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BIOFORTIFICATION: HOW CAN WE EXPLOIT PLANT SCIENCE TO REDUCE MICRONUTRIENT DEFICIENCIES?

Topic Editors
Laura De Gara, Michael A. Grusak and Irene Murgia

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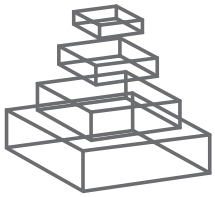

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BIOFORTIFICATION: HOW CAN WE EXPLOIT PLANT SCIENCE TO REDUCE MICRONUTRIENT DEFICIENCIES?

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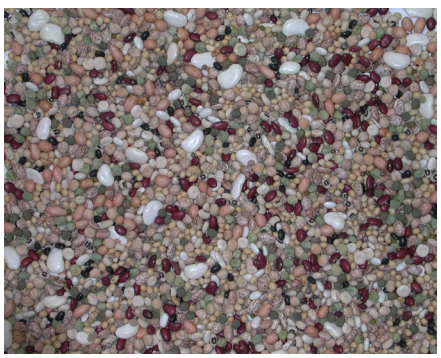

Legumes are an example of important food crops that provide nutritional value in diets of people throughout the world. They are one of many food crop groups that have been targeted for nutritional improvement through biofortification. Included in the image are seeds of Glycine max (soybean), Phaseolus vulgaris (black bean, red bean, pinto bean, great northern bean), P. lunatus (lima bean), Vigna radiata (mung bean), V. unguiculata (cowpea), Pisum sativum (pea), Cicer arietinum (chickpea), Lens culinaris (lentil), Lupinus albus (lupin), Medicago sativa (alfalfa), and M. truncatula (barrel medic). Photograph contributed by Michael A. Grusak (USDA-ARS Children's Nutrition Research Center, Houston, TX, USA).

In many areas of the world, inadequate amounts of micronutrients in the human diet have more devastating consequences than low energy intake. As examples, deficiencies in zinc, iodine, iron and vitamin A account for 11% of deaths of all pre-school aged children; also, sub-optimal intakes of vitamin C and folate present significant health threats in several developing countries, as well as in developed ones.

Micronutrients are involved in all aspects of growth and physiology of the human body, even as early as during embryonic development. Micronutrient deficiencies can cause birth defects, permanent physical and mental impairment and increased risk of death by infectious diseases.

As plant scientists, we are now facing ever more challenging and urgent goals with respect to the quality of our food supply. As important players in the global effort to improve crops, we should ask ourselves: how do we feed the world's increasing population and how do we feed it better? In other words, we need to increase food crop productivity in a sustainable manner, and such food should be of high nutritional value.

To reach the goal of nutritional adequacy, it is not sufficient to merely increase the concentration of a given micronutrient in the edible part of a plant of interest, but also to increase concentrations to appropriate, knowledge-based levels (i.e. nutritionally adequate, yet non-toxic) and to ensure that the micronutrient is effectively absorbed (i.e. bioavailable). These types of decisions and assessments, with the long-term goal of improving quality of life, will require a stronger interaction between plant scientists, human nutritionists, social scientists, and epidemiologists. Communication among these disciplines is critical to understand which nutritional/health targets should be addressed, what are the possibilities from a food-based approach, and how can all sides come together to deliver new improved varieties to farmers and consumers.

Moreover, the increasing evidence of the relevance of dietary patterns on the fitness of the human gut and its microbiome opens new promising lines of research that could contribute to the micronutrient issue. For instance, changes in the intake of dietary prebiotics could enhance and/or alter the diversity of gut microbial populations and hence contribute to the absorption of micronutrients.

We believe that interest in the topic of biofortification is expanding, and it is indeed gaining attention from several fields of science and from various governmental and policy-based organizations. Thus, we believe a continued dialogue and sharing of ideas amongst plant scientists is critical to see sustained growth and success in the area of micronutrient biofortification. Our intent, therefore, is to bring together the most recent experimental advances in the field, along with comprehensive reviews of the different approaches, new ideas, and thought-provoking opinions.

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EDITORIAL
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Biofortification: how can we exploit plant science and biotechnology to reduce micronutrient deficiencies?

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Throughout the developing world, the long-term consequences of insufficient amounts of essential micronutrients in the human diet can be more devastating than low energy intake. Micronutrients are involved in all aspects of development, growth, and physiology of the human body (including from early embryonic stage), and their deficiencies can cause birth defects, permanent physical and mental impairment, as well as an increased risk of death by infectious and chronic diseases. As plant scientists, we are now facing challenging and urgent goals: how to feed the world's increasing population and how to feed it better. In other words, we need to produce more plant food in sustainable ways and such food should be of the highest nutritional value.

This e-Book aims to provide the most recent advances on plant biofortification for micronutrients as well as a comprehensive overview of the different approaches that can be pursued for producing micronutrient-rich staple plants. We list here some of the major points arising from these papers.

VITAMINS

Vitamins are organic compounds that are required in limited amounts for normal human growth and activity and that cannot be synthesized by the human body. Various genetic engineering approaches for increasing the concentrations of vitamin B_1, B_6, C, or D in edible parts of the plant are discussed, together with the possible effects of such increases on plant tolerance to stress.

Acute deficiency in Vitamin B_1 (thiamin) can result in fatal neurological and cardiovascular disorders. Pourcel et al. (2013) highlight the fact that although the main source of vitamin B_1 in the diet is plants, there is still a need for enhancing thiamin levels in crop plants because a human diet based mainly on the edible portions of the staple crops, rice, wheat, or maize, is poor in vitamin B_1. In addition, the refining process for producing white flour and polished rice further reduces their final vitamin B_1 content. The thiamin biosynthetic pathway is quite complex in plants, utilizing enzymes regulated in a highly complicated fashion, and involving RNA sequences called riboswitches. The most updated pathway scheme achieved in the model plant *Arabidopsis thaliana* is reported in Pourcel et al. (2013), where the various biofortification approaches are discussed in light of the complexity of the pathway itself.

Vitamin B_6 is involved in different biochemical pathways and in diverse physiological roles in humans that are related to hormones, the immune system, and vascular functions. Adequate vitamin B_6 intake is beneficial for humans since it reduces the incidence of various health threats, including several multifactorial neurological disorders (Vanderschuren et al., 2013). Differently from vitamin B_1, *de novo* plant vitamin B_6 biosynthesis involves only two enzymes, PDX1 and PDX2 (pyridoxin synthesis gene 1 and 2). Vanderschuren et al. (2013) comment on the first successful results in engineering vitamin B_6 biofortification by overexpression of both enzymes in Arabidopsis. These authors suggest that next steps will be the full understanding of the regulation of vitamin B_6 biosynthesis, the selection of target food crops, and use of biofortification strategies (including the one applied in Arabidopsis) to such food crops.

Vitamin D maintains and regulates calcium levels in the body. Vitamin D_3 biosynthesis requires UV-B rays for its photochemical conversion from pro-vitamin D_3, a process occurring in the human body; therefore, depending on latitude or season, people may be at risk for vitamin D deficiency if sufficient vitamin D is not consumed through supplementation or through a vitamin D-rich diet. Fish are good sources of vitamin D, whereas only small amounts of vitamin D can be found in plants (Jäpelt and Jakobsen, 2013). Nonetheless, Jäpelt and Jakobsen (2013) argue that increased knowledge of vitamin D and of its metabolites in plants, as well as improvements in analytical methods for detection of vitamin D and of its derivatives, can offer new tools for increasing vitamin D content in edible parts of plants. Furthermore, a hypothetical biosynthetic pathway for vitamin D in plants is presented by these authors.

Plant foods are the main dietary source of vitamin C (ascorbic acid; ASC) for humans, for whom severe deficiency causes scurvy and can lead to death. Sub-optimal vitamin C intake, by leading to increased susceptibility to infections and diseases, can represent a health threat for both developing and developed world populations. In the review by Locato et al. (2013), possible plant biofortification strategies for this vitamin are examined, while acknowledging that ASC levels in plants should be considered a complex quantitative trait. Interestingly, ASC levels are tightly linked not only to plant tolerance to stress, but also to sugar biosynthesis and to the regulation of the fluxes of ASC precursors among metabolic pathways (Locato et al., 2013). Transgenic tomato plants overepressing a bacterial pyrophosphatase show increased expression of some genes coding for enzymes involved in the major ASC biosynthetic route in plants (Smirnoff-Wheeler pathway) and such transgenic plants show increased ASC content

in fruits, as well as an increase of major sugars (sucrose and glucose) and a decrease in starch content (Osorio et al., 2013).

METAL MICRONUTRIENTS

Iron (Fe) and zinc (Zn) are two essential metal micronutrients for human health; their deficiencies in the human diet contribute to high rates of mortality in developing countries. Manganese (Mn) deficiency, though less prevalent than Fe and Zn deficiency, can also lead to serious health problems, including birth defects (Bashir et al., 2013). A review of progress in biofortification of rice with such micronutrients is presented in Bashir et al. (2013), where a list of possible bottlenecks for biofortification are also presented, including the avoidance of accumulation of the toxic metal cadmium. The need for better knowledge of seed physiology and morphology is also discussed, in order to ensure successful increases in micronutrient concentrations in the edible parts of plant seeds, especially after the polishing and refining processes are completed. Along these lines, Blair et al. (2013) studied seed coats of common bean seeds to characterize variations in Fe and Zn concentrations in individual lines of a recombinant inbred population. They use these results to identify some of the underlying genetic loci responsible for seed coat accumulation of these metals, which should be useful for future biofortification efforts with bean.

Success in increasing Fe and Zn concentrations in polished seeds of a major rice variety in Myanmar, through a transgenic approach targeting metal transport and accumulation, is presented by Aung et al. (2013). In another study, increases in Fe concentration in a *Japonica* rice variety were also achieved by stacking a set of Fe homeostasis genes (Masuda et al., 2013). Because several iron-related processes were altered (from uptake to chelation and storage), the authors were careful to elevate seed Fe levels without inducing symptoms of Fe deficiency at the whole-plant level. Moreover, they used a marker-free vector that could serve to increase public acceptance of the transformed lines (Masuda et al., 2013). This so-called "push-pull" mechanism of augmenting Fe uptake at one end of the system, along with the storage sink for Fe at the other end of the system (by means of overexpressing Fe transport and storage genes), was investigated in detail in a different transgenic rice line and demonstrated not to interfere with general Fe homeostasis in such plants, relative to non-transgenic controls (Wang et al., 2013). Similar non-target effects of metal-related genes were studied in maize plants expressing a soybean ferritin gene (iron storage protein) in the endosperm (Kanobe et al., 2013). These authors demonstrate that the levels of some metal-related gene transcripts and proteins were indeed changed in the transgenic line, emphasizing the need to take a holistic approach when evaluating transgenic events.

Three different gene families have been described in detail and proposed as possible targets for future Zn or Fe biofortification strategies: the NAC transcription factors (Ricachenevsky et al., 2013a), the metal tolerance proteins (MTP's) (Ricachenevsky et al., 2013b) and the ZIP family of metal transporters (Astudillo et al., 2013). Notably, a candidate gene for increasing Zn concentration in common bean, *PvZIP12*, is proposed in Astudillo et al. (2013) and a model has been proposed for the role of the rice *OsNAC5* gene in senescence and metal re-mobilization (Ricachenevsky et al., 2013a).

IODINE

Iodine is a non-metal micronutrient that is essential for human health and whose deficiency impairs thyroid functions. When severe deficiency occurs, fetal development can be affected with consequent irreversible brain damage and mental retardation. A possible approach to produce plants biofortified with iodine is to administer exogenous iodine salts to the soil during plant growth. Kato et al. (2013) demonstrate that rice roots possess the ability to reduce IO_3^- to I^- and that such reduction is dependent, at least in part, on external iodine conditions. Such results suggest the existence of an iodate reductase in plant roots (Kato et al., 2013). Moreover, Kiferle et al. (2013) demonstrate that tomato fruits can be good targets for iodine fortification, by means of plant irrigation with KIO_3 during growth.

MOVING FORWARD

The papers presented in this e-Book demonstrate how knowledge in plant metabolism, physiology, and molecular biology can provide approaches for increasing the nutritional value of plant derived foods. The contributions also draw attention to the need for multidisciplinary efforts to cope with the challenges of food security and micronutrient malnutrition. We believe that the information presented in this e-Book will provide several novel ideas and will stimulate new directions amongst researchers in the field of plant biofortification. We also hope that these contributions can serve as a good source of background knowledge to educate and bring new scientists into the field. We are indebted to the many authors who have contributed to this e-Book and for their continued work in this important field of science. We feel privileged to have had the opportunity to oversee and edit this fine group of papers.

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The *Phaseolus vulgaris* ZIP gene family: identification, characterization, mapping, and gene expression

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Zinc is an essential mineral for humans and plants and is involved in many physiological and biochemical processes. In humans, Zn deficiency has been associated with retarded growth and reduction of immune response. In plants, Zn is an essential component of more than 300 enzymes including RNA polymerase, alkaline phosphatase, alcohol dehydrogenase, Cu/Zn superoxidase dismutase, and carbonic anhydrase. The accumulation of Zn in plants involves many genes and characterization of the role of these genes will be useful in biofortification. Here we report the identification and phlyogenetic and sequence characterization of the 23 members of the ZIP (ZRT, IRT like protein) family of metal transporters and three transcription factors of the bZIP family in *Phaseolus vulgaris* L. Expression patterns of seven of these genes were characterized in two bean genotypes (G19833 and DOR364) under two Zn treatments. Tissue analyzed included roots and leaves at vegetative and flowering stages, and pods at 20 days after flowering. Four of the genes, *PvZIP12*, *PvZIP13*, *PvZIP16*, and *Pv bZIP1*, showed differential expression based on tissue, Zn treatment, and/or genotype. *PvZIP12* and *PvZIP13* were both more highly expressed in G19833 than DOR364. *PvZIP12* was most highly expressed in vegetative leaves under the Zn (−) treatment. *PvZIP16* was highly expressed in leaf tissue, especially leaf tissue at flowering stage grown in the Zn (−) treatment. *Pv bZIP1* was most highly expressed in leaf and pod tissue. The 23 *Pv*ZIP genes and three bZIP genes were mapped on the DOR364 × G19833 linkage map. *PvZIP12*, *PvZIP13*, and *PvZIP18*, *Pv bZIP2*, and *Pv bZIP3* were located near QTLs for Zn accumulation in the seed. Based on the expression and mapping results, *PvZIP12* is a good candidate gene for increasing seed Zn concentration and increase understanding of the role of ZIP genes in metal uptake, distribution, and accumulation of zinc in *P. vulgaris*.

Keywords: biofortification, *Phaseolus vulgaris*, QTL mapping, gene expression, zinc transporter

INTRODUCTION

Dry beans (*Phaseolus vulgaris* L) are the most highly consumed whole food legume in the world. Beans are a food security crop for small farmers and urban poor in many African and Latin American countries (Siddiq and Uebersax, 2012). In contrast to many other staple crops, beans are rich in a variety of nutrients, including protein, fiber, folate, and minerals (Juliano, 1999). Beans are also a good source of dietary iron and zinc. According to the USDA Nutrient Database, a 100 g of cooked beans provides an average of 2 mg Fe and 1 mg Zn and the Estimated Average Requirement for Fe ranges from 3 to 23 mg per day and 2.5–10.9 mg per day per Zn depending on age and gender (Ahuja et al., 2012). Meeting the Fe and Zn dietary requirements is a challenge for many people. An estimated two billion people suffer from iron deficiency, which is a major cause of anemia (Rastogi and Mathers, 2002; Balarajan et al., 2011). Zinc deficiency is also widespread, with an estimated 48% of humans at risk, especially populations consuming vegetarian diets rich in unrefined cereals (Sandstead, 1991). In humans, Zn deficiency can be expressed through diverse symptoms including reduced immune function, fetal brain cell development and child's growth, reproductive, and cognitive development (Hambidge, 2000). Biofortification of staple foods, including dry beans, with Fe and Zn is one agricultural based approach being developed and applied to combat micronutrient malnutrition (Bouis et al., 2011). While average dry bean Fe and Zn levels are 55 mg kg^{-1} and 34 mg kg^{-1} respectively, three-fold genotypic variation in both Fe and Zn levels exist within the species (Blair et al., 2009 and Islam et al., 2002).

This existing variation makes breeding common beans a viable biofortification approach. Significant progress has been achieved in Fe biofortification of beans through conventional breeding as illustrated in the recent release of five high Fe bean varieties in Rwanda (Saltzman et al., 2013). Zinc biofortification has lagged behind that of Fe-biofortification perhaps because of lower quantities of Zn in the seeds but also perhaps less incentive because of the difficulty in assessing Zn nutritional status in humans. While there are biomarkers to asses Fe deficiency readily in humans,

no such biomarkers are yet available for Zn, although recently a potential biomarker (dematin) has been identified (Ryu et al., 2012).

In addition to relying solely on phenotypic selection to increase seed Fe and Zn levels, there has been an effort to understand the genetic control of seed Zn and Fe accumulation. Since 2009, at least five QTL studies have been published for seed micronutrient levels. In total, 38 QTLs were associated with zinc accumulation, explaining 15–40% of the variability. These studies have been in inter gene pool populations (Blair et al., 2009, 2010a,b), Andean populations (Cichy et al., 2009; Blair et al., 2011) and Mesoamerican populations (Blair et al., 2010a). QTL studies have yet to be applied to marker assisted selection. There has also been limited effort in identifying genes underlying QTL for Fe and Zn. Discovery of genes involved in increased seed Fe and Zn levels would be useful for biofortification efforts in beans and possibly also as targets for transgenic biofortification approach in other crops.

The Zrt and Irt-like protein (ZIP) family is well characterized for its role in Zn transport and to a lesser extent it role in Fe transport (Eide et al., 1996). The ZIP family is well conserved among bacteria, fungi, protozoa, animals, and plants (Grotz et al., 1998; Chen et al., 2008). ZIP proteins are predicted to have eight transmembrane domains with a histidine motif which may be part of an intramembranous heavy metal binding site that plays a role in the transport pathway for the minerals that are transferred (Eng et al., 1998). ZIP transporters have been implicated in Zn uptake, transport of Zn in leaves and translocation to seeds, embryo, endosperm, and seed coat (Waters and Sankaran, 2011). Previous information on the role of ZIP genes in Zn movement throughout the plant come from expression analysis, yeast complementation and Zn hyper accumulator mutants. In *A. thaliana* 15 members have been identified and characterized, revealing a wide variety of localization and function (Milner et al., 2012). *At*ZIPs have been detected mainly in the roots, shoots (Milner et al., 2012). In rice, 17 ZIP coding sequences (CDS) were identified. They have been evaluated in roots, shoots, and panicles of efficient and inefficient genotypes (Grotz et al., 1998; Guerinot, 2000; Connolly et al., 2002; Weber et al., 2004; Chen et al., 2008; Shanmugam et al., 2011; Milner et al., 2012). In *Medicago truncatula,* six genes were identified in roots and leaves which were upregulated under Zn deficiency and three of them restored yeast growth on Zn-limited media (Lopez-Millan et al., 2004). In *Glycine max*, *GmZIP1* has been detected in nodules and was highly selective for Zn in a functional complement in yeast (Moreau et al., 2001). In *Vitis vinifera*, *VvZIP3* was expressed in developing flowers and its expression was correlated with high Zn accumulation in this tissue (Gainza-Cortes et al., 2012 and Afoufa-Bastien et al., 2010). Analysis of this family in different species demonstrates the importance of these genes in Zn transport.

Another important gene family related with Zn transport is the bZIP family. This family has been well characterized in *Arabidopsis* with 75 members divided in 10 groups based on conserved motifs that reflect functional similarities (Jakoby et al., 2002). Group F includes bZIP19, bZIP23, and bZIP24. These transcription factors contain a DNA binding domain, a leucine zipper dimerization motif and histidine-rich motif which are essential for responding to low Zn supply in *Arabidopsis* (Assuncao et al., 2003; Assunção et al., 2010).

With the recent release of the *P. vulgaris* genome sequence (*Phaseolus vulgaris* v1.0, DOE-JGI and USDA-NIFA, http://www.phytozome.net/commonbean), it is possible to identify candidate genes for seed Fe and Zn levels. Characterization of genes related to Zn homeostasis in *P. vulgaris* will provide useful information on specific target genes in the biofortification breeding effort. This research has identified and characterized of 23 members of the *Pv*ZIP gene family. Three members of a second family of genes, bZIP transcription factors, were also characterized similarly. The relative expression of genes from both the ZIP and bZIP families was characterized in various tissues and stages of development in two *P. vulgaris* genotypes, DOR 364 and G19833 grown under two Zn treatments. Selected ZIP and bZIP genes were also located on a linkage map overlaid with QTL locations for Zn accumulation in seed.

MATERIALS AND METHODS

PLANT MATERIAL AND PHENOTYPIC DATA

Two bean genotypes were evaluated in this study, DOR364, a small seeded, high yielding improved cultivar from the Middle American genepool and G19833, a large seeded landrace from the Andean genepool known for its tolerance to low P soils (Beebe et al., 2006). These genotypes also exhibit contrasting seed mineral levels as shown in field trials in Darien, Colombia. DOR364 had 49 mg kg^{-1} Fe while G19833 had 75.5 mg kg^{-1}, and DOR364 had 21.7 mg kg^{-1} Zn while G19833 had 29.9 mg kg^{-1} (Blair et al., 2009). DOR364 and G19833 were specifically chosen for this study because valuable genetic information exists for the lines. A recombinant inbred line (RIL) between these parents was developed by single seed descent at the International Center for Tropical Agriculture (CIAT), Colombia. It consists of 87 individuals and has a linkage map of 499 single copy markers with a coverage of 2306 cM (Galeano et al., 2011). This population has been used by different research groups for map saturation and QTL identification associated to biotic and abiotic traits (Blair et al., 2009) and QTL positions for seed minerals (Blair et al., 2003, 2009; Beebe et al., 2006; Galeano et al., 2011).

IDENTIFICATION OF *Pv* ZIP AND *Pv* bZIP GENES AND PHYLOGENETIC ANALYSIS

ZIP genes in *P. vulgaris* were identified using the sequences of 18 *Arabidopsis thaliana* ZIP genes (http://www.arabidopsis.org/). The program tBlastn was used to compare the *Arabidopsis* ZIP genes against the bean genome (*Phaseolus vulgaris* v1.0, DOE-JGI and USDA-NIFA, http://www.phytozome.net/commonbean). These sequence data were produced by the US Department of Energy Joint Genome Institute. Conserved domains in each predicted transcript was verified using Pfam 26.0 protein database (http://pfam.sanger.ac.uk/) to confirm the reliability of the match with the ZIP family. The CDS for each gene was aligned with genomic DNA sequence to confirm splice signals in boundaries between introns and exons. The *P. vulgaris* ZIP genes were assigned unique names from *PvZIP1* to *PvZIP19* and *PvIRT1 to PvIRT4*. These names do not relate to naming of ZIP genes in others species. Since this gene family characterization

is based on an incomplete genome sequence, the existence of additional ZIP genes in the bean genome is a possibility.

Three *Pv* bZIP genes were identified in the *P. vulgaris* genome based on sequences of *bZIP19*, *bZIP23*, and *bZIP24* reported by Assunção et al. (2010) in *Arabidopsis*. Identification of the new bZIP genes was based on the homology with the Basic Leucine Zipper Domain (bZIP domain).

Sequence alignments, phylogenetic analysis, tree estimation using bootstrapping and graphs of each gene were performed using ClustalW (Larkin et al., 2007) using the program Geneious® 6.0.3, created by Biomatters (build 2012-11-06 10:52).

In silico MAPPING OF *Pv* ZIP AND *Pv* bZIP GENES

Each of the 23 putative ZIP transport protein genes and 3 putative bZIP transcription factor genes were mapped *in silico* to a location on the DOR364 x G19833 linkage map based on sequence homology with the *P. vulgaris* genome. This alignment was conducted with an MS Excel based program MapSynteny (Fernandez et al., 2011).

GENETIC MAPPING OF SELECT MEMBERS OF THE PvZIP AND Pv bZIP FAMILY GENES

Five ZIP genes were also mapped genetically in the DOR364 × G19833 population. These five genes were chosen for genetic mapping based on their location near QTLs for seed Fe and Zn concentration on chromosomes 1, 3, 6, and 8 (Blair et al., 2009). The ZIP genes located *in silico* in these regions were mapped genetically in the full set of RILs of the DOR364 × G19833 population. These include *PvZIP2*, *PvZIP6*, *PvZIP8*, *PvZIP13*, and *PvIRT3*. Primers were designed to flank ZIP gene intron sequence (**Table 1**). PCR was conducted on DOR364 and G19833 as a first step to test for polymorphisms. The PCR mix contained 2.0 mM Mg, 0.2 μM dNTPs, and 0.3 μM of each primer. PCR reactions were carried out for 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55 or 60°C (based on the annealing temperature of each primer), and a final period of 5 min at 72°C. Products were visualized on agarose gels to verify amplification and identify insertion/deletions that had potential to serve as molecular markers. To increase the possibility of finding polymorphisms for monomorphic products, the SSCP technique (from single strand conformational polymorphism) was used, which is based on detection of conformational differences of single stranded DNA fragments due mobility shifts in non-denaturing polyacrylamide gel electrophoresis (Orita et al., 1989) such as MDE acrylamide gels (MDE Gel Solution 250ML Lonza NJ, USA) as described in Galeano et al. (2009). For genetic mapping, Mapdisto software version 1.7 Beta 132 (Lorieux, 2012) was used to locate the position of the ZIP genes on the DOR364 × G19833 genetic map reported by Galeano et al. (2011). The command *place locus* was used to located the ZIP genes, using as criteria the highest LOD value and lowest recombination rate. The position of each ZIP gene was confirmed using the *Ripple order* command.

QTL DATA AND ANALYSIS

Phenotypic data for seed Fe and Zn concentration from Popayan and Darien Colombia in 1998 and 2003 were reported for this population in Blair et al. (2009). Additionally, seed Fe and Zn concentration from the same locations in 2006 (not previously reported) were used for QTL analysis with the linkage map reported in Galeano et al. (2011). QTL cartographer v. 2.5 (Wang et al., 2012) was used to find QTLs following the same parameters described in Blair et al. (2009).

EXPRESSION ANALYSIS OF SELECT *Pv ZIP* AND *Pv bZIP*

Plant growing conditions

Seeds of DOR364 and G19833 were surface sterilized and planted in 500 ml clay pots with 3:1 Sunshine Brand premium grade vermiculite (Sunshine Brand, TX, USA) and horticultural grade perlite (Industries, Inc., MA, USA). Half strength Hoagland solution (3 mM KNO_3, 2 mM Ca $(NO_3)_2 \times 4H_2O$, sequestrene DTPA 10% Fe, 1.0 mM $MgSO_4 \times 7H_2O$, 23.1 mM H3BO3, 0.38 mM ZnSO4 × 7H2O, 0.16 mM CuSO4 × 5H2O, 4.6 mM MoO4 × 2H2O, 1M KH2PO4 (pH to 6.0) was applied to pots a rate of 400 ml three times per week. Two Hoagland solution treatments were employed: (1) Zn (+) where Zn was added as $ZnSO_4 \times 7H_2O$ and (2) Zn (−). A total of three pots per genotype were planted and each one was designated as a biological replicate. The experiment was arranged as a randomized complete block design. Plants were grown in a growth chamber (1.86 m^2) with a photoperiod of 16 h light and 8 h dark and an average of temperature of 29°C/20°C (day/night). For the vegetative samples, roots, and leaves were collected from the vegetative 3 stage (V3), when the third trifoliate leaf was unfolded at node 5. Leaf and

Table 1 | Primer list for gene expression analysis via RT-qPCR and genetic mapping.

Gene	Sequence		Approach
PvZIP12	GGGCAGAGGCAAGTGCAGGG	GGGCGTGATGGAGATGCAGGA	RT qPCR
PvZIP13	CGCGCTCTTCGATTGCCAGGT	CCACCGGCGTGTAGTGCGTA	RT qPCR
PvZIP13	GCGGTGGCTCGTTGAGTATT	TGCTATGAGGTCAACAAGAGCC	Mapping
PvZIP16	TGCACGGTTGATGGCGACGG	ACGGAACTCCTTCGCCATCGT	RT qPCR
PvIRT3	AGAATAACACCATCCCCAAAATTA	AGTCACTATGGGAATGTCACAGAA	RT qPCR
PvIRT3	AATGCACATCGTGGGGATGC	GGCTTTAAACTGCGCTTGGG	Mapping
bZIP1	ATGCAACCCACCTGGCCCTGATGCT	TGCCTGCCCTTGTAGTTTCCTCGCT	RT qPCR
bZIP2	ATCGGGAGAAGAAGAAGGCTCGCGC	TCCGGCCCCTTATGTCCACCAGCAA	RT qPCR
bZIP3	GCAGCAGTTCTTGAGCGTGGAGGCT	TGAAGGTGGTGTTGCCGAAACCTGCA	RT qPCR
PvactinII	TGCCATCCAGGCCGTTCTTTCA	GGGGACTGTGTGGCTGACACC	RT qPCR

root samples collected at flowering were harvested at the R2 stage when 30% of the flowers were opened. Pod samples were collected at 20 days after flowering. Plant tissue was collected in labeled sterilized tubes of 50 ml in liquid nitrogen and stored at −80°C.

RNA EXTRACTION AND REAL-TIME QUANTITATIVE PCR

About 2 g of tissue from each sample collected was ground in liquid N2. Total RNA from root and leaf tissue of two developmental stages was extracted by RNeasy Plant Mini Kit (Qiagen). Pods were extracted following a protocol optimized for high starch samples (Li and Trick, 2005). Total RNA was stored in aliquots at −80°C. The concentration of RNA was quantified through Quant-iT™ RiboGreen (Invitrogen). Two μg of RNA of each sample were treated with DNase I and purified by 0.1 vol of 3M sodium acetate (pH 5.2) and 3 vol of 100% ethanol. cDNA synthesis was carried out by High Capacity cDNA Reverse Transcription Kits (Applied Biosystem), using 1 μg of RNA. cDNA concentration was measured by Quant-iT™ PicoGreen (Invitrogen).

The relative expression levels of eighth ZIP genes; *PvZIP2, PvZIP7*, *PvZIP6*, *PvZIP12*, *PvZIP13*, *PvZIP16*, *PvZIP18, and PvIRT3* and three transcription factors belonging to the bZIP family, *bZIP1*, *bZIP2*, *bZIP3* were measured using RT-qPCR. Primers for RT-qPCR were designed for each gene in such a way that they spanned one or two exons in genes with intronic regions to detect genomic DNA contamination (**Table 1**). Quantification of all transcripts was performed using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In total three technical replicates of 50 ng of cDNA for each biological replicate of all tissues were used as template. Ten-fold serial dilutions were used to determine the efficiencies of each primer. RT-qPCR master-mix was prepared as follows: 1 μl of diluted cDNA, 5 μl of 2X SYBR Green Reaction Mix, 0.5 μl 3 pmol of each primer and nuclease-free water in a final volume of 10 μl. The StepOnePlus^{Ů} Real-Time PCR System (Applied Biosystems) was used for amplification and fluorescence measurement of each transcript at each temperature step and cycle during the reaction. Thermal cycling conditions consisted of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 45 s at 60°C. The identity and purity of the amplified product was checked through analysis of the melting curve carried out at the end of amplification. Relative gene expression was calculated using the comparative CT method (Livak and Schmittgen, 2001). bActin was used as a reference gene and root in vegetative stage Zn (−) treatment as a calibrator (Wen et al., 2005). Fold changes of greater than 2 was used as criteria to determine if genes were differentially expressed. Statistical analysis was performed using SAS V 9.3 (SAS Institute Inc., NC, USA). A repeated measurement analysis (Proc Mixed) was performed. Main effects were tested by ANOVA and a probability of $P < 0.05$ was chosen as the level of significance for the statistical test.

QUANTIFICATION OF Zn CONCENTRATIONS IN TISSUE

Plant tissue from two biological replicates of DOR364 and G19833 under two zinc treatments was quantified for Zn concentration. Tissue was freeze dried and ground to powder using a Geno Grinder 2000 (Spex CertiPrep, Metuchen, NJ) and zircon grinding balls. Two grams were sent to A&L Great Lakes Labs, Inc. Fort Wayne, IN, for mineral analysis by induced coupled plasma spectroscopy.

RESULTS

IDENTIFICATION OF ZIP FAMILY MEMBERS AND COMPARISON WITH HOMOLOGS IN OTHER SPECIES

Twenty three sequences, including 19 ZIP and four IRT genes were identified in the *P. vulgaris* genome sequence based on similarity to ZIP genes in *A. thaliana* and/or *Medicago truncatula*. All new genes have full-length CDS containing open reading frames (ORF) ranging from 153 to 655 amino acids in length. Sequences identified were confirmed in the PFAM database based on ZIP transmembrane domain and had E-values higher than −10. Peptide sequences of all new ZIP genes identified in common bean were aligned with 18 ZIP genes reported in the *A. thaliana*, and *M. truncatula* (**Table 2**). A phylogenetic neighbor joining tree shows the relationship among ZIP genes in *P. vulgaris*, *A. thaliana*, and *M. truncatula* (**Figure 1**). Alignments at the amino acid level predicted eight highly conserved transmembrane domains (**Figure 2**) and a potential metal binding motif containing histidine residues implicated in metal transport which are highly conserved throughout the entire family (Guerinot, 2000; Lopez-Millan et al., 2004). All ZIP genes contained a histidine motif between transmembrane domain III and IV except *PvZIP6*, *PvZIP7*, and *PvZIP18*. The ZIP gene family members in *P. vulgaris* shared 3–81.4% homology to each other. Of all ZIP genes found *PvIRT3* was the most closely related to *Arabidopsis*, sharing 59 and 57.3% similarity with genes AtIRT3_AT1G60960.1 and AtZIP4_AT1G10970, respectively. *PvZIP14* also showed high similarity with AtZIP6_AT3G30080.1 at 53.8%.

Gene structure analysis of ZIP genes in *P. vulgaris* revealed that the 23 genes have different intron-exon structures with a wide range of lengths. *PvZIP2*, *PvZIP6*, *PvZIP7*, *PvZIP15,* and *PvIRT1*, are composed of three exons and two introns. *PvZIP3*, *PvZIP5*, *PvZIP9*, *PvZIP10*, *PvZIP11*, *PvZIP13*, *PvZIP16, PvZIP19,* and *PvIRT2* each have four exons and three introns. *PvZIP17* and *PvIRT3* have five exons and four introns. Seven exons were identified in *PvZIP1* and *PvIRT4*. Many exons (10–14) were present in *PvZIP4*, *PvZIP8*, *PvZIP12*, *PvZIP14, and PvZIP18*.

Given the importance of some members of the bZIP gene family in the regulation of ZIP genes and in turn plant Zn homeostasis, their sequences were also characterized in the *P. vulgaris* genome. The common bean genes *bZIP1*, *bZIP2* and *bZIP3* were 261, 266 and 154 amino acids long, respectively. None of the bZIP genes contained introns. The three amino acids sequences encoding the bZIP genes shared 4.0–38.5% similarity among each other and 15–55.4% similarity with *bZIP19*, *bZIP23*, and *bZIP24* genes described in *A. thaliana* (Assunção et al., 2010).

MAPPING OF PvZIP GENES AND QTL FOR SEED Fe AND Zn CONCENTRATION

ZIP and bZIP were mapped *in silico* on the DOR364 × G19833 genetic map by aligning ZIP gene sequences and molecular

Table 2 | The Zrt and Irt -like protein (ZIP) family genes and bZIP genes identified in the *P. vulgaris* genome.

Sequence ID	Gene	Chrom	Pos ition	Homology to *A. thaliana*		*Homology to M. truncatula*	
Phvulv091010812m	PvZIP1	Chr01	3,442,406	ATZIP4_Zinc transporter 4	3E-63	ZIP-like zinc transporter -Medtr1g016120.1	4E-145
Phvulv091015745m	PvZIP2	Chr01	49,770,995	ATZIP4_Zinc transporter 4	0	Zinc transporter 5 -Medtr3g082050.1	4E-21
Phvulv091015614m	PvZIP3	Chr01	49,839,431	ZIP3_Zinc transporter 3	4E-37	Zinc transporter 5 -Medtr3g082050.4	2E-22
Phvulv091019402m	PvZIP4	Chr02	33,735,220	IAR1_ZIP metal ion transporter	9.5E-42	Zinc transporter 5	
Phvulv091012034m	PvZIP5	Chr05	5,645,010	ZIP1_Zinc transporter 1	3E-156	Zinc transporter - Medtr3g082050.3	2E-109
Phvulv091029608m	PvZIP6	Chr05	37,426,497	ZIP2_ZRT/IRT-like protein 2	2.1E-46	Iron regulated transporter -Medtr2g097580.1	5E-99
Phvulv091029689m	PvZIP7	Chr05	37,431,839	ZIP2_ZRT/IRT-like protein 2	2E-43	Iron regulated transporter -Medtr2g097580.1	2E-161
Phvulv091029664m	PvZIP8	Chr05	37,715,863	ZTP29_ZIP metal ion transporter	6.5E-23	Zinc transporter 5 -Medtr4g065640.1	2E-130
Phvulv091026664m	PvZIP9	Chr06	200,959	ZIP5_Zinc transporter 5	1E-132	Zinc transporter -Medtr3g082050.1	1E-150
Phvulv091009317m	PvZIP10	Chr06	1,033,953	ZIP5_Zinc transporter 5	4E-130	Zinc transporter -Medtr3g082050.1	4E-44
Phvulv091009315m	PvZIP11	Chr06	1,040,964	ZIP5_Zinc transporter 5	1E-129	Zinc transporter zupT -Medtr3g082050.1	7E-147
Phvulv091018095m	PvZIP12	Chr06	17,174,396	ZIP11_Zinc transporter 11	5.7E-52	Zinc transporter 5 -Medtr2g097580.1	3E-113
Phvulv091002113m	PvZIP13	Chr06	18,954,219	ATZIP6_ZIP metal ion transporter	1.5E-56	Zinc transporter 5 -Medtr5g071990.1	7E-141
Phvulv091007436m	PvZIP14	Chr08	7,634,926	ZIP metal ion transporter family	1.2E-14	Zinc transporter 5 -Medtr7g074060.1	0
Phvulv091022274m	PvZIP15	Chr08	57,181,509	ATZIP6_ZIP metal ion transporter	2E-158	Zinc transporter -Medtr5g071990.1	2E-139
Phvulv091004709m	PvZIP16	Chr08	59,351,699	ZIP1_Zinc transporter 1	3.2E-33	Zinc transporter 6-Medtr3g082050.3	7E-91
Phvulv091010505m	PvZIP17	Chr10	9,817,594	ZIP metal ion transporter family	0	ZIP transporter -Medtr7g074060.1	0
Phvulv091003125m	PvZIP18	Chr11	5,071,268	ZTP29_ZIP metal ion transporter	1.3E-20	Zinc transporter 6, -Medtr4g065640.1	3E-124
Phvulv091030363m	PvZIP19	Chr02	19,642,824	ZIP10_Zinc transporter 10	1E-102	Zinc transporter	3E-174
Phvulv091011372m	PvIRT1	Chr03	49,001,506	IRT1_Iron-regulated transporter 1	4E-126	Zinc transporter	3E-169
Phvulv091011626m	PvIRT2	Chr03	49,013,793	IRT1_Iron-regulated transporter 1	5E-112	Zinc/iron permease-Medtr8g105030.1	6E-129
Phvulv091000876m	PvIRT3	Chr09	12,670,278	ATIRT3_Iron regulated transporter 3	2.2E-68	ZIP transporter - Medtr3g 104400.1	2E-131
Phvulv091000875m	PvIRT4	Chr09	12,670,315	ATIRT3_Iron regulated transporter 3	9.5E-42	Zinc transporter 4—Medtr4g083570.1	3E-174
Phvulv091018638m	bZIP1	Chr05	3,213,447	bZIP23—transcription factor family	7E-49	Basic leucine zipper—Medtr4g073100.1	
Phvulv091015330m	bZIP2	Chr11	3,134,797	bZIP19—transcription factor family	1E-109	Basic leucine zipper—Medtr4g073100.1	5E-57
Phvulv091015414m	bZIP3	Chr11	3,709,270	bZIP44 |basic leucine-zipper 44	2E-52	bZIP transcription factor—Medtr4g070860.1	4E-71

Chromosome and position in base pairs indicate the location of each gene. Their respective homologs in A. thaliana and M. truncatula are shown. The program tBlastn was used to compare the A. thaliana ZIP genes against the bean genome. Homology was based on E–^{10}.

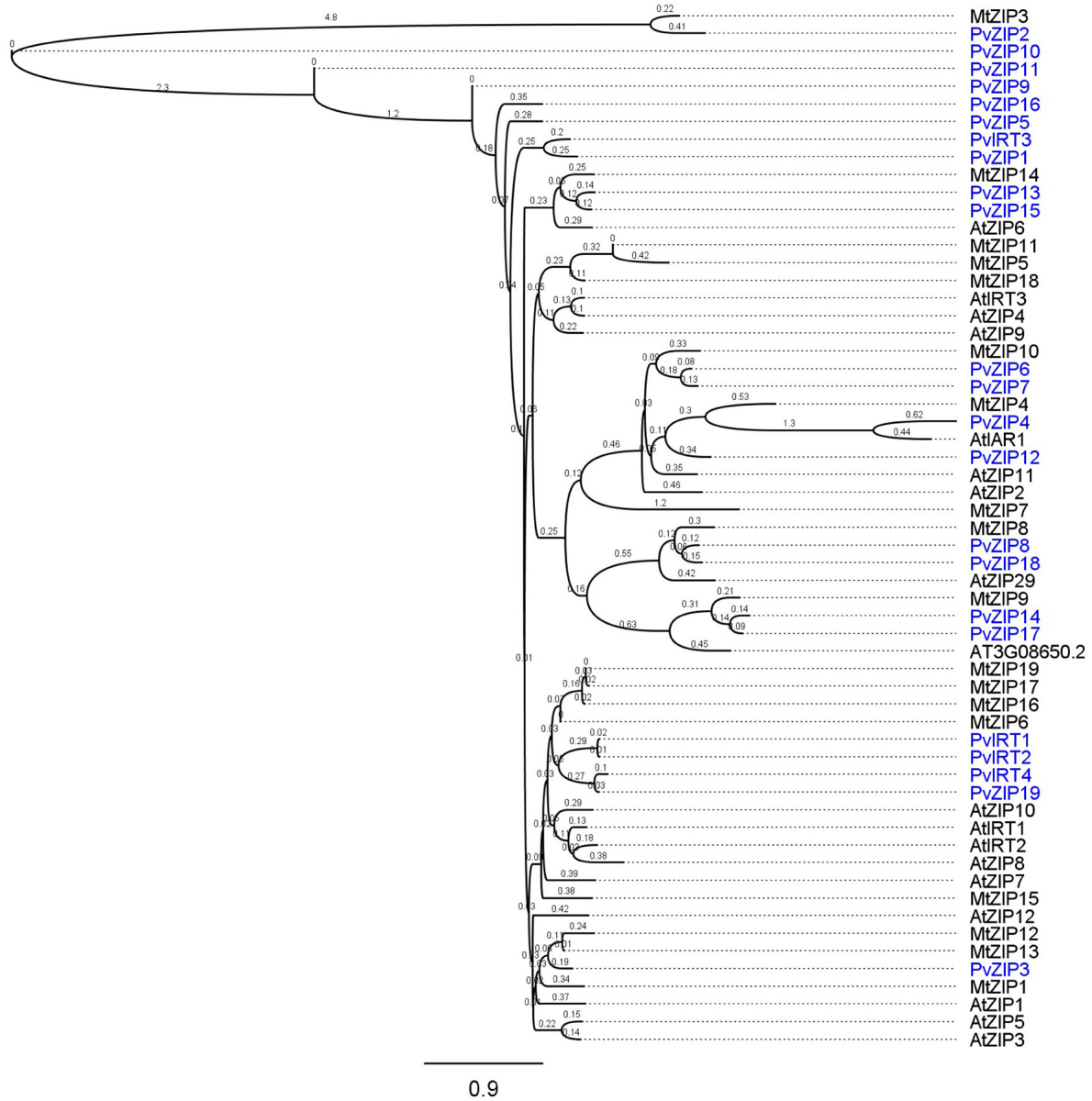


FIGURE 1 | Phylogenetic tree of homologs ZRT, IRT-like protein family in *Phaseolus vulgaris, Arabidopsis thaliana,* and *Medicago truncatula*. Analysis was based on alignment of amino acid sequences using Geneious program v. 6.0.3 and N-J trees were generated. Arabidopsis genes are indicated with the ZIP and IRT number used on TAIR database. ZIP names used in Medicago were according to Lopez-Millan et al. (2004) (ZIP1 to ZIP7). ZIP8 in front were assigned with a consecutive number.

marker sequence in DOR 364 × G19833 against the *P. vulgaris* genome sequence. The results of the *in silico* mapping indicate that ZIP genes are distributed on all *P. vulgaris* chromosomes except 4 and 7. There was a tendency for ZIP genes to cluster together, most notably on chromosome 5 and 6 (**Figure 3**).

Through *in silico* mapping, *bZIP1* was located on chromosome 5 and *bZIP2* and *bZIP3* were located near each other on chromosome 11. The *bZIP3* gene location was expected based on the position and sequence of the SNP marker g785 which contains the bZIP domain. This marker was described as PvMcCleanNDSU2007_11_g785 (http://cmap.comparative-legumes.org). Selected ZIP genes were also mapped genetically via DNA polymorphisms in the DOR364 × G19833 population using the *P. vulgaris* reference genetic map published by Galeano et al. (2011). This map consists of 499 single copy markers and 2306 cM of coverage (**Figure 3**). *PvZIP2*, *PvZIP6—PvZIP8, PvZIP13*, and *PvIRT3* mapped to chromosomes 1, 5, 6, and 9, respectively.

Once gene markers were mapped, QTLs for seed Fe and Zn were also identified on this map. These QTLs include previously

FIGURE 2 | Alignment of the predicted ZRT, IRT-like protein using CLUSTAL W. Identical amino acids are indicated with dark shading and similar amino acids are indicated with light shading. The histidine-rich sequence located in the variable region between transmembrane domains III and IV and fully conserved histidine motifs are indicated by gray lines. The eight domains are shown as a red line above the sequences.

published data for two sites (Blair et al., 2009) as well as QTL identified in whole and cotyledon seed mineral evaluation from a 2006 planting of the same population in Darien, Colombia. QTL analysis in the 2006 evaluation identified new QTLs for Zn concentration on chromosomes 1 and 2 and also confirmed the QTLs identified by Blair et al. (2009) (**Table 3**). For seed Fe concentration, 13 QTLs were found on chromosomes 2, 3, 6, 8, and 11. For seed Zn concentration 11 QTLs were found on 1, 2, 3, 6, 9, and 11 (**Table 3**). ZIP genes mapped on chromosomes 3, 6, 8, and 11 mapped within the regions of QTL for Fe and/or Zn. On chromosome 11 two bZIP genes (with genomic position 3,134,797 and 3,709,270 bp) were mapped *in silico* within the region of two QTLs for seed Fe and one QTL for seed Zn (**Figure 3**). Two *Pv*IRT genes are present on chromosome 3 (at 49,001,506 and 49,013,793 bp) and three QTLs for seed Fe concentration mapped between the QTLs (**Figure 3**). **Table 3** shows specifically which ZIP genes are located within or nearby QTL for seed mineral concentration.

EXPRESSION ANALYSIS OF *PvZIP* GENES

Studies in *Arabidopsis*, *Glycine*, *Vitis* and *Medicago* indicate that ZIP genes may be expressed in roots, leaves, and reproductive tissue (Grotz et al., 1998; Lopez-Millan et al., 2004). Many studies so far have focused on expression in roots and shoots (Grotz et al., 1998; Lopez-Millan et al., 2004; Milner et al., 2012). From the perspective of biofortification, it is necessary for a bean plant not only to efficiently take up Zn from the soil, but also transport and accumulate it in vegetative tissue, pods, and ultimately seeds. In order to determine the expression profile of members of ZIP family and their relevance during the development of common bean, relative expression levels were measured by RT qPCR. *PvZIP2*, *PvZIP7*, *PvZIP6*, *PvZIP12*, *PvZIP13*, *PvZIP16*, *PvZIP18*, and *PvIRT3* genes were selected for this analysis based on their location in the genome in relation to presence of QTLs for Zn and Fe in the DOR364 × G19833 population. Three tissue types were analyzed for gene expression in DOR364 and G19833: roots, leaves, and pods. Roots and leaves were collected at two time points, one during vegetative growth, and one during flowering. Pods were sampled 20 days after flowering. Each tissue type was selected from plants with two Zn treatments. At four weeks after planting, DOR364 and G19833 plants in the Zn (−) treatment exhibited some Zn deficiency symptoms such as interveinal chlorosis, bronzing, and shortening of the internode (Brown and Leggett, 1967). The ZIP genes *PvZIP12*, *PvZIP13*, *PvZIP16*, and *PvIRT3* were expressed in all tissue analyzed (**Figure 4**). However, *PvZIP2*, *PvZIP6*, *PvZIP7*, and *PvZIP18* were undetectable under RT qPCR in all tissue types. This finding is also supported by pod transcriptome data which also found low to no expression for *PvZIP2*, *PvZIP6*, *PvZIP7*, and *PvZIP18* (Astudillo et al., in preparation).

Gene expression of *PvZIP12* and *PvZIP16* was induced upon Zn status in leaf tissue. *PvZIP12* was most highly expressed in leaves at vegetative stage under Zn (−) treatment, especially in G19833 (**Figure 4**). For *PvZIP13*, G19833 was more highly expressed than DOR364 especially in leaves sampled during flowering. Of each of the ZIP genes studied, *PvZIP16* showed the highest differential expression based on tissue type, stages, and zinc treatment but not between genotypes. It was 139–848-fold more expressed in the leaves than the roots for both genotypes and developmental stages.

EXPRESSION ANALYSIS OF THREE TRANSCRIPTION FACTORS *bZIP*

RNA from the same samples described above were also used to determine the relative expression of three transcription factors *Pv bZIP1*, *Pv bZIP2* and *Pv bZIP3*, which are homologous to *Arabidopsis* bZIP genes in the zinc homeostasis network (**Table 2**). The common bean homologue *bZIP1* was detected in roots, leaves, (at vegetative stages) and pods but expression pattern did not change based on stages and Zn treatment. This gene was more highly expressed in leaf tissue sampled during flowering than

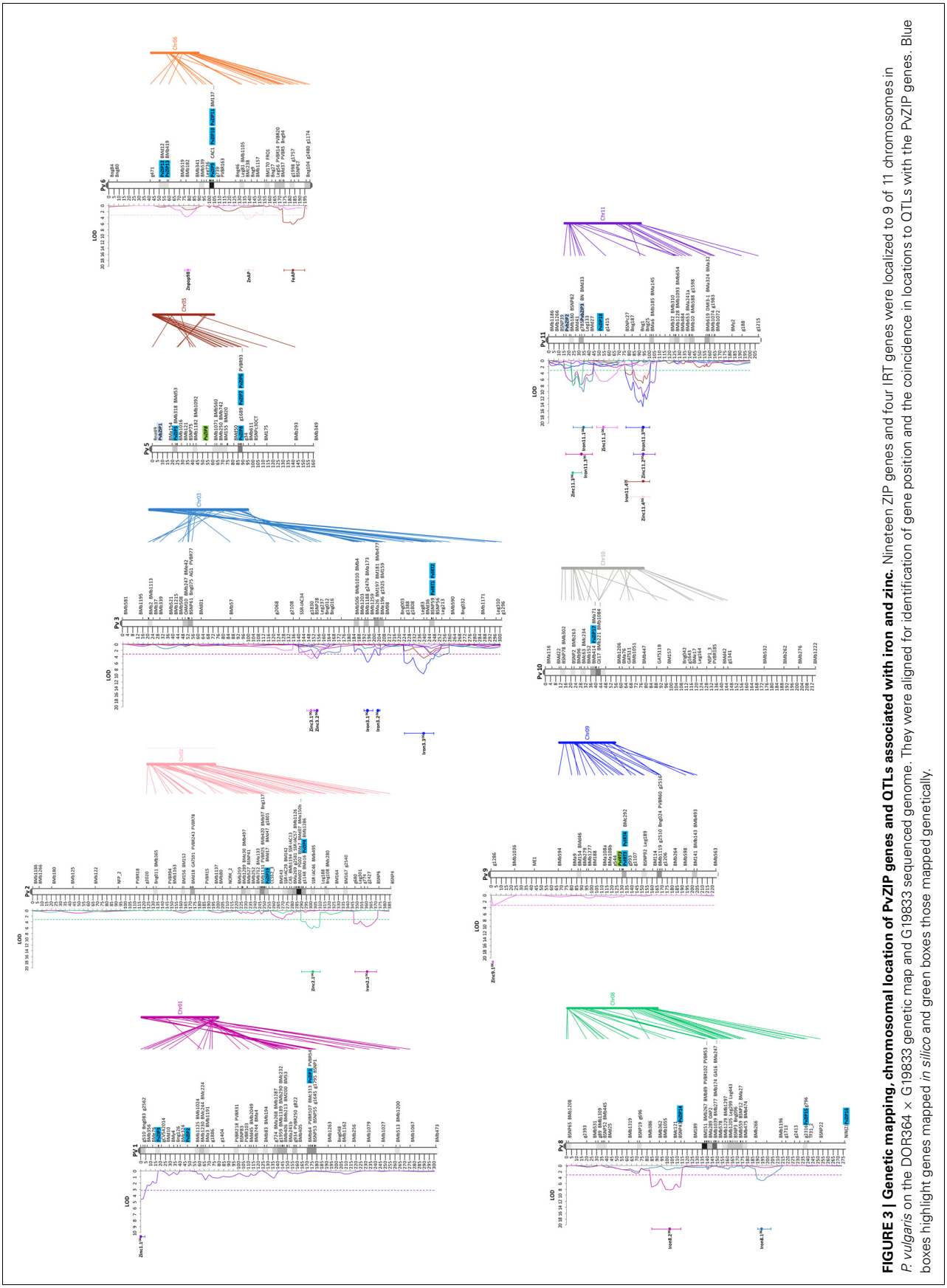


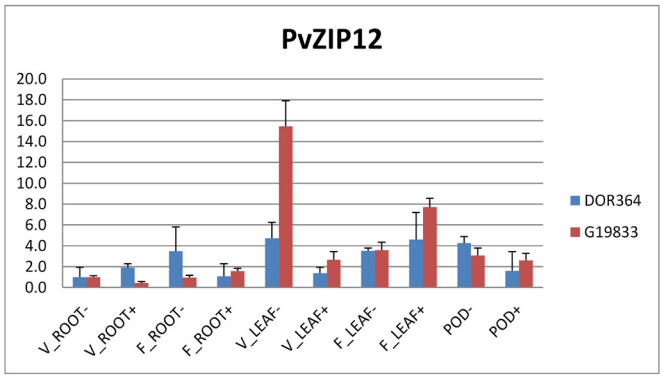
FIGURE 3 | Genetic mapping, chromosomal location of PvZIP genes and QTLs associated with iron and zinc. Nineteen ZIP genes and four IRT genes were localized to 9 of 11 chromosomes in *P. vulgaris* on the DOR364 × G19833 genetic map and G19833 sequenced genome. They were aligned for identification of gene position and the coincidence in locations to QTLs with the PvZIP genes. Blue boxes highlight genes mapped *in silico* and green boxes those mapped genetically.

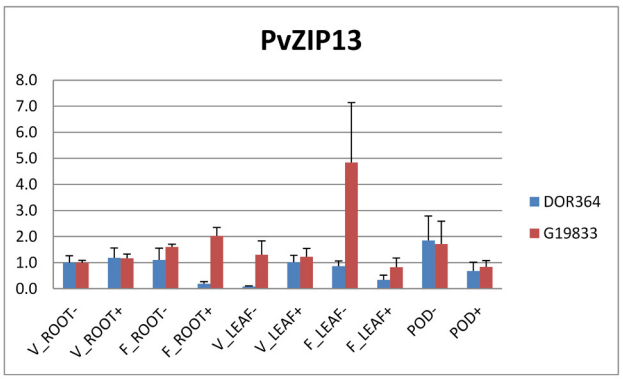
Table 3 | Quantitative trait loci (QTL) for iron and zinc concentration identified with composite interval mapping in the DOR364 × G19833 population.

