLC chromatogram of vitamin B6 extract from cassava leaves.
(b) HPLC chromatogram of vitamin B6 extract from cassava storage roots.
(c) Free (white), phosphorylated (black), glycosylated (gray) forms of vitamin B6 in cassava leaves.
(d) Free (white), phosphorylated (black), glycosylated (gray) forms of vitamin B6 in cassava storage roots.

The data was obtained by an HPLC analysis and values are averages ± SE of three biological replicates. 35S-3 and 35S-5 indicate two independent lines of 35S-PDX1-35S-PDX2. Pat-2 and Pat-12 indicate two independent lines of PAT-PDX1-PAT-PDX2. wt, wild-type plants.
Table 1
B6 vitamer and glycosylated derivative composition in transgenic and wild-type cassava leaves (a) and storage roots (b) under field conditions

### a. Field-grown cassava leaves (µg/g FW)

<table>
<thead>
<tr>
<th>Leaves</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.65 ± 0.06</td>
<td>1.05 ± 0.67</td>
<td>0.31 ± 0.02</td>
<td>0.97 ± 0.09</td>
<td>0.26 ± 0.02</td>
<td>0.09 ± 0.01</td>
<td>1.08 ± 0.52</td>
<td>3.32 ± 1.05</td>
<td>1.07 ± 0.25</td>
<td>8.79 ± 1.28</td>
</tr>
<tr>
<td>35S-3</td>
<td>3.39 ± 0.26</td>
<td>16.41 ± 1.72</td>
<td>0.54 ± 0.10</td>
<td>0.94 ± 0.09</td>
<td>0.38 ± 0.04</td>
<td>0.16 ± 0.02</td>
<td>9.35 ± 0.36</td>
<td>62.20 ± 1.24</td>
<td>1.28 ± 0.18</td>
<td>94.65 ± 3.74</td>
</tr>
<tr>
<td>35S-5</td>
<td>2.39 ± 0.57</td>
<td>14.98 ± 5.32</td>
<td>0.65 ± 0.21</td>
<td>0.86 ± 0.07</td>
<td>0.63 ± 0.08</td>
<td>0.50 ± 0.31</td>
<td>6.78 ± 0.60</td>
<td>64.70 ± 2.87</td>
<td>1.30 ± 0.24</td>
<td>92.79 ± 6.32</td>
</tr>
<tr>
<td>Pat-2</td>
<td>2.27 ± 0.06</td>
<td>3.36 ± 0.58</td>
<td>0.65 ± 0.04</td>
<td>1.16 ± 0.04</td>
<td>0.56 ± 0.08</td>
<td>0.25 ± 0.02</td>
<td>5.08 ± 0.21</td>
<td>40.32 ± 1.51</td>
<td>0.95 ± 0.20</td>
<td>54.60 ± 1.84</td>
</tr>
<tr>
<td>Pat-12</td>
<td>1.62 ± 0.31</td>
<td>8.42 ± 3.94</td>
<td>0.53 ± 0.08</td>
<td>0.88 ± 0.02</td>
<td>0.30 ± 0.08</td>
<td>0.11 ± 0.02</td>
<td>4.15 ± 0.21</td>
<td>31.60 ± 1.35</td>
<td>0.93 ± 0.09</td>
<td>48.54 ± 4.53</td>
</tr>
</tbody>
</table>

The data was obtained by HPLC analysis and values are averages ± SE of three biological replicates. Numbers in bold indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).

### b. Field-grown cassava storage roots (µg/g FW)

<table>
<thead>
<tr>
<th>Roots</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.13 ± 0.01</td>
<td>0.03 ± 0.00</td>
<td>0.27 ± 0.05</td>
<td>0.18 ± 0.04</td>
<td>0.03 ± 0.00</td>
<td>trace</td>
<td>0.32 ± 0.02</td>
<td>0.50 ± 0.06</td>
<td>0.38 ± 0.05</td>
<td>1.84 ± 0.08</td>
</tr>
<tr>
<td>35S-3</td>
<td>0.38 ± 0.13</td>
<td>0.04 ± 0.01</td>
<td>0.32 ± 0.06</td>
<td>0.29 ± 0.03</td>
<td>0.04 ± 0.02</td>
<td>trace</td>
<td>0.79 ± 0.27</td>
<td>2.40 ± 0.07</td>
<td>0.43 ± 0.07</td>
<td>4.70 ± 0.61</td>
</tr>
<tr>
<td>35S-5</td>
<td>0.37 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>0.31 ± 0.01</td>
<td>0.39 ± 0.06</td>
<td>0.04 ± 0.00</td>
<td>trace</td>
<td>1.32 ± 0.06</td>
<td>4.47 ± 0.21</td>
<td>0.60 ± 0.02</td>
<td>7.54 ± 0.35</td>
</tr>
<tr>
<td>Pat-2</td>
<td>1.13 ± 0.21</td>
<td>0.20 ± 0.33</td>
<td>0.37 ± 0.07</td>
<td>0.33 ± 0.07</td>
<td>0.11 ± 0.02</td>
<td>trace</td>
<td>3.40 ± 0.90</td>
<td>11.82 ± 0.81</td>
<td>0.65 ± 0.02</td>
<td>17.99 ± 1.95</td>
</tr>
<tr>
<td>Pat-12</td>
<td>1.68 ± 0.15</td>
<td>0.33 ± 0.02</td>
<td>0.49 ± 0.08</td>
<td>0.54 ± 0.01</td>
<td>0.15 ± 0.03</td>
<td>trace</td>
<td>4.78 ± 0.35</td>
<td>13.82 ± 1.54</td>
<td>0.78 ± 0.11</td>
<td>22.56 ± 1.49</td>
</tr>
</tbody>
</table>

The data was obtained by HPLC analysis and values are averages ± SE of three biological replicates. Numbers in bold indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
In field-grown cassava leaves, HPLC quantification revealed a substantial increase of total vitamin B6, up to 10- and 6-fold of wild-type levels in selected lines of 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 vectors, respectively (Figure 3c, Supplementary Table 2a). Free forms of vitamin B6 were consistently increased in transgenic cassava leaves of both 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 transgenic lines. No consistent changes in total phosphorylated derivatives content between transgenic and wild-type leaves were observed. The glycosylated derivatives were the major contributors to vitamin B6 enrichment in transgenic leaves, which showed 13- and 8-fold increase over wild-type in transgenic 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 lines, respectively (Supplementary Table 3a). In wild-type leaves, the free forms, phosphorylated derivatives, and glycosylated derivatives were 22.82%, 15% and 62.18% of the total vitamin B6 pool, respectively (Supplementary Table 4a). The data suggests that glycosylated derivatives are the major storage forms of vitamin B6 in wild-type cassava leaves. Glycosylated derivatives in transgenic cassava leaves accounted for more than 75% of the total vitamin B6 content independently from the transformation vector used. The free forms of vitamin B6 were the second most abundant forms, accounting for 10-20% of the total vitamin B6 content in both transgenic 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 lines. Phosphorylated derivatives content was low, accounting for less than 5% of the total vitamin B6 content in transgenic cassava leaves.

In field-grown storage roots, HPLC quantification revealed an increase of total vitamin B6 up to 4- and 12-fold of wild-type levels in selected lines of 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 vectors, respectively (Figure 3d, Supplementary Table 2b). The amount of free forms of vitamin B6 was increased more than 4-fold in transgenic storage roots of PAT-PDX1-PAT-PDX2 vector. We observed a 1.7-fold increase in one transgenic 35S-PDX1-35S-PDX2 line (35S-5), but could not observe a significant increase in a second transgenic 35S-PDX1-35S-PDX2 line (35S-3) (Supplementary Table 3b). A 3.3-fold increase of total phosphorylated derivatives content was observed in storage roots of transgenic line Pat-12, but could not be observed in the other selected lines (35S-3, 35S-5, Pat-2). The total glycosylated derivatives content was the major contributor of vitamin B6 accumulation in transgenic storage roots, which showed up to 5- and 16-fold increases compared to wild-type levels in transgenic 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 lines, respectively (Supplementary Table 3b). In wild-type storage roots, free forms, phosphorylated derivatives, and glycosylated derivatives were 23.49%, 11.59% and 64.92% of the total vitamin B6 pool, respectively (Supplementary Table 4b). The B6 vitamers distribution in wild-type storage roots resembled the one observed in wild-type leaves. The total glycosylated derivatives content in transgenic storage roots in both transgenic 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 lines accounted for around 85% of the total vitamin B6 pool. The amount of free forms of vitamin B6 was higher than the phosphorylated derivatives in transgenic storage roots (Figure 3d). The relative contribution of the three forms to vitamin B6 in transgenic storage roots was similar in transgenic lines of both transformation vectors (Supplementary Table 4b).
A closer look at the B6 vitamers and their glycosylated derivatives revealed that PM and PN as well as PMG and PNG, were substantially increased in transgenic cassava leaves of both 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 vectors (Table 1a; Supplementary Table 2a). However, the levels of PN showed a high variation between biological replicates. The PNG level in transgenic cassava leaves displayed the largest increase 20- and 12-fold in transgenic 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 lines, respectively, compared to wild-type, accounting for 70% of the total vitamin B6 pool. PMG was the second most abundant vitamin B6 derivative, accounting for nearly 10% of the total vitamin B6 pool. PMP and PLG levels in leaves were not significantly different between transgenic and wild-type plants. A vitamer analysis of PL, PNP and PLP showed high variation among leaves of transgenic lines, and the amount of these vitamers was relative low with in the total vitamin B6 content.

Ectopic expression of AtPDX1.1 and AtPDX2 in transgenic storage roots had a similar relative distribution of B6 vitamers and glycosylated derivatives to the one observed in leaves (Supplementary Table 4). Both constitutive and root-specific expression led to a significant increase of PM and PN as well as PMG and PNG (Table 1b; Supplementary Table 2b). The PNG level in transgenic cassava storage roots displayed the largest increase, up to 9- and 28-fold of wild-type levels in transgenic 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2 cassava, respectively, accounting for 60% of the total vitamin B6 pool. PMG ranked as the second most abundant vitamin B6 derivative in transgenic storage roots, accounting for nearly 20% of the total vitamin B6 pool. PL, PMP, PNP, PLP, and PLG levels had no or little increase in transgenic storage roots.

An analysis of free B6 vitamers as well as phosphorylated and glycosylated derivative accumulation in transgenic cassava revealed that PNG and PMG were the main contributors to total vitamin B6 enrichment in both leaves and storage roots of transgenic cassava. Despite its high variation, the PN content was the third most abundant form in leaves of transgenic lines. PM was the third most abundant form in storage roots of transgenic lines particularly in transgenic PAT-PDX1-PAT-PDX2 lines. We observed either no or very little fold increases of PL, PLG and phosphorylated derivatives in leaves and storage roots of transgenic lines. The amount of PL, PLG and phosphorylated derivatives were relative low, which only had a minor contribution to the total vitamin B6 enrichment in both transgenic leaves and storage roots.

**Retention of vitamin B6 during thermal processing**

The bioavailability of micronutrients is an essential parameter to assess the potential of biofortified crops in addressing deficiencies. Due to its solubility in water, vitamin B6 content in plant tissues may change during cooking (Lešková et al., 2006). We performed baking and
boiling of vitamin-enriched cassava plant material to determine vitamin B6 stability and retention in cassava after thermal processing.

Following the field harvest, transgenic plant material was selected for thermal processing based on their respective vitamin B6 accumulation profile (Supplementary Figure 7). The vitamin B6 content was measured before and after thermal treatment. Cassava leaves were boiled, while cassava storage roots were submitted to two distinct treatments, i.e. boiling and baking.

The total vitamin B6 content significantly decreased in leaves of wild-type cassava as well as in transgenic leaves after boiling treatment (Supplementary Table 5). Total vitamin B6 was partially released into water during boiling. The vitamin B6 was detected in the water which was used for boiling. However, transgenic leaves retained vitamin B6 levels over 14-fold higher than the ones observed in wild-type leaves after boiling (Figure 4a; Supplementary Table 5b; Supplementary Table 7b). Transgenic storage roots retained vitamin B6 levels 4-fold higher than the ones observed in wild-type after boiling (Supplementary Figure 8b). Moreover, transgenic storage roots retained vitamin B6 levels 5-fold higher than the ones observed in wild-type after baking (Supplementary Table 8b).

After the boiling process, B6 vitamers and glycosylated derivatives decreased significantly, and PNG remained the predominant derivative in boiled transgenic leaves, accounting for over 75% of the total vitamin B6 content (Supplementary Table 5). The total glycosylated derivatives content was the most abundant and the amount of free forms of vitamin B6 was the second most abundant in boiled transgenic leaves (Figure 4b). Intriguingly, three phosphorylated derivatives in boiled transgenic leaves had similar levels with boiled wild-type leaves (Supplementary Table 5b). In baked storage roots, the relative distribution of vitamers was comparable with boiled leaves. PNG was the predominant derivative in baked storage roots, accounting for over 45% of the total vitamin B6 content (Supplementary Table 6). The total glycosylated derivatives content was the most abundant and the amount of free forms was the second most abundant in baked transgenic storage roots (Figure 4b). In general, PNG and PMG were the two predominant forms in boiled leaves and baked storage roots, accounting for around 80% of the total vitamin B6 content. Levels of free forms of vitamin B6 remained higher in transgenic plant material compared to wild-type after processing. Phosphorylated derivatives were at a low level after processing.
Figure 4. Free, phosphorylated and glycosylated forms of vitamin B6 in transgenic cassava expressing Arabidopsis PDX1.1 and PDX2 under field conditions after thermal processing. (a) Boiled leaves (b) Baked storage roots. The data was obtained by HPLC analysis and values are averages ± SE of three biological replicates except for samples of Pat-2 and Pat-12 in (b) which are with two biological replicates. 35S-3 and 35S-5 indicate two independent lines of 35S-PDX1-35S-PDX2. Pat-2 and Pat-12 indicate two independent lines of PAT-PDX1-PAT-PDX2. wt, wild-type plant.

Discussion

In the present study, we report the successful genetic engineering of vitamin B6 enriched cassava, one of the most important staple crops in the tropics. To our knowledge, it is the first example of vitamin B6 biofortification in a crop plant. We achieved a 10- and 12-fold increase in transgenic cassava leaves and storage roots, respectively, expressing the same PDX gene combination under constitutive and root-specific promoters.

We identified three AtPDX1 homologs in the cassava genome, while two cassava PDX1 copies appeared to be more homologous to AtPDX1.3. Both AtPDX1.3 homologs were transcriptionally active in cassava. The other cassava PDX1 copy is more homologous to AtPDX1.2, which was not transcriptionally active in cassava under the tested conditions. The contribution of each PDX1 protein to vitamin B6 biosynthesis in cassava remains to be elucidated. An analysis of PDX1 and PDX2 transcripts in cassava demonstrated that expression of AtPDX1.1 and AtPDX2 genes in transgenic cassava does not alter the transcript levels of endogenous PDX1 and PDX2 genes. Additionally, increased vitamin B6 levels did not appear to alter endogenous PDX1 and PDX2 genes at the transcriptional level in cassava.

In order to facilitate the screening process, we used a yeast microbiological quantification to perform a quick estimation of the total vitamin B6 content. We precisely determined vitamin B6 content and B6 vitamer profiles using an HPLC method. However, the vitamin B6 content
from microbiological quantification was not comparable with the HPLC assay. We found higher values of vitamin B6 using HPLC. The difference between the two vitamin B6 quantification methods has been reported earlier as well (Kall, 2003). Because other metabolites in the cassava extracts may impede yeast growth, the vitamin B6 content may be underestimated using the microbiological assay. Therefore, we adopted the HPLC assay for an accurate value of vitamin B6 content.

The profile of B6 vitamers and glycosylated derivatives in cassava substantially differs from the one in Arabidopsis. In Arabidopsis shoots, phosphorylated derivatives are the major contributors to the vitamin B6 pool and over-expression of PDX genes further increase the contribution of phosphorylated derivatives to total vitamin B6 content in transgenic Arabidopsis (Raschke et al., 2011). On the contrary, expression of the Arabidopsis PDX genes in cassava further accentuated the contribution of glycosylated derivatives to total vitamin B6 content. No significant change of phosphorylated derivatives was observed in transgenic cassava.

The substantial increase in vitamin B6 content in leaves has been postulated to account for physiological differences as illustrated by increased organ size in transgenic Arabidopsis over-expressing PDX genes (Raschke et al., 2011). However, we could not consistently observe a similar biomass increase in our transgenic cassava under greenhouse conditions. One explanation is that the major provider of vitamin B6 enrichment in transgenic Arabidopsis is phosphorylated derivatives rather than glycosylated derivatives in transgenic cassava. It must be noted that PLP was substantially increased up to 4-fold in transgenic Arabidopsis (Raschke et al., 2011), but the level of PLP was not changed in transgenic cassava. PLP and PMP are the active cofactor forms of vitamin B6 in plants. Glycosylated derivatives of vitamin B6 are postulated to be the storage forms and are without vitamin B6 cofactor function in plants (Gregory III, 1998). While transgenic cassava produces PLP de novo consistently, excess PLP may be converted into glycosylated derivatives subsequently by the salvage pathway or other unknown mechanisms. The accumulation of glycosylated derivatives may be a mechanism in transgenic cassava to avoid interfering with cellular metabolism homeostasis.

Cooking experiments were performed at day 7 after harvest. It must be noted that we observed an increase of the total vitamin B6 content during the 7 days storage (Table 1; Supplementary Table 5; Supplementary Table 6). Compared to the freshly harvested cassava, the vitamin B6 content was significantly increased in both leaves and storage roots at day 7 after harvest. It may be caused by certain forms of vitamin B6, particularly the glycosylated derivatives release from the plant tissue matrix gradually after harvest. A similar observation was made in potato, which shows a 40% increase of vitamin B6 content after 7 months of storage (Mooney et al., 2013). The mechanism of vitamin B6 increase during postharvest needs to be further investigated. This also suggests that the stability of total vitamin B6
content in fresh cassava leaves and storage roots could maintain few days at room temperature after harvest.

Vitamin B6 deficiency is a universal problem for human nutrition particularly in tropical regions where many populations rely on cassava as a major calorie source. Based on our quantification results from cooking experiments, boiled leaves and baked storage roots of transgenic cassava retain a maximal 17- and 5-fold increase, respectively, in the total vitamin B6 content compared to that in wild-type plants. After thermal processing, glycosylated derivatives remained the predominant forms (more than 80% of total vitamin B6) in both boiled and baked cassava tissues. Despite a lower bioavailability of glycosylated derivatives compared to free and phosphorylated forms of vitamin B6, the increase of PNG in transgenic cassava, with its reported 50% bioavailable for human consumption (Gregory III, 2012), is substantial enough to confer improved nutritional value. We assume that the baked transgenic storage roots could offer a maximal 7.7 mg bioavailable vitamin B6 per day in a typical adult cassava meal (500 g). However, the absorption of nutrients in food matrices for humans is dependent on many factors (Martin, 2013). The accurate value of bioavailable vitamin B6 in transgenic cassava tissue needs to be subjected to further experimental validation.

Plants accumulating high vitamin B6 levels represent novel opportunities for industrial vitamin production (Eggersdorfer et al., 2012). Transgenic plants accumulating high levels of vitamin B6 could be a cheap source of vitamin B6 supply. Since cassava is a perennial plant with high biomass productivity, it could offer an interesting alternative for vitamin B6 production to replace its expensive chemical vitamin synthesis. Moreover, because cassava is used both for human food and animal feed, cassava enriched in vitamin B6 represents an interesting alternative to food biortification and vitamin supplementation. The recent success of orange flesh sweet potato distributed in Africa to tackle vitamin A deficiencies in African populations demonstrates the potential of biofortified crops to address micronutrient deficiency issues (Blancquaert et al., 2013; Low et al., 2007; van Jaarsveld et al., 2005).

We anticipate that physiological changes occur in transgenic cassava accumulating high levels of vitamin B6. Further characterization, including metabolite profiling and genome-wide gene expression analysis of transgenic cassava lines will help us to decipher altered traits associated with heterologous PDX expression and enhanced levels of B6 vitamers. An evaluation of agronomic performance will require multi-year and multi-location field trials with sufficient numbers of plant replicates and adequate experimental design.
Materials and Methods

Plant materials

Cassava (*Manihot esculenta* Crantz cv. 60444) was used in this study. *In vitro* cassava plantlets were kept under a 16/8h light and dark regime at 28°C, multiplied and propagated in tissue culture conditions for one month, before transfer to soil in a greenhouse. Cassava plants were kept under greenhouse conditions (16h light at 26°C and 60% humidity, 8h dark at 17°C and 50% humidity) and the effect of natural illumination for 6 months. Fully expanded leaves on the top of plant and storage roots were harvested for analysis.

The field trial was conducted in China in collaboration with Prof. Peng Zhang in Shanghai Institutes for Biological Sciences, CAS, China. Cassava plant cuttings from the greenhouse were planted in the field. Fully expanded leaves on the top of plant and storage roots were harvested for analysis 6 months after planting.

*In silico* identification of cassava *PDX* genes

Cassava sequences cassava4.1_012402m.g (hereafter named *MePDX1.3a*) and cassava4.1_012325m.g (hereafter named *MePDX1.3b*) were identified as putative orthologs of *AtPDX1.3*, cassava4.1_034454m.g (hereafter named *MePDX1.2*) as ortholog of *AtPDX1.2* and cassava4.1_014591m.g (hereafter named *MePDX2*) as ortholog of *AtPDX2* using the OMA browser (Altenhoff et al., 2011) and sequence alignment.

Alignments of PDX1 and PDX2 amino acid sequences were performed with the Clustal W method (DNASTAR Lasergene). Cassava PDX1 and PDX2 sequences were obtained from phytozome (www.phytozome.net/cassava) (Prochnik et al., 2012).

Transformation vector construction

Constitutive expression vector

The 35S promoter fused with *AtPDX1.1* (At2g38230) in *pCAMBIA1300.1-PDX1* (Tambasco-Studart et al., 2005) was linearized with EcoRI and Xbal, and inserted into pBlueScript, generating the *pBS-35S-PDX1.1* vector. The octopine synthase terminator of *AtPDX1.1* in *pCAMBIA1300.1-PDX1* was synthesized by PCR with primers polyA-F (5' - TCTAGAGTCTCGACCCCTGAGG-3') and polyA-R (5'-ACAATCAGTAATTGAACGGGAG-3'), and subcloned into the *PCR2.1 TOPO*® vector (TOPO TA cloning kit; Invitrogen), to generate the *pTOPO-polyA* vector. The octopine synthase terminator in *pTOPO-polyA* was linearized with Xbal and NotI, and inserted into *pBS-35S-PDX1.1* to generate the *pBS-35S-PDX1.1-pA* vector.
The *pBlueScript* vector was digested with EcoRI, blunted and self-ligated, generating a *pBS-EcoRI* vector without the EcoRI cutting site. The expression cassette 35S promoter::*AtPDX2*::terminator containing the *AtPDX2* gene (At5g60540) was linearized from the *pBIN19-PDX2* vector (Tambasco-Studart et al., 2005) with HindIII and inserted into the *pBS-EcoRI* vector, generating the *pBS-PDX2* vector. The *pBS-PDX2* vector was digested with EcoRI, blunted and self-ligated, to generate the *pBS-PDX2-EcoRI* vector without the EcoRI cutting site. The expression cassette, 35S promoter::*AtPDX2*::terminator, in *pBS-PDX2-EcoRI* was linearized with HindIII and inserted into the *pCAMBIA1300* (CSIRO, GI:7638064) binary vector, generating the *p1300-35S-PDX2* vector. The expression cassette, 35S promoter::*AtPDX1.1*::terminator, in *pBS-35S-PDX1.1-PA* was linearized with EcoRI, and inserted into *p1300-35S-PDX2*, generating the constitutive expression transformation vector 35S-PDX1-35S-PDX2.

**Root-specific expression vector**

The root-specific *patatin* promoter in the *Pat::GUS* vector was PCR amplified by primers Patatin-F-EcoRI (5’-GAATTCTGCAGTTGTAGTTAATGCG-3’) and Patatin-R-KpnI (5’-GGTACCAGGATCAAGCAT-3’), and subcloned into the *pJET1.2/blunt* Cloning Vector (CloneJET PCR cloning Kit; Thermo Scientific), generating the *pJ-Patatin* vector. The *patatin* promoter in *pJ-Patatin* was linearized with EcoRI and KpnI, and inserted into *pCAMBIA1300.1-PDX1.1* by replacement of the 35S promoter to generate the *p1300-Pat-PDX1* vector. The *patatin* promoter in *pJ-Patatin* was linearized with EcoRI and KpnI and inserted into *p1300-35S-PDX2* by replacement of the 35S promoter to generate the *p1300-Pat-PDX2* vector. The expression cassette, *patatin* promoter::*AtPDX1.1*::terminator, in *p1300-Pat-PDX1* was linearized with PvuII, and inserted into *p1300-Pat-PDX2* digested with EcoRI and blunted, to generate the root-specific expression transformation vector PAT-PDX1-PAT-PDX2.

Primer sequences used for vector construction are in Supplementary Table 9.

**Cassava transformation**

Transformation vectors, 35S-PDX1-35S-PDX2 and PAT-PDX1-PAT-PDX2, were introduced into *Agrobacterium tumefaciens* strain LBA4404, and transgenic cassava were produced according to an optimized protocol previously established in the laboratory (Bull et al., 2009).
**Molecular characterization**

DNA extraction was conducted as previously described (Sheu et al., 1996) and the T-DNA integration copy number in transgenic plants was determined by Southern blot (SB) analysis (Vanderschuren et al., 2012). SB membranes were hybridized with a DIG-labeled hptII probe.