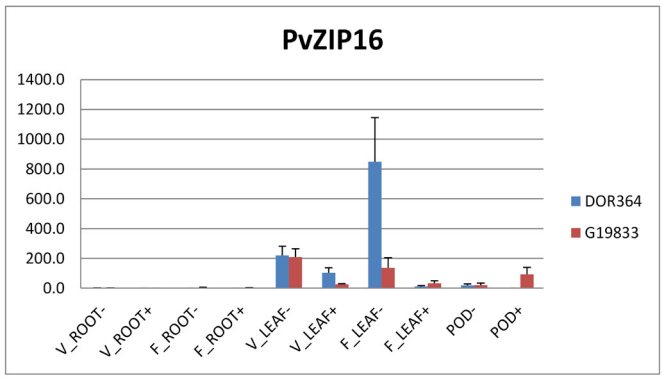
Trait	QTL	Tissue	Environment	Chromosome	Marker interval	Position (cM)	ZIP genes nearby[a]	Genomic position (bp)	LOD	R2	Adaptive effect	Source
Iron	Iron2.1^{DG*}	Cotyledon	Darien 2003	2	g680—BSNP6	361.1			5.7	10.6	3.1	G19833
	Iron3.1^{DG}	Whole seed	Darien 2003	3	BMb1188—BMb1259	194.8			5.7	11.1	1.8	G19833
	Iron3.2^{DG}	Whole seed	Darien 2003	3	g1388—Leg213	226.2	PvIRT1—PvIRT2		9.4	19.7	2.4	DOR364
	Iron3.3^{DG}	Whole seed	Darien 2006	3	G1388—BSNP59	239.5	PvIRT1—PvIRT2	49,001,506–49,013,793	4.5	10.5	1.3	DOR364
	Iron 3.4^{DG*}	Cotyledon	Darien 2003	3	BSNP59	245.5	PvIRT1—PvIRT2		3.3	5.9	2.3	DOR364
	Iron3.5^{DG}	Whole seed	Popayan 1998	3	BSNP56—BMb59O	251.1			4.0	9.4	3.4	G19833
	Iron6.1^{DG*}	Whole seed	Darien 2006	6	PVBR5—Bng104	183.4			6.1	15.3	1.6	G19833
	Iron8.1^{DG}	Whole seed	Popayan 1998	8	BMb266—BMb196	192.1			5.0	13.4	3.4	G19833
	Iron8.2^{DG*}	Cotyledon	Darien 2003	8	BMb386—BSNP43	100.8	PvZIP14	7,634,926	8.2	17.7	4.1	G19833
	Iron11.1^{DG}	Whole seed	Popayan 1998	11	BSNP82—BMd27	22.0	Pv bZIP3	3,709,270	9.9	24.5	4.4	G19833
	Iron11.2^{DG}	Whole seed	Darien 2003	11	BSNPc27—BMa145	81.4			14.7	34.8	3.0	G19833
	Iron11.3^{DG*}	Cotyledon	Darien 2003	11	BSNP39—BMd27	30.8	Pv bZIP2—Pv bZIP3	3,134,797–3,709,270	7.6	15.0	3.6	G19833
	Iron11.4^{DG*}	Whole seed	Darien 2006	11	BSNPc27—BMa6	79.4			7.2	20.1	1.8	G19833
Zinc	Zinc1.1^{DG*}	Whole seed	Darien 2003	1	g510—BMb356	0.0	PvZIP3	49,839,431	4.6	8.8	0.6	G19833
	Zinc2.1^{DG*}	Cotyledon	Darien 2003	2	BMb1286—Leg188	302.5	PvZIP4	33,735,220	6.2	14.1	1.7	DOR364
	Zinc3.1^{DG}	Whole seed	Popayan 1998	3	IAC34—BSNP28	150.1			4.6	10.1	1.2	G19833
	Zinc3.2^{DG}	Whole seed	Darien 2003	3	g1830—Bng012	154.7			4.5	8.5	0.6	G19833
	Zinc6.1^{DG*}	Whole seed	Darien 2006	6	BMc238—Bng009	139.3			4.4	13.1	0.7	G19833
	Zinc6.2^{DG}	Whole seed	Popayan 1998	6	BMb182	78.8	PvZIP12—PvZIP13	17,174,396–18,954,219	3.6	9.8	1.0	DOR364
	Zinc9.1^{DG}	Whole seed	Popayan 1998	9	G1286	0.0			3.2	6.7	1.0	G19833
	Zinc11.1^{DG}	Whole seed	Popayan 1998	11	BMd27—BSNPc27	53.4	PvZIP18	5,071,268	6.6	17.7	1.5	G19833
	Zinc11.2^{DG}	Whole seed	Darien 2003	11	Bng187—BMa145	92.7			11.5	28.0	1.1	G19833
	Zinc11.3^{DG*}	Cotyledon	Darien 2003	11	BSNP82—BN	22.0	Pv bZIP2	3,134,797	7.8	17.5	1.8	G19833
	Zinc11.4^{DG*}	Whole seed	Darien 2006	11	Bng001—BMa6	92.7			5.5	16.0	0.8	G19833

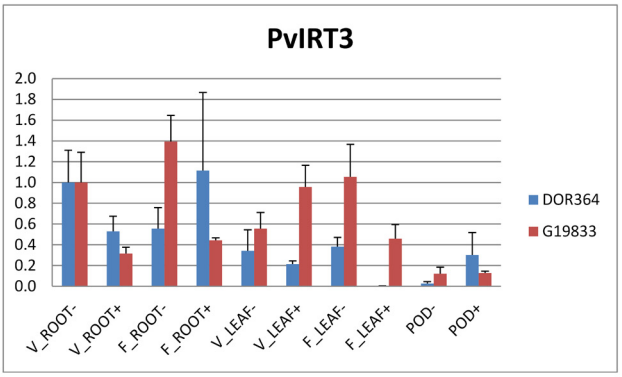
[a] *PvZIP genes and Pv bZIP transcription factor coinciding with QTLs found.*

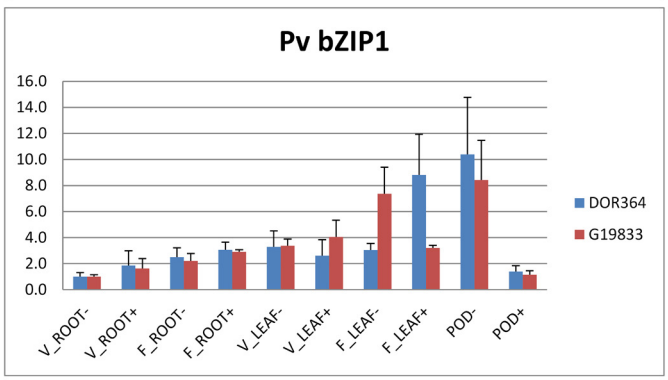
** New QTLs found.*

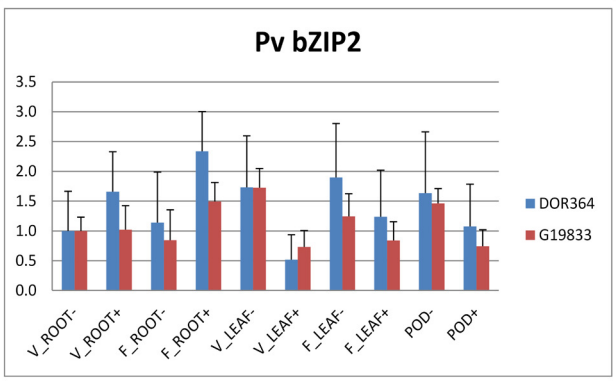


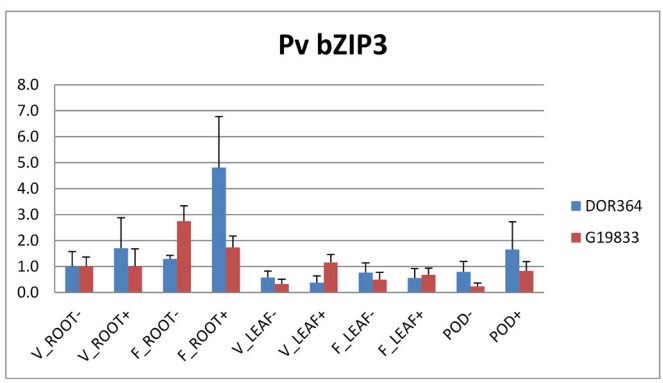












	Tissue	Stage	Zn treatment	Genotype
PvZIP12	*	*	*	*
PvZIP13	ns	ns	ns	*
PvZIP16	*	*	*	ns
PvIRT3	ns	ns	ns	ns
PvbZIP1	*	ns	ns	*
PvbZIP2	ns	ns	ns	ns
PvbZIP3	ns	ns	ns	ns

*: Significance level P<0.05

ns: No significant

FIGURE 4 | Relative expression level of PvZIP gene transporters and three bZIP transcription factors in genotypes Dor364 and G19833 in different tissues and two Zn treatment. (i) roots at vegetative stage (V_ROOT− and V_ROOT+), (ii) roots at flowering stage (F_ROOT− and F_ROOT+); (iii) leaves at vegetative stage (V_LEAF− AND V_LEAF+) stage; (iv) leaves at flowering stage (F_LEAF− and F_LEAF+); and (v) pods (POD− and POD+) of plants under Zn (−) and Zn (+) treatment.

vegetative tissue in both G19833 and DOR364. Transcripts of *Pv bZIP2* were detected in roots, leaves, and pods and its expression pattern was not influenced by tissue type, developmental stage or Zn treatment. *Pv bZIP3* was expressed in roots during vegetative and flowering with a barely expression in leaves and pods.

TISSUE ZINC CONCENTRATION

Zn concentration was determined for DOR364 and G19833 for all tissues, developmental stages, and Zn treatments (**Figure 5**). The highest Zn concentration was observed in roots for both genotypes and no significant differences were observed in leaves. Significant tissue × Zn treatment × genotype interaction was found. Plants grown under the Zn (+) treatment had higher levels of Zn in pods and seeds than those grown under the Zn (−) treatment. Seed Zn levels were 26 and 53% less in the Zn (−) treatment in DOR364 and G19833, respectively. G19833 had higher seed Zn levels that DOR364 under the Zn (+) treatment but not under the Zn (−) treatment (**Figure 5**).

DISCUSSION

Common bean is becoming an alternative to dietary supplements as a way to improve human health. ZIP metal transporters are one of the most important gene families for Zn and Fe cellular uptake and translocation in plants (Guerinot, 2000; Chen et al., 2008; Wu et al., 2009; Adams et al., 2012). Identification of ZIP members in *P. vulgaris* and characterization of their expression patterns is useful to increase the understanding of uptake, transportation, and storage of Zn. This study is a unique combination of gene family characterization with physical and genetic mapping and functional expression data that has utility in common bean improvement.

Twenty-three ZIP genes were identified in the *P. vulgaris* genome and genes were annotated and characterized based on similarity to other ZIP family members in *A. thaliana* and *M. truncatula*. According to the total number ZIP family members across species the family origin may be from a common ancestor that has undergone sequence duplication followed by divergence events (D'Ovidio et al., 2004). PvZIP genes clustered on chromosomes 3, 5, 6, and 9 showed high sequence similarity. The close proximity and sequence similarity of many of the ZIP gene family members might suggest of gene duplication followed by diversification (Yang et al., 2009). On the other hand, heterogeneity in structure and expression in each *Pv*ZIP gene suggests diversity in functionality. Four of the eight genes evaluated were not expressed in none of the tissue analyzed. Loss of function in these proteins might be compensated for by duplicate genes (D'Ovidio et al., 2004).

It is important to consider the link between functional variation and gene structural differences among ZIP family members. In many cases, Zn interacts with cysteines and histidines in proteins and may determine the ionic selectivity of ion transporters (Ramesh et al., 2003; Lopez-Millan et al., 2004). The motif of histidine in variable regions between transmembrane domain III and IV in many ZIPs has been postulated to serve as a potential metal ion binding site (Eide et al., 1996; Zhao and Eide, 1996; Grotz et al., 1998). For *Pv*ZIPs identified in this study, all contained this motif except *PvZIP6*, *PvZIP7*, and *PvZIP18*, interestingly these ZIP genes were also not expressed in all tissue analyzed, suggesting without the motif they are not functional.

In *Arabidopsis*, ZIP genes have been shown to regulate and also contribute to the uptake, transport and accumulation of Zn (Grotz et al., 1998; Weber et al., 2004; Talke et al., 2006; Lin et al., 2009; Milner et al., 2013). Here we used RT-qPCR approach to obtain a picture of ZIP gene transcription in roots and leaves at vegetative and flowering stages, and pods at 20 days after flowering in *P. vulgaris*. Some processes such as Zn uptake, have been studied in detail, while others such as remobilization of Zn from vegetative to reproductive tissues are less well understood

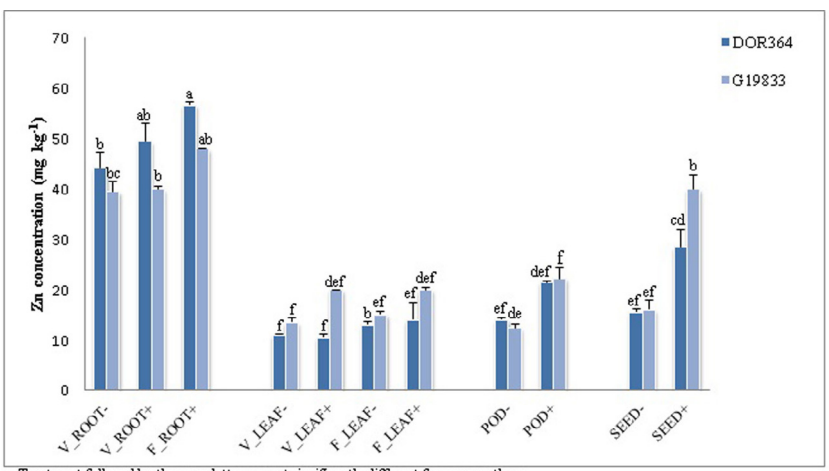


FIGURE 5 | Zinc concentration in DOR364 and G19833. Zn concentration (ppm) in (i) roots at vegetative stage (V_ROOT− and V_ROOT+), (ii) roots at flowering stage (F_ROOT+); (iii) leaves at vegetative stage (V_LEAF− AND V_LEAF+) stage; (iv) leaves at flowering stage (F_LEAF− and F_LEAF+); (v) pods (POD− and POD+) and seeds (SEED− and SEED+) of plants under Zn (−) and Zn (+) treatment.

(Genc et al., 2006). The evaluation of gene expression patterns based on tissue, Zn treatments, and genotype not only provides information on the functionality of the ZIP family genes but also may help explain genotypic differences in seed Zn accumulation. These data indicate differential gene regulation associated with the nutritional requirements and possible mechanism of partitioning of Zn along the plant. According to ZIP genes characterization in *Arabidopsis* approximately half of the genes characterized are induced in response to Zn deficiency (Grotz et al., 1998; Talke et al., 2006).

ZIP gene expression differences in *P. vulgaris* were related to Zn treatments, genotype, and tissue type. Genotypic differences in Zn translocation capacity in different organs may be an important factor in Zn accumulation in seeds (Hacisalihoglu et al., 2004). Observed differences between genotypes could also be due to genetic differences and diversity among Andean and Mesoamerican gene pools (Blair et al., 2009). Similarly to previous studies, G19833 had higher seed Zn level than DOR364 (Blair et al., 2009). However DOR364 had higher Zn in its roots as compared to G19833 suggesting that G19833 can translocate more Zn from roots to seeds.

Zinc plays a specific role in fertilization and pollen grains contain very high concentrations of Zn (Fageria et al., 2011). At flowering most of the Zn taken up is incorporated into the developed seed (Jiang et al., 2008) so genes highly expressed at flowering and in pods such as *PvZIP12*, *PvZIP16*, and *bZIP1* could be directly related to Zn remobilization to seeds.

Although leaves are known as the major source of remobilized micronutrients in common bean (Sekara et al., 2005) in rice stems are the major source of Zn in the seed (Waters and Sankaran, 2011). With this study it was not possible to determine how much and the source of Zn remobilization. Future studies with radio labeled Zn would be warranted to asses Zn remobilization.

Based on the relative expression values established via RT-qPCR, the high Zn concentration in roots did not reflect expression values for the ZIP genes evaluated in this tissue. In *Arabidopsis* at least 10 different members of the ZIP family play a role in zinc uptake in roots, including ZIP1, 2, 3, 4, 5, 9, 10, 11, 12, and IRT3 (Van De Mortel et al., 2006). We evaluated four of their respective homologous in *P. vulgaris* and found that they were only weakly expressed in roots.

The DOR364 × G19833 RILs mapping population consists of 86 individuals, which are adequate for identifying QTL with moderately large effects based on QTLs previously detected (Blair et al., 2009, 2011; Galeano et al., 2011). *In silico* mapping of ZIP genes was a successful strategy to locate *Pv*ZIP genes aligned with QTL for seed Fe and Zn in the bean genome. QTL analysis was carried out in the current reference bean map (Galeano et al., 2011). It is worth noting that on chromosomes 2 and 6 where *PvZIP4* and *PvZIP12* and *PvZIP13* are located, there are QTL for seed Zn concentration. For Fe, the IRT genes are considered to be the main transporters for high-affinity iron uptake in roots in *Arabidopsis* (Connolly et al., 2002; Henriques et al., 2002; Lin et al., 2009). In this study, *PvIRT1* and *PvIRT2* were located on chromosome 3 within an important QTL region associated with seed Fe concentration. The *Pv bZIP2* and *Pv bZIP3* genes were located on chromosome 11 and aligned with the most important QTL for Fe and Zn reported in *P. vulgaris*. There are no obvious genotypic differences in expression of these genes in G19833 and DOR 364, however. The QTL in this region has been found in at least three mapping populations, including Mesoamerican and Andean intra and inter genepool crosses (Blair et al., 2009, 2010a,b, 2011). The bZIP transcription factors analyzed correspond to genes in *Arabidopsis* responsible for response and adaptation to low Zn supply. In general, *Pv*ZIP, *Pv*IRT, and *Pv* bZIPs co-localization with QTLs for Fe and Zn levels suggest that their function is important in Fe and Zn homeostasis in *P. vulgaris*. In *Arabidopsis*, the bZIP transcription factors that interacted with ZIP genes were found directly upstream of the ZIP genes (Assunção et al., 2010). In the case of *P. vulgaris* none of the bZIP genes were adjacent to ZIP genes.

This study is the first to characterize the ZIP gene family, report the expression profile in various tissues with two genotypes and fertilization treatments. It provides evidence of the relationship among level of transcripts and QTLs in dry bean seed as was identified in *PvZIP12* gene. This contribution will be particularly useful for advancing bean breeding programs. The use of such gene marker encoding proteins associated with transport and accumulation of Zn and Fe could increase the efficiency and accuracy in the selection of breeding materials for biofortification.

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Iron biofortification of Myanmar rice

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Iron (Fe) deficiency elevates human mortality rates, especially in developing countries. In Myanmar, the prevalence of Fe-deficient anemia in children and pregnant women are 75 and 71%, respectively. Myanmar people have one of the highest per capita rice consumption rates globally. Consequently, production of Fe-biofortified rice would likely contribute to solving the Fe-deficiency problem in this human population. To produce Fe-biofortified Myanmar rice by transgenic methods, we first analyzed callus induction and regeneration efficiencies in 15 varieties that are presently popular because of their high-yields or high-qualities. Callus formation and regeneration efficiency in each variety was strongly influenced by types of culture media containing a range of 2,4-dichlorophenoxyacetic acid concentrations. The Paw San Yin variety, which has a high-Fe content in polished seeds, performed well in callus induction and regeneration trials. Thus, we transformed this variety using a gene expression cassette that enhanced Fe transport within rice plants through overexpression of the nicotianamine synthase gene *HvNAS1*, Fe flow to the endosperm through the Fe(II)-nicotianamine transporter gene *OsYSL2*, and Fe accumulation in endosperm by the Fe storage protein gene *SoyferH2*. A line with a transgene insertion was successfully obtained. Enhanced expressions of the introduced genes *OsYSL2*, *HvNAS1*, and *SoyferH2* occurred in immature T_2 seeds. The transformants accumulated 3.4-fold higher Fe concentrations, and also 1.3-fold higher zinc concentrations in T_2 polished seeds compared to levels in non-transgenic rice. This Fe-biofortified rice has the potential to reduce Fe-deficiency anemia in millions of Myanmar people without changing food habits and without introducing additional costs.

Keywords: iron, anemia, biofortification, nicotianamine, ferritin, OsYSL2, Myanmar rice, rice transformation

INTRODUCTION

Iron (Fe) deficiency is one of the most prevalent micronutrient deficiency problems in humans. According to a World Health Organization (WHO) report (WHO, 2002), Fe deficiency affects an estimated two billion people, causing almost one million deaths annually worldwide. Among risk factors for human health, Fe deficiency ranks sixth in developing countries, where it contributes to high rates of mortality (WHO, 2002). Myanmar is one of the countries with critical levels of Fe-deficiency anemia (IDA) (WHO, 2008). The prevalence of IDA is ∼75% in Myanmar children under 5 years of age, ∼26% in adolescent schoolgirls, ∼45% in non-pregnant women, and ∼71% in pregnant women (MOH, 2003). It shows many population groups are strongly impacted by IDA. Young children and women in pregnancy or breast feeding are most frequently and severely affected (WHO, 2002). IDA causes disruptions in brain development in young children and deaths of women in pregnancy and childbirth. It threatens national productivities and lowers the intellectual capacity of human populations (UNICEF and The Micronutrient Initiative, 2004a) in addition to causing coma and death in severe cases of anemia (WHO, 2002). Mineral deficiency lowers the annual productivity of the Myanmar adult work force by an estimated 0.7% of GDP (UNICEF and The Micronutrient Initiative, 2004b).

The main cause of anemia is inadequate dietary Fe intake. Applications exist that address Fe deficiency, including Fe supplementation, Fe fortification, and dietary diversification. Because of financial, cultural, regional, or religious restrictions, these applications are not always successful (Graham et al., 2001; Bouis et al., 2003; Lyons et al., 2003; Timmer, 2003), but a complementary approach to mineral malnutrition termed "biofortification" is proving to be an efficient solution for developing countries (Graham et al., 2001; Bouis, 2003). Biofortification is a process that increases bioavailable concentrations of essential elements in edible portions of crop plants through agronomic intervention or genetic selection (White and Broadley, 2005). Arguments exist to indicate that once mineral-dense lines have been developed, little additional cost will be incurred in incorporating them into ongoing breeding programs. Therefore, biofortification is sustainable and cost-effective, and reaches remote rural populations (Bouis et al., 2003; Timmer, 2003; Welch and Graham, 2005).

Rice is the main staple food crop in Myanmar. The country was the sixth largest rice producer in the world in 2010 (IRRI, 2013). The national rice growing area occupies 8 million ha, representing 63% of the total cultivated landscape (MOAI, 2010); annual rice production is ∼32 million t year^{-1} (MOAI, 2010). Myanmar people are among the highest rice-consumers globally. Average

per capita rice consumption is ∼578 g day^{-1} (Kennedy et al., 2002; Maclean et al., 2002), and represents 75% of the caloric intake. Thus, Fe biofortification of Myanmar rice varieties would provide an effective solution to Fe deficiency in the country.

Among available processes that might be used for Fe biofortification, transgenic methodology has potential to be the fastest and most efficient. There were some reports about improved Fe content in rice grain through transgenic procedures. In a first approach, Goto et al. (1999) generated a *japonica* cv. Kitaake transgenic rice with twofold increase in Fe concentration in endosperm (edible part of the rice grain that includes the polished seed) by expressing the soybean *ferritin* gene (*SoyferH1*) in the endosperm under the control of the rice *glutelin* gene (*OsGluB1*) promoter. Furthermore, through this endosperm-specific expression of *ferritin*, twofold increase in Fe in a transgenic *japonica* cv. Taipei 309 (Lucca et al., 2001), 3.7-fold increase in *indica* cv. IR68144 (Vasconcelos et al., 2003) and 2.1-fold increase in *indica* cv. Pusa Sugandhi II (Paul et al., 2012) were reported. In a second approach, overexpression of the nicotianamine synthase gene (*NAS*) increased Fe concentration in polished rice seeds threefold under greenhouse conditions using *japonica* cv. Tsukinohikari (TK) (Masuda et al., 2009a), *japonica* cv. Dongjin (Lee et al., 2009), and *japonica* cv. Nipponbare (Johnson et al., 2011). In a third approach, Fe concentration in polished rice seeds of TK was increased up to threefold through enhancement of Fe(II)-nicotianamine transporter gene (*OsYSL2*) expression under the control of the rice sucrose transporter gene (*OsSUT1*) promoter, leading to elevated expression in the panicle and immature seeds during seed maturation (Ishimaru et al., 2010). Moreover, by combining these three approaches, Masuda et al. (2012) generated "Fer-NAS-YSL2" rice, in which the Fe content was elevated sixfold in greenhouse-grown T_2 polished seeds and fourfold in paddy field-grown T_3 polished seeds of TK. Accordingly, we applied this combined approach to the currently cultivated and consumed Myanmar rice variety.

Hiei et al. (1994) reported the first efficient *Agrobacterium*-mediated transformation of *japonica* rice. Using this published procedure, an *Agrobacterium*-mediated transformation methodology with minor modifications has been developed for many rice varieties other than *japonica*, *indica*, and tropical *japonica*, including *indica* cv. Kasalath (model *indica* rice; Saika and Toki, 2010), *indica* cv. RD6 (Thai commercial rice cultivar; Pipatpanukul et al., 2004), *indica* cv. IR64 (IRRI, a well-known high-yield rice cultivar; Rajesh et al., 2008), and *indica* cv. MR219 (Malaysian rice; Sivakumar et al., 2010). Many studies have also demonstrated that transformation efficiencies depend on culture responses of individual varieties (Pipatpanukul et al., 2004; Nishimura et al., 2007; Summart et al., 2008). Therefore, optimization of tissue culture conditions is essential for varieties that regenerate with difficulty (Hiei et al., 1994, 1997). Relevant reports have been published on callus induction and regeneration in *indica* cv. BR-8 (Bangladesh *indica* rice; Amin et al., 2004) and *indica* cv. KDML 105 (Thai aromatic rice; Summart et al., 2008). Nevertheless, no current reports have described transformations of Myanmar rice varieties, and thus no relevant information exists to aid in the selection of Myanmar varieties that would be suitable for this procedure. Similarly, no information exists to guide selection of callus induction and regeneration media that are efficient for regeneration. Accordingly, production of high-Fe Myanmar rice through Fe-biofortification methodology requires analyses of callus induction and regeneration ability in each variety before performing actual rice transformation.

In the present study, we first tested callus induction and regeneration efficiency in 15 popular Myanmar rice varieties. The Paw San Yin variety produced calli readily and had moderate regeneration efficiency when we used the modified methods of Hiei et al. (1994). The main purpose of our research is to produce Fe biofortified Myanmar rice using the currently cultivated and consumed rice variety and come closer to practical application for the needs. Therefore, we used Paw San Yin variety, which is a famous high-quality variety, and presently cultivated and consumed rice variety in Myanmar. We produced high-Fe Myanmar rice through introduction of the Fer-NAS-YSL2 vector, which includes multiple genes for enhancing Fe transportation and accumulation in rice plants (Masuda et al., 2012). The procedures produced elevated gene expression in T_2 immature seeds, and the Fe concentration in T_2 polished seeds increased 3.4-fold in comparison with the non-transgenic line.

MATERIALS AND METHODS

PLANT MATERIALS

We used seeds of 15 Myanmar high-yield or high-quality rice cultivars, which were provided by the Myanmar Rice Research Center (MRRC), Hmawbi, Myanmar, for the regeneration trials. These were Kyaw Zay Ya (V1), Ayar Min (V2), Paw San Yin (V3), Shwe War Htun (V4), Sin Thwe Lat (V5), Yezin Lone Thwe (V6), Thu Kha Yin (V7), Thee Htet Yin (V8), Sin Nwe Yin (V9), Yadana Toe (V10), Hmawbi 2 (V11), Hmawbi 3 (V12), Hmawbi 4 (V13), Hmawbi 5 (V14), and Hmawbi Kauk Nyin Hmwe (V15) (**Table 1**). *Japonica* rice cv. TK, a well-known variety for efficient rice transformation, was used as the control for callus induction and regeneration trails.

We used Myanmar rice *tropical Japonica* cv. Paw San Yin (V3) (**Table 1**; Figure S1 in Supplementary Material) for rice transformation and as the non-transgenic control. Seeds from MRRC were sown in a greenhouse and grown to maturity to provide fresh seeds for transgenic procedures.

STERILIZATION OF RICE SEEDS

Husks were removed from the seeds manually. The brown seeds were first surface-sterilized in 70% ethanol for 5 min and then rinsed thoroughly with distilled water (DW). Then, the seeds were washed in a 2.5% solution of sodium hypochlorite (NaClO; The Clorox Co., Oakland, CA, USA) containing 0.2% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) as a wetting agent for 30 min in a rotator. After surface sterilization, seeds were rinsed five times with sterile water and used for induction of calli.

MEDIA USED FOR CALLUS INDUCTION AND REGENERATION TEST

We used N6D, N6D4, N6D6, 2N6, MSre, and MS media for callus induction and regeneration trials. Components of 2N6 and MS media were from Hiei et al. (1994). Components of N6D media were from Ishizaki and Kumashiro (2008). MSre (MS regeneration) media was modified from Toki (1997), and 2 mg l^{-1} of α-naphthaleneacetic acid and 1 mg l^{-1} of kinetin were applied as

Table 1 | Myanmar rice varieties used in callus induction and regeneration trials.

Variety number	Variety name	Variety type
V1	Kyaw Zay Ya	HYV
V2	Ayar Min	HYV
V3	Paw San Yin	HQV
V4	Shwe War Htun	HYV
V5	Sin Thwe Lat	HYV
V6	Yezin Lone Thwe	HQV
V7	Thu Kha Yin	HYV
V8	Thee Htet Yin	HYV
V9	Sin Nwe Yin	HYV
V10	Yadana Toe	HYV
V11	Hmawbi 2	HQV
V12	Hmawbi 3	HYV
V13	Hmawbi 4	HQV
V14	Hmawbi 5	HYV
V15	Hmawbi Kauk Nyin Hmwe	HYV

HYV, high-yield variety; HQV, high-quality variety. Seeds were provided by the Myanmar Rice Research Center (MRRC) and source of data were from the Ministry of Agriculture and Irrigation (MOAI), Myanmar.

hormones for regeneration of plants in all regeneration trials and rice transformation. Components of N6D4 and N6D6 medium matched those in N6D except that 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations were 4 and 6 mg l^{-1} instead of 2 mg l^{-1}, respectively. Five media combinations tested through the developmental steps from callus induction to plant regeneration were coded as follows: N6D-N6D-MSre-MS, N6D-2N6-MSre-MS, 2N6-2N6-MSre-MS, N6D4-N6D4-MSre-MS, and N6D6-N6D6-MSre-MS. Thus, the code N6D-N6D-MSre-MS indicates the following sequence of media: (i) N6D medium used for callus induction; (ii) induced calli transferred to N6D medium for subsequent callus growth; (iii) calli transferred to MSre medium for regeneration; (iv) final transfer to MS medium for root and shoot growth.

CALLUS INDUCTION TEST

After surface sterilization, 15 seeds of each variety were transferred to various media, such as N6D, N6D4, N6D6, or 2N6, for callus induction and kept in darkness in a growth chamber at 28°C. After 28–36 days, about 30–40 calli that were growing well were transferred to new N6D, N6D4, N6D6, or 2N6 medium and cultured for 10–19 days for subsequent callus growth. During both callus induction and growth stages, we observed callus condition daily and photographed the specimens twice a week. We then assessed callus quantity (the amount of callus induction) and quality (callus yellowness, size, and hardness) for each variety on N6D, N6D4, N6D6, and 2N6 media to determine the best medium combination.

PLANT REGENERATION TEST

After callus induction, we transferred all calli except those that were brownish onto MSre medium and cultured them for 11–32 days under constant light at 28°C in a growth chamber until green spots appeared; green spots indicated the germination of somatic embryos. Subsequently, all calli were transferred to MS medium and kept for 5–22 days under constant light conditions at 28°C in a growth chamber until plant length exceeded 3–5 cm. We then opened the culture dishes and added water, after which plants were acclimated for about 3 days in a growth chamber at 28°C with illumination. We counted the numbers of regenerated plants and also recorded the duration of time from callus induction to the appearance of green spots. The condition of each callus and regenerated plant was observed daily and photographed twice a week. Once the plant development stage had been reached, we calculated regeneration efficiency for each variety as follows: regeneration efficiency = total number of regenerated plants/number of callus-induced seeds

TRANSFORMATION OF THE PAW SAN YIN RICE VARIETY

We used the Fer-NAS-YSL2 vector (Masuda et al., 2012) for rice transformation (Figure S2 in Supplementary Material). *Agrobacterium tumefaciens* (strain C58) was used to introduce the construct into *Oryza sativa* L. cv. Paw San Yin (V3) following the modified method outlined in Hiei et al. (1994) and Akiyama et al. (1997).

For Paw San Yin-Fer-NAS-YSL2 transformation, calli were induced for 29 days and preincubated for 8 days in N6D medium. We performed *Agrobacterium*-infection on 2N6-AS medium for 3 days following Hiei et al. (1994) with *Agrobacterium* optical density (OD) concentrations of 0.1 or 0.01. Calli were then treated with first, second, and third media selected by the hygromycin check test: N6D-CH10 (N6D medium containing 500 mg l^{-1} claforan; Sanofi K. K., Tokyo, Japan and 10 mg l^{-1} hygromycin B; Wako, Osaka, Japan) medium for 14 days, N6D-CH30 (N6D medium containing 500 mg l^{-1} claforan and 30 mg l^{-1} hygromycin B) medium for 14 days, and N6D-CH50 (N6D medium containing 500 mg l^{-1} claforan and 50 mg l^{-1} hygromycin) medium for 7 days at 28°C in darkness. High-quality calli were selected and transferred to MSre-CH50 (MSre medium containing 500 mg l^{-1} Claforan and 50 mg l^{-1} hygromycin B) medium and incubated for 48 days at 28°C under illumination. High-quality calli were transferred to MS-C medium (MS medium containing 500 mg l^{-1} claforan and no hygromycin B) and maintained until green spots and regenerated plants appeared after 15 days. Four regenerated plants were obtained and one independent Fer-NAS-YSL2 line of T_0 transgenic specimens with all inserted genes was selected.

DETECTION OF THE GENE INSERTION IN TRANSGENIC LINES

Leaves of T_0 transgenic and non-transgenic lines were cut off with scissors and crushed in a Multi-beads Shocker (Yasui Kikai, Osaka, Japan). Subsequently, we prepared total DNA following Ikeda et al. (2001). We performed genomic PCR analysis to check for the inserted gene. Rice endogenous *OsActin1* was detected as a positive control using *OsActin1* forward primer (5′-ACA CCG GTG TCA TGG TCG G-3′) and *OsActin1* reverse primer (5′-ACA CGG AGC TCG TTG TAG AA-3′). The *OsGlb* promoter–*SoyferH2* cassette was detected using the *OsGlb1* promoter forward primer (5′-CCG ATC GCC ATC ATC TCA TCA TCA G-3′) and *SoyferH2* reverse primer (5′-GCT TCC ACC AAC ACC CTT AGA AAG-3′).

The *OsActin1* promoter–*HvNAS1* cassette was detected using the *OsActin1* promoter forward primer (5′-GGG TAG AAT TTG AAT CCC TCA GCA-3′) and *HvNAS1* reverse primer (5′-CGA TCT TCT CGA TCA GAG CAG CGA-3′). *HPT* was detected using the *HPT* forward primer (5′-CGG CAT CTA CTC TAT TCC TTT GC-3′) and *HPT* reverse primer (5′-GTC TCC GAC CTG ATG CAG CTC-3′). *NPTII* was detected using the *NPTII* forward primer (5′-GAT GGA TTG CAC GCA GGT TCT C-3′) and *NPTII* reverse primer (5′-GCC AAC GCT ATG TCC TGA TAG C-3′). *iGUS* was detected using the *iGUS* forward primer (5′-CTG TGG AAT TGA TCA GCG TTG G-3′) and *iGUS* reverse primer (5′-CGC AAG TCC GCA TCT TCA TGA C-3′).

GREENHOUSE CULTIVATION

Paw San Yin T_0 transgenic and T_1 plants were cultivated in a mixture of 3 kg/pot of commercially supplied soil used in Japanese rice nurseries (Bonsolichigou; Sumitomo Chemicals, Tokyo, Japan) and 210 g/pot of vermiculite (Buiesu-Kakou Co., Ltd., Tokyo, Japan). The nutrient content of the bonsol soil were 1.8 g/pot of nitrogen (N), 4.8 g/pot of phosphate (P), and 2.6 g/pot of potassium (K). Slow-release fertilizer (LongTotal-70 and -140; JCAM AGRI. Co., Ltd., Tokyo, Japan) was applied at planting time and panicle initiation time. The nutrients included in the slow released fertilizers per one application time were as follows: N: 0.91 g/pot, P: 0.77 g/pot, K: 0.91 g/pot, Mn: 7 mg/pot, boron (B): 4.2 mg/pot, Fe: 14 mg/pot, Cu: 3.5 mg/pot, Zn: 1.05 mg/pot, magnesium (Mg): 140 mg/pot and molybdenum (Mo): 1.4 mg/pot. Plants were grown in a greenhouse at 30°C with 14 h day^{-1} of natural light, and at 25°C with 10 h day^{-1} of natural light. Specimens were grown in plant cultivation pots (size: 1/5000; Fujiwara-Seisakusho, Tokyo, Japan). One plant was transferred to each pot.

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

Total RNA was extracted from immature T_2 seeds (seeds at an early milky stage, 10 days after fertilization) harvested from each subline and NT line grown in a greenhouse. Seeds were crushed in a Multi-beads Shocker®. We subsequently extracted RNA using an RNeasy Plant Mini Kit (Qiagen KK, Tokyo, Japan). First-strand cDNA was synthesized using a ReverTra Ace kit (Toyobo, Osaka, Japan) with oligo-$d(T)_{30}$. Real-time RT-PCR was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Tokyo, Japan) with SYBR Premix Ex Taq II reagent (Takara, Shiga, Japan). The primers used were as follows: *OsYSL2* forward (5′-GAG GGA CAA CGG TGT CAT TGC TGG T-3′) and *OsYSL2* reverse (5′-TGC AGA AAA GCC CTC GAC GCC AAG A-3′) for *OsYSL2* expression, *HvNAS1* forward (5′-GGA CGT CGC CGA CCT CAC CCA G-3′) and *HvNAS1* reverse (5′-CAG GGA CGC CCC CTC CAC C-3′) for *HvNAS1* expression, and *SoyferH2* forward (5′-GCT TTT ATC TCT CGC CCG TTG-3′) and *SoyferH2* reverse (5′-CAT TGT GTG CAA TCG GAA CAG C-3′) for *SoyferH2* expression. Transcript levels were normalized to the expression levels of alpha-*Tubulin* determined using the primers alpha-*Tubulin* forward (5′-TCT TCC ACC CTG AGC AGC TC-3′) and alpha-*Tubulin* reverse (5′-AAC CTT GGA GAC CAG TGC AG-3′). The sizes of the amplified fragments were confirmed by agarose gel electrophoresis.

METAL CONCENTRATION ANALYSIS OF SEEDS

We harvested T_1 and T_2 seeds from the greenhouse and measured metal concentrations in brown seeds, polished seeds, and husks. We converted brown seeds to polished seeds using a Multi-Beads Shocker, as previously described by Masuda et al. (2009a) with modification for *indica* rice polishing. We transferred 20 seeds to a 2-ml plastic tube (Yasui Kikai, Osaka, Japan) and machine-shook them vigorously through four cycles of 2500 rpm for 300 s. We used three replicates of 10 well-polished seeds each for subsequent analyses. Each 10-seed replicate was dried overnight and weighed before digestion. Subsequently, we added 1 ml of 13 M HNO_3 (Wako) and 1 ml of 8.8 M H_2O_2 (Wako) to each liner to digest the seeds inside. We set all the liners in a MARSS XPRESS digester (CEM Japan, Tokyo, Japan) and digested seeds at 220°C for 20 min. After digestion, samples were collected and made up to 5 ml volume with 0.1 mM HCl and filtered as previously described by Masuda et al. (2009a). Subsequently, we measured metal concentrations in digested seed samples using an inductively coupled plasma atomic emission spectrometer (ICPS-8100; Shimadzu, Kyoto, Japan) and calculated seed metal concentrations.

RESULTS

CALLUS INDUCTION AND GROWTH AT STANDARD 2,4-D CONCENTRATIONS

Fifteen Myanmar rice varieties were included in the callus induction and regeneration trials. Calli were induced on both N6D and 2N6 media. Callus induction capacity differed greatly among the varieties (Figure S3 in Supplementary Material). Within varieties, culture conditions also influenced callus formation. In most varieties, callus induction and callus growth were better on N6D media than on 2N6 media (Figures S3 and S4 in Supplementary Material). Callus induction and formation of Paw San Yin (V3) (especially on N6D-N6D medium) was among the best in Myanmar varieties tested, with performance matching that of the *japonica* cultivar TK (**Figure 1**, Figures S3 and S4 in Supplementary Material). Callus initiation in the Paw San Yin variety began 8 days after germination (DAG) on all media, and callus proliferation increased after transfer to new medium, especially on N6D (**Figures 1A,B**).

REGENERATION TESTING AFTER CALLUS INDUCTION AT STANDARD 2,4-D CONCENTRATIONS

We observed the regeneration efficiency of 15 Myanmar rice varieties in various medium combinations. For many varieties, including V2, V4, V6, V9, and V13, calli obtained by 2N6-2N6 treatment turned brown and died on MSre media 15–31 days after transferring (DAT), and no regenerated plants were obtained (Figures S5–S9 in Supplementary Material). Plants regenerated only on the N6D-N6D-MSre-MS medium combination. Regenerated plants of varieties V3 and V14 were obtained on both N6D-N6D-MSre-MS and 2N6-2N6-MSre-MS medium combinations (**Figure 1**, Figure S10 in Supplementary Material). Callus quantity and quality of the Paw San Yin high-quality variety (V3) were similar to those of the TK cultivar in both the callus induction and growth stages, though were somewhat inferior after transfer to MSre regeneration media (**Figure 1**). However, green spots appeared afterward, and callus proliferation increased

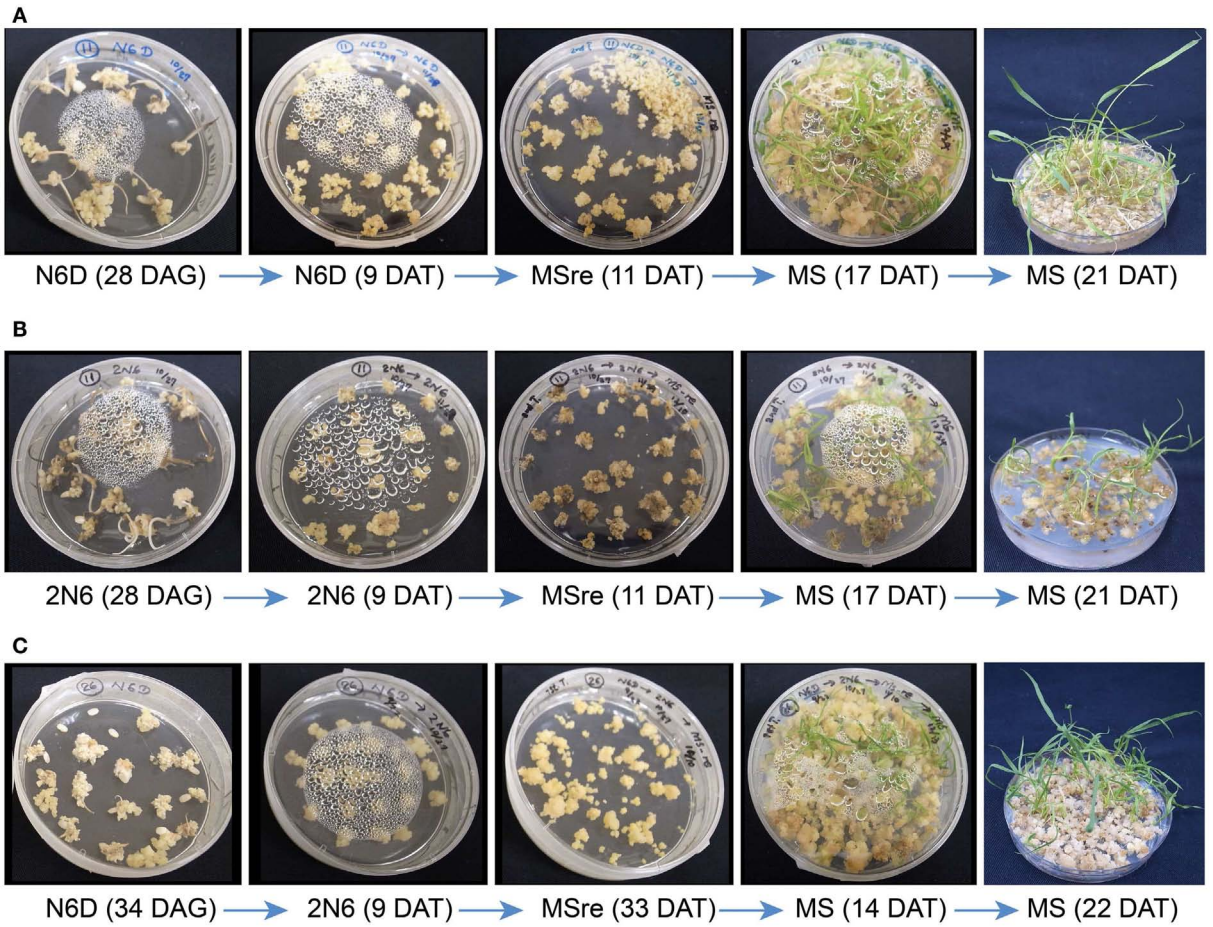

FIGURE 1 | Callus induction and regeneration efficiency in rice varieties Paw San Yin (V3) and Tsukinohikari in different medium combinations. **(A)** Tissue culture of Paw San Yin (V3) in the N6D-N6D-MSre-MS medium combination. **(B)** Tissue culture of Paw San Yin (V3) in the 2N6-2N6-MSre-MS medium combination. **(C)** Tissue culture of Tsukinohikari in the N6D-2N6-MSre-MS medium combination. See "Media Used for Callus Induction and Regeneration Test" in the Section "Materials and Methods" for medium combination. DAG, days after germination; DAT, days after transferring. The numerals shown inside parentheses mean DAG or DAT when photograph was taken on each medium.

considerably after transfer to MS medium. Like several other varieties, many more regenerated plants of V3 developed 21 DAT under the N6D-N6D-MSre-MS medium combination than under the 2N6-2N6-MSre-MS combination (**Figure 1**).

EFFECTS OF 2,4-D CONCENTRATIONS ON CALLUS INDUCTION AND REGENERATION ON N6D AND 2N6 MEDIA

Callus inductions of Myanmar rice varieties V1, V8, V10, and V11 were not adequate on either 2N6 or N6D medium (Figures S11A,B–S14A,B in Supplementary Material). 2,4-D concentration is an important callus induction factor in all rice varieties (Bajaj, 1991). Accordingly, we tested the effects of 2,4-D concentrations on callus induction and growth of selected varieties, aiming to improve callus induction and regeneration efficiency. We tested various 2,4-D concentrations, e.g., 4 and 6 mg l^{-1} in addition to the standard concentration of 2 mg l^{-1}.

Some elevated 2,4-D concentrations in N6D media enhanced callus induction and growth, and raised the regeneration efficiency in V1 (N6D6-N6D6-MSre-MS), V8 (N6D4-N6D4-MSre-MS), V10 (N6D4-N6D4-MSre-MS), and V11 (N6D6-N6D6-MSre-MS) (Figures S11–S14 in Supplementary Material). V5 and V7 were also tested at 2,4-D concentrations of 4 and 6 mg l^{-1}, but callus production was poor and no regenerated plants were produced (data not shown).

IDENTIFICATION OF THE BEST MEDIUM COMBINATION AND REGENERATION EFFICIENCY

Best culture types in which regenerated plants were obtained for each variety, period kept in each medium from callus induction to acclimation, and numbers of regenerated plants are presented in Table S1 in Supplementary Material. During both callus induction and growth stages, we assessed callus quantity (degree of callus induction) and quality (callus yellowness, size, and hardness). Scores for diverse culture media within and among varieties were used to identify the best culture conditions. Among the 15 Myanmar rice varieties tested, Paw San Yin produced best calli and scored best in these assessments. Relationships between callus condition and the number of regenerated plants in the best medium for each variety are presented in Figure S15 in Supplementary Material.

After examining performance in five types of medium combination, we identified the best medium combination for each variety (**Figure 2**). Among the 15 Myanmar rice varieties tested, 13 varieties were able to regenerate (**Figure 2**; Table S1 in Supplementary Material). We calculated regeneration efficiency for each variety in the best medium among those tested (see Plant Regeneration Test in the Materials and Methods; **Figure 3**). Regeneration efficiency was very different among varieties. Most regenerated well and some exceeded rates of the cultivar TK. Yezin Lone Thwe (V6), Yadana Toe (V10), Hmawbi 4 (V13), and Hmawbi 5 (V14) regenerated poorly; Ayar Min (V2), Hmawbi 3 (V12), and Hmawbi Kauk Nyin Hmwe (V15) regenerated well. Paw San Yin (V3) had intermediate regeneration efficiency that was higher than TK.

We also recorded time to green spot appearance on regeneration media (MSre). All 13 regenerating Myanmar rice varieties produced green spots on calli sooner than cultivar TK (**Figure 4**). Green spots developed most quickly in Ayar Min (V2).

TRANSFORMATION OF PAW SAN YIN-FER-NAS-YSL2

We selected the high-quality variety Paw San Yin (V3) for transformation based on its superior callus induction capacity and moderate regeneration efficiency. In addition, Fe content in its polished seeds was also the third highest ($2.2\,\mu g\,g^{-1}$) among 15 high-yield and high-quality Myanmar varieties tested (data not shown). Callus induction of Paw San Yin on N6D media took 29 days (**Figure 5A**). Callus condition of Paw San Yin was the best among the varieties tested. Paw San Yin calli were hard, yellow, and larger than those of most other Myanmar rice varieties (**Figures 1A** and **5A,B**, Figures S3 and S4 in Supplementary Material). Paw San Yin calli were smaller than those of TK, but callus induction rates were similar in the two varieties (**Figures 1** and **5A**). *Agrobacterium*-infection was performed at OD concentrations of 0.1 and 0.01 because the effect of bacterial density on callus infection time is a key effect on transgenic efficiency, and excess proliferation of *Agrobacterium* decreases the transformation frequency during co-cultivation step (Ozawa, 2009). Callus appearance differed little between the two *Agrobacterium* concentrations (**Figures 5C–F**), except that calli were slightly browner at OD 0.1 during the first 2 weeks in the first selection media (**Figure 5D**). After selection steps, calli were transferred to MSre-CH50 (MS regeneration media supplemented with $500\,mg\,l^{-1}$ claforan and $50\,mg\,l^{-1}$ hygromycin) for ∼1.0 month

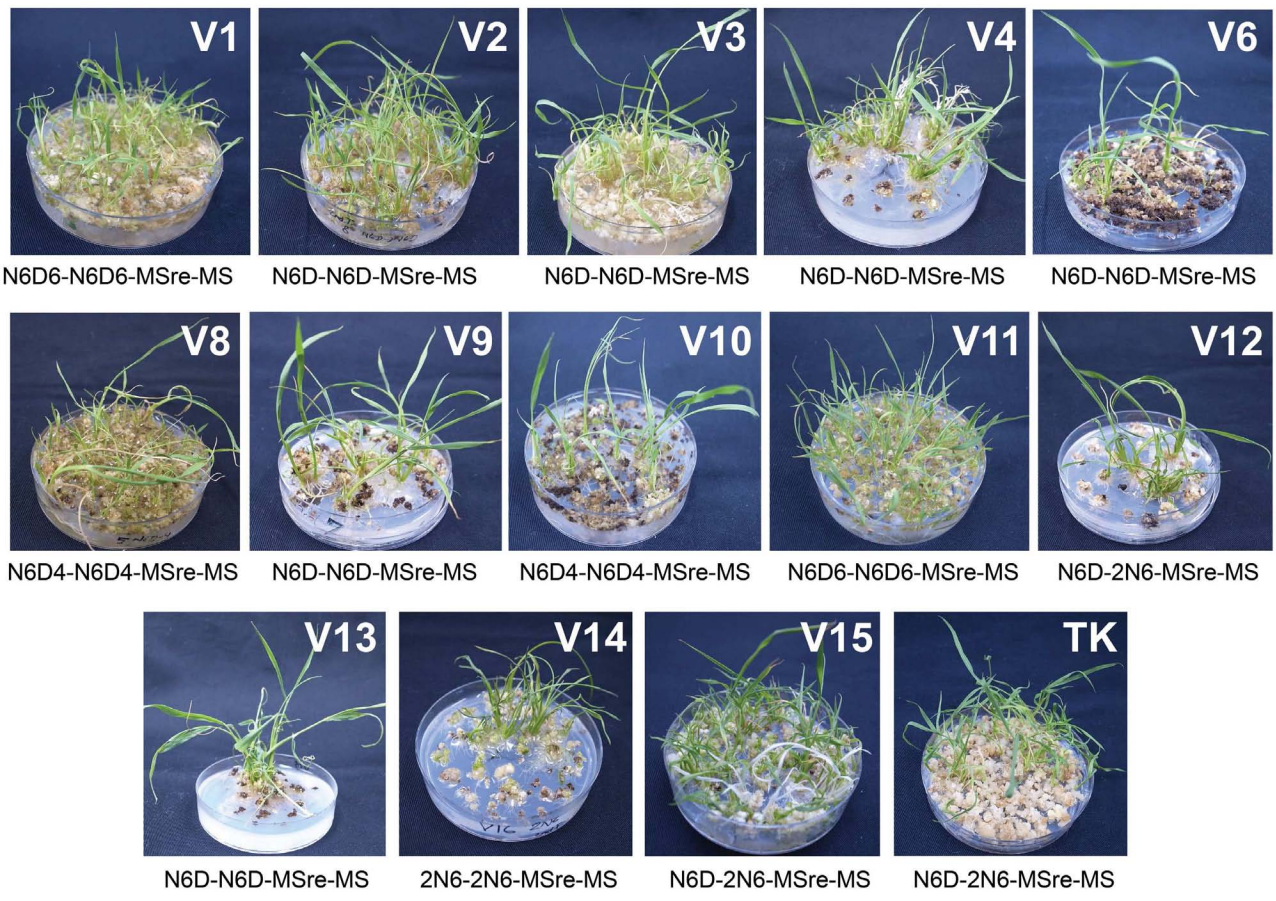

FIGURE 2 | Identification of the best medium combination for each variety. V1–V15 shown on upper right part of each picture represent the variety numbers described in **Table 1** and TK means Tsukinohikari. The media combination described under each picture showed the best media combination for each variety. Pictures of regenerated plants were taken at 3 days after acclimation. Pictures of V5 and V7 were not shown as regenerated plants were not obtained from these two varieties.

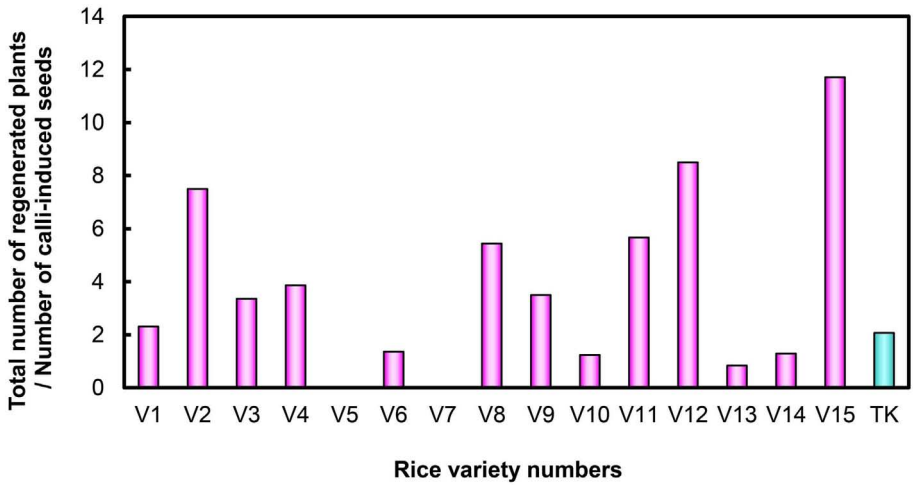

FIGURE 3 | Regeneration efficiency of Myanmar rice varieties and the Tsukinohikari variety. V1–V15 under horizontal bar represent the rice variety numbers described in **Table 1** and TK means Tsukinohikari. Numbers on vertical bars are quotients of total numbers of regenerated plants divided by numbers of callus-induced seeds, which represent regeneration efficiency.

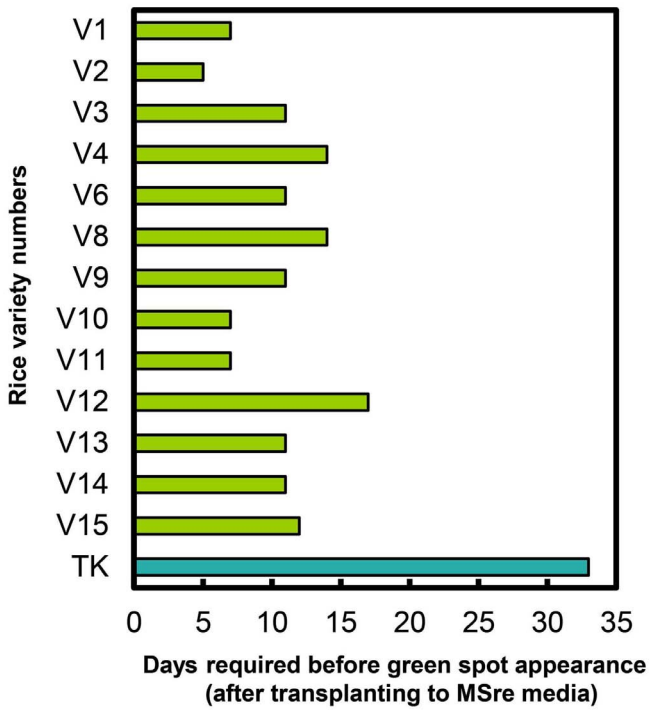

FIGURE 4 | Durations of time required for green spot appearance in Myanmar rice varieties and in the variety Tsukinohikari. V1–V15 on vertical bar represent the variety numbers described in **Table 1** and TK means Tsukinohikari. Durations of time from transfer to MSre media to green spot appearance are indicated. Regenerated plants were obtained from these green spots.

(**Figures 5G,H**). However, no green spots appeared on calli from MSre-CH50 media 45 DAT. After transferring calli onto MS-C media, many green spots appeared and callus shooting occurred (**Figure 5I**). Four regenerated plants were obtained and subjected to acclimation for 10 days to produce hardier seedlings (**Figure 5J**). Subsequently, T_0 plants were grown in a greenhouse to check for inserted genes and to obtain T_1 seeds for further analyses (**Figure 5K**).