Transgene (AtPDX2) integration in transgenic 35S-PDX1-35S-PDX1 lines was confirmed by PCR using primers AtPDX2-F (5'-CTACGTGAGTTTGTTAAGATG-3') and AtPDX2-R (5'-CTCTTTCTGACCAACTGCTC-3'). Transgene (AtPDX2) integration in transgenic PAT-PDX1-PAT-PDX2 lines were confirmed by PCR using primers Pat-F2 (5'-ATGACAGTTCGGTGCAAAAG-3') and PDX2-seq (5'-AATCTCGACCGCTTGGAC-3').

**RT-qPCR analysis**

Total RNA was extracted according to an established method (Cazzonelli et al., 1998) with minor modifications. cDNA was generated using the RevertAid First Strand cDNA Synthesus Kit (Thermo Scientific) with 1 μg total RNA according to the manufacturer’s instruction using random hexamer primers. RT-qPCR was performed using the 7500 FAST real-time PCR system and Fast SYBR® Green Master Mix (Applied Biosystems). The relative target gene expression levels were normalized to the reference gene PP2A (Moreno et al., 2011) using the delta-delta CT method (Livak and Schmittgen, 2001). Primer sequences used for RT-qPCR are reported in Supplementary Table 1.

**Vitamin B6 quantification**

*Yeast microbiological assay*

Total vitamin B6 was quantified by a yeast microbiological assay as described previously (Tambasco-Studart et al., 2005). The fresh leaves and parenchyma tissues of storage roots were used for vitamin B6 quantification. Vitamin B6 was extracted in 1 mL of 0.22 M sulphuric acid at 100°C for 1 h, and then treated with acid phosphatase and β-glucosidase for 12-15 h at 37°C to convert phosphorylated and glycosylated forms of vitamin B6 to free forms. The quantity of total vitamin B6 content was extrapolated from a standard curve of known amounts of commercial pyridoxine.
**HPLC analysis**

The free forms of vitamin B6 and their phosphorylated and glycosylated derivatives were quantified by an HPLC method according to an established protocol (Szydlowski et al., 2013). Two hundred mg of plant tissue was ground in liquid nitrogen, and 400 μL of 50 mM ammonium acetate, pH 4, was added before centrifugation at 16100 g for 15 min. The supernatant was subsequently heated at 100°C for 3 min, before centrifugation at 16100 g for 15 min, and then analyzed by HPLC. The extract was injected to determine the free forms and phosphorylated derivatives content of vitamin B6. An equivalent amount of original extract was treated with β-glucosidase to hydrolyze glycosylated derivatives for additional HPLC analysis. The increase of PM, PN, and PL was extrapolated to define the quantity of glycosylated derivatives.

*The HPLC analysis was performed by Dr. Michæel Moulin in the Plant Biochemistry and Physiology group at University of Geneva.

**Thermal processing**

Before thermal processing, cassava leaves and storage roots were kept at room temperature for 7 days after harvest.

* **Cassava leaves**

Field-grown cassava leaves were placed into 50 mL plastic tubes and covered with water. After 30 min of boiling, cassava leaves were dried and ground into a fine powder for vitamin B6 extraction.

* **Cassava storage roots**

Field-grown cassava storage roots were sliced into approximately 1.5 cm sections. Each slice was peeled and quartered into four pieces. One piece of cassava root was wrapped in aluminum foil and baked in an incubator at 180°C for 30 min prior to analysis. One piece of cassava root was placed into 50 mL plastic tube and covered with water and then boiled for 30 min prior to analysis.

*The thermal processing was performed by Ms. Nathalie Mangel in the Plant Biotechnology Lab at ETH Zürich.*
Supplementary Figure 1. Molecular characterization of transgenic cassava.
Southern blot analysis of transgenic cassava plants using an hptII probe. (a) 35S-PDX1-35S-PDX2 vector (b) PAT-PDX1-PAT-PDX2 vector. Analysis of AtPDX2 transgene integration using PCR (c) 35S-PDX1-35S-PDX2 vector (d) PAT-PDX1-PAT-PDX2 vector.
35S-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16 indicate 15 putative selected transgenic 35S-PDX1-35S-PDX2 plants. Pat-1, 2, 3, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16 indicate 14 putative selected transgenic PAT-PDX1-PAT-PDX2 plants; wt, wild-type plant; vector control, 35S-PDX1-35S-PDX2 in (c), PAT-PDX1-PAT-PDX2 in (d).
Supplementary Figure 2. Biomass of 6-month-old transgenic cassava under greenhouse conditions. (a) Below ground fresh weight. (b) Above ground fresh weight. Values are averages ± SD of four biological replicates except for Pat-2 which is with three biological replicates.
Supplementary Figure 3. Analysis of transgenic cassava expressing Arabidopsis PDX1.1 and PDX2 under greenhouse conditions.

Relative total vitamin B6 content in cassava leaves (a) and storage roots (b) of independent transgenic lines and wild-type plants. Total vitamin B6 was quantified by a yeast microbiological assay (Tambasco-Studart et al., 2005). Values are averages ± SD of at least three biological replicates. wt (white), 35S-PDX1-35S-PDX2 (gray), PAT-PDX1-PAT-PDX2 (black).
Supplementary Figure 4. RT-qPCR analysis of transgene expression in transgenic cassava under greenhouse conditions. (a) AtPDX1.1 (b) AtPDX2. Values are averages ± SD of three biological replicates. Leaves (black), storage roots (gray).
Supplementary Figure 5. Amino acid sequence alignment of Arabidopsis and cassava PDX1 (a) and PDX2 (b). Consensus residues are marked with black boxes. *AtPDX1.1*, At2g38230; *AtPDX1.2*, At3g16050; *AtPDX1.3*, At5g01410; *AtPDX2*, At5g60540; *MePDX1.2*, cassava4.1_034454m.g; *MePDX1.3a*, cassava4.1_012402m.g; *MePDX1.3b*, cassava4.1_012325m.g; *MePDX2*, cassava4.1_014591m.g.
Supplementary Figure 6. RT-qPCR analysis of endogenous PDX gene expression in transgenic and wild-type cassava (a) leaves and (b) storage roots under greenhouse conditions. MePDX1.3a (black), MePDX1.3b (gray), MePDX2 (white). Values are averages ± SD of three biological replicates. Asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
Supplementary Figure 7. Analysis of transgenic cassava expressing Arabidopsis PDX1.1 and PDX2 under field conditions.

(a) Relative total vitamin B6 content in cassava leaves. 35S-3, 35S-4, 35S-5 indicate 3 independent lines of 35S-PDX1-35S-PDX2. Pat-2, Pat-7 and Pat-12 indicate three independent lines of PAT-PDX1-PAT-PDX2. wt, wild-type plant.

(b) Relative total vitamin B6 content in cassava storage roots of independent transgenic cassava lines and wild-type plants.

Total vitamin B6 content was quantified using a yeast microbiological assay. Values are averages ± SD of three biological replicates. wt (white), 35S-PDX1-35S-PDX2 (gray), PAT-PDX1-PAT-PDX2 (black).
Supplementary Figure 8. Analysis of transgenic cassava expressing Arabidopsis PDX1.1 and PDX2 under field conditions after boiling. Relative total vitamin B6 content in storage roots (a) before boiling and (b) after boiling. Total vitamin B6 content quantified using a yeast microbiological assay. Values are averages ± SD of three biological replicates. wild-type (white), Pat-2 and Pat-12 indicate two independent lines of PAT-PDX1-PAT-PDX2 (gray).
### Supplementary Table 1

**Primer sequences of RT-qPCR analysis**

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Supplementary Table 2

Relative levels of vitamer and glycosylated derivative to the total vitamin B6 content in cassava leaves (a) and storage roots (b) under field conditions

**a. Field-grown cassava leaves**

<table>
<thead>
<tr>
<th></th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.64</td>
<td>1.00 ± 0.07</td>
<td>1.00 ± 0.09</td>
<td>1.00 ± 0.09</td>
<td>1.00 ± 0.48</td>
<td>1.00 ± 0.32</td>
<td>1.00 ± 0.23</td>
<td>1.00 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>35S-3</td>
<td>5.24 ± 0.40</td>
<td>15.64 ± 1.64</td>
<td>1.76 ± 0.34</td>
<td>0.97 ± 0.10</td>
<td>1.45 ± 0.17</td>
<td>1.83 ± 0.24</td>
<td>8.63 ± 0.33</td>
<td>18.76 ± 0.37</td>
<td>1.20 ± 0.16</td>
<td>10.77 ± 0.43</td>
</tr>
<tr>
<td>35S-5</td>
<td>3.69 ± 0.88</td>
<td>14.28 ± 5.07</td>
<td>2.09 ± 0.69</td>
<td>0.89 ± 0.07</td>
<td>2.40 ± 0.29</td>
<td>5.83 ± 3.58</td>
<td>6.27 ± 0.56</td>
<td>19.51 ± 0.87</td>
<td>1.22 ± 0.23</td>
<td>10.56 ± 0.72</td>
</tr>
<tr>
<td>Pat-2</td>
<td>3.51 ± 0.10</td>
<td>3.20 ± 0.55</td>
<td>2.11 ± 0.14</td>
<td>1.20 ± 0.04</td>
<td>2.15 ± 0.29</td>
<td>2.89 ± 0.29</td>
<td>4.69 ± 0.19</td>
<td>12.16 ± 0.46</td>
<td>0.89 ± 0.19</td>
<td>6.21 ± 0.21</td>
</tr>
<tr>
<td>Pat-12</td>
<td>2.51 ± 0.48</td>
<td>8.02 ± 3.76</td>
<td>1.70 ± 0.24</td>
<td>0.91 ± 0.02</td>
<td>1.13 ± 0.32</td>
<td>1.30 ± 0.21</td>
<td>3.84 ± 0.19</td>
<td>9.53 ± 0.41</td>
<td>0.87 ± 0.08</td>
<td>5.52 ± 0.52</td>
</tr>
</tbody>
</table>

**b. Field-grown cassava storage roots**

<table>
<thead>
<tr>
<th></th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.07</td>
<td>1.00 ± 0.18</td>
<td>1.00 ± 0.22</td>
<td>1.00 ± 0.13</td>
<td>1.00 ± 0.40</td>
<td>1.00 ± 0.05</td>
<td>1.00 ± 0.12</td>
<td>1.00 ± 0.14</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>35S-3</td>
<td>3.01 ± 1.05</td>
<td>1.30 ± 0.27</td>
<td>1.19 ± 0.22</td>
<td>1.60 ± 0.14</td>
<td>1.29 ± 0.50</td>
<td>0.55 ± 0.11</td>
<td>2.47 ± 0.86</td>
<td>4.80 ± 0.14</td>
<td>1.15 ± 0.19</td>
<td>2.55 ± 0.33</td>
</tr>
<tr>
<td>35S-5</td>
<td>2.95 ± 0.20</td>
<td>1.37 ± 0.11</td>
<td>1.13 ± 0.03</td>
<td>2.18 ± 0.34</td>
<td>1.24 ± 0.12</td>
<td>1.21 ± 0.29</td>
<td>4.12 ± 0.20</td>
<td>8.94 ± 0.43</td>
<td>1.59 ± 0.04</td>
<td>4.10 ± 0.19</td>
</tr>
<tr>
<td>Pat-2</td>
<td>8.93 ± 1.63</td>
<td>5.71 ± 0.78</td>
<td>1.34 ± 0.24</td>
<td>1.82 ± 0.42</td>
<td>3.58 ± 0.76</td>
<td>1.35 ± 0.17</td>
<td>10.62 ± 2.80</td>
<td>23.64 ± 1.62</td>
<td>1.73 ± 0.07</td>
<td>9.77 ± 1.06</td>
</tr>
<tr>
<td>Pat-12</td>
<td>13.31 ± 1.16</td>
<td>9.54 ± 0.67</td>
<td>1.79 ± 0.30</td>
<td>3.01 ± 0.03</td>
<td>4.77 ± 0.90</td>
<td>1.28 ± 0.30</td>
<td>14.93 ± 1.11</td>
<td>27.63 ± 3.07</td>
<td>2.08 ± 0.29</td>
<td>12.25 ± 0.81</td>
</tr>
</tbody>
</table>

The data was obtained by an HPLC analysis and values are expressed relative to wild-type. Values are averages ± SE of three biological replicates. Numbers in bold indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
**Supplementary Table 3**

Relative contribution of free, phosphorylated and glycosylated derivatives to total vitamin B6 content in cassava leaves (a) and storage roots (b) under field conditions

a. Field-grown cassava leaves

<table>
<thead>
<tr>
<th>Leaves</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.31</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.28</td>
</tr>
<tr>
<td>35S-3</td>
<td>10.14 ± 1.03</td>
<td>1.13 ± 0.07</td>
<td>13.33 ± 0.29</td>
</tr>
<tr>
<td>35S-5</td>
<td>8.98 ± 3.02</td>
<td>1.51 ± 0.34</td>
<td>13.32 ± 0.55</td>
</tr>
<tr>
<td>Pat-2</td>
<td>3.13 ± 0.34</td>
<td>1.50 ± 0.08</td>
<td>8.48 ± 0.29</td>
</tr>
<tr>
<td>Pat-12</td>
<td>5.27 ± 2.16</td>
<td>0.98 ± 0.06</td>
<td>6.71 ± 0.29</td>
</tr>
</tbody>
</table>

b. Field-grown cassava storage roots

<table>
<thead>
<tr>
<th>Roots</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.13</td>
<td>1.00 ± 0.20</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>35S-3</td>
<td>1.73 ± 0.46</td>
<td>1.54 ± 0.19</td>
<td>3.03 ± 0.31</td>
</tr>
<tr>
<td>35S-5</td>
<td>1.68 ± 0.09</td>
<td>2.02 ± 0.26</td>
<td>5.34 ± 0.22</td>
</tr>
<tr>
<td>Pat-2</td>
<td>3.90 ± 0.42</td>
<td>2.08 ± 0.46</td>
<td>13.27 ± 1.41</td>
</tr>
<tr>
<td>Pat-12</td>
<td>5.76 ± 0.27</td>
<td>3.25 ± 0.14</td>
<td>16.20 ± 1.13</td>
</tr>
</tbody>
</table>

The data was obtained by an HPLC analysis and values are expressed relative to wild-type. Values are averages ± SE of three biological replicates. Numbers in bold indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
Supplementary Table 4

Percentage of free, phosphorylated and glycosylated forms of total vitamin B6 content in cassava leaves (a) and storage roots (b) under field conditions

a. Field-grown cassava leaves

<table>
<thead>
<tr>
<th>Leaves</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>22.82%</td>
<td>15.00%</td>
<td>62.18%</td>
</tr>
<tr>
<td>3SS-3</td>
<td>21.49%</td>
<td>1.57%</td>
<td>76.94%</td>
</tr>
<tr>
<td>3SS-5</td>
<td>19.41%</td>
<td>2.15%</td>
<td>78.44%</td>
</tr>
<tr>
<td>Pat-2</td>
<td>11.51%</td>
<td>3.62%</td>
<td>84.87%</td>
</tr>
<tr>
<td>Pat-12</td>
<td>21.77%</td>
<td>2.66%</td>
<td>75.57%</td>
</tr>
</tbody>
</table>

b. Field-grown cassava storage roots

<table>
<thead>
<tr>
<th>Roots</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>23.49%</td>
<td>11.59%</td>
<td>64.92%</td>
</tr>
<tr>
<td>3SS-3</td>
<td>15.92%</td>
<td>6.98%</td>
<td>77.10%</td>
</tr>
<tr>
<td>3SS-5</td>
<td>9.65%</td>
<td>5.73%</td>
<td>84.63%</td>
</tr>
<tr>
<td>Pat-2</td>
<td>9.38%</td>
<td>2.46%</td>
<td>88.16%</td>
</tr>
<tr>
<td>Pat-12</td>
<td>11.04%</td>
<td>3.07%</td>
<td>85.89%</td>
</tr>
</tbody>
</table>

The numbers were calculated from the HPLC results.
Supplementary Table 5

B6 vitamer and glycosylated derivative composition in cassava leaves after boiling. (a) Before boiling and (b) After boiling.

a. Field-grown cassava leaves before boiling (µg/g FW)

<table>
<thead>
<tr>
<th>Leaves</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.29 ± 0.21</td>
<td>0.39 ± 0.06</td>
<td>0.42 ± 0.06</td>
<td>1.53 ± 0.15</td>
<td>0.10 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>3.54 ± 0.39</td>
<td>6.78 ± 0.81</td>
<td>0.98 ± 0.35</td>
<td>15.08 ± 2.05</td>
</tr>
<tr>
<td>35S-3</td>
<td>6.55 ± 0.17</td>
<td>16.51 ± 6.45</td>
<td>0.55 ± 0.16</td>
<td>1.16 ± 0.07</td>
<td>0.12 ± 0.02</td>
<td>0.07 ± 0.01</td>
<td>18.00 ± 0.16</td>
<td>71.45 ± 1.08</td>
<td>3.44 ± 0.56</td>
<td>117.85 ± 6.32</td>
</tr>
<tr>
<td>35S-5</td>
<td>5.86 ± 0.18</td>
<td>20.70 ± 5.78</td>
<td>0.55 ± 0.13</td>
<td>0.79 ± 0.06</td>
<td>0.18 ± 0.01</td>
<td>0.09 ± 0.03</td>
<td>15.73 ± 0.17</td>
<td>67.63 ± 2.88</td>
<td>3.26 ± 0.44</td>
<td>114.78 ± 7.10</td>
</tr>
</tbody>
</table>

b. Field-grown cassava leaves after boiling (µg/g FW)

<table>
<thead>
<tr>
<th>Leaves</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.27 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.58 ± 0.08</td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.80 ± 0.08</td>
<td>1.20 ± 0.21</td>
<td>0.19 ± 0.05</td>
<td>3.20 ± 0.40</td>
</tr>
<tr>
<td>35S-3</td>
<td>1.42 ± 0.14</td>
<td>2.96 ± 1.67</td>
<td>0.18 ± 0.05</td>
<td>0.69 ± 0.15</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.00</td>
<td>4.52 ± 0.77</td>
<td>34.53 ± 6.49</td>
<td>0.99 ± 0.29</td>
<td>45.31 ± 6.95</td>
</tr>
<tr>
<td>35S-5</td>
<td>1.54 ± 0.32</td>
<td>1.84 ± 0.60</td>
<td>0.29 ± 0.03</td>
<td>0.62 ± 0.09</td>
<td>0.07 ± 0.02</td>
<td>0.01 ± 0.00</td>
<td>4.68 ± 0.85</td>
<td>45.94 ± 1.02</td>
<td>1.08 ± 0.06</td>
<td>56.06 ± 2.04</td>
</tr>
</tbody>
</table>

The data was obtained by an HPLC analysis and values are averages ± SE of three biological replicates.
Supplementary Table 6

B6 vitamer and glycosylated derivative composition in cassava storage roots after baking. (a) Before baking and (b) After baking.

a. Field-grown cassava storage roots before baking (µg/g FW)

<table>
<thead>
<tr>
<th>Roots</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.48 ± 0.05</td>
<td>0.06 ± 0.01</td>
<td>0.14 ± 0.03</td>
<td>0.20 ± 0.02</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>1.37 ± 0.12</td>
<td>2.57 ± 0.17</td>
<td>0.45 ± 0.04</td>
<td>5.29 ± 0.31</td>
</tr>
<tr>
<td>Pat-2</td>
<td>1.21 ± 0.02</td>
<td>0.23 ± 0.03</td>
<td>0.18 ± 0.05</td>
<td>0.27 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.00</td>
<td>3.51 ± 0.06</td>
<td>25.78 ± 4.33</td>
<td>0.86 ± 0.07</td>
<td>32.06 ± 4.33</td>
</tr>
<tr>
<td>Pat-12</td>
<td>2.07 ± 0.20</td>
<td>0.37 ± 0.04</td>
<td>0.48 ± 0.06</td>
<td>0.56 ± 0.12</td>
<td>0.05 ± 0.01</td>
<td>trace</td>
<td>6.68 ± 0.63</td>
<td>28.73 ± 3.94</td>
<td>1.17 ± 0.21</td>
<td>40.11 ± 3.93</td>
</tr>
</tbody>
</table>

b. Field-grown cassava storage roots after baking (µg/g FW)

<table>
<thead>
<tr>
<th>Roots</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.44 ± 0.08</td>
<td>0.08 ± 0.02</td>
<td>0.28 ± 0.03</td>
<td>0.49 ± 0.08</td>
<td>0.01 ± 0.00</td>
<td>trace</td>
<td>1.23 ± 0.23</td>
<td>2.18 ± 0.25</td>
<td>0.52 ± 0.05</td>
<td>5.23 ± 0.67</td>
</tr>
<tr>
<td>Pat-2</td>
<td>1.67 ± 0.06</td>
<td>0.32 ± 0.02</td>
<td>0.55 ± 0.02</td>
<td>0.69 ± 0.05</td>
<td>0.01 ± 0.01</td>
<td>trace</td>
<td>4.77 ± 0.01</td>
<td>14.24 ± 7.98</td>
<td>1.29 ± 0.10</td>
<td>23.55 ± 8.11</td>
</tr>
<tr>
<td>Pat-12</td>
<td>2.79 ± 0.01</td>
<td>0.50 ± 0.04</td>
<td>0.50 ± 0.06</td>
<td>1.38 ± 0.20</td>
<td>0.07 ± 0.02</td>
<td>0.01 ± 0.00</td>
<td>8.15 ± 0.42</td>
<td>12.11 ± 7.76</td>
<td>0.94 ± 0.04</td>
<td>26.45 ± 7.89</td>
</tr>
</tbody>
</table>

The data was obtained by an HPLC analysis and values are averages ± SE of three biological replicates except for Pat-2 and Pat-12 in (b) which are with two biological replicates.
Supplementary Table 7

Relative levels of free, phosphorylated and glycosylated forms of vitamin B6 in cassava leaves after boiling. (a) Before boiling and (b) After boiling.

a. Field-grown cassava leaves before boiling

<table>
<thead>
<tr>
<th>Leaves</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.15</td>
<td>1.00 ± 0.11</td>
<td>1.00 ± 0.14</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td>35S-3</td>
<td>11.21 ± 3.10</td>
<td>0.80 ± 0.06</td>
<td>8.22 ± 0.03</td>
<td>7.82 ± 0.42</td>
</tr>
<tr>
<td>35S-5</td>
<td>12.86 ± 2.73</td>
<td>0.63 ± 0.06</td>
<td>7.67 ± 0.31</td>
<td>7.61 ± 0.47</td>
</tr>
</tbody>
</table>

b. Field-grown cassava leaves after boiling

<table>
<thead>
<tr>
<th>Leaves</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.04</td>
<td>1.00 ± 0.12</td>
<td>1.00 ± 0.15</td>
<td>1.00 ± 0.12</td>
</tr>
<tr>
<td>35S-3</td>
<td>11.29 ± 4.35</td>
<td>1.19 ± 0.24</td>
<td>18.29 ± 3.40</td>
<td>14.18 ± 2.17</td>
</tr>
<tr>
<td>35S-5</td>
<td>9.09 ± 1.26</td>
<td>1.15 ± 0.12</td>
<td>23.62 ± 0.88</td>
<td>17.54 ± 0.64</td>
</tr>
</tbody>
</table>

The data was obtained by an HPLC analysis and values are expressed relative to wild-type. Values are averages ± SE of three biological replicates.