INSERTED GENE CHECK BY GENOMIC PCR

We checked four regenerated plants for selected gene insertions using genomic PCR. Paw San Yin-Fer-NAS-YSL2 line 1 (L1) contained insertions, including *OsGlb* promoter-*SoyferH2*, *OsAct* promoter-*HvNAS1*, *HPT*, *NPTII*, and *iGUS*, which was also the case in the Fer-NAS-YSL2 vector itself (**Figure 6**). The inserted genes were lacking in non-transgenic Paw San Yin (NT). We detected the endogenous sequence for the rice *OsActin1* gene in L1 and NT.

METAL CONCENTRATIONS IN T_1 POLISHED SEEDS

The mean Fe concentration in T_1 polished seeds of L1 was $6.3\,\mu g\,g^{-1}$, a value double that in NT ($3.2\,\mu g\,g^{-1}$) (**Figure 7A**). The mean Zn concentration in L1 seeds was $\sim 34.2\,\mu g\,g^{-1}$, slightly higher than in NT seeds ($32.4\,\mu g\,g^{-1}$) (**Figure 7B**). L1 had a higher manganese (Mn) concentration than NT, its copper (Cu) concentration was similar to that of NT, and its calcium (Ca) concentration in polished seeds was lower than in NT (Figures S16A–C in Supplementary Material). In T_1 brown seeds, L1 had higher Fe and Mn concentrations and lower Cu concentration than NT (Figures S17A,D,E in Supplementary Material). Zn and Ca concentrations in brown seeds were not different between L1 and NT (Figures S17B,C in Supplementary Material). In T_1 husk, L1 had lower Zn concentration and higher Ca and Cu concentrations than NT (Figures S18B–D in Supplementary Material). The cadmium (Cd) concentration was lower in polished seeds, brown seeds and husk of L1 than in those of NT (Figures S16D, S17F, and S18E in Supplementary Material).

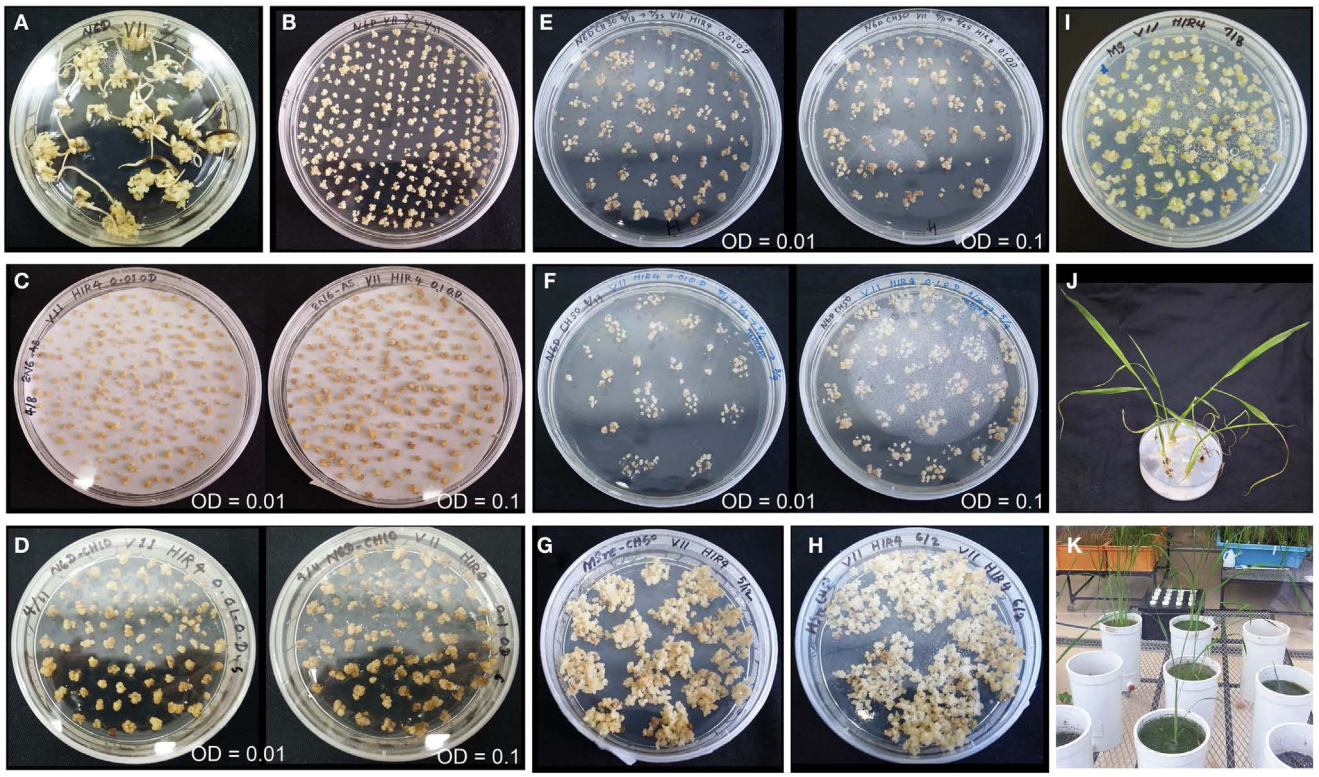

FIGURE 5 | Transformation of Paw San Yin-Fer-NAS-YSL2. **(A)** Callus induction (at 29 DAG). **(B)** Pre-incubation (at 8 DAT). **(C)** Three days following *Agrobacterium*-infection. **(D)** Calli on 1st selection medium of N6D-CH10 (at 7 DAT). **(E)** Calli on 2nd selection medium of N6D-CH30 (at 14 DAT). **(F)** Calli on 3rd selection medium of N6D-CH50 (at 7 DAT). **(G)** Calli on MS regeneration medium (MSre-CH50) (at 20 DAT). **(H)** Calli on MSre-CH50 (at 32 DAT). **(I)** Green spots appearance of calli and shoots emergence on MS medium (at 4 DAT). **(J)** Regenerated plants on MS medium. **(K)** Greenhouse-grown T_0 plants. Left panels of **(C–F)** show transformation with *Agrobacterium* at a concentration of OD = 0.01; right panels of **(C–F)** shows transformation at a concentration of OD = 0.1. The numerals shown with DAG or DAT mean DAG or DAT when photograph was taken on each medium.

GENE EXPRESSION IN IMMATURE T_2 SEEDS

T_1 seeds of L1 were germinated on MS-CH50 media. Subsequently, we cultivated T_1 plants (sublines) in a greenhouse. No significant differences were observed in plant growth and morphology between L1 sublines and NT under these cultivation conditions (**Figure 8**). We confirmed the expressions of *HvNAS1*, *OsYSL2*, and *SoyferH2* in immature T_2 seeds of L1 sublines by real-time RT-PCR analysis (**Figure 9**). Enhanced expressions of *HvNAS1*, *OsYSL2*, and *SoyferH2* genes were detected in L1 sublines compared with NT (**Figure 9**). *HvNAS1* expression in NT was barely detectable because sequences of *OsNAS1* and *HvNAS1* were very similar, and *OsNAS1* expression may be detectable in this line (**Figure 9A**). Expression of *OsYSL2* was very low in NT (**Figure 9B**). *SoyferH2* expression was undetectable in the NT line (**Figure 9C**). In contrast, *HvNAS1*, *OsYSL2*, and *SoyferH2* were strongly expressed in the L1-1, L1-2, and L1-3 sublines.

METAL CONCENTRATIONS IN T_2 SEEDS

We measured metal concentrations in T_2 polished seeds from the L1-1, L1-2, and L1-3 sublines and in NT (**Figures 10A,B**, Figure S19 in Supplementary Material). Mean Fe concentrations in polished seeds of L1-1, L1-2, and L1-3 sublines were 5.02, 4.31, and 3.96 $\mu g\,g^{-1}$, respectively, values that were 3.0–3.4-fold higher than values in NT seeds (1.46 $\mu g\,g^{-1}$) (**Figure 10A**). Mean Zn concentrations in polished seeds of L1-1, L1-2, and L1-3 sublines were 38.6, 39.2, and 36.1 $\mu g\,g^{-1}$, respectively, values that were 22–33% higher than that in NT seeds (29.5 $\mu g\,g^{-1}$) (**Figure 10B**). No clear differences were observed in polished seed concentrations of Ca, Mn, and Cu between the L1-1, L1-2, and L1-3 sublines and NT (Figures S19A–C in Supplementary Material). Polished seed Cd concentrations in the L1-1, L1-2, and L1-3 sublines were reduced by ∼20% compared to NT seeds (Figure S19D in Supplementary Material).

Mean T_2 brown seed Fe concentrations in the L1-1, L1-2, and L1-3 sublines were 30.4, 25.4, and 26.0 $\mu g\,g^{-1}$, respectively; these values were 15–37% higher than that in NT seeds (22.1 $\mu g\,g^{-1}$) (**Figure 10C**). Mean T_2 brown seed Zn concentrations in the L1-1, L1-2, and L1-3 sublines were 50.9, 51.9, and 46.6 $\mu g\,g^{-1}$, respectively; these values were 12–24% higher than that in NT seeds (41.7 $\mu g\,g^{-1}$) (**Figure 10D**). Ca, Mn, and Cu polished rice concentrations in the L1-1, L1-2, and L1-3 sublines were little different from that in NT (Figures S20A–C in Supplementary Material). Mean T_2 brown seed Cd concentrations in the L1-1, L1-2, and L1-3 sublines were 0.07, 0.04, and 0.03 $\mu g\,g^{-1}$, respectively; these values were two to fourfold lower than that in NT (0.1 $\mu g\,g^{-1}$) (Figure S20D in Supplementary Material).

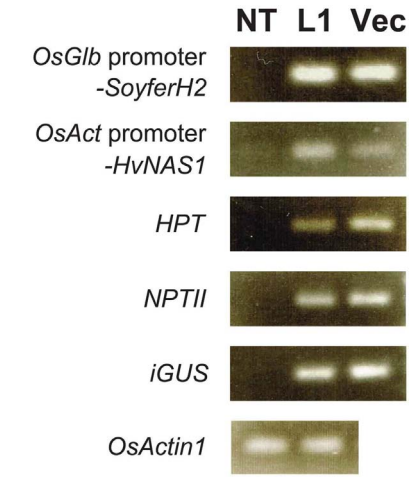

FIGURE 6 | Confirmation of gene insertion in transgenic Paw San Yin-Fer-NAS-YSL2 line 1. NT, non-transgenic Paw San Yin line; L1, Paw San Yin-Fer-NAS-YSL2 line 1; Vec, Fer-NAS-YSL2 vector (positive control). *OsGlb* promoter-*SoyferH2*, soybean *Ferritin* gene *SoyferH2* with promoter region of the 26 kDa *OsGlb1* gene; *OsAct* promoter-*HvNAS1*, barley nicotianamine synthase 1 gene with promoter region of the rice *OsActin1* gene; *HPT*, hygromycin phosphotransferase gene; *NPTII*, neomycin phosphotransferase II gene; *iGUS*, β-glucuronidase gene *with an intron*; *OsActin1*, endogenous rice *actin* gene.

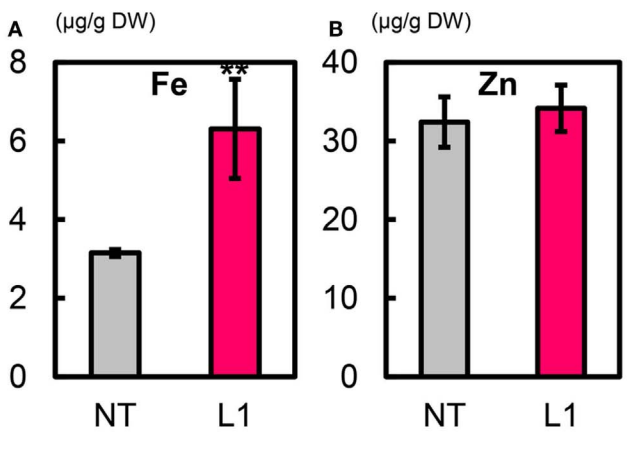

FIGURE 7 | Metal concentrations in T_1 polished seeds of Paw San Yin-Fer-NAS-YSL2. **(A)** Fe concentration. **(B)** Zn concentration. NT, non-transgenic Paw San Yin. L1, Paw San Yin-Fer-NAS-YSL2 transgenic line 1. Bars represent means ± SE, $n = 3$. Asterisks (**) above the bars indicate significant differences at $P < 0.01$ between NT and L1 (determined by *t*-test).

T_2 husk Fe and Zn concentrations were similar between the L1 sublines and NT (Figures S21A,B in Supplementary Material). Ca, Mn, and Cu were higher in the sublines than in NT (Figures S21C–E in Supplementary Material).

FE AND ZN CONTENTS PER SEED

We estimated the partitioning of Fe and Zn among components of the seed structure (**Figure 11**, Figure S22 in Supplementary Material). Within T_2 seeds of the L1-1 and L1-2 sublines, the total mean Fe contents were 590 and 450 ng seed^{-1} (including endosperm, bran, and husk), respectively; the value for NT was 455 ng seed^{-1} (**Figure 11A**). Mean Fe contents in endosperm (polished seed) of the L1-1 and L1-2 sublines (93.4 and 72.2 ng seed^{-1}, respectively) were three to fourfold higher than that of NT (23.9 ng seed^{-1}). Thus, the new lines had higher Fe allocation to edible seed portions than to either bran or husk (**Figure 11A**).

Total mean Zn contents per seed in the L1-1 and L1-2 sublines (1016 and 911 ng seed^{-1}, respectively) were higher than in NT (808 ng seed^{-1}) (**Figure 11B**). The endosperm Zn contents in the L1-1 and L1-2 sublines were 1.4–1.5-fold higher than in NT seeds. We observed similar trends in differences between the L1 line and NT in T_1 seeds (Figure S22 in Supplementary Material).

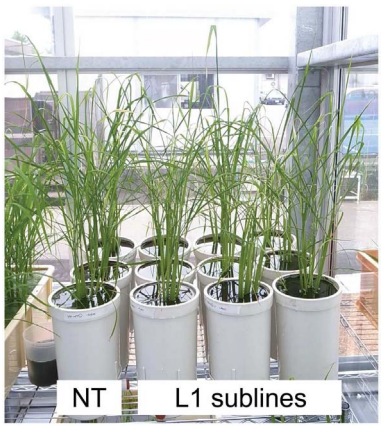

FIGURE 8 | Greenhouse-grown T_1 plants. Photograph was taken during tillering stage at 30 days after transplanting. NT, non-transgenic Paw San Yin. L1 sublines, Paw San Yin-Fer-NAS-YSL2 transgenic line 1 sublines.

DISCUSSION

PAW SAN YIN WAS SELECTED FOR RICE TRANSFORMATION

Calli of Myanmar rice varieties were smaller than those of cultivar TK. The small, soft calli had limited tolerance to *Agrobacterium*-infection during transformation in our transgenic trials. However, callus inductions were good in V3, V6, and V14, and proliferations were closely similar to that of TK (Figure S4 in Supplementary Material). Particularly, V3 had a good callus induction response, and induced calli of V3 were largest; they were clear yellow and had the highest hardness index, traits that were closely similar to those of TK calli (**Figure 1**). These callus attributes make V3 suitable for rice transformation. *Agrobacterium*-infection is an important step in practical transformation, and callus tolerance of this infection is required for a successful outcome. Importantly, V3 calli in the N6D-N6D-MSre-MS medium combination maintained good condition in MS rooting media and produced many regenerated plantlets in the regeneration test (**Figure 1A**).

Myanmar people have one of the highest rice consumption rates in the world (FAO, 2002). The estimated polished seed Fe concentration needed to increase for these people is ∼8.6 μg g^{-1} (Supplementary Material). Our premise was that by using a high-Fe rice variety, we would be able to produce lines with much higher

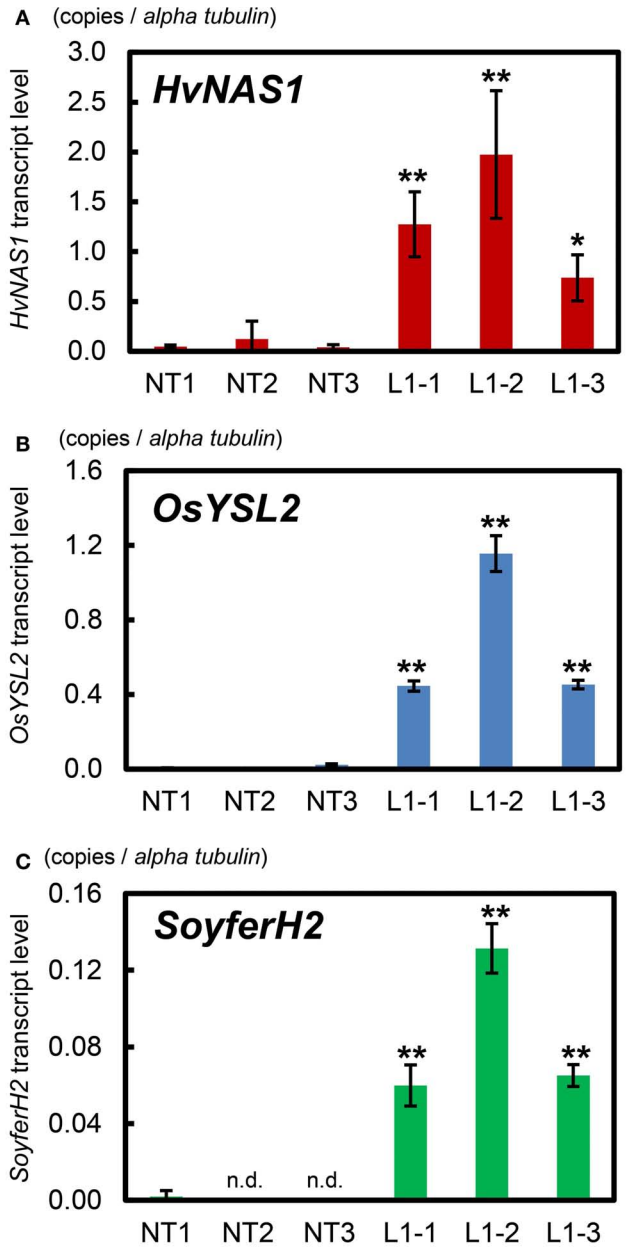

FIGURE 9 | Quantitative real-time RT-PCR analysis of *HvNAS1, OsYSL2,* and *SoyferH2*. (A) *HvNAS1*, **(B)** *OsYSL2*, and **(C)** *SoyferH2* expression levels. T_1 plants were cultivated in commercial soil (Bonsolichigou) in a greenhouse. Total RNA was extracted from immature T_2 seeds (seeds at the early milky stage, 10 days after fertilization) of each line ($n = 3$). NT, non-transgenic Paw San Yin. L1-1, L1-2, and L1-3, Paw San Yin-Fer-NAS-YSL2 transgenic line 1 sublines. Bars represent means ± SE of three independent real-time RT-PCR reactions. Asterisks (*) and (**) above the bars indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, between NT and L1 sublines (determined by *t*-test). n.d., not detected.

levels of Fe biofortification through transgenic transformations using the Fer-NAS-YSL2 vector (Masuda et al., 2012). Myanmar people will probably readily accept a novel Fe-biofortified rice produced from a native variety, and it was for this reason that we screened diverse Myanmar varieties for genetic variation in micronutrient concentrations (Aung et al., unpublished data). This screening identified Paw San Yin (V3) as a variety with high-Fe concentration ($\sim$2.2 $\mu g\, g^{-1}$) in relation to other popular rice strains tested (Aung et al., unpublished data): V3 had more than double the Fe content of the variety with the lowest concentration (Ma Naw Htun) (Aung et al., unpublished data) and double the content of field-grown TK rice (Masuda et al., 2012). Moreover, harvested Paw San Yin seed converts readily to a high-quality, aromatic rice product for human consumption. Grusak and Cakmak (2005) demonstrated that aromatic rice genotypes contain consistently more Fe and Zn than non-aromatic genotypes, indicating that the aromatic germplasm has promise as a genetic resource for improving rice micronutrient levels. Therefore, we selected Paw San Yin (V3) for practical transformation to produce Fe-biofortified rice.

TRANSFORMATION OF PAW SAN YIN-FER-NAS-YSL2

Our transformation procedure followed, except for a few modifications, an established protocol for *japonica* variety cv. TK developed by Hiei et al. (1994) and Akiyama et al. (1997). Hiei et al. (1994) reported that prior to infection, pre-cultivation of calli on fresh medium for 4 days is an important first step in *japonica* rice transformation. We demonstrated that a longer 8-day pre-incubation period was required for production of hard V3 calli for transformation. Callus condition was good during induction and *Agrobacterium* co-cultivation, though calli size were smaller than those of TK (**Figures 5A–C**).

Transformation efficiency was low in Paw San Yin-Fer-NAS-YSL2. Two possible explanations exist for this outcome. Although Paw San Yin calli were in adequate condition for transformation, they were negatively affected and damaged by *Agrobacterium*-infection to a greater extent than TK calli, where *Agrobacterium* presence was minimal in calli after the *Agrobacterium* wash (**data not shown**). Therefore, low regeneration rates from selected calli in this Paw San Yin variety may have resulted from adverse effects of *Agrobacterium*-infection when the bacterium further propagated on calli during the selection periods (**Figures 5D–F**). Hence, we applied a claforan concentration double that was used for TK transformation when selecting media that prevented *Agrobacterium* propagation. Green spots did not appear on MSre-CH50 through 1 month following transfer. The large size of the vector that we introduced may also have been responsible for the low rate of transformation we obtained (Masuda et al., 2012) (Figure S2 in Supplementary Material).

GENE EXPRESSION ANALYSIS AND FUNCTIONS OF INTRODUCED GENES

Real-time RT-PCR analyses confirmed elevated expressions of *HvNAS1*, *OsYSL2*, and *SoyferH2* transgenes in Paw San Yin-Fer-NAS-YSL2 L1-1, L1-2, and L1-3 T_2 sublines (**Figure 9**). *HvNAS1* encodes barley nicotianamine synthase, which biosynthesizes the divalent metal chelator nicotianamine (Higuchi et al., 1999), and this gene was overexpressed by the constitutive rice *Actin1* promoter in Fer-NAS-YSL2 vector (Figure S2 in Supplementary Material) (Masuda et al., 2012). As is the case for TK transformants with introduced Fer-NAS-YSL2 (Masuda et al., 2012),

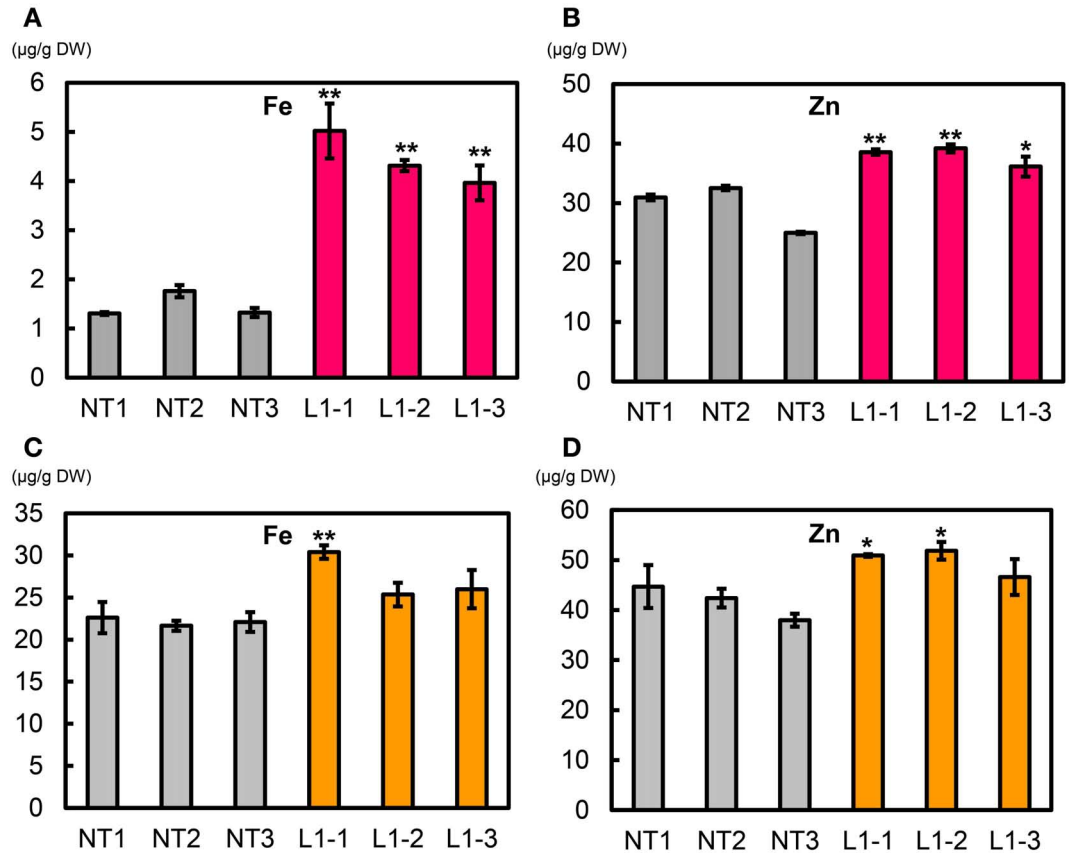

FIGURE 10 | Metal concentrations in T_2 polished and brown seeds of Paw San Yin-Fer-NAS-YSL2. **(A)** Fe concentration in T_2 polished seeds. **(B)** Zn concentration in T_2 polished seeds. **(C)** Fe concentration in T_2 brown seeds. **(D)** Zn concentration in T_2 brown seeds. NT, non-transgenic Paw San Yin. L1-1, L1-2, and L1-3, Paw San Yin-Fer-NAS-YSL2 transgenic line 1 sublines. Bars represent means ± SE, $n = 3$. Asterisks (*) and (**) above the bars indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, between NT and L1 sublines demonstrated by *t*-tests.

HvNAS1 was strongly expressed in Paw San Yin-Fer-NAS-YSL2 T_2 seeds during the seed milky stage 10 days after fertilization (early seed maturation stage) (**Figure 9A**). *OsYSL2* encodes an Fe(II)-nicotianamine and Mn(II)-nicotianamine transporter, which are responsible for internal metal transport in rice (Koike et al., 2004; Ishimaru et al., 2010). The Fer-NAS-YSL2 vector contains two cassettes for *OsYSL2* expression (Figure S2 in Supplementary Material), each driven by an *OsSUT1* promoter that drives expression in the early seed maturation stage beginning 7 days after fertilization (Hirose et al., 2002) and by an endosperm-specific *OsGlb1* promoter (Qu and Takaiwa, 2004). As a result, *OsYSL2* was strongly expressed in Paw San Yin-Fer-NAS-YSL2 T_2 seeds during the seed maturation stage 10 days after fertilization (**Figure 9B**). *SoyferH2* encodes the soybean Fe storage protein ferritin, which was driven by endosperm-specific *OsGlb1* and *OsGluB1* promoters in the Fer-NAS-YSL2 vector (Figure S2 in Supplementary Material). Qu and Takaiwa (2004) demonstrated that the GUS reporter protein accumulates in the early maturation stage 7 days after fertilization in *OsGlb1* promoter-*GUS* and 2.3-kb *OsGluB1* promoter-*GUS* plants. Hence, it was assumed that *ferritin* expression under the control of the *OsGlb1* and 2.3-kb *OsGluB1* also initiates ferritin protein accumulation in the early seed maturation stage 7 days after fertilization. We clearly detected *SoyferH2* expression in Paw San Yin-Fer-NAS-YSL2 10 days after fertilization (**Figure 9C**). Thus, we assumed that coordination occurred in the timing of enhancement in nicotianamine production through *HvNAS1* overexpression, the enhancement of Fe(II)-nicotianamine transport by *OsYSL2* expressed under the control of the *OsSUT1* and *OsGlb1* promoters, and the enhancement of Fe accumulation by the *OsGlb1* promoter-*ferritin* and 2.3-kb *OsGluB1* promoter-*ferritin* (Masuda et al., 2012).

Fe contents in polished seeds of Paw San Yin-Fer-NAS-YSL2 sublines were three to fourfold higher than those of NT (**Figure 11A**). We supposed that Fe transport into the plant body and into grain was enhanced by elevated production of nicotianamine by overexpression of *HvNAS1* (Lee et al., 2009; Masuda et al., 2009a; Johnson et al., 2011) and improved expression of the *OsSUT1 promoter-OsYSL2* (Koike et al., 2004; Ishimaru et al., 2010). Moreover, the Fe storage protein ferritin, which accumulated in the endosperm, may have worked cooperatively in Fe translocation to the endosperm component of grain. We demonstrated that genes involved in Fe transport and accumulation in the endosperm (*HvNAS1*, *OsYSL2*, and *SoyferH2*) work efficiently together to increase Fe content in the endosperm, the edible component of the rice grain.

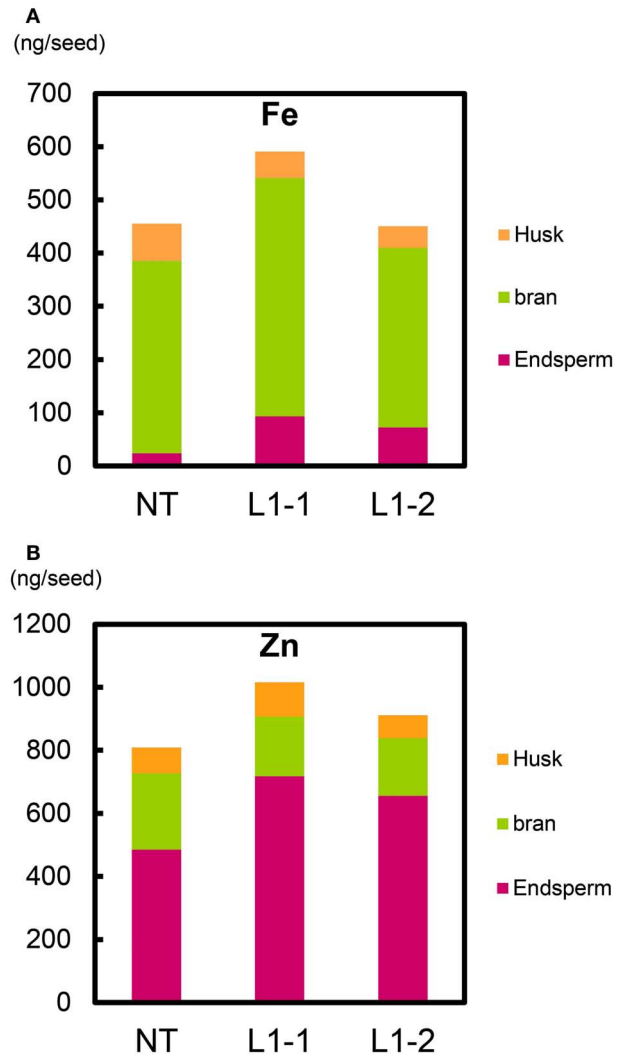

FIGURE 11 | Fe and Zn content per T_2 seed of Paw San Yin-Fer-NAS-YSL2. **(A)** Fe content in husk, bran, and endosperm per T_2 seed. **(B)** Zn content in husk, bran, and endosperm per T_2 seed. NT, non-transgenic Paw San Yin; L1-1 and L1-2, Paw San Yin-Fer-NAS-YSL2 transgenic line 1 sublines. Metal content of polished seed is shown as endosperm. Metal content of bran is calculated by subtracting metal content of polished seed from that of brown seed.

SEED FE CONCENTRATION INCREASED IN PAW SAN YIN-FER-NAS-YSL2

Fe concentration in Paw San Yin-Fer-NAS-YSL2 brown seed was 15–37% higher than that in NT brown seed (**Figure 10C**) and 3.4-fold higher in polished grain (**Figure 10A**), clearly demonstrating that Fe was effectively translocated into the endosperm. There were reports of other high-Fe-content rice, showing two to over three times increase in Fe concentration of various rice varieties (Goto et al., 1999; Lucca et al., 2001; Vasconcelos et al., 2003; Lee et al., 2009; Masuda et al., 2009a; Johnson et al., 2011; Paul et al., 2012). Using the transgenic approaches, most trials reported the rice varieties efficient for *in vitro* and molecular research, or the varieties which have already established transgenic procedures. There are still only a few trials working with popular rice varieties for Fe biofortification. In our present research, we generated the Fe biofortified Paw San Yin rice, which is a high-quality and currently cultivated and consumed rice variety in Myanmar, with 3.4-fold increase in Fe concentration in rice endosperm.

We found that the transgenic efficiency of Paw San Yin was low and difficult to obtain many transgenic lines. The main limiting factor to increase in Fe-density of the transgenic Paw San Yin is the difficulty in obtaining many transgenic lines which include all inserted genes. In our previous report by Masuda et al. (2012), the line with six times increase in Fe concentration of TK rice was selected among 45 lines. If many more lines of Paw San Yin were obtained, there is a possibility to obtain higher Fe lines.

In the present study, our objective was to increase the polished rice Fe concentration about 4.5-fold in the high Fe variety, Paw San Yin, based on a per capita rice consumption of 578 g day^{-1} in Myanmar (Supplementary Material). T_2 seed analysis demonstrated that the polished seed Fe concentration was 3.4-fold higher than in NT line (**Figure 10A**). Thus, we closely approached the estimated target level. Therefore, it can be assumed that 3.4-fold increase in Fe concentration of Paw San Yin variety is remarkable.

Fer-NAS-YSL2 gene improved Fe accumulation and transport, but Fe uptake was not enhanced yet. Mugineic acid is known as a natural Fe(III) chelator used in Fe acquisition from the rhizosphere in barley and some other graminaceous plants. Masuda et al. (2008) analyzed the transgenic rice line carrying barley *IDS3* gene, which is the mugineic acid synthase gene. The transformants showed 1.4-fold increase in Fe concentration in polished seeds of rice grown in field cultivation. If barley *IDS3* is introduced to this Fer-NAS-YSL2 gene to enhance Fe uptake from soil, there is a possibility to increase more Fe accumulation in grain.

ZN CONCENTRATION INCREASED IN PAW SAN YIN-FER-NAS-YSL2

Zn deficiency is a serious problem in Myanmar (Hotz and Brown, 2004). In polished seeds produced from rice grown in the MRRC field, Paw San Yin had the highest Zn concentration ($\sim$19.1 $\mu g\,g^{-1}$), which was almost double those in a range of other popular rice varieties (Aung et al., unpublished data). The Zn concentration in polished seeds was elevated by 30% in Paw San Yin-Fer-NAS-YSL2 sublines (**Figure 10B**), which may meet the target concentration for Zn biofortification in the Myanmar diet.

Zn content in the endosperm of Paw San Yin-Fer-NAS-YSL2 was 1.4-fold higher than that in NT endosperm (**Figure 11B**). This outcome may have resulted from *HvNAS1* overexpression and endosperm-specific expression of the *ferritin* gene. *NAS* overexpression increases both Fe and Zn concentrations in seeds (Lee et al., 2009; Masuda et al., 2009a; Johnson et al., 2011). Endosperm-specific expression of *ferritin* also increases Zn concentrations in seeds (Goto et al., 1999; Vasconcelos et al., 2003). In comparison with Fe, Zn levels changed little, likely because more Zn occurs naturally in endosperm than in bran; hence, polishing has less of an effect on Zn levels than on Fe levels (**Figure 11**).

OTHER METAL CONCENTRATIONS IN SEEDS OF PAW SAN YIN-FER-NAS-YSL2

Concentrations of other metals in both polished and brown seeds, including Ca, Mn, and Cu, were little different between Paw San Yin-Fer-NAS-YSL2 sublines and NT, although levels were

somewhat higher in husks of transformed sublines (Figures S19A–C, S20A–C, and S21C–E in Supplementary Material). This lack of an effect may be attributable to specific targeting of the Fer-NAS-YSL2 vector toward accumulation of an elevated Fe concentration in endosperm. Thus, the Fer-NAS-YSL2 construct increased Fe concentrations in grain without reducing concentrations of other required minerals.

Cadmium is a toxic metal present in soil. It was detected in seeds of both NT and the Fer-NAS-YSL2 line (Figures S16D, S17F, S18E, S19D, and S20D in Supplementary Material). Cd concentrations in our samples were low compared to the level at which the metal becomes toxic in polished rice ($\sim$0.4 μg g^{-1}) (CODEX, 2012). Notably, Cd concentrations in brown and polished seeds of Paw San Yin-Fer-NAS-YSL2 T_1 and T_2 generations were remarkably lower than levels in Paw San Yin-NT (Figures S16D, S17F, S18E, S19D, and S20D in Supplementary Material). The Cd concentration in T_2 polished seeds was about 20% lower in Paw San Yin-Fer-NAS-YSL2 than in NT (Figure S19D in Supplementary Material). One may reasonably argue that when Fe transportation to seed was increased in this transgenic rice, the expression levels of Fe transporters that also transport Cd, such as *OsIRT1* (Nakanishi et al., 2006), *OsNRAMP5* (Ishimaru et al., 2012; Sasaki et al., 2012), or other unknown Fe and Cd transporters, may decrease. Thus, it was assumed that Cd concentration in Paw San Yin-Fer-NAS-YSL2 seeds was lowered in the transgenic sublines. The seed Cd concentration in the *HvNAS1* overexpression line also decreased (Masuda et al., 2012). Hence, rice with an Fe content elevated through insertion of the Fer-NAS-YSL2 construct will likely be useful in reducing dietary Cd levels, especially in crops grown on Cd contaminated soil.

CONCLUSION

Our starting premise was that currently consumed rice varieties with high Fe and Zn contents would be suitable starting material for the production of Fe-fortified grain. We screened methods for regenerating diverse Myanmar rice varieties and identified those varieties with good callus induction and high regeneration efficiency, which might contribute to transgenic development.

Paw San Yin is a high-quality, popular rice variety. The first Myanmar transgenic rice was produced successfully from this variety. Through our program, we achieved Fe-biofortified Paw San Yin rice with an Fe concentration 3.4-fold higher than the concentration in control seeds. The degree of Fe increase closely approached the dietary target for Myanmar people. The Zn concentration in the new transgenic sublines may also fulfill Zn requirements for the Myanmar population.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2013.00158/abstract

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The road to micronutrient biofortification of rice: progress and prospects

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Biofortification (increasing the contents of vitamins and minerals through plant breeding or biotechnology) of food crops with micronutrient elements has the potential to combat widespread micronutrient deficiencies in humans. Rice (*Oryza sativa* L.) feeds more than half of the world's population and is used as a staple food in many parts of Asia. As in other plants, micronutrient transport in rice is controlled at several stages, including uptake from soil, transport from root to shoot, careful control of subcellular micronutrient transport, and finally, and most importantly, transport to seeds. To enhance micronutrient accumulation in rice seeds, we need to understand and carefully regulate all of these processes. During the last decade, numerous attempts such as increasing the contents/expression of genes encoding metal chelators (mostly phytosiderophores) and metal transporters; Fe storage protein ferritin and phytase were successfully undertaken to significantly increase the micronutrient content of rice. However, despite the rapid progress in biofortification of rice, the commercialization of biofortified crops has not yet been achieved. Here, we briefly review the progress in biofortification of rice with micronutrient elements (Fe, Zn, and Mn) and discuss future prospects to mitigate widespread micronutrient deficiencies in humans.

Keywords: biofortification, biosafety, iron, micronutrient transport, *Oryza sativa* L., zinc

INTRODUCTION

Micronutrients are not only essential for plant growth and development but are also integral to human and animal health. In the last two decades, the concept of hidden hunger (deficiency of certain vitamins and micronutrient nutrients despite eating enough calories) has been well established (Nilson and Piza, 1998). As a result, the importance of micronutrient nutrition is increasing at a great pace. The micronutrients iron (Fe), zinc (Zn), and manganese (Mn) are of particular interest, given that all three are essential micronutrients for all higher organisms and we will focus on these three micronutrients in this review. Fe serves as an important cofactor for various enzymes performing basic functions in humans. Fe deficiency results in anemia and is also reported to have pathological consequences (Stoltzfus, 2003; Hentze et al., 2004). Zn deficiency causes growth retardation, hypogonadism, immune dysfunction, and cognitive impairment (Prasad, 2009). Fe and Zn deficiencies are among the most prevalent micronutrient deficiencies in humans, affecting two billion people and causing more than 0.8 million deaths annually (World Health Organization, 2003). Mn deficiency is also a serious problem and can lead to asthma and severe birth defects, however, it is relatively less prevalent compared to Fe and Zn deficiency. The combined effects of these micronutrient deficiencies pose a significant threat to human health.

Biofortification (increasing the contents of vitamins and micronutrients through plant breeding or biotechnology) of food crops with vitamins and micronutrients is not a new concept and was suggested more than a decade ago as a way to significantly ameliorate deficiencies (Ye et al., 2000; Guerinot, 2001; Clemens et al., 2002b). Biofortification is an effective and cheaper alternate to traditional ways of combating micronutrient deficiencies, i.e., healthy food (which is often expensive), micronutrient supplementation, and food fortification. These conventional methods are difficult to afford for a large proportion of the world's population, especially those with limited resources and low incomes (Haas et al., 2005). Increasing the micronutrient content of grain by biofortification offers great potential to combat micronutrient deficiency and dramatically impact human health. Another definition of the problem is nutritional genomics which is described as manipulating plant micronutrients to improve human health by the interface of plant biochemistry, genomics, and human nutrition (DellaPenna, 1999).

Fe, Zn, and Mn are also essential in plants. Fe participates in various cellular events such as respiration, chlorophyll biosynthesis, and photosynthetic electron transport. Low chlorophyll content (chlorosis) of young leaves is the most obvious visible symptom of Fe deficiency (Marschner, 1995). Fe deficiency also seems to triggers oxidative stress (Tewari et al., 2005; Bashir et al., 2007). Fe is essential for the function of chloroplast and mitochondria. Fe is transported to mitochondria through mitochondrial Fe transporter (MIT; Bashir et al., 2011a,c). Additional Fe may be diverted to vacuole (Kim et al., 2006; Zhang et al., 2012). In rice the vacuolar Fe transporter 1 (OsVIT1) and OsVIT2 are suggested to play an important role in subcellular Fe transporter (Zhang et al., 2012).

Zn plays diverse roles in different cellular processes (Ishimaru et al., 2011b). Protein, nucleic acid, carbohydrate, and lipid metabolism depend to a great extent on Zn (Rhodes and Klug, 1993; Vallee and Falchuk, 1993). Zn uptake must be tightly

regulated to ensure that the correct amount of Zn is present at all times (Ishimaru et al., 2011b). In plants, Zn deficiency results in the accumulation of starch and inactive RNases, suggesting that RNA degradation could be regulated by the availability of Zn in the cell (Suzuki et al., 2012). Mn serves as a cofactor or activator of enzymes with different functional groups and diverse activities. For example, oxalate oxidase, Mn superoxide dismutase, RNA polymerase, malic enzyme, isocitrate dehydrogenase, and phosphoenolpyruvate carboxykinase use Mn as a cofactor (Bowler et al., 1991; Marschner, 1995; Requena and Bornemann, 1999; Alscher et al., 2002). Mn is also important in photosynthetic oxygen evolution in chloroplasts (Britt, 1996; Clemens et al., 2002a; Rutherford and Boussac, 2004). Mn-deficient plants are more susceptible to low-temperature stress and pathogen infection, leading to significant decreases in crop yield (Marschner, 1995; Hebbern et al., 2005). The transport of Mn is believed to share an entry route with Fe and cadmium (Cd). Fe, Zn, and Mn transport overlaps in plant biological systems, and Fe, Zn, and Mn deficiencies are particularly severe when plants are grown in alkaline soils. As alkaline soils account for approximately 30% of the world's cultivated soils (Chen and Barak, 1982), the development of plants able to grow in these soils may greatly benefit agriculture. Furthermore, plants that accumulate more micronutrients (e.g., Fe, Zn, and Mn) would contribute significantly to combating micronutrient deficiencies in humans.

SEED MICRONUTRIENT LOCALIZATION AND BIOFORTIFICATION OF RICE

In rice seeds Fe localizes to dorsal vascular bundle, aleurone layer, and endosperm as well as it localizes to the scutellum and vascular bundle of the scutellum of embryo. During germination localization of Fe changes significantly, particularly in embryo and 36 h after sowing, Fe localizes to the scutellum, coleoptile, and epithelium, as well as to the leaf primordium and radicle (Takahashi et al., 2009). Unlike Fe, Zn is unevenly distributed to all of parts of the seed, with a significantly high value for the aleurone layer and embryo (Takahashi et al., 2009). During germination, Zn flow is quite dynamic compared to Fe and Mn. Mn is observed both in the endosperm and embryo, and during germination accumulation of Mn increases in the scutellum at the cost of Mn accumulation in coleoptile. Besides micronutrients, rice seed also contain nicotianamine (NA) and 2′-deoxymugineic acid (DMA; Masuda et al., 2009; Usuda et al., 2009), which are suggested to chelate and mobilize micronutrients during germination (Takahashi et al., 2009).

Significant variation for seed Fe concentration has been reported in rice (Gregorio et al., 2000). Fe is abundant in mineral soils and the major problem with its acquisition is solubility, thus application of soil Fe as fertilizer is not an effective strategy for increasing seed Fe. The application of Zn as fertilizer is effective in promoting plant growth and also in the fortification of crops with Zn. Variation in different rice genotypes in terms of Zn use efficiency and grain Zn concentration has been reported (Neue et al., 1998; Yang et al., 1998; Graham et al., 1999; Wissuwa et al., 2006, 2008). For controlling grain Zn concentrations, the native soil Zn status is the dominant factor, followed by genotype and fertilizer, while for Fe pH of the soil and the concentration of carbonate as well as field conditions like submergence or dry filed are more important. Rice grain Zn concentrations may be as low as 15.9 and as high as 58.4 mg kg^{-1} depending upon the genotype (Graham et al., 1999), and within a single variety, the grain Zn concentration may vary from 8 to 47 mg kg^{-1} depending on soil Zn status (Wissuwa et al., 2006, 2008). Different labs have demonstrated the potential to increase the Zn concentration of rice grains through soil–plant interactions, traditional breeding, and marker-assisted breeding (reviewed by Impa and Johnson-Beebout, 2012); however, traditional breeding has thus far been an unsuccessful approach for increasing the seed Fe concentration.

BIOFORTIFICATION THROUGH BIOTECHNOLOGY

Upon storage, especially in tropical environments, rice seeds rapidly deteriorate. As a conventional practice for maintaining the quality and improving the shelf-life of rice grains, the embryo, pericarp, testae, and aleurone layers are removed during milling, leaving only the endosperm as the edible part (Matsuo and Hoshikawa, 1993). Thus, biofortification would only be effective when metal concentrations are increased in the rice endosperm. Biotechnology techniques such as plant transformation offer great opportunities for increasing the amounts of trace metals in the endosperm. **Table 1** reviews the types of genes that have been used for plant transformation in attempts to increase the metal content of rice endosperm. The genes used for biofortification of rice are mainly those encoding metal chelators (mostly phytosiderophores) and metal transporters; genes encoding the Fe storage protein ferritin and phytase have also been used.

BIOFORTIFICATION THROUGH INCREASING THE AMOUNT OF METAL CHELATORS

Graminaceous plants, which include rice, have sophisticated mechanisms for acquiring micronutrients from soil and transporting them from roots to shoots and grains by secreting small molecules called mugineic acid family phytosiderophores (MAs). MAs have the potential to solubilize Fe, Zn, Cu, and Mn (Treeby et al., 1989). The synthesis of MAs has been studied at the molecular level and has been extensively reviewed (Bashir et al., 2006, 2010, 2012; Bashir and Nishizawa, 2006; Nagasaka et al., 2009; Ishimaru et al., 2011b; Kobayashi and Nishizawa, 2012; Suzuki et al., 2012); thus, it will be discussed very briefly here. MAs are synthesized from L-Met (Mori and Nishizawa, 1987) via a conserved pathway comprising the trimerization of *S*-adenosyl methionine to NA by NA synthase (NAS; e.g., OsNAS1-3), conversion of NA to a keto-intermediate by NA aminotransferase (NAAT; e.g., OsNAAT1), and transformation of the keto-intermediate to 2′-DMA by DMA synthase (DMAS; e.g., OsDMAS1). NA is also a metal chelator and is found in all plants. MAs are released into rhizosphere through transporter of mugineic acid 1 (TOM1), a member of major facilitator superfamily antiporter (Nozoye et al., 2011) and the metal-MA complex is taken up by transporters belonging to the yellow stripe-like (YSL) family (Curie et al., 2001; Inoue et al., 2009). Exporter of NA 1 (ENA1) is NA transporter suggested to be localized to vacuole (Nozoye et al., 2011). ENA1 is similar to the *Arabidopsis thaliana* zinc-induced facilitator 1 (AtZIF1) and AtZIFL2. AtZIF1 also localizes to vacuolar membrane and is suggested to be involved in Zn detoxification

Table 1 | Summary of different approached undertaken for mineral biofortification of rice.

Gene	Promoter	Cultivar	Fold increase in Fe/Zn	Reference
AtNAS1+, Pvferritin+, Afphytase	CaMV 35S, Glb-1, Glb-1	*Japonica* cv. Taipei 309	6.3/1.6[1]	Wirth et al. (2009)
HvNAS1	Actin	*Japonica* cv. Tsukinohikari	3.4/2.3[2]	Masuda et al. (2009)
HvNAS1, HVNAS1+HvNAAT, IDS3	Genomic fragments	*Japonica* cv. Tsukinohikari	1.0/1.0[2], 1.1/1.1, 1.4/1.3	Masuda et al. (2008)
OsNAS1, OsNAS2, OsNAS3	CaMV 35S	*Japonica* cv. Nipponbare	2.2/1.4, 4.2/2.2, 2.2/1.4	Johnson et al. (2011)
OsNAS2	Activation tagging	*Japonica* cv. Dongjin	3/2.7	Lee et al. (2011, 2012b)
OsNAS3	Activation tagging	*Japonica* cv. Dongjin	/2.2	Lee et al. (2009b)
OsNAS1	GluB1	*Japonica* cv. Xiushui 110	1.0/1.3	Zheng et al. (2010)
SoyferH-1	Glu-B1	*Japonica* cv. Kitaake	3.0	Goto et al. (1999)
PvFerritin+ rgMT	Glb-1	*Japonica* cv. Taipei 309	2.0[3]	Lucca et al. (2001)
SoyFer	Glu-B1	*Indica* cv. IR68144	3.7/1.4	Vasconcelos et al. (2003)
SoyFer	Glu-B1; Glb-1	*Japonica* cv. Kitaake	3.0/1.1	Qu et al. (2005)
OsFer2	*OsGluA2*	Basmati rice (*Indica* cv. Pusa-Sugandh II)	2.1/1.4	Paul et al. (2012)
TOM1	CaMV 35S	*Japonica* cv. Tsukinohikari	1.2/1.6	Nozoye et al. (2011)
OsYSL2	OsSUT1	*Japonica* cv. Tsukinohikari	4.4	Ishimaru et al. (2010)
OsIRT1	Ubi	*Japonica* cv. Dongjin	1.1/1.1[3]	Lee and An (2009)
OsYSL2+, SoyFerH2+, HvNAS1	OsSUT1, Glb-1, Glb-1. Glu-B1, Act	*Japonica* cv. Tsukinohikari	6 or 4[4]/1.6	Masuda et al. (2012)

[1] *Mn concentration also increased by two fold.*
[2] *Plants were tested in isolated filed.*
[3] *Concentration in brown rice.*
[4] *Six times in T2 seeds while 4.2 times in T3 seeds.*

through sequestration into the vacuole (Haydon and Cobbett, 2007). Increasing the concentrations of NA and DMA in rice plants has been shown to effectively increase the metal concentration in rice grains. The Fe and Zn concentrations in rice grains were significantly increased by the overexpression of *HvNAS1* (Masuda et al., 2008, 2009) or *OsNAS1-3* (Johnson et al., 2011), the simultaneous overexpression of *AtNAS1* and *Pvferritin* (Wirth et al., 2009), and the activation of *OsNAS2* and *OsNAS3* (Lee et al., 2009b, 2011, 2012b). The overexpression of *TOM1* slightly increased seed Fe, Zn, and Cu concentrations (Nozoye et al., 2011), and the overexpression of barley iron deficiency-specific clone 3 (*IDS3*), whose product converts DMA to MA and 3-epihydroxy-2′-deoxymugineic acid (epiHDMA) to 3-epihydroxymugineic acid (epiHMA) (Nakanishi et al., 2000), increased Fe accumulation in rice grains (Masuda et al., 2008). These results suggest that an increase in NA and/or DMA/MA synthesis could increase Fe and Zn translocation to rice grains and that the increase in Fe is positively correlated with the accumulation of NA or DMA (Johnson et al., 2011; Masuda et al., 2012).

EXPLOITING METAL TRANSPORTERS FOR BIOFORTIFICATION

Metal transporters belonging to different families have been reported to play significant roles in metal uptake in rice; these have been discussed extensively (Koike et al., 2004; Ishimaru et al., 2007, 2011a,b,c, 2012a,b; Lee and An, 2009; Lee et al., 2009a, 2010a,b, 2012a,c; Bashir et al., 2010, 2011a,b,c, 2012; Kakei et al., 2012; Suzuki et al., 2012). In short, iron-regulated transporter-like protein 1 (OsIRT1; Fe and Cd), OsIRT2 (Fe and Cd), natural resistance-associated macrophage protein 1 (OsNRAMP1; Fe and Cd), and OsNRAMP5 (Mn, Fe, and Cd) transport metals (Bughio et al., 2002; Ishimaru et al., 2006, 2012a,b; Nakanishi et al., 2006; Takahashi et al., 2011; Sasaki et al., 2012), while OsYSL2 (Mn–NA or Fe–NA), OsYSL15, OsYSL16, and OsYSL18 [Fe(III)–DMA] transport NA- or DMA-bound metals (Koike et al., 2004; Aoyama et al., 2009; Inoue et al., 2009; Lee et al., 2009a, 2012c; Ishimaru et al., 2010; Kakei et al., 2012). Moreover, the rice phenolics efflux transporters, phenolics efflux zero 1 and 2 (PEZ1 and PEZ2), secrete phenolics in to apoplasm (in roots and in xylem) to solubilize apoplasmic Fe for transport (Bashir et al., 2011b; Ishimaru et al., 2011a,c). All these transporters localize to plasma membrane and transport apoplasmic micronutrients into cytoplasm. Although OsIRT1, OsIRT2, OsYSL15, and OsNRAMP5 are mainly involved in micronutrient uptake from soil, these transporters are also suggested to play a role in micronutrient translocation to seeds. On the other hand, OsYSL2, OsYSL16, and OsYSL18 are suggested to be involved in xylem to phloem transport (phloem loading) in rice while TOM1 may also play a role in xylem loading (Kobayashi and Nishizawa, 2012).

Increases in the uptake of Fe from the soil and its translocation through the plant are reported to increase the Fe content of rice endosperm. Rice lines that overexpress *OsIRT1* accumulated more Fe and Zn in the seeds (Lee and An, 2009), and similar results were observed for lines overexpressing *OsYSL15* (Lee et al., 2009a). The overexpression of *HvYS1* in rice enhanced the tolerance to alkaline soil, but did not increase the Fe concentration in the grain (Gómez-Galera et al., 2012). *OsYSL2* overexpression resulted

in decreased root-to-shoot translocation of metals, whereas *OsYSL2* overexpression driven by a sucrose transporter promoter (*OsSUT1*) which specifically expresses in phloem, significantly increased Fe and Mn concentrations in rice seeds (Ishimaru et al., 2010). Thus, especially for metal transporters, control of the spatial and temporal expression of genes may significantly increase Fe flow to rice grains.

Additional transporters also appear to be good candidates for metal biofortification. Overexpression of rice plasma membrane zinc-regulated transporter (ZRT) IRT like protein 4 (OsZIP4), *OsZIP5*, and *OsZIP8* resulted in decreased root-to-shoot translocation of Zn and reduced seed Zn concentrations (Ishimaru et al., 2007; Lee et al., 2010a,b). The inclusion of tissue-specific promoters such as the *OsSUT1* promoter, which was used in the case of *OsYSL2* (Ishimaru et al., 2010) may increase the Zn concentration in rice grains. Rice plants overexpressing rice *heavy metal ATPase 2* (*OsHMA2*) under the control of *OsSUT1* were reported to accumulate slightly more Zn in seeds (Takahashi et al., 2012).

OTHER APPROACHES FOR MICRONUTRIENT BIOFORTIFICATION

Ferritin, a globular protein found in prokaryotes and eukaryotes, has the ability to store and keep Fe in a soluble and non-toxic form (Harrison and Arosio, 1996). In plants ferritin is mainly localized to plastids (Briat et al., 2010). The overexpression of ferritin was the first attempt at increasing the metal content of rice grains. Ferritin-overexpressing plants accumulated Fe in seed endosperm at a level threefold that in wild-type plants (Goto et al., 1999; Lucca et al., 2001; Vasconcelos et al., 2003; Qu et al., 2005). Recent approaches employed to increase the solubility of Fe include the development of rice plants expressing *Aspergillus fumigatus* thermotolerant phytase and a cysteine-rich metallothionein-like protein (Lucca et al., 2001). Phytases are abundant in grains and oil seeds and play an important role in the degradation of phosphates containing organic molecules (such as phytate; Li et al., 2010), thus increasing the solubility of Fe. Metallothioneins are low molecular weight proteins rich in cysteine and are suggested to play a role in regulation of metals by binding to these metals (Grennan, 2011).