Supplementary Table 8

Relative levels of free, phosphorylated and glycosylated forms of vitamin B6 in cassava storage roots after baking (a) Before baking and (b) After baking.

a. Field-grown cassava storage roots before baking

<table>
<thead>
<tr>
<th>Roots</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.12</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.06</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>Pat-2</td>
<td>2.40 ± 0.06</td>
<td>1.36 ± 0.09</td>
<td>6.86 ± 0.98</td>
<td>6.06 ± 0.82</td>
</tr>
<tr>
<td>Pat-12</td>
<td>4.33 ± 0.19</td>
<td>2.77 ± 0.57</td>
<td>8.32 ± 0.91</td>
<td>7.58 ± 0.74</td>
</tr>
</tbody>
</table>

b. Field-grown cassava storage roots after baking

<table>
<thead>
<tr>
<th>Roots</th>
<th>Free</th>
<th>Phosphorylated</th>
<th>Glycosylated</th>
<th>Total vitamin B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.00 ± 0.15</td>
<td>1.00 ± 0.17</td>
<td>1.00 ± 0.12</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>Pat-2</td>
<td>3.15 ± 0.12</td>
<td>1.40 ± 0.10</td>
<td>5.18 ± 2.06</td>
<td>4.50 ± 1.55</td>
</tr>
<tr>
<td>Pat-12</td>
<td>4.71 ± 0.10</td>
<td>2.89 ± 0.36</td>
<td>5.41 ± 1.71</td>
<td>5.06 ± 1.23</td>
</tr>
</tbody>
</table>

The data was obtained by an HPLC analysis and values are expressed relative to wild-type. Values are averages ± SE of three biological replicates except for Pat-2 and Pat-12 in (b) with 2 biological replicates.
**Supplementary Table 9**

**Primers used for vector construction and PCR screening**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>polyA-F</td>
<td>5'-TCTAGAGTCGACCCTGCAGG-3'</td>
</tr>
<tr>
<td>polyA-R</td>
<td>5'-ACAATCAGTAAATTGAACGGAG-3'</td>
</tr>
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<td>Patatin-F-EcoRI</td>
<td>5'-GAATTCTGCAGTTGTAGTTAATGC-3'</td>
</tr>
<tr>
<td>Patatin-R-KpnI</td>
<td>5'-GGTACCATGGCATATAACAAGCAT-3'</td>
</tr>
<tr>
<td>AtPDX2-F</td>
<td>5'-CTACGTGAGTTTGTTAAGATG-3'</td>
</tr>
<tr>
<td>AtPDX2-R</td>
<td>5'-CTCTTCTGACCAACTGCTC-3'</td>
</tr>
<tr>
<td>Pat-F2</td>
<td>5'-ATGACAGTTGCCTGGCAAAG-3'</td>
</tr>
<tr>
<td>PDX2-seq</td>
<td>5'-AATCTCGACGCTTTGGAC-3'</td>
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Chapter 3 Vitamin B6 biofortification of rice by metabolic engineering

Abstract
Vitamin B6 is an essential micronutrient to maintain metabolism in all organisms including humans. It serves as a cofactor for enzymes in various metabolic pathways, and is a potent antioxidant to scavenge reactive oxygen species and promote human health. The recent characterization of genes essential for vitamin B6 accumulation in Arabidopsis opens new perspectives to increase vitamin B6 in crop plants through genetic engineering. Here, we report genetic engineering of rice expressing two key genes from Arabidopsis, AtPDX1.1 and AtPDX2, essential for biosynthesis de novo of vitamin B6 in plants. Transgenic rice showed up to 2- and 1.3-fold increase of total vitamin B6 in unpolished and polished rice grains, respectively. The increase of vitamin B6 in transgenic rice was largely due to glycosylated derivatives. Our results demonstrate the potential of vitamin B6 biofortification in rice.

Introduction
The vitamin B6 biosynthesis pathway has been recently characterized in plants. Three genes involved in the salvage pathway have been characterized in Arabidopsis. SOS4 is a kinase which phosphorylates free B6 vitamers producing phosphorylated derivatives (González et al., 2007; Rueschhoff et al., 2013; Shi and Zhu, 2002). PDX3 is an oxidase of pyridoxine and pyridoxamine (González et al., 2007; Sang et al., 2007). PLR1 was reported to function as a pyridoxal reductase (Herrero et al., 2011). Additionally, there may have non-specific phosphatases to dephosphorylate the phosphorylated derivatives (Fitzpatrick et al., 2007). All organisms use a salvage pathway to interconvert six different forms of B6 vitamers. The de novo biosynthesis pathway in planta involves only two genes, AtPDX1 and AtPDX2, and occurs in the cytosol (Tambasco-Studart et al., 2007; Tambasco-Studart et al., 2005). The limited number of enzymes involved in vitamin B6 de novo biosynthesis has facilitated the development of strategies to increase vitamin B6 accumulation in crop plants. Genetic engineering of vitamin B6 has been successfully conducted in Arabidopsis by over-expressing AtPDX1 and AtPDX2 together. Transgenic Arabidopsis over-expressing AtPDX1.3 and AtPDX2 under control of the seed-specific 12S promoter have achieved a 3-fold increase of vitamin
B6 in seeds (Chen and Xiong, 2009). The aforementioned transgenic plant was reported to have an equivalent phenotype to wild-type plants. Nevertheless, transgenic Arabidopsis transformed with AtPDX1.3 showed retarded growth or limited increases of vitamin B6 in two other independent studies (Leuendorf et al., 2010; Raschke et al., 2011). It has been demonstrated that over-expression of AtPDX1.1 in combination with AtPDX2 could increase vitamin B6 content up to 4-fold in Arabidopsis shoots and seeds, respectively, under constitutive expression (Raschke et al., 2011). To date, over-expression of the adequate combination of AtPDX1.1 and AtPDX2 transgenes has resulted in the best strategy to increase vitamin B6 accumulation in Arabidopsis.

Genetic engineering of vitamin B6 in plants has so far only been reported in model plants, i.e. Arabidopsis and tobacco (Chen and Xiong, 2009; Herrero and Daub, 2007; Leuendorf et al., 2010; Raschke et al., 2011). The vitamin B6 content in polished rice grains provides only 31% of adult daily requirements and a 3-fold increase in vitamin B6 content has been estimated to be theoretically sufficient to address the issue of vitamin B6 deficiency (Fitzpatrick et al., 2012). Presently, the vitamin B6 content of polished rice grains is not sufficient to meet human daily requirements.

To investigate the possibility of genetic engineering to improve vitamin B6 content in rice, two Arabidopsis genes AtPDX1.1 and AtPDX2, previously shown to be sufficient to increase vitamin B6 levels in Arabidopsis, were expressed in rice. In the present study, both constitutive and endosperm-specific expression strategies were pursued. Our results indicate that the total vitamin B6 content can be significantly enhanced in rice leaves and unpolished seeds and that vitamin B6 levels correlate with the accumulation of AtPDX1.1 transcripts. Analysis of rice endosperm revealed limited vitamin B6 accumulation. We further discuss the strategy of combining AtPDX1.1 and AtPDX2 for increased vitamin B6 accumulation and other possibilities for vitamin B6 biofortification through genetic engineering of rice.

Results

Generation of transgenic rice

Two strategies were deployed to enhance vitamin B6 in rice. In the first transformation vector, both AtPDX1.1 and AtPDX2 were driven by the constitutive 35S promoter; this construct is referred to as 35S-PDX1-35S-PDX2 hereafter (Figure 1a). In a second transformation vector, both AtPDX1.1 and AtPDX2 were under the control of an endosperm-specific globulin promoter (Qu and Takaiwa, 2004), which was generated to drive over-expression of PDX transgenes in rice endosperm; this is later referred to as Glo-PDX1-Glo-PDX2 (Figure 1b).
Figure 1. Schematic diagram of transformation vectors T-DNA regions. 
(a) Constitutive expression vector, 35S-PDX1-35S-PDX2 (b) Endosperm-specific expression vector, Glo-PDX1-Glo-PDX2. 35S, CaMV 35S promoter; Glo, globulin promoter; AtPDX1.1, At2g38230; AtPDX2, At5g60540; HPT, hygromycin phosphotransferase; LB, left border of T-DNA; RB, right border of T-DNA; T1, CaMV 35S terminator; T2, Octopine synthase terminator.

Transgenic rice plants were generated using Agrobacterium-mediated transformation of both transformation vectors and putative transgenic lines were selected for molecular characterization. The number of T-DNA integration events in the rice genome was confirmed by Southern blot analysis (Supplementary Figure 1). Transgene integration (AtPDX1.1) was confirmed by PCR (Supplementary Figure 2).

Four transgenic 35S-PDX1-35S-PDX2 lines with 1-3 T-DNA insertions and three transgenic Glo-PDX1-Glo-PDX2 lines with a single T-DNA insertion were selected for further evaluation. The T1 seeds were germinated on hygromycin-containing medium to obtain transgene-containing seedlings. It must be noted that three of the four independent transgenic lines (35S-7, 35S-27, and 35S-29) containing the 35S-PDX1-35S-PDX2 vector showed growth inhibition or retardation when germinating on hygromycin-containing medium (Supplementary Figure 3a,c). The transgenic line 35S-12 was the only transgenic 35S-PDX1-35S-PDX2 line that grew normally on selective medium (Supplementary Figure 3b). The Glo-PDX1-Glo-PDX2 transgenic seedlings did not display any growth defect or retardation on selective medium (Supplementary Figure 3d,e).

Transgenic rice lines accumulate high levels of vitamin B6 in leaves and grains under greenhouse conditions

We then determined the amounts of vitamin B6 in both transgenic 35S-PDX1-35S-PDX2 and Glo-PDX1-Glo-PDX2 rice under greenhouse conditions. Selected transgenic lines of both transformation vectors at the T3 generation were grown together including wild-type and one transgenic pCAMBIA1300 line as an empty vector control. Total vitamin B6 content was quantified using a yeast microbiological assay (Tambasco-Studart et al., 2005), which offers an effective way to estimate the total vitamin B6 content. Rice extracts were subjected to phosphatase and β-glucosidase treatment in order to convert phosphorylated and glycosylated derivatives into free forms to allow total vitamin B6 quantification using a microbiological assay.
Figure 2. Analysis of total vitamin B6 content in transgenic rice expressing AtPDX1.1 and AtPDX2 under greenhouse conditions. (a) Leaves at reproductive stage. (b) Dry unpolished seeds. (c) Dry polished seeds. The data was measured using a yeast microbiological assay and values are averages ± SD of three biological replicates. 35S-7, 35S-12, 35S-27 and 35S-29 indicate four independent lines of 35S-PDX1-35S-PDX2. Glo-13, Glo-41 and Glo-47 indicate three independent lines of Glo-PDX1-Glo-PDX2. pCAMBIA1300, transgenic pCAMBIA1300 line; wt, wild-type plant. wt and pCAMBIA1300 control (white), 35S-PDX1-35S-PDX2 (gray), Glo-PDX1-Glo-PDX2 (black). Asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
In mature rice leaves at the reproductive stage, the transgenic control line (pCAMBIA1300) showed no change of the vitamin B6 content compared to wild-type plants. Amongst all selected transgenic lines, one transgenic line (35S-12) showed over 10-fold increased levels of total vitamin B6 compared to the levels measured in wild-type and empty vector control plants (Figure 2a). Three transgenic lines (35S-7, 35S-27 and 35S-29) of 35S-PDX1-35S-PDX2 vector and three transgenic lines (Glo-13, Glo-41 and Glo-47) of Glo-PDX1-Glo-PDX2 vector did not have significantly increased levels of vitamin B6 compared to wild-type.

We further quantitated total vitamin B6 content in rice grains. As it is difficult to quantify vitamin B6 in embryos and the aleurone layer directly, we performed quantification in unpolished and polished seeds. In contrast to leaves, an increase of total vitamin B6 content was observed among transgenic lines of both 35S-PDX1-35S-PDX2 and Glo-PDX1-Glo-PDX2 vectors. Three out of four transgenic 35S-PDX1-35S-PDX2 lines showed up to a 2.3-fold increase of vitamin B6 in unpolished seeds compared to wild-type (Figure 2b). No change of total vitamin B6 content was observed in transgenic line 35S-27. Two out of three transgenic Glo-PDX1-Glo-PDX2 lines (Glo-41 and Glo-47) displayed a substantial increase of total vitamin B6 content up to nearly 2-fold. Transgenic line Glo-13 displayed no change of total vitamin B6 content compared to wild-type. As expected, the empty vector control pCAMBIA1300 line had no difference of total vitamin B6 content in unpolished seeds compared to wild-type.

Total vitamin B6 content in polished seeds showed a limited increase in transgenic 35S-PDX1-35S-PDX2 lines (Figure 2c). The best performing transgenic line (35S-12) of the 35S-PDX1-35S-PDX2 vector showed a 1.2-fold increase in polished seeds. Transgenic lines 35S-7, 35S-27 and 35S-29 showed slight increases compared to wild-type. We detected only slight increases of vitamin B6 up to 1.3-fold in the three transgenic Glo-PDX1-Glo-PDX2 lines. No change in the total vitamin B6 content was observed in the pCAMBIA1300 line. The rice bran (the embryo and aleurone layer) weight is 10% of the entire unpolished seed (Tanaka et al., 2006). Compared to the quantification of unpolished seeds, it was revealed that vitamin B6 was highly accumulated in the embryo and/or aleurone layer of transgenic rice.

**Total vitamin B6 is stable during drying**

To investigate the effect of moisture content reduction during postharvest drying on vitamin B6, we performed quantification in fresh rice grains of selected transgenic lines at the dough stage of grain filling. Transgenic line 35S-12 showed a 2.7-fold increase and transgenic line 35S-7 had no change of total vitamin B6 content in fresh unpolished seeds (Supplementary Figure 4a). Three transgenic Glo-PDX1-Glo-PDX2 lines showed around 2-fold increase of total vitamin B6 in fresh unpolished seeds, but the increase of total vitamin B6 in transgenic line Glo-47 was not statistically significant. No or little increase was observed in fresh polished
seeds of transgenic 35S-PDX1-35S-PDX2 lines and transgenic Glo-PDX1-Glo-PDX2 lines (Supplementary Figure 4a). Based on the observations (Supplementary Figure 4a,b), the relative vitamin B6 levels remained stable before and after the dry processing. It revealed that vitamin B6 accumulation in the endosperm of transgenic lines was limited during seed development. The drying did not alter the fold change of vitamin B6 between transgenic and wild-type rice.

Expression of PDX transgenes correlate with vitamin B6 accumulation in transgenic rice

To confirm whether the expression of transgenes was in accordance with vitamin B6 accumulation, RT-qPCR was performed to quantify the levels of transgene expression in selected lines. Expression of AtPDX1.1 and AtPDX2 was observed in rice leaves of transgenic line 35S-12 accumulating high levels of vitamin B6 (Figure 3a). The levels of AtPDX1.1 and AtPDX2 transcripts in leaves of transgenic line 35S-7 and in three transgenic Glo-PDX1-Glo-PDX2 lines were significantly lower than transgenic line 35S-12. Expression levels of AtPDX1.1 and AtPDX2 in leaves of transgenic line 35S-12 were 60- and 15-fold higher than transgenic lines which had no increase of vitamin B6, respectively. Expression analysis of AtPDX2 revealed that AtPDX2 transcripts accumulated at lower levels in leaves of transgenic lines compared to AtPDX1.1. Expression levels of PDX transgenes correlated with vitamin B6 content in transgenic leaves.

We subsequently analyzed the expression levels of transgenes in rice grains. AtPDX1.1 transcripts accumulated at lower levels in polished seeds of transgenic 35S-PDX1-35S-PDX2 lines compared to AtPDX2 (Figure 3b). In contrast to transgenic 35S-PDX1-35S-PDX2 lines, AtPDX1.1 transcripts in polished seeds of transgenic Glo-PDX1-Glo-PDX2 lines were 1.5-fold higher than AtPDX2. The average transcript level of AtPDX1.1 in transgenic Glo-PDX1-Glo-PDX2 lines was 5-fold higher than the average transcript level of AtPDX1.1 in transgenic 35S-PDX1-35S-PDX2 lines (Figure 3b; Supplementary Figure 5a). AtPDX2 transcripts accumulated in polished seeds were not considerably different between transgenic lines of both transformation vectors. We did not observe a clear correlation between expression levels of PDX transgenes and vitamin B6 accumulation in polished seeds (Figure 3b; Supplementary Figure 4a).

Expression patterns of AtPDX1.1 and AtPDX2 were similar in polished and unpolished seeds of transgenic 35S-PDX1-35S-PDX2 lines (Figure 3b,c). AtPDX1.1 transcripts accumulated at lower levels in unpolished seeds of transgenic 35S-PDX1-35S-PDX2 lines compared to AtPDX2 (Figure 3c). AtPDX1.1 transcripts accumulated at a similar level to AtPDX2 in unpolished seeds of transgenic Glo-PDX1-Glo-PDX2 lines (Figure 3c). The levels of AtPDX2 transcripts in unpolished seeds of transgenic 35S-PDX1-35S-PDX2 lines were significantly higher than transgenic Glo-PDX1-Glo-PDX2 lines. AtPDX2 transcripts accumulated in
Figure 3. RT-qPCR analysis of AtPDX1.1 and AtPDX2 expression in transgenic rice. (a) Leaves at the reproductive stage (b) Fresh polished seeds and (c) Fresh unpolished seeds at the dough stage. Values are averages ± SD of three biological replicates. AtPDX1.1 (white), AtPDX2 (gray).
transgenic line 35S-12 were 10-fold higher than transgenic Glo-PDX1-Glo-PDX2 lines. It revealed that AtPDX2 mainly accumulated in rice bran of transgenic 35S-PDX1-35S-PDX2 lines (Supplementary Figure 5b). The vitamin B6 content in unpolished seeds was in accordance with expression levels of the AtPDX1.1 transgene (Figure 3c; Supplementary Figure 4a). The expression level of AtPDX2 in unpolished seeds was not related to vitamin B6 content.

**Distribution of B6 vitamers is altered in transgenic rice and vitamin B6 increase is largely due to PNG**

To further quantify B6 vitamers and glycosylated derivatives, we performed measurements using HPLC. The best performing transgenic line for each transformation vector (35S-12 and Glo-41) and wild-type plants were selected for HPLC analysis.

In wild-type plants, glycosylated derivatives were the most abundant forms in both leaves and polished seeds, accounting for 78% and 87% of the total vitamin B6 content, respectively (Figure 4; Table 1). Phosphorylated derivatives were the second most abundant forms in wild-type leaves. The amount of phosphorylated derivatives was 2-fold higher than free forms of vitamin B6 in leaves. Similar levels of free forms and phosphorylated derivatives of vitamin B6 were observed in wild-type polished seeds.

We found different compositions of vitamin B6 between wild-type and transgenic rice. First, total vitamin B6 content in leaves of transgenic line 35S-12 was substantially increased up to 14-fold compared to wild-type (Figure 4a). Transgenic line Glo-41 also showed a slight increase of vitamin B6 in leaves. Second, transgenic lines 35S-12 and Glo-41 showed a 1.5- and 2.2-fold increase in polished seeds over wild-type plants, respectively (Figure 4b). The fold increase of total vitamin B6 content in polished seeds measured by HPLC was not equal to the measurements using a yeast microbiological quantification (Figure 2c; Figure 4b). The HPLC analysis found significantly higher increase in total vitamin B6 content than the microbiological quantification.

Glycosylated derivatives of vitamin B6 were the major contributors to vitamin B6 accumulation in either transgenic or wild-type rice leaves (Figure 4a; Table 1a). Transgenic line 35S-12 showed approximately an 18-fold increase of glycosylated derivatives compared to wild-type and contributed to 95% of total vitamin B6 in this best performing line. Transgenic line Glo-41 showed a 1.8-fold increase of glycosylated derivatives over wild-type in leaves and contributed to 88% of the total vitamin B6 pool. PNG levels in transgenic rice leaves displayed the largest increase, 20- and 2-fold in transgenic line 35S-12 and Glo-41, respectively, over wild-type. Phosphorylated derivatives were the second most abundant forms in wild-type leaves. However, compared to wild-type, no change of phosphorylated derivatives content was observed in leaves of transgenic line 35S-12. PNP content was
decreased in leaves of transgenic line Glo-41. In transgenic line 35S-12, free forms of vitamin B6 were the second most abundant forms in leaves. Substantial increases of PM, PN and PL were observed in leaves of transgenic line 35S-12. PN content in leaves of transgenic line 35S-12 was 4 times higher than PM and PL. Compared to wild-type leaves, the level of PN was increased 36-fold in transgenic line 35S-12. Free forms of vitamin B6 in leaves of transgenic line Glo-41 showed no difference compared to wild-type plants.

Figure 4. Free, phosphorylated and glycosylated vitamin B6 in transgenic and wild-type rice. (a) Mature leaves (b) Polished seeds. 35S-12, transgenic 35S-PDX1-35S-PDX2 line; Glo-41, transgenic Glo-PDX1-Glo-PDX2 line; wt, wild-type plant. Values are averages ± SE of three biological replicates. Free (white), glycosylated (gray), and phosphorylated (black) forms of vitamin B6.

A vitamer analysis revealed that glycosylated derivatives were also the major contributors to vitamin B6 accumulation in polished seeds as well as in leaves (Figure 4b). Despite its high variation, an increase of glycosylated derivatives was observed in transgenic line 35S-12 and Glo-41 (Table 1b). However, the increase of total glycosylated derivatives in transgenic
polished seeds was not statistically significant. The content of phosphorylated derivatives in polished seeds of transgenic line 35S-12 and Glo-41 showed no difference compared to wild-type. The level of PN in transgenic polished seeds was increased 4- and 2.3-fold in transgenic line 35S-12 and Glo-41, respectively. PM and PL in both transgenic lines were unchanged compared to wild-type plants.

Due to some technical issues, PNP and PMG have not yet been measured in polished seeds, and PMG has not yet been measured in leaves. In addition, we only selected one line of each transformation vector to perform the HPLC quantification. A larger sample size would be required to derive robust conclusions about the alteration of B6 vitamers in transgenic rice.
### Table 1

B6 vitamer and glycosylated derivative composition in transgenic and wild-type rice leaves (a) and polished seeds (b)

#### (a) Rice leaves (µg/g FW)

<table>
<thead>
<tr>
<th>Leaves</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.51 ± 0.06</td>
<td>0.21 ± 0.02</td>
<td>0.45 ± 0.03</td>
<td>2.22 ± 0.11</td>
<td>0.64 ± 0.08</td>
<td>0.07 ± 0.00</td>
<td>n.m.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35S-12</td>
<td>1.91 ± 0.07</td>
<td>7.56 ± 0.13</td>
<td>1.59 ± 0.04</td>
<td>1.72 ± 0.16</td>
<td>0.75 ± 0.03</td>
<td>0.19 ± 0.04</td>
<td>n.m.</td>
<td>226.02 ± 25.82</td>
<td>0.81 ± 0.43</td>
</tr>
<tr>
<td>Glo-41</td>
<td>0.48 ± 0.18</td>
<td>0.33 ± 0.08</td>
<td>0.67 ± 0.09</td>
<td>1.73 ± 0.06</td>
<td>0.26 ± 0.05</td>
<td>trace</td>
<td>n.m.</td>
<td>20.40 ± 2.12</td>
<td>3.19 ± 0.53</td>
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</table>

#### (b) Polished rice seeds (µg/g DW)

<table>
<thead>
<tr>
<th>Polished seeds</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
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<tr>
<td>wt</td>
<td>0.15 ± 0.01</td>
<td>0.03 ± 0.00</td>
<td>0.05 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td>n.m.</td>
<td>trace</td>
<td>n.m.</td>
<td>2.30 ± 0.01</td>
<td>0.37 ± 0.04</td>
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<tr>
<td>35S-12</td>
<td>0.18 ± 0.01</td>
<td>0.12 ± 0.00</td>
<td>0.05 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>n.m.</td>
<td>trace</td>
<td>n.m.</td>
<td>2.80 ± 0.14</td>
<td>1.27 ± 0.20</td>
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<tr>
<td>Glo-41</td>
<td>0.14 ± 0.05</td>
<td><strong>0.07 ± 0.01</strong></td>
<td>0.07 ± 0.02</td>
<td>0.26 ± 0.04</td>
<td>n.m.</td>
<td>0.12 ± 0.09</td>
<td>n.m.</td>
<td>3.87 ± 0.43</td>
<td>2.14 ± 0.48</td>
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The data was obtained by HPLC analysis and values are averages ± SE of three biological replicates. n.m., not measured. The numbers in bold indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
Discussion

In the present study, we genetically engineered increased vitamin B6 accumulation in one of the most important staple crops in the world, particularly in Asian countries. Transgenic rice showed a maximal two-fold increase in vitamin B6 content in unpolished seeds using both constitutive and endosperm-specific expression strategies. Slight increases of vitamin B6 (20-30%) were observed in polished seeds of both transformation vectors using the microbiological quantification. Together, transgenic rice using the constitutive expression strategy also displayed more than 10-fold increase of vitamin B6 content in rice leaves.

Examining more closely the expression levels of transgenes, high vitamin B6 transgenic line (35S-12) had a substantial increase of AtPDX1.1 and AtPDX2 transcripts in leaves. Transgenic lines which displayed no or limited increase of vitamin B6 in leaves showed significantly lower levels of PDX transcripts accumulation. In unpolished seeds, vitamin B6 accumulation was correlated with the expression of AtPDX1.1. Expression levels of AtPDX2 appeared not to be the determining factor of vitamin B6 production in unpolished seeds. This is further confirmed by the same observation in transgenic Arabidopsis (Raschke et al., 2011). However, vitamin B6 accumulation in polished seeds was different comparing the observations in transgenic rice leaves and unpolished seeds. The expression levels of AtPDX1.1 in polished seeds of transgenic Glo-PDX1-Glo-PDX2 lines are 5-fold higher than the ones in transgenic 35S-PDX1-35S-PDX2 lines, but vitamin B6 content in transgenic Glo-PDX1-Glo-PDX2 lines is equal to the ones in transgenic 35S-PDX1-35S-PDX2 lines. Compared to the quantification results from unpolished and polished seeds, it suggests that vitamin B6 is highly accumulated in the embryo and/or aleurone layer. We found little increase of vitamin B6 in endosperm, whereas AtPDX1.1 and AtPDX2 transcripts were highly accumulated. This reveals that there are currently unknown factors modulating vitamin B6 accumulation in polished seeds. We suspected that vitamin B6 may be unstable during the drying process. To explore the effect of moisture content reduction on vitamin B6 content, we quantified vitamin B6 levels in fresh polished seeds. The results suggested that vitamin B6 accumulation was limited in fresh polished seeds as well as dry seeds. Another possible explanation is that precursors of vitamin B6 biosynthesis (e.g. glutamine, ribose 5-phosphate, glyceraldehyde) may be restricted in rice endosperm. Further characterization of metabolite profiling in the endosperm will help us to understand the bottleneck of vitamin B6 production in polished seeds.

While germinating transgenic seeds of transgenic 35S-PDX1-35S-PDX2 lines on hygromycin-containing medium, we noticed that the hpt selectable marker may be silenced in seedlings, while it was previously active at the callus stage during the transformation procedure. Growth inhibition of germinating transgenic seeds on hygromycin-containing medium appeared in three out of four selected transgenic 35S-PDX1-35S-PDX2 lines, therefore it seems unlikely that the growth inhibition was due to position effects of T-DNA integration in the rice genome. We later confirmed that these growth retarded plants did indeed contain
integrated transgenes. Though we did not quantify hpt transcripts directly, expression analysis revealed that transgene expression levels in transgenic line 35S-7 were significant lower than the ones in transgenic line 35S-12 in both leaves and seeds. It must be noted that the 35S promoter was used repetitively (for expression of the selectable marker gene, AtPDX1.1 and AtPDX2) in the 35S-PDX1-35S-PDX2 vector. It has been reported that repetitious use of the 35S promoter can have adverse effects on transgene expression in transgenic plants (Matzke and Matzke, 1995; Meyer and Saedler, 1996; Peremarti et al., 2010; Vaucheret et al., 1998). Therefore, to optimize the transformation vector for genetic engineering of rice, careful selection of promoters for the expression of multiple transgenes in a single T-DNA construct should be taken into consideration.

We used two different methods (HPLC and yeast microbiological quantification) to quantify the vitamin B6 content. The quantification result of the vitamin B6 content by a microbiological assay did not correspond to the HPLC analysis. The value of vitamin B6 content using HPLC analysis is higher than the values obtained by microbiological assay. It has been reported previously that results obtained using the two methods had differed by up to 70% in the same samples (Kall, 2003). Our preliminary results using the HPLC method displayed a 2-fold increase in vitamin B6 in polished seeds of transgenic line Glo-41. However, the same sample only showed a 20-30% increase using the yeast microbiological assay. Similar results were also observed in transgenic line 35S-12. Because the other metabolites in rice extracts may impede yeast growth for microbiological assay, we will adopt the HPLC quantification method to obtain an accurate value of vitamin B6 in later analyses. The correlation between two quantification methods needs further determination.

Glycosylated derivatives were the predominant forms in rice leaves and seeds, accounting for more than 90% of the total vitamin B6 pool in transgenic plants (Figure 4). It has been reported that the PNG form is only 50% bioavailable for human consumption (Gregory III, 2012). Because PNG was the major contributor of vitamin B6 accumulation, transgenic rice seeds may have lower vitamin B6 bioavailability than would be expected based on the measurement of total vitamin B6. The bioavailability of vitamin B6 content in transgenic rice grains is still subject to experimental validation. For example, an in vitro solubility method and the Caco-2 cell model have been reported for evaluating the bioavailability of vitamin B6 for human nutrition (Etcheverry et al., 2012). In the future, we can utilize these two methods to further examine the bioavailability of vitamin B6 in transgenic rice grains.

The pathway responsible for the formation of glycosylated derivatives in plants is still an open question. The conversion procedure between B6 vitamers and glycosylated derivatives must be active in transgenic rice because of the high levels of PNG and other vitamers accumulation. Further characterization including proteomics and genome-wide expression analysis of transgenic and wild-type plants could identify the genes involved in interconversion of B6 vitamers and glycosylated derivatives. These candidate genes may be blocked by genetic engineering, i.e. RNAi technology, in order to reduce the formation of
glycosylated derivatives. This knowledge will assist in developing new strategies to produce predominantly free and phosphorylated forms of vitamin B6 in transgenic plants. Both free and phosphorylated forms of vitamin B6 were reported to be fully bioavailable for human consumption (Gregory III, 2012). Moreover, transgenic Arabidopsis with enhanced levels of phosphorylated derivatives has been demonstrated to have higher biomass accumulation and tolerance to oxidative stress (Raschke et al., 2011). Increased levels of phosphorylated derivatives in transgenic rice may improve biomass accumulation and raise stress tolerance.

The limited success of increasing vitamin B6 accumulation in rice endosperm could be improved by combining other strategies. A synergistic approach to transport specific nutrients from source organ to edible organ has been proven in rice iron biofortification studies (Masuda et al., 2012; Wirth et al., 2009). Transgenic 35S-PDX1-35S-PDX2 rice displayed abundant amounts of vitamin B6 in leaves and embryos. The synergistic approach provides an opportunity to develop novel strategies to transport vitamin B6 from highly accumulating tissues into the endosperm. Expression of vitamin B6 transporters in plants may alter the dynamics of vitamin B6 in different tissues, but the candidate transporters need further elucidation. If vitamin B6 could be potentially transported to endosperm by certain transporters, engineering the vitamin B6 transporter together with constitutive expression of PDX transgenes may result in successful transport of the vitamin from leaves or embryo into the endosperm. We speculate that vitamin B6 could be highly accumulated in rice endosperm through this type of approach. Moreover, parboiled rice is consumed worldwide particularly in south Asian countries such as India, Pakistan, and Bangladesh (Kar et al., 1999). During the parboiling process, micronutrients in the rice bran could diffuse into the endosperm at the steaming step. The vitamin B6 content in transgenic rice endosperm can be enriched through this existing postharvest technology of rice production. Additionally, rice bran is used to feed poultry and livestock generally in rice production countries. The by-product of rice grains after milling (rice bran) with high vitamin B6 content could also be used for feed additives to improve livestock nutritional status.

In conclusion, we have developed transgenic rice accumulating increased levels of vitamin B6 in leaves and unpolished seeds (more than 10- and 2-fold, respectively). Our results demonstrate a proof-of-principle strategy to increase vitamin B6 in transgenic rice. These findings could provide a sustainable solution to address malnutrition issue in populations who are suffering from vitamin B6 deficiency. Because of its antioxidant activity, the high vitamin B6 rice grains can also promote human health through daily consumption even in regions where malnutrition is not a significant problem. Rice is consumed by more than half of the world’s population. We foresee that the transgenic rice accumulating high vitamin B6 can make a substantial impact on global health.
Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* ssp. japonica cv. Taipei 309) was used in this study. Dehusked rice seeds were sterilized with 3% (w/v) sodium hypochlorite for 30 min and washed thoroughly by sterile distilled water. Transgenic and wild-type rice seeds were germinated on MS medium [1x MS salt, 3% sucrose, 0.3% gelrite] with or without hygromycin B (50mg/L; Carl Roth, Switzerland) in darkness at 37°C for 2 d to have homogenous germination, and then moved to a climate chamber (16h light/8h dark at 28°C) for 10 d. The uniformly growing transgenic and wild-type seedlings were transferred to soil under greenhouse conditions (12 h light at 30 °C and 70% humidity, 12 h dark at 20 °C and 60% humidity) and the effects of the natural illumination.

Transformation vector construction

**Constitutive expression vector**

The same vector (*35S-PDX1-35S-PDX2*) described in Chapter 2 used for cassava transformation was used here.

**Endosperm-specific expression vector**

The endosperm-specific *globulin* promoter was amplified from the NFP vector (Wirth et al., 2009) by PCR with primers Globulin-F-EcoRI (5'-GAATTCAGATGATGAGAGAGGA-3') and Globulin-R-KpnI (5'-GGTACCTGATGATCAGAGGAGAAGG-3'), and subcloned into pJET1.2/blunt cloning vector (CloneJET PCR cloning Kit; Thermo Scientific), to generate the pJ-Globulin vector. The *globulin* promoter in pJ-Globulin was linearized with EcoRI and KpnI, and inserted into pCAMBIA1300.1-PDX1.1 by replacement of the 35S promoter to generate the p1300-Glo-PDX1 vector. The *globulin* promoter in pJ-Globulin was linearized with EcoRI and KpnI, and inserted into pCAMBIA1300.1-PDX1 by replacement of the 35S promoter to generate the p1300-Glo-PDX2 vector. The *globulin* promoter::PDX1 fragment in p1300-Glo-PDX1 was linearized with EcoRI and XbaI, and inserted into the pBlueScript vector to generate the pBS-Glo-PDX1 vector. The *octopine* synthase terminator in pTOPO-polyA was linearized with XbaI and NotI, and inserted into pBS-Glo-PDX1 to generate the pBS-Glo-PDX1-pA vector. The expression cassette, *globulin* promoter::AtPDX1.1::terminator, in pBS-Glo-PDX1-pA vector was linearized with EcoRI, and inserted into p1300-Glo-PDX2 to generate the endosperm-specific expression transformation vector Glo-PDX1-Glo-PDX2.
**Rice transformation**

Two transformation vectors, 35S-PDX1-35S-PDX2 and Glo-PDX1-Glo-PDX2, were introduced into *Agrobacterium tumefaciens* strain LBA4404, and transgenic rice was generated as previously described (Nishimura et al., 2007).

**Molecular characterization**

DNA isolated from rice leaves was extracted according to an established method (Sheu et al., 1996). To determine the T-DNA integration copy number in transgenic plants, 10 μg of genomic DNA was digested with HindIII, run by gel electrophoresis on an agarose gel, and transferred onto a nylon membrane (GE Healthcare LifeScience). Southern blot analysis was subsequently performed according to the manufacturer’s instructions (Roche, Switzerland). Membranes were hybridized with a DIG-labeled hptII probe.

Transgene (AtPDX1.1) integration in transgenic 35S-PDX1-35S-PDX2 lines and Glo-PDX1-Glo-PDX2 lines was confirmed by PCR using primers PDX1.1 (5'-GTGAGGAGTGTGAACGGAGC-3') and polyA-R (5'-ACAATCAGTAAATTGAACGGAG-3').

**Preparation of samples for RNA extraction and vitamin B6 quantification**

Mature rice leaves were sampled at the reproductive stage for RT-qPCR and vitamin B6 quantification. Fresh seeds were sampled at the dough stage for RT-qPCR and vitamin B6 quantification. Fresh seeds were dehusked to obtain fresh unpolished seeds, and subsequently the aleurone layer and embryo manually removed to obtain fresh polished seeds.

For each sample, eight seeds were ground into a fine powder for RNA extraction and vitamin B6 quantification.

Mature rice seeds were dried at 37°C for 4 d after harvest. Dry grains were dehusked to obtain dry unpolished seeds, and then were polished with a rice milling machine (Pearleat, Kett Electric Laboratory, Japan) according to the manufacturer’s instructions to obtain polished seeds. Twenty seeds of each sample were ground into a fine powder for vitamin B6 quantification.

**RT-qPCR analysis**

Total RNA extraction was conducted as described (Cazzonelli et al., 1998) from mature leaves at the reproductive stage, and fresh unpolished and polished seeds at dough stage.
cDNA was generated using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with 2 μg of total RNA according to the manufacturer’s instructions using random hexamer primers. RT-qPCR was performed using the 7500 FAST real-time PCR system and Fast SYBR® Green Master Mix (Applied Biosystems). Relative target gene expression levels were normalized to the reference gene, Ubiquitin-conjugating enzyme E2 (UBC) (Jain et al., 2006) using the delta-delta CT method (Livak and Schmittgen, 2001). Primers sequences used for RT-qPCR are reported in Supplementary Table 2.

**Vitamin B6 quantification**

*Yeast microbiological assay*

Total vitamin B6 was quantified according an established protocol (Tambasco-Studart et al., 2005). Total vitamin B6 was extracted from 50-100 mg plant material in 1 mL of 0.22 M sulphuric acid at 100°C for 1 h, and then treated with acid phosphatase and β-glucosidase for 12-15 h at 37°C to convert phosphorylated and glycosylated derivatives of vitamin B6 to free forms of vitamin B6. The quantity of total vitamin B6 in plant extracts was extrapolated from a standard curve of known amounts of commercial pyridoxine.

**HPLC analysis**

The composition of B6 vitamers and its glycosylated derivatives were determined by HPLC as previously described (Szydlowski et al., 2013). In brief, plant material was ground into a fine powder in liquid nitrogen. 400 μL of 50 mM ammonium acetate (pH 4) was added to 200 mg of plant material before centrifugation at 16100 g for 15 min. The supernatant was subsequently heated at 100°C for 3 min to inactivate protein activity, before centrifugation at 16100 g for 15 min, and the supernatant collected in a new tube for HPLC analysis. The extract was injected to determine the free forms and phosphorylated derivatives of vitamin B6. An equivalent amount of original extract was treated with β-glucosidase to hydrolyse glycosylated derivatives for further HPLC analysis. The increase of free vitamers was extrapolated to determine the quantity of glycosylated derivatives.

*The HPLC assay was performed by Dr. Michäel Moulin in the Plant Biochemistry and Physiology group at University of Geneva.*
Supplementary Figures and Tables

**Supplementary Figure 1.** Southern blot analysis of transgenic rice plants using an *hptII* probe. 35S-7, 35S-12, 35S-27, 35S-29 indicate 4 independent lines of 35S-PDX1-35S-PDX2. Glo-13, Glo-41, Glo-47 indicate 3 independent lines of Glo-PDX1-Glo-PDX2. wt, wild-type plant. M, DIG marker.

**Supplementary Figure 2.** Analysis of transgene (*AtPDX1.1*) integration in transgenic rice by PCR. 35S-7, 35S-12, 35S-27, 35S-29 indicate 4 independent lines of 35S-PDX1-35S-PDX2. Glo-13, Glo-41, Glo-47 indicate 3 independent lines of Glo-PDX1-Glo-PDX2. wt, wild-type plant. Plasmid, transformation vector 35S-PDX1-35S-PDX2. M, marker.
Supplementary Figure 3. Transgenic seeds germinating on hygromycin-containing medium after 12 days. (a) 35S-7 (b) 35S-12 (c) 35S-29 (d) Glo-13 (e) Glo-47.
Supplementary Figure 4. Analysis of vitamin B6 content in transgenic rice expressing \textit{AtPDX1.1} and \textit{AtPDX2} under greenhouse conditions. (a) Fresh rice seeds at the dough stage (b) Dry rice seeds after drying processing. Total vitamin B6 content was measured using a yeast microbiological assay. Values are averages ± SD of three biological replicates. Unpolished rice seeds (white), polished rice seeds (black).
Supplementary Figure 5. RT-qPCR analysis of transgene expression in transgenic rice under greenhouse conditions. (a) AtPDX1.1 (b) AtPDX2. Values are averages ± SD of three biological replicates. Polished seeds (white), unpolished seeds (gray), leaves (black).
### Supplementary Table 1

**Primer sequences for vector construction and PCR screening**

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<tr>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>polyA-F</td>
<td>5'-TCTAGAGTCGACCCTGCAGG-3'</td>
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<tr>
<td>polyA-R</td>
<td>5'-ACAATCAGTAAATTGAACGGAG-3'</td>
</tr>
<tr>
<td>Globulin-F-EcoRI</td>
<td>5'-GAATTCAGAGATGGTGAGAGAGGA-3'</td>
</tr>
<tr>
<td>Globulin-R-KpnI</td>
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</tr>
<tr>
<td>PDX1.1</td>
<td>5'-ACAATCAGTAAATTGAACGGAG-3'</td>
</tr>
<tr>
<td>polyA-R</td>
<td>5'-ACAATCAGTAAATTGAACGGAG-3'</td>
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### Supplementary Table 2

**Primer sequences for RT-qPCR analysis**

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<tr>
<td></td>
<td>PDX1.1-R</td>
<td>5'-GCACAACCAAATCATACTGGC-3</td>
</tr>
<tr>
<td><em>AtPDX2</em></td>
<td>AtPDX2-F</td>
<td>5'-CTACGTGAGTTTTGTTAAGATG-3</td>
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<tr>
<td></td>
<td>AtPDX2-R</td>
<td>5'-CTCTTTCTGACCAACTGCTC-3'</td>
</tr>
<tr>
<td><em>UBC</em></td>
<td>UBC-F</td>
<td>5'-CCGTTTGTAGAGCCATAATTGCA-3'</td>
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<tr>
<td></td>
<td>UBC-R</td>
<td>5'-AGGGTGCTGAGTCAGTTAAGTG-3</td>
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Chapter 4 Vitamin B1 biofortification of cassava and rice by metabolic engineering

Abstract

Vitamin B1 is an essential micronutrient, which plays a central role in carbohydrate and amino acid metabolism. Vitamin B1 deficiency is common in populations having a limited diversity in their diet. In addition to its essential role in mammals, exogenous application of vitamin B1 in plants was reported to enhance biotic and abiotic stress tolerance. Strategies to increase vitamin B1 content based on metabolic engineering were conducted in cassava and rice. Vectors for expression of exogenous AtTHIC and endogenous THI1 and THIC genes were elaborated and transgenic plants were generated. The total vitamin B1 content in transgenic plants was quantified. Analysis of transgenic cassava showed no increase or even decreased levels of vitamin B1 content in both leaves and storage roots. Transgenic rice expressing AtTHIC showed a slight increase (up to 1.4- and 1.6-fold, respectively) in vitamin B1 content at the seedling stage and in polished seeds. Transgenic rice over-expressing endogenous THI1 or THIC did not show a significant increase of vitamin B1 content in mature plants or seeds. Moreover, adverse effects on plant growth were recorded in several transgenic cassava and rice lines. Our results indicate that over-expression of both THI1 and THIC alone is not sufficient to increase vitamin B1 accumulation and may interfere with the regulation of vitamin B1 biosynthesis in transgenic cassava and rice.

Introduction

Vitamin B1 is generated by condensation of a pyrimidine moiety (hydroxymethylpyrimidine; HMP) and a thiazole moiety (hydroxyethylthiazole; HET) (Figure 1) (Goyer, 2010). Three different mechanisms for the production de novo of vitamin B1 have been characterized in fungi, bacteria and plants (Helliwell et al., 2013). Vitamin B1 biosynthesis in fungi is metabolically expensive because it uses two single turnover enzymes THI4 (Chatterjee et al., 2011) and THI5 (Lai et al., 2012) to generate the thiazole moiety from vitamin B3 (NAD⁺) and the pyrimidine moiety from vitamin B6 (pyridoxal 5’-phosphate), respectively. These single turnover enzymes mean that one molecule of protein can only produce one molecule of product. In bacteria, the THIC protein regulates pyrimidine moiety formation while thiazole formation requires an intermediate precursor from the isoprenoid pathway (Jurgenson et al., 2009). Genes associated with the de novo vitamin B1 biosynthesis in planta including THIC, THI1 and TH1, were initially identified in the model plant Arabidopsis (Ajjawi et al., 2007;
The general mechanism of vitamin B1 biosynthesis in plants has been well reviewed (Goyer, 2010; Pourcel et al., 2013). Hydroxyethylthiazole phosphate (HET-P) and hydroxymethylpyrimidine pyrophosphate (HMP-PP) are coupled in a condensation reaction to form TMP. Dephosphorylation of TMP generates thiamin. Thiamin is subsequently pyrophosphorylated to the active cofactor form, TPP. In the HET-P biosynthesis pathway, the THI1 protein is postulated to catalyze NAD$^+$, glycine, and sulfur which is from a particular cysteine residue in the THI1 protein, to generate HET-P (Chatterjee et al., 2011). The THI1 protein is a single turnover enzyme as one molecule of the THI1 protein only produces one molecule of HET-P. In the HMP-PP biosynthesis pathway, the THIC protein is used to convert aminoimidazole ribonucleotide (AIR) to HMP-P, and then HMP-P is subsequently phosphorylated to HMP-PP. The vitamin B1 biosynthesis pathway in plants occurs in the chloroplast (Pourcel et al., 2013). Additionally, a riboswitch regulatory element is encoded in the 3′-UTR of the THIC gene in plants and bacteria (Helliwell et al., 2013). Riboswitch elements are RNA-based sensors of small metabolites (e.g. vitamins, amino acids) located within noncoding regions of a mRNA (Serganov and Nudler, 2013; Wachter, 2010). Riboswitch elements sense the cellular concentration of metabolites to control the expression levels of the target genes. THIC is the only reported gene under the control of a riboswitch in higher plants. The THIC riboswitch has been demonstrated to be involved in feedback inhibition by TPP in Arabidopsis, which is mediated by a splicing and differential processing of 3′-UTR of THIC mRNAs (Bocobza et al., 2007; Wachter et al., 2007). Under low TPP concentrations in cells, TPP does not interact with the riboswitch motif so the transcript processing site located in the upstream of riboswitch motif is retained, resulting in a short 3′-UTR. THIC transcripts containing the short 3′-UTR are stable and their translation results in higher expression levels of the THIC protein. Excess TPP binds the riboswitch element, which triggers a structural change of the pre-mRNA, resulting in the removal of the riboswitch-containing intron by splicing. The transcript processing site located upstream of the riboswitch motif is spliced out under high TPP concentrations, yielding an unstable long 3′-UTR transcript. The unstable transcripts are degraded, resulting in reduced expression of the THIC protein. Moreover, it has been demonstrated that the activity of the THIC promoter is controlled by the circadian rhythm regulation (Bocobza et al., 2013). The comprehensive mechanism and regulation of vitamin B1 biosynthesis in plants still needs further characterization.

Strategies to enhance vitamin B1 in plants were recently examined (Pourcel et al., 2013). Exogenous application of HMP together with HET to plants on growth medium can significantly increase thiamin content in Arabidopsis seedlings (Pourcel et al., 2013). However, exogenous application of HMP or HET alone does not lead to an increase of thiamin content. This suggests that enhancing vitamin B1 content in plants requires accumulation of both pyrimidine and thiazole moieties. The thiazole moiety is produced through the action of the THI1 protein in the HET-P biosynthesis pathway. The THIC protein is the key regulator of pyrimidine moiety accumulation in the HMP-PP biosynthesis pathway.
Transgenic Arabidopsis constitutively over-expressing the coding region of *AtTHIC* showed a slight increase of total vitamin B1 content (up to 1.5-fold) in leaves under short day conditions together with undesirable effects on plant growth (Bocobza et al., 2013). The strategy combining over-expression of *THI1* together with *THIC* genes has not yet been subjected to experimental validation in plants by genetic engineering.

Here we report several strategies to investigate the feasibility of vitamin B1 biofortification through genetic engineering. First, transgenic plants over-expressing *AtTHIC* were generated to investigate the impact of expression of exogenous *THIC* on vitamin B1 production in cassava and rice. Because the protein specificity of THI1 and THIC may differ in different species, we then cloned endogenous homologs from cassava and rice for genetic transformation. Transformation vectors expressing endogenous *THI1* or *THIC* singly were generated to explore the contribution of single moiety accumulation to vitamin B1 biosynthesis. A transformation vector expressing endogenous *THI1* and *THIC* is assumed to increase vitamin B1 accumulation in both cassava and rice. Transgenic lines of all transformation vectors were generated to perform vitamin B1 quantification.

![Figure 1. The vitamin B1 biosynthesis pathway in plants](image)

Enzymes of the vitamin B1 biosynthesis pathway (i.e. THIC, HMP-P synthase; THI1, HET-P synthase; TH1, HMP-P kinase/TMP-PPase; TPK, thiamin pyrophosphokinase). Three B1 vitamers: TMP, thiamin monophosphate; thiamin; TPP, thiamin pyrophosphate. AIR, 5-Aminoimidazole ribonucleotide; HMP-P, 4-amino-2-methyl-5-hydroxymethylpyrimidine monophosphate; HMP-PP, 4-amino-2-methyl-5-hydroxymethylpyrimidine diphosphate; NAD⁺, nicotinamide adenine dinucleotide; HET-P, 4-methyl-5-B-hydroxyethylthiazole phosphate. (Figure was modified from Goyer 2010.)
Results

Cloning of endogenous THI1 and THIC genes involved in the vitamin B1 biosynthesis pathway in cassava and rice

It has been recognized that plants use the THI1 and THIC genes to produce the thiazole and pyrimidine moieties separately, and then vitamin B1 is generated by a condensation of these two moieties (Goyer, 2010; Pourcel et al., 2013). Using AtTHI1 and AtTHIC sequences, one THI1 and one THIC homolog was identified in the rice genome (www.phytozome.net/rice) (Ouyang et al., 2007). Through the same approach, two THI1 homologs and two THIC homologs were predicted in silico in the cassava genome (www.phytozome.net/cassava) (Prochnik et al., 2012). The rice gene LOC_Os07g34570 (hereafter named OsTHI1) was identified as a putative ortholog of AtTHI1. The rice gene LOC_Os03g47610 (hereafter named OsTHIC) was identified as a putative ortholog of AtTHIC. Based on an old version of the cassava genome sequence in phytozome4, the cassava genes cassava24565 (hereafter named MeTHI1) and cassava37020 (hereafter named MeTHI1-2) were identified as putative orthologs of AtTHI1. However, only one THI1 homolog (cassava4.1_010620m) is identified in the latest version of phytozome v9.1. The cassava gene cassava4.1_003348m (hereafter named MeTHIC1) and cassava4.1_003360m (hereafter named MeTHIC2) were identified as putative orthologs of AtTHIC.

Following the in silico identifications of THI1 and THIC homologs, primers were designed to clone their coding regions from cassava (cv. 60444) and rice (cv. Taipei 309), and to sequence each of them. The sequences of OsTHI1 (Figure 2a) and OsTHIC (Figure 2b) in rice cv. Taipei 309 are identical to the ones in the published rice genome (cv. Nipponbare). An amino acid sequence alignment shows that MeTHI1 and MeTHI1-2 in cassava cv. 60444 are highly homologous to MeTHI1 in cv. AM560-2 (93.4% and 99.4% similarity, respectively) (Figure 3a), while the sequence of MeTHIC1 in cassava cv.60444 is identical to the one in cassava cv. AM560-2 (Figure 3b). An amino acid sequence alignment shows that MeTHIC2 in cassava cv. 60444 has 99.4% similarity to the one in cassava cv. AM560-2 (Figure 3c).