In addition, phytase overexpression with the aim of degrading phytic acid was attempted in order to increase micronutrient bioavailability in rice (Wirth et al., 2009). Basmati rice (Pusasugandh II) plants overexpressing rice ferritin (*OsFer2*) under the control of the endosperm-specific *GlutelinA2* (*OsGluA2*) promoter were established (Paul et al., 2012). The expression of ferritin in these T_3 transgenic plants was 7.8-fold than that in wild-type plants, and the transgenic plants accumulated Fe and Zn at levels 2.1- and 1.4-fold, respectively, compared to wild-type plants (Paul et al., 2012).

Furthermore, the simultaneous overexpression of ferritin under the control of the endosperm-specific promoters *globulin b1* (*OsGlb1*) and *glutelin B1* (*OsGluB1*), *NAS* under the control of the *OsActin1* promoter, and *OsYSL2* under the control of *OsGlb1* promoter and the *OsSUT1* transporter promoter have been demonstrated to significantly increase the Fe, Zn, Mn, and Cu concentrations in T_3 polished seeds of field grown rice (Masuda et al., 2012).

BOTTLENECK OF BIOFORTIFICATION

The application of Fe transporters for micronutrient biofortification of rice is problematic because many transporters, including OsIRT1, OsNRAMP1, and OsNRAMP5, also transport other metals such as Cd. Cd is a toxic metal in humans (World Health Organization, 2003) and affects cellular metabolism (Singh and McLaughlin, 1999). Thus, the use of these transporters for biofortification may also increase the grain Cd concentration in rice grown in soils contaminated with Cd. The knockout of *PEZ1* and *PEZ2* increased the seed Cd content, and *PEZ1* overexpression significantly reduced plant growth, probably due to Fe toxicity (Bashir et al., 2011b; Ishimaru et al., 2011a,c). It is possible to use mutated versions of these proteins that have narrow substrate specificity and do not transport Cd. It has been demonstrated that replacing specific amino acids in *Arabidopsis* IRT1 changed its substrate specificity (Rogers et al., 2000). Moreover, Podar et al. (2012) recently revealed the key components controlling metal selectivity, thus offering a strategy for the modification of transporters for effective biofortification.

The physiology and seed morphology of a particular crop plant should be carefully considered before utilizing any gene for biofortification. A gene that is effectively used in one crop may not be suitable for use in other crops, and vise versa. This is especially true in the case of *OsVIT1* and *OsVIT2*. Although *osvit1-1* and *osvit2-1* mutants accumulate significantly higher levels of Fe and Zn in brown rice, most of the Fe localizes to the embryo and aleurone layer (Zhang et al., 2012), which are removed during the processing and polishing of rice grains. Moreover, although OsVIT1 and OsVIT2 do not transport Cd, the grain Cd concentration increased by 60% in rice seeds of *osvit1-1* and *osvit2-1* mutants compared with the concentration in wild-type plants when grown in Cd-contaminated soils. Thus, these genes do not offer an advantage for the biofortification of rice. In rice the role of other subcellular metal transporters, such as MIT, in regulating seed metal concentration is not clear. It may be better to consider regulating the expression of these transporters to exploit their full potential for increasing the micronutrient concentration of grains.

Careful examination and understanding of the expression patterns of the genes involved in micronutrient transport throughout plant development are important before selecting a gene for use in a biofortification program. This information would help to identify candidate genes that could significantly enhance the micronutrient content in rice grains as well as to develop rice plants that are tolerant to low micronutrient availability under diverse environmental conditions. Based on the expression patterns of genes in rice, it appears that OsYSL16 and its promoter may be good candidate for increasing the micronutrient concentration in rice grains.

FUTURE PROSPECTS

The ultimate objective of developing biofortified crops is to utilize these crops in the field; however, no serious efforts have been reported for the commercialization of biofortified rice. For example, although sufficient scientific research and development have led to the establishment of stable transgenic lines of golden rice, a rice biofortified with β-carotene, the commercialization of golden rice has suffered from serious delays (Potrykus, 2012). Thus far,

transgenic rice has not been commercialized in any country. It is possible that after the initial commercialization of golden rice, commercialization of micronutrient-biofortified rice will proceed at a faster rate, depending upon its acceptance by farmers and its performance in the field. Furthermore, the use of genes from rice or other plants to produce biofortified rice such as golden rice may meet with consumer approval more easily than the use of *Bacillus thuringiensis* (*Bt*) genes. It is unfortunate that exploitation of non-transgenic mutants, such as Tos17 mutants or mutants generated through chemical or radiation treatment, has not been examined for the micronutrient biofortification of rice.

Rice feeds more than half of the world's population and is a staple food in most parts of Asia. When considering the commercialization of biofortified rice, it should be remembered that most rice is consumed in Asia and that many of the potential beneficiaries belong to poor countries and poor families. Thus, regulatory laws that prevent the mixing of genetically modified rice with traditional rice may pose practical impediments. Many countries export rice to other countries, and it may be difficult to enforce regulatory laws in the field, which could lead to problems for rice exporters. In addition, the suitability of sites for the cultivation of biofortified transgenic rice must be evaluated with regard to maintaining biodiversity and fully exploiting the potential of transgenic rice. Biosafety issues concerning different traits of transgenic rice have been discussed extensively (Husnain et al., 2003, 2004; Bashir et al., 2004a,b, 2005; Riaz et al., 2006). One important issue is the development of marker-free lines. Recently, rice lines defective in the MIT (Bashir et al., 2011a,c) were complemented using a mutated rice acetolactate synthase gene as a selectable marker (Ogawa et al., 2008). Markers that are originally cloned from rice may be easily accepted by farmers. In addition, lines harboring the *HvNAS1* gene and accumulating higher amounts of NA have been developed (Usuda et al., 2009), and the Cre/*loxP* DNA excision (CLX) system was successfully used to remove the hygromycin resistant (HPT) marker gene in these lines. As a result, the final transformants were marker-free and contained the *HvNAS1* gene alone (Usuda et al., 2009). Moreover, these transgenic lines were crossed with a cleistogamous mutant to prevent gene transfer through cross-pollination. Although the rate of cross-pollination in transgenic rice is very low (Bashir et al., 2004a; Mahmood-ur-Rahman et al., 2007), it is still wise to utilize cleistogamous mutants to further reduce this possibility. The development of marker-free and cleistogamous rice would help to minimize public concern with regard to the use of transgenic rice in biofortification programs. The number of transgenes in a line may also create public objections. Transgenic lines containing one gene (e.g., *OsNAS2*; Johnson et al., 2011) appear to be more easily accepted than lines containing numerous genes (e.g., *OsYSL2–HvNAS1–ferritin*; Masuda et al., 2012).

A feeding test of biofortified rice in mice and Caco-2 (human epithelial colorectal adenocarcinoma cells) cell lines confirmed that the increased metal content of rice owing to increased NAS expression is highly bioavailable (Lee et al., 2009b, 2011, 2012b; Zheng et al., 2010). These results suggest that at least in the case of NAS overexpression lines, there is no need to co-express phytase or metalloproteins to increase bioavailability. Recently it was shown that biofortification of rice with Zn significantly increases Zn uptake in Caco-2 as well as in rat pups, and are suggested to be the same in human populations (Jou et al., 2012). Feeding tests in humans may be the next step toward the release of biofortified lines. In addition to the genes and transgenic lines already available in different breeding programs, new genes and new lines with better combinations are being sought. Micronutrient-biofortified rice may be bred with golden rice and/or folate-rich rice to provide even more essential nutrients and vitamins.

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A legume biofortification quandary: variability and genetic control of seed coat micronutrient accumulation in common beans

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Common beans (*Phaseolus vulgaris* L.), like many legumes, are rich in iron, zinc, and certain other microelements that are generally found to be in low concentrations in cereals, other seed crops, and root or tubers and therefore are good candidates for biofortification. But a quandary exists in common bean biofortification: namely that the distribution of iron has been found to be variable between the principal parts of seed; namely the cotyledonary tissue, embryo axis and seed coat. The seed coat represents ten or more percent of the seed weight and must be considered specifically as it accumulates much of the anti-nutrients such as tannins that effect mineral bioavailability. Meanwhile the cotyledons accumulate starch and phosphorus in the form of phytates. The goal of this study was to evaluate a population of progeny derived from an advanced backcross of a wild bean and a cultivated Andean bean for seed coat versus cotyledonary minerals to identify variability and predict inheritance of the minerals. We used wild common beans because of their higher seed mineral concentration compared to cultivars and greater proportion of seed coat to total seed weight. Results showed the most important gene for seed coat iron was on linkage group B04 but also identified other QTL for seed coat and cotyledonary iron and zinc on other linkage groups, including B11 which has been important in studies of whole seed. The importance of these results in terms of physiology, candidate genes and plant breeding are discussed.

Keywords: advanced backcross breeding method, cotyledon, embryo axis, iron and zinc concentration, use of wild beans, seed coat

INTRODUCTION

Biofortification is a relatively recent addition to breeding goals in plants based on improving the nutritional quality of the edible portion of the plant through traditional or transgenic approaches (Dwivedi et al., 2012). To date, most biofortification work has concentrated on micronutrients and vitamins, although conceivably protein content, amino acid distribution and beneficial secondary metabolites could be considered to be goals of biofortification (Welch, 1999). The stated goals of most biofortification work is to reduce mineral or vitamin deficiencies and where needed protein deficiencies. These deficiencies manifest themselves in conditions of iron deficiency anemia (IDA) but are not in themselves actual diseases but rather imbalances in the diet that need to be approached through modifications in the diet (Pfeiffer and McClafferty, 2007).

Micronutrient deficiencies are predicted to affect half the world's human population, with IDA being an especially common health concern affecting at least 2 billion people. IDA is caused by low consumption of iron especially in reproductive age women and developing adolescents (Welch, 1999). Zinc deficiency is suspected to be equally as common but has not been as well documented as IDA (Welch and Graham, 2002). While IDA causes losses in work productivity and developmental problems, zinc deficiency causes lowered disease immunity and stunting. These types of deficiencies are sometimes difficult to address through supplementation or fortification for technical, societal or economic reasons and therefore these minerals do not always reach the poor consumer (Dwivedi et al., 2012). How to reach the bottom of the pyramid of societies' economic strata (people making less than 2 dollars a day) with food containing sufficient micronutrients has been debated but one way is through biofortification of staple crops that are consumed in high amounts by the poor, such as rice, wheat and beans (seed crops) or potato and cassava (root and tuber crops).

Common bean is a highly nutritious food because of its balance of carbohydrates to protein (between 4:1 and 3:1), content of important vitamins and its micronutrient concentration, especially for iron which is at much higher levels than in the starchy staples of barley, corn or wheat. Common bean is widely grown in many parts of Asia, Africa, Europe and North, Central and South America and has two major types, the large seed Andean beans and the small seed Mesoamerican beans (Broughton et al., 2003). Consumption of common beans generally is higher than 20 to 30 kg/year in rural northeast

Brazil, Central America and Mexico and can be as high as 40 to 60 kg/year in regions where meat is scarce such as in the Great Lakes of Africa. In Europe and North America consumption is low at 5 kg/year or less. Like other legumes, common bean is usually combined in the diet with a starch based food either in a mixture of two components (pulse + grain) or as a side dish such as "dals" made of lentils or "Frijoladas" made of beans. In some places common bean is the national dish as in Brazil where "Feijoada" is served daily or in the Dominican Republic where the "Bandera" is a twice-a-day meal made of rice with a side of beans. Certain regions of Central and South America consume mashed or re-heated "Calentao" beans as breakfast.

One reason for the micronutrient density of legumes is their anatomical seed structure, where a thick, maternally-derived seed coat or testa surrounds the expanded cotyledons of a quiescent but fairly large and well developed embryo. This structure differs radically from cereals where a thin, maternally-derived aleurone layer surrounds specialized endosperm tissue with a less well developed embryo in the bran. Breeding programs for cereals have had little concern for the sub-compartmentalization of micronutrients given that the bran is removed in milling processes and the major target tissue is the endosperm. However, in legumes, for the most part, the seed is consumed whole after a process of boiling, rather than by any processes of milling or grinding. In addition, the seed coat makes up 7 to 10% or more of the total seed weight of beans and is a source of consumer preference, the cotyledon makes up 85% or more of total seed weight and the embryo is only 2 or 3% of seed weight but is dense in nutrients (Ariza-Nieto et al., 2007).

Seed size varies more than seed coat thickness so smaller seeded legumes especially wild relatives of beans tend to have even higher percentages of seed coat compared to cotyledonary tissue of cultivated beans. The presence of these different seed tissues means that all three components of the common bean seed should be targets of biofortification: seed coat, cotyledons and embryo. An understanding of mineral distribution, loading and inheritance into each tissue is essential for making progress in breeding of this crop.

The goal of this research, therefore, was to evaluate the concentration of iron and zinc, in particular, and other minerals more generally in seed coats that were separated from cotyledons for a common bean population derived from a wild (small-seeded) common bean crossed with a cultivated (large-seeded) common bean. This analysis was part of a biofortification breeding program using the advanced backcross (AB) quantitative trait loci (QTL) approach as outlined in Tanksley and Nelson (1996); Blair et al. (2006) and Blair and Izquierdo (2012). The identification of QTL for seed coat *versus* cotyledonary mineral concentrations can be used in molecular breeding of common bean as regular peeling of seed coats for analysis is time consuming and onerous for the researcher. In addition, we were interested in determining the value of wild beans as a source of higher iron concentration as they have been found to have very high levels of this mineral (Guzmán-Maldonado et al., 2004) but have a disadvantage of small seed size.

MATERIALS AND METHODS

SEED SOURCE AND FIELD CONDITIONS

An advanced backcross (AB) population of 138 $BC_2F_{2:5}$ lines (cross NH 21154_ (F_2)−1−M−M) was developed by crossing the cultivated recurrent parent Cerinza with the wild donor parent G10022, a source of high seed mineral concentration as described in Blair and Izquierdo (2012). Cerinza as the recurrent or recipient parent is large-seeded and of red color, and is rounder than a kidney bean.

Cerinza, like other Andean large-seeded beans, weighing approximately 50 g *per* 100 seed, has a good baseline of iron concentration averaging 60 ppm in multiple field trials (Astudillo and Blair, 2008). Meanwhile, the wild donor parent G10022 from Mexico is small seeded, weighing approximately 5 g *per* 100 seed and was discovered in a screening program for nutritional traits in wild beans and was found to have a very high concentration of 110 ppm iron concentration (Islam et al., 2002). Zinc concentration was less contrasting in previous studies of the two parents with G10022 having 38 ppm and Cerinza having 25 to 27 ppm.

The advantage of using the AB population instead of a simple cross between wild and cultivated beans for the nutritional analysis was that most of the genotypes were of similar seed size and adaptation (Blair et al., 2006; Blair and Izquierdo, 2012). They could therefore be grown together in the same field experiment at a site in Darién, Colombia (3°54′N, 76°30′W, 1485 m above sea level, average yearly temperature 20°C, average relative humidity 80%, average yearly rainfall 1288 mm) with moderately acid, loam soils (pH 5.6, Andisol). The field experiment (a randomized complete block design with three replicates and 3 m long single-row plots) was managed with two foliar applications of zinc and boron as microelements (300 g ha^{-1} as chelates) carried out at 14 and 21 days after planting. Harvesting was by hand into clean plastic buckets to avoid soil contamination. Foliar disease pathogens were controlled with the fungicides, mancozeb, and benomyl, at doses of 1.0 and 0.3 kg ha^{-1}, respectively.

A sample of the harvest from the first two replicates was carefully placed separately into two paper bags to keep them clean through transport. Back in seed laboratory, the seeds were hand washed with 70% ethanol to remove dust and dried in a stationary oven. The seed were all weighed upon reaching 10% moisture and the seed color was recorded, although most of the genotypes had medium to large red seed (45 to 55 g *per* 100 seed) like the recurrent parent Cerinza. The similarity of the lines was a result of backcrosses of the recurrent cultivated parent as a recipient of the wild donor parent genes.

MINERAL EVALUATION AND DATA ANALYSIS

For each repetition, 12 g of seed were washed with sterile double-distilled water and peeled by hand using a sterile scalpel to remove the seed coat from the cotyledons. The separate seed coat and cotyledon samples were placed in separate envelopes and then dried for 24 h at 45°C in a bench-top oven, before grinding in a modified Retsh mill with 24 sample slots using zirconium grinding balls and Teflon grinding chambers. Seed coat and cotyledonary tissues were dried and weighed out into two replicates of ∼0.25 g dry weight each which were analyzed separately before averaging. These samples were analyzed with

nitric acid-perchloric acid digestion as described in Blair et al. (2009).

Digested samples were taken to dryness and re-suspended in 15 mL of trace-metal grade nitric acid (2% v/v), prior to multi-element analysis by inductively coupled plasma—optical emission spectroscopy (ICP-OES) (CIROS ICP Model FCE12; Spectro, Kleve, Germany) at the USDA-ARS Children's Nutrition Research Center at the Baylor College of Medicine (Houston TX). The instrument was calibrated daily with certified standards. Tomato leaf standards (SRM 1573A; National Institute of Standards and Technology, Gaithersburg, MD) were digested and analyzed along with the seed samples to ensure accuracy of the instrument's calibration. Because of the large amount of work involved in peeling sufficient seed, only 60 of the full set of 138 AB representing a random selection of lines for full-seed iron concentration were evaluated. These included 20 high, 20 moderate and 20 low seed iron containing lines for the cross covering the full range from 60 to 95 ppm (Table S1) as measured for Darien and Palmira by Blair and Izquierdo (2012). In addition, the two parents, Cerinza and G10022 were used as control genotypes twice in the analysis. Because of the higher seed coat ratio in G10022 only 4.5 g of this wild bean was needed to obtain sufficient seed coat for analysis.

All quantitative data were analyzed using a general linear model and an analysis of variance for a split plot design for genotypes within tissue types analyzed in the software package Statistix v. 8.0 (Analytical Software Inc.) and means were estimated to use for subsequent analysis. Population distributions were evaluated for normality using the same software. Transformations were carried out with natural logarithm in cases were population results were skewed before use for QTL analysis. Pearson's correlations were estimated based on the non-transformed data. QTL analysis was carried out with the means described above and the genetic map built by Blair and Izquierdo (2012). QTL were identified using two software programs QTL Cartographer v. 2.5 (Basten et al., 2001) for composite interval mapping analysis (CIM) and MapDisto v. 1.7 (http://mapdisto.free.fr/) for single point analysis (SPA). In the CIM analysis we performed the analysis every 1 cM (walkspeed) with a window size of 10 cM and using ten background markers in a forward-backward stepwise multiple linear regression model. In terms of population type, the B_{12} genetic model was assumed for the CIM analysis. Meanwhile a simple regression model was assumed for the SPA analysis. In both cases the homozygous donor parent allele class was combined with the heterozygous genotypic class. Significance thresholds were set at LOD 3.0 ($P \leq 0.001$).

The phenotypic variance controlled by a given QTL was determined by its determination coefficient (R2), as defined by the software program. QTL for micronutrient concentrations were named using the mineral name, the abbreviation for seed coat or cotyledon and a two number code derived from the linkage group and the number of the QTL identified on that linkage group, separated by a period. Genetic maps and QTL locations were drawn with MapChart v. 1.0 software program (Voorips, 2002) where map distances were reported in centiMorgans (cM) estimated with the Kosambi mapping function.

RESULTS

MINERAL VARIABILITY IN SEED COAT vs. COTYLEDONS

A total of ten minerals were analyzed by the ICP-OES experiment for the two subsamples namely the seed coat and cotyledon (**Table 1**). These minerals were boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), sulfur (S), zinc (Zn). The concentrations were calculated in part per million (ppm) equivalent to g/kg or μg/g. The seed coat was found to have low concentrations compared to the cotyledon for B, Cu, K, Mn and especially P and S. Meanwhile the seed coat had higher concentrations of Ca, Fe, Mg, and Zn.

For the minerals of greatest interest to our study, average Fe and Zn concentrations of the cotyledon were similar to that of seed coat across the entire population (**Table 1**). However, the range of Fe in seed coat was very large with a minimum of 20 ppm to a maximum of 263 ppm, while Zn in seed coat ranged from 17 to maximum of 54.5 ppm. By comparison cotyledonary values were less variable ranging from 54.5 to 93 ppm for Fe and 27 to 41 ppm for Zn. For other nutritionally important minerals, Ca ranged from 12500 to 21500 ppm in seed coat which was surprising given average of 284 ppm in cotyledon. The opposite was true for *P* which averaged 4332 in cotyledon but only 424 ppm in seed coat. Similarly S averaged 1982 ppm in cotyledons but only 350 ppm in seed coat. The differences in the concentration averages between seed coat and cotyledon for each of these minerals were significant in each case.

Observation of population distribution types for iron in cotyledons and zinc in both tissues showed normality and a lack of high kurtosis or skewing (**Figure 1**, **Table 1**). The exception to this was the binomial distribution and high *K* and *S* values for seed coat iron suggesting simpler inheritance and perhaps a major QTL for this trait. This was in contrast to the normal population distributions for zinc concentrations in seed coat or for iron concentration in the cotyledons. Therefore the inheritance of these minerals in these tissues appears to be controlled by multigenic or quantitative. The other minerals were also graphed for their population distribution (data not shown). The range, median, means and *K* or *S* values all indicate normal distributions and quantitative inheritance for the most part, although the *K* and *S* values were high for seed coat Cu and for cotyledonary B and Mg. Kurtosis was also observed for cotyledonary S (**Table 1**).

STATISTICAL ASSOCIATIONS BETWEEN MINERALS

In the ANOVA results, genotype effects for the advanced backcross lines were highly significant ($P = 0.01$–0.001) in each case except B, for which the effect was lower ($P = 0.05$) while tissue effects were either highly or moderately significant (**Table 2**). Tissue × genotype effects were also highly significant for all minerals. Replication differences were minor as evidenced by low sums of squares values (data not shown), which is explained by the good repeatability of the study, with separate samples managed from the field to the seed room to lab analysis with very similar treatment. Coefficients of variation (CVs) were below 3%, except for B and Ca which had higher CVs for the two error terms used Error (Replication × Tissue) to test significance of Tissue differences and Error (Replication × Tissue × Genotype) to

Table 1 | Descriptive statistics for seed coat vs. cotyledonary minerals evaluated as measured by inductively coupled plasma (ICP) analysis for the advanced backcross population of common beans.

Seed coat values	**Minerals evaluated[1]**									
	B coat	**Ca coat**	**Cu coat**	**Fe coat**	**K coat**	**Mg coat**	**Mn coat**	**P coat**	**S coat**	**Zn coat**
Minimum (ppm)[2]	7.0	12500	1.0	**20.0**	2000	2000	2.0	325	298	**17.0**
Maximum (ppm)	21.0	21500	3.0	**263.0**	9000	4000	5.5	635	440	**54.0**
Range (ppm)	14.0	9000	2.0	**243.0**	7000	2000	3.5	310	141	**37.5**
Average (ppm)	13.25	16638	1.19	**69.795**	6377	2884	2.684	424	350	**32.428**
SD (ppm)	2.5	1365	0.307	**56.305**	934	265	0.574	56	26	**6.436**
Kurtosis	−0.245	0.217	7.084	**1.532**	1.862	2.281	2.656	0.513	0.340	**−0.018**
Skewing	0.015	0.382	2.670	**1.799**	−0.558	−0.704	1.267	1.048	0.832	**0.582**
CV (%)	23.4	10.8	38.2	**18.4**	19.2	14.0	26.6	16.5	9.3	**25.6**
Cotyledonary values	**B coty**	**Ca coty**	**Cu coty**	**Fe coty**	**K coty**	**Mg coty**	**Mn coty**	**P coty**	**S coty**	**Zn coty**
Minimum (ppm)[2]	11.0	189	6.0	**54.5**	12500	1000	12.0	3500	1000	**27.0**
Maximum (ppm)	38.5	374	9.0	**93.0**	15000	1281	21.0	6000	3000	**41.0**
Range (ppm)	27.5	185	3.0	**38.5**	2500	281	9.0	2500	2000	**14.0**
Average (ppm)	15.598	284	7.577	**75.362**	13971	1012	15.576	4332	1982	**32.358**
SD (ppm)	3.028	34	0.751	**5.903**	582	24	1.375	435	79	**2.188**
Kurtosis	10.177	−0.510	−0.763	**0.461**	−1.104	18.066	1.234	0.393	15.181	**0.546**
Skewing	2.868	−0.057	0.105	**−0.553**	−0.064	4.390	0.862	1.074	−1.230	**0.626**
CV (%)	30.5	14.5	11.7	**10.3**	5.2	5.5	11.5	11.6	11.8	**8.8**

[1]Mineral abbreviations: B, boron; Ca, calcium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; P, phosphorus; S, sulfur; Zn, zinc. Tissue Abbreviations: coat, seed coat; coty, cotyledon (and embryo). The minerals of most interest, iron and zinc are indicated in the columns which are in bold text.
[2]Mineral concentrations given in part per million (ppm); note difference between micronutrients (B, Cu, Fe, Mn, Zn) vs. other minerals (Ca, K, Mg, P and S).

test significance of Genotype and Tissue × Genotype differences (**Table 2**).

Parental comparisons showed that for the most part tissue differences were highly to moderately significant as were genotype differences, except in the case of the first mineral, B ($P > 0.05$), which was non-significant for any of the sources of variation (**Table 2**). Tissue × genotype effects were also non-significant for Cu and Zn amongst the parents but were significant for all other minerals although to a lesser extent and lower probability than for the advanced backcross lines.

Similarly, probability values for differences between the tissues were less significant than for the lines in most cases although this was more notable for the minerals B and Fe among the lines (differences at $P \leq 0.05$) with all other minerals surpassing the level of high significance (differences at $P \leq 0.001$). Some of the genotype comparisons for minerals were very highly significant (differences at $P \leq 0.0000$). Tissue × genotype effects were less observable for B but were also of very high significance for the remainder of the minerals (differences at $P \leq 0.0000$).

The average parental values for Fe and Zn (**Figure 1**, arrows) were of interest given our attention to these micronutrients. Most noticeably, the wild donor parent (G10022) had more than double the seed coat Fe concentration (93.28 ppm) of the cultivated recurrent parent (Cerinza, 40.52 ppm). Meanwhile the cotyledonary Fe concentrations were similar but reversed with Cerinza having higher iron in the cotyledons (80.16 ppm) than the cotyledons of G10022 (67.51 ppm). Differences were significant based on paired *t*-tests, but higher for seed coats ($P = 0.0039$) than for cotyledons ($P = 0.0180$). For Zn concentrations the cotyledons were about 6–8 ppm richer in this mineral than the seed coats. However, in both cases G10022 had more Zn (28.7 ppm in seed coat, 36.2 ppm in cotyledons) than Cerinza (26.5 ppm in seed coat, 32.16 ppm in cotyledons). Paired *t*-tests showed high significance for the contrast in seed coat ($P = 0.0033$) but non-significance for the contrast in the cotyledon ($P = 0.0684$).

The Pearson's correlation coefficient results (**Table 3**) confirmed tissue × genotype interaction and that the relationships among minerals was different in the two types of tissues, the seed coat and the cotyledon. For cotyledonary tissue, Mn was correlated with P and S just as K was correlated with Mn but none of the negative correlations were highly significant. Less significant ($P \leq 0.05$) and negative correlations of Ca and K or Fe and Mg were observed in the cotyledons. Likewise positive correlations ($P \leq 0.01$) for Cu and K or Mg and Mn were observed. It was surprising that Fe was not correlated with P or Zn in the cotyledon.

Meanwhile, for seed coat tissues Fe was positively correlated with P and S (at $P \leq 0.01$ and $P \leq 0.001$, respectively) but not with Zn. Potassium and Cu levels were positively and highly

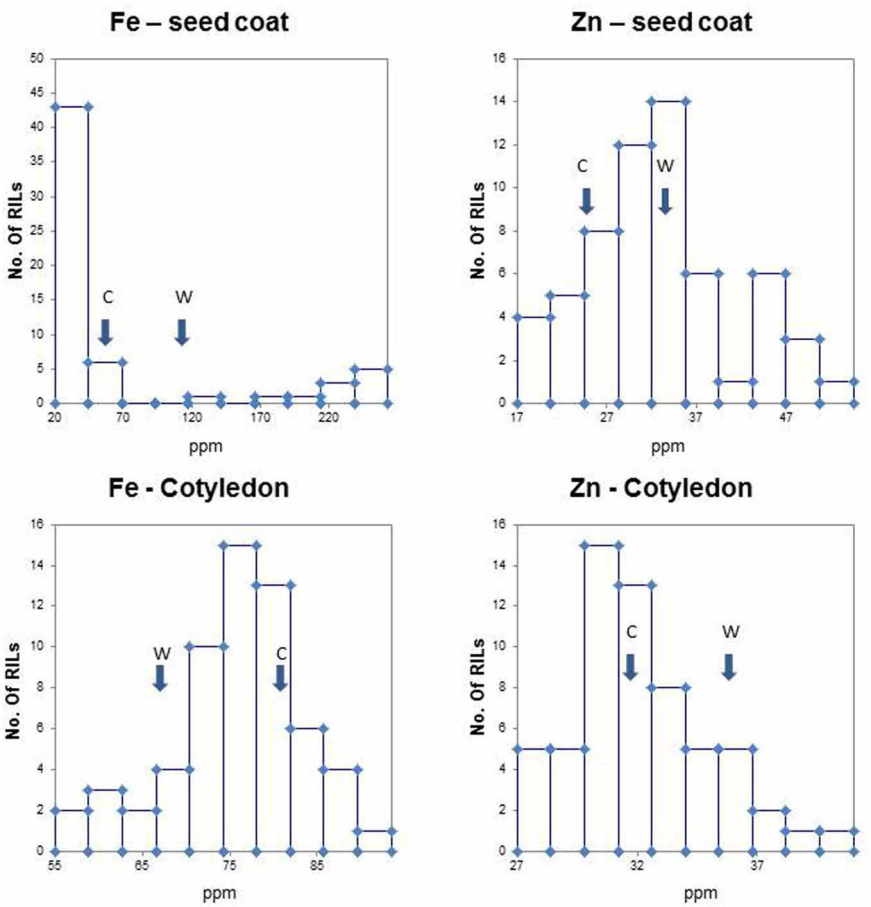

FIGURE 1 | Population frequency distributions for iron (Fe) and zinc (Zn) concentration in seed coats and cotyledonary tissues in the (Cerinza × (Cerinza × (Cerinza × G10022))) advanced backcross population as determined by ICP analysis. Arrows indicate phenotypic value of recurrent parent Cerinza (C) and the wild donor parent G10022 (W). The mineral concentration in parts per million (ppm) is found on the x-axis, while the number (no.) of recombinant inbred lines (RILs) is found on the y-axis.

correlated ($P \leq 0.001$) with those of *P* and *S* but less so with Mn ($P \leq 0.01$). Highly significant negative associations existed between Fe and B as well as between Mn and Ca in the seed coat. Ca was also negatively correlated with K and Mg in seed coat but at lower significance ($P = 0.01$ and $P = 0.05$, respectively).

INHERITANCE OF SEED COAT AND COTYLEDONARY IRON AND ZINC

Using only the Fe and Zn datasets, a total of 7 QTL were found with CIM analysis and permutations for significance threshold (**Table 4** and **Figure 2**) and 49 for significance at $P = 0.05$ with SPA analysis (**Table 4** and **Figure 2**). Among the QTL identified with CIM all were for seed coat Fe and Zn and none were found for cotyledonary Fe and Zn. The seed coat CIM—QTL were divided into six Zn loci on linkage groups B01, B02, B07, and B11 and one Fe locus on linkage group B04 based on linkage group assignments. LR values ranged from 9.49 for the Fe QTL to 29.53 for one of the Zn QTL. It was notable that the Fe QTL and three of the Zn QTL, including one with the second highest LR value, were derived from the high mineral wild donor parent, G10022. Three other QTL for Zn concentration, including one with the highest LR values and one with the third highest LR value were from the cultivated recurrent parent, Cerinza. Two linkage groups contained more than one QTL for Zn concentration but in each case the two QTL were derived from the same parental allele. For example on linkage group B01, both QTL had positive alleles from G10022; while on B02, both QTL had positive alleles from Cerinza. The additive effect of the Fe QTL showed that this represents a major gene that can provide up to 110 ppm increased seed coat mineral concentration, which would change the balance and total concentration of Fe in the whole seed. The additive effects of the Zn QTL were smaller, ranging from 3.6 to 12.1 ppm increases which are also important in total seed mineral concentration considering the range of variability for Fe (243 ppm) and Zn (32.43) in seed coat. For example the additive effect of 12.1 ppm for Zn is 44.53% increase and very similar to that observed for Fe (45.27%).

Although many SPA—QTL were identified for iron and zinc (**Table 5**) these results agreed with the results from CIM—QTL analysis in terms of major locations and source of high iron or zinc alleles, validating the previous analysis. SPA results for minerals other than Fe and Zn are shown in Table S2. Of the

Table 2 | Significance in terms of probability (P) of *F*-tests for the analyses of variance for each mineral evaluated by inductively coupled plasma (ICP) analysis for seed coat vs. cotyledonary tissue mineral concentration measured for the advanced backcross genotypes (lines) and parents (Par.) of the advanced backcross population of common beans.

Sources[1]	Df[2]	B-Lines	Ca-Lines	Cu-Lines	Fe-Lines	K-Lines	Mg-Lines	Mn-Lines	P-Lines	S-Lines	Zn-Lines
Tissues	1	0.0395*	0.0057**	0.0010***	0.0273*	0.0024**	0.0018**	0.0017**	0.0008***	0.0002***	0.0020**
Genotypes	59	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***
Tissues × Genotypes	59	0.0246*	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***	0.0000***
C.V. (%) Rep × Tissue		7.55	13.34	1.58	2.53	2.08	1.70	2.78	1.67	0.39	0.03
CV (%) Rep × Tissue × Genotype		24.89	4.00	2.18	2.89	1.13	2.12	2.30	2.10	2.15	3.31
Sources[1]	**Df[2]**	**B-Par.**	**Ca-Par.**	**Cu-Par.**	**Fe-Par.**	**K-Par.**	**Mg-Par.**	**Mn-Par.**	**P-Par.**	**S-Par.**	**Zn-Par.**
Tissues	1	0.0918^{ns}	0.0060**	0.0007**	0.0347*	0.0021**	0.0067**	0.0010***	0.0012**	0.0035**	0.0322*
Genotypes	59	0.3790^{ns}	0.0275*	0.0031**	0.0003**	0.0013**	0.0146*	0.0047**	0.0022**	0.0038**	0.0197*
Tissues × Genotypes	59	0.0676^{ns}	0.0270*	0.0711^{ns}	0.0001***	0.0185*	0.0022**	0.0020**	0.0037**	0.0077**	0.1736^{ns}
C.V. (%) Rep × Tissue		3.25	2.59	0.21	0.76	0.28	0.93	0.33	0.47	1.17	1.52
CV (%) Rep × Tissue × Genotype		7.14	5.86	2.92	0.67	1.08	1.40	1.21	1.55	2.74	2.03

[1]Mineral abbreviations for sources of variation: B, boron; Ca, calcium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; P, phosphorus; S, sulfur; Zn, zinc.
*Tissue Abbreviations: coat, seed coat; coty, cotyledon (and embryo). Significance codes: *** = 0.001, ** = 0.01, * = 0.05, ns, not significant.*
[2]Df, Degrees of freedom, based on the ANOVA analysis.

biofortification SPA - QTL, a total of 16 were for Fe in the seed coat and 10 were for Fe in the cotyledon. All of the seed coat Fe QTL were the result of alleles from G10022, the very high seed coat iron parent, and four of the associated markers with highest additive effect were on B04. The phenotypic variance explained by each of the markers ranged from 7.2 to 25.3 % with the marker Pv-at03 and the surrounding markers on linkage group B04 being the most important, confirming the results of a major locus detected with CIM analysis. The additive effects of substitution of the G10022 allele for any of these markers was an amazing 113 to 171 ppm of iron with BMd9 the most significant marker in terms of additivity.

Other slightly less important SPA-QTL seed coat Fe were detected on B02 (at ATA16) and B03 (at ATA26), but these still had major additive effects of up to 172 and 100 ppm iron concentration, respectively, for the best marker in each region. The phenotypic effects explained by these regions averaged 10 and 9%, respectively. A more minor QTL was found on B01 with smaller additive effects but still contributing over 55 ppm which is more than the average seed concentration of most common beans. The phenotypic variances explained by these markers (PV54, 107, and 233) were around 10%.

Iron in the cotyledon presented a total slightly smaller number of QTL (10) than seed coat QTL (16) with the SPA method. However, in contrast to seed coat QTL, most of these SPA-QTL for cotyledonary Fe (8) were derived from Cerinza and only 2 were from G10022. The position of the QTL for cotyledon Fe were different than those for the seed coat Fe on some linkage groups for example B07 and B10. On the other hand seed coat and cotyledon QTL overlapped on some linkage groups (B01, B02, B03, B04, and B08). On linkage group B04, in both cases QTL were linked to BMd16 marker for seed coat and cotyledon, although from a different source (Cerinza instead of G10022, respectively). The effects of these QTL were significant as seen in the additivity values of 6.6 to 21.9 ppm (up to 56.9% more iron) considering the range of variability for cotyledon Fe (38.5 ppm). The QTL explained 6.9–15.1% of phenotypic variance.

In terms of Zn QTL, the SPA method detected 10 QTL for seed coat concentration and 12 QTL for cotyledon concentration. Among the first of these QTL the associated markers were distributed across linkage groups B02, B03, B05, B06, B08, B10, and B11 with most of the high Zn alleles (9) coming from G10022, except for the first one on B02. Their importance however in terms of additivity was more minor (6.4–12.7 ppm) although the explained variance ranged from 7.3 to a high of 24.6%. In the case of the cotyledonary zinc SPA-QTL, 4 were derived from Cerinza and 8 from G10022 and their additive effect ranged from 2.3 to 9.0 ppm zinc and were found on B01, B03, B04, B06, B09, and B11. In terms of overlap, the Zn SPA-QTL on linkage group B02 were different than the CIM-QTL identified on this same linkage group. In summary some seed coat Fe or Zn were in similar locations as cotyledonary QTL while others were in different locations than for cotyledonary QTL.

DISCUSSION

The major results of this study present a quandary for biofortification breeding: namely, that both the distribution

Table 3 | Pearsons correlation coefficients and significance for ten minerals[1] evaluated by inductively coupled plasma (ICP) analysis for seed coat and cotyledonary tissue in the advanced backcross population of common beans.

Coat	B	Ca	Cu	Fe	K	Mg	Mn	P	S
Ca	−0.1035^{ns}								
Cu	0.0482^{ns}	0.0096^{ns}							
Fe	−0.4808***	0.1085^{ns}	0.0968^{ns}						
K	0.2303*	−0.2054*	0.2173*	0.0011^{ns}					
Mg	−0.1274^{ns}	−0.2740**	0.0909^{ns}	−0.0663^{ns}	−0.2546**				
Mn	−0.0133^{ns}	−0.2959***	0.2196*	0.1159^{ns}	0.2208*	0.1239^{ns}			
P	−0.2396**	0.0315^{ns}	0.3075***	0.7342***	0.4418***	0.0613^{ns}	0.1758^{ns}		
S	0.0250^{ns}	0.0613^{ns}	0.5226***	0.2927**	0.3577***	0.1733^{ns}	0.1404^{ns}	0.6848***	
Zn	−0.0487^{ns}	−0.0799^{ns}	0.0559^{ns}	0.1538^{ns}	−0.2975**	0.1154^{ns}	0.4137***	−0.0767^{ns}	0.0152^{ns}
Coty	**B**	**Ca**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**P**	**S**
Ca	0.1024^{ns}								
Cu	−0.1461^{ns}	−0.1233^{ns}							
Fe	0.1192^{ns}	0.3660***	0.1424^{ns}						
K	0.0615^{ns}	−0.1990*	0.2513**	−0.0915^{ns}					
Mg	0.1419^{ns}	−0.0495^{ns}	0.0254^{ns}	−0.2811**	0.1431^{ns}				
Mn	0.0971^{ns}	0.0631^{ns}	0.2243*	−0.0924^{ns}	0.3531***	0.2431**			
P	−0.2117*	−0.1024^{ns}	0.6508***	0.1367^{ns}	0.3421***	−0.0848^{ns}	0.4886***		
S	−0.2208*	0.0595^{ns}	0.5806***	0.1610^{ns}	0.0948^{ns}	−0.0020^{ns}	0.5513***	0.7512***	
Zn	−0.1665^{ns}	0.1791^{ns}	0.5358***	0.1746^{ns}	0.1425^{ns}	−0.0095^{ns}	0.4922***	0.7235***	0.8094***

[1]Mineral abbreviations for correlation coefficients: B, boron; Ca, calcium; Cu, copper; Fe, iron; K, potassium; Mg, magnesium; Mn, manganese; P, phosphorus; S, sulfur; Zn, zinc.

*Tissue Abbreviations: coat, seed coat; coty, cotyledon (and embryo). Significance codes: *** = 0.001, ** = 0.01, * = 0.05, ns, not significant, as indicated by dark to light shaded boxes, with no significance non-shaded.*

Table 4 | Quantitative trait loci for seed coat iron and zinc concentration identified by composite interval (CIM) mapping analysis in the advanced backcross population derived from the wild donor parent (G10022) and the recurrent cultivated parent (Cerinza).

Trait	QTL name	Chr.	Position	LR[1]	Additivity	Source	R2	Nearest marker[2]
SEED COAT QTL								
Fe		4	0.0901	9.49	109.8173	G10022	0.087679	PV-gaat1
Zn		1	0.1701	27.93	5.7301	G10022	0.062669	PV139
Zn		1	1.3701	12.41	3.6300	G10022	0.035964	PV54
Zn		2	2.4201	22.69	4.9477	Cerinza	0.042576	ATA133
Zn		2	3.3001	29.53	5.4577	Cerinza	0.042892	PV78
Zn		7	2.4801	13.95	12.1286	Cerinza	0.274638	PV35
Zn		11	0.6001	13.83	5.4419	G10022	0.077907	BMd33
COTYLEDON QTL								
Fe	None found	0	NA	NA	NA	NA	NA	NA
Zn	None found	0	NA	NA	NA	NA	NA	NA

[1]LR, likelihood ratio test statistic for H_0:H_1 where H_0 is the hypothesis of no QTL effect at test position and H_1 is the hypothesis of a QTL effect at the test position; R2, proportion of variance explained by the QTL at test site; Values have significance at 0.5% probability after 1000-fold permutation tests.

[2]The nearest marker is the marker closest to the peak LR score.

and the inheritance of micronutrients is different in the maternally derived seed coat tissue versus in the cotyledonary tissues which are part of the embryo which will germinate in the next sporophytic generation. These results confirm the variability in seed coat Fe found by Ariza-Nieto et al. (2007) and Moraghan et al. (2002): however, our genetic results are new and differ from previous inheritance studies that evaluated micronutrient concentrations in whole seed in other populations (Blair et al., 2009,

2010a, 2011) or with the same population (Blair and Izquierdo, 2012). When dealing with maternally-derived seed coat tissue we must think about the seed-producing generation in terms of genetics, plant growth and seed development. For any seed trait the previous season's growing conditions are important too.

The importance of differences in micronutrient distribution in seed coat and the rest of the seed resides in the interaction of minerals between each other and with secondary metabolites such as phytates or tannins that vary in concentration as well as between seed coat and cotyledonary tissues (Moraghan, 2004; Ariza-Nieto et al., 2007). In terms of inheritance of the

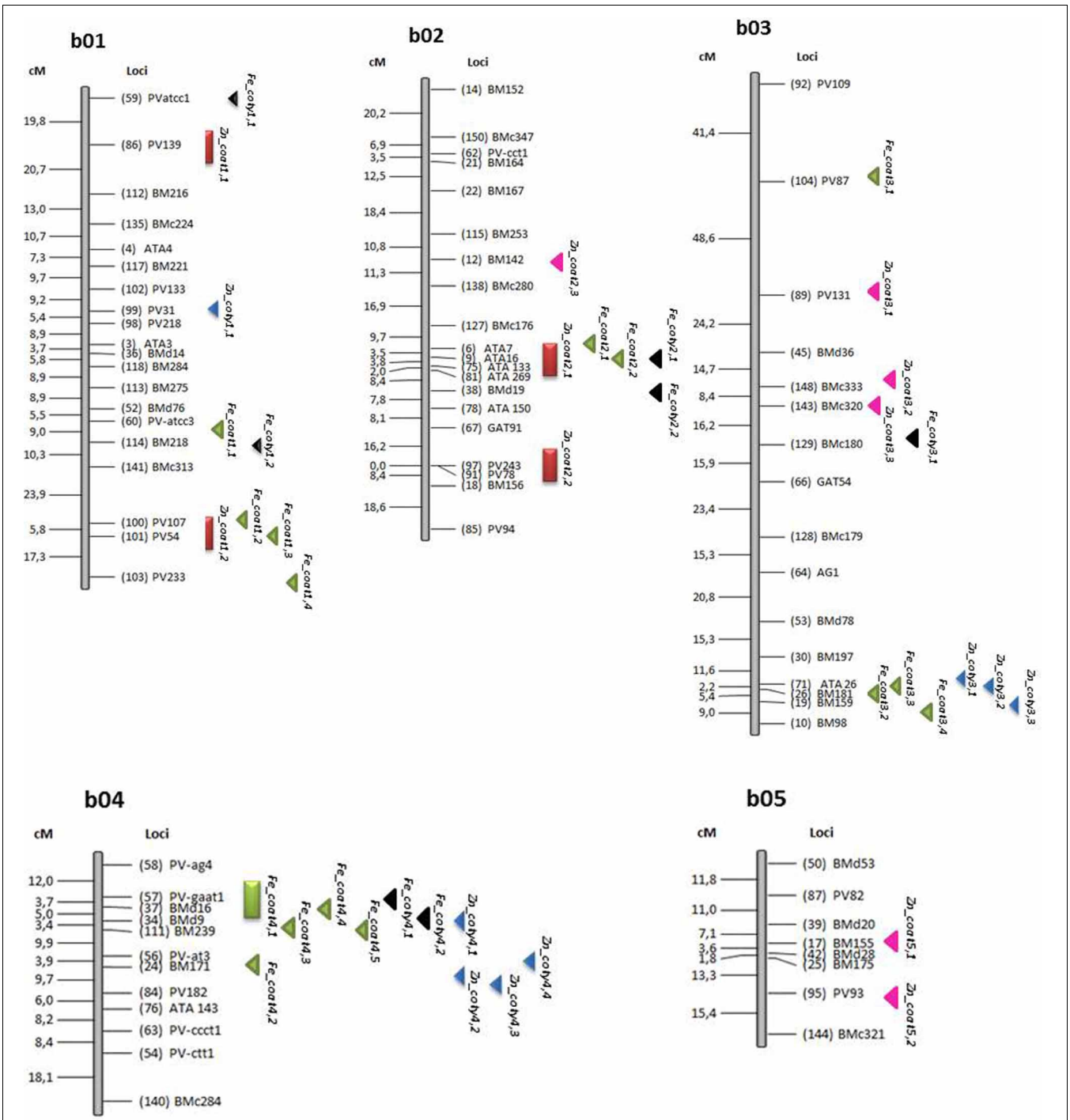

FIGURE 2 | Continued

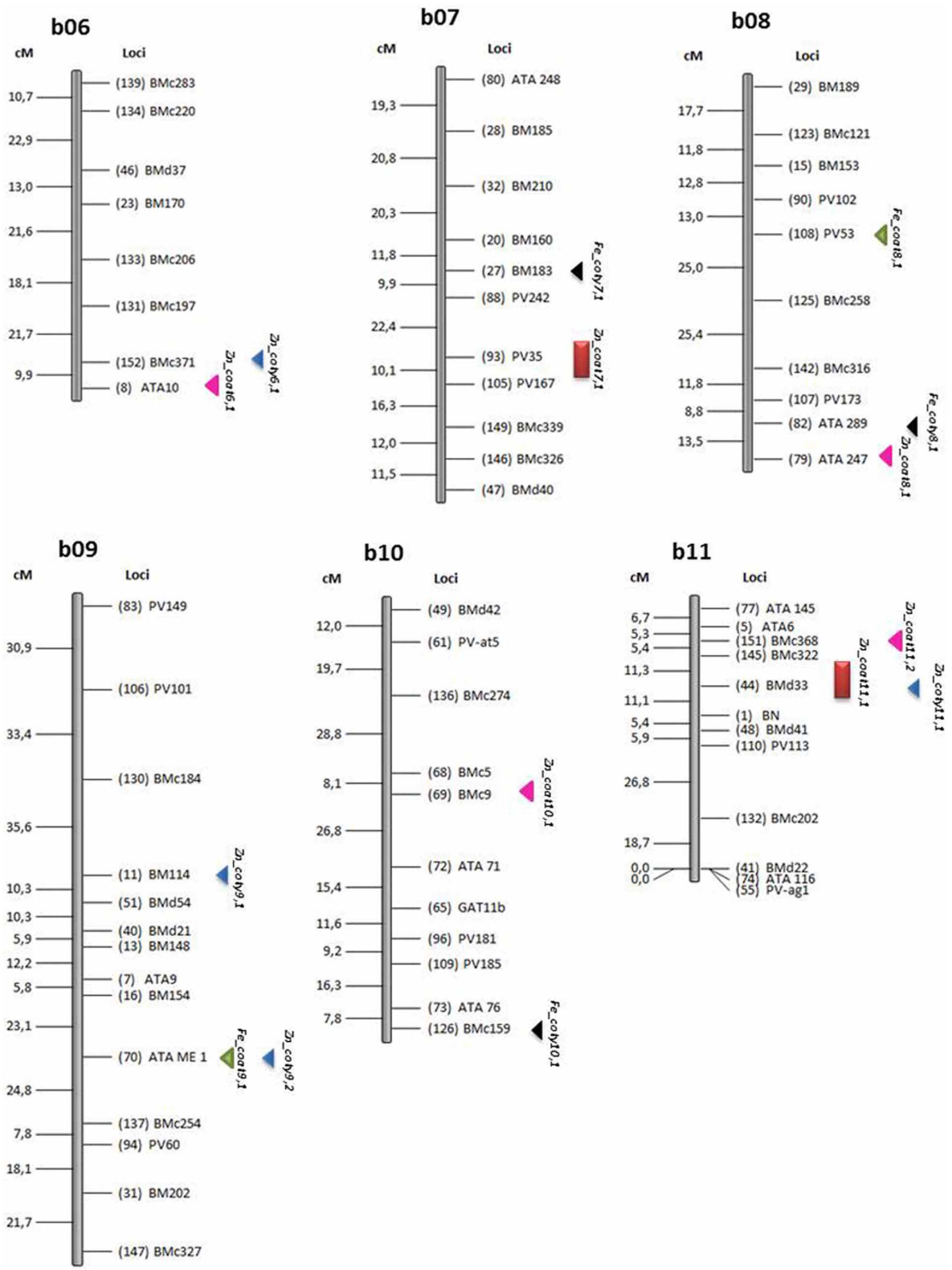

FIGURE 2 | Location of quantitative trait loci (QTL) for iron (Fe) and zinc (Zn) concentration in seed coat. Those QTL identified by CIM analysis in the (Cerinza × (Cerinza × (Cerinza × G10033))) advanced backcross population are indicated by thick bars acompanied by a QTL name as indicated in **Table 4**. QTL identified for Fe and Zn with SPA analysis as indicated in **Table 5** are shown as left pointing arrow heads pointing towards the most significant markers. Abbreviations for QTL are based on Fe, iron; Zn, zinc in seed coat; coat or cotyledon, coty.

Table 5 | Quantitative trait loci for seed coat or cotylendon iron (Fe) and zinc (Zn) concentration identified by single point analysis (SPA) in the advanced backcross population derived from the wild donor parent (G10022) and the recurrent cultivated parent (Cerinza).

LG	Marker	Significance			Additivity	Source
		F	R^2	*P*		
Fe COAT						
1	BM218	5.23	0.083	0.026	79.16	G10022
1	PV107	5.63	0.094	0.021	57.80	G10022
1	PV54	5.06	0.090	0.029	59.94	G10022
1	PV233	6.81	0.118	0.012	81.59	G10022
2	BMc176	7.51	0.128	0.008	82.73	G10022
2	ATA16	5.55	0.090	0.022	172.43	G10022
3	PV87	6.19	0.100	0.016	54.30	G10022
3	ATA 26	5.05	0.101	0.029	100.33	G10022
3	BM159	4.12	0.070	0.047	58.88	G10022
3	BM98	4.96	0.083	0.030	54.60	G10022
4	Pv-at3	17.36	0.258	0.000	127.50	G10022
4	PV182	10.59	0.172	0.002	113.25	G10022
4	BMd16	6.32	0.098	0.015	132.87	G10022
4	BMd9	5.26	0.083	0.025	171.37	G10022
8	PV53	4.17	0.072	0.046	73.31	G10022
9	ATA ME1	5.03	0.093	0.029	74.97	G10022
Fe COTYLEDON						
1	PVatcc1	4.74	0.076	0.033	6.61	Cerinza
1	BMc313	6.15	0.116	0.017	8.54	Cerinza
2	ATA7	4.22	0.069	0.044	15.78	Cerinza
2	GAT91	6.36	0.104	0.015	9.58	G10022
3	BMc180	4.02	0.072	0.050	7.15	G10022
4	PV-ag4	4.95	0.084	0.030	7.85	Cerinza
4	BMd16	5.27	0.083	0.025	12.42	Cerinza
7	BM183	8.82	0.143	0.004	14.44	Cerinza
8	ATA 289	7.64	0.128	0.008	21.08	Cerinza
10	BMc159	9.23	0.151	0.004	21.89	Cerinza
Zn COAT						
2	BM142	5.03	0.088	0.029	7.43	Cerinza
3	PV131	4.57	0.089	0.038	6.40	G10022
3	BMc333	4.98	0.092	0.030	10.84	G10022
3	BMc320	4.74	0.079	0.034	10.34	G10022
5	BMd20	4.59	0.073	0.036	10.34	G10022
5	PV93	5.60	0.096	0.021	8.44	G10022
6	ATA10	7.95	0.124	0.007	10.62	G10022
8	ATA 247	13.07	0.246	0.001	12.73	G10022
10	BMc9	4.26	0.074	0.044	6.75	G10022
11	BMc368	4.98	0.082	0.030	17.09	G10022
Zn COTYLEDON						
1	PV133	5.53	0.091	0.022	3.12	Cerinza
3	ATA 26	15.75	0.259	0.000	5.66	G10022
3	BM159	4.93	0.082	0.030	2.48	G10022
3	BM98	6.33	0.103	0.015	2.29	G10022
4	BMd9	11.36	0.164	0.001	9.03	G10022
4	PV182	9.94	0.163	0.003	4.43	G10022
4	ATA 143	8.10	0.135	0.006	3.47	G10022
4	BM171	5.85	0.098	0.019	4.93	Cerinza
6	BMc371	4.94	0.088	0.031	2.96	Cerinza
9	ATA ME 1	10.37	0.175	0.002	3.58	G10022
9	BM114	4.22	0.081	0.045	2.51	G10022
11	BMd33	4.64	0.077	0.035	3.17	Cerinza

micronutrient accumulation traits, the importance of a difference in seed coat and cotyledon resides in the presumably different genes involved in each tissue's mineral accumulation. It was not surprising therefore that we found a different set of QTL for seed coat Fe and Zn in this study compared to our previous study working with whole seed (Blair and Izquierdo, 2012), especially in terms of the major CIM-QTL. Seed coat mineral accumulation is important to consider for breeding of micronutrient rich beans due to the bioavailability of micronutrients of this portion of the seed (Ariza-Nieto et al., 2007) and the GxE effects that are found to affect mineral accumulation (Blair et al., 2010b).

The genetic results described above showed that most minerals were normally distributed both in seed coat and cotyledon or at least in cotyledon across the population. These data proved the multi-genic or quantitative inheritance of mineral concentrations. One exception was the set of values for Fe in seed coat, which had a bimodal distribution indicating a major CIM-QTL and qualitative inheritance which was found to be on linkage group B04. This was in contrast to seed coat Zn concentration which was normally distributed and had six CIM-QTL, which were found on four different linkage groups. SPA–QTL were more abundant and will be discussed as well.

Blair and Izquierdo (2012), with the same population but analysis of whole seed, found the same CIM based QTL for Zn accumulation on B07 near marker PV35 but different QTL for Fe concentration or content on B07 and B08, respectively. Some SPA based QTL for Fe and for Zn from Blair and Izquierdo (2012) overlapped with the seed coat or cotyledon based SPA - QTL on B01, B04, B10, and B11.

The QTL for zinc concentration on B11 were especially important in another Andean × Mesoamerican cross study (Blair et al., 2009). The QTL for zinc concentration on B01 near PV139 may have been influenced by the *fin* locus as was found by Cichy et al. (2009) for another cross involving a determinate Andean bean, similar in growth habit to Cerinza. The *fin* locus was shown to control the determinacy but not the height of bean plant growing shoots (Chavarro and Blair, 2010) and in the advanced backcross population analyzed here was observed to affect total biomass production and plant size (Izquierdo et al., unpublished results). One possible hypothesis could be that larger indeterminate *Fin Fin* plants accumulated a large amount of zinc in vegetative tissue and this zinc was available for translocation to the seed. Meanwhile, the shorter and smaller biomass *fin fin* plants that are determinate in growth habit would accumulate less zinc in both vegetative and reproductive tissues. A different Zn QTL found on linkage group B02 near PV78 and derived from Cerinza was in close proximity to the QTL *ZnPoAAS2.1* found by Blair et al. (2010a) in a Mesoamerican x Mesoamerican cross and another QTL *ZnAAS2c* found by Blair et al. (2011) in an Andean × Andean cross.

On the other hand, many additional SPA-QTL from this study appear to be novel based on their evaluation in the different tissues. For example, the seed coat Fe QTL on B04 derived from G10022 was not detected near any Fe concentration QTL in the cultivated Andean × Andean or Mesoamerican × Mesoamerican populations studied by (Blair et al., 2010a, 2011) and may be specific to wild bean sources. This major QTL from linkage group B04 probably influenced the observation of binomial population distribution in the population for seed coat iron concentration.

The lack of cotyledonary CIM-QTL for iron or zinc in the present study may have been a reflection of the smaller differences between parents for the Fe and Zn concentrations in this tissue, although SPA-QTL analysis identified a good number of markers with significant effects. This may show that epistatic interactions are sometimes important in identification of QTL for the minerals due to the dependence of iron translocation to the seed on the amount of iron uptake by the roots into the plant. More QTLs were found with the SPA method due to the lower probability threshold ($P \leq 0.05$) used in that analysis compared to the CIM method where thresholds were determined with permutations. The lack of cotyledonary CIM iron QTL was a result of high thresholds found for this variable's analysis. Despite this, the cotyledonary QTL where in similar locations to QTL found in the whole seed for the same population by Blair and Izquierdo (2012).

From a breeding perspective, the lines with high seed coat Fe concentration might be of interest if this Fe is shown to be bioavailable although initial results suggest that seed coat iron especially in colored beans is not very bioavailable (Ariza-Nieto et al., 2007). It was notable that some lines of the population had seed coat Fe concentrations of more than 250 ppm, while seed coat Zn concentrations above 50 ppm might be of interest. The microsatellite that were associated with QTL for seed coat mineral concentration could be used for marker assisted selection (MAS) of these traits as was suggested for whole seed concentration QTL by Blair and Izquierdo (2012).

Considering that the seed coat makes up approximately 10% of the seed the amount of iron in the seed coat can have a large impact on the amount of iron in the entire seed. In populations like the advanced backcross lines, the amount of seed coat is fairly uniform but the amount of seed coat Fe was not. In a typical recombinant inbred line population that segregates for seed size, the percentage seed coat, the ratio of seed coat to total seed and the amount of seed coat Fe would all be variables to study. The result of the high iron concentration in the seed coat and the high percentage of seed coat to seed weight is that seed coat Fe at high concentrations affects the overall average Fe concentration of the seed quite substantially. For example, from this study and our previous results (Blair and Izquierdo, 2012), the total amount of seed iron can increase or decrease by up to 40 ppm based mostly on seed coat Fe content.

The results we observed for correlation among minerals for cotyledons are different than Fe-Zn relationships observed previously for whole seed in several populations (Blair et al., 2009, 2010a, 2011) and in the same advanced backcross population (Blair and Izquierdo, 2012). Meanwhile seed coat results showed interactions of Fe and P which are new information for seed coat,

but there was no correlation for Fe and Zn, in contrast to Blair et al. (2009). A relationship of Fe and P in seed coat was surprising considering that phytate which binds Fe should not be found in high concentrations in the seed coat but rather in the cotyledons (Ariza-Nieto et al., 2007).

The Fe and P correlation in seed coat but not in cotyledons can be influenced by the fact that concentrations of P in the seed coat are only one-tenth that of P concentrations in the cotyledons and that in seed coat P may be binding with tannins and other seed coat substances that also bind Fe (Blair et al., 2012). The lack of correlation in the cotyledon between P and Fe might be because Fe in legumes is targeted to vascular cells where starch also accumulates in amyloplast and is found bound to ferritin (Cvitanich et al., 2010). Perhaps phytates are not important in sequestering iron in the seed but rather only as anti-nutrients in the human digestive system (Ariza-Nieto et al., 2007). On the other hand P fertilization is known to affect Zn uptake by plants (Moraghan and Grafton, 2002) and Zn QTL detection (Cichy et al., 2009). The number of Zn QTL contrasts with single gene inheritance found by Cichy et al. (2005) in navy beans, but agrees with multiple gene inheritance found in later studies (Blair et al., 2009, 2010a, 2011; Cichy et al., 2009).

Our hypothesis of a major gene for Fe in seed coat on B04 is the first time a single gene has been proposed for control of this mineral's accumulation in the seed coat of common bean and therefore might be a target for gene cloning and characterization. It would be interesting to know if the seed coat accumulation of Fe is based on a seed coat expressed gene or a gene that diminishes loading of iron into the seed's embryo/cotyledons. Based on co-localization or the lack thereof, ferritin is unlikely to be the mechanism of iron accumulation in seed coat but binding with seed coat tannins could be possible (Ariza-Nieto et al., 2007; Cvitanich et al., 2010). The location of the QTL on the long arm of B04 is interesting as nearby genes for phytohemaglutinnins are expressed only in seeds (Blair et al., 2010c). The clustering of genes expressed in the same tissue is often typical.

Whatever the case, the specificity of the gene to the wild bean source is interesting and could be relevant for gene discovery in other legumes, such as the model species *Medicago truncatula*, where transcriptome analysis has shown the large number of genes (over 30,000) expressed in the seed coat (Verdier et al., 2013). Before this study, the high iron of wild beans could be questioned as an artifact based on the high ratio of seed coat to total seed size in these very small seeded seeds (less than 10 g per 100 seed). If this is the case it would be difficult to use wild beans as a source of high iron. However, it appears that wild beans might preferentially accumulate iron in their seed coats compared to cultivars. Alternatively, cultivars might have been selected to accumulate less iron in their seed coats than wild beans as part of domestication and development of large seed sized domesticates. Selection processes may have been different in large-seeded Andean vs. small-seeded Mesoamerican beans or in very thick-seed coated species such as scarlet runner bean and year-long bean (Singh, 2001).

The idea that human selection for these invisible micronutrient traits has been active is intriguing. We could postulate that this selection was conducted through proxy mechanisms such as seed coat color, palatability, cooking time or noticeable health benefits. Seed coat color is an obvious trait that differs greatly in common bean cultivars with some of these colors associated with tannin or anthocyanin accumulation (Caldas and Blair, 2009; Díaz et al., 2010). Perhaps humans instinctively were attracted to low tannin varieties of white and yellow beans in some cultures or developed methods to remove tannin in red and black beans by soaking, thus also affecting palatability.

Palatability is hard to measure, but cooking time parameters have been evaluated in various studies that also have looked at micronutrient concentration. For example, Saha et al. (2009) found that zinc concentration was negatively correlated with swelling and hydration capacity as components of cooking quality. A few high iron genotypes from that study were also hard to cook. The amount of Fe in the seed coat might have implications for the cooking method recommended when aiming to preserve the nutritional quality of common beans (Ariza-Nieto et al., 2007; Carvalho et al., 2012). As discussed, some of the QTL specific to the seed coat concentration for Fe and Zn were the same as those identified for overall mineral content and might lead Fe to be more likely to stay in the seed when cooked.

The idea that farmer-consumers selected for micronutrient density in the parts of the seed that were more bioavailable in their cultivars compared to their wild relatives is not far-fetched: iron consumption may be noticeable in a feeling of more energy from avoiding anemic status which would have been connected to the foods eaten. The diet of Amerindians from the tropical New World who domesticated common beans was unlike the diets of Indigenous people to the north or south which were very low in animal protein sources. Therefore, the traditional diet of pre-Colombian Middle American and Andean cultures are almost exclusive in vegetable protein sources with basic staples such as maize, beans and squash that were better in amino acids, micronutrients and vitamins than the staples of "Old World" consumers, who had carbohydrate-rich millets, sorghum, rice and wheat. Beans are known as the "meat of the poor." Perhaps they should be better known as "meat of the Americas" and as "a substitute for animal protein" as many poor people or vegetarians in Africa, Asia and the Americas are well aware of, based on their high consumption of beans.

In conclusion, further molecular and physiological approaches are needed to explain the mechanism for iron accumulation in the seed coat versus the cotyledons but this study provides a starting point for gene and tissue analysis of seed coat minerals. Additional work should look into why there is a very different genetic control of seed iron and zinc tissue distribution in wild vs. cultivated beans, with higher concentrations of iron accumulating in the seed coat of wild beans, but lower concentrations in their cotyledons, and the reverse being the case for cultivated beans. In addition to a major gene on B04 for seed coat Fe in both CIM and SPA analysis, it was notable that the most

important of the seed coat Zn SPA-QTL was from a region of the genome on linkage group B11, that has been important in other studies (Blair et al., 2009; Cichy et al., 2009) and that contains ZIP type transporters, which have a role in the uptake and transport of iron in plants (K. Cichy, pers. Communic). Some other regions on B01, B02, B03, B04, B09, and B10 overlap for QTL with those of studies of Fe and Zn accumulation in whole beans (Blair et al., 2009, 2010a, 2011; Cichy et al., 2009). The distribution of QTL for other minerals or the effect of seed weight QTL on B02, B03, and B09 might be worth looking at in more detail in respect to Fe and Zn accumulation. The implications of all this work for biofortification of beans should all be considered in molecular breeding and physiological analysis. Genetic studies along with candidate gene analysis provide the framework to understand the physiology of iron uptake into the plants, transfer to the pod and seed and accumulation in different seed tissues.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2013.00275/abstract

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Vitamin D in plants: a review of occurrence, analysis, and biosynthesis

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The major function of vitamin D in vertebrates is maintenance of calcium homeostasis, but vitamin D insufficiency has also been linked to an increased risk of hypertension, autoimmune diseases, diabetes, and cancer. Therefore, there is a growing awareness about vitamin D as a requirement for optimal health. Vitamin D_3 is synthesized in the skin by a photochemical conversion of provitamin D_3, but the necessary rays are only emitted all year round in places that lie below a 35° latitude. Unfortunately, very few food sources naturally contain vitamin D and the general population as a results fail to meet the requirements. Fish have the highest natural content of vitamin D expected to derive from an accumulation in the food chain originating from microalgae. Microalgae contain both vitamin D_3 and provitamin D_3, which suggests that vitamin D_3 exist in the plant kingdom and vitamin D_3 has also been identified in several plant species as a surprise to many. The term vitamin D also includes vitamin D_2 that is produced in fungi and yeasts by UVB-exposure of provitamin D_2. Small amounts can be found in plants contaminated with fungi and traditionally only vitamin D_2 has been considered present in plants. This review summarizes the current knowledge on sterol biosynthesis leading to provitamin D. It also addresses the occurrence of vitamin D and its hydroxylated metabolites in higher plants and in algae and discusses limitations and advantages of analytical methods used in studies of vitamin D and related compounds including recent advances in analytical technologies. Finally, perspectives for a future production of vitamin D biofortified fruits, vegetables, and fish will be presented.

Keywords: plants, algae, biosynthesis, vitamin D, 25-hydroxy vitamin D, 1,25-dihydroxy vitamin D, sterols, detection

INTRODUCTION

The main function of vitamin D is in maintenance and regulation of calcium levels in the body and vitamin D is, therefore, critically important for the development of a healthy skeleton. Thus, vitamin D insufficiency increases the risk of osteoporosis, but has also been linked to an increased risk of hypertension, autoimmune diseases, diabetes, and cancer (Hyppönen et al., 2001; Cantorna and Mahon, 2004; Holick, 2004; Lappe et al., 2007; Pittas et al., 2007; Kendrick et al., 2009). As a result, there is a growing awareness about vitamin D as a requirement for optimal health. Vitamin D_3 is synthesized in the skin by a photochemical conversion of provitamin D_3, but the necessary UVB rays (290–315 nm) are only emitted all year round in places that lie below a 35° latitude. Thereby, a dietary intake of vitamin D becomes essential, but very few food sources naturally contain vitamin D. The consequence of a low dietary intake and limited vitamin D derived from the sun is that the general populations fail to meet their vitamin D requirements (Brot et al., 2001; Bailey et al., 2010). Fish have the highest natural amount of vitamin D_3, which is expected to derive from a high content of vitamin D_3 in planktonic microalgae at the base of the food chain (Takeuchi et al., 1991; Sunita Rao and Raghuramulu, 1996a). The occurrence of vitamin D_3 in algae suggests that vitamin D_3 may exist in the plant kingdom and vitamin D_3 has also been identified in several plant species as a surprise to many (Boland et al., 2003). The term vitamin D also includes vitamin D_2 that is produced in fungi and yeast by UVB-exposure of provitamin D_2 and small amounts can be found in plants contaminated with fungi. Traditionally, only vitamin D_2 has been considered present in plants.

Two reviews exist on vitamin D compounds in plants (Boland, 1986; Boland et al., 2003). Boland (1986) focused on plant species with vitamin D-like activity, possible functions of vitamin D_3 in these plants and metabolism of $1,25(OH)_2D_3$ glycosides in animals. Boland et al. (2003) dealt with the detection, presence, and tissue distribution of vitamin D_3 compounds in flowering plants, the production of vitamin D_3 and derived metabolites in plant cultures, and biological functions of vitamin D_3 in flowering plants. However, important questions still remain, especially regarding the biosynthesis of vitamin D in plants and the present review, therefore, summarizes current knowledge on sterol biosynthesis leading to provitamin D. Before discussing this subject, essential information on vitamin D synthesis, metabolism, biological functions, as well as dietary sources and recommended intake of vitamin D are described. This review also considers the occurrence of all vitamin D active compounds existing in plants and algae and discusses the advantages and disadvantages of analytical methods applied for studying vitamin D and related compounds including recent advances

in analytical technologies. Finally, perspectives for the future production of vitamin D biofortified fruits, vegetables, and fish will be presented.

VITAMIN D

SYNTHESIS AND ACTIVATION OF VITAMIN D

Vitamin D is classified into five different classes numbered 2–6. The two main forms of vitamin D are cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2), which differ structurally in the side chain, where vitamin D_2 has a C22–C23 double bound and an additional methyl group at C24 (**Figure 1**). The vitamins are secosteroids, i.e., steroids with a broken ring. Vitamin D_2 is produced in fungi and yeast by a UVB-exposure of ergosterol (provitamin D_2), whereas vitamin D_3 is produced by UVB-exposure of 7-dehydrocholesterol (provitamin D_3) in the skin (**Figure 1**). The conversion to the previtamin D happens by an exposure to sunlight at 290–315 nm (UVB) (**Figure 1**). Conversion also happens at lower wavelengths, but solar radiation below 290 nm is prevented from reaching the earth's surface by the ozone layer in the stratosphere (MacLaughlin et al., 1982). High-energy photons are absorbed in the conjugated 6,7-diene in the B-ring of ergosterol and 7-dehydrocholesterol, which results in a ring opening at C9 and C10 to form previtamin D (Havinga, 1973). Previtamin D is biologically inactive and thermodynamically unstable and undergoes a transformation to vitamin D in a temperature-dependent manner (Havinga, 1973). Previtamin D_3 will by prolonged UVB-exposure be converted to the inactive forms lumisterol and tachysterol to protect the organism from vitamin D toxicity (Holick et al., 1981). Synthesis of vitamin D in the skin depends on, e.g., season and latitude. The solar zenith angle increases during the winter months and with latitude. When the solar zenith angle is large, filtration of sunlight through the ozone layer takes place through an increased path length, decreasing the UVB photons that penetrate into the earth's surface. As a result, the rays necessary for the vitamin D production are only emitted all year round in places that lie below 35° latitude (Holick, 2003). In the northern hemisphere this is, e.g., Northern Africa and Los Angeles.

Vitamin D from the skin diffuses into the blood, where it is transported by vitamin D binding protein (DPB) to the liver, whereas vitamin D from the diet is absorbed in the small intestine and transported to the liver via chylomicrons and DBP. Vitamin D is biologically inactive and the activation involves two hydroxylations (**Figure 1**). First, vitamin D is hydroxylated in the liver at C-25 by a 25-hydroxylase to yield 25-hydroxyvitamin D (25OHD) (Jones et al., 1998; Prosser and Jones, 2004). The activity of 25-hydroxylase is poorly regulated and dependent primarily on the concentration of vitamin

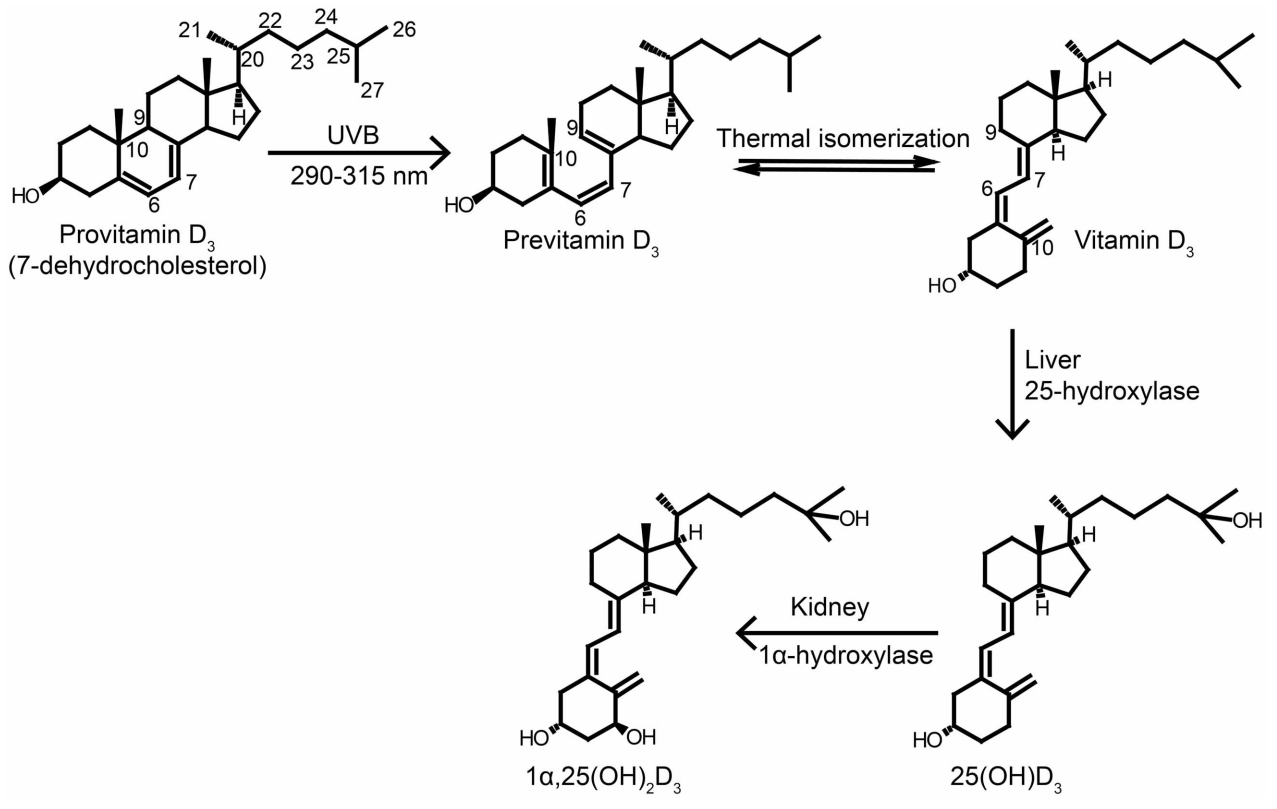

FIGURE 1 | Synthesis and activation of vitamin D. Vitamin D_3 is synthesized in the skin upon UVB exposure. The UVB exposure of provitamin D_3 (7-dehydrocholesterol) in the skin breaks the B-ring to form previtamin D_3, which undergoes thermally induced rearrangement to vitamin D_3. Vitamin D_3 is transported to the liver where it is hydroxylated at C-25 by the enzyme 25-hydroxylase producing $25OHD_3$, which is the major circulating form in vertebrates. The $25OHD_3$ is hydroxylated a second time at C-1 in the kidneys to the active metabolite $1,25(OH)_2D_3$. Figure adapted from Jäpelt et al. (2012).

D (Bhattacharyya and DeLuca, 1973). The circulating concentration of 25OHD is the accepted biomarker for vitamin D status, as this reflects both dietary intake and skin production. The optimal vitamin D status has been a subject of debate and there is no general standard (Dawson-Hughes et al., 2005). Some studies indicate that vitamin D_2 and vitamin D_3 acts equally in maintaining vitamin D status (Rapuri et al., 2004; Holick et al., 2008), while others indicate that vitamin D_2 is less effective than vitamin D_3 (Trang et al., 1998; Armas et al., 2004). After production of 25OHD in the liver, it is transported, to the kidneys bound to DPB. In passing through the kidneys, 25OHD is hydroxylated at the α-position of C-1 by 1α-hydroxylase to generate 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D] (Jones et al., 1998; Prosser and Jones, 2004). The bioconversion of 25OHD to 1,25$(OH)_2$D is strictly regulated by serum calcium and serum phosphorus levels, 1,25$(OH)_2$D blood levels and parathyroid hormone (PTH) (Prosser and Jones, 2004).

BIOLOGICAL EFFECT OF VITAMIN D

The main function of vitamin D is in maintenance and regulation of calcium and phosphorus levels in the body (DeLuca, 2004). Low blood calcium stimulates release of PTH from the parathyroid gland. In turn, PTH stimulates 1α-hydroxylase in the kidneys to produce 1,25$(OH)_2$D, which then increases serum calcium and phosphorus concentrations by acting on three targets: increased absorption from the intestine, reabsorption in the kidneys and mobilization from bones (DeLuca, 2004). The active metabolite, 1,25$(OH)_2$D, mediates its biological effect by binding to the vitamin D receptor (VDR). The mechanisms by which 1,25$(OH)_2$D performs its biological effect can be divided into two: a genomic and a non-genomic (Norman et al., 1992). The genomic mechanism is mediated by nuclear VDRs that on binding to 1,25$(OH)_2$D interacts with DNA to modulate gene expression, while the non-genomic pathway includes interactions with VDRs in the cell membrane (Norman et al., 1992). The non-genomic pathway is usually working very fast, i.e., within seconds and minutes, whereas genomic responses typically take a few hours to days (Norman, 2006).

DIETARY INTAKE AND RECOMMENDED DAILY INTAKE OF VITAMIN D

Because the body produces vitamin D_3, vitamin D does not meet the classical definition of a vitamin. Generally, fish have the highest natural amount of vitamin D_3, e.g., salmon contains 30 μg/100 g and tuna 2.9 μg/100 g (Danish Food Composition Databank, revision. 7, 2008). Other sources of vitamin D_3 are meat (∼0.6 μg/100), egg (∼1.75 μg/100) and milk products (∼0.1 μg/100) (Danish Food Composition Databank, revision. 7, 2008). The content of vitamin D in food of animal origin depends on what the animal has been fed (Mattila et al., 1999; Graff et al., 2002; Jakobsen et al., 2007). The main compound in food is vitamin D_3, but the metabolites, which are part of the metabolic pathway in vertebrates also exist (Mattila et al., 1995a,b; Clausen et al., 2003; Jakobsen and Saxholt, 2009). The potency of 25OHD has often been attributed to possess five times the potency of vitamin D (Reeve et al., 1982; Cashman et al., 2012). This value is implemented in food composition databases. However, there is no consensus on the conversion factor that should be used for 25OHD to calculate the vitamin D activity mainly because of very limited human data (Jakobsen, 2007; Cashman et al., 2012). The potency of 1,25$(OH)_2$D has been attributed to ten relative to vitamin D (Tanaka et al., 1973), but this value is not implemented in food composition tables, as there is no specific composition data available for 1,25$(OH)_2$D. Food sources of vitamin D_2 are very limited and wild mushrooms are one of the only significant sources of vitamin D_2 (Mattila et al., 1994, 2002; Teichmann et al., 2007). However, milk from dairy cows contains a significant although low amount of vitamin D_2, which is expected to derive from grass and hay (Jakobsen and Saxholt, 2009). Vitamin D fortification of selected foods has been accepted as a strategy to improve the vitamin D status of the general population both in the United States and in many European countries. Milk and margarine are the primary products that are enriched with vitamin D (Natri et al., 2006), but also orange juice (Calvo et al., 2004), bread (Natri et al., 2006; Hohman et al., 2011), cheese and yoghurt may be enriched (Holick, 2011). This area is regulated differently in each country. Fortification may either be voluntary or mandatory and the levels added vary accordingly.

The American dietary vitamin D recommendations are 15 μg/day for the age group 1–70 years including women who are pregnant or lactating and increases to 20 μg/day for adults older than 70 years (Institute of Medicine, 2011). An adequate intake is estimated to 10 μg/day for infants (Institute of Medicine, 2011). Without sufficient vitamin D humans will develop a deficiency disease. Growing children develop rickets because of failure in calcification of cartilaginous growth plates. Osteomalacia develops in adults during prolonged vitamin D deficiency, where the newly formed uncalcified bone tissue gradually replaces the old bone tissue with weakened and soft bones as a consequence. Excessive vitamin D consumption can result in toxicity. Toxic levels are not obtained by a usual diet, but by excessive consumption of vitamin D supplements or over-fortification of food. Vitamin D intoxication is primarily due to hypercalcemia caused by increased intestinal absorption of calcium, together with increased resorption of bones. If the vitamin D exposure is prolonged, deposition of calcium in soft tissues particularly in arterial walls and in the kidney occurs. An upper intake level for vitamin D has been set to 100 μg for adults and children aged 9 years and older (Institute of Medicine, 2011).

THE DISCOVERY OF VITAMIN D IN PLANTS

In 1924 two groups independently discovered that light exposure of inert food could result in antirachitic activity (could cure rickets) (Hess and Weinstock, 1924; Steenbock and Black, 1924). Otherwise, inert foods such as linseed oil, cottonseed oil, wheat and lettuce were made antirachitic when exposed to light from a mercury lamp (Hess and Weinstock, 1924, 1925). The question at that time was: "What was the substance in vegetables and crops that could be activated by light exposure?" Later, vitamin D_2 was identified from solutions of irradiated ergosterol (Askew et al., 1930; Windaus, 1931). The high concentrations of ergosterol in fungi and as a result in plants contaminated with fungi

led to a general perception of vitamin D_2 as a plant form of vitamin D. Vitamin D_3 has on the other hand been considered absent from plants. However, grazing animals in several parts of the world develop calcium intoxication, similar to that caused by vitamin D toxicity, from consuming particular plants (Mello, 2003). This is believed to be due to vitamin D_3 or a metabolite of vitamin D_3 present in the plants that stimulate calcium absorption producing hypercalcemia and deposition of calcium in soft tissue including aorta, heart, kidneys, intestines, and uterus (Mello, 2003). Most work has been made on the plant *Solanum glaucophyllum* Desf. (*S. glaucophyllum*) that causes calcium intoxication of livestock in South America (Mello, 2003). Controlled studies with various animals including rabbits (Mautalen, 1972; Humphreys, 1973; Dallorso et al., 2008), chickens (Wasserman et al., 1976a; Weissenberg et al., 1989) and rats (Uribe et al., 1974; Basudde and Humphreys, 1976) demonstrated that *S. glaucophyllum* leaves or extracts caused increased absorption of calcium and phosphorus similar to vitamin D. *Cestrum diurnum* L. (*C. diurnum*) and *Trisetum flavescens* Beauv. (*T. flavescens*) are also known to cause calcium intoxication very similar to *S. glaucophyllum* (Wasserman et al., 1975; Peterlik et al., 1977; Rambeck et al., 1979). Studies with these plants later led to the identification of vitamin D_3 and related compounds in plant tissue.

STEROLS—PRECURSORS OF VITAMIN D

Sterols are essential for all eukaryotes. They are components of membranes and have a function in regulation of membrane fluidity and permeability (Piironen et al., 2000). Sterols also play an important role as precursors of many steroid hormones including vitamin D and brassinosteroids as well as for a wide range of secondary metabolites such as saponins and glycoalkaloids. Sterols are made up of four rings designated A, B, C, and D with one or more double bonds, a long flexible side chain at C17, a hydroxyl group attached to C3 and a variety of substituents (**Figure 2**). The hydroxy group at C3 can be esterified with either a long-chain fatty acid or a phenolic acid to give a steryl ester (**Figure 3**). Steryl esters are present in all plants, most often localized in the cytoplasm of plant cells (Benveniste, 2002), and represent a storage form of sterols (Piironen et al., 2000). The 3-hydroxy group can also be linked to a carbohydrate forming a steryl glycoside (**Figure 3**). Steryl glycosides usually consist of a mixture differing in carbohydrate moiety and esterification of the sugar by a fatty acid can give rise to an acetylated steryl glycoside (**Figure 3**). Especially, plants from the *Solanaceae* family show a unique abundance of glycosides (Moreau et al., 2002; Potocka and Zimowski, 2008).

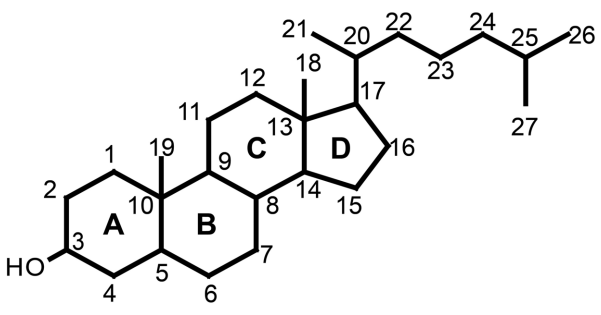

FIGURE 2 | The core structure of sterols is a fused four ring (A,B,C, and D ring). Various groups are added to the core structure to generate a variety of sterols. Numbering of the carbon atoms is according to the 1989 IUPAC-IUB recommendations.

Generally, sterols can be divided into three groups according to the alkylations at the C24 position in the side chain: 24-desmethylsterols (without an alkyl group), 24-demethylsterols (with one methyl group) and 24-ethylsterols (with one ethyl group). 24-desmethylsterols are typical for animals, whereas the 24-demethylsterols and 24-ethylsterols are typical for plants and fungi. Animals and fungi accumulate the major end product sterols, cholesterol (24-desmethylsterol) and ergosterol (24-demethylsterol), whereas the plant kingdom in comparison produces a wide range of sterols. More than 250 sterols have been found in plants (Hartmann, 2004), but sitosterol, campesterol, and stigmasterol normally predominates (Lagarda et al., 2006). Plant sterols typically have a double bond between C5 and C6 in the B ring and are called Δ5-sterols. Sterols with a Δ5 nucleus are the most common, but Δ7-sterols, Δ5,7-sterols, and Δ22-sterols also occur (Piironen et al., 2000). Plant tissues contain an average quantity of 1–3 mg sterols per gram dry weight (Schaller, 2004). The sterol composition of plant species is genetically determined and varies considerably (Schaller, 2003). The model plant, *Arabidopsis thaliana*, e.g., has a sterol composition of 64% sitosterol, 11% campesterol, 6% stigmasterol, 3% isofucosterol, 2% brassicasterol and 14% of other minor sterols (Schaeffer et al., 2001). Cholesterol is the major sterol in animals, but is also present in plants. Usually, cholesterol accounts for 1–2% of total plant sterols, but higher levels are present in especially *Solanaceae* (Whitaker, 1988, 1991; Zygadlo, 1993; Moreau et al., 2002; Jäpelt et al., 2011b). It has been suggested that cholesterol serves as a precursor of glycoalkaloids (Bergenstråhle et al., 1996) and ecdysteroids (Dinan, 2001) in these plants.

VITAMIN D BIOSYNTHESIS

Vitamin D biosynthesis is taking place along the normal sterol pathway, i.e., vitamin D_2 is formed by UVB exposure of ergosterol and vitamin D_3 by UVB exposure of 7-dehydrocholesterol. Therefore, we need to understand how its sterol precursors are formed in order to understand how vitamin D synthesis takes place in plants. Sterol biosynthesis can be divided into two parts. The first part is the mevalonic acid pathway. All isoprenoid compounds, including sterols, are formed via the mevalonic acid pathway from the common C5 isoprene building blocks isopentyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (**Figure 4**). One molecule DMAPP and two molecules IPP is assembled to farnesyl pyrophosphate (FPP) (**Figure 4**). Finally, two molecules FPP are combined to make squalene (Hartmann, 2004). Cyclization of squalene is via the intermediate 2,3-oxidosqualene, that forms either lanosterol or cycloartenol via a series of enzymatic cyclizations (**Figures 4**, **5**). Animals and fungi forms lanosterol catalyzed by lanosterol synthase (LAS) and plants form cycloartenol catalyzed by cycloartenol synthase (CAS) (**Figure 5**). Several reviews have covered the enzymes and

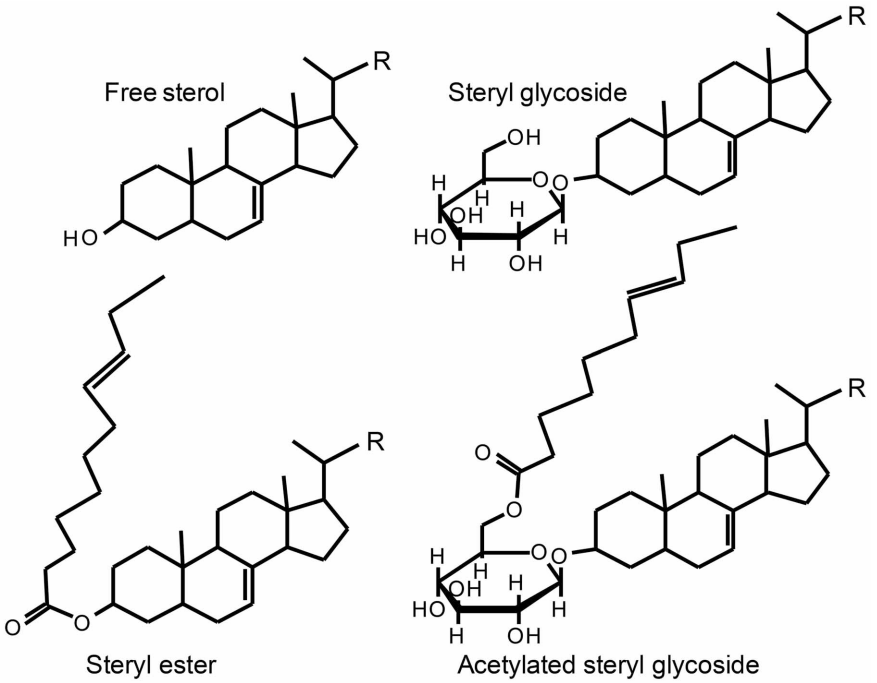

FIGURE 3 | Basic structures of free sterol and its conjugates. The side chain R varies between various sterols. Figure Adapted from Toivo et al. (2001).

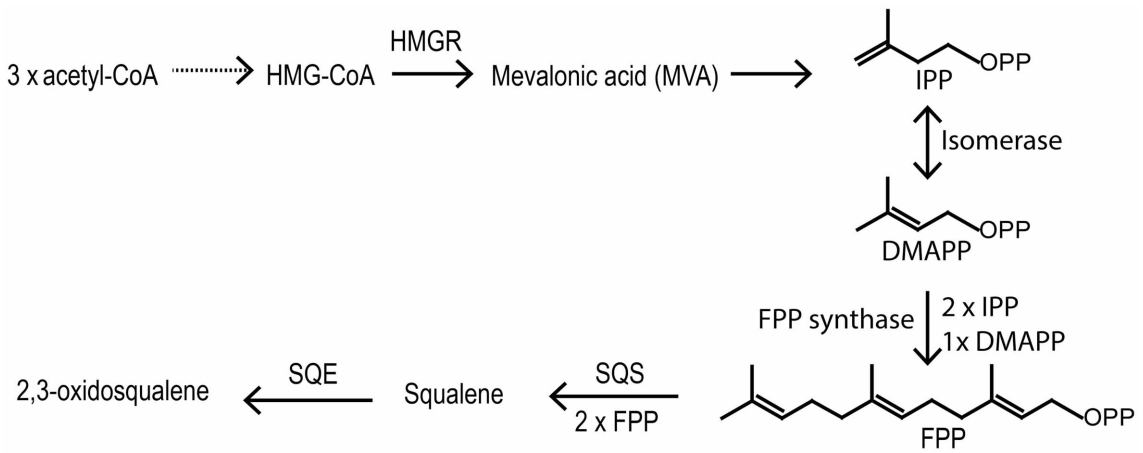

FIGURE 4 | First part of the biosynthetic pathway of sterols. Solid arrows mean one reaction and doted arrow multiple steps. HMG-CoA, 3-hydroxymethyl-3-glutaryl coenzyme A; HMGR, HMG-CoA reductase; IPP, Isopentyl pyrophosphate; DMAPP, Dimethylalkyl pyrophosphate; FPP, Farnesyl pyrophosphate; SQS, squalene synthase; SQE, squalene epoxidase.

genes involved in the sterol pathway (Benveniste, 1986, 2002, 2004; Bach and Benveniste, 1997; Schaller, 2003, 2004; Hartmann, 2004; Nes, 2011). Therefore, only the steps downstream from 2,3-oxidosqualene relevant for the biosynthesis of vitamin D_2 and vitamin D_3 will be included in the present review.

STEROL BIOSYNTHESIS LEADING TO VITAMIN D_3—ANIMALS

The major end product of the animal sterol pathway is cholesterol synthesized via lanosterol (**Figure 5**). The conversion of lanosterol to cholesterol requires nine different enzymes (Risley, 2002; Nes, 2011) and involves removal of three methyl groups, reduction of double bonds and migration of a double bond in lanosterol to a new position in cholesterol (Waterham et al., 2001). Two intersecting routes to cholesterol have been postulated (Nes, 2011). The direction of the pathway is determined by the stage at which the double bond at C24 in the sterol side chain is reduced (Nes, 2011). In the Kandutsch–Russell pathway, the reduction of the C24 double bond happens as the first step

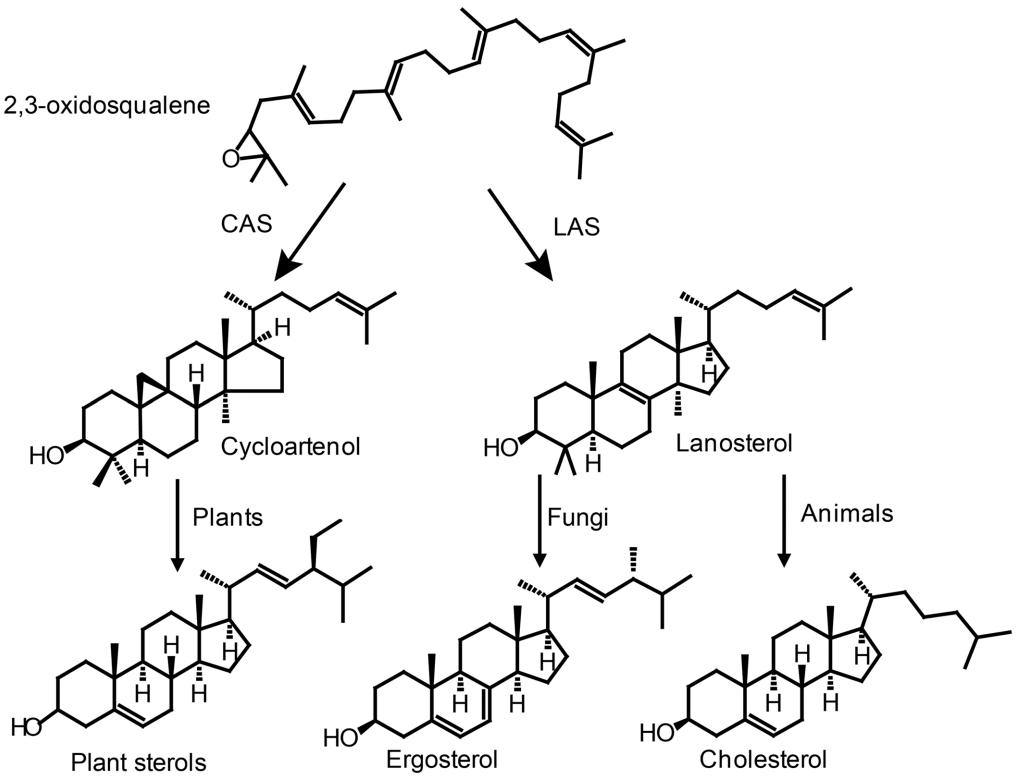

FIGURE 5 | Cyclization of 2,3-oxidosqualene forms either lanosterol or cycloartenol via a series of enzymatic cyclizations leading to sterols in plants, fungi and animals. CAS, cycloartenol synthase; LAS, lanosterol synthase.

(Kandutsch and Russell, 1960). The final precursor for cholesterol in the Kandutsch–Russell pathway is 7-dehydrocholesterol and the last step a reduction of the Δ7 double bond by a $\Delta^{5,7}$-sterol-Δ^{7}-reductase (7-dehydrocholesterol reductase) to give cholesterol (**Figure 6**). Desmosterol is the ultimate precursor of cholesterol in the Bloch pathway (Bloch, 1983) (**Figure 6**). Desmosterol is converted to cholesterol in the final step of the pathway by a sterol-Δ^{24}-reductase. However, the sequence of reactions in the cholesterol biosynthetic pathway may vary (Waterham et al., 2001). Alternate routes exist because reduction of the C24–C25 double bond in the side chain by sterol Δ^{24}-reductase can occur on all intermediates between lanosterol and desmosterol in the Bloch Pathway, giving rise to various intermediates (Bae and Paik, 1997). These intermediates can serve as substrates in the Kandutsch-Russell pathway as shown for 7-dehydrodesmosterol in **Figure 6**.

STEROL BIOSYNTHESIS LEADING TO VITAMIN D_2—FUNGI

The major sterol end product in fungi is ergosterol synthesized via lanosterol (**Figure 5**). The yeast *Saccharomyces cerevisiae* has been used as a model system for the elucidation of the ergosterol pathway and all enzymes involved have been identified (Lees et al., 1995). Cholesterol and ergosterol share the pathway until zymosterol (**Figure 6**) (Lees et al., 1995). However, sterols from fungi differ from animal sterols by the presence of a methyl group at C24. The alkylation of the side chain is catalyzed by *S*-adenosylmethionine sterol methyltransferase (ERG6) that in *S. cerevisiae* converts zymosterol into fecosterol (**Figure 7**) (Bach and Benveniste, 1997). Plants are not known to produce ergosterol, and any vitamin D_2 present is probably derived from endophytic fungi or a fungal infection.

STEROL BIOSYNTHESIS LEADING TO VITAMIN D_3—PLANTS

The enzymes involved in 24-demethylsterol and 24-ethylsterol synthesis have been identified in the model plant *Arabidopsis thaliana*. However, the biosynthetic pathway for 24-desmethylsterols, such as cholesterol and 7-dehydrocholesterol, remains unknown. This is probably due to the fact that these are minor sterols in Arabidopsis as well as in most other plants. Experiments with biosynthetic mutants and transgenic plants indicate that the enzymes regulating 24-demethylsterols and 24-ethylsterols also are involved in the regulation of 24-desmethylsterols. Within this chapter a hypothetical biosynthetic route to 24-desmethylsterols with cholesterol as end product will be presented (**Figure 8**).

Lanosterol as an alternative precursor for sterols

Plant sterols are synthesized via cycloartenol catalyzed by CAS (**Figure 5**). However, evidence exists of the presence of putative LAS genes in *Arabidopsis thaliana* (Kolesnikova et al., 2006; Suzuki et al., 2006; Ohyama et al., 2009), *Panax Ginseng* (Suzuki

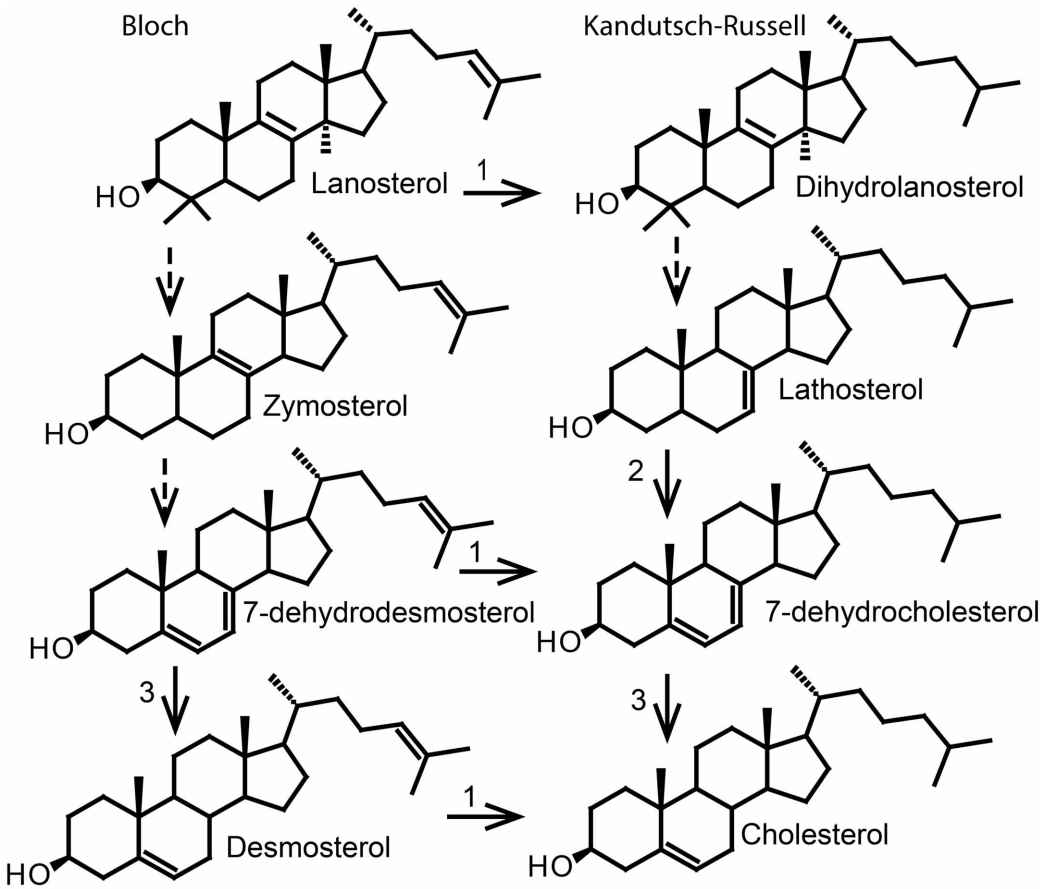

FIGURE 6 | Simplified cholesterol biosynthesis. Lanosterol is converted to cholesterol in a series of enzyme reactions. Dashed arrows indicate more than one biosynthetic step. Solid arrows indicate a biosynthetic step regulated by: (1), sterol-Δ^{24}-reductase; (2), lathosterol 5-desaturase; (3), 7-dehydrocholesterol reductase.

et al., 2006) and *Lotus japonica* (Kolesnikova et al., 2006; Sawai et al., 2006). Consequently, lanosterol synthesized by LAS in plants may act as an alternative intermediate for sterol synthesis. A lanosterol pathway to plant sterols has been demonstrated in Arabidopsis (Ohyama et al., 2009). The lanosterol pathway only contributed to 1.5% of the sitosterol biosynthesis, but this was increased to 4.5% by LAS overexpression (Ohyama et al., 2009). Thus, sterols in plants may be synthesized by two biosynthetic routes, via cycloartenol and/or via lanosterol. As a result cholesterol and 7-dehydrocholesterol may be formed in plants through lanosterol as is known from animals (**Figure 6**). In future experiments, it has to be confirmed if plants producing high amounts of these sterols such as *Solanaceae*, have a more efficient LAS enzyme.

S-adenosylmethionine sterol methyltransferases (SMTs)

Sterols from plants differ from animal sterols by the presence of a methyl or an ethyl group at C24. *S*-adenosylmethionine sterol methyltransferases (SMTs) catalyze the transfer of two carbon atoms from *S*-adenosyl methionine to make the 24-alkylations and are considered important regulatory steps in the biosynthesis of sterols in plants (Schaller, 2003). The alkyl substituent at C24 is the product of either one single carbon addition or two single carbon additions. The two methyl additions are performed as distinct steps in the pathway and two classes of SMTs exist: SMT1 and SMT2 (**Figure 8**). SMT1 preferably catalyze the first methylation of cycloartenol to give 24-methylenecycloartenol (Hartmann, 2004) (**Figure 8**). The ratio of cholesterol and the major plant sterols sitosterol, stigmasterol and campesterol has been shown to be controlled by the activity of SMT1 (Hartmann, 2004). In *Arabidopsis thaliana* plants, bearing a SMT1 knockout, cholesterol was the major sterol, composing 26% of total sterols, compared with 6% in wild-type plants (Diener et al., 2000). The *smt*1 mutant displayed poor growth and fertility, root sensitivity to Ca^{2+} and loss of proper embryo morphogenesis (Diener et al., 2000). SMT1 overexpressing tobacco plants do in contrast have a reduced content of cholesterol and no visual phenotype (Schaeffer et al., 2000; Sitbon and Jonsson, 2001; Holmberg et al., 2002). Similar results have been observed in transgenic potato (*Solanum tuberosum* cv Désirée) overexpressing SMT1 (Arnqvist et al., 2003). These results indicate that the production of high amounts of cholesterol in plants results from

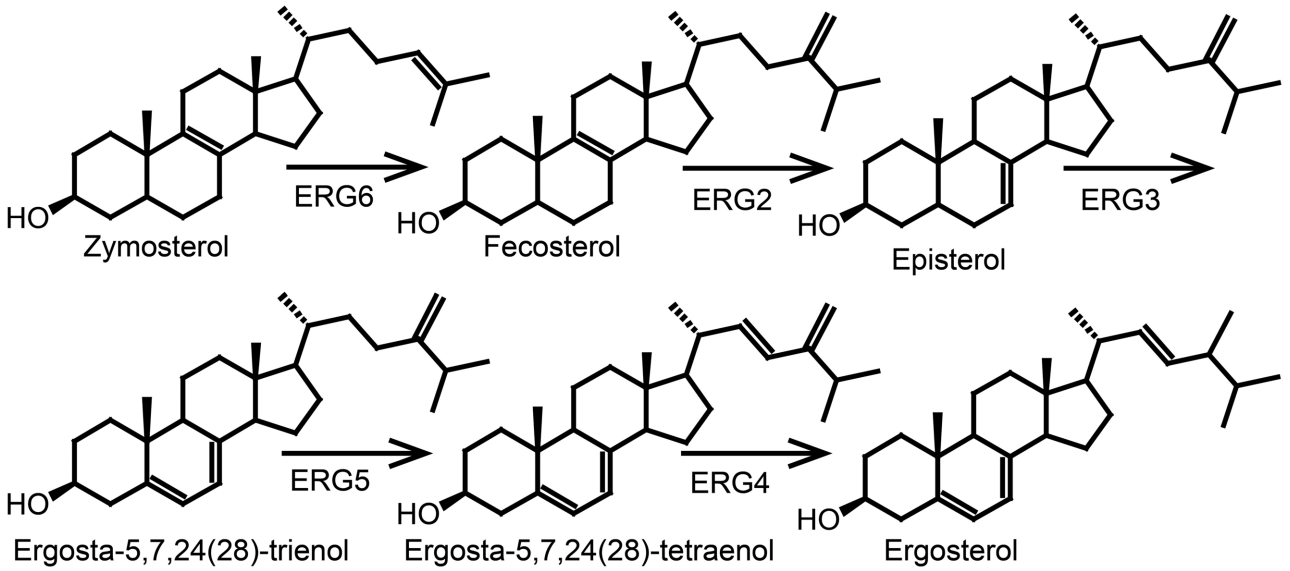

FIGURE 7 | The last five steps of the ergosterol biosynthetic pathway in *Saccharomyces cerevis*. ERG6, *S*-adenosylmethionine sterol methyltransferase; ERG2, C8-C7 sterol isomerase; ERG3, Δ^5-desaturase; ERG5, Δ^{22}-desaturase; ERG4, Δ^{24}-reductase. Figure adapted from Lees et al. (1995).

a by-pass of SMT1. Thus, manipulation of SMT1 might be a tool to increase the 7-dehydrocholesterol and cholesterol content in plants.

Proposed steps of the 24-desmethylsterol biosynthesis

Several enzymes involved in the sterol biosynthesis can be found across plants and animals, e.g., $\Delta^{5,7}$-sterol-Δ^7-reductase called DWARF5 in plants and 7-dehydrocholesterol reductase in animals. Several of these enzymes do not have absolute substrate specificity (Benveniste, 1986). The possibility, therefore, exist that plant biosynthetic enzymes could be involved in 24-desmethyl sterol biosynthesis. Application of cycloartanol to growing tobacco plants generates cholesterol (Devys et al., 1969) and we hypothesize that the reduction of the Δ24 double bond of cycloartenol to yield cycloartanol is the first step of cholesterol synthesis in plants (**Figure 8**). In Arabidopsis, the Δ24-reduction step is catalyzed by Δ5-sterol-Δ24-reductase (DIM/DWARF1) (Klahre et al., 1998). Interestingly, the Arabidopsis *dim* mutant (Klahre et al., 1998) and also the rice *dim* mutant has decreased levels of cholesterol compared to the wild type (Hong et al., 2005). These results indicate a role of DIM/DWARF1 in cholesterol biosynthesis. Production of 7-dehydrocholesterol in animals involves a Δ^7-sterol-C-5-desaturase (lathosterol 5-desaturase) that introduces a double bond at C5 (**Figure 6**). A similar Δ^7-sterol-C-5-desaturase (DWARF7/STE1) exists in plants, which converts episterol/avanesterol into 5-dehydroepisterol/5-dehydroavanesterol by a removal of two protons (**Figure 8**). An Arabidopsis mutant (*ste1/dwarf7*) defective in the Δ^7-sterol-C-5-desaturase has been identified, which only produces limiting amounts of $\Delta^{5,7}$-sterols (Gachotte et al., 1995, 2002; Choe et al., 1999; Husselstein et al., 1999). It can be hypothesized that a *ste1/dwarf7* mutant would be defective in converting cholesta-7-enol to 7-dehydrocholesterol and further to cholesterol (**Figure 8**). However, no significant decrease in cholesterol levels was observed in *ste1* mutants (Gachotte et al., 1995; Husselstein et al., 1999). The enzymatic step after C5 reduction is mediated by a $\Delta^{5,7}$-sterol-Δ^7-reductase called DWARF5 in plants (**Figure 8**) and 7-dehydrocholesterol reductase in animals (**Figure 6**). DWARF5 e.g., catalyze the reduction of the Δ7 double bond in 5-dehydroepisterol to give the Δ5 sterol 24-methylenecholesterol (Choe et al., 2000). We propose that DWARF5 also act on 7-dehydrocholesterol to form cholesterol in plants (**Figure 8**). An Arabidopsis *dwarf5* mutant accumulating $\Delta^{5,7}$-sterols has been identified (Choe et al., 2000). The DWARF5 mutant display a characteristic dwarf phenotype, which includes short robust stems, reduced fertility, prolonged life cycle and dark-green curled leaves when grown in light (Choe et al., 1999). The special dwarf phenotype is explained by a deficiency in brassinosteroids, which are important growth hormones for plants (Klahre et al., 1998; Choe et al., 1999, 2000; Hong et al., 2005). It is possible that vitamin D_3 producing plants have a less efficient DWARF5 enzyme that allows for accumulation of 7-dehydrocholesterol and later vitamin D_3 by photoconversion.

OCCURRENCE OF VITAMIN D_3 AND ITS METABOLITES IN PLANTS

PROVITAMIN D_3 AND VITAMIN D_3

Vitamin D_3 and its provitamin 7-dehydrocholesterol have been identified in the leaves of several plant species mostly belonging to *Solanaceae* (Esparza et al., 1982; Prema and Raghuramulu, 1994, 1996; Aburjai et al., 1998; Curino et al., 1998; Skliar et al., 2000) (**Table 1**). Huge variations exist in the content of vitamin D_3 and 7-dehydrocholesterol (**Table 1**). Some studies used plant cell cultures instead of whole plants (Aburjai et al., 1996; Curino et al., 1998, 2001; Skliar et al., 2000), which may explain some

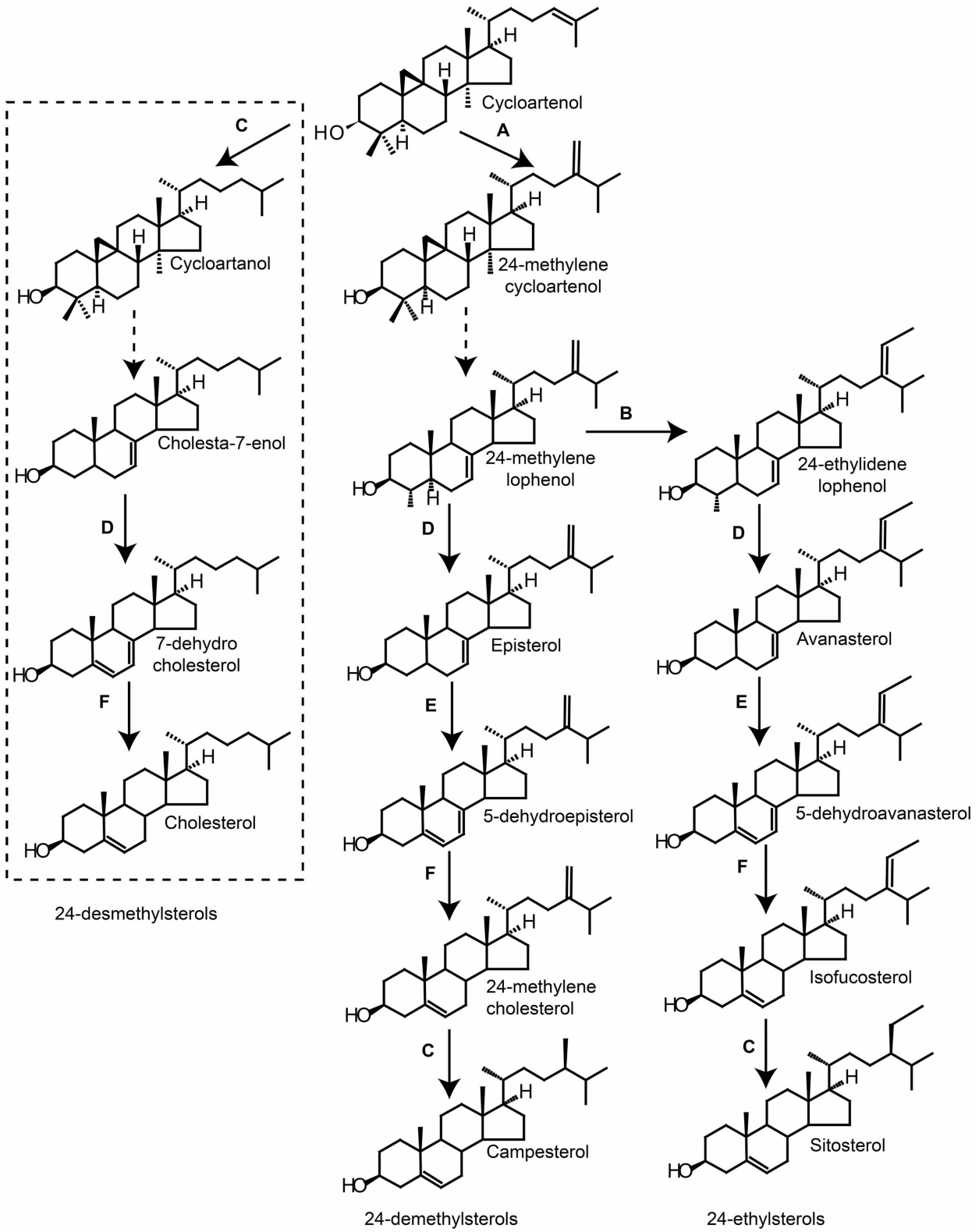

FIGURE 8 | The figure represents the biosynthetic pathways to sterols downstream from cycloartenol. Hypothetical pathway for 24-desmethylsterols (left) marked with dashed box. Simplified biosynthetic pathways for 24-demethylsterols (middle) and 24-ethylsterols (right). Dashed arrows indicate more than one biosynthetic step. Solid arrows indicate a biosynthetic step regulated by: **(A)** SMT1, **(B)** SMT2, **(C)** DIM/DWARF1, **(D)** DWARF7/STE1, **(E)** C4-demethylase and **(F)** DWARF5.