An amino acid sequence alignment shows that cassava MeTHI1 and MeTHI1-2 proteins have 78.1% and 79% similarity to the Arabidopsis homolog, respectively, and rice OsTHI1 has 76.6% similarity to the Arabidopsis homolog (Figure 4a). The sequence alignment of THI1 proteins reveals that the cysteine residue that acts as a putative sulfur donor and that other important catalytic residues are highly conserved amongst Arabidopsis, cassava and rice THI1 proteins (Chatterjee et al., 2011; Godoi et al., 2006; Moulin et al., 2013). Another amino acid sequence alignment shows that cassava MeTHIC1 and MeTHIC2 proteins are 87.4% and 87.5% similar to the Arabidopsis homolog, respectively, and the rice OsTHIC protein has 81% similarity to the Arabidopsis homolog (Figure 4b). A sequence alignment of THIC proteins shows that the consensus amino acid motif CX_2CX_4C (where X is any amino acid)
corresponding to iron-sulfur cluster function (Raschke et al., 2007; Zhao et al., 2011), and is conserved in Arabidopsis, cassava and rice. Subcellular localization analysis of THI1 and THIC proteins in cassava and rice using the TargetP (Emanuelsson et al., 2007) webserver predicts that the proteins are targeted to the chloroplast, except for the MeTHI1-2 that is targeted to the secretory pathway. These subcellular localization predictions are consistent with vitamin B1 production in plastids (Bocobza et al., 2013; Pourcel et al., 2013). Altogether, these observations suggest that the cassava and rice THIC and THI1 genes could be used to increase vitamin B1 content in plants.

Figure 2. Amino acid sequence alignment of genes involved in the vitamin B1 biosynthesis in two rice varieties (cv. Nipponbare and cv. Taipei 309). (a) OsTHI1, LOC_Os07g34570 (b) OsTHIC, LOC_Os03g47610
Figure 3. Amino acid sequence alignment of genes involved in the vitamin B1 biosynthesis in two cassava varieties (cv. AM560-2 and cv. 60444). (a) MeTHI1. MeTHI1 (AM560-2), cassava4.1_010971m; MeTHI1 (60444), cassava24565 (phytozome4); MeTHI1-2, cassava37020 (phytozome4). (b) MeTHIC1, cassava4.1_003348m. (c) MeTHIC2, cassava4.1_003360m.
Figure 4. Amino acid sequence alignment of genes involved in the vitamin B1 biosynthesis from Arabidopsis, rice and cassava. (a) Sequence alignment of THI1 homologs. The red asterisk indicates a putative sulfur donor from the cysteine residue, required for thiazole biosynthesis. The blue asterisks indicate conserved residues of catalytic sites. (b) Sequences alignment of THIC homologs. The blue box indicates a functional consensus motif.
Vitamin B1 biofortification of cassava by metabolic engineering

I. Metabolic engineering of transgenic cassava expressing AtTHIC

Generation of transgenic cassava expressing AtTHIC

In an initial step to modulate vitamin B1 content in cassava, AtTHIC was selected for genetic engineering. AtTHIC was demonstrated to be necessary for pyrimidine moiety formation in Arabidopsis (Kong et al., 2008; Raschke et al., 2007). To avoid the effect of feedback regulation by excess vitamin B1, the riboswitch motif in the 3'UTR was removed from the AtTHIC gene sequence. The coding region of AtTHIC was used as transgene. The absence of a riboswitch regulatory element should prevent any negative feedback regulation by TPP binding. The AtTHIC transgene was driven by the 35S promoter in the 35S-AtTHIC vector for constitutive expression and a nos terminator was used to provide a polyadenylation signal in this transgene expression cassette (Figure 5a). In second transformation vector, the AtTHIC gene was driven by the patatin promoter (Kim et al., 1994) and later referred to as Pat-AtTHIC vector (Figure 5b). The Pat-AtTHIC vector was generated to drive expression of the AtTHIC transgene in cassava roots.

Figure 5. Schematic representation of cassava transformation vector T-DNA regions containing AtTHIC. (a) Constitutive expression vector, 35S-AtTHIC (b) Root-specific expression vector, Pat-AtTHIC. 35S, CaMV 35S promoter; Pat, patatin promoter; AtTHIC, At2g29630; HPT, hygromycin phosphotransferase; LB, left border of T-DNA; RB, right border of T-DNA; T1, nos terminator.

35S-AtTHIC

Sixteen putative transgenic lines resulting from transformation of the 35S-AtTHIC vector into cassava were selected for molecular characterization. Southern blot analysis confirmed T-DNA integration in all putative transgenic 35S-AtTHIC lines (Figure 6a). Four independent transgenic lines (35S-AtTHIC-1, 35S-AtTHIC-6, 35S-AtTHIC-8 and 35S-AtTHIC-15) with single copy and one transgenic line (35S-AtTHIC-7) with two copies of T-DNA were selected for further analysis.
**Pat-AtTHIC**

Fourteen putative transgenic *Pat-AtTHIC* lines were selected to determine T-DNA integration events by Southern blot analysis (Figure 6b). Five independent transgenic lines (Pat-AtTHIC-2, Pat-AtTHIC-3, Pat-AtTHIC-4, Pat-AtTHIC-5 and Pat-AtTHIC-11) with single copy and one transgenic line (Pat-AtTHIC-13) with two full copies of T-DNA were selected for further analysis.

\[\text{a.} \]

![Southern blot analysis of transgenic cassava plants using an hptII probe. (a) 35S-AtTHIC vector. The numbers indicate 16 putative selected transgenic cassava plants. (b) Pat-AtTHIC vector. The number denotes putative selected transgenic cassava plants. M, DIG-labeled marker.}

Selected transgenic lines with wild-type controls were transferred to a greenhouse to evaluate their performance. Transgenic cassava and wild-type controls were harvested 5 months after transferring to soil. The height of transgenic 35S-AtTHIC and Pat-AtTHIC lines was on average equal to the wild-type control (Figure 7a). A significant decrease of biomass (fresh weight) was observed in above ground part of cassava in two of five transgenic 35S-AtTHIC lines (35S-AtTHIC-6 and 35S-AtTHIC-7), but transgenic Pat-AtTHIC lines had no significant change compared to wild-type except for transgenic line Pat-AtTHIC-5 (Figure 7b). Storage root weight of transgenic lines from both transformation vectors was not significantly different from wild-type control. Despite its high variation, transgenic line 35S-AtTHIC-6 had a decrease in storage root weight in two of three plants used as biological replicates (Figure 7c).
Figure 7. Agronomic performance of 6-month-old transgenic cassava expressing AtTHIC under greenhouse conditions. (a) Height (b) Aboveground fresh weight (c) Storage roots fresh weight. Values are averages ± SD of three biological replicates. wt (white), 3SS-AtTHIC (gray), Pat-THIC (black). Asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests ($p<0.05$).
Vitamin B1 content of transgenic cassava expressing AtTHIC under greenhouse conditions

The total vitamin B1 content was quantified in cassava leaves of transgenic 35S-AtTHIC lines, and in storage roots of transgenic 35S-AtTHIC and Pat-AtTHIC lines under greenhouse conditions. Two out of the five transgenic 35S-AtTHIC lines (35S-AtTHIC-7 and 35S-AtTHIC-8) showed a significant decrease of the vitamin B1 content in leaves (Figure 8a). A 2.5- and 5-fold reduction in vitamin B1 content was observed in leaves of 35S-AtTHIC-7 and 35S-AtTHIC-8, respectively. Transgenic line 35S-AtTHIC-6 showed no significant difference in vitamin B1 content compared to wild-type levels. However, the vitamin B1 content in two of three plants of transgenic line 35S-AtTHIC-6 used as biological replicates were 20-30% higher than the average of wild-type. The leaves of these two increased vitamin B1 accumulation plants showed a chlorotic phenotype in vascular areas (Figure 8c). A similar chlorotic leaf phenotype was also observed in transgenic line Pat-AtTHIC-5.

The vitamin B1 content in storage roots from transgenic 35S-AtTHIC and Pat-AtTHIC lines showed a general reduction, but the biological variation was too high to make a solid conclusion (Figure 8b). The results suggested that transgenic cassava over-expressing AtTHIC could not increase vitamin B1 accumulation in either leaves or storage roots. Additionally, storage roots of the two plants (35S-AtTHIC-6) with chlorotic leaf phenotype contained vitamin B1 levels that were in the same range as the other transgenic plants. However, a decrease in storage root weight was observed in these two chlorotic leaf plants (35S-AtTHIC-6) (Figure 7c). The slight increase of vitamin B1 was observed in chlorotic leaves, but it was associated with adverse effects on storage roots formation. A 3.5-fold reduction in storage roots weight (fresh weight) was observed in the transgenic line 35S-AtTHIC-6 with chlorotic leaves. However, the same reduction of storage root weight was not observed in the transgenic line Pat-AtTHIC-5.
Figure 8. Analysis of transgenic cassava expressing AtTHIC under greenhouse conditions. (a) Total vitamin B1 content in leaves (b) Total vitamin B1 content in storage roots (c) Chlorotic leaf phenotype of transgenic line expressing AtTHIC (35S-AtTHIC-6). Vitamin B1 quantification was performed using a yeast microbiological assay. Values are averages ± SD of three biological replicates except for 35S-AtTHIC-15 in leaves and 35S-AtTHIC-7 in storage roots which are with two biological replicates. wt (white), 35S-AtTHIC (gray), Pat-AtTHIC (black). Asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
II. Metabolic engineering of transgenic cassava expressing MeTHI1 and/or MeTHIC

Generation of transgenic cassava expressing MeTHI1 and/or MeTHIC

To explore whether the combination of MeTHI1 and MeTHIC can increase vitamin B1 content in cassava, transformation vectors for constitutive over-expression of MeTHI1, MeTHIC1, MeTHIC2 as well as MeTHI1 combined with MeTHIC1 were constructed. Transformation vectors were designed to over-express MeTHI1 or MeTHIC only and to examine the contribution of single moiety accumulation on vitamin B1 synthesis. The schematic representations of T-DNA regions are shown in Figure 9. All transgenes were driven by a constitutive promoter (i.e. 35S promoter).

![Figure 9. Schematic representation of cassava transformation vectors T-DNA regions](image)

(a) 35S-MeTHI1  (b) 35S-MeTHIC1  (c) 35S-MeTHIC2  (d) 35S-MeTHI1-35S-MeTHIC1. 35S, CaMV 35S promoter; MeTHI1, cassava24565; MeTHIC1, cassava4.1_003348m; MeTHIC2, cassava4.1_003360m; HPT, hygromycin phosphotransferase; LB, left border of T-DNA; RB, right border of T-DNA; T1, nos terminator.

35S-MeTHI1

Nineteen putative transgenic 35S-MeTHI1 lines were selected to determine T-DNA integration events by Southern blot analysis (Figure 9a). Two transgenic lines (35S-MeTHI1-4 and 35S-MeTHI1-18) with a single T-DNA copy were transferred to a greenhouse for further analysis. However, it appeared that transgenic 35S-MeTHI1 lines had adverse effects on cassava growth. Three of the six transgenic plants (three plants per independent transgenic line) did not survive the transfer from in vitro jars to soil in the greenhouse. The remaining transgenic 35S-MeTHI1 plants showed early flowering (Figure 10b), early branching (Figure 10c) and dieback of the top (Figure 10d). It must be noted that the male flowers withered away before developing a complete flower structure. The limited number of biological replicates did not allow continuing analysis.
Figure 10. Analysis of transgenic 35S-MeTH11 cassava under greenhouse conditions. (a) Southern blot analysis of transgenic cassava plants using a hptII probe from three independent blottings. The numbers indicate 19 selected putative transgenic lines. M, DIG-labeled marker. (b) Early flowering (c) Early branching (d) Dieback of the top shoot portion.

35S-MeTH1C1

Three putative transgenic 35S-MeTHIC1 lines (35S-MeTHIC1-1, 35S-MeTHIC1-2, 35S-MeTHIC1-3) were selected to determine T-DNA integration events by Southern blot analysis (Figure 11). Transgenic lines 35S-MeTHIC1-1 and 35S-MeTHIC1-2 had an identical T-DNA integration pattern and therefore were considered as belonging to the same line.

Figure 11 Southern blot analysis of transgenic 35S-MeTHIC1 cassava using a hptII probe. The numbers indicate three selected putative transgenic lines. M, DIG-labeled marker.
35S-MeTHIC2

Fifteen putative transgenic 35S-MeTHIC2 lines were selected to perform Southern blot analysis to confirm T-DNA integration events (Figure 12). Three transgenic 35S-MeTHIC2 lines (35S-MeTHIC2-1, 35S-MeTHIC2-3 and 35S-MeTHIC2-15) with a single copy of the T-DNA integration were transferred to a greenhouse.

Figure 12. Southern blot analysis of transgenic 35S-MeTHIC2 cassava using a hptII probe. The numbers indicate 15 putative selected transgenic lines. M, DIG-labeled marker.

35S-MeTHI1-35S-MeTHIC1

Twenty putative transgenic 35S-MeTHI1-35S-MeTHIC2 lines resulting from transformation were selected to determine T-DNA integration events by Southern blot analysis (Figure 13). Transgenic 35S-MeTHI1-35S-MeTHIC1 lines (35S-MeTHI1-35S-MeTHIC1-6, 35S-MeTHI1-35S-MeTHIC1-7 and 35S-MeTHI1-35S-MeTHIC1-12) with a single copy of the T-DNA integration were transferred to a greenhouse.

Figure 13. Southern blot analysis of transgenic 35S-MeTHI1-35S-MeTHIC1 cassava using a hptII probe. The numbers indicate 20 putative selected transgenic lines. M, DIG-labeled marker.
Together with two transgenic lines (pCambia1301-2K and pCambia1301-3K) transformed with the pCambia1301 vector control and wild-type plants, all cassava plants were grown to assess their performance under greenhouse conditions.

**Agronomic performance of transgenic cassava expressing endogenous THI1 and/or THIC under greenhouse conditions**

The average plant height did not differ between transgenic 35S-METHIC1, 35S-METHIC2 and 35S-METHIC1-35S-METHIC2 lines and the control line (Figure 14a). The biomass of the above ground part of all transgenic cassava was on average equal to the wild-type control (Figure 14b). In this experiment, wild-type plants showed the highest storage root weight under greenhouse conditions (Figure 14c). The storage root weight of one transgenic 35S-METHIC1 line (35S-METHIC1-2) was comparable to wild-type levels, but another transgenic line 35S-METHIC1-3 showed a reduction. The storage root weight of two of three transgenic 35S-METHIC2 lines (35S-METHIC2-1 and 35S-METHIC2-15) was equal to wild-type. A 50% reduction of storage root weight in the transgenic line 35S-METHIC2-3 was observed. Moreover, all three transgenic 35S-METHI1-35S-METHIC1 lines showed a 50% reduction of storage root weight compared to wild-type plants.

**Vitamin B1 content of transgenic cassava expressing MeTHI1 and MeTHIC under greenhouse conditions**

The total vitamin B1 content in leaves of transgenic lines and the wild-type control was quantified. To facilitate the screening procedure, the fluorescence intensity of thiochrome was measured to get a relative value of vitamin B1 content in tested samples. Cassava extracts were treated with ferricyanide in order to convert thiamin into thiochrome (Fujiwara and Matsui, 1953). Transgenic 35S-METHIC1 and 35S-METHIC2 lines showed lower levels of vitamin B1 content in leaves compared to wild-type (Figure 14d). All three transgenic 35S-METHI1-35S-METHIC1 lines showed lower levels of vitamin B1 content. However, the vector control plants (pCambia1301-2K) also displayed a slight decrease of vitamin B1 content compared to wild-type plants. These results revealed that over-expressing THI1 together with THIC could not enhance vitamin B1 content in transgenic cassava.
Figure 14. Analysis of transgenic 35S-MeTHIC1, 35S-MeTHIC2, 35S-MeTHI1-35S-MeTHIC1 cassava under greenhouse conditions. (a) Height (b) Above ground fresh weight (c) Storage roots fresh weight (d) Relative vitamin B1 content in leaves using a fluorescence intensity quantification. wt, wild-type (white); transgenic pCAMBIA1301 lines (gray); transgenic 35S-MeTHIC1 lines (green); transgenic 35S-MeTHIC2 lines (blue); transgenic 35S-MeTHI1-35S-MeTHIC1 lines (black). Values are averages ± SD of four biological replicates and asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests ($p<0.05$).
Vitamin B1 biofortification of rice by metabolic engineering

I. Metabolic engineering of transgenic rice expressing AtTHIC

Generation of transgenic rice expressing AtTHIC

First, the same transformation vector that was used for cassava transformation (Figure 5a), 35S-AtTHIC, was also used to transform rice. Transformation vector 35S-AtTHIC was generated for constitutive expression of the AtTHIC transgene (Figure 15a). In second transformation vector, the AtTHIC transgene was driven by the endosperm-specific globulin promoter (Qu and Takaiwa, 2004) and later referred as Glo-AtTHIC. Transformation vector Glo-AtTHIC was generated to drive expression of AtTHIC in rice endosperm (Figure 15b). The 35S promoter is active in most plant tissues in transgenic rice (Furtado et al., 2008). Experimental evidence showed that the globulin promoter is active in the whole endosperm, but the expression level is higher in the central region of the endosperm (Furtado et al., 2008). The 35S-AtTHIC vector was used to assess the correlation between expression levels of AtTHIC and vitamin B1 content in different plant tissues. The Glo-AtTHIC vector was used to evaluate vitamin B1 accumulation in the endosperm.

Figure 15. Schematic representation of rice transformation vector T-DNA regions expressing AtTHIC. (a) Constitutive expression vector, 35S-AtTHIC (b) Endosperm-specific expression vector, Glo-AtTHIC. 35S, CaMV 35S promoter; Glo, globulin promoter; AtTHIC, At2g29630; HPT, hygromycin phosphotransferase; LB, left border of T-DNA; RB, right border of T-DNA; T1, nos terminator.

Vitamin B1 content of in vitro transgenic rice seedling expressing AtTHIC

Transgenic rice plants of both 35S-AtTHIC and Glo-AtTHIC vectors were generated by Agrobacterium-mediated transformation. In an initial step, T1 transgenic seeds were germinated on MS medium with hygromycin to obtain transgene-containing seedlings, and then vitamin B1 content was determined in 5-week-old in vitro seedlings. Three independent transgenic 35S-AtTHIC lines (35S-AtTHIC-30, 35S-AtTHIC-31 and 35S-AtTHIC-32) together
with two transgenic Glo-AtTHIC lines (Glo-AtTHIC-3 and Glo-AtTHIC-14) were selected to perform vitamin B1 quantification. Due to technical problems, two samples of wild-type plants were lost in this experiment, so only one replicate of wild-type is presented. The two transgenic 35S-AtTHIC lines (35S-AtTHIC-31 and 35S-AtTHIC-32) tended to have a higher vitamin B1 content in the in vitro seedlings compared to transgenic Glo-AtTHIC lines and the single replicate of wild-type plant (Figure 16a). Additionally, several albino seedlings were randomly observed in transgenic line Glo-AtTHIC-14 (Figure 16b), but the vitamin B1 quantification was not further pursued.

Figure 16. Analysis of 5-week-old in vitro rice seedlings expressing AtTHIC. (a) Total vitamin B1 content in rice seedlings. Total vitamin B1 quantification using a yeast microbiological assay. wt, wild-type (gray); transgenic 35S-AtTHIC lines (green); transgenic Glo-AtTHIC lines (black). Values are averages ± SD of three biological replicates except for wt which is with a single replicate. (b) Albino phenotype in transgenic line Glo-AtTHIC.
**Vitamin B1 content of transgenic rice expressing AtTHIC under greenhouse conditions**

Two transgenic Glo-AtTHIC lines (Glo-AtTHIC-14 and Glo-AtTHIC-15) and one transgenic 35S-AtTHIC line (35S-AtTHIC-32) were transferred to a greenhouse in order to grow plants to maturation and harvest rice grains to quantify their vitamin B1 content. First, the total vitamin B1 content in unpolished and polished seeds of wild-type plants was quantified (Figure 17a). The quantification results from wild-type seeds showed vitamin B1 accumulated to higher levels (5.5-fold) in unpolished seeds than the ones in polished seeds. Because the weight of rice bran accounts for about 10% of the entire seed weight (Tanaka et al., 2006), vitamin B1 was postulated to be accumulated at high levels in the embryo and/or aleurone layer. Transgenic line Glo-AtTHIC-14 showed a 1.6-fold increase of vitamin B1 in polished seeds compared to wild-type (Figure 17b). Transgenic line 35S-AtTHIC-32 and Glo-AtTHIC-15 had a higher amount of vitamin B1, but the difference was not statistically significant. Intriguingly, the vector control transgenic line pCAMBIA1301 showed the highest amount of vitamin B1 which was a 2-fold increase over wild-type.

![Graph showing vitamin B1 content comparison](image)

**Figure 17.** Analysis of the total vitamin B1 content in transgenic rice expressing AtTHIC under greenhouse conditions. (a) Total vitamin B1 content in wild-type unpolished (white) and polished seeds (gray) (b) Total vitamin B1 content in polished seeds. wt, wild-type (gray); transgenic pCAMBIA1301 line (blue); transgenic 35S-AtTHIC line (green); transgenic Glo-AtTHIC lines (black). Total vitamin B1 quantification using a yeast microbiological assay. Values are averages ± SD of three biological replicates. Asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests ($p<0.05$).
II. Metabolic engineering of transgenic rice expressing OsTHI1 and/or OsTHIC

Generation of transgenic rice expressing OsTHI1 and/or OsTHIC

Transformation vectors designed to over-express OsTHI1 or OsTHIC alone would help to understand the impact of single moiety accumulation on vitamin B1 biosynthesis in rice. To examine whether the combination of OsTHI1 and OsTHIC can increase vitamin B1 content in rice, the transformation vector constitutively over-expressing OsTHI1 and OsTHIC1 was constructed. The schematic representations of the T-DNA regions of generated vectors are shown in the Figure 18. All transgenes were driven by the constitutive 35S promoter. Transgenic rice plants were generated from Agrobacterium-mediated transformation.

![Figure 18. Schematic representation of rice transformation vector T-DNA regions expressing endogenous THI1 and/or THIC.](image)

35S-OsTHI1

Twenty-one putative transgenic 35S-OsTHI1 lines at T0 generation were selected to determine T-DNA integration events by Southern blot analysis (Figure 19). Seven independent transgenic lines with a single copy of T-DNA integration (35S-OsTHI1-2, 35S-OsTHI1-5, 35S-OsTHI1-6, 35S-OsTHI1-7, 35S-OsTHI1-8, 35S-OsTHI1-9 and 35S-OsTHI1-18) were transferred to a greenhouse to produce progeny seeds.
Figure 19. Southern blot analysis of transgenic 35S-OsTHI1 rice using a hptII probe. The numbers indicate 21 selected putative transgenic rice plants.

**35S-OsTHIC**

Eight putative transgenic 35S-OsTHIC lines at T0 generation were selected to conduct Southern blot analysis (Figure 20). All putative transgenic lines showed a single copy of T-DNA integration except for 35S-OsTHIC-14, which had two copies of T-DNA integration.

Figure 20. Southern blot analysis of transgenic 35S-OsTHIC rice using a hptII probe from two independent blottings. The numbers indicate eight selected putative transgenic rice plants. M, DIG-labeled marker.

**35S-OsTHI1-35S-OsTHIC**

Thirty-five putative transgenic 35S-OsTHI1-35S-OsTHIC lines at T0 generation were selected to determine T-DNA integration events by Southern blot analysis (Figure 21). All putative transgenic lines of 35S-OsTHI1-35S-OsTHIC vector with a single insertion were transferred to a greenhouse to produce progeny seeds.
Figure 21. Southern blot analysis of transgenic 35S-OsTHI1-35S-OsTHIC rice using a hptII probe from two independent blottings. The numbers indicate 35 selected putative transgenic rice plants.

**Vitamin B1 content of soil-grown transgenic rice seedlings expressing OsTHI1 and/or OsTHIC under greenhouse conditions**

To investigate the impact of transgenic rice expressing THI1 and/or THIC, four independent transgenic lines from vectors 35S-AtTHIC, 35S-OsTHI1, 35S-OsTHIC and 35S-OsTHI1-35S-OsTHIC were selected to quantify vitamin B1 content at the seedling stage under greenhouse conditions. Transgenic lines pCAMBIA1300 and pCAMBIA1301 were included as vector controls. Three week old soil-grown rice seedlings were sampled to perform the vitamin B1 quantification using a yeast microbiological assay.