Table 1 | Content of vitamin D_3 and provitamin D_3 (μg/g) in various plants determined with chemical methods.

Species	Reference no.	Vitamin D_3	Provitamin D_3	Method
Solanum lycopersicum	1	0.28 μg/g dry wt.	0.61–0.76 μg/g dry wt.	Not stated
	2	0.09 μg/g dry wt.	0.23–0.47 μg/g dry wt.	LC-APCI-MS/MS
	3	1.1 μg/g fresh wt.	n.a	HPLC-UV and identification by H NMR and MS
	4	0.8 μg/g dry wt.	n.a.	HPLC-UV
	5	0.1 μg/g dry wt. (+UVB) 0.002 μg/g dry wt. (−UVB)		LC-ESI-MS/MS with derivatization
Solanum tuberosum	3	0.15 μg/g fresh wt.	n.a	HPLC-UV and identification by H NMR and MS
Cucurbita pepo	3	0.23 μg/g fresh wt.	n.a.	HPLC-UV and identification by H NMR and MS
Solanum glaucophyllum	2	0.21 μg/g dry wt.	0.67–1.26 μg/g dry wt.	LC-APCI-MS/MS
	5	0.1–0.2 μg/g dry wt. (+UVB) 0.0032-0.0055 μg/g dry wt. (−UVB)	n.a.	LC-ESI-MS/MS with derivatization
	6	2.2–42.1 μg/g fresh wt.	5–58 μg/g fresh wt.	HPLC-UV and identification by H NMR and MS
	7,8	ID	ID	HPLC-UV and identification by MS
Nicotiana glauca	9	ID	ID	HPLC-UV and identification by MS
Cestrum diurnum	10	0.1 μg/g dry wt.	n.a.	HPLC-UV
Medicago sativa	11	0.00062-0.001 μg/g dry wt.	n.a.	HPLC-UV with identification by MS
Trisetum flavescens	12	0.1 μg/g dry wt.	n.a.	GC-MS
Capsicum annuum	2	<LOD	0.03 μg/g	LC-APCI-MS/MS
	5	0.0029–0.0063 μg/g dry wt (+UVB)	n.a.	LC-ESI-MS/MS with derivatization

n.a., not analysed; ID, identified not quantified; <LOD, below detection limit.

1, (Björn and Wang, 2001); 2, (Jäpelt et al., 2011b); 3, (Aburjai et al., 1998); 4, (Prema and Raghuramulu, 1996); 5, (Jäpelt et al., 2012); 6, (Aburjai et al., 1996); 7, (Curino et al., 2001); 8, (Curino et al., 1998); 9, (Skliar et al., 2000); 10, (Prema and Raghuramulu, 1994); 11, (Horst et al., 1984); 12, (Rambeck et al., 1979).

of the variability between studies. Growth conditions are easily controlled when using plant cells, but discrepancies between *in vitro* and *in vivo* can be seen due to transformations occurring in the culture medium (Curino et al., 2001). Differences in growth conditions, e.g., the intensity of the light source and length of exposure will have a significant impact on the vitamin D_3 content, but unfortunately growth conditions are poorly described in most studies (Prema and Raghuramulu, 1994, 1996; Aburjai et al., 1998). However, vitamin D_3 has been studied in *S. lycopersicum* grown in greenhouse with or without UVB exposure (Björn and Wang, 2001) and in *S. lycopersicum*, *S. glaucophyllum* and *C. annuum* using growth chambers, an UVB lamp and controlled temperature and light/day settings (Jäpelt et al., 2011b, 2012). Vitamin D_3 has in most studies been identified after UVB exposure (Zucker et al., 1980; Aburjai et al., 1996; Björn and Wang, 2001; Jäpelt et al., 2011b), but vitamin D_3 synthesis without the action of UVB has also been reported (Curino et al., 1998; Jäpelt et al., 2012). Recently, we compared vitamin D_3 in UVB- and non-UVB-exposed plants using a sensitive liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method. The content of vitamin D_3 in the UVB-exposed plants was 18–64 times higher than for the non-UVB-exposed plants (Jäpelt et al., 2012). Previously failure to detect vitamin D_3 in non-UVB-exposed plants could be due to the use of relative insensitive analytical methods. Since the isomerization of previtamin D_3 to vitamin D_3 is a temperature-dependent reaction an effect of growth temperature could be expected. Therefore, the effect of elevated temperature and a combination of elevated temperature and UVB light were investigated in *S. lycopersicum*, *S. glaucophyllum* and *C. annuum* (Jäpelt et al., 2012). Plants were kept in a growth chambers for 7 days at 32°C either exposed to UVB light or not, but no consistent effect was seen. In *S. glaucophyllum* the value for elevated temperatures combined with UVB was half the value for UVB alone, whereas in *S. lycopersicum* and *C. annuum* a small increase for elevated temperatures combined with UVB compared to UVB alone was observed (Jäpelt et al., 2012).

HYDROXYLATED METABOLITES OF VITAMIN D_3

Hydroxylated metabolites of vitamin D_3 have been found in various plants (**Table 2**). The highest content of $1,25(OH)_2D_3$ has been found in *S. glaucophyllum* and not only in the leaves, but also in fruits, stems and roots (Weissenberg et al., 1989; Curino et al., 2001). The level of $1,25(OH)_2D_3$ found in cell cultures varied according to the origin of the culture, i.e., stem > leaf > fruit (Curino et al., 2001). However, vitamin D like activity could not been found in tomatoes (Prema and Raghuramulu, 1996). Earlier work using cell cultures indicates that the production of hydroxylated metabolites is influenced by the calcium concentration (Aburjai et al., 1997; Curino et al., 2001; Burlini et al., 2002). The level of $25OHD_3$ (Aburjai et al., 1997) and $1,25(OH)_2D_3$ (Burlini et al., 2002) in *S. glaucophyllum* cell suspensions increased markedly when incubated in a Ca^{2+} free media compared to if Ca^{2+} was present. However, another study performed with *S. glaucophyllum* cells showed that media deprived from calcium contained low levels of $1,25(OH)_2D_3$ (Curino et al., 2001).

The biosynthesis of $1,25(OH)_2D_3$ is finely regulated in vertebrates and the question is if this also is the case in plants. The $25OHD_3/1,25(OH)_2D_3$ ratio has either been reported to be >1 (Prema and Raghuramulu, 1994, 1996), ∼1 (Jäpelt et al., 2012) or <1 (Aburjai et al., 1996). This indicates that the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ not is as tightly regulated as in vertebrates. Enzymatic activities involved in formation of $25OHD_3$ and $1,25(OH)_2D_3$ have been identified in *S. glaucophyllum* (Esparza et al., 1982). Vitamin D 25-hydroxylase activity has been localized in the microsomes, whereas the 1α-hydroxylase activity has been localized in mitochondria and microsomes (Esparza et al., 1982). However, no enzymes have been isolated from plants showing vitamin D 25-hydroxylase or 1α-hydroxylase catalytic activity. For the biological role of vitamin D_3 and its hydroxylated metabolites in plant physiology is referred to Boland et al. (2003).

VITAMIN D_3 CONJUGATES

Several studies of *S. glaucophyllum* identified $1,25(OH)_2D_3$ after enzymatic hydrolysis with glycosidases (Haussler et al., 1976; Wasserman et al., 1976b; Hughes et al., 1977; Napoli et al., 1977; Esparza et al., 1982; Jäpelt et al., 2012) and also in *C. diurnum* (Hughes et al., 1977). Similarly, vitamin D_3 and $25OHD_3$ were identified in *S. glaucophyllum* after incubation with glycosidases (Esparza et al., 1982). Ruminal fluids contain glycosidases and research show that aqueous extracts of *S. glaucophyllum* leaves incubated with bovine ruminal fluid (de Boland et al., 1978) and ovine ruminal fluid (Esparza et al., 1983) exhibit more vitamin D activity than extracts not incubated. Later vitamin D_3 and its metabolites were identified in *S. glaucophyllum* extracts incubated with ovine ruminal fluid (Skliar et al., 1992). These studies indicate that vitamin D_3 and its metabolites exist as glycosides in plants. However, the existence of glycosides is debated and other

Table 2 | Content of the hydroxylated metabolites of vitamin D_3 in various plants determined by immunoassays or chemical methods.

Species	Reference no.	$25OHD_3$	Method	$1,25(OH)_2D_3$	Method
Solanum lycopersicum	1	0.15 μg/g fresh wt.	HPLC-UV and MS identification	<LOD	HPLC-UV
	2	0.022 μg/g dry wt.	HPLC-UV and biological activity	0.10 μg/g dry wt.	HPLC-UV and biological activity
	3	0.004 μg/g dry wt	LC-ESI-MS/MS with derivatization	<LOD	LC-ESI-MS/MS with derivatization
Solanum glaucophyllum	3	0.011–0.031 μg/g dry wt	LC-ESI-MS/MS with derivatization	0.012–0.032 μg/g dry wt	LC-ESI-MS/MS with derivatization
	4	ID	HPLC-UV and MS identification	ID	Radioreceptor assay, HPLC-UV and MS identification
	5	1.0 μg/g fresh wt.	HPLC-UV and MS and HNMR identification	0.1 μg/g fresh wt.	HPLC-UV and MS and HNMR identification
Capsicum annuum	3	0.0004–0.0005 μg/g dry wt	LC-ESI-MS/MS with derivatization	<LOD	LC-ESI-MS/MS with derivatization
Cestrum diurnum	6	0.102 μg/g dry wt.	HPLC-UV and biological activity	1 μg/g dry wt.	HPLC-UV and biological activity
Nicotiana glauca	7	ID	HPLC-UV with MS identification	0.3–1 μg/g fresh wt.	Radioreceptor assay, HPLC-UV and MS identification

ID, identified not quantified; <LOD, below detection limit.

1, (Aburjai et al., 1998); 2, (Prema and Raghuramulu, 1996); 3, (Jäpelt et al., 2012); 4, (Curino et al., 1998); 5, (Aburjai et al., 1996); 6, (Prema and Raghuramulu, 1994); 7, (Skliar et al., 2000).

studies only quantified the free forms (Prema and Raghuramulu, 1994, 1996; Aburjai et al., 1996, 1997, 1998). It has been proposed that the glycoside content depends on the collection, drying and storage of the plant material, which may explain some differences between studies (Peterlik et al., 1977; Prema and Raghuramulu, 1994).

The site of glycosylation, the type of bond and the identity of the sugar unit have not been completely determined. The number of sugar units seems to differ as the vitamin D active glycosides of *S. glaucophyllum* and *T. flavescens* are soluble in water (Humphreys, 1973; Uribe et al., 1974; Wasserman et al., 1976b; Napoli et al., 1977; Morris and Levack, 1982), whereas the glycoside of *C. diurnum* are soluble in a mixture of chloroform and methanol (Wasserman et al., 1976a). Vidal et al. (1985) isolated the $1,25(OH)_2D_3$ glycoside from *S. glaucophyllum* and found that $1,25(OH)_2D_3$ was bound to a series of fructoglucosides of variable molecular weights.

The formation of glycosides may cause dramatic changes in the chemical, nutritional and metabolic properties (Gregory, 1998). Rambeck et al. (1984) studied the biological activity of $1\alpha(OH)D_3$ 3-β-cellobioside, $1\alpha(OH)D_3$ 3-β-glucoside and vitamin D_3 3-β-glucoside and the corresponding parent molecules in bioassays using rats, chickens and quails. Glucosidation did not reduce the activity of the parent vitamin D (Rambeck et al., 1984). In constrast the β-D-glucoside of $1\alpha(OH)D_3$ exhibited only 10% activity compared to $1\alpha(OH)_2D_3$ in all bioassays and the disaccharide (1α(OH)vitamin D_3 3-β-cellobioside) showed no vitamin D activity in the chicken bioassay (Rambeck et al., 1984). No study on glycosylated forms of $25OHD_3$ or $1,25(OH)_2D_3$ has been performed. The existence of esters of vitamin D and the hydroxylated metabolites in plant material seems likely, but has not been shown.

VITAMIN D_2 IN PLANT MATERIAL

Ergosterol is a cell membrane component of fungi, but is also the provitamin of vitamin D_2. Thus, vitamin D_2 can be found in plants contaminated with fungi. Conversion to vitamin D_2 occurs by sun-exposure of the plant material during growth and in the curing process. The antirachitic activity of grass and hay was studied intensively 50–80 years back using rat assays (Steenbock et al., 1925; Russell, 1929; Wallis, 1938, 1939; Moore et al., 1948; Newlander, 1948; Thomas and Moore, 1951; Newlander and Riddell, 1952; Thomas, 1952; Keener, 1954; Henry et al., 1958; Wallis et al., 1958). Most of these studies were on alfalfa (*Medicago sativa* L.) and the activities ranged from 0–3800 IU/kg, equivalent to 0–95 μg vitamin D/kg. However, some studies were on hay and others on fresh grass and dry matter was not stated in all cases, which makes comparisons difficult. The assumption at that time was that the antirachitic activity was due to vitamin D_2 produced from ergosterol (Newlander and Riddell, 1952). Later vitamin D_2 was identified in crops using chemical methods (Horst et al., 1984; Jäpelt et al., 2011a). Horst et al. (1984) analyzed sun-cured field grown alfalfa using high performance liquid chromatography (HPLC) with UV detection and found 48 μg vitamin D_2/kg. Jäpelt et al. (2011a) studied ergosterol and vitamin D_2 in six varieties of perennial ryegrass (*Lolium perenne* L.) harvested four times during the season. The content of vitamin D_2 and ergosterol was analyzed by LC atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS). An average content of vitamin D_2 of 2 μg/kg fresh weight (0.07–6.4 μg/kg fresh weight) was found (Jäpelt et al., 2011a). The vitamin D_2 content was maximum 2‰ of the ergosterol content (Jäpelt et al., 2011a). The vitamin D_2 content in these two studies is almost similar if we take into account the difference in dry matter between hay and fresh grass. Compared to results obtained by rat assays, the latter is slightly higher, which could be due the contribution of other vitamin D metabolites, or to a natural decline during the last 50–80 years.

The content of vitamin D_2 in the plant material has been shown to increase with the level of sun exposure, and for crops also curing method (Hess and Weinstock, 1924; Steenbock et al., 1925; Russell, 1929; Newlander and Riddell, 1952). However, inconsistent results were obtained regarding the importance of sun exposure, which indicates that other factors may be important (Moore et al., 1948; Newlander, 1948; Henry et al., 1958; Wallis et al., 1958). Several studies observed that plants at later stage of maturity were higher in vitamin D than at early stage (Thomas and Moore, 1951; Keener, 1954; Henry et al., 1958). Especially, dead leaves were high in vitamin D and the proportion of dead leaves was observed to increase with maturity of the plant (Thomas and Moore, 1951). Newell et al. (1996) measured the ergosterol content in grass and found that it increased with time and with increasing fungal damage. Consequently, larger vitamin D activities are observed with time if the plant is exposed to sunlight. Recently, a systematic study looking at vitamin D_2 and ergosterol in perennial ryegrass during the season was performed (Jäpelt et al., 2011a). The content of both ergosterol and vitamin D_2 changed more than a factor of 10 during the season (Jäpelt et al., 2011a). Weather factors were recorded and a principal component analysis (PCA) was performed to study, which factors that were important for the formation of vitamin D_2. The PCA revealed that both sun/temperature and ergosterol/precipitation was important. This suggested that a combination of weather factors was involved as observed previously (Moore et al., 1948; Newlander, 1948; Henry et al., 1958; Wallis et al., 1958). Precipitation and high humidity are essential for ergosterol synthesis, whereas sunlight is necessary for vitamin D_2 synthesis (Jäpelt et al., 2011a).

VITAMIN D IN ALGAE

Fish are known to be rich sources of vitamin D_3, but the origin of vitamin D_3 in fish has not been clarified. Both a non-photochemical pathway and a photochemical pathway for vitamin D_3 synthesis in fish are doubted (Mattila et al., 1997). The latter due to limited UVB-light in their natural habitats combined with low amounts of 7-dehydrocholesterol in fish skin (Bills, 1927; Sugisaki et al., 1974; Takeuchi et al., 1991; Sunita Rao and Raghuramulu, 1996b; Rao and Raghuramulu, 1997). Evidence, on the other hand, exist that microalgae as the basis of the food chain is the origin of the high content of vitamin D_3 in fish (Takeuchi et al., 1991; Sunita Rao and Raghuramulu, 1996a). However, data for vitamin D in algae are limited and not consistent (De Roeck-Holtzhauer et al., 1991; Takeuchi et al.,

1991; Sunita Rao and Raghuramulu, 1996a; Brown et al., 1999). Takeuchi et al. (1991) found significant amounts of vitamin D_2 (1.9–4.3 μg/100 g), vitamin D_3 (5.0–15 μg/100 g) and their provitamins (260–1450 μg/100 g) in microalgae. Sunita Rao and Raghuramulu (1996a) also reported high concentrations of ergosterol (390 μg/100 g), 7-dehydrocholesterol (2400 μg/100 g), vitamin D_2 (5.3 μg/100 g) and vitamin D_3 (80 μg/100 g) in freshwater microalgae. The content of vitamin D_2 and vitamin D_3 in four Australian microalgae studied by Brown et al. (1999) were in all cases below the detection limit (35 μg/100 g) of the method used. De Roeck-Holtzhauer et al. (1991) studied vitamin D_2 in several algae including the macroalgae *Sargassum multicum* and found very high amounts (90–3900 μg/100 g). No studies on vitamin D_3 in macroalage have been performed. Both vitamin D_2 and vitamin D_3 are available for fish in their diet, but vitamin D_2 is almost absent in fish (Lock et al., 2010). This suggests that the bioavailability of vitamin D_2 is lower than for vitamin D_3 (Andrews et al., 1980; Barnett et al., 1982; Takeuchi et al., 1991).

Microalgae usually live at the surface of the water and vitamin D is probably synthesized by sun exposure of provitamins D (Takeuchi et al., 1991). Takeuchi et al. (1991) observed that microalgae caught in August were higher in vitamin D than in October and December, which supports that vitamin D is synthesized from sun exposure of provitamin D. To synthesize vitamin D_3 by UVB exposure, microalgae should be able to synthesize 7-dehydrocholesterol if using the same pathway as vertebrates. However, the sterols found in microalgae display a great diversity as may be expected from the large number of classes and species combined with a long evolutionary history (Volkman, 2003). Red algae (*Rhodophyta*) primarily contain cholesterol, although several species contain large amounts of desmosterol. Fucosterol is the dominant sterol of brown algae (*Phaeophyta*) (Patterson, 1971). Generalizations about the sterols in most other algae, e.g., diatoms (*Bacillariophyta*) and green algae (*Chlorophyta*) cannot be made as they are much more varied (Patterson, 1971). The most common sterol in diatoms are 24-methylcholesta-5,24(28)-dien-3β-ol, but cholesterol and sitosterol are also common (Rampen et al., 2010). The green algae are very variable, they contain significantly amounts of 24-ethyl sterols (Volkman, 2003), but also cholesterol and ergosterol (Patterson, 1974). Microalgae are an extremely diverse group, as also seen from the large variability in the sterol content. It is, therefore, difficult to make any conclusions about algae's production of vitamin D_2 and vitamin D_3. Species differences and geographic differences may be expected.

ANALYTICAL METHODS TO STUDY THE VITAMIN D FORMS IN PLANTS

Research into vitamin D in plants is limited, presumably due to limitations in selectivity and sensitivity of the analytical methods available. Determination of vitamin D in food has always been a challenge due to low amounts of vitamin D combined with the existence of multiple vitamin D active compounds. Plants are a complex matrix, which makes the analysis of vitamin D even more challenging. Selective and sensitive methods are, therefore, a prerequisite. Each step in the analytical methods used in the research of vitamin D in plants will be discussed in the following chapter.

BIOLOGICAL METHODS FOR VITAMIN D

The official method for vitamin D was for many years the line test using animals. Either a rat or a chicken was put on a vitamin D deficient diet until the animal developed rickets. Afterwards, they were fed plants or plant extracts and it was estimated to which extent the rickets were cured (Wallis, 1938, 1939; Moore et al., 1948; Thomas and Moore, 1951; Thomas, 1952; Keener, 1954; Henry et al., 1958; Wallis et al., 1958). This method is time-consuming as it takes 5 weeks, and precision and accuracy may be discussed. However, an advantage of this method may be that the amount of quantified vitamin D corresponds to the total vitamin D activity independent of the specific metabolites and their difference in activity. The interest in the $1,25(OH)_2D$ metabolite initiated the use of more specific methods utilizing that a high strontium intake by chickens block the conversion of 25OHD to $1,25(OH)_2D$ by suppressing 1α-hydroxylase activity (Wasserman, 1974; Weissenberg et al., 1989). This means that the inhibitory effect of strontium can be overcome by the administration of $1,25(OH)_2D$, but not by 25OHD and vitamin D. Studies of calcium absorption in nephrectomized rats that possess a suppressed 1α-hydroxylase activity (Walling and Kimberg, 1975) and assays with organ-culture systems such as cultured duodenum have also been used to study $1,25(OH)_2D$ specifically in plants (Corradino and Wasserman, 1974). However, the biological activity measured in these methods could be due to other compounds that interfere with vitamin D metabolism, calcium absorption or to other compounds present, e.g., calcium and phosphorus that increase or inhibit the activity of vitamin D. More specific methods are, therefore, needed to study vitamin D and its metabolites in details.

CHEMICAL METHODS FOR VITAMIN D—SAMPLE PREPARATION

Proper sample preparation is crucial for reliable analysis and should optimally release all vitamin D active compounds. Glycosylation and acetylation is general metabolic processes that occur in plants and vitamin D and related compounds are expected to be found as glycosides, esters and acetylated glycosides (**Figure 3**). Saponification followed by liquid-liquid extraction is typically used to liberate esters, where cold saponification is preferred over hot saponification due to reversible and temperature-dependent equilibration between vitamin D and pre-vitamin D (Buisman et al., 1968; Hanewald et al., 1968). However, saponification fails to hydrolyze the bond between vitamin D and the carbohydrate moiety in the glycosides. Both direct and indirect analysis (with or without hydrolysis) can be used for glycosides (Van Hoed et al., 2008). Direct analysis is fast, as a sample preparation step is omitted, but complicated as the needed conjugated standards is non-available. For indirect analyses, acid hydrolysis has been used to release glycosidic forms (Toivo et al., 2001; Liu et al., 2007; Nyström et al., 2007). Acid hydrolysis is typical performed under relatively harsh conditions, e.g., 60 min at 80°C with 6 M ethanolic hydrochloric acid solution (Kamal-Eldin et al., 1998; Toivo et al., 2001; Nyström et al., 2007). This is not optimal due to risk

of isomerization of certain sterols (Kamal-Eldin et al., 1998) including 5,7-dienes as 7-dehydrocholesterol (Dolle et al., 1988) as well as vitamin D_3 (Jin et al., 2004). An alternative to acid hydrolysis is the gentler enzymatic hydrolysis. Kesselmeier et al. (1985) used β-glucosidase in the hydrolysis of steryl glycosides in oat leaves and seeds, but other researchers have not been successful in repeating these results (Moreau and Hicks, 2004; Nyström et al., 2008). A hypothesis is that the observation by Kesselmeier et al. (1985) may be due to impurities of minor enzymes rather than the actual β-glucosidase, whereas similar secondary activities not are present in modern highly purified enzyme preparations (Moreau and Hicks, 2004; Nyström et al., 2008).

The extraction of liberated vitamin D compounds from the non-saponifiable matter is usually performed by liquid/liquid extraction using non-polar organic solvents (CEN, 2008). Further clean-up of the extracts is usually needed to remove interfering compounds and to avoid contamination of the analytical column by other co-extracted substances, e.g. chlorophyll and other lipophilic pigments (Jäpelt et al., 2011b). Combinations of column chromatography or/and preparative HPLC have been used for purification of plant extracts before vitamin D analysis (Rambeck et al., 1979; Esparza et al., 1982; Morris and Levack, 1982; Prema and Raghuramulu, 1994; Curino et al., 1998, 2001; Skliar et al., 2000). However, fractionation by column chromatography is time-consuming and not suitable for routine analysis and has recently been replaced by solid phase extraction (SPE) (Jäpelt et al., 2011a,b).

If total vitamin D activity is required, the sum of vitamin D and any other metabolites that may have vitamin D activity must be quantified. The hydroxylated metabolites have higher polarity than vitamin D, but despite the difference in polarity are vitamin D and 25OHD extracted in the same run (Mattila et al., 1995a; Jakobsen et al., 2004). Only few studies have included quantification of $1,25(OH)_2D$ in food (Kunz et al., 1984; Takeuchi et al., 1988; Montgomery et al., 2000). These studies omitted the saponification step, which seems to question whether conjugated forms of $1,25(OH)_2D$ will be quantified. Our recent study, included saponification in the analysis of $1,25(OH)_2D$ in plant material, but poor extraction efficiency from the non-saponifiable matter was observed, which increased the detection limit (Jäpelt et al., 2012). Therefore, optimization of the extraction procedure is needed.

QUANTIFICATION OF VITAMIN D FORMS

An internal standard is essential for quantification of vitamin D due to reversible isomerization with the corresponding previtamin D (Schlatmann et al., 1964). An internal standard is also needed to eliminate analytical errors due to losses of vitamin D during sample preparation and to compensate for signal variation if using mass spectrometry (MS) detection (Dimartino, 2007). Vitamin D_2 and vitamin D_3 are chemically very similar and vitamin D_2 has been used as internal standard when determining vitamin D_3 and *vice versa*. However, this is not the best approach when vitamin D_2 and vitamin D_3 occur simultaneously as could be the case in plants (Horst et al., 1984). For quantification by MS isotopic labeled compounds are ideal internal standards, because of the complete resemblance with the analyte.

SEPARATION AND DETECTION PRINCIPLES FOR VITAMIN D AND ITS STEROL PRECURSORS

Gas chromatography flame ionization detection (GC-FID) and gas chromatography mass spectrometry (GC-MS)

Sterols act as precursors of vitamin D so sterol analysis is essential to investigate the biosynthesis of vitamin D in plants. Sterols are typically measured by gas chromatography (GC) as trimethylsilyl (TMS) ether derivates (Piironen et al., 2000), which are detected either by flame ionization detection (FID) (Phillips et al., 2005; Brufau et al., 2006; Liu et al., 2007) or MS (Toivo et al., 2001; Nyström et al., 2007). GC was also the first chromatography principle used to replace the biological assay for analysis of vitamin D (Bell and Christie, 1973), but while GC is a good separation method for sterols it is not the best choice for vitamin D. Vitamin D undergoes thermal cyclization in a GC split/splitless injector (>125°C) resulting in formation of the corresponding pyro and isopyro compounds with a concomitant decrease in sensitivity (Yeung and Vouros, 1995). However, early studies did use GC for identification of vitamin D_3 in plants (Rambeck et al., 1979; Suardi et al., 1994).

High performance liquid chromatography with UV detection (HPLC-UV)

HPLC with UV detection (265 nm) is used in official methods for vitamin D in food (Staffas and Nyman, 2003; CEN, 2008) and has also been used in recent studies on vitamin D in plants (Prema and Raghuramulu, 1994, 1996; Aburjai et al., 1996, 1997, 1998; Curino et al., 1998, 2001). Nevertheless, these methods are laborious as high degree of purification of the extracts is needed. Analysis of vitamin D in complex matrices such as plants is especially challenging due to co-eluting interferences.

GC is generally considered superior to HPLC for sterol analysis (Lagarda et al., 2006), but introduction of columns with particle sizes of 1–2 μm improve resolution of co-eluting sterols and may bring HPLC ahead of GC (Lu et al., 2007). Furthermore, HPLC have compared to GC the advantage of analysis without derivatization and gentler conditions suitable for thermally unstable sterols. Even though HPLC may be combined with UV for detection of sterols (Careri et al., 2001; Sanchez-Machado et al., 2004), this is not the most sensitive method, as sterols adsorb UV between 200 and 210 nm where most organic solvents have low transparency.

Liquid chromatography mass spectrometry (MS, LC-MS, LC-MS/MS)

Detection of vitamin D by MS detection is challenging due to low ionization efficiency. The most used ionization source for LC-MS is ESI, which works best when the analyte already is in its ionic form in solution (Cech and Enke, 2001). The ionization efficiency of vitamin D and its sterol precursors are as a result low in most ESI methods (Dimartino, 2007). APCI is a much more efficient ionization technique for neutral and apolar substances such as vitamin D and has been used several times for vitamin D analysis (Dimartino, 2007; Byrdwell, 2009; Jäpelt et al., 2011b). Atmospheric pressure photoionization (APPI) is

another ionization method suitable for lipophilic compounds, which also has been used for detection of vitamin D (Soldin et al., 2009). MS has been used for identification of vitamin D_3 in plants in several studies, but in most cases not coupled to liquid chromatography (LC) (Aburjai et al., 1996; Curino et al., 1998; Skliar et al., 2000). We recently used liquid chromatography tandem mass spectrometry (LC-MS/MS) for selective detection of vitamin D in various plant matrices (Jäpelt et al., 2011a,b). LC-MS/MS improves both selectivity and sensitivity compared to LC-MS in particular by using selected reaction monitoring (SRM). In SRM both a precursor and a product ion is selected, which reduce background noise resulting in a good signal to noise ratio. SRM increases selectivity, but more than one transition is needed for reliable confirmation, which preferable is combined with other evidence such as relative intensities of product ions in the mass spectra, accurate mass, retention time and peak shape to positively identify the compound as vitamin D (Jäpelt et al., 2011b). LC-MS and LC-MS/MS have also been used several times for analysis of sterols in plant matrices (Mezine et al., 2003; Rozenberg et al., 2003; Ruibal-Mendieta et al., 2004; Cañabate-Díaz et al., 2007; Lu et al., 2007; Jäpelt et al., 2011b). To study vitamin D and its sterol precursors in plants LC-MS/MS is the method of choice. However, a significant challenge is that the content of various sterols span several orders of magnitude. The major sterols such as sitosterol and campesterol is between 10 and 200 μg/g fresh weight, whereas minor sterols and vitamin D_3 are present at less than 0.1 μg/g fresh weight (Jäpelt et al., 2011b; Schrick et al., 2011). This requires a huge dynamic range of the analytical method or fractionation of the extracts.

Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) is a powerful tool for structure elucidation and identification and offer valuable information in addition to UV and MS detection. NMR can discriminate between compounds that only differ in terms of local chemical environment, e.g., compounds with same mass, but different locations of functional groups. However, in general NMR analyses require extensive purified samples, and possess low sensitivity (Eisenreich and Bacher, 2007). Nevertheless, ^{1}H NMR has been used for identification of vitamin D_3 in plants, but extraction of as much as 2 kg fresh plant leaves was required (Aburjai et al., 1998).

ANALYTICAL METHODS FOR QUANTIFICATION OF HYDROXYLATED METABOLITES OF VITAMIN D

Analysis of the hydroxylated metabolites of vitamin D represents a challenge because they exist in even lower concentrations than vitamin D (Aburjai et al., 1996, 1998; Prema and Raghuramulu, 1996). They have been detected in plants using both protein-binding assays (Skliar et al., 2000; Curino et al., 2001) and chemical methods such as HPLC with UV detection (Aburjai et al., 1998, 1996; Prema and Raghuramulu, 1994, 1996) and MS detection (Jäpelt et al., 2012). Protein-binding assays, including RIA (radioimmunoassay) and RRA (radioreceptor binding assay), are widely used in clinical laboratories for analysis of 25OHD and $1,25(OH)_2D$ in serum due to the simplicity (Hollis and Horst, 2007). RIAs are commercially available and have been used for extracts and cell cultures of *S. glaucophyllum* and *C. diurnum* (Weissenberg et al., 1988; Gil et al., 2007). RRA has been applied for identification of $1,25(OH)_2D_3$ in *S. glaucophyllum* (Curino et al., 2001) and *Nicotiana glauca* Graham (Skliar et al., 2000). However, the lipophilic nature of vitamin D makes it difficult to analyze in any protein-binding assay due to solubility problems (Hollis and Horst, 2007). Matrix effects are also common due to interfering compounds found in the assay tube but not in the standard that compete with binding to the protein. The most common chemical detection principle used for the detection of the hydroxylated metabolites in plants has been HPLC-UV, but this is not totally specific. Specific quantification of vitamin D metabolites can on the other hand be obtained by using MS methods. However, direct LC-MS/MS analysis of especially $1,25(OH)_2D$ is challenging because of poor ionization efficiency, low concentration and an extensive product ion spectra by most soft ionization techniques (Aronov et al., 2008). Attempts to increase ionization efficiency have been reported several times mostly for serum samples, these include adduct formation (Kissmeyer and Sonne, 2001; Casetta et al., 2010), derivatization with Cookson-type reagents (Higashi and Shimada, 2004; Gao et al., 2005; Kamao et al., 2007; Aronov et al., 2008; Higashi et al., 2011) and microflow LC-MS together with derivatization (Duan et al., 2010). Microflow LC improve sensitivity 15-fold compared to normal LC, but has a small loading capacity that counteracts the sensitivity gain, especially when analyzing complex matrices (Duan et al., 2010). The advantage of using microflow LC may, therefore, be limited for analysis of plant extracts. Recently, LC-ESI-MS/MS in combination with Diels-Alder derivatization was used to study $25OHD_3$ and $1,25(OH)_2D_3$ in the leaves of *S. glaucophyllum, S. lycopersicum* and *C. annuum* (Jäpelt et al., 2012).

CONCLUDING REMARKS

Vitamin D deficiency is a problem in populations with limited sun exposure where a dietary intake of vitamin D becomes essential. However, dietary recommendations for vitamin D are difficult to meet because few food items naturally contain vitamin D and it would, therefore, be valuable to increase the food sources of vitamin D in the human diet or to optimize the content by biofortification. Traditionally, only animal products have been considered a source of vitamin D_3, but today we know that vitamin D_3 and its metabolites are formed in certain plants. Accordingly, fruits and vegetables have the potential to serve as a source of vitamin D. Especially, the *Solanaceae* family contains high amounts of vitamin D_3, which is of special interest considering the importance of this family in human nutrition. The *Solanaceae* family includes important vegetables such as potato, tomato and pepper all of which have been found to contain vitamin D_3. Our current knowledge is limited to the content in leaves, but future investigation will elucidate if also the edible portions contain vitamin D_3. It would be valuable to screen a variety of crops and vegetables for vitamin D, but to carry out a larger screening development of less time-consuming and preferably more sensitive analytical methods are needed. A further challenge is to improve methods to study and quantify vitamin D conjugates in details.

Planktonic microalgae, inhabiting the sea, are another large group of photosynthetic organisms that contain vitamin D. Microalgae are, as part of the aquatic food chain, identified as a source of vitamin D for fish. Currently, the world's wild fish stocks are being overexploited and there has been a growth in the aqua-culture industry. The current trend is to replace fish meals or fish oil partly by vegetable feed substitutes when feeding cultured fish will reduce the content of vitamin D compared to wild fish (Bell and Waagbø, 2008). Microalgae with a high natural amount of vitamin D may be used as a natural vegetable form for the bio-fortification of aqua-cultured fish.

Basic knowledge about the biosynthesis of vitamin D_3 in photosynthetic organisms is still lacking and any increase in our knowledge will help us to manipulate the content to produce plants with a higher natural amount of vitamin D_3. Vitamin D_3 is only synthesized in minute amounts, which makes it challenging to study the pathways and enzymes involved. However, it also means that even small changes in vitamin D_3 can have a significant impact on human health. Biosynthesis of 24-desmethylsterols in plants is complex and poorly understood and makes the final goal to produce plants with a higher natural amount of vitamin D_3 a great challenge. Currently, the key biosynthetic steps and the enzymes involved are unknown. These need to be identified before we even can begin to modify the content of vitamin D_3 in plants. In the present review, a hypothetical biosynthetic pathway for 7-dehydrocholesterol and cholesterol is presented. The steps catalyzed by SMT1 and DWARF5 seem to be promising targets to manipulate the level of 7-dehydrocholesterol in plants. A block in SMT1 will force the biosynthetic pathway in the direction of 7-dehydrocholesterol and cholesterol. Further increase in 7-dehydrocholesterol can probably be achieved by a block in $\Delta^{5,7}$-sterol-Δ^{7}-reductase (DWARF5). However, any increase in provitamin D_3 should be viewed in the context of the overall changes in the metabolic profile and a significant challenge will be selective to accumulate vitamin D_3 in edible organs such as fruits, without affecting plant growth and the development of the plant and consequently yields. An important thing to consider before putting a lot of energy into producing plants with a high amount of vitamin D is the bioavailability, as low bioavailability of vitamin D from plants may diminish the potential of plants as a new vitamin D source.

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Changes in endogenous gene transcript and protein levels in maize plants expressing the soybean ferritin transgene

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Transgenic agricultural crops with increased nutritive value present prospects for contributing to public health. However, their acceptance is poor in many countries due to the perception that genetic modification may cause unintended effects on expression of native genes in the host plant. Here, we tested effects of soybean ferritin transgene (SoyFer1, M64337) on transcript and protein levels of endogenous genes in maize. Results showed that the transgene was successfully introduced and expressed in the maize seed endosperm. mRNA abundance of seven tested iron homeostasis genes and seed storage protein genes differed significantly between seed samples positive and negative for the transgene. The PCR negative samples had higher zein and total protein content compared to the positive samples. However, PCR positive samples had significantly higher concentrations of calcium, magnesium, and iron. We have shown that the soybean ferritin transgene affected the expression of native iron homeostasis genes in the maize plant. These results underscore the importance of taking a holistic approach to the evaluation of transgenic events in target plants, comparing the transgenic plant to the untransformed controls.

Keywords: soybean ferritin, transgenic maize, endogenous genes, gene expression, transcription, iron

INTRODUCTION

Genetic engineering has been used to improve the performance and nutritional qualities of many economically important crops world-wide. Maize (*Zea mays*) is no exception and since it is a major source of dietary calorie intake in many countries, a lot of research focusing on its nutritional enhancement has been done. With most of the world's population dependent on cereal crops as basal sources of their diet (Minihane and Rimbach, 2002), it is imperative that these crops contain adequate levels of nutrients required for good health.

Iron is one of the major micronutrients required in the human body. However, cereals are very poor sources of iron as it is not readily bioavailable. This is to a large extent due to the presence of phytates that bind with dietary iron, making it unavailable for absorption (Hallberg et al., 1989; Minihane and Rimbach, 2002). Cereal grains contain 0.2–2.8 mg/100 g of iron (Glahn et al., 2002) but less than 2% is absorbed in the human gut. Therefore, increasing the quantity and bioavailability of iron in cereals presents one way to fight anemia-related problems, especially in populations where cereals are a major staple crop. This could be achieved through classical breeding or by genetic engineering. However, traditional breeding is limited by inadequate genetic variation for iron content and bioavailability in the natural maize populations.

Genetic manipulation through over expression of ferritin genes from different sources as a means to increase the amount of iron in plants has been successful (Goto et al., 1999; Drakakaki et al., 2000, 2005; Lucca et al., 2001, 2002; Vasconcelos et al., 2003; Qu et al., 2005; Aluru et al., 2011; Borg et al., 2012). While these results are promising from the nutritional perspective, only one of these studies examined gene expression and transcription profiles of native genes in ferritin-transformed plants compared to non-transformed plants. As such, the impact of foreign genes on expression and transcription of native genes remains relatively uncharacterized in this system. In Arabidopsis, El Ouakfaoui and Miki (2005) reported a small number of genes differing in expression levels between T-DNA-insertion mutants and their non-transformed counterparts. On the other hand, Baudo et al. (2006) compared changes in mRNA levels of conventionally generated and genetically modified wheat lines expressing glutenin with the traditional wheat lines using 9K cDNA microarray technique and they concluded that breeding through conventional means causes more variation in expression levels than using genetic engineering.

Introduction of genes involved in iron storage is likely to alter the regulation of expression and transcription of native genes for iron storage or those genes required for other iron homeostasis processes. In transgenic tobacco expressing soybean ferretin, for example (Van Wuytswinkel et al., 1999; Vansuyt et al., 2003) physiological and biochemical changes were observed that mirrored iron-deficient plants, possibly due to sequestration of iron in ferritin. Knowledge of the transgene effect on expression of

native genes in the host genome is required in order to predict unintended effects of these foreign genes on the levels and stability of the native genes. If event evaluation and selection is focused on identifying presence of superior transgene phenotypes, it is possible that internal changes in expression levels of other genes within the host genome as a result of transformation will be overlooked.

In maize, the 27 kDa zein promoter has been used to regulate endosperm specific expression in transgenic cereal crops and indeed, the same promoter was used in this study to drive the expression of the soybean ferritin gene to the maize seed endosperm. Since maize contains endogenous zein storage proteins, it would be interesting to examine the effect of the introduced transgene driven by the zein promoter on expression and transcription of other endogenous genes involved in iron sequestering and storage as well as endogenous zein protein genes in the maize seed endosperm, since the zeins have been reported to share promoter regions (Kodrzycki et al., 1989). Our main objective was therefore to over express the soybean ferritin gene in maize seed endosperm and study the transgene effects on the transcript and expression levels of selected endogenous maize genes in roots, leaves and seed endosperm.

MATERIALS AND METHODS

CONSTRUCT DEVELOPMENT AND TRANSFORMATION

The soybean ferritin construct (pMNK01) is the same construct used in Aluru et al. (2011) and consisted of the super gamma zein promoter sequence (979 bp) (Aluru et al., 2008) for endosperm-specific expression, the soybean ferritin coding sequence (753 bp) with a plastid transit peptide to direct protein accumulation to the amyloplast (Lescure et al., 1991), and the Tvsp terminator sequence (515 bp) from the soybean vegetative storage protein gene (Aluru et al., 2008). The construct was developed using the polymerase chain reaction (PCR)-based cloning and it is illustrated in **Figure 1**. Plasmid delivery into maize HiII line (A188 × B73) (Armstrong et al., 1991) was done by the Iowa State University Plant Transformation Facility according to Frame et al. (2000). The plasmid construct pMKN01 was co-bombarded with pBAR184 (Frame et al., 2000), which has the maize ubiquitin promoter that drives the expression of the *Streptomyces hygroscopicus* gene for the bialophos resistance to enable selection of successful transformation events. Successful transformations were confirmed in biolophos-resistant calli by PCR, using primer pairs specific for the soybean ferritin gene [5′GCCATGGCTCTTGCTCCATCC3′ (forward primer) and 5′CAAAGTGCCAAACACCGTGACCC3′ (reverse primer)]. The plantlets regenerated were transferred to the greenhouse, grown to maturity and the seeds were harvested and separated by ear. A total of eight transformation events were harvested after bombardment and selection at the callus level.

GREENHOUSE AND FIELD PRODUCTION OF TRANSGENIC INBRED LINES

Plants regenerated from the transformed callus were crossed to the *Zea mays* L. inbred B73 to produce F_1 seeds. The F_1 plants were backcrossed to B73 to produce BC_1F_1 seeds for the second generation. The BC_2F_1 and BC_3F_1 generation seeds were produced by backcrossing the BC_1F_1 and BC_2F_1 generation plants to B73 in subsequent third and fourth generations, respectively. Thus, the genomic composition of all the seeds planted for analysis was 93.75% B73 by pedigree, with the remainder being from A188. The selection of which plants to advance to the next generation was done based on presence and expression of the soybean ferritin transgene in individual seeds. The first two generations were grown in a greenhouse, while the third and fourth generations were grown in the field at the Iowa State University's transgenic nursery at Woodruff farm near Ames, Iowa.

FIELD PLOT DESIGN FOR PRODUCTION OF MAIZE PLANT TISSUES

Tissues used in zein and mineral analyses were produced in the field in 2009 while those for DNA detection, western blots and mRNA transcript analyses were produced in the field in 2010.

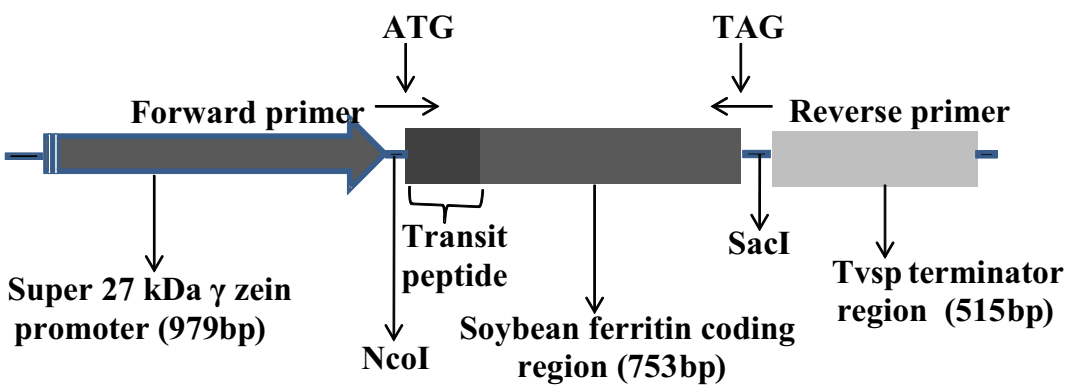

FIGURE 1 | Organization of the soybean ferritin transformation construct. The construct consisted of the super gamma zein promoter, the soybean ferritin coding sequence and the Tvsp terminator from soybean vegetative storage protein gene. The corresponding sizes for each construct component are indicated in brackets below the construct component name. The ATG and TAG indicate the start and stop sites, respectively, while NcoI and SacI represent the enzyme restriction sites flanking the soybean ferritin sequence with the plastid transit peptide. The forward and the reverse primer directions are indicated by arrows pointing in opposite directions.

Each transformation event was randomly assigned to a plot. A plot consisted of four rows. The first three rows contained PCR positive seeds planted ear to row from three different ears within the same transformation event. The fourth row contained a bulk of PCR negative seeds from the same three ears.

FERRITIN DNA DETECTION IN TRANSFORMED MAIZE SEED ENDOSPERMS

Four transformation events (P344-2-4-1, P344-4-1-6, P344-5-2-1 and P344-6-6-1) were selected and used in this analysis. PCR was used to test for presence of soybean ferritin DNA in individual seeds. Test samples were obtained using a non-destructive method where a 10 mg sample of the endosperm was drilled from each of the seeds. DNA was extracted from each sample using the phenol-chloroform manual extraction method. The extraction buffer consisted of 200 mM Tris-HCl (PH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS, all in water. The PCR reaction was performed with the EconoTaq plus Green 2x master mix (Lucigen Corporation, Middleton, WI) following the manufacturer's instructions. The primers used were designed to amplify a 753 bp soybean ferritin fragment and these were 5′GCCATGGCTCTTGCTCCATCC3′ (forward primer) and 5′CAAAGTGCCAAACACCGTGACCC3′ (reverse primer). The PCR reaction cycle consisted of initial denaturation for 2 min at 95°C, denaturation for 30 s at 95°C, annealing for 30 s at 57°C and extension for 1 min at 72°C for a total of 35 cycles. The PCR products were run on an 1% agarose gel containing ethidium bromide which was photographed under UV light.

COMPARISON OF SOYBEAN FERRITIN PROTEIN IN TRANSGENIC MAIZE SEEDS POSITIVE OR NEGATIVE FOR THE SOYBEAN FERRITIN DNA

Western blot analysis was carried out to determine the expression of the soybean ferritin transgene in maize seed endosperm. Mature transgenic seeds were dried and a 10 mg sample was drilled from individual seeds and collected into a 1.5 ml tube. The remaining portion of each of the seeds was kept separate. DNA was extracted from the samples and PCR performed as described in the previous section. The PCR positive and negative samples were noted and their seeds separated. An endosperm sample was drilled from the saved portion of the seed and used for protein extraction and detection. Total protein was extracted with 100 µl of extraction buffer (200 mM Tris-HCl pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM 2-mercaptaethanol and 0.05% Tween-20) for a 10 mg sample of the endosperm powder. 20 µl of total protein were analyzed by electrophoresis using sodium dodecyl sulphate (Laemmli, 1970) on a 12% polyacrylamide gel. After the run was completed, the proteins were transferred to a nitrocellulose membrane for 2 h using a biorad transblot apparatus according to the manufacturer's directions. Polyclonal antibodies for the soybean ferritin gene were raised in rabbit against soybean ferritin peptide (PQVSLARQNYADEC) produced by GenScript Company, Piscataway, NJ. Protein detection was performed using standard western blot analysis protocols (Anti-rabbit-AP secondary antibody with AP conjugate substrate, Biorad Laboratories, Hercules, CA) following the manufacturer's instructions. The blot was probed with 1:300 and 1:3000 dilutions of the primary and secondary antibodies, respectively.

COMPARISON OF TRANSCRIPT LEVELS OF MAIZE ENDOGENOUS GENES IN MAIZE PLANT TISSUES POSITIVE OR NEGATIVE FOR THE SOYBEAN FERRITIN TRANSGENE

The aim of this experiment was to compare mRNA transcripts of endogenous maize genes in roots, leaves and seed endosperms using a quantitative polymerase chain reaction (qPCR) method. The presence or absence of the soybean ferritin transgene in maize plants was first determined by PCR using primers specific for the transgene (see previous sections). Transcript levels of PCR positive plants were compared to those of PCR negative plants in maize roots, leaves and seed endosperm tissues. Maize plants in BC_2F_1 and BC_3F_1 generations segregating for the soybean ferritin transgene were grown in the field in 2009 and 2010, respectively. The first leaf and root samples were collected from individual plants 1 month after planting (MAP) and at 18 days after pollination (DAP), leaf, root, and seed endosperm samples were collected. The 1 MAP and 18 DAP data collection points were included in the analysis in order to capture differences in mRNA transcript levels of endogenous maize genes before and after onset of soybean ferritin transgene expression, respectively. Leaf and root samples were collected from one plant in each PCR positive row and three plants from each PCR negative row for a total of six plants per transformation event for each of the four events (P344-2-4-1, P344-4-1-6, P344-5-2-1, and P344-6-6-1). Similarly, twenty immature seed endosperms were collected from each of the three ferritin PCR positive and negative ears from six plants for each of the four transformation events. Each endosperm sample was divided into two portions, one for DNA analysis in order to identify positive and negative endosperms and the second for mRNA analysis. Tissues were immediately frozen in liquid nitrogen while in the field and upon arrival in the laboratory, they were stored at −80°C until processing. DNA was extracted from each of the endosperms and subjected to PCR analysis for soybean ferritin transgene detection. Soybean ferritin positive and negative seeds were identified and mRNA was extracted from the saved endosperms using the PolyATract mRNA Isolation System kit (Promega Madison, WI). Likewise, mRNA was extracted from the root and leaf tissues using the same method as above. Quantitative PCR analysis was performed with the Brilliant II SYBR Green QRT-PCR Master Mix kit (Stratagene, Santa Clara, CA).

Genes used for the analysis

A complete list of genes and tissues in which these genes were tested in addition to the primer information for each gene is presented in **Table 1**. Among the selected genes were the zeins, the major group of seed storage proteins genes in the maize seed endosperm. These included 27 kDa γ and 16 kDa γ-zeins, 15 kDa β-zein, 19 kDa and 22 kDa α-zeins, and 18 kDa δ zein. Also, genes involved in the iron homeostasis pathway (Maize ferritin, maize ferredoxin-1, *Zea mays* 4 iron—4 sulphur ferredoxin (ferredoxin), *maize nicotianamine amino transferase*

Table 1 | Details on gene, primer information, and maize plant tissue in which the gene was tested in the QPCR experiment.

Gene name	Accession no.	Maize plant tissue tested	Sequence (5′–3′)	Product (bp)	References
27kDa γ-zein	EU976420	Seed endosperm	CCACCATGCCACTACCCTAC ACTGATGCCTCAGGAACTCG	166	Alexandrov et al., 2009
16kDa γ-zein	AF371262	Seed endosperm	TGGAGAACTCGACACCATGA GGTGGTTGAGTGGGGTATTG	201	Woo et al., 2001
15kDa β-zein	AF371264	Seed endosperm	TCAGTAGTAGGGCGGAATGG TGTACGAGCCAGCTCTGATG	179	Woo et al., 2001
19kDa α-zein	AF371269	Seed endosperm	CGTGGGTGAGACCAACTAGC GAAGACACCGCTGGTGAGAG	203	Woo et al., 2001
18kDa δ-zein	AF371265	Seed endosperm	CTCTGATTCCATCTCGCACA GGCATGCCGACTTCATTATT	161	Woo et al., 2001
22kDa α-zein / Floury2	L34340	Seed endosperm	CAACAGTTTCTGCCATCACTG GGCTAGTTGACTGAGCACTGG	162	Coleman et al., 1995
Soybean ferritin	M64337	Seed endosperm	AACTGCTCCCCAAGTCTC G CAGCGTGCTCTCTTTCTTCC	200	Lescure et al., 1991
Maize ferritin	X61392	Root, leaf, Seed endosperm	CGACCCCCACGCGCCTATATC GCGGCCCCACCAGAGAGATG	200	Lobreaux et al., 1992
ferredoxin-1	EU958223	Root, leaf, seed endosperm	GCCTGTCGTCGCAGTAGTGA AGGAGTAGGGCAGGTCCAAC	172	Alexandrov et al., 2009
4Fe-4S ferredoxin	EU969603	Root, leaf, seed endosperm	AGAGCTGTGTCCTATGTTCGAG TAACTGCAACCAGCTTCACG	145	Alexandrov et al., 2009
Zm*NAAT1*	AB375372	Root	TGGCCACATTGCTCCTGTCTTG GAAGTGCTCCCTGAAAGTTGCTGA	184	Inoue et al., 2008
yellow stripe 1	NM_001111482	Root	GATGCAAGTCCGAGGGTTCCTC CGAGGTGCGAACAGATCATCCC	180	Ueno et al., 2009
ZmNAS1	AB061270	Root, leaf, seed endosperm	TGTTCACCAGCCTCGTCATG CTGAAGTAGGGAAAGCGGCC	180	Mori and Mizuno, 2001a
ZmNAS2	AB061271	Root, leaf, seed endosperm	GCTATGTCACGGGCATCGCA GAACATTTGCTTGCGCAGGC	172	Mori and Mizuno, 2001b
ZmNAS3	AB042551	Root, leaf, seed endosperm	TCGCTCTTCCCGTACATCAA CCGCAGATGTCGTAGTTGTC	191	Mori and Mizuno, 2002

Soybean ferritin is the transgene used in this study.

1 (*ZmNAAT1*), *maize yellow stripe* 1 (*ZmYS1*), *maize nicotianamine synthase* 1 (*ZmNAS1*), *maize nicotianamine synthase* 2 (*ZmNAS2*) and *maize nicotianamine synthase* 3 (*ZmNAS3*) were included in this study. These genes were chosen for this study because they are known to respond to or be involved with determining iron homeostasis and are well-characterized in maize. The reference gene used for normalization was the 18S ribosomal RNA.

COMPARISON OF ZEIN PROTEINS IN MAIZE SEED ENDOSPERMS POSITIVE OR NEGATIVE FOR THE SOYBEAN FERRITIN TRANSGENE

This experiment was carried out to compare zein protein levels in maize seeds, positive or negative for the soybean ferritin transgene using the high performance liquid chromatography (HPLC). The materials used for this analysis were obtained from plants harvested in the third generation. Three transformation events (P344-2-4-1, P344-4-1-6, and P344-5-2-1) and three ears per event were used for this study. In brief, a 10 mg sample was drilled from each of the several kernels from the three ears from each of the three transformation events. DNA was extracted from each of the sample and tested with PCR to identify positive and negative maize seeds for the soybean ferritin transgene. The PCR reaction primers and conditions were as described in the previous sections. The remaining portion of the seed endosperm (positive or negative) was used to obtain another 10 mg sample for zein extraction and HPLC analysis.

Extraction of zein proteins and HPLC analysis

Maize zeins were extracted from each 10 mg sample with 400 μl of extraction buffer (70% Ethanol and 61 mM sodium acetate solution) in a 1.5 m centrifuge tube. The tubes were shaken for 1 h at room temperature before they were centrifuged for 10 min at 14,000 rpm. The supernatant was removed and transferred into a fresh tube. One hundred micro liters of supernatant were transferred into a vial (Alltech 8 × 30 mm shell vial clear glass, catalog 97029) and used for HPLC analysis using the Waters Alliance HPLC system. The solvents used were trifluoroacetic acid (TFA) (0.1%) in ultrapure water, TFA (0.1%) in acetonitrile (Paulis et al., 1991). Samples were separated with a c-18 column (Vydac 218TP54) and the UV absorbance was monitored at 210 nm. Levels of individual zeins were determined by integration of the resulting peaks.