Figure 22. Analysis of the total vitamin B1 content in transgenic soil-grown rice seedlings under greenhouse conditions. wt, wild-type (white); transgenic pCAMBIA1300 line and transgenic pCAMBIA1300 line (gray); transgenic 35S-AtTHIC lines (green); transgenic 35S-OsTHIC lines (blue); transgenic 35S-OsTHI1 lines (orange); transgenic 35S-OsTHI1-OsTHIC lines (black). Total vitamin B1 content quantification using a yeast microbiological assay. Values are averages ± SD of three biological replicates and asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
The vector control line pCAMBIA1300 had similar levels of vitamin B1 compared to wild-type (Figure 22). Intriguingly, another vector control line pCAMBIA1301 showed higher levels of vitamin B1 (1.3-fold) in seedlings. To examine the impact of over-expression of exogenous THIC genes in transgenic rice, three transgenic 35S-AtTHIC lines were included. The total vitamin B1 content in transgenic 35S-AtTHIC lines showed up to a 1.4-fold increase over wild-type (Figure 22). The result further confirmed the observed vitamin B1 enrichment in the transgenic 35S-AtTHIC lines previously measured in the in vitro transgenic rice seedlings (Figure 15). Three transgenic 35S-OsTHIC lines showed no change of vitamin B1 content compared to wild-type (Figure 22). One of three transgenic 35S-OsTHI1 lines (35S-OsTHI1-9) had a 1.2-fold increase of vitamin B1 content. However, a closer look of transgenic line 35S-OsTHI1-8 revealed that two of the three plants as biological replicates had a 2-fold increase of vitamin B1 content over the average of wild-type. Additionally, three of five transgenic 35S-OsTHI1-35S-OsTHIC lines showed a slight increase of vitamin B1 content up to 1.4-fold. These results revealed that the total vitamin B1 content can be potentially increased at the seedling stage of several transgenic rice lines but the increase is limited to 20-40%.

Furthermore, the transgenic 35S-OsTHI1 lines tended to have a reduction of plant height compared to both wild-type and transgenic line pCAMBIA1300 plants (Figure 23a). The height of transgenic 35S-OsTHI1-35S-OsTHIC lines was comparable to both wild-type and transgenic line pCAMBIA1300 (Figure 23b).

Figure 23. Phenotype of 3-week-old soil-grown rice seedlings under greenhouse conditions. (a) Transgenic 35S-OsTHI1 lines (b) Transgenic 35S-OsTHI1-35S-OsTHIC lines. wt, wild-type; pCAMBIA1300, transgenic pCAMBIA1300 line.
**Vitamin B1 content of transgenic rice over-expressing OsTHI1 and OsTHIC under greenhouse conditions**

To investigate whether the over-expression of OsTHI1 and OsTHIC can increase vitamin B1 content, five transgenic 35S-OsTHI1-35S-OsTHIC lines were selected to perform further analysis under greenhouse conditions. The T2 seeds of selected lines were grown under greenhouse conditions. Transgenic lines pCAMBIA1300 and pCAMBIA1301 were included as vector controls. Both vector control lines accumulated similar levels of vitamin B1 in leaves and unpolished seeds compared to wild-type (Figure 24a,b). Four of the five selected transgenic 35S-OsTHI1-OsTHIC lines had an equal amount of total vitamin B1 content in mature leaves compared to wild-type (Figure 24a). Transgenic line 35S-OsTHI1-OsTHIC-2 showed lower vitamin B1 content but the decrease was not statistically significant. Total vitamin B1 content in unpolished seeds was further quantified. Transgenic 35S-OsTHI1-OsTHIC lines had lower levels of vitamin B1 compared to wild-type, but the decrease was not statistically significant (Figure 24b). The result suggested that over-expression of THI1 combined with THIC did not increase vitamin B1 accumulation in maturation transgenic rice leaves and grains.

Expression levels of the coding region of OsTHIC in leaves of selected transgenic lines were quantified by RT-qPCR (Figure 24c). As expected, vector control line pCAMBIA1300 had equal levels of OsTHIC transcripts compared to wild-type. Two of four transgenic 35S-OsTHI1-OsTHIC lines (35S-OsTHI1-OsTHIC-2 and 35S-OsTHI1-OsTHIC-29) showed higher expression levels of OsTHIC over wild-type (1.8- and 3.1-fold, respectively). However, the vitamin B1 content was not correlated to expression levels of OsTHIC.
Figure 24. Analysis of transgenic rice over-expressing endogenous THI1 combined with THIC under greenhouse conditions. (a) Total vitamin B1 content in leaves (b) Total vitamin B1 content in unpolished seeds. (c) Transcripts levels of the coding region of OsTHIC in leaves. wt, wild-type (white). Transgenic lines pCambia1300 and pCambia1301 (gray). Transgenic 35S-OsTHI1-35S-OsTHIC lines (black). Total vitamin B1 content quantification using a yeast microbiological assay. Values are averages ± SD of three biological replicates except for 35S-OsTHI1-35S-OsTHIC1-30 in (a), which is represented by two replicates. Asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
**Distribution of B1 vitamers in the in vitro transgenic rice seedling**

To further investigate the B1 vitamers content in transgenic rice, an HPLC assay was used to quantify three B1 vitamers, namely thiamin, TMP and TPP. Initially, two transgenic lines (35S-OsTHI1-OsTHIC-29 and 35S-OsTHI1-OsTHIC-35) and wild-type plants were selected to perform the analysis. The HPLC analysis revealed similar levels of total vitamin B1 in transgenic lines and wild-type controls. An analysis of B1 vitamers revealed that TPP was the most abundant form in rice seedling, accounting for more than 70% of the total vitamin B1 pool (Figure 25). Thiamin was the second most abundant form, accounting for more than 20% of total vitamin B1 pool. The amount of TMP was low, accounting for less than 5% of the total vitamin B1 pool. No significant differences of B1 vitamer distribution were observed in transgenic rice.

![Figure 25. Analysis of B1 vitamer content in in vitro transgenic rice seedlings by HPLC.](image)

wt, wild-type. 35S-OsTHI1-35S-OsTHIC-29 and 35 indicate two independent lines of 35S-OsTHI1-35S-OsTHIC vector. Values are averages ± SE of three biological replicates and asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests (p<0.05).
Discussion

A summary of the transgenic cassava and rice lines performance is shown in Table 1 and 2, respectively. All transgenic cassava not only failed to display increased levels of vitamin B1, rather several of them had an unexpected reduction of vitamin B1. The different strategies employed for rice were not successful. A limited success was only observed in transgenic rice in particular when using expression of the AtTHIC gene. It has been estimated that a 5.7-fold increase in vitamin B1 content in polished rice would be sufficient to address vitamin B1 deficiency issues for rice consumption populations (Fitzpatrick et al., 2012). The limited increase of vitamin B1 content in transgenic rice expressing AtTHIC (1.6-fold in the endosperm of the best performing line Glo-AtTHIC-14; Figure 16b) is not sufficient to meet human daily requirements.

The vector control line pCAMBIA1300 had levels of vitamin B1 equivalent to wild-type rice plants. However, another vector control line pCAMBIA1301 showed a 1.8-fold increase in vitamin B1 in polished rice seeds (Figure 17b). The increase of vitamin B1 content in the same transgenic line pCAMBIA1301 was also observed at the seedling stage (Figure 22), but it is not consistent in all experiments (Figure 24a,b). Because this is the only transgenic pCAMBIA1301 line used in this thesis, the vitamin B1 content in transgenic rice of additional lines of pCAMBIA1301 needs to be further determined.

The functionality of different orthologs is an important consideration in transgenic approaches. It has been reported that the specificity of regulatory proteins may be different in different species (Butelli et al., 2008). In the Golden Rice study, for example, transgenic rice transformed with the PSY transgene from rice or maize displayed the highest levels of carotenoid content, which was 12-fold higher than using the PSY gene from daffodil (Paine et al., 2005). In addition, in the vitamin B6 de novo biosynthesis pathway, the interaction of two essential genes, PDX1 and PDX2, was reported to be under a species-specific regulation (Tambasco-Studart et al., 2007). Both THI1 and THIC homologs in cassava and rice show high similarity to the ones in Arabidopsis and therefore it is anticipated that both species use the same mechanism as Arabidopsis to synthesize vitamin B1. Despite this, an additional low-risk approach that would avoid the species specificity constraint, if present at all, was included by cloning the endogenous THI1 and THIC genes from cassava and rice for genetic transformation.

Using an in silico prediction of subcellular localization, the MeTHI1 protein is predicted to be targeted to the chloroplast and the MeTHI1-2 protein is predicted to be targeted to the secretory pathway. THI1 was reported to participate not only in vitamin B1 biosynthesis in the chloroplast but also in mitochondria DNA damage repair (Chabregas et al., 2001; Machado et al., 1996). The Arabidopsis THI1 gene has two alternatively spliced transcripts encoding one protein targeted to the chloroplast and the other targeted to mitochondria (Chabregas et al., 2003). The THI1 protein in cassava may be involved in various metabolic
pathways, but the different functions have not yet been characterized. The MeTHI1 protein is postulated to participate in the vitamin B1 biosynthesis pathway because of its subcellular localization. The MeTHI1-2 protein may be involved in the other metabolic pathways in cassava cells, but this needs further investigation. Only MeTHI1 was used to generate transgenic cassava. The amino acid sequence of the MeTHIC1 protein presents a similarity of 95% to the MeTHI2 and the functional motif CX2CX4C (where X is any amino acid) is conserved. Given that an in silico analysis failed to predict the primary homolog for vitamin B1 biosynthesis, both cassava THIC genes were used to generate transgenic cassava.

As shown previously, over-expression of AtTHIC is not sufficient to enhance vitamin B1 content, rather it may lead to negative effects in the plant. A chlorotic leaf phenotype was observed in a few individual plants of both transgenic line 3SS-AtTHIC-6 and Pat-AtTHIC-5 lines (Figure 8c). Because the chlorotic leaf phenotype appeared in transgenic lines generated with two independent binary vectors, we can exclude that the observed phenotype is due to a position effect of the T-DNA integration site in the cassava genome. The rice seedlings also presented randomly the albino leaf phenotype in the transgenic line Glo-AtTHIC-14 (Figure 16b). The AtTHIC transgene in transgenic Glo-AtTHIC lines was driven by the endosperm-specific globulin promoter, but the leaky activity of the globulin promoter in leaves was observed in transgenic Glo-PDX1-Glo-PDX2 lines (Chapter 2). A similar chlorotic phenotype was reported recently in transgenic Arabidopsis with constitutive over-expression of the coding region of endogenous THIC gene, suggesting a connection between THIC gene regulation and the chlorosis (Bocobza et al., 2013). The chlorotic transgenic Arabidopsis over-expressing AtTHIC had higher levels of vitamin B1 (up to 1.5-fold). We observed that the chlorotic cassava leaves of transgenic line 3SS-AtTHIC-6 had up to a 1.3-fold increase of vitamin B1 content over the average of wild-type plants using a microbiological assay (Figure 8a). This suggests that the chlorotic leaves may be linked to vitamin B1 accumulation. Because the vitamin B1 content in chlorotic leaves of transgenic cassava line Pat-AtTHIC-5 and albino leaves of transgenic rice seedlings were not quantified, the levels of vitamin B1 in other chlorotic leaves need to be further determined. In addition, the quantification of vitamin B1 was performed by yeast microbiological assay. Other metabolites in cassava extracts may impede yeast growth. The chlorotic leaves should have a different metabolite profile compared to the green leaves. The slight increase of vitamin B1 could be due to the influence of different metabolites composition in cassava extracts. The vitamin B1 content in chlorotic leaves should be further confirmed using an HPLC assay. Furthermore, it was reported that the THIC protein is associated with the thioredoxin/ferredoxin system in the chloroplast (Raschke et al., 2007). The chlorotic leaves may be a consequence of the over-accumulation of the THIC protein in leaves, perturbing the homeostasis balance in the chloroplast. Exogenous application of different B1 vitamers on leaf and the quantitative analysis of THIC protein accumulation would allow investigating this possibility. Exogenous application experiments suggest that increased levels of thiamin in Arabidopsis seedlings do not alter the morphology of plant (Pourcel et al., 2013).
The increased levels of total vitamin B1 content in transgenic Arabidopsis using a constitutive expression strategy were mainly due to TPP accumulation (Bocobza et al., 2013). TPP is the active cofactor form of thiamin involved in various metabolic pathways. Increased levels of TPP have been demonstrated to lead to higher activity of TPP-dependent enzymes, which resulted in enhanced carbohydrate oxidation and perturbation of the homeostasis of central metabolism. Adverse effects of TPP on plant growth were observed in transgenic Arabidopsis (Bocobza et al., 2013). If the exogenous application of TPP can lead to the same phenotype, it will suggest that increased levels of TPP lead to these undesirable effects on plant growth. Over-accumulation of the THIC protein may be another factor contributing to the chlorotic phenotype. The levels of the THIC protein could be quantified by Western blot. Characterization of B1 vitamers content and the THIC protein in leaves can help to understand the cause of the chlorotic phenotype. In addition, the chlorotic phenotype appeared inconsistently in the same transgenic cassava line. It may be influenced by the external environment. The chlorotic leaf phenotype in transgenic Arabidopsis was photoperiod-dependent and appeared when plants were grown under short day conditions (Bocobza et al., 2013). In contrast to transgenic Arabidopsis, the in vitro albino rice seedlings and chlorotic leaf cassava were grown under long day conditions in the growth chamber and greenhouse, respectively. Conditions leading to the chlorotic phenotype in transgenic cassava expressing AtTHIC are currently being investigated. The interaction between B1 vitamers content and the AtTHIC protein may also be under the regulation of the external environment.

A reduction of storage root weight (fresh weight) was observed in the chlorotic leaf cassava lines (transgenic line 35S-AtTHIC-6 and Pat-AtTHIC-5) (Figure 7c). The decreased levels in chlorophyll content in the chlorotic leaves may lead to a lower photosynthetic rate of the transgenic cassava. The photosynthetic activity of cassava leaves is positively correlated to the storage root yield (El-Sharkawy et al., 1990). The decreased weight of storage roots can be linked to the phenotype of chlorosis. The biomass difference between transgenic and wild-type cassava plants was mostly observed in below ground parts. The cassava plant was grown in a small pot (9cmx9cmx10cm) under greenhouse conditions. It has been reported that the small pot can restrict the capacity of cassava growth (Rosenthal et al., 2012). The growth of above ground parts of cassava may reach the capacity earlier than the below ground parts in a small pot. This might explain why the biomass of above ground parts of transgenic lines is indistinguishable from wild-type. A proper agronomic trait evaluation of transgenic cassava should be conducted in confined field trials with a large sample size.

To investigate the impact of single moiety accumulation on vitamin B1 biosynthesis, transgenic plants expressing endogenous THI1 or THIC alone were generated. Transgenic cassava 35S-MeTHI1 lines showed undesirable phenotypes (early flowering, early branching and dieback of top), which are related to the stress response or under unfavorable conditions (Figure 10 b,c,d) (Lian and Cock, 1979; Roux et al., 2006). We can exclude that
these stress-related phenotypes were due to the greenhouse conditions, since these phenotypes were not observed in the other transgenic cassava plants grown in parallel. In addition, a reduction of plant height of transgenic 35S-OsTHI1 lines was observed in 3-week-old soil-grown seedlings compared to wild-type and the transgenic pCAMBIA1300 line (Figure 23a). Altogether, these results suggest a toxic effect related to the over-expression of the THI1 gene. Several studies have demonstrated that the THI1 gene is associated with plant development and stress responses. Defective shoot apical meristems were observed in a maize thi2 loss-of-function mutant, which is the ortholog of Arabidopsis THI1 (Woodward et al., 2010). In spite of the reports showing a correlation between THI1 protein levels and heat-tolerance in wild rice (Scafaro et al., 2010), and the involvement of the THI1 homolog in DNA protection and stress response in microorganisms (Chatterjee et al., 2011), the over-expression of THI1 gene in plants through genetic engineering has not yet been reported. It would not be surprising that previous attempts to engineer such transgenic lines must have been unsuccessful as well, because of the negative effects on plants of the single thiazole moiety or the THI1 protein accumulation in transgenic plants. The co-expression of the THIC gene may neutralize these negative effects on transgenic plants expressing the THI1 gene directly or indirectly: transgenic cassava 35S-MeTHI1-35S-MeTHIC1 lines did not show the same phenotype as transgenic cassava 35S-MeTHI1 lines, and transgenic rice 35S-OsTHI1-35S-OsTHIC lines had a similar level of plant height compared to wild-type and control plants (Figure 23b). Further characterization of transgenic plants including the expression analysis of transcripts and proteins, quantification of pyrimidine and thiazole moieties, and phenotype evaluation can help us to understand these gene functions in plants.

The total vitamin B1 content was decreased in leaves of all transgenic cassava lines of both 35S-MeTHIC1 and 35S-MeTHIC2 vectors (Figure 14d). Transgenic rice expressing OsTHIC did not show increased levels of vitamin B1 (Figure 22). The coding region of the THIC gene was used as a transgene. The absence of the riboswitch motif in the 3'-UTR of the transgene would lead to a transcript completely insensitive to the feedback regulation by TPP. The levels of the THIC protein and the pyrimidine moiety in the transgenic plants should be quantified to confirm the transgene functionality. On the contrary, a slight increase of vitamin B1 was observed in seedlings and endosperm (up to 1.4- and 1.6-fold, respectively) of transgenic rice expressing AtTHIC. One possible reason is the endogenous OsTHIC may be regulated by other unknown factors in rice, but the regulatory factors cannot recognize the exogenous THIC. The existence of another mechanism to regulate pyrimidine moiety production in plants is likely, since no THIC ortholog was found in the Medicago truncatula genome (http://www.medicagohapmap.org) with Blast searches. And last but not least: the species-specific effect could be exploited to bypass the endogenous feedback regulation, e.g., the co-expression of AtTHI1 and AtTHIC in rice or cassava may lead to an increase in vitamin B1. Both AtTHI1 and AtTHIC in Arabidopsis have been functionally characterized (Kong et al., 2008; Machado et al., 1996; Raschke et al., 2007).
Additionally, the gene function of endogenous THI1 and THIC in cassava and rice has not yet been validated. These transgenes may not be the functional forms to synthesize vitamin B1. Arabidopsis THI1 and THIC genes were reported to functionally complement in their respective loss-of-function mutants of *E. coli* (Kong et al., 2008; Machado et al., 1996). Furthermore, the Arabidopsis THIC gene could successfully complement Arabidopsis *thic* loss-of-function mutant (Kong et al., 2008; Tambasco-Studart et al., 2007). Similar experiments with the THI1 and THIC orthologs from cassava and rice would validate their functions. Moreover, quantitative analysis of pyrimidine and thiazole moieties in transgenic plants expressing either the THI1 or the THIC gene alone can also further confirm gene function.

Furthermore, two out of three plants of transgenic rice line 35S-OsTHI1-8 as biological replicates had increased levels of vitamin B1 accumulation (Figure 22). It seems that high vitamin B1 plants with a normal phenotype appeared randomly in the transgenic rice population. In contrast to the observation in transgenic Arabidopsis and cassava with increased levels of vitamin B1, these two high vitamin B1 rice plants did not show a chlorotic phenotype. The transgenic rice plants of line 35S-OsTHI1-8 accumulating increased levels of vitamin B1 are currently growing in the greenhouse to further determine vitamin B1 content in mature plants and progeny seeds. It could be used to investigate vitamin B1 accumulation in rice by analyzing expression levels of the transgene and alteration of endogenous genes associated with the vitamin B1 biosynthesis pathway. Together, the balance between pyrimidine and thiazole moieties to generate vitamin B1 is still elusive. Quantitative analysis of these two moieties in transgenic plants with increased levels of vitamin B1 can characterize the proper ratio to increase this vitamin. The analysis will assist in identifying the limiting factors of vitamin B1 production in plants without undesirable effects.

Different strategies to increase vitamin B1 have been discussed (Pourcel et al., 2013). Transgenic plants engineered for the over-expression of THI1 and THIC genes is assumed to be a successful strategy, since the single moiety accumulation cannot increase vitamin B1 content in plant tissues. A similar observation was made in a heat-tolerance wild rice variety which showed increased levels of the THI1 protein at a higher temperature but the vitamin B1 content was stable (Scafaro et al., 2010). However, we did not observe increased levels of vitamin B1 in both transgenic cassava and rice over-expressing endogenous THI1 and THIC. Biofortification of iron has been successfully proven in rice endosperm through over-expression of the iron storage protein (ferritin) (Goto et al., 1999) and the same strategy was recommended for vitamin B1 biofortification (Pourcel et al., 2013). Several thiamin storage proteins were reported in legumes and cereal grains including rice (Gołda et al., 2004). Root specific-expression of thiamin storage proteins in cassava and in rice using endosperm-specific expression strategies may lead to enhanced levels of vitamin B1 in the edible organs. In addition, engineering a whole biochemical pathway from microorganisms into plants has been demonstrated to be a feasible strategy. Plants and microorganisms may use different
precursors to produce the same metabolite. Engineering a biochemical pathway from microorganisms into plants would avoid the disturbance of endogenous pathways and adverse effects. This was illustrated with the introduction of the *E. coli* glycolate catabolic pathway into Arabidopsis for improving biomass accumulation (Kebeish et al., 2007). There are three different mechanisms of vitamin B1 biosynthesis in fungi, plants and bacteria (Helliwell et al., 2013). In fungi, two single turnover enzymes are used to produce pyrimidine and thiazole moieties. The THI4 protein is used to catalyze the transformation of vitamin B3 (NAD$^+$) to the thiazole moiety (HET-P) (Chatterjee et al., 2011). The *THI4* gene is the ortholog of Arabidopsis *THI1*. The THI5 protein uses vitamin B6 (pyridoxal-5'-phosphate) as precursor to produce the pyrimidine moiety (HMP-PP) (Lai et al., 2012). In this thesis, both transgenic cassava and rice enriched with vitamin B6 in the storage roots and grains have been developed, respectively. Therefore, over-expression of *THI4* and *THI5* genes in transgenic 35S-PDX1-35S-PDX2 lines may lead to cassava and rice lines improved in both vitamin B1 and vitamin B6 levels.
### Table 1

**Summary of transgenic cassava performance**

<table>
<thead>
<tr>
<th>Transformation vectors</th>
<th>Total vitamin B1 content</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exogenous transgene</strong></td>
<td><strong>Promoter</strong></td>
</tr>
<tr>
<td>35S-AtTHIC</td>
<td>constitutive</td>
</tr>
<tr>
<td>Pat-AtTHIC</td>
<td>root-specific</td>
</tr>
<tr>
<td><strong>Endogenous transgene</strong></td>
<td><strong>promoter</strong></td>
</tr>
<tr>
<td>35S-MeTHI1</td>
<td>constitutive</td>
</tr>
<tr>
<td>35S-MeTHIC1</td>
<td>constitutive</td>
</tr>
<tr>
<td>35S-MeTHIC2</td>
<td>constitutive</td>
</tr>
<tr>
<td>35S-MeTHI1-35S-MeTHIC1</td>
<td>constitutive</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Transformation vectors</th>
<th>Total vitamin B1 content</th>
<th>Exogenous transgene</th>
<th>Promoter</th>
<th>Line number</th>
<th>In vitro seedling</th>
<th>Soil-grown seedling</th>
<th>Mature leaves</th>
<th>Unpolished seeds</th>
<th>Polished seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>35S-AtTHIC</td>
<td>constitutive</td>
<td>30, 31, 32, 85, 86, 88</td>
<td>tend to increase</td>
<td>up to 1.4-fold increase</td>
<td>-</td>
<td>-</td>
<td>no change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glo-AtTHIC</td>
<td>endosperm-specific</td>
<td>3, 14</td>
<td>no change</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.6-fold increase (no.14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35S-OsTHI1</td>
<td>constitutive</td>
<td>7, 8, 9</td>
<td>-</td>
<td>up to 1.2-fold increase*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35S-OsTHIC</td>
<td>constitutive</td>
<td>3, 5, 16</td>
<td>-</td>
<td>no change</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35S-OsTHI1-35S-OsTHIC</td>
<td>constitutive</td>
<td>2, 13, 29, 30, 35</td>
<td>-</td>
<td>up to 1.4-fold increase</td>
<td>no change</td>
<td>no change</td>
<td>-</td>
</tr>
<tr>
<td>Vector control</td>
<td></td>
<td>pCAMBIA1300</td>
<td>-</td>
<td>pCAMBIA1300</td>
<td>-</td>
<td>no change</td>
<td>no change</td>
<td>no change</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pCAMBIA1301</td>
<td>-</td>
<td>pCAMBIA1301</td>
<td>1.3-fold increase</td>
<td>no change</td>
<td>no change</td>
<td>no change</td>
<td>1.8-fold increase</td>
</tr>
</tbody>
</table>

*Two of three plants as biological replicates in transgenic line 35-OsTHI1-8 showed a 2-fold increase.
Materials and methods

Plant materials and growth conditions

Cassava

Cassava (*Manihot esculenta* Crantz cv. 60444) was used in the present study. *In vitro* cassava plantlets were kept under a 16/8 h light and dark regime at 28°C for one month, before transferring to soil under greenhouse conditions. Cassava plants were grown under greenhouse conditions (16 h light at 26°C and 60% humidity, 8 h dark at 17 °C and 50% humidity) and the effects of natural illumination for 6 months. Fully expanded leaves on the top of the plant and storage roots were harvested for analysis.

Rice

Greenhouse-grown rice

Rice (*Oryza sativa* ssp. japonica cv. Taipei 309) was used in this study. Dehulled rice seeds were sterilized with 3% (w/v) sodium hypochlorite for more than 30 min and washed thoroughly with sterile distilled water. Transgenic and wild-type rice seeds were germinated on MS medium [1x MS salt, 3% sucrose, 0.3% gelrite] with or without hygromycin B (50 mg/L; Carl Roth, Switzerland) in darkness at 37°C for 2 d to have homogenous germination. Germinated rice seed on selection medium was moved to a climate chamber (16/8 h light and dark regime at 28°C) for 7 d. The uniformly growing transgenic and wild-type seedlings were transferred to soil under greenhouse conditions (12 h light at 30°C and 70% humidity, 12 h dark at 20°C and 60% humidity) and the effects of natural illumination for 5 months.

*In vitro* seedlings

Dehulled transgenic and wild-type rice seeds were sterilized with 3% (w/v) sodium hypochlorite for more than 30 min and washed thoroughly with sterile distilled water. Transgenic and wild-type rice seeds were germinated on MS medium [1x MS salt, 3% sucrose, 0.3% gelrite] with or without hygromycin B (50 mg/L; Carl Roth, Switzerland) in darkness at 37°C for 2 d to have homogenous germination and then moved to climate chamber (16/8h light and dark regime at 28°C) for 7d. After that, growing rice seedlings were transferred to MS medium [1x MS salt, 3% sucrose, 0.3% gelrite] in big jars without hygromycin in a climate chamber (16/8h light and dark regime at 28°C) for 4 weeks.
Soil-grown seedlings

Dehulled transgenic and wild-type rice seeds were sterilized with 3% (w/v) sodium hypochlorite for more than 30 min and washed thoroughly by sterile distilled water. Transgenic and wild-type rice seeds were germinated on MS medium [1x MS salt, 3% sucrose, 0.3% gelrite] with or without hygromycin B (50 mg/L; Carl Roth, Switzerland) in darkness at 37°C for 2 d to have homogenous germination and then moved to a climate chamber (16/8 h light and dark regime at 28°C) for 7 d. After that, growing rice seedlings were transferred to soil under greenhouse conditions (16 h light at 26°C and 60% humidity, 8 h dark at 17°C and 50% humidity) and the effects of natural illumination for 14 d.

**In silico** identification of THIC and THI1 genes in cassava and rice

Using AtTHIC (At2g29630) and AtTHI1 (At5g54770) sequences from Arabidopsis, homologs in the cassava and rice genome was identified by Blast searches on phytozome (www.phytozome.net).

**Accession number**

THI1: Arabidopsis, At5g54770 (hereafter named AtTHI1); rice, LOC_Os07g34570 (hereafter named OsTHI1); cassava, cassava24565 (hereafter named MeTHI1) (phytozome4), cassava37020 (hereafter named MeTHI1-2) (phytozome4).

THIC: Arabidopsis, At2g29630 (hereafter named AtTHIC); rice, LOC_Os03g47610 (hereafter named OsTHIC); cassava, cassava4.1_003348m (hereafter named MeTHIC1) and cassava4.1_003360m (hereafter named MeTHIC2).

**Sequence alignments**

Alignments of THI1 and THIC amino acid sequences were performed with the Clustal W method (DNASTAR Lasergene).

**Transformation vectors construction**

Cassava transformation vectors

1. **35S-AtTHIC**

The AtTHIC (At2g29630) in pCAMBIA1302-AtTHIC (Raschke et al., 2007) was amplified by PCR with primers THIC-F-KpnI (5'-GGTACCATGGCTGCTTCA GTACAC-3') and THIC-R-KpnI (5'-GGTACCTTATTTCTGAGCAGCTTTGAC-3'). The AtTHIC DNA fragment was digested with KpnI and inserted into p1380-35S, generating the transformation vector 35S-AtTHIC.
2. **Pat-AtTHIC**

The *patatin* promoter in the *pJ-Patatin* vector was linearized with EcoRI and KpnI, and inserted into p1380-35S which 35S promoter was replaced, to generate the p1380-Pat vector. The AtTHIC DNA fragment was digested with KpnI and inserted into p1380-Pat, generating the transformation vector *Pat-AtTHIC*.

3. **35S-MeTHI1**

The *MeTHI1* gene was amplified from cassava cDNA by PCR with primers cassava24565-F (5'-TTTCAGTCTTTTCTGC-3') and cassava24565-R (5'-AGCCAGACTCATTTTCTACAG-3'), and subcloned into the *pJET1.2/blunt* Cloning Vector (CloneJET PCR cloning Kit; Thermo Scientific), generating the *pJ-MeTHI1* vector. The *MeTHI1* DNA fragment in *pJ-MeTHI1* was linearized with BgIII, and inserted into p1380-35S, generating the transformation vector 35S-MeTHI1.

4. **35S-MeTHIC1**

The *MeTHIC1* gene was amplified from cassava cDNA by PCR with primers cassava23361-F (5'-ATGGGCGTCTGTGCAAGCTACT-3') and cassava23361-R (5'-TCATATGCTCTTCTAGAAGATC-3'), and subcloned into the *pJET1.2/blunt* Cloning Vector, generating *pJ-MeTHIC1* vector. The *MeTHIC1* DNA fragment in *pJ-MeTHIC1* was linearized with BgIII, and inserted into p1380-35S, generating the transformation vector 35S-MeTHIC1.

5. **35S-MeTHIC2**

The *MeTHIC2* gene was amplified from cassava cDNA by PCR with primers cassava23286-F (5'-ATGGCATCGGTGCAAGCTACTAG-3') and cassava23286-R (5'-TCACATGCTCTTCTAGAAGATT-3'), and subcloned into the *pJET1.2/blunt* Cloning Vector, generating *pJ-MeTHIC2* vector. The *MeTHIC2* DNA fragment in *pJ-MeTHIC2* was linearized with BgIII, and inserted into p1380-35S, generating the transformation vector 35S-MeTHIC2.

6. **35S-MeTHI1-35S-MeTHIC1**

The expression cassette, 35S promotor::*MeTHI1*::terminater, in 35S-MeTHI1 was synthesised by PCR with primers clone-35S-F (5'-GGC CGCGCCAATATCGGTGCAAGCTACT-3') and clone-polyA-R (5'-GGC CGCGCCAATATGCTCTTCTAGAAGATT-3'), and sub cloned into the *pJET1.2/blunt* Cloning Vector, generating *pJ-35ScassavaTHI1* vector. The expression cassette, 35S promotor::*MeTHI1*::terminater, in *pJ-35ScassavaTHI1* was linearized with Asci, and inserted into 35S-MeTHIC1, generating the transformation vector 35S-MeTHI1-35S-MeTHIC1.
Rice transformation vectors

1. **35S-AtTHIC**

   The same transformation vector (35S-AtTHIC) described in this chapter for cassava transformation was used here.

2. **Glo-AtTHIC**

   The *globulin* promoter in the *pJ-Globulin* vector was linearized with EcoRI and KpnI, and inserted into p1380-35S which 35S promoter was replaced, to generate the p1380-Glo vector. The AtTHIC DNA fragment was digested with KpnI and inserted into p1380-Glo, generating the transformation vector Glo-AtTHIC.

3. **35S-OsTHI1**

   The OsTHI1 gene was amplified from rice cDNA by PCR with primers rice-THI1-F2 (5'-AGAGCAAGAAGCTCAGCTCC-3') and rice-THI1-R (5'-TCAGGCGCCAGACGATCTGC-3'), and subcloned into the *pJET1.2/blunt* Cloning Vector, generating the *pJ-OsTHI1* vector. The OsTHI1 DNA fragment in *pJ-OsTHI1* was linearized with BglII, and inserted into p1380-35S, generating the transformation vector 35S-OsTHI1.

4. **35S-OsTHIC**

   The OsTHIC gene was amplified from rice cDNA by PCR with primers rice-THIC-F1 (5'-ATGGCTGCCCTGCAACCCTC-3') and rice-THIC-R1 (5'-AGATGGACCAAGACGATC-3'), and subcloned into the *pJET1.2/blunt* Cloning Vector, generating the *pJ-OsTHIC* vector. The OsTHIC DNA fragment in *pJ-OsTHIC* was linearized with BglII, and inserted into p1380-35S, generating the transformation vector 35S-OsTHIC.

5. **35S-OsTHI1-35S-OsTHIC**

   The expression cassette, 35S promoter::OsTHI1::terminator, in 35S-OsTHI1 was amplified by PCR with primers clone-35S-F (5'-GGCGCGCCGAATTCATGGAGT-3') and clone-polyA-R (5'-GGCGCGCCAATATATCTGCA-3'), and subcloned into the *pJET1.2/blunt* Cloning Vector, generating the *pJ-35SriceTHI1* vector. The expression cassette, 35S promoter::OsTHI1::terminator, in *pJ-35SriceTHI1* was linearized with Ascl to perform partial digestion, and the full cassette DNA fragment was chosen to insert into 35S-OsTHIC, generating the transformation vector 35S-OsTHI1-35S-OsTHIC.

Primer sequences used for gene cloning and vector construction is given in Table 3.
Plant transformation

Cassava

Six transformation vectors (35S-AtTHIC, Pat-AtTHIC, 35S-MeTHI1, 35S-MeTHIC1, 35SMeTHIC2, and 35S-MeTHI1-MeTHIC1) were introduced into Agrobacterium tumefaciens strain LBA4404, and transgenic cassava was produced using an established protocol for cassava transformation (Bull et al., 2009).

*The cassava transformation of four vectors (35S-MeTHI1, 35S-MeTHIC1, 35SMeTHIC2, and 35S-MeTHI1-MeTHIC1) was done by Ms. Kim Schlegel in the Plant Biotechnology Lab at ETH Zürich.

Rice

Five transformation vectors (35S-AtTHIC, Glo-AtTHIC, 35S-OsTHIC, 35S-OsTHI1, and 35S-OsTHI1-OsTHIC) were introduced into Agrobacterium tumefaciens strain LBA4404, and transgenic rice was generated as described (Nishimura et al., 2007).

Molecular characterization

DNA was extracted from cassava and rice leaves according to an established protocol (Sheu et al., 1996). In brief, plant tissue was ground in liquid nitrogen, and extraction buffer was added (7 M Urea, 0.3 M NaCl, 50 mM Tris-HCl, 20 mM EDTA, pH 8), before centrifugation 16100 g for 2 min. Then, one volume of 25:24:1 phenol:chloroform:isoamyl alcohol was added and mixed with supernatant, before centrifugation at 16100 g for 15 min. The aqueous phase was recovered, and one-tenth volume of 3 M sodium acetate, 1μL of RNase A (20mg/mL) and one volume of isopropanol were added, before incubating for 15 min at -80°C. The DNA pellet was resuspended in water after centrifugation 16100 g for 20 min.

To determine the T-DNA integration copy number in transgenic cassava or rice plants, 20 μg or 10 μg of genomic DNA was digested with HindIII, electrophoresed on an agarose gel, and transferred onto a nylon membrane (GE Healthcare Lifescience, UK). Southern blot analysis was performed subsequently following manufacturer’s instructions (Roche, Switzerland). Membranes were hybridized with a DIG-labeled hptII probe.

RNA extraction and cDNA synthesis

Total RNA was isolated from mature rice leaves at the reproductive stage or in vitro cassava leaves as in an established protocol (Cazzonelli et al., 1998) with slight modifications. In brief, plant tissue was ground in liquid nitrogen, and lysis buffer (150 mM Tris, 50 mM EDTA, 2%
SDS, pH 7.5) was added. Then, one-fourth volume of absolute ethanol was added and mixed. 0.1 volume of 5 M potassium acetate was added, and the mixture was vortexed for 1 min. One volume of 24:1 chloroform:isoamyl alcohol was added and mixed, before centrifugation at 16100 g for 10 min. The aqueous phase was recovered, and extracted with one volume of 25:24:1 phenol:chloroform:isoamyl alcohol. The aqueous phase was recovered and precipitated with 2 volumes of absolute ethanol for 30 min at -80°C, before centrifugation at 16100 g for 30 min at 4°C. RNA was precipitated by incubation in 2 M LiCl 30 min at -80°C, before centrifugation at 16100 g for 30 min at 4°C. The RNA pellet was resuspended in DEPC-water.

cDNA was generated by RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with 2 μg total RNA using random hexamer primers according to the manufacturer’s instructions. RT-qPCR was performed using the 7500 FAST real-time PCR system and Fast SYBR® Green Master Mix (Applied Biosystems). Relative target gene expression levels were normalized to the reference gene, Ubiquitin-conjugating enzyme E2 (UBC) (Jain et al., 2006), using the delta-delta CT method (Livak and Schmittgen, 2001). Primers sequences used for RT-qPCR are reported in Table 4.

**Vitamin B1 quantification**

Yeast microbiological assay

Total vitamin B1 was quantified according to an established protocol (Raschke et al., 2007). Total vitamin B1 was extracted from 50-100 mg of homogenized plant material in 1 mL of 0.22 M sulphuric acid at 100°C for 1 h, and then treated with acid phosphatase for 12-15 h at 37°C to convert phosphorylated forms to thiamin. The quantity of total vitamin B1 in plant extracts was extrapolated from a standard curve of known amounts of commercial thiamin hydrochloride.

Fluorescence assay

Total vitamin B1 extraction was performed according to an established protocol (Moulin et al., 2013). Total vitamin B1 was extracted from 150 mg of homogenized plant material in 300 μL 1% TCA and vortexed at room temperature for 30 min. 200 μL of clear supernatant was transferred to a new tube after centrifugation at 16100 g for 10 min and neutralized with 20 μL of 3 M sodium acetate. Then 44μL of 30 mM potassium ferricyanide was added which was dissolved in 15% sodium hydroxide and incubated in the dark for 10 min. After that, 66 μL of 1 M sodium hydroxide and 110 μL of methanol were added sequentially before centrifugation at 16100 g for 10 min. The fluorescence intensity of clear supernatant was measured by a spectrofluorometer (Infinite® M1000 PRO, Tecan) with an excitation wavelength of 375 nm and an emission wavelength of 450 nm at room temperature.
The vitamin B1 extraction was performed as described in the fluorescence assay. The B1 vitamers content was determined by HPLC according to an established protocol (Moulin et al., 2013).

*The HPLC assay was performed by Dr. Michäel Moulin in the Plant Biochemistry and Physiology group at University of Geneva.

Table 3
Primer sequences for gene cloning and vector construction

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<th>Target gene</th>
<th>Primer name</th>
<th>Sequence</th>
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<tr>
<td>AtTHIC</td>
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Chapter 5 Concluding remarks and perspectives

In this thesis, strategies to increase vitamin B1 and vitamin B6 content in staple crops have been examined in cassava and rice. These results demonstrate the feasibility of vitamin B6 biofortification in both cassava and rice through genetic engineering. To our knowledge, this is the first successful example of vitamin B6 biofortification in staple crops. However, no significant increase of vitamin B1 content was observed in both transgenic cassava leaves and storage roots. Transgenic rice also displayed no consistent increase of vitamin B1 content, suggesting that the selected strategy alone is not sufficient for vitamin B1 biofortification.

_Vitamin B6 biofortification of cassava by metabolic engineering_

The strategy to increase vitamin B6 content in cassava was based on the expression of two key genes from Arabidopsis, \textit{AtPDX1.1} and \textit{AtPDX2}, under the control of constitutive or root-specific promoters. In transgenic cassava, we achieved a maximal 10-fold increase in vitamin B6 in leaves using a constitutive expression strategy \((35S-PDX1-35S-PDX2)\) and a maximal 12-fold increase in storage roots using a root-specific expression strategy \((PAT-PDX1-PAT-PDX2)\) under field conditions. The fold-increase in vitamin B6 in transgenic cassava is much higher than in the two reported screenings of natural variation in potato and wheat \((1.5-\text{and} \, 2.3\text{-fold, respectively})\) (Mooney et al., 2013; Shewry et al., 2011). This reveals that biofortification of vitamin B6 through genetic engineering may overcome the limitation of natural variation in plants. Both the greenhouse- and field-grown transgenic cassava are phenotypically indistinguishable from wild-type plants. We did not observe adverse effects on transgenic cassava growth under greenhouse and field conditions. Additionally, transgenic cassava expressing \textit{AtPDX1.1} and \textit{AtPDX2} does not alter the transcript level of endogenous \textit{PDX1} and \textit{PDX2} genes. The increase of vitamin B6 in both transgenic cassava leaves and storage roots is largely due to glycosylated derivatives. Evaluation of agronomic traits under field conditions will need to be performed in multi-year and multi-location confined field trials with an adequate experimental design to validate the improved plants.

Glycosylated derivatives of vitamin B6 are postulated to be the storage forms and lack the functionality of vitamin B6. Transgenic cassava mainly accumulating glycosylated derivatives may be a mechanism to maintain homeostasis of metabolic pathways, which are related to vitamin B6. To test this hypothesis, we can apply exogenous vitamin B6 (e.g. pyridoxine) to transgenic cassava and therefore determine the vitamers content in tissues. If exogenous application of pyridoxine on transgenic cassava results in conversion into glycosylated derivatives mostly, it may suggest that cassava cannot store high levels of free and
phosphorylated forms of vitamin B6. Furthermore, we did not observe a biomass increase in transgenic cassava, which was reported for high vitamin B6 transgenic Arabidopsis. In contrast to transgenic cassava, the phosphorylated derivatives were the major contributors of total vitamin B6 in transgenic Arabidopsis (Raschkke et al., 2011). The composition of vitamin B6 vitamers might explain why we did not observe a biomass increase in transgenic cassava.

Pyridoxine-β-glucoside (PNG) was the main contributor of vitamin B6 accumulation in transgenic cassava, accounting for 70% and 60% of total vitamin B6 pool in leaves and storage roots, respectively. PNG was reported to be 50% bioavailable for human consumption (Gregory III, 2012). The retention of different forms of vitamin B6 is also affected by cooking processes. Transgenic cassava retained a maximum of 17- and 5-fold increase in total vitamin B6 content in boiled leaves and baked storage roots, respectively, compared to wild-type plants. Glycosylated derivatives remained the most predominant forms in cooked transgenic cassava, accounting for more than 80% of the total vitamin B6 pool in both boiled leaves and baked storage roots. The baked transgenic storage roots are estimated to have a maximum of 7.7 mg bioavailable vitamin B6 in a 500 g cassava meal. It should be substantial enough to meet daily requirements of adults (1.3 mg/per day). However, the actual bioavailable vitamin B6 in transgenic cassava for human consumption needs further investigations. Because the efficiency of vitamin B6 absorption in model animals is not the same as in humans, evaluation of the vitamin B6 bioavailability is difficult to conducted in rat or mouse (Gregory III, 2012; Ink et al., 1986). Two in vitro methods have been applied to examine vitamin B6 bioavailability including in vitro solubility and the Caco-2 cell model (Etcheverry et al., 2012). The bioavailability of vitamin B6 in transgenic cassava with different cooking styles is currently being investigated with the Caco-2 cell assay. It will help us to assess the actual bioavailability of vitamin B6 in transgenic cassava.

It is also interesting to further evaluate the antioxidant property of transgenic cassava with high vitamin B6 content. Transgenic Arabidopsis that accumulates high vitamin B6 content has increased tolerance to oxidative damage (Raschke et al., 2011). Post-harvest physiological deterioration (PPD) is a major constraint of storing cassava storage roots. PPD is caused by an oxidative burst within 15 min after roots harvest and is related to reactive oxygen species (ROS) accumulation (Reilly et al., 2004). It has been demonstrated that increased levels of antioxidant activity in transgenic cassava storage roots can delay the occurrence of PPD (Xu et al., 2013b; Zidenga et al., 2012). In addition, transgenic cassava with enhanced antioxidant content has been reported to have improved cold and drought tolerance (Xu et al., 2013a). It is worth to explore the PPD occurrence and abiotic stress responses in transgenic cassava accumulating high levels of vitamin B6. Further characterization including genome-wide gene expression analysis of transgenic cassava will assist in examining the agronomic and stress-tolerance traits related to the increased levels of vitamin B6.
Due to its high vitamin B6 content, transgenic cassava is more nutritious than the variety cv.60444, which is a model variety for genetic transformation. The farmer-preferred cultivars from TME series and three local varieties in Kenya with good agronomic traits have been successfully transformed in our lab and another laboratory in Kenya (Nyaboga et al., 2013; Zainuddin et al., 2012). It is therefore important to implement this proof-of-concept strategy from the model variety cv. 60444 to farmer-preferred varieties with local research institutions in vitamin B6 deficiency areas. Recently, the epidemic disease nodding syndrome was reported in northern Uganda and South Sudan (Donnelly, 2012; Williams, 2012). This nodding syndrome has been linked to vitamin B6 deficiency (Foltz et al., 2013). Cassava is the major staple crop in these areas where people suffer from a deficiency of vitamin B6. Transgenic cassava with increased levels of vitamin B6 can potentially address this micronutrient deficiency issue sustainably and cost-effectively through daily diet consumption.

Additionally, in our first attempt to increase the vitamin B6 content in staple crops, hygromycin phosphotransferase was used as a selectable marker in the transformation vectors. The selectable marker is of no use after the transformation procedure in transgenic plant. The retained selectable marker in the commercialized transgenic crops may bring public concerns regarding the biosafety to all organisms and gene flow in the nature (Manimaran et al., 2011). The methods to remove selectable markers in transgenic crops have been well reviewed (Darbani et al., 2007; Miki and McHugh, 2004; Zuo et al., 2002). Emerging technologies in other plant species provide new opportunities to remove the selectable marker in transgenic cassava (i.e. TAL effector nucleases and CRISPR) (Li et al., 2012; Shan et al., 2013). The intellectual property right of the promoters and genes used in genetic engineering should be also taken into consideration. For example, the intellectual property right of the 35S promoter is owned by a private company Monsanto (Odell et al., 1985). It should be considered to use alternative promoters which were developed in public sectors for the new transformation vectors. Several alternative promoters are available for public use such as the ubiquitin promoter, the FMV 34S promoter, and the octopine synthase promoter (Graff et al., 2003). However, the activity of these promoter in transgenic cassava needs further investigations.

Furthermore, stacking of multiple transgenes has been successfully demonstrated in transgenic maize with enhanced multivitamin content including vitamin A, vitamin C and folate (Naqvi et al., 2009). The technical feasibility of transgene stacking was proven in transgenic maize. Progress for biofortification of iron and vitamin A has been made in transgenic cassava separately (Sayre et al., 2011). Several improved traits associated with biotic and abiotic stress tolerance in cassava have been developed through genetic engineering (e.g. virus-resistant, insect-resistant, cold tolerance and shelf-life extension) (Duan et al., 2013; Vanderschuren et al., 2012; Xu et al., 2013a; Xu et al., 2013b; Zidenga et al., 2012). This demonstrates the potential of stacking multiple traits to develop stress-
tolerant cassava varieties together with multi-nutrient traits through genetic engineering. The biofortified cassava variety combined with stress tolerance traits has the potential to improve nutritional status and food production of the poorest people in the developing countries. In addition, transgenic cassava accumulating high levels of vitamin B6 could be a potential alternative for chemical vitamin synthesis. Because of its good agronomic performance, transgenic cassava can grow on suboptimal environments to produce feedstock for industrial vitamin B6 production. Unlike maize cultivation for biofuel, cassava plantations in the marginal environment do not compete for food production systems.

**Vitamin B6 biofortification of rice by metabolic engineering**

The same strategy to increase vitamin B6 in cassava was also applied to rice. Transgenic rice displayed up to 2-fold increase in vitamin B6 content in unpolished grains and more than a 10-fold increase in leaves. A limited increase of vitamin B6 content (up to 1.3-fold) was observed in polished seeds using a yeast microbiological assay. However, the same polished seed samples displayed a 2-fold increase using an HPLC assay. The actual value of vitamin B6 content needs further investigation. It has been estimated that a 3.2-fold increase in vitamin B6 content in polished seeds can meet the human daily requirements (Fitzpatrick et al., 2012). Additional strategies should be developed to increase vitamin B6 content in rice endosperm. An expression analysis showed both AtPDX1.1 and AtPDX2 were highly expressed in the endosperm. The transcript level of AtPDX1.1 in the transgenic Glo-PDX1-Glo-PDX2 lines were higher than in the transgenic 35S-PDX1-35S-PDX2 lines, but the levels of vitamin B6 were equal in transgenic lines of both vectors. The vitamin B6 content in rice endosperm did not correlate with the expression levels of the transgenes. A maximal 1.3-fold increase in vitamin B6 in endosperm was observed in all transgenic rice plants using a microbiological assay. This reveals that available precursors of vitamin B6 biosynthesis may be another limiting factor to restrict vitamin B6 production in the endosperm. Metabolite profiling of the rice endosperm is currently being done. It will help us to investigate the bottleneck of vitamin B6 biosynthesis in rice endosperm.

In this thesis, we observed that only one out of four transgenic 35S-PDX1-35S-PDX2 lines that displayed high levels of vitamin B6 in leaves. The repetitious use of the 35S promoter might have silenced the transgene in leaves of the transgenic rice lines. The issue of promoter use in transgenic plants has been extensively discussed (Peremarti et al., 2010). Furthermore, it has been reported that increased levels of vitamin B6 in transgenic Arabidopsis can improve the tolerance to oxidative stress and increase biomass (Raschke et al., 2011). Transgenic rice leaves accumulating high levels of vitamin B6 could have increased potential of abiotic stress tolerance. It is worth to produce additional lines with high vitamin B6 content in leaves or whole plants to examine its stress resistant ability. Moreover, the total biomass and grain yield of transgenic rice should be further determined. To avoid
transgene silencing, careful selection of promoters used in transformation vectors is important. The actin and ubiquitin promoters have been proven useful in constitutive expression of transgenes in rice transformation (Cornejo et al., 1993; Zhang et al., 1991). The expression levels of a transgene under the control of the ubiquitin promoter should be more stable and stronger than the one under the control of the 35S promoter in transgenic rice. To develop a new transformation vector, the 35S promoter driving the PDX transgenes in the 35S-PDX1-35S-PDX2 vector could be replaced with the actin or ubiquitin promoter. This might lead to a stable expression of PDX transgenes in transgenic rice leaves. Transgenic rice with increased levels of vitamin B6 in leaves could be further examined for their tolerance to different stresses.