COMPARISON OF TOTAL MINERAL CONCENTRATIONS IN FERRITIN PCR POSITIVE AND NEGATIVE MAIZE SEEDS

Previous studies have shown that activation of enzymes related to iron uptake also increases the uptake of other divalent metal cations (Welch, 1993; Delhaize, 1996; Vansuyt et al., 2000). In light of these previous findings we sought to carry out mineral quantification in maize seeds transformed with the soybean ferritin transgene. The minerals that were analyzed in this study included calcium, copper, magnesium, manganese, zinc and iron. Three transformation events (P344-2-4-1, P344-4-1-6, and P344-5-2-1) were analyzed and samples were obtained from three maize ears for each of the events. The analysis was done on ground whole maize seeds that tested positive or negative using PCR, for the soybean ferritin transgene. Samples of 1.5 g were prepared from 20–45 seeds (depending on kernel size) from a bulk of ears from the same transformation event. Six samples, three PCR positives and three PCR negatives were prepared for each of the transformation events. Samples were analyzed for mineral content using inductively coupled plasma atomic emission spectrometry (ICAP) at the Soil and Plant Analysis Laboratory, Iowa State University. A total of eighteen samples were analyzed in duplicates.

COMPARISON OF PERCENT TOTAL NITROGEN IN MAIZE SEED SAMPLES POSITIVE OR NEGATIVE FOR THE SOYBEAN FERRITIN DNA

Percent total nitrogen (as a measure of percentage total protein) was measured using the LECO CN combustion elemental analyzer (LECO Corporation, St. Joseph, MI) and using the same materials as those in the previous section on mineral analysis. As described above, samples negative or positive for the soybean ferritin transgene were compared in three transformation events (P344-2-4-1, P344-4-1-6, and P344-5-2-1).

STATISTICAL ANALYSIS

For zein and nitrogen data, analysis of variance (ANOVA) was carried out using JMP SAS-based statistical software (Version 8.0.1) in which the variance was partitioned among the effects listed in **Tables 2** and **3** by least squares fitting to a linear model. All effects were considered fixed, limiting the inference space to observations in this experiment and allowing us to estimate the magnitudes and significance probabilities of all effects. The significance of the transgene PCR + or − effect indicates whether a significant difference was between transgenic and non-transgenic plants and is therefore the effect of greatest interest and wherever significant differences were obtained, means were compared by the student's *t*-test. For the qPCR analyses, the delta cycle threshold values were used and these were calculated as discussed in Pfaffl (2001).

RESULTS

EXPRESSION OF SOYBEAN FERRITIN TANSGENE IN MAIZE SEED ENDOSPERM

PCR was used to test for the presence of soybean ferritin transgene DNA in maize leaves and seed endosperm. A band of the expected size was detected in some plants in the first through the fourth generation plants. This indicated successful integration and sexual transmission of the soybean ferritin transgene into the maize genome. The PCR product was not detected in non-transgenic B73, the negative control (**Figure 2A**). Of the 272 seeds tested from 6 transformation events of F_1 plants, 141 were positive for the soybean ferritin transgene following PCR screening (Data not shown).

After DNA detection, we analyzed the ferritin protein expression using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses. As anticipated, the 28 kDa soybean ferritin protein was detected in PCR positive samples but not in the PCR negative samples implying that the transgene was effectively expressed in the maize seed endosperm. In addition to the targeted protein, the antibody used also cross-reacted with another protein of a higher molecular weight in both PCR positive and negative samples for the soybean transgene. No immune reactive 28 kDa band was detected in untransformed A188 and B73 samples (**Figure 2B**).

Table 2 | Transcript ratios of transgene PCR positive plants relative to PCR negative plants averaged across four transformation events (P344-2-4-1, P344-4-1-6, P344-5-2-1, and P344-6-6-1) in three tissues (roots, leaves, and seed endosperm) at two developmental stages (1 MAP and 18 DAP).

Gene	Tissue				
	Root		Leaf		Endosperm[1]
	1 MAP	18 DAP	1MAP	18 DAP	18 DAP
Transgene: Soybean ferritin[2]	n.a.	n.a.	n.a.	n.a.	32.21***
ZmNAS1	0.94	1.02	0.99	1.08	4.76**
ZmNAS2	0.93	1.02	0.98	0.90	5.88**
ZmNAS3	1.08	0.95	0.76	0.39	4.23**
Maize ferritin	1.10	1.82	1.10	1.04	4.13*
Yellow stripe 1	0.92	1.01	n.a.[3]	n.a.	n.a.
ZmNAAT1	4.35**	5.00**	n.a.	n.a.	n.a.
Maize ferredoxin	n.a.	n.a.	1.49	1.49	4.50*
4Fe-4S ferredoxin	n.a.	n.a.	1.08	6.67*	5.29***
27kDa γ-zein	n.a.	n.a.	n.a.	n.a.	1.36
16kDa γ-zein	n.a.	n.a.	n.a.	n.a.	1.05
15kDa β-zein	n.a.	n.a.	n.a.	n.a.	1.35
19kDa α-zein	n.a.	n.a.	n.a.	n.a.	8.34**
18kDa δ-zein	n.a.	n.a.	n.a.	n.a.	1.10
22kDa α-zein/ Floury2	n.a.	n.a.	n.a.	n.a.	8.35**

[1] *Endosperm was examined only at 18 DAP because it was not formed yet at 1 MAP.*

[2] *Since the transgene is not present in the PCR negative samples, these ratios are represent of the PCR positive samples to the background level.*

[3] *n.a.: measurement not taken because genes are not expressed in tissue.*

, **, and * indicate significance probabilities at $P < 0.05$, $P < 0.001$, and $P < 0.0001$, respectively.*

Table 3 | ANOVA Table showing the sums of squares for the mean HPLC peak areas of maize zein proteins in maize seed samples positive or negative for the soybean ferritin transgene.

		Model effects				
		Event[1]	Transgene PCR + or −[2]	Event × Transgene PCR + or −	Error	Total
	DF[3]	2	1	2	12	17
HPLC Peak[4]	1	2.39E+12	9.87E+12***	1.16E+11	5.70E+12	1.81E+13
	2	4.88E+12	2.15E+12	2.74E+12	1.27E+13	2.24E+13
	3	7.91E+12	7.47E+12*	1.00E+12	1.23E+13	2.87E+13
	4	1.62E+11	3.47E+12***	2.93E+11	1.06E+12	4.90E+12
	5	1.79E+12	1.32E+13**	1.17E+12	1.14E+13	2.75E+13
	6	1.59E+11	5.27E+12***	4.53E+11	2.10E+12	7.98E+12
	7	9.40E+11	2.27E+13**	1.39E+12	8.82E+12	3.39E+13
	8	1.30E+13	1.885E+14***	1.61E+13	1.12E+14	3.29E+14
	9	1.45E+12	2.7973E+13***	9.25E+11	5.19E+13	1.08E+14
	10	1.84E+12	4.7691E+13**	6.14E+12	5.19E+13	1.08E+14
	11	3.96E+11	9.98E+12	4.25E+11	8.98E+11	1.17E+13
	12	5.11E+11	1.297E+11**	2.38E+11	1.13E+12	2.01E+12
	13	1.28E+12	3.3112E+12***	1.26E+12	2.02E+13	2.60E+13
	14	1.00E+12	1.39E+13	2.53E+12	1.49E+13	3.24E+13
	15	4.34E+09	3.3112E+11*	1.58E+11	9.45E+11	1.44E+12

Significant differences between PCR positive and negative seed samples are indicated with one, two, or three stars before each value to represent significant differences at 5, 0.1, and 0.01% significance levels, respectively.

[1] Three events were used in this study: P344-2-4-1, P344-4-1-6 and P344-5-2-1.

[2] Samples were screened with PCR to identify transgene-encoded ferritin positive and negative seeds.

[3] Degrees of freedom. Read across row.

*[4] HPLC peak assignments are shown in **Figure 7**.*

, **, and * indicate significance probabilities at $P < 0.05$, $P < 0.001$, and $P < 0.0001$, respectively.*

EXPRESSION OF ENDOGENOUS GENES IN LEAVES, ROOTS, AND SEED ENDOSPERM OF MAIZE TRANSFORMED WITH THE SOYBEAN FERRITIN TRANSGENE

In this experiment, differential expression of endogenous genes in roots, leaves, and seed endosperms was examined in maize lines transformed with the soybean ferritin transgene. Generally, the soybean ferritin transgene only affected expression of a few genes of those tested in maize leaves and roots measured during the vegetative and reproductive stages. The vegetative stage represents period before transgene expression (1 MAP) while reproductive stage represents period after transgene expression (18 DAP).

In maize roots, the only significant differences ($P = 0.0303$ and $P = 0.0097$; vegetative and reproductive stages, respectively) in transcript levels were observed for the *NAAT1* gene between the PCR positive and negative plants (**Figure 3A**). PCR positive plants had 4.35 and 5 times more *NAAT1* mRNA before and after soybean ferritin transgene expression, respectively, relative to the PCR negative plants (**Table 2**). The transcript accumulation of other genes (*ZmNAS1*, *ZmNAS2*, *ZmNAS3, maizeYS1*, and maize ferritin) did not differ significantly ($P > 0.05$) between soybean ferritin PCR negative and positive maize root samples (**Figure 3A**; **Table 2**). Comparing expression levels of all the genes tested relative to 18S RNA, we observed that *ZmNAS2, NAAT1, ZmNAS1*, and *Maize YS1* were the most highly expressed while *ZmNAS3* and maize ferritin were the least expressed before transgene expression (Data not shown). After transgene expression, the transcript level of maize ferritin increase along the others but *ZmNAS3* did not change. In either case, however, it was only *NAAT1* that was significantly higher in transgene PCR positive samples compared to the control.

Gene expression results from leaf samples were almost identical to those observed from the root samples. Prior to transgene expression, there were no significant differences in mRNA levels of all the tested genes between transgene PCR positive and negative samples (**Figure 3B**). However, after transgene expression, the amount of ferredoxin binding protein mRNA levels significantly ($P < 0.05$) increased 6.67 fold in transgene PCR positive samples compared to the PCR negative samples (**Figure 3B**; **Table 2**). Comparing changes in the expression levels of all genes relative to 18S RNA showed that *ZmNAS1* had the highest mRNA levels compared to other genes during the vegetative stage, while maize ferritin and maize ferredoxin and ferredoxin binding protein were highly expressed compared to others during the reproductive stage.

More dramatic expression differences were observed for genes endogenous to maize seed endosperm (**Figures 4A**,**B**). The presence of soybean ferritin transgene in the seed endosperm significantly ($P < 0.001$) reduced mRNA accumulation of the 19 and 22 kDa zeins but did not affect ($P > 0.05$) mRNA levels of the 27, 16, 15, and 18 kDa zeins (**Figure 4A**). The 19 and 22 kDa zeins mRNA was reduced 8.34 and 8.35-fold, respectively, in

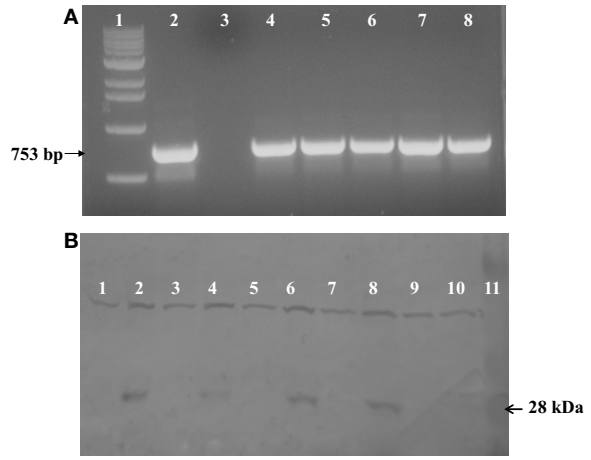

FIGURE 2 | (A) One percent agarose gel stained with ethidum bromide showing PCR analysis results of soybean ferritin DNA detection in transformed maize lines and the non-transformed line B73 used as a negative control. Lanes 1 and 2 refer to 1 kb molecular DNA size marker and soybean ferritin plasmid (pMNK01) positive control, respectively. Lane 3 represents the negative control while lanes 4 through 8 represent the transformed maize lines (P344-2-4-1, P344-4-1-6, P344-5-2-1, P344-6-6-1, and P344-7-4-1, respectively). The black arrow shows the specific 753 bp fragment of soybean ferritin coding sequence. **(B)** Western blot showing the 28 kDa soybean ferritin protein band in maize seed endosperms. Lanes 1 through 8 represents transformed maize seeds that were PCR, negative (lanes 1, 3, 5, and 7) or PCR positive (lanes 2, 4, 6, and 8) for the soybean ferritin transgene. Each positive/negative pair of lanes are from a different transformation event (P344-2-4-1, P344-4-1-6, P344-5-2-1, and P344-6-6-1). Lanes 1 and 3 are from event P344-2-4-1; lanes 3 and 4 are from event, P344-4-1-6; lanes 5 and 6 are from event P344-5-2-1 and lanes 7 and 8 are from event P344-6-6-1. Lanes 9 and 10 non-transgenic A188 and B73 seeds that were used as negative controls, respectively, while lane 11 contains the protein molecular size marker.

PCR positive samples compared to the negative ones (**Table 2**). Similar to the zein transcripts, the mRNA abundance of the seven tested iron homeostasis genes differed between seed samples positive and negative for the soybean ferritin transgene in the maize seed endosperm. The samples positive for the transgene had significantly ($P < 0.0001$) more transcripts of the soybean ferritin, ferredoxin ferrodoxin, maize ferredoxin, maize ferritin, and *ZmNAS3* genes than the negative samples (**Figure 4B**). Ferredoxin, maize ferritin, maize ferredoxin and *ZmNAS3* showed atleast four times more expression in the soybean ferritin PCR positive seed endosperms than in the negative ones (**Table 2**). On the contrary, the presence of the transgene significantly ($P < 0.001$) reduced the accumulation of *ZmNAS1* and 2 mRNA in PCR positive seed endosperms compared to their negative counterparts (**Figure 4B**).

When we analyzed mRNA accumulation levels of all zein genes relative to 18S RNA, results showed that the 16 kDa was the most highly expressed compared to all other genes. However, the analysis of iron homeostasis genes' mRNA accumulation in soybean ferritin PCR positive and negative samples relative to 18S RNA showed that *ZmNAS2* had significantly ($P < 0.0001$) higher expression levels compared to all other genes, although significant differences were also observed among individual PCR positive and negative samples (Data not shown).

ZEIN PROTEIN PROFILES IN MAIZE, POSITIVE AND NEGATIVE FOR THE SOYBEAN FERRITIN TRANSGENE

This experiment was carried out to determine if presence of the soybean ferritin transgene affected the overall profile of zein proteins in the transformed maize. The chromatograms showed some qualitative and quantitative differences between soybean ferritin PCR positive and negative samples (**Figure 5** and **Table 3**). Zein profiles for each group are given numbers (1–15) depending on the elution time (peaks). For already described zeins i.e., 18δ, 27γ, and 16γ zeins, individual protein peaks were identified and their molecular sizes (kDa) indicated. Most of the peaks within the PCR negative and positive samples had similar elution patterns, indicating same protein profile in PCR negative and positive samples (**Figure 5**). However, in some of the PCR positive samples, the elution time for peak 12 was shifted resulting in position 13 (see expanded portion of the chromatogram, **Figure 5**). The occurrence of samples whether positive or negative for the transgene influenced the magnitude of the mean peak area. The peak areas in PCR negative samples were significantly ($P < 0.0001$) higher than their PCR positive counterparts. This was true mostly for the alpha zeins (area peaks 4–15) while the relative proportions of the delta (area peak 1) and the gamma (peaks 2 and 3) zeins were similar within the PCR positive and negative seed endosperm samples (**Figure 6**). Alpha peaks 8, 10, 12, 13, and 15 had significantly higher peak areas in PCR negative samples compared to the positive samples (**Figure 6**). The mean differences between peak areas for events one and two were significantly ($P < 0.05$) different among PCR positive and negative samples with the PCR negative samples having higher peak areas in all the transformation events (**Figure 7**).

EFFECTS OF THE SOYBEAN FERRITIN TRANSGENE ON MINERAL COMPOSTION IN THE MAIZE SEED ENDOSPERM

The presence of the soybean ferritin transgene significantly ($P < 0.05$) affected the concentration of calcium, magnesium, and iron but not copper, manganese, and zinc. The mineral concentrations of calcium, magnesium and iron were higher in samples positive for the soybean ferritin transgene compared to the negative samples (**Figure 8**). Although the iron concentration in samples positive for the transgene differed significantly from that in the negative samples, they were only 0.2 times higher. While the mean mineral concentrations of copper, manganese and zinc were higher in the soybean ferritin PCR positive samples compared to the negative samples, their mean differences were not significant at $P = 0.05$.

PERCENT TOTAL PROTEIN DECREASED IN MAIZE SEED ENDOSPERMS TRANSFORMED WITH THE SOYBEAN FERRITIN TRANSGENE

The mean percentage total protein differences between PCR positive and negative maize seed endosperm samples were highly significant ($P < 0.0001$) (**Table 4**). Percentage total protein was higher in transgene PCR negative samples than PCR positive samples. On the other hand, transformation events (P344-2-4-1, P344-4-1-6, and P344-5-2-1) had significant differences in

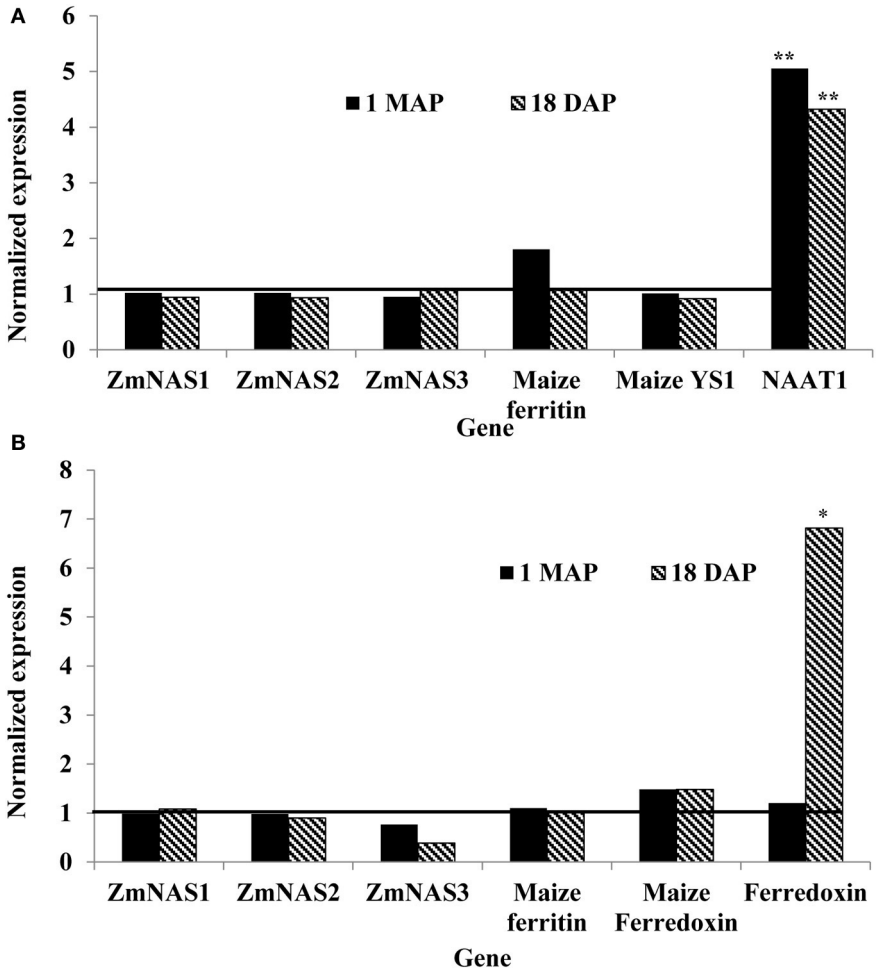

FIGURE 3 | Relative mRNA changes in maize plant roots (A) and leaves (B) at 1 month after planting (MAP) and 18 days after pollination (DAP). Maize plants were selected based on presence and absence of the soybean ferritin DNA using PCR. Data collected at 1 MAP and 18 DAP represent time before and after soybean ferritin transgene expression, respectively. For each gene, the delta cycle threshold (ct) values were normalized using 18S RNA. mRNA transcript levels in both PCR negative and positive samples were compared after normalization with respect to the ferritin PCR negative samples. The horizontal line on Figure represents values for PCR negative samples, normalized to one relative to the PCR negatives. Significant differences at $P < 0.05$ and $P < 0.001$) between transgene PCR positive and negative samples are indicated with one star and two stars, respectively, on top of the bar.

total nitrogen measurements between samples positive and negative for the soybean ferritin transgene (**Table 4**). Transformation events 1 (P344-2-4-1) and 3 (P344-5-2-1) had significantly higher total nitrogen ($1.678 \pm 1.354\%$ and $1.666 \pm 1.354\%$, respectively) compared to transformation event 2 (P344-4-1-6) ($1.592 \pm 1.354\%$). In this experiment, the presence of the soybean ferritin transgene was associated with decreased percentage total protein in maize seed endosperms.

DISCUSSION

Increasing the nutritive value of plants for human benefit has been an area of interest to many research groups. Maize is an excellent target crop because it serves as a major dietary source to many nations, especially those in the developing world, who cannot afford the cost of food supplements. The main objective of this study was to determine the effect of the soybean ferritin transgene on endogenous gene transcripts and protein levels in maize roots, leaves, and seed endosperms. Maize plants expressing the soybean ferritin gene directed to the seed endosperm by the super gamma zein promoter were produced. Soybean ferritin DNA was successfully detected in the leaves and seed endosperm samples, with a corresponding increase in the transgene protein expression in only the ferritin PCR positive seed samples, indicating a successful transformation and integration process. One of the strategies for alleviating mineral or iron deficiency health problems is to produce transgenic plants with increased ferritin levels as reported in findings by different research groups (Goto et al., 1999; Brinch-Pedersen et al., 2000; Drakakaki et al., 2000, 2005; Lucca et al., 2001, 2002; Vasconcelos et al., 2003; Qu et al., 2005; Aluru et al., 2011).

Transgene introduction in plants can potentially lead to unintended modifications in transcription and translation of native genes in the host plants. It is therefore necessary to assess the likelihood of these occurrences by measuring transcript changes

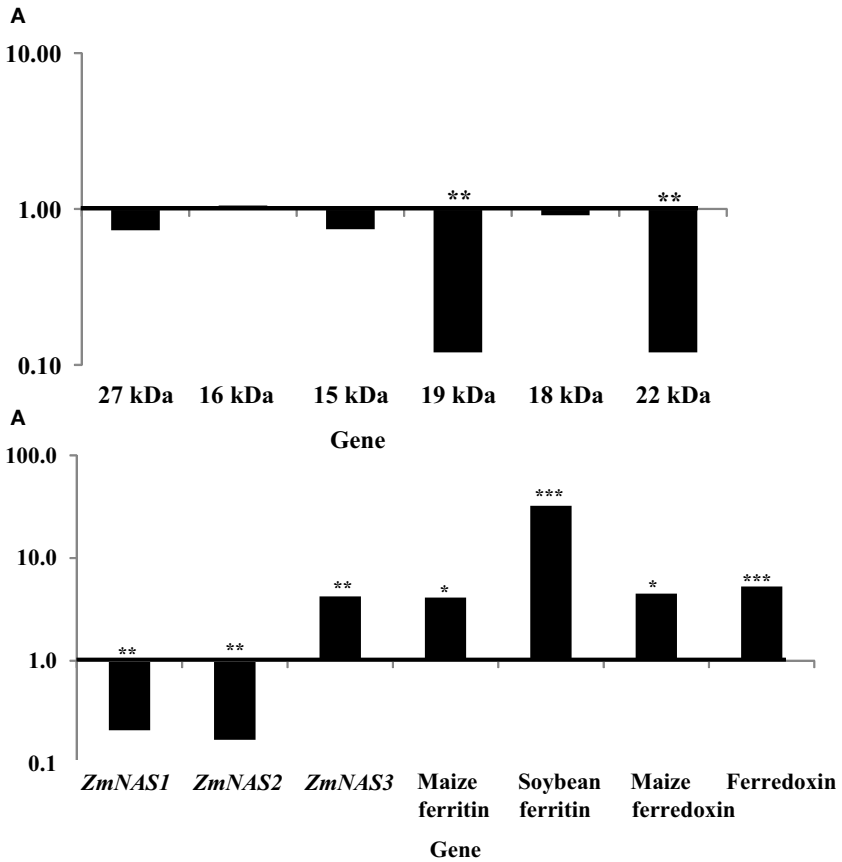

FIGURE 4 | mRNA transcript changes in maize seed endosperm samples harvested at 18 days after pollination (DAP). Maize seed endosperms were selected based on presence and absence of the soybean ferritin DNA. The 18 DAP represents the time after the soybean ferritin transgene expression. Panels **(A)** and **(B)** represent qPCR analysis results for the maize seed storage protein genes (zeins) and iron homeostasis genes, respectively. For each gene, the delta cycle threshold (delta ct) values were normalized using 18S RNA, with respect to the ferritin PCR negative samples. The horizontal lines on the panels represent normalized values for the PCR negative samples. Significant differences ($P < 0.0001$, $P < 0.001$, and $P < 0.05$) are indicated with three, two, and one star(s), respectively, on top of each bar for which the soybean ferritin PCR positive and negative samples differ.

of native genes especially those in tissues where the transgene is inserted or is likely to cause significant changes. Transgene insertion into the host genome is a random process which can result in the transgene landing in the coding sequence of a native gene. When this happens, it can cause changes in the stability or levels of mRNA accumulation of the native gene. Therefore, one of the ways in which we can determine whether or not the transgene causes some accidental effects is to look at mRNA levels of native genes that control the routine activities or that are directly involved in the regulation of specific metabolic processes where the transgene is to be acting. We made an effort in this study to assess the effects of soybean ferritin transgene on the transcript accumulation of native genes in maize roots, leaves and seed endosperm and at two developmental stages. Our results showed that the presence of the soybean ferritin transgene had no significant effects ($P > 0.05$) on most of the genes in the maize roots and leaves, before (1 MAP) and after (18 DAP) transgene expression in the target tissue. Measurement of mRNA transcripts for *ZmNAAT1* gene, however, showed significantly ($P < 0.05$) higher mRNA amounts for the positive PCR root samples than the negative ones at the two data collection points. *ZmNAAT1* is an enzyme that is required in the homeostasis of mugineic acid (MA), an important metal chelator that is involved in the transportation of metal ions, including iron ions across the plasma membrane in both grass and non-grass plants (Higuchi et al., 1996). In rice, Cheng et al. (2007) reported that a mutation in the rice *NAAT1* gene led to substantial accumulation of nicotianamine in roots and shoots but the mutant plants could not effectively absorb Fe (III) because of the failure to produce deoxymugineic acid. Nicotianamine is a chelator that occurs in plants and it is involved in internal iron transport (Stephan et al., 1994, 1996). In our experiment, in maize roots, presence of the soybean ferritin transgene was associated with increased levels of *ZmNAAT1* mRNA. This increase would make sense if the plant was experiencing iron deficiency because iron was sequestered in the seed in the transgene-encoded ferritin. It has been reported that overexpression of soybean ferritin in transgenic tobacco caused plants to respond as if they were iron deficient due to illegitimate sequestration of iron (Van Wuytswinkel et al., 1999; Vansuyt et al.,

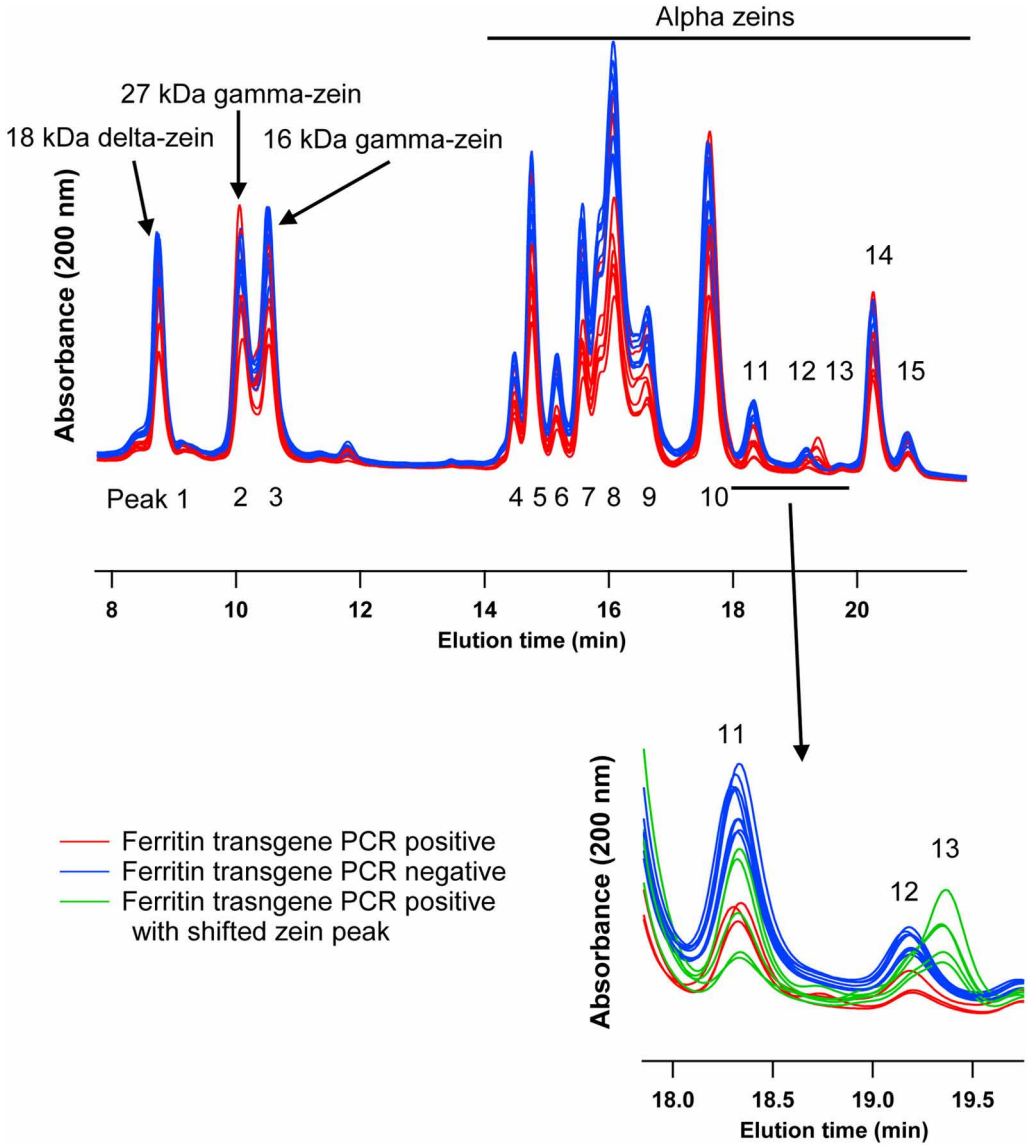

FIGURE 5 | HPLC chromatograms of maize zeins for maize seed endosperms transformed with the soybean ferritin transgene. Maize seed endosperms were screened with PCR to identify soybean ferritin positive (Red or bottom) and negative (blue or top) seed samples. Samples were separated with a c-18 column. Peaks are labeled 1–15, depending on elution time. When known, the peaks (1, 2, and 3) are labeled with their respective sizes (kDa) and 4–15 refer to the unassigned alpha zeins. The inset emphasizes a peak that appeared in some transgene PCR positive samples, peak 13 in green traces.

2003). This explanation is not completely satisfying, however, because the *ZmNAAT1* transcript is up-regulated in transgenic plants prior to seed set as well as after seed set. Our results are in contrast to those of Aluru et al. (2011) who found that in roots of maize plants transformed with the same transgene used in this study, NAAT mRNA was reduced two to four fold. Accumulation of this message may therefore be influenced by the environment or the genetic background of the plants used in the study. Earlier studies also indicated that the presence of iron increased the amount of ferritin mRNA in bean leaves and soybean cell cultures by more than 30 fold (Proudhon et al., 1989). The fact that transcript levels of other genes in maize roots were not different in presence or absence of the soybean ferritin transgene illustrates that there was no detectable interaction between these genes and the soybean ferritin transgene in these tissues.

Gene expression results from leaf samples were almost identical to those observed with the root samples, with only the ferredoxin or Iron–sulphur (4Fe-4S) proteins showing increased transcript levels in samples where the soybean ferritin transgene was detected. 4Fe-4S proteins contain iron and sulphur bound to the polypeptide chain by 4 cysteinyl sulfur linkages to the iron atoms (Sweeney and Rabinowitz, 1980). These proteins are well-known for their involvement in electron transport and gene

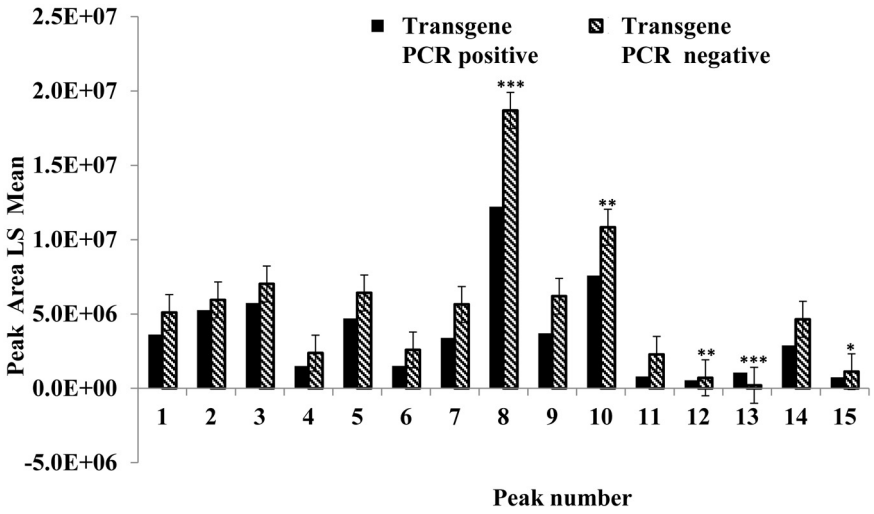

FIGURE 6 | Peak area least square means for the maize seed storage protein genes (zeins). The horizontal axis shows the HPLC protein peaks, named 1–15 depending on the elution time (see **Figure 5**). Significant differences between transgene-encoded ferritin PCR positive and negative maize seed samples are indicated with one, two, and three stars on top of each bar to represent significant differences at 5, 1, and 0.1% significance levels, respectively.

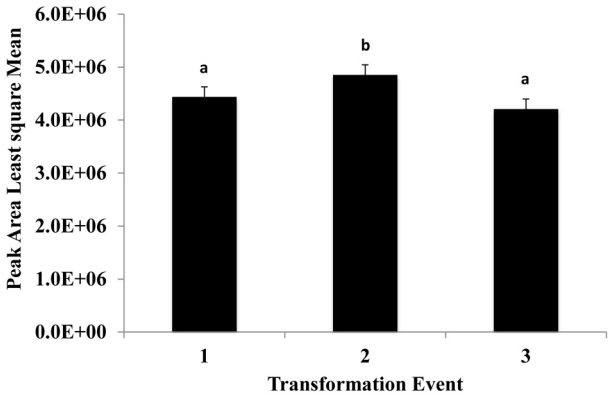

FIGURE 7 | The effect of three transformation events, 1, 2, and 3 (P344-2-4-1, P344-4-1-6, and P344-5-2-1, respectively) on mean peak areas of maize seed storage protein genes for maize seed endosperms transformed with the soybean ferritin transgene. Significant differences ($P < 0.05$) are indicated with a different letter on top of each error bar.

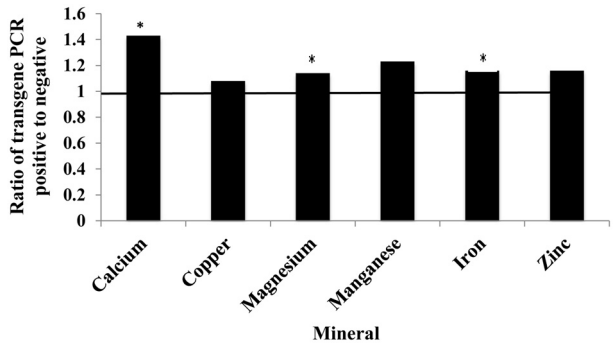

FIGURE 8 | Fold change in mean mineral concentration in maize seed samples transformed with the soybean ferritin transgene. The samples were screened with PCR to categorize them into those positive and negative for the soybean ferritin transgene. Three transformation events (P344-2-4-1, P344-4-1-6, and P344-5-2-1) were used for this test. The event effect was not significant (data not shown), therefore, the results presented are from combined data. The horizontal line represents normalized values for the transgene PCR negative samples. Significant differences ($P < 0.05$) are indicated with a star on top of each error bar for which the PCR positive and negative samples differ.

expression regulation (Lill and Muehlenhoff, 2006). It is likely that the increased transcription of ferritin, an iron storage protein, led to increased transcript levels of this gene. Because the amount of iron present in a given tissue regulates the transcription of the ferritin gene (Savino et al., 1997; De Domenico et al., 2008), it is possible for the iron to similarly have the same effects on genes that act in the same pathway as the ferritin gene and this could explain the relative increase in the transcripts of the genes in samples where the ferritin transgene was detected.

Different endogenous maize genes showed differential gene expression patterns in maize seed endosperm samples with or without the soybean ferritin transgene. Two of the alpha zein protein transcripts (19 and 22 kDa α-zeins) were significantly lower in transgenic plants than in non-transgenic plants. We found more than 8-fold decrease in mRNA accumulation in the 19 and 22 kDa in the PCR positive samples. Conversely, no significant mRNA changes were observed in the gamma and delta zeins as well as other alpha zein proteins tested. These results were consistent with those obtained in the samples harvested and analyzed in earlier previous year (data not shown). These observations are also supported by the zein protein analysis results that showed increased accumulation of these same proteins in soybean ferritin PCR negative compared to PCR positive samples. In addition, the percent total nitrogen levels were

Table 4 | ANOVA for the least square means for percentage total nitrogen in transformed maize seeds, positive or negative for the soybean ferritin transgene.

Least square area means (total nitrogen)				
Effect	**DF[1]**	**Sums of squares**	***F*-ratio**	**Prob > *F***
Replicate	1	0.0022	0.3471	0.5613
[2]Transgene PCR + or −	1	0.4984	73.0392	<0.0001***
[3]Event	2	0.0525	3.8465	0.0356*
Transgene PCR + or − × replicate	1	0.0007	0.1095	0.7436
Transgene PCR + or − × Event	2	0.0194	1.4206	0.2612
Event × replicate	2	0.0064	0.4696	0.6309
Transgene PCR + or − × Event × replicate	2	0.0028	0.2080	0.8137

[1] Degrees of freedom.

[2] The transformed maize seed samples were screened with PCR to identify those positive and negative for the soybean ferritin transgene. Comparisons are made based on significant differences in percentage total nitrogen between transgene PCR positive and negative samples.

[3] Three transformation events were examined in duplicate for this test (P344-2-4-1, P344-4-1-6, and P344-5-2-1).

** and *** indicate significance probabilities at $P < 0.05$ and $P < 0.0001$, respectively.*

also significantly higher in ferritin PCR negative samples compared to the PCR positive samples. The use of the super gamma zein promoter is a possible explanation for the observed differences. This promoter shares regulatory sequences with endogenous genes and could compete for transcription factors, which could result in reduction in endogenous gene transcription levels. It is somewhat surprising that differences were not observed in the zein most closely related to the transgene, the 27 kDa gamma zein, but were observed in other zeins. Peak 13 was present only in PCR positive samples and these samples lacked Peak 12. We don't know the identity of this peak, but it's co-occurrence with the transgene is intriguing. Several of the alpha peaks had significantly higher peak areas in PCR negative compared to the positive samples. The amount of zein protein depended on both transformation event and presence or absence of the soybean ferritin transgene in the maize. In fact, measurement of the percent total protein in maize seeds without the soybean ferritin transgene showed that they had significantly higher percent total protein than their corresponding positive ones. The high values for zein transcript and protein levels in soybean ferritin negative samples are consistent with percentage total protein content results.

On the other hand, transcripts of iron homeostasis genes in maize seed endosperm accumulated to higher levels in ferritin PCR positive samples compared to their negative counterparts. The regulation of iron homeostasis genes depends on the iron status in the tissue of interest (Savino et al., 1997; De Domenico et al., 2008). It has been reported that overexpression of soybean ferritin in transgenic tobacco caused plants to respond as if they were iron deficient due to an illegitimate sequestration of iron (Van Wuytswinkel et al., 1999; Vansuyt et al., 2003). Thus, changes seed ferritin levels will most likely correlate with up or down-regulation of iron homeostasis genes. In this study, the mRNA transcripts of the soybean ferritin gene accumulated to high levels in maize endosperm samples. In plants, ferritin production is regulated at both transcriptional and post transcriptional levels (Lescure et al., 1991; Kimata and Theil, 1994; Savino et al., 1997). This is dependent on presence of iron that results in the relative induction of ferritin mRNA and protein levels. Protein analysis revealed soybean ferritin protein accumulated to detectable levels only in PCR positive samples. Kimata and Theil (1994) reported stable ferritin mRNA levels in soybean plants during development, with a corresponding decrease in ferritin protein content. Measurement of iron levels in maize seed endosperm samples indicated a direct correlation between presence of ferritin transgene and iron concentrations. The soybean ferritin PCR positive samples in general contained higher mineral concentrations with significant differences observed for calcium, magnesium and iron. The amount of iron that accumulated in PCR positive samples was 0.2 fold higher than in the PCR negative samples. Previous studies (Goto et al., 1999; Vasconcelos et al., 2003; Drakakaki et al., 2005) reported even higher correlations between soybean ferritin expression levels and total iron content. On the contrary, Drakakaki et al. (2000), observed no significant increases in seed iron content in rice and wheat samples transformed with a ferritin gene and whose expression was controlled by the ubiquitin promoter. Iron concentration levels have been reported to differ from one generation to another (Qu et al., 2005) and this could possibly have happened in this case. The fact that different lines will likely have differences in their expression patterns could be another reason why we might expect differences in iron levels. However, in our findings, the different transformation events did not have significant effects on the amount of iron obtained. Changes in environmental factors have also been reported to influence iron uptake and storage (Vansuyt et al., 2000), a factor we cannot rule out in explaining this observation.

Significant increases in the concentrations of other metals (magnesium and calcium) in ferritin PCR positive seed samples could be due to increased iron accumulation resulting from the activation of the ferritin transgene. Vasconcelos et al. (2003) reported a similar finding in rice seeds with enhanced expression levels of the ferritin transgene. Previous research groups (Vansuyt et al., 2000; Welch, 1993) showed that it was possible to increase the uptake of other divalent metal cations with the activation of enzymes involved in iron uptake and use. Since other main factors and their interactions did not seem to have significant effects on metal accumulation, it seems very likely that the observed differences in metal concentrations were due to changes in iron status in maize seed samples. Qu et al. (2005), however, reported no significant changes in concentrations of calcium, copper, cadmium, magnesium, manganese, and zinc, a result that is contrary to our observations.

CONCLUSION

This work describes efforts to assess the effect of soybean ferritin transgene on transcript and protein levels of maize endogenous genes. Differential expression patterns were observed between

maize samples with or without the soybean ferritin transgene. The iron homeostasis genes selected for this study were up-regulated in soybean ferritin PCR positive samples compared to negatives ones. Only two of the zein protein genes were down-regulated in soybean ferritin PCR positive samples while the rest of the zeins remained unchanged. The transformation event did not affect transcript or protein levels of the different genes, but some genes were affected by the presence or absence of the transgene. By knowing transgene effects that can lead to changes in endogenous gene activities and levels, researchers will be able to find viable explanations for the unexpected results and be able to target specific stages controlled by specific genes in iron homeostasis.

ACKNOWLEDGMENTS

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Rice (*Oryza sativa* L.) roots have iodate reduction activity in response to iodine

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Although iodine is not an essential nutrient for higher plants, their roots take up and transport the element. However, the exact mechanisms involved in iodine uptake and metabolism in higher plants have yet to be elucidated. In this study, we compared two cultivars differing in iodine tolerance ("Nipponbare" and "Gohyakumangoku") to increasing levels of I^- and IO_3^- in the root solutions of water-cultured rice (*Oryza sativa* L.). We found that IO_3^- added to the root solutions was converted to I^- in the presence of roots. Iodate reduction occurred over the course of several hours. Furthermore, the iodate reduction activity of "Nipponbare" (iodine-sensitive) and "Gohyakumangoku" (iodine-tolerant) roots increased after adding IO_3^- or I^-. The roots of barley and soybean also showed iodate reduction activity and the activity responded to iodine treatment either with IO_3^- and I^-. This study suggests that plant roots biologically reduce iodate to iodide and indicates that the iodate reduction activity of roots responds to external iodine conditions.

Keywords: iodine, reduction, rice, barley, soybean, root

INTRODUCTION

Iodine, an essential element for humans, is an important component of thyroid hormones. About 15.8% of the world's population suffers from goiter, which is primarily caused by iodine deficiency. Moreover, an additional one-third of the world's population has been estimated as being at risk of iodine deficiency (De Benoist et al., 2004). One approach to addressing this problem is to increase the iodine content of the edible portions of crops. Recently, iodination of the irrigation waters or fertilizers was investigated to produce iodine-enriched crops (Cao et al., 1994; Weng et al., 2008b; Hong et al., 2009). However, growing of plants with high iodine levels in the irrigation waters is problematic as iodine-toxicity symptoms can develop under these conditions. In addition, inasmuch as iodine is not an essential nutrient for higher plants, detailed mechanisms of iodine uptake and its subsequent metabolism in higher plants have yet to be elucidated.

The major chemical form of soluble iodine in soil solutions is I^- under flooded conditions (Muramatsu et al., 1989; Yuita, 1992) and IO_3^- under non-flooded conditions (Yuita, 1992). Iodine uptake and metabolism in plants is dependent on the chemical species present in the irrigation solution. Previous reports have indicated that I^- is more phytotoxic than IO_3^- (Umaly and Poel, 1971; Mackowiak and Grossl, 1999; Zhu et al., 2003). Plants have been hypothesized to reduce IO_3^- to I^- in culture media (Böszörményi and Cseh, 1960; Muramatsu et al., 1983) and subsequently take up I^- from the media (Muramatsu et al., 1983). However, to date, the reduction of IO_3^- and the exact chemical species of iodine taken up by the plant have not been fully established.

In the present study, we investigated any changes in the chemical species of inorganic iodine (I^- and IO_3^-) in buffer solutions caused by rice root to illuminate the detailed mechanism of iodine uptake by higher plants. Furthermore, we examined the role of rice roots in regard to iodine reduction. From these data, we concluded that iodate reduction is a primary physiological response of rice roots to the presence of iodine.

MATERIAL AND METHODS

PLANT MATERIALS

For the test of tolerance to iodine excess, three cultivars of rice were used: *Oryza sativa* L. cv. "Nipponbare," "Koshihikari" and "Gohyakumangoku." Rice seeds were sown to the pots with perlite. Rice plants were cultured with the culture media in a green house under natural light condition. Fourteen days-old rice seedlings were subjected to iodine treatment (1 mmol L^{-1} of IO_3^-) for 17 days. Composition of the culture media (pH 5.5) was 1.5 mmol L^{-1} NH_4NO_3, 1.5 mmol L^{-1} K_2SO_4, 0.25 mmol L^{-1}

$Ca(NO_3)_2$, 0.5 mmol L^{-1} $NH_4H_2PO_4$, 1 mmol L^{-1} $MgSO_4$ and adequate levels of micronutrients. The media was renewed three times a week. At the end of the iodine treatment, shoot length was measured. For the measurement of iodine concentration in shoots of soil-cultured rice, rice seedlings were subjected to iodine treatment (IO_3^- was mixed with the soil at 1 mmol kg $soil^{-1}$) in the green house under natural light condition. After the IO_3^- treatment, shoots were harvested for the determination of iodine concentration.

For the measurement of iodate reduction activity using rice roots without iodine treatment, rice seedlings (cv. "Koshihikari") were water-cultured for 27 days in a growth chamber (28/23°C, 12/12 h) with culture solution (pH 5.5) mentioned above. The media were renewed once a week.

For the measurement of iodate reduction activity using rice roots with iodine treatment, water-cultured 21 days-old seedlings (cv. "Nipponbare" and "Gohyakumangoku") were subjected to iodine treatment for 1 week in a temperature-controlled green house (32/27°C, 12/12 h). I^- treatment was performed at 0, 0.025, 0.25, 2.5, 25 μmol L^{-1}, and IO_3^- treatment was at 0, 0.25, 2.5, 25, 50, 100 μmol L^{-1}. Iodine containing media were renewed twice a week.

For the comparison of iodate reduction activity with barley and soybean roots, water-cultured 10 days-old barley seedlings (*Hordeum vulgare* L. cv. "Mikamogolden") were subjected to iodine treatment in a temperature-controlled (27/22°C, 12/12 h) green house under natural light condition. Water-cultured 15 days-old soybean seedlings (*Glycine max* cv. "Tachinagaha") were subjected to iodine treatment in the temperature-controlled (27/22°C, 12/12 h) green house under natural light condition. Twenty-one days-old rice seedlings (cv. "Gohyakumangoku") were subjected to iodine treatment in the temperature-controlled green house (32/27°C, 12/12 h). I^- treatment was performed at 0, 5, 10, 20, 50 μmol L^{-1}, and IO_3^- treatment was at 0, 50, 100, 200, 500 μmol L^{-1}. Iodine containing media were renewed twice a week after the start of iodine treatment.

DETERMINATION OF IODATE REDUCTION ACTIVITY

Rice roots were rinsed with ion-exchanged water and cut at the basal parts. For the determination of iodate reduction activity using roots without iodine treatment, excised roots were immersed in Tris-HCl buffer (50 mmol L^{-1}, pH 8.0) containing 0.16 μmol L^{-1} (20 μg L^{-1} as I) of I^- or IO_3^-. After 24 h of incubation in the dark at 20°C, the buffer solutions were filtered (0.2 μm) and frozen at −80°C immediately. pH of assay buffer was determined to prevent loss of produced I^- by automatical change 2 $I^- \rightarrow I_2$ at lower pH. The concentrations of I^- and IO_3^- in the buffer solutions were determined by ion chromatography and inductively coupled plasma-mass spectrometry system (IC-ICP-MS) (Yoshida et al., 2007) described as follows.

For the determination of iodate reduction activity using roots subjected to iodine treatment, excised roots were immersed to Tris-HCl (5 mmol L^{-1}, pH 8.0) containing IO_3^- 0.1 mmol L^{-1}, followed by 6 h' incubation at 25°C in the dark. After the incubation, the buffer solution was filtered (0.2 μm) and frozen immediately at −80°C until the measurement. Iodate reduction activity was evaluated by the amount of I^- reduced from IO_3^- by 1 g (FW) of excised roots per hour. I^- concentration in the buffer was determined by 4,4′-methylenebis(*N*,*N*-dimethylaniline)-chloramine T reaction (Yonehara et al., 1991). To evaluate the net I^- concentration reduced from IO_3^- by excised roots, the concentration of I^- in the IO_3^--free buffer incubated with excised roots was also determined as I^- derived from excised roots during incubation, and subtracted from I^- concentration in the IO_3^- buffer incubated with excised roots.

THE SEPARATE DETERMINATION OF CONCENTRATION OF I^- AND IO_3^- IN THE BUFFER SOLUTIONS

The concentrations of I^- and IO_3^- in the buffer solutions were determined by ion chromatography (IC: IC7000S, Column: EXCELPAK ICS-A23, Yokogawa Analytical Systems Inc.) and inductively coupled plasma-mass spectrometry (7500, Agilent) system (IC-ICP-MS) (Yoshida et al., 2007). The detection limit of the system was 8 nmol L^{-1} of I^- and IO_3^-.

DETERMINATION OF TOTAL IODINE CONCENTRATION IN PLANT TISSUE

About 0.1 g of plant samples (shoots and roots) dried at 80°C were digested by 25% tetramethyl ammonium hydroxide (TAMAPURE-AA TMAH, Tama Chemicals Co., Ltd.) in a 6 ml PFA vial (Savillex Co.) overnight at 80°C (Tagami et al., 2006). Concentration of ^{127}I in the solution was determined by ICP-MS (7500, Agilent).

RESULTS

TOLERANCE AMONG THREE RICE CULTIVARS TO HIGH SOLUTION LEVELS OF IODINE

To confirm the tolerance levels of our three rice cultivars, "Nipponbare," "Koshihikari" and "Gohyakumangoku" to high iodine concentrations, we treated each cultivar with 1 mmol L^{-1} IO_3^-. **Figure 1A** shows plants treated with IO_3^- in the pots filled with perlite and culture media. Visible symptoms of iodine toxicity were observed in all three cultivars. The basal part of the stem developed a reddish-brown color. In addition, reddish-brown puncta were observed in the lower leaves. The symptoms were the most visible in "Nipponbare" and the least apparent in "Gohyakumangoku." Chlorosis was also observed in leaves of both "Nipponbare" and "Gohyakumangoku." **Figure 1B** shows the shoot length of the three rice cultivars treated with or without IO_3^- for 17 days. In our IO_3^- treatment, the average shoot lengths of "Nipponbare," "Koshihikari" and "Gohyakumangoku" were 145, 176, and 193 mm, respectively.

Figure 1C illustrates the tolerance of levels among three cultivars grown with IO_3^--mixed soil under flooded conditions. Growth retardation by iodine treatment was most apparent in "Nipponbare." Iodine concentration in shoots of "Nipponbare" was 1.7 and 1.8 times as much as that of "Koshihikari" and "Gohyakumangoku," respectively (**Figure 1D**). The tolerance levels among the three cultivars to excess iodine in I^- treatment were similar to those in IO_3^- treatment (**Figure A1A**). Based on these results, "Nipponbare" was used as our iodine-sensitive cultivar and "Gohyakumangoku" as our iodine-tolerant cultivar in this study.

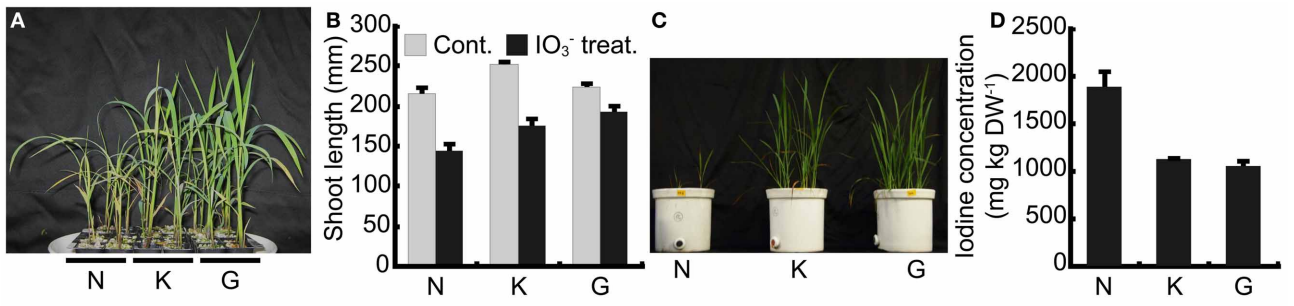

FIGURE 1 | Tolerance among three rice cultivars, "Nipponbare" (N), "Koshihikari" (K), and "Gohyakumangoku" (G) to iodine excess. (A) Rice plants treated with IO_3^-. Rice plants were cultured in pots filled with perlite and Kasugai's nutrient solution (pH 5.5) containing IO_3^-. **(B)** Shoot length of rice plants treated with or without IO_3^-. Cont.: rice plants treated without IO_3^-, IO_3^- treat.: rice plants treated with IO_3^-. Data are mean ± standard error ($n = 6$). **(C)** Shoots of soil-cultured rice cultivars after the iodine treatment. Rice seedlings were grown in 1/5000a Wagner pots with soil mixed with IO_3^-. **(D)** Concentration of iodine in the shoots of soil-cultured rice **(C)** after the IO_3^- treatment. Data are mean ± standard error ($n = 4$).

IODATE REDUCTION BY RICE ROOTS

To investigate any changes in chemical species of iodine caused by rice roots, excised rice roots were immersed in solutions of I^- or IO_3^-. The roots of "Koshihikari" were used because it showed medium-tolerance to iodine excess between "Nipponbare" and "Gohyakumangoku." **Figure 2A** shows the concentrations of I^- or IO_3^- in the solutions of I^- or IO_3^- after a 24-h incubation period with or without root tissue. The concentrations of I^- or IO_3^- were not changed in the solutions of I^- or IO_3^- without roots after the incubation. The concentration of I^- was also unchanged in the I^- solution with roots after the incubation. On the other hand, the concentration of I^- was increased in the IO_3^- solution with roots after the incubation.

A time course of changes in chemical species of iodine was also analyzed in the solutions of IO_3^- with roots (**Figure 2B**). The concentration of IO_3^- in IO_3^- solution was decreased over 24 h. Meanwhile, I^- concentrations in IO_3^- solution were increased over the same time period. This result indicated that the chemical form of iodine was changed between I^- and IO_3^- and suggested that almost all the IO_3^- in the solution were reduced to I^- by rice roots.