A synergistic approach to transport vitamin B6 from leaves or the embryo to the endosperm could be another potential strategy. Plants can produce vitamin B6 de novo, but also can take up vitamin B6 from the external environment. How the rice endosperm accumulates vitamin B6 is still an open question. To investigate the feasibility, several strategies could be deployed. In an initial step, transgenic rice expressing the vitamin B6 transporters could be used to investigate the dynamics of vitamin B6. If the vitamin B6 distribution in tissues of transgenic rice expressing vitamin B6 transporter could be altered, it may reveal the potential to translocate the vitamin B6 in different plant tissues. We can then use endosperm-specific expression strategy to restrict the expression of the candidate transporter to the endosperm. Both constitutive and endosperm-specific strategies can help us to examine the feasibility of vitamin B6 biofortification in endosperm using the vitamin B6 transporter. Given that this is feasible, transgenic rice consisting of expression of vitamin B6 transporter and PDX transgenes through genetic engineering will be generated to accumulate vitamin B6 in rice endosperm.

**Vitamin B1 biofortification of cassava and rice by metabolic engineering**

The strategies deployed in this thesis research were not sufficient to increase vitamin B1 in either transgenic cassava or rice to meet the human daily requirements. The endogenous THI1 and THIC genes of cassava and rice used in genetic transformation have not yet been characterized. It needs experimental validation to confirm their function. It has been reported that both pyrimidine and thiazole moieties are equally important to biosynthesize vitamin B1 (Pourcel et al., 2013). To increase vitamin B1 in plant tissues, this is essential to determine the stoichiometric amounts of pyrimidine and thiazole moieties for vitamin B1 production. Transgenic plants with increased or decreased levels of vitamin B1 could be a valuable material to investigate the balance of pyrimidine and thiazole moieties for vitamin B1 accumulation. Further characterization including transcriptional and translational levels of transgene and endogenous genes expression, metabolite profiling, and B1 vitamers
content in these transgenic plants will assist in developing new strategies for vitamin B1 biofortification.

Moreover, transgenic Arabidopsis accumulating increased levels of vitamin B1 only appeared under short day conditions and an oscillation of vitamin B1 content was observed (Bocobza et al., 2013). In this study, transgenic cassava was grown under long day conditions (16 h light/8 h dark), and the transgenic rice was grown under short day conditions (12 h light/12 h dark) in the greenhouse. The vitamin B1 content of transgenic cassava and rice under different photoperiod conditions needs further investigation. It is also important to quantify the vitamin B1 content under the circadian rhythm. This will help us to decide the most suitable sampling time point to quantify vitamin B1 in plants.

Additional strategies of vitamin B1 biofortification in plants have been reviewed (Pourcel et al., 2013). Several thiamin binding proteins that serve as a thiamin reserve in seeds have been characterized in different plant species such as maize, oat, and garden pea (Gołda et al., 2004). Over-expression of thiamin storage proteins in edible organs of plants was recommended for vitamin B1 biofortification (Pourcel et al., 2013). In addition, genetically engineering the vitamin B1 biosynthesis pathway from microorganisms into plants can be another potential strategy. Yeast (*Saccharomyces cerevisiae*) uses vitamin B3 and vitamin B6 as precursors to produce vitamin B1 (Helliwell et al., 2013). THI4 and THI5 are two single turnover enzymes in yeast producing the pyrimidine and thiazole moieties, respectively (Chatterjee et al., 2011; Lai et al., 2012). The vitamin B3 content in plants is relatively higher than the other B vitamins (Fitzpatrick et al., 2012). For example, the vitamin B3 content is 10-fold higher than vitamin B6 in both cassava storage roots and rice polished grains, suggesting vitamin B3 is relatively abundant in cassava and rice edible organs. We have developed transgenic cassava and rice accumulating high levels of vitamin B6. It may therefore be possible to genetically engineer the vitamin B1 biosynthesis pathway (*THI4* and *THI5* genes) from yeast into transgenic plants accumulating increased levels of vitamin B6. Transgenic approaches consisting of expression of *THI4*, *THI5*, *AtPDX1.1* and *AtPDX2* can be a potential strategy to increase vitamin B1 and vitamin B6 together in plants.

Furthermore, the genetic variation of vitamin B1 content among natural varieties may assist in investigating vitamin biosynthesis in plants. Natural variation of vitamin B1 content was observed in several crops. Vitamin B1 contents within the ranges (1.17-17.4 µg/g) were reported in rice grains of 79 varieties (Kennedy and Burlingame, 2003). A 2.5-fold difference of vitamin B1 contents was observed in 24 winter wheat varieties (Shewry et al., 2011). In a screening of fifty-four wild potato clones, the vitamin B1 contents were within a wide range (2.9-16.4 µg/g DW) (Goyer and Sweek, 2011). Further characterization of expression levels of genes associated with vitamin B1 biosynthesis amongst these contrasting varieties can help to understand the limiting factors of this vitamin production. This knowledge could be useful to increase vitamin B1 content in staple crops for both conventional breeding and genetic engineering.

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Exogenous application of vitamin B1 has been demonstrated to improve biotic and abiotic stress tolerance by triggering the endogenous defense systems in several important crops including rice, wheat, and barley (Al-Hakimi and Hamada, 2001; Bahuguna et al., 2012; Hamada and Jonsson, 2013). The mechanism needs to be further explored. Transgenic plants accumulating high levels of vitamin B1 could be potentially useful to improve stress resistance. This needs to be further examined in transgenic plants accumulating increased levels of vitamin B1. Biofortification of vitamin B1 in staple crops can be a useful strategy to alleviate malnutrition together with improving food production.
Appendix

In this biofortification of vitamin B6 project, transgenic cassava expressing *AtPDX1.1* or *AtPDX2* alone using constitutive and root-specific expression strategies were also generated. All transformation vectors are shown in Figure A1. In this thesis, we only continued to analyze transgenic cassava harboring 3SS-*PDX1*-3SS-*PDX2* and PAT-*PDX1*-PAT-*PDX2* vectors. Initially, screening of total vitamin B6 content in transgenic cassava was conducted in *in vitro* plantlets on CBM medium with vitamins (2.43 µM pyridoxine). We did not observe increased levels of vitamin B6 in all transgenic cassava leaves and fibrous roots compared to wild-type plants (Figure A2). One possible explanation is that cassava can acquire vitamin B6 from the mediums so wild-type cassava also accumulated high levels of vitamin B6. We therefore performed all experiments on soil-grown cassava plants under greenhouse and field conditions. The total vitamin B6 quantification results of greenhouse- and field-grown cassava using yeast microbiological assays are shown in Figure A3 and Figure A4. An HPLC analysis was also conducted in greenhouse-grown cassava (Table A1). A consistent increase of vitamin B6 in transgenic cassava was observed under both greenhouse and field conditions using the two quantification methods.

Because antioxidant accumulation in cassava storage roots as well as ectopic application of ascorbate have previously been reported to correlate with reduced PPD, we anticipated that vitamin B6 could delay the occurrence of PPD due to its antioxidant properties (Morante et al., 2010; Reilly et al., 2004; Sánchez et al., 2006). Using our established PPD quantification assay, we tested the impact of vitamin application on the onset of PPD in wild-type cassava storage roots (Owiti et al., 2011). An image-based PPD score analysis revealed that application of pyridoxine (100 mM) or thiamin (100 mM) significantly delayed the development of PPD symptoms at 24h after PPD initiation (Figure A5). Our evaluation also indicated that ascorbate requires higher concentration (500 mM) to delay the onset of PPD in our quantification assay (Figure A6). We subsequently performed PPD assays with the storage roots of selected greenhouse-grown transgenic cassava lines. We did not observe significant differences of PPD symptoms between transgenic and wild-type cassava at 24h and 48h after PPD initiation (Figure A7). However, PPD is a highly variable symptom especially in greenhouse-grown storage roots. We cannot derive a solid conclusion in this experiment with three biological replicates. A proper evaluation should be conducted in field-grown material with large sample size.

Transgenic rice expressing *AtPDX1.1* or *AtPDX2* alone using constitutive and endosperm-specific expression strategies were also generated. All transformation vectors are shown in Figure A8. Another independent experiment was conducted and included transgenic 3SS-*PDX1*-3SS-*PDX2*, Glo-*PDX1*-Glo-*PDX2*, Glu-*PDX1*-Glu-*PDX2*, *p1300*-3SS-*PDX1* and *p1300*-3SS-
Transgenic rice expressing AtPDX1.1 alone showed increased levels of vitamin B6 in leaves, but transgenic rice expressing AtPDX2 did not (Figure A9a). This reveals that AtPDX1.1 is the limiting factor for vitamin B6 biosynthesis. The best performing transgenic line 35S-12 had higher levels of vitamin B6 in leaves, suggesting the combination of AtPDX1.1 and AtPDX2 is the best strategy for vitamin B6 biofortification. We observed similar results of transgenic 35S-PDX1-35S-PDX2 and Glo-PDX1-Glo-PDX2 lines in the rice seeds (Figure A9b,c). However, transgenic Glu-PDX1-Glu-PDX2 lines, which employ the endosperm-specific expression strategy showed lower vitamin B6 content in unpolished seeds compared to transgenic Glo-PDX1-Glo-PDX2 lines. Further characterization of expression levels of transgenes could assist in investigating the differences between transgenic plants of two endosperm-specific expression vectors. Agronomic traits were also evaluated in this experiment. Transgenic rice displayed decreased grain weight compared to wild-type (Figure A10d). Because transgenic line pCAMBIA1301 also showed decreased grain weight, the impact of expression of AtPDX1.1 and AtPDX2 on agronomic traits needs further investigation.

A screening of total vitamin B1 content in transgenic cassava expressing AtTHIC using constitutive and root-specific expression strategies was conducted in *in vitro* plantlets on CBM medium with vitamins (0.3 µM thiamin). One out of the three transgenic 35S-AtTHIC lines in leaves and all three transgenic 35S-AtTHIC lines in fibrous roots showed increased levels of total vitamin B1 content (Figure A11). Transgenic Pat-AtTHIC lines did not show differences in vitamin B1 content in leaves and fibrous roots compared to wild-type plants.

The feedback regulation of in the 3′-UTR of the THIC gene by TPP was demonstrated in Arabidopsis (Bocobza et al., 2007; Wachter et al., 2007). Three types of transcript of AtTHIC were characterized. According to the classification (Wachter et al., 2007), the type I transcript is the pre-mature RNA of the THIC gene. The type II transcript splice out the TPP aptamer (riboswitch motif) with a shorter 3′-UTR of the THIC gene and the type III transcript containing the TPP aptamer with a long the 3′-UTR after differentially processing (Wachter et al., 2007). The levels of type II transcript would decrease and the levels of type III transcript would increase in Arabidopsis under high thiamin conditions. The levels of type I transcript are stable in different concentrations of thiamin. A similar regulation mechanism of the THIC gene was anticipated in rice (Wachter et al., 2007). The levels of type-II transcript of the THIC gene decrease and the levels of type-III transcript increase under high TPP concentrations in the cell. To test this hypothesis in rice, we designed primers to quantify the transcript levels of the three types 3′-UTR of OsTHIC according to the predicated structure of OsTHIC gene. RT-qPCR was conducted in transgenic 35S-OsTHI1-35S-OsTHIC lines and wild-type rice leaves under greenhouse conditions. A high variation was observed amongst the three types of transcripts (Figure A12). The type I and II transcripts did not show significant differences between transgenic and wild-type plants (Figure A12a,b). Transgenic lines 35S-OsTHI1-35S-OsTHIC-2 and 35S-OsTHI1-35S-OsTHIC-29 showed decreased levels of type III transcripts.
(Figure A12c). Compared to the quantification results of total vitamin B1 in the same samples (Figure 24a, Chapter 4), we did not observe any correlation between the expression levels of transcripts and the total vitamin B1 content due to the high variations. However, the comprehensive regulation of THIC in rice has not yet been reported, there may be other types of THIC transcripts in rice. It needs further investigation to elucidate the mechanism.

Figure A1. Schematic diagram of transformation vector T-DNA regions generated for vitamin B6 biofortification in cassava. (a) pCAMBIA1300.1-PDX1.1 (p1300-35S-PDX1) (b) p1300-35S-PDX2 (c) 35S-PDX1-35S-PDX2 (d) p1300-Pat-PDX1 (e) p1300-Pat-PDX2 (f) PAT-PDX1-PAT-PDX2. 35S, CaMV 35S promoter; Pat, patatin promoter; AtPDX1.1, At3g18780; AtPDX2, At5g60540; HPT, hygromycin phosphotransferase; LB, left border of T-DNA; RB, right border of T-DNA; T1, CaMV 35S terminator; T2, Octopine synthase terminator.
Figure A2. Analysis of the total vitamin B6 content in \textit{in vitro} transgenic cassava on CBM medium (with vitamins). (a) Leaves, (b) Fibrous roots. 35S-2, 35S-4, and 35S-7 indicate three independent lines of 35S-PDX1-35S-PDX2; Pat-2, Pat-7, and Pat-13 indicate 3 independent lines of PAT-PDX1-PAT-PDX2; wt, wild-type plants. Total vitamin B6 content was quantified by microbiological assay and values are averages ± SD of three biological replicates. wt (white), 35S-PDX1-35S-PDX2 (gray), PAT-PDX1-PAT-PDX2 (black).

Figure A3. Analysis of the total vitamin B6 content in transgenic cassava under greenhouse conditions. (a) Leaves (b) Storage roots. 35S-1, 2, 3, 5, and 7 indicate five independent lines of 35S-PDX1-35S-PDX2. Pat-1, 2, 3, and 12 indicate four independent lines of PAT-PDX1-PAT-PDX2. wt, wild-type plants. Total vitamin B6 content was quantified by a microbiological assay and values are averages ± SD of three biological replicates. wt (white), 35S-PDX1-35S-PDX2 (gray), PAT-PDX1-PAT-PDX2 (black).
Figure A4. Analysis of the total vitamin B6 content in transgenic cassava under field conditions. (a) Leaves (b) Storage roots. 35S-3, 4, and 5 indicate three independent lines of 35S-PDX1-35S-PDX2. Pat-2, 7, and 12 indicate three independent lines of PAT-PDX1-PAT-PDX2. wt, wild-type plants. Total vitamin B6 content was quantified by a microbiological assay and values are averages ± SD of three biological replicates. wt (white), 35S-PDX1-35S-PDX2 (gray), PAT-PDX1-PAT-PDX2 (black).

Figure A5. Impact of exogenous application of vitamins (100 mM) on PPD in wild-type cassava storage roots. (a) PPD score of entire root slices at 24 h after PPD initiation. The PPD scores were generated by gray-value distribution using MATLAB processing. Values are averages ± SD of three biological replicates and asterisks indicate significant differences between samples treated with vitamins and H2O control as determined by t-tests (p<0.05). (b) Storage root slices treated with pyridoxine (time point: 24 h after PPD initiation). (c) Storage root slices treated with water as control (time point: 24 h after PPD initiation). The scale bar represents 1 cm.
Figure A6. Impact of exogenous application of ascorbate (500 mM) on PPD in wild-type cassava storage roots. The PPD scores of entire root slices at 50 h after PPD initiation were generated by gray-value distribution using MATLAB processing. Values are averages ± SD of three biological replicates and asterisks indicate significant differences between samples treated with vitamins and H$_2$O control as determined by t-tests (p<0.05).

Figure A7. Analysis of the onset of PPD in greenhouse-grown transgenic cassava roots. The PPD scores were entire root slices at 24 h and 48 h after PPD initiation generated by gray-value distribution using MATLAB processing. 35S-1, 2, and 7 indicate three independent lines of 35S-PDX1-35S-PDX2. Pat-2, and 12 indicate two independent lines of PAT-PDX1-PAT-PDX2. wt, wild-type plants. Values are averages ± SD of three biological replicates.
Figure A8. Schematic diagram of transformation vectors T-DNA regions generated for vitamin B6 biofortification in rice. (a) pCAMBIA1300.1-PDX1.1 (p1300-35S-PDX1) (b) p1300-35S-PDX2 (c) 35S-PDX1-35S-PDX2 (d) p1300-Glo-PDX1 (e) p1300-Glo-PDX2 (f) Glo-PDX1-Glo-PDX2 (g) p1300-Glu-PDX1 (h) p1300-Glu-PDX2 (i) Glu-PDX1-Glu-PDX2. 35S, CaMV 35S promoter; Glo, globulin promoter; Glu, glutelin promoter; AtPDX1.1, At2g38230; AtPDX2, At5g60540; HPT, hygromycin phosphotransferase; LB, left border of T-DNA; RB, right border of T-DNA; T1, CaMV 35S terminator; T2, Octopine synthase terminator.
Figure A9. Analysis of the total vitamin B6 content in transgenic rice expressing *AtPDX1.1* and/or *AtPDX2* under greenhouse conditions. (a) Leaves at the reproductive stage (b) Unpolished seeds (c) Polished seeds. Total vitamin B6 content was quantified by a microbiological assay and values are averages ± SD of three biological replicates except for 35S-P1-24 which is represented by two biological replicates. 35S-7 and 12 indicate two independent lines of 35S-*PDX1-35S-PDX2* (gray); Glo-13, 41, and 47 indicate three independent lines of *Glo-PDX1-Glo-PDX2* (black); Glu-35, 36, 62 and 63 indicate four independent lines of *Glu-PDX1-Glu-PDX2* (green); 35S-P1-24, 26 and 65 indicate three independent lines of *p1300-35S-PDX1* (blue); 35S-P2-53 indicates one transgenic line of *p1300-35S-PDX2* (orange); pCAMBIA1300 indicates transgenic *pCAMBIA1300* line (white); pCAMBIA1301 indicates transgenic *pCAMBIA1301* line (white); wt, wild-type plants (white).

*The vitamin B6 quantification of rice seeds was performed by Ms. Nathalie Mangel in the Plant Biotechnology group at ETH Zürich.*
Figure A10. Agronomic traits evaluation of transgenic rice expressing \textit{AtPDX1.1} and/or \textit{AtPDX2} under greenhouse conditions. (a) Height (b) Number of panicles (c) Biomass of leaves (d) Grain weight. 35S-7 and 12 indicate two independent lines of 35S-\textit{PDX1}-35S-\textit{PDX2} (gray); Glo-13, 41 and 47 indicate three independent lines of Glo-\textit{PDX1}-Glo-\textit{PDX2} (black); Glu-35, 36, 62 and 63 indicate four independent lines of Glu-\textit{PDX1}-Glu-\textit{PDX2} (green); 35S-P1-24, 26 and 65 indicate three independent lines of \textit{p1300-35S-PDX1} (blue); 35S-P2-53 indicates one transgenic line of \textit{p1300-35S-PDX2} (orange); pCAMBIA1300 indicates transgenic pCAMBIA1300 line (white); pCAMBIA1301 indicates transgenic pCAMBIA1301 line (white); wt, wild-type plants (white). Values are averages ± SD of three biological replicates except for 35S-P1-24 which is with two biological replicates.

*The agronomic traits evaluation was performed by Ms. Nathalie Mangel in the Plant Biotechnology group at ETH Zürich.
Figure A11. Analysis of the total vitamin B1 content in in vitro transgenic cassava on CBM medium (with vitamins). (a) Leaves (b) Fibrous roots. 35S-AtTHIC-1, 7 and 15 indicate three independent lines of 35S-AtTHIC; Pat-AtTHIC-4, 5 and 13 indicate three independent lines of Pat-AtTHIC; wt, wild-type plants. Total vitamin B1 content was quantified by a microbiological assay and values are averages ± SD of three biological replicates. Asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests ($p<0.05$).

Figure A12. RT-qPCR analysis of OsTHIC transcript types in transgenic rice leaves under greenhouse conditions. (a) Type I (b) Type II (c) Type III. RT-qPCR was conducted on 3'-UTR of OsTHIC transcripts from rice leaves under greenhouse conditions. Three types of 3’-UTR of OsTHIC are classified according to published report in Arabidopsis. 35S-OsTHI1-35S-OsTHIC-2, 13, 29 and 35 indicate four independent lines of 35S-OsTHI1-35S-OsTHIC (black); pCAMBIA1300 indicates the transgenic pCAMBIA1300 line (gray); wt, wild-type plants (white). Values are averages ± SD of three biological replicates and asterisks indicate significant differences between transgenic lines and wild-type as determined by t-tests ($p<0.05$).
Table A1 B6 vitamer and glycosylated derivative composition in transgenic and wild-type cassava leaves (a) and storage roots (b) under greenhouse conditions.

The data was measured by HPLC analysis and values are averages ± SE of three biological replicates. n.m., not measured. Numbers in bold indicate significant differences between transgenic lines and wild-type as determined by t-tests (p < 0.05). Blue color indicates increased levels and red color indicates decreased levels of B6 vitamers and glycosylated derivatives.

*The HPLC analysis was performed by Dr. Michäel Moulin in the Plant Biochemistry and Physiology group at University of Geneva.

### a. Greenhouse-grown cassava leaves (µg/g FW)

<table>
<thead>
<tr>
<th>Leaves</th>
<th>PM</th>
<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.50 ± 0.12</td>
<td>0.45 ± 0.08</td>
<td>0.07 ± 0.00</td>
<td>0.52 ± 0.10</td>
<td>n.m.</td>
<td>0.02 ± 0.00</td>
<td>n.m.</td>
<td>3.80 ± 1.11</td>
<td>n.m.</td>
</tr>
<tr>
<td>35S-1</td>
<td>1.83 ± 0.03</td>
<td>9.61 ± 1.95</td>
<td>0.12 ± 0.01</td>
<td>0.62 ± 0.04</td>
<td>n.m.</td>
<td>0.01 ± 0.00</td>
<td>n.m.</td>
<td>52.60 ± 4.73</td>
<td>n.m.</td>
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<tr>
<td>35S-2</td>
<td>1.29 ± 0.09</td>
<td>4.85 ± 0.37</td>
<td>0.11 ± 0.01</td>
<td>0.46 ± 0.02</td>
<td>n.m.</td>
<td>0.01 ± 0.00</td>
<td>n.m.</td>
<td>40.32 ± 1.79</td>
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<tr>
<td>35S-5</td>
<td>1.75 ± 0.27</td>
<td>9.21 ± 0.70</td>
<td>0.08 ± 0.01</td>
<td>0.49 ± 0.03</td>
<td>n.m.</td>
<td>0.01 ± 0.00</td>
<td>n.m.</td>
<td>55.08 ± 7.99</td>
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<td>35S-7</td>
<td>0.51 ± 0.09</td>
<td>0.51 ± 0.08</td>
<td>0.06 ± 0.01</td>
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<tr>
<td>Pat-2</td>
<td>1.23 ± 0.08</td>
<td>1.94 ± 0.31</td>
<td>0.03 ± 0.00</td>
<td>0.72 ± 0.05</td>
<td>n.m.</td>
<td>0.01 ± 0.00</td>
<td>n.m.</td>
<td>24.99 ± 2.16</td>
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<tr>
<td>Pat-12</td>
<td>1.26 ± 0.04</td>
<td>2.24 ± 0.17</td>
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<td>0.72 ± 0.10</td>
<td>n.m.</td>
<td>0.01 ± 0.00</td>
<td>n.m.</td>
<td>23.29 ± 2.00</td>
<td>n.m.</td>
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### b. Greenhouse-grown cassava storage roots (µg/g FW)

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<tr>
<th>Roots</th>
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<th>PN</th>
<th>PL</th>
<th>PMP</th>
<th>PNP</th>
<th>PLP</th>
<th>PMG</th>
<th>PNG</th>
<th>PLG</th>
<th>Total vitamin B6</th>
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</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.24 ± 0.02</td>
<td>0.12 ± 0.00</td>
<td>0.58 ± 0.04</td>
<td>0.34 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>0.59 ± 0.05</td>
<td>1.16 ± 0.03</td>
<td>1.09 ± 0.05</td>
<td>4.25 ± 0.18</td>
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<td>35S-1</td>
<td>2.71 ± 0.47</td>
<td>1.12 ± 0.12</td>
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<td>5.61 ± 1.06</td>
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<td>1.15 ± 0.17</td>
<td>42.72 ± 10.42</td>
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<td>0.49 ± 0.04</td>
<td>0.29 ± 0.01</td>
<td>0.02 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>1.42 ± 0.11</td>
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<td>1.05 ± 0.03</td>
<td>9.13 ± 0.37</td>
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<td>0.43 ± 0.03</td>
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<td>0.03 ± 0.01</td>
<td>0.03 ± 0.00</td>
<td>2.18 ± 0.06</td>
<td>8.10 ± 0.43</td>
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<td>0.03 ± 0.00</td>
<td>0.04 ± 0.00</td>
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<td>0.06 ± 0.00</td>
<td>3.02 ± 0.16</td>
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Reference


Morante N, Sánchez T, Ceballos H, Calle F, Pérez JC, Egesi C, Cuanbe CE, Escobar AF, Ortiz D, Chávez AL and Fregene M (2010) Tolerance to Postharvest Physiological Deterioration in Cassava Roots All rights reserved. No part of this periodical may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without permission in writing from the publisher. Permission for printing and for reprinting the material contained herein has been obtained by the publisher. *Crop Science* **50**:1333-1338.


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Publications
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