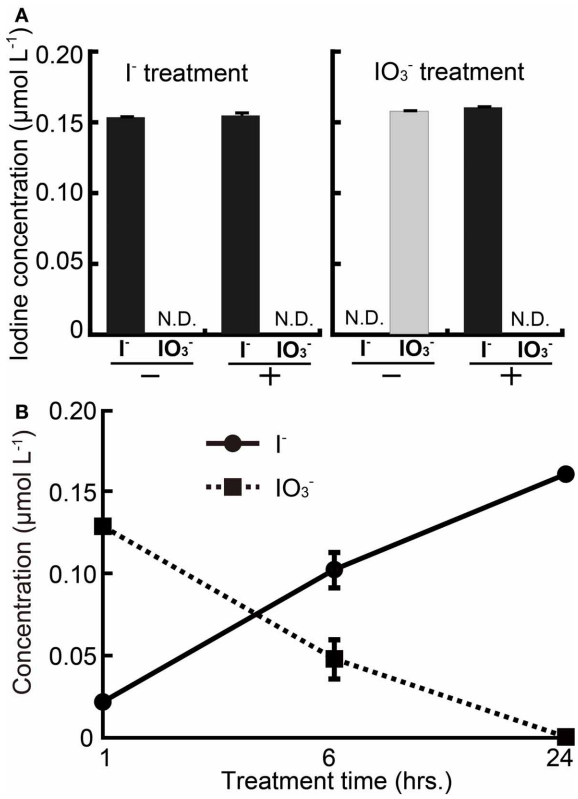

FIGURE 2 | (A) Changes in concentration of I^- or IO_3^- after the 24 h' incubation with or without rice roots. I^- treatment: incubation using the buffer containing I^-, IO_3^- treatment: incubation using the buffer containing IO_3^-, +: incubation with roots, −: incubation without roots. **(B)** Time course analysis of I^- and IO_3^- concentration in IO_3^- solution with excised rice roots. Excised rice roots were immersed to the incubation buffer containing IO_3^- for 1, 6, and 24 h. Data are mean ± standard error ($n = 3$).

EFFECT OF IODINE TREATMENT ON THE GROWTH OF AN IODINE-SENSITIVE AND AN IODINE-TOLERANT CULTIVAR

Figure 3 shows the third leaves of "Nipponbare" and "Gohyakumangoku" 1 week after IO_3^- treatment at increasing iodine concentrations. At the iodine concentration below 25 µmol L^{-1} IO_3^-, visible iodine-toxicity symptoms were not observed in the shoots of "Nipponbare." However, mild chlorosis and reddish-brown puncta were observed in the shoot of "Nipponbare" at 50 µmol L^{-1} IO_3^-, and severe symptoms at 100 µmol L^{-1} IO_3^-. Conversely, visible changes were not observed in the shoots of "Gohyakumangoku" at all concentrations of the IO_3^- treatment.

Table 1 shows the length and fresh weight of both the shoots and roots of "Nipponbare" and "Gohyakumangoku" after IO_3^- treatment. The shoot length of "Nipponbare" was decreased in our IO_3^- treatment in a concentration-dependent manner. The shoot length of "Nipponbare" at 100 µmol L^{-1} IO_3^- was 85% that of the control. Significant changes were not observed in shoot fresh weight, root length, or root fresh weight of "Nipponbare" by IO_3^- treatment. The length and fresh weight of the shoot and root

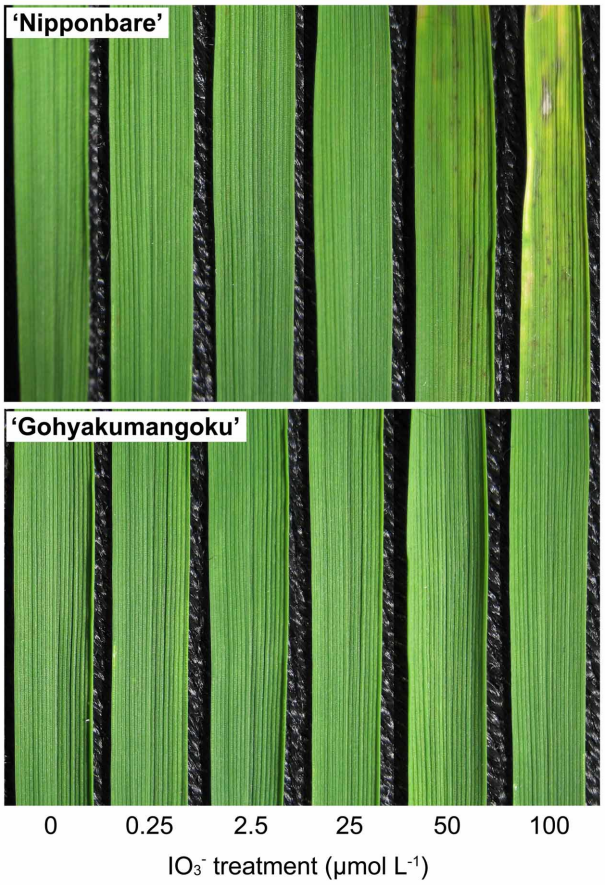

FIGURE 3 | The third leaves of "Nipponbare" and "Gohyakumangoku" after the IO_3^- treatment at varying concentration. Rice seedling were treated with IO_3^- at 0, 0.25, 2.5, 25, 50, 100 μmol L^{-1}.

Table 1 | Effect of the IO_3^- treatment on length and fresh weight of shoots and roots of A "Nipponbare" and B "Gohyakumangoku."

IO_3^- treatment (μmol L^{-1})	Length (mm)		Fresh weight (g)	
	Shoot	Root	Shoot	Root
(A)				
0	$292 \pm 5^{a,b}$	116 ± 8^{a}	$1.00 \pm 0.06^{a,b}$	0.28 ± 0.02^{a}
0.25	$290 \pm 12^{a,b}$	112 ± 4^{a}	$1.06 \pm 0.07^{a,b}$	0.29 ± 0.03^{a}
2.5	301 ± 3^{a}	106 ± 5^{a}	1.09 ± 0.06^{a}	0.29 ± 0.02^{a}
25	$287 \pm 7^{a,b}$	114 ± 2^{a}	$1.04 \pm 0.02^{a,b}$	0.30 ± 0.01^{a}
50	$260 \pm 11^{b,c}$	120 ± 4^{a}	0.84 ± 0.04^{b}	0.25 ± 0.01^{a}
100	249 ± 4^{c}	122 ± 2^{a}	$0.92 \pm 0.05^{a,b}$	0.25 ± 0.01^{a}
(B)				
0	297 ± 8^{a}	142 ± 5^{a}	1.27 ± 0.07^{a}	0.37 ± 0.02^{a}
0.25	297 ± 3^{a}	131 ± 2^{a}	1.21 ± 0.07^{a}	0.32 ± 0.02^{a}
2.5	318 ± 16^{a}	132 ± 5^{a}	1.23 ± 0.03^{a}	0.37 ± 0.01^{a}
25	308 ± 2^{a}	138 ± 4^{a}	1.18 ± 0.04^{a}	0.34 ± 0.01^{a}
50	305 ± 2^{a}	133 ± 5^{a}	1.27 ± 0.07^{a}	0.41 ± 0.03^{a}
100	302 ± 3^{a}	144 ± 4^{a}	1.19 ± 0.03^{a}	0.37 ± 0.02^{a}

Data in the columns are mean ± standard error (n = 5). Values in each column followed by the same letter are not significantly different (Tukey's multiple range test, $P < 0.05$).

of "Gohyakumangoku" were not significantly affected by IO_3^- treatment.

The third leaves of "Nipponbare" and "Gohyakumangoku" plants subjected to varying I^- concentrations for 7 days are shown in **Figure 4**. In the leaves of "Nipponbare," mild and severe iodine-toxicity symptoms appeared in our 2.5 and 25 μmol L^{-1} I^- treatments, respectively. When subjected to 25 μmol L^{-1} of I^- treatment, the leaves of "Gohyakumangoku" plants also exhibited visible iodine-toxicity symptoms.

The growth of "Nipponbare" shoots decreased in the plants grown under I^- treatment. This decrease was also in a concentration-dependent manner (**Table 2A**). In our 2.5 and 25 μmol L^{-1} treatments, we measured a 20% and 26% decrease, respectively, in shoot length compared to the controls. The shoot fresh weight was only 70% and 58% that of the controls at 2.5 and 25 μmol L^{-1} I^-, respectively. In the case of "Nipponbare" root growth, the fresh weight of the roots under 2.5 and 25 μmol L^{-1} I^- treatment decreased to 68 and 58%, respectively, of the control value. Meanwhile, root length did not appear to be significantly affected by I^- treatment. With respect to our tolerant cultivar, we found no significant effects on length and fresh weight by I^- treatment in both shoot and roots (**Table 2B**).

EFFECT OF IODINE TREATMENTS ON IODATE REDUCTION ACTIVITY IN ROOT TISSUE

To investigate the effect of iodine in rhizosphere on iodate reduction activity in root tissue, iodate reduction activity was measured after treatment with two kinds of species of iodine, IO_3^- and I^-, at varying concentrations.

Figure 5A shows the iodate reduction activity of roots of "Nipponbare" and "Gohyakumangoku" treated with IO_3^- for 7 days at increasing concentrations. Iodate reduction by the roots was higher in "Nipponbare" than in "Gohyakumangoku" at all concentrations tested. Iodate reduction increased in "Nipponbare" treated with IO_3^-. Iodate reduction increased in a concentration-dependent manner, even at concentrations resulting in the appearance of symptoms indicating iodine toxicity. Iodate reduction activity increased up to 2.8-fold at 100 μmol L^{-1} IO_3^-. On the other hand, root iodate reduction activity did not increase at concentrations higher than 2.5 μmol L^{-1} IO_3^- in the "Gohyakumangoku" cultivar. However, at 2.5 μmol L^{-1} IO_3^-, reduction activity increased 1.6-fold.

Iodine concentrations in both the shoots and roots of "Nipponbare" and "Gohyakumangoku" after IO_3^- treatment are shown in **Figures 5B**, **C**, respectively. The concentrations of iodine in the shoots and roots of both cultivars increased with IO_3^- treatment in a concentration-dependent manner. The difference in the shoot iodine concentrations between the two cultivars was magnified at the highest iodine treatment. The shoot iodine concentrations were higher in the shoots of "Gohyakumangoku."

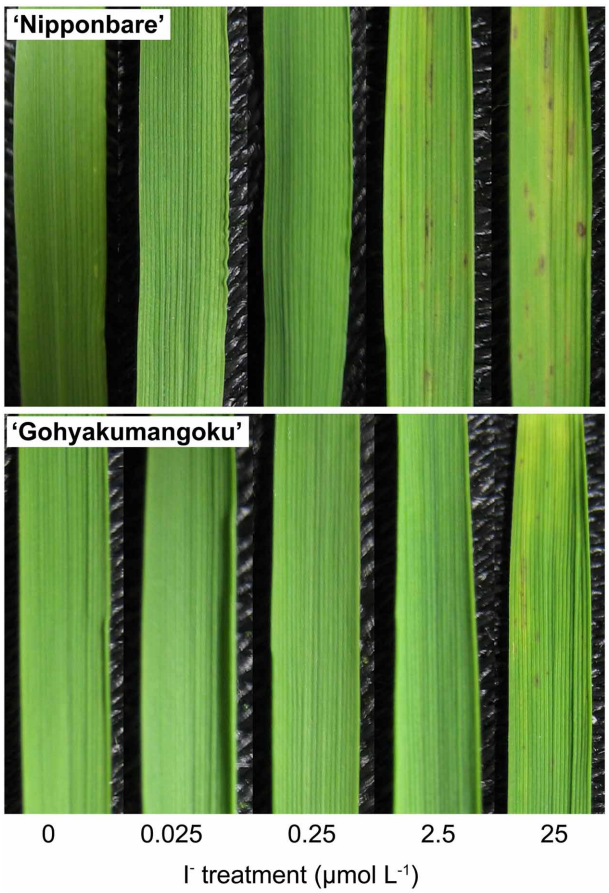

FIGURE 4 | The third leaves of "Nipponbare" and "Gohyakumangoku" after the I^- treatment at varying concentration. Rice seedlings were treated with I^- at 0, 0.025, 0.25, 2.5, 25 μmol L^{-1}.

Table 2 | Effect of the I^- treatment on length and fresh weight of shoots and roots of A "Nipponbare" and B " Gohyakumangoku."

I^- treatment (μmol L^{-1})	Length (mm)		Fresh weight (g)	
	Shoot	Root	Shoot	Root
(A)				
0	261 ± 12^{a}	137 ± 8^{a}	0.89 ± 0.04^{a}	0.40 ± 0.02^{a}
0.025	255 ± 7^{a}	145 ± 14^{a}	0.87 ± 0.03^{a}	$0.37 \pm 0.01^{a,b}$
0.25	$235 \pm 4^{a,b}$	135 ± 11^{a}	0.83 ± 0.05^{a}	0.33 ± 0.01^{b}
2.5	$209 \pm 3^{b,c}$	112 ± 6^{a}	0.62 ± 0.02^{b}	0.27 ± 0.01^{c}
25	193 ± 3^{c}	131 ± 7^{a}	0.52 ± 0.03^{b}	0.23 ± 0.01^{c}
(B)				
0	219 ± 5^{a}	$110 \pm 2^{a,b}$	$0.70 \pm 0.02^{a–c}$	0.32 ± 0.01^{a}
0.025	223 ± 4^{a}	$111 \pm 3^{a,b}$	$0.72 \pm 0.02^{a,b}$	0.31 ± 0.00^{a}
0.25	239 ± 9^{a}	$108 \pm 3^{a,b}$	0.76 ± 0.02^{a}	0.33 ± 0.01^{a}
2.5	230 ± 6^{a}	124 ± 6^{a}	$0.66 \pm 0.02^{b,c}$	0.30 ± 0.00^{a}
25	237 ± 5^{a}	105 ± 3^{b}	0.62 ± 0.03^{c}	0.30 ± 0.01^{a}

Data in the columns are mean ± standard error (n = 6). Values in each column followed by the same letter are not significantly different (Tukey's multiple range test, $P < 0.05$).

Unexpectedly, we found no significant difference in iodine concentration in the roots of our two cultivars.

Root iodate reduction activities of rice treated with I^- are shown in **Figure 6A**. Root iodate reduction was higher in "Nipponbare" at all concentrations tested. Even at concentrations resulting in iodine-toxicity symptoms, we found that root iodate reduction increased twofold in "Nipponbare." Our tolerant cultivar, "Gohyakumangoku" also demonstrated an increase in reduction activity due to I^- treatment in a concentration-dependent manner. However, reduction activity decreased at treatment levels resulting in toxicity symptoms.

Figures 6B, **C** shows the total iodine concentrations in both the shoot and roots of rice plants after 7 days of I^- treatment. The concentrations of iodine in shoots and roots of both cultivars increased in a concentration-dependent manner with I^- treatment. Also, we found that the iodine levels were higher in the shoots and lower in the roots of "Gohyakumangoku" than those of "Nipponbare." The difference in the concentration levels in these tissues between the two cultivars increased in a concentration-dependent manner with I^- treatment. At 25 μmol L^{-1} I^-, the iodine concentrations in the shoots of "Gohyakumangoku" was 1.8 times greater than that of "Nipponbare."

Figure 7 shows the iodate reduction activity of roots of "Nipponbare" and "Gohyakumangoku" treated with 0 and 100 μmol L^{-1} of IO_3^- for 7 days. Iodate reduction activity of roots was increased with age, and induced apparently by 7 days' IO_3^- treatment.

IODATE REDUCTION ACTIVITY IN ROOT TISSUE OF BARLEY AND SOYBEAN

To compare these phenomena of rice with those of upland crops, iodate reduction activity of roots was investigated in barley and soybean. Barley and soybean showed also iodate reduction activity (**Figures 8A**, **9A**). In IO_3^- treatment, iodate reduction activity was the highest in rice among these three plant species (**Figure 8A**). Iodine concentrations in the shoots and roots of rice were higher than those of barley and soybean (**Figure 8B**). In I^- treatment, the iodate reduction activity and iodine concentrations in the shoots and roots were the highest in barley, and the lowest in soybean (**Figure 9**).

DISCUSSION

Rice plants often display iodine-toxicity symptoms, known as *reclamation Akagare disease* or Akagare type III, when grown in upland fields that have been converted to lowland fields in some volcanic ash soils (Baba et al., 1964; Tensho, 1970; Watanabe and Tensho, 1970). In soil solutions, the major chemical species of soluble iodine are generally thought to be I^- under flooded conditions (Muramatsu et al., 1989; Yuita, 1992) and IO_3^- under non-flooded conditions (Yuita, 1992). At similar concentrations, I^- appears to be more phytotoxic than IO_3^- (Umaly and Poel, 1971; Mackowiak and Grossl, 1999; Zhu et al., 2003; Weng et al.,

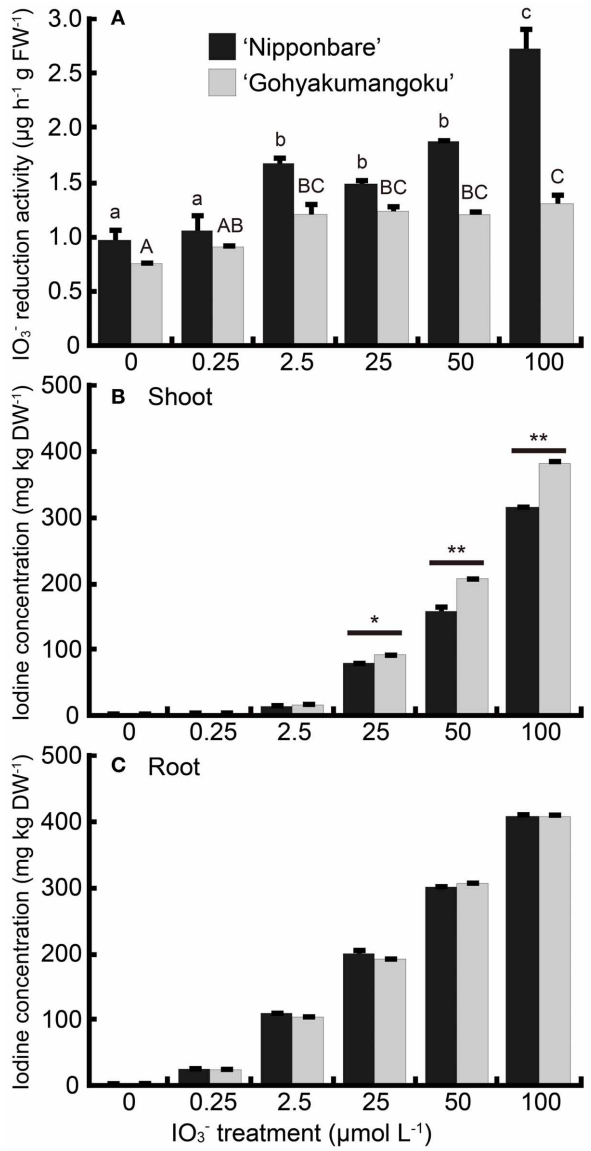

FIGURE 5 | (A) Effect of the IO_3^- treatment on iodate reduction activity of excised rice roots. After the IO_3^- treatment at 0, 0.25, 2.5, 25, 50, 100 μmol L^{-1}, iodate reduction activity (the amount of I^- reduced from IO_3^- by 1 g (FW) of excised roots per hour) was measured. Data are mean ± standard error ($n = 3$). Bars with the same letter are not significantly different among each iodine treatments in each cultivar (Tukey's multiple range test, $P < 0.05$). **(B, C)** Concentration of total iodine in **(B)** shoots and **(C)** roots of "Nipponbare" and "Gohyakumangoku" after the IO_3^- treatment. Data are mean ± standard error ($n = 3$). $^*P < 0.05$; $^{**}P < 0.01$, *t*-test.

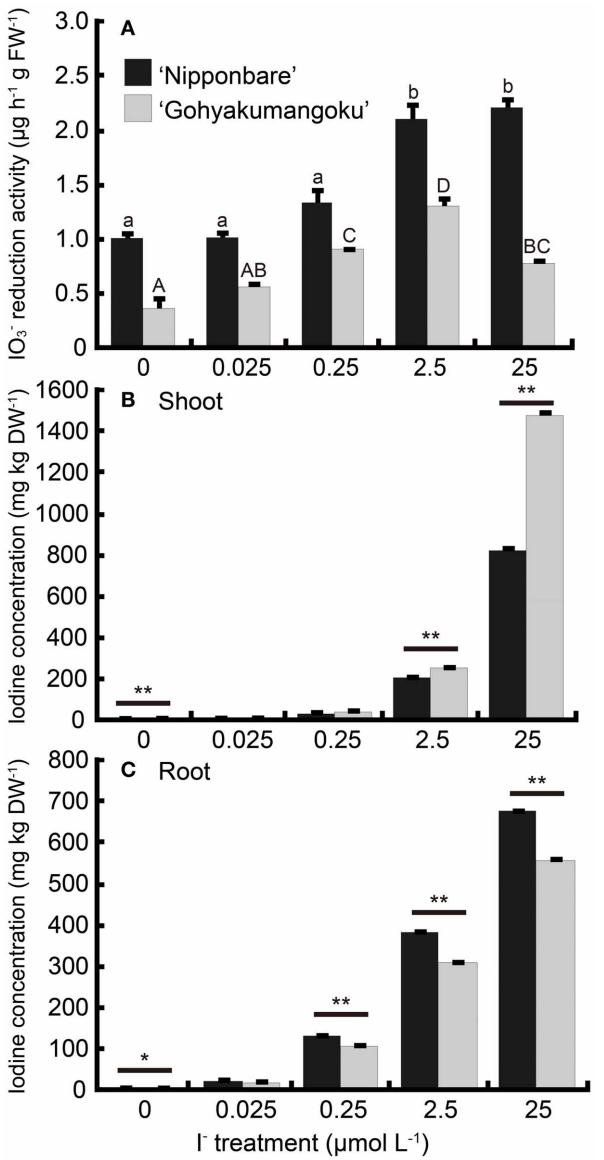

FIGURE 6 | (A) Effect of the I^- treatment on iodate reduction activity of excised rice roots. After the I^- treatment at 0, 0.025, 0.25, 2.5, 25 μmol L^{-1}, iodate reduction activity was measured. Data are mean ± standard error ($n = 3$). Bars with the same letter are not significantly different among each iodine treatments in each cultivar (Tukey's multiple range test, $P < 0.05$). **(B, C)** Concentration of total iodine in **(B)** shoots and **(C)** roots of "Nipponbare" and "Gohyakumangoku" after the I^- treatment. Data are mean ± standard error ($n = 3$). $^*P < 0.05$; $^{**}P < 0.01$, *t*-test.

2008a). Böszörményi and Cseh (1960) suggested that IO_3^- is reduced to I^- electrochemically before uptake by wheat roots. Muramatsu et al. (1983) reported that the Komatsuna plant, *Brassica rapa* var. *pervidis*, can accelerate the conversion of IO_3^- to I^- in culture solution. Together, these reports indicate that in culture media, plants can reduce IO_3^- to I^- and would take up I^-. Recently, Yamada et al. (2005) further proposed that rice roots might even take up I_2 oxidized from I^-.

To date, however, whether plant roots physiologically function to change the chemical species of iodine in the rhizosphere remains unclear. To address this question, we performed a set of experiments aimed at investigating the role, if any, of rice roots in changing the chemical species of iodine. Additionally, we examined any changes occurring in the iodate reduction activity of roots by the presence of external iodine.

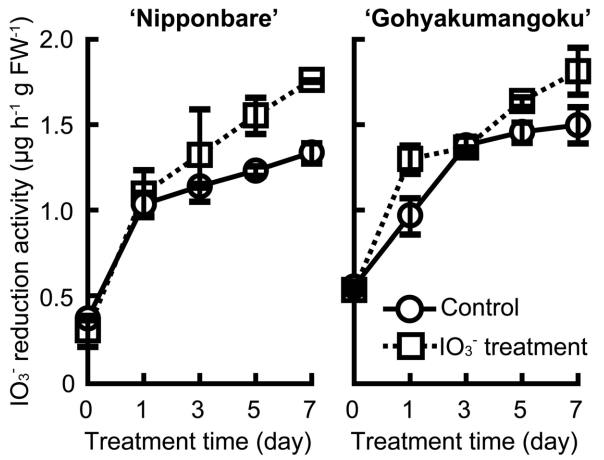

FIGURE 7 | Effect of the IO_3^- treatment time on iodate reduction activity of excised rice roots. Rice seedlings (cv. "Nipponbare" and "Gohyakumangoku") were subjected to 0 and 100 μmol L^{-1} of IO_3^- for 0, 1, 3, 5, and 7 days. After the IO_3^- treatment, iodate reduction activity was measured. Data are mean ± range ($n = 2$) Control: iodate reduction activity of rice roots treated without IO_3^-, IO_3^- treatment: iodate reduction activity of rice roots treated with IO_3^-.

DIFFERENCES IN IODINE TOLERANCE IN RICE

To confirm iodine tolerance among rice cultivars, we selected three rice cultivars and cultured them under the excess condition of two forms of iodine, i.e., iodide (I^-) and iodate (IO_3^-). Our results clearly demonstrate the degree of tolerance to excess iodine (I^- and IO_3^-) among these cultivars: "Nipponbare" < "Koshihikari" < "Gohyakumangoku" (**Figures 1**, **A1**). These results are in agreement with those reported by Yamada et al. (2006). However, it was considered that the tolerance levels among three rice cultivars to excess iodine had no obvious relation to iodine concentration in shoots as described below.

IODATE REDUCTION BY ROOT TISSUE

To elucidate whether rice roots convert IO_3^- or I^- to some other chemical species of iodine, concentrations of IO_3^- or I^- were determined in iodine solutions incubated with the excised roots of "Koshihikari." Almost all of IO_3^- added to the buffer was reduced to I^- by the excised roots during a 24-h incubation period. However, the concentration of I^- was unchanged in the I^- solution with roots after the incubation (**Figure 2A**). In addition, our time-course analysis indicated that iodate reduction occurred over the course of several hours (**Figure 2B**). Collectively, these results indicate that rice roots have the ability

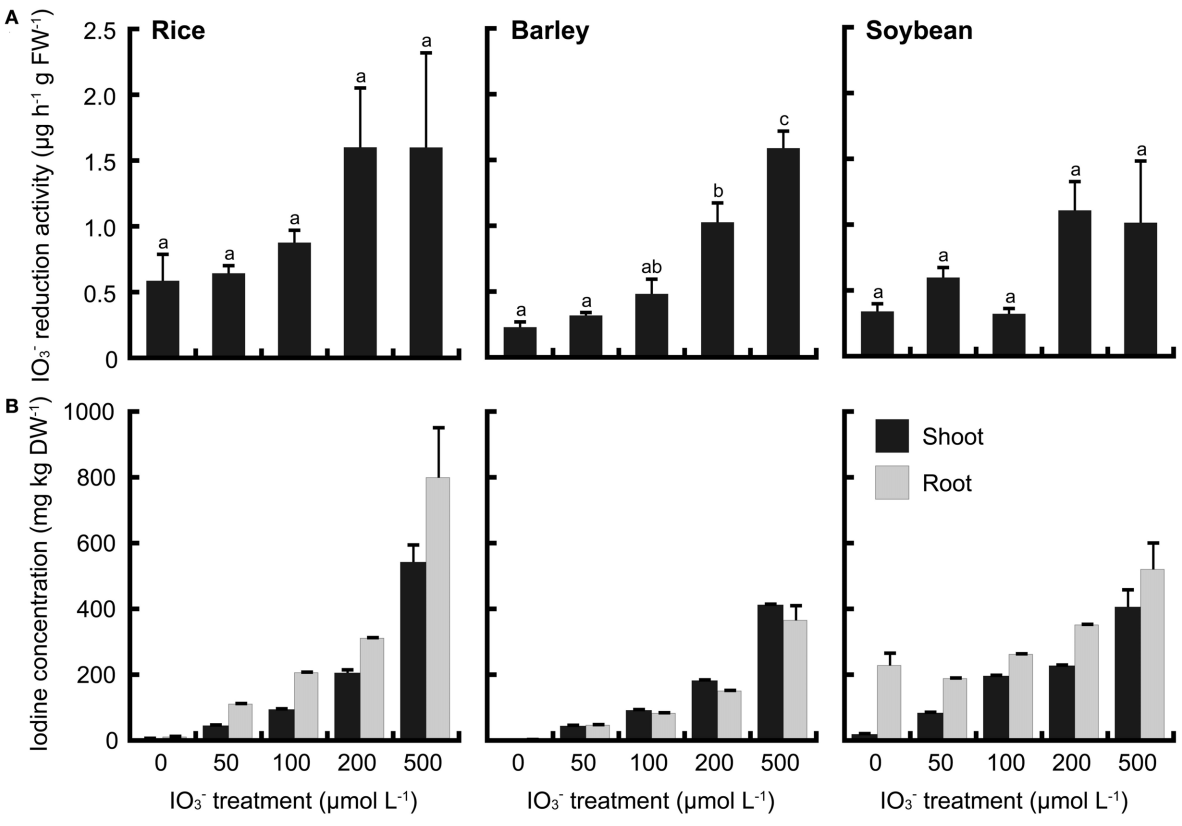

FIGURE 8 | (A) Effect of the IO_3^- treatment on iodate reduction activity of excised roots in rice, barley and soybean. After the IO_3^- treatment at 0, 50, 100, 200, and 500 μmol L^{-1}, iodate reduction activity was measured. Data are mean ± standard error ($n = 3$). Bars with the same letter are not significantly different among each iodine treatments (Tukey's multiple range test, $P < 0.05$). **(B)** Concentration of total iodine in shoots and roots of rice, barley and soybean after the IO_3^- treatment. Data are mean ± standard error ($n = 3$).

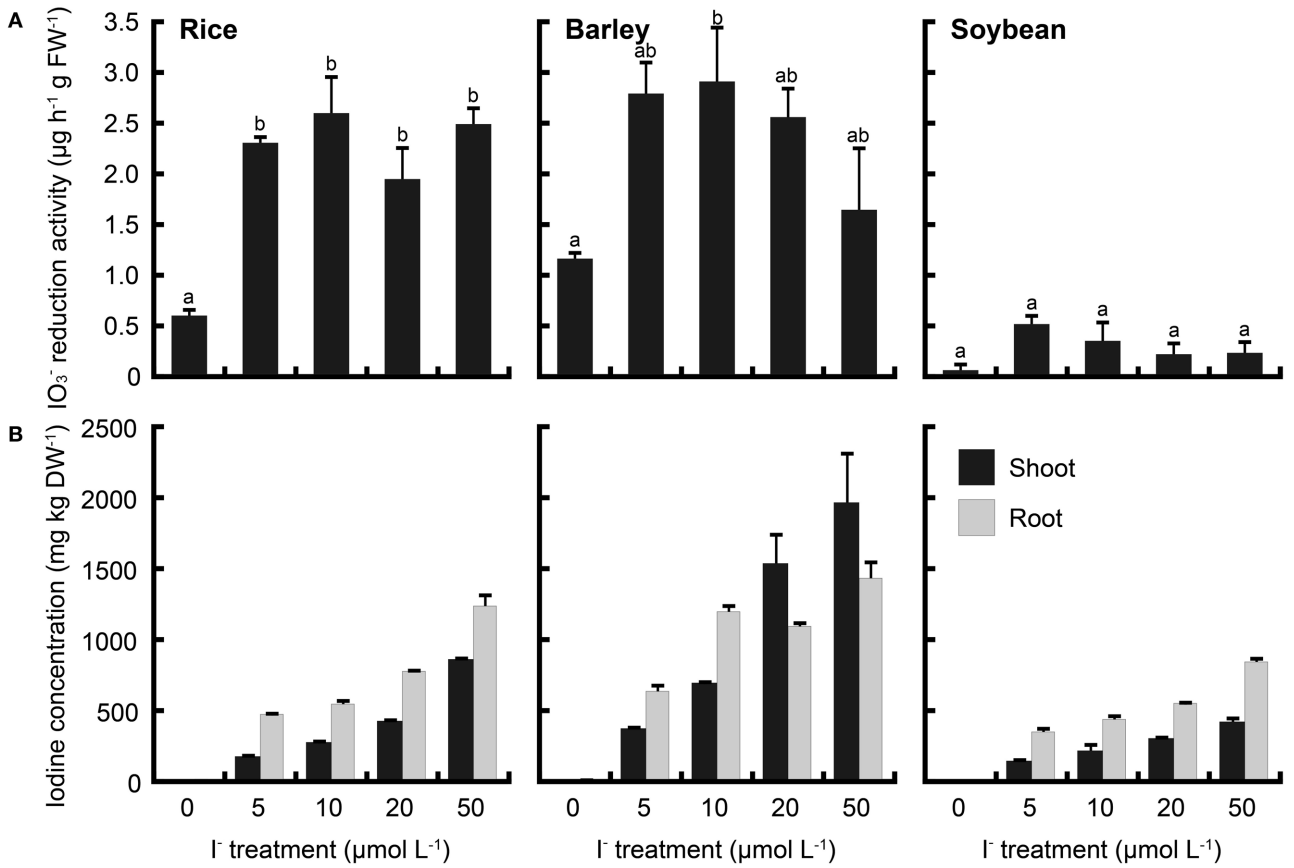

FIGURE 9 | (A) Effect of the I^- treatment on iodate reduction activity of excised roots in rice, barley and soybean. After the I^- treatment at 0, 5, 10, 20, and 50 μmol L^{-1}, iodate reduction activity was measured. Data are mean ± standard error (rice and soybean; $n = 3$, barley; $n = 6$). Bars with the same letter are not significantly different among each iodine treatments (Tukey's multiple range test, $P < 0.05$). **(B)** Concentration of total iodine in shoots and roots of rice, barley and soybean after the I^- treatment. Data are mean ± standard error ($n = 3$).

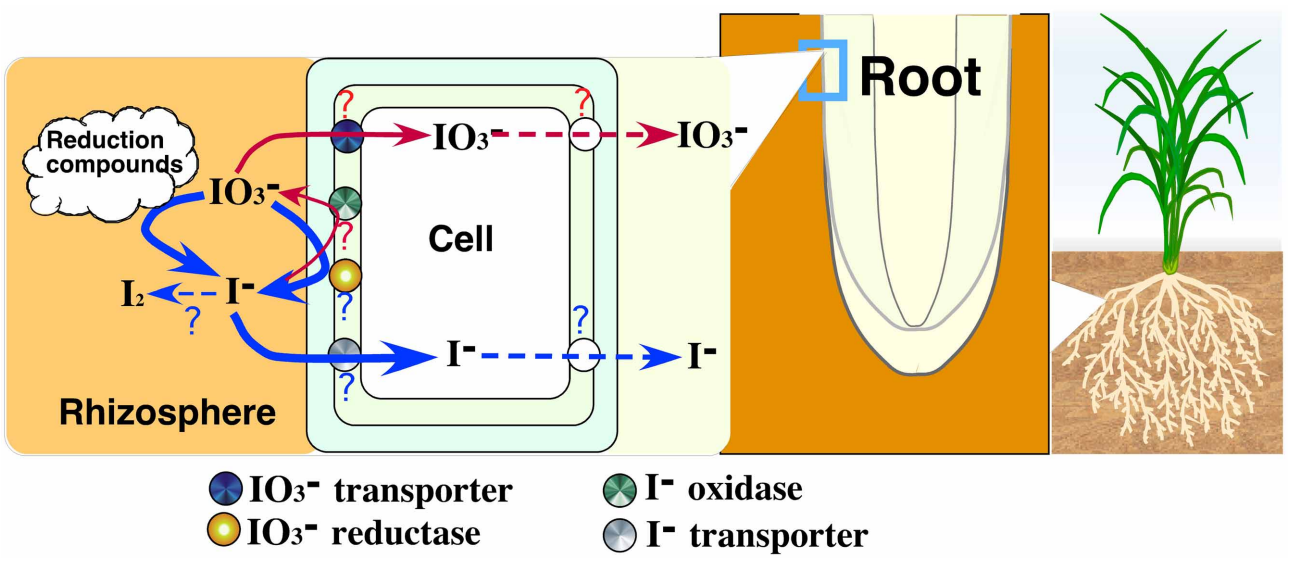

FIGURE 10 | The model diagram showing our hypothesis of uptake of iodine by higher plants.

to reduce IO_3^- to I^-. Earlier, a report stated that when grown at similar iodine levels, I^--treated plants had a higher iodine content than IO_3^--treated plants (Muramatsu et al., 1983; Mackowiak and Grossl, 1999; Zhu et al., 2003; Weng et al., 2008a; Voogt et al., 2010). Our results suggest that plants would take up I^- reduced from IO_3^-.

EFFECT OF IODINE EXCESS ON RICE SHOOTS AND ROOTS

From our observations, the concentration of iodine in our I^- treatment that caused adverse effects on growth of both the sensitive and tolerant cultivars was much lower than that of our IO_3^- treatment (**Tables 1**, **2**). This result is consistent with previous reports in the literature (Umaly and Poel, 1971; Mackowiak and Grossl, 1999; Zhu et al., 2003; Weng et al., 2008a). Plant height decreased with high iodine treatment in both the soil culture and water culture (**Figures 1A–C**, **A1A**, **Tables 1**, **2**). On the other hand, root length was not significantly affected, although root fresh weight decreased under the high iodine treatment, especially I^- excess. This may in part have been due to the age of the treated plants. Also, the emergence of new roots appeared to be suppressed at high iodine levels in the growth solutions. Decrease in root fresh weight is related to the inhibition of elongation of new roots. High iodine levels would also inhibit the elongation of roots as with any other excessive nutrient condition.

IODINE CONCENTRATION IN PLANT BODY AND TOLERANCE TO IODINE EXCESS

Under the water-cultured condition, iodine concentration in the shoots of "Gohyakumangoku" (tolerant) was higher than that of "Nipponbare" (sensitive) in both the IO_3^- and I^- treatments (**Figures 5B**, **6B**, **A1B**). On the other hand, under the soil-cultured condition, iodine concentration in the shoots of "Gohyakumangoku" (tolerant) was lower than that of "Nipponbare" (sensitive) in the IO_3^- treatments (**Figure 1D**). However, "Gohyakumangoku" showed higher tolerance to excess iodine than "Nipponbare" regardless of iodine concentration in shoots under both conditions. Yamada et al. (2006) also reported that iodine concentration in shoots of "Gohyakumangoku" was much lower than that of "Nipponbare" under soil culture. The discrepancy between these results was considered due to the different culture condition employed, namely soil-cultured (**Figure 1D**) or water-cultured (**Figure 5B**). Therefore, it is considered that the tolerance levels among three rice cultivars to excess iodine had no obvious relation to iodine concentration in shoots. In our study, higher iodine concentration in "Gohyakumangoku" (tolerant) shoots suggests that the form or localization of iodine within the plant body is more important than the total concentration of iodine. The higher tolerance of "Gohyakumangoku" to iodine stress might correlate with the species of iodine transported or stored within the plant body (e.g., vacuole and intercellular space).

INDUCTION OF IODATE REDUCTION ACTIVITY IN RESPONSE TO EXTERNAL IODINE

Iodate reduction activity displayed by "Nipponbare" and "Gohyakumangoku" roots increased with either IO_3^- or I^- treatment. In "Gohyakumangoku," iodate reduction activity decreased at concentrations of iodine that resulted in visible iodine-toxicity symptoms (**Figures 5A**, **6A**).

The induction of iodate reduction by I^- treatment was an unexpected phenomenon since our original hypothesis predicted that plants would take up I^- reduced from IO_3^-. The mechanism of this induction is still unclear. Unidentified oxidation of I^- might be related to this induction since the oxidation activity of roots detected by ∂-naphtylamine was also increased by iodine treatment (data not shown).

The decrease in iodate reduction under high iodine conditions seems to correlate with iodine tolerance. Because lower iodate reduction activity will be helpful under excess iodine condition since high iodate reduction activity can contribute to uptake of I^-, more toxic form of iodine. Lower concentration of iodine in roots of "Gohyakumangoku" might be related to lower iodate reduction activity in I^- treatment. Further detailed studies are needed to fully clarify these responses.

However, our results suggest that iodate reduction in rice roots is related, at least in part, to external iodine conditions. The induction of iodate reduction activity in IO_3^- treatment was suppressed by removing iodine from culture media (data not shown). We also demonstrated that barley and soybean roots converted IO_3^- to I^-, and that iodate reduction activity of barley and soybean roots increased with both IO_3^- and I^- treatment (**Figures 8**, **9**).

THE MECHANISM OF UPTAKE AND TRANSPORT OF IODINE IN PLANTS

This is the first report demonstrating that plant roots have an ability to reduce IO_3^- to I^- and that iodate reduction activity in roots could respond to external iodine. We show our hypothesis of uptake of iodine by roots in **Figure 10**. Conversion of IO_3^- to I^- by the excised roots suggests the existence of iodate reductase in roots. Iodate would be reduced by iodate reduction compounds released from the roots treated with iodine (data not shown). Under excess iodine condition, it is considered that I^- is mainly absorbed by roots due to high iodate reduction activity of iodate reductase. The existence of iodate reductase induced by iodine would be suggested by the induction of iodate reduction activity of crude proteins extracted from rice roots treated with iodine (data not shown). Iodide oxidase might also exist and be related to the iodate reduction in I^- treatment. The uptake of iodine could be regulated by transporters in plasma membrane. Transport of iodine from roots to shoots could be also regulated by transporters. In barley treated with higher I^-, iodine concentration in shoots reversed that of roots. In IO_3^- treatment, iodine concentration in shoots was higher than that of roots only in barley. These results suggest the existence of iodine transporters. The major chemical form of soluble iodine in soil solutions is I^- under flooded conditions (Muramatsu et al., 1989; Yuita, 1992) and IO_3^- under non-flooded conditions (Yuita, 1992). Soybean and barley showed the difference in the iodate reduction activity and iodine concentrations in plant body between in I^- and IO_3^- treatment. This might suggest the difference in the mechanism of uptake and transport of iodine between paddy-rice and upland crops.

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APPENDIX

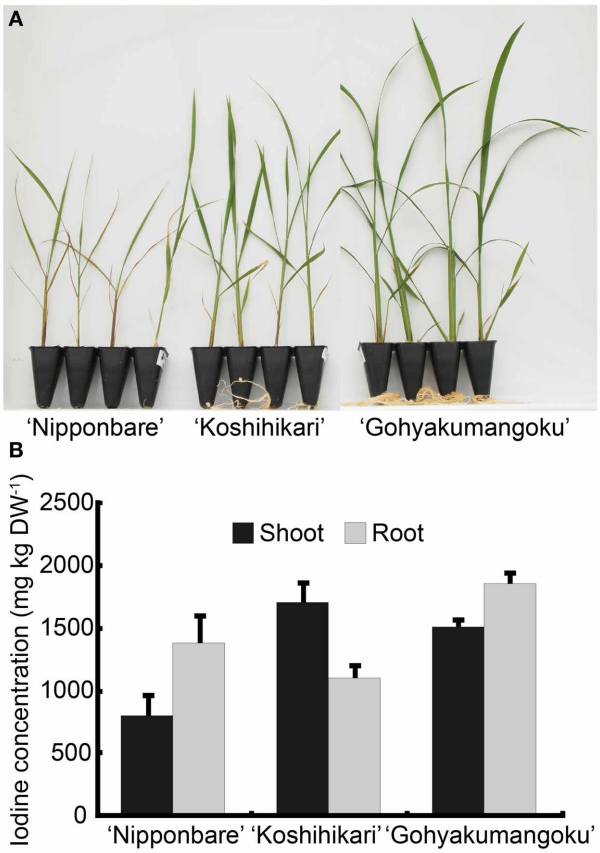

FIGURE A1 | (A) Rice plants of three rice cultivars (cv. "Nipponbare," "Koshihikari," and "Gohyakumangoku") after the I^- treatment in the pots filled with perlite. Seedlings were cultured with Kasugai's nutrient solution (pH 5.5) in the green house under natural light condition. Twelve days-old rice seedings were subjected to iodine treatment (I^- 100 μmol L^{-1}) for 21 days. **(B)** Concentration of iodine in shoots and roots of rice ("Nipponbare," "Koshihikari," and "Gohyakumangoku") after the I^- treatment. Data are mean ± standard error ($n = 4$).

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Tomato fruits: a good target for iodine biofortification

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Iodine is a trace element that is fundamental for human health: its deficiency affects about two billion people worldwide. Fruits and vegetables are usually poor sources of iodine; however, plants can accumulate iodine if it is either present or exogenously administered to the soil. The biofortification of crops with iodine has therefore been proposed as a strategy for improving human nutrition. A greenhouse pot experiment was carried out to evaluate the possibility of biofortifying tomato fruits with iodine. Increasing concentrations of iodine supplied as KI or KIO_3 were administered to plants as root treatments and the iodine accumulation in fruits was measured. The influences of the soil organic matter content or the nitrate level in the nutritive solution were analyzed. Finally, yield and qualitative properties of the biofortified tomatoes were considered, as well as the possible influence of fruit storage and processing on the iodine content. Results showed that the use of both the iodized salts induced a significant increase in the fruit's iodine content in doses that did not affect plant growth and development. The final levels ranged from a few mg up to 10 mg iodine kg^{-1} fruit fresh weight and are more than adequate for a biofortification program, since 150 μg iodine per day is the recommended dietary allowance for adults. In general, the iodine treatments scarcely affected fruit appearance and quality, even with the highest concentrations applied. In contrast, the use of KI in plants fertilized with low doses of nitrate induced moderate phytotoxicity symptoms. Organic matter-rich soils improved the plant's health and production, with only mild reductions in iodine stored in the fruits. Finally, a short period of storage at room temperature or a 30-min boiling treatment did not reduce the iodine content in the fruits, if the peel was maintained. All these results suggest that tomato is a particularly suitable crop for iodine biofortification programs.

Keywords: biofortification, iodine, iodine deficiency, potassium iodate, potassium iodide, *Solanum lycopersicum* L., tomato

INTRODUCTION

The health and well-being of a population are significantly influenced by their nutritional status. A healthy and well-balanced diet, with a variety of high-quality foods ensuring the right proportions of different types of nutrients, is important both in the prevention and in the treatment of several diseases. Not only do daily calorie requirements need to be carefully met, but also the consumption of a number of specific elements, the lack of which may promote or lead to serious pathologies, needs to be guaranteed in order to prevent nutritional deficiencies.

Iodine (I) is a trace element used in the synthesis of thyroid hormones (Arthur and Beckett, 1999). It is naturally present in fish, eggs, meat, dairy products, and, to a lesser extent, in grains, fruits, and vegetables. For an adult the recommended daily allowance (RDA) for iodine is 150 μg (Institute of Medicine, Food and Nutrition Board, 2001), a very minute quantity. Nevertheless, its deficiency is one of the most serious public health issues worldwide and nearly one-third of the human population still has an insufficient iodine intake (Andersson et al., 2012). This is due to the fact that iodine deficiency is largely related to the environment. In many regions of the world, mountainous areas and flood plains in particular, soils contain very low amounts of iodine, which negatively affects the iodine content of crops, thus increasing the risk of iodine deficiency among people who consume foods primarily produced there.

Inadequate iodine intake impairs the thyroid function, with the onset of a wide spectrum of disorders negatively affecting growth and development at various levels. All age groups can be susceptible, and in cases of severe deficiency, damage to the fetus, perinatal and infant mortalities, endemic goitre, irreversible mental retardation and brain damage can occur (Delange, 2000; Zimmermann et al., 2008). Such problems are widespread in all the world's least industrialized nations, with South Asia and sub-Saharan Africa particularly affected (Zimmermann, 2009). However, even in developed countries some groups of people remain at risk, especially children and pregnant women, resulting in minor cognitive and neuropsychological deficits (Haddow et al., 1999).

The main strategy for controlling and preventing iodine deficiency is the universal fortification of salt with iodine (Andersson et al., 2010). "Universal" is the key word in this strategy because it highlights that all the salt consumed by the population should be iodized, including salt used in food processing and for animal feed. This strategy has been implemented by many countries over the past few decades and has dramatically

reduced the prevalence of iodine deficiency worldwide (Zimmermann, 2009; Andersson et al., 2010). However, a boost to the consumption of iodized salt is becoming increasingly untenable, as it conflicts with other important public health objectives, such as the prevention of cardiovascular diseases. Other strategies have been adopted, including the addition of iodine to oils, bakery products, or even to drinking water, but none of these alternatives has proved effective by itself as a means of prevention.

The biofortification of edible crops, based on the production of micronutrient-rich plants destined for human consumption, is a more recent alternative approach to controlling mineral malnutrition, especially in poor countries (Nestel et al., 2006). Biofortified crops may contain higher amounts of specific micronutrients due to their improved ability to take up and accumulate them or through a lower content of antinutrient compounds. These crops can be obtained by selecting superior genotypes through the use of traditional breeding or modern biotechnology. In alternative, improved agronomic approaches can be developed and applied (White and Broadley, 2009).

Although necessary for humans and animals, the importance of iodine for higher plants and a possible role in their metabolism have not yet been demonstrated. Usually fruits and vegetables are poor sources of iodine, although with large variations due to the differences in the iodine content of soils. However, several studies indicate that plants can accumulate iodine, and there is generally a positive correlation between applications to the soil and the final accumulation in plants (Zhu et al., 2003; Dai et al., 2004; Blasco et al., 2008; Weng et al., 2008a). The iodine biofortification of crops might thus be a cost-effective strategy for increasing iodine levels in plant-derived food, and thus improve human nutrition.

Several methods of iodine plant enrichment have been proposed, but none of these can be considered as optimal and each species requires a careful and specific evaluation. Although the positive results obtained in trials carried out with some leafy vegetables (e.g., spinach, lettuce), particularly in hydroponic culture, have suggested that they are good candidates for iodine biofortification programs (Zhu et al., 2003; Dai et al., 2004; Blasco et al., 2008; Hong et al., 2008; Weng et al., 2008b; Voogt et al., 2010), the fortification of other kinds of cultivated plants appears more difficult. Cereals, in particular, seem to be less suitable for such approaches, due to the scarce iodine accumulation levels in the grains (Mackowiak and Grossl, 1999), which in turn may be due to an insufficient phloematic route for iodine and/or a high volatilization rate of iodine from the plant to the atmosphere (Redeker et al., 2000; Landini et al., 2012).

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown and commercially important vegetable crops, with a worldwide cultivation covering more than four million hectares (FAOSTAT, 2011). It is cultivated as an annual crop in open fields and under greenhouse conditions for both fresh consumption and industrial processing. The nutraceutical properties of tomato are well-known and are mainly related to the antioxidant potential of its fruits, due to the presence of a mix of bio-molecules such as lycopene, ascorbic acid, polyphenols, potassium, folate, and α-tocopherol (Basu and Imrhan, 2007).

Recent studies have proposed tomato as a possible candidate for iodine biofortification programs (Landini et al., 2011). Both its widespread distribution and possible consumption as a fresh fruit make it a good target crop for a fortification study. Indeed, positive results in terms of effective iodine accumulation within the fruits, representing the edible part of the plant, have been achieved (Landini et al., 2011).

In the present study an iodine biofortification approach was attempted using a commercial variety of tomato grown in potting soil in a greenhouse. Various agronomic aspects that may or may not influence the availability of iodine for plant uptake were analyzed, for example the iodine source and dose, the type of soil, and the concentration of other nutrients. The final effects were also analyzed in terms of quantitative yield and qualitative properties of the biofortified tomatoes, as well as the possible influence of fruit storage and processing.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

The tomato variety SUN7705 (Nunhems, Parma, ID 83660, USA) was used in all the experiments. Seeds were sown in soil (Hawita Flor, Vechta, Germany) in plastic plugs and in a growth chamber under the following conditions: 25°C temperature, 55% relative humidity, 80 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation). From germination to transplanting, plants were watered once a week with a nutritive solution, whose composition was the same as that used in the pot cultivation (see later in this paragraph). About 40 days after germination, tomato plants were transplanted to 24 cm diameter plastic pots (volume = 8 dm^3) containing a mixture of soil and pumice (70:30, by volume), and transferred to a glass greenhouse. Pumice was used in order to facilitate water drainage. The main characteristics of the soil were: clay 8.4%, silt 32.0%, sand 59.6%; C/N 8.5; organic matter 1.31%; and electrical conductivity 0.44 mS cm^{-1}. Throughout the trial, day/night temperatures ranged from 25 to 31°C, and from 15 to 21°C, respectively. The composition of the nutritive solution, supplied to plants for 1 min three times per day, was: (in mM) N-NO_3 11; N-NH_4 0.5; P 1.2; K 7; Ca 4; Mg 0.94; Na 10; Cl 9.5; S-SO_4 2.16; and (in μM) Fe 45; B 23; Cu 1; Zn 5; Mn 10; Mo 1; EC 2.79 mS cm^{-1}, and pH between 5.7 and 6.0. The moderate sodium and chloride content was due to the use of slightly saline irrigation water.

For pest management, a foliar application of copper was performed before transplanting to prevent tomato blight. Confidor (Bayer, Germany) was applied as a foliar application against aphids and white flies, once a week from transplanting to flowering. In addition, a systemic fungicide (Ridomil Gold® EC, Novartis, NY, USA) was applied to the soil every 10 days from transplant to harvest.

Different experimental trials were performed, as later described. In all the trials, iodine treatment administrations were carried out, supplying iodine to pots as KI or KIO_3, dissolved in a volume of 200 ml water per plant. KI or KIO_3 concentrations ranged from 0 to 10 mM, depending on the type of experiment. Treatment applications were carried out weekly, starting from the development of the first branch of fruits.

EXPERIMENT 1: EFFECT OF IODINE DOSE AND FORM ON IODINE UPTAKE AND ACCUMULATION

Tomato plants were grown in soil in a glass greenhouse, fertirrigated with a nutritive solution, as above described. Starting with the set of the first truss of fruits, plants were root-treated with KI or KIO_3 once a week. Eight iodine administrations were performed.

Following a preliminary trial, with a very wide iodine dose–response curve (KI and KIO_3 concentrations ranging from 0 to 60 mM), performed to find out the most suitable doses of iodine without phytotoxicity symptoms, KI was supplied in concentrations of 1, 2, and 5 mM, while KIO_3 in concentrations of 0.5, 1, and 2 mM. Ten replicates for each experimental condition were carried out.

After the first four iodine administrations (total effective iodine supplied per plant: 0, 50.76, 101.52, 203.04, 507.6, and 1,015.2 mg I, corresponding, respectively, to 0, 0.5, 1, 2, 5 and 10 mM I applied as KI or KIO_3), the iodine content was measured in fruits from both the first and the second trusses. Other four iodine treatments were then carried out and the iodine content was measured in fruits collected from the first truss and used for the qualitative analyses.

EXPERIMENT 2: EFFECT OF SOIL ORGANIC MATTER ON IODINE UPTAKE AND ACCUMULATION

Plants were grown in pots in a glass greenhouse, fertirrigated with a nutritive solution, as above described, and divided into two groups, according to the organic matter content of the soil mixture used. Two different soil mixtures, characterized by a low and a high organic matter content, respectively, were used. The composition of the soil mixture with the low organic matter content (approximately 1% on a weight base) was the same described above (soil:pumice, 70:30 by volume). The soil mixture with the high organic matter content was obtained by mixing soil, commercial peat (Hawita Flor) and pumice (28:41:30, by volume), considering the main characteristics of the different substrates, that were, respectively: organic matter content: 1.31, 40, and 0% on a dry matter basis; apparent density (kg/L): 1.5, 0.5, and 0.85 on a dry matter basis; dry matter content: 90, 72, and 90%. In the mixture soil enriched with peat the final organic matter content was approximately 10% (determined on a weight base).

Starting from the development of the first branch of fruits, four weekly administrations of 10 mM KI or KIO_3 (total effective iodine supplied per plant: 1,015.2 mg I) were performed in both the two groups of plants. Control plants, untreated with iodine, were also grown in the two types of soils. Ten replicates for each experimental condition were carried out.

Fruits were collected from the first fruit cluster at the end of the iodine treatments and analyzed for the iodine content. At the end of the trial, some plant growth parameters (fruit and shoot dry weight, fruit yield) were measured.

EXPERIMENT 3: EFFECT OF THE NITRATE LEVEL OF THE NUTRITIVE SOLUTION ON IODINE UPTAKE AND ACCUMULATION

Plants were grown in soil in a glass greenhouse, as above described, and divided into three different groups, according to the nitrate level of the nutritive solution. Three different nutritive solutions, containing, respectively, a low (2 mM), medium (10 mM), and high (20 mM) nitrate content, were used. The medium nitrate nutritive solution had the following composition: (in mM) $N\text{-}NO_3$ 10; P 1.2; K 8; Ca 6; Mg 1; Na 10; Cl 9.5; $S\text{-}SO_4$ 4.97; and (in μM) Fe 56; B 23; Cu 1; Zn 5; Mn 11; Mo 1; EC 3.32 mS cm^{-1}, and pH between 5.7 and 6.0. The low nitrate nutritive solution contained 2 mM $N\text{-}NO_3$, while the high nitrate one 20 mM $N\text{-}NO_3$. Furthermore, some adjustments were made to the low and high nitrate solutions to maintain comparable macronutrient levels. The low nitrate nutritive solution contained additional 7 mmol l^{-1} chloride, while in the high nitrate solution the sulfate content was reduced to 0.8 mM.

Starting from the development of the first branch of fruits, four weekly administrations of 10 mM KI or KIO_3 (total effective iodine supplied per plant: 1,015.2 mg I) were performed in all the three groups of plants. Control plants, untreated with iodine, were also grown with each of the three different nutritive solutions. Ten replicates for each experimental condition were carried out.

Fruits were collected from the first fruit cluster at the end of the iodine treatments and analyzed for the iodine content. At the end of the trial, some plant growth parameters (fruit and shoot dry weight, fruit yield) were measured.

EXPERIMENT 4: EFFECT OF SHELF-LIFE AND COOKING ON THE IODINE ACCUMULATED IN TOMATO FRUITS

For this experiment, fruits collected from plants of the Experiment 1, treated for 4 weeks with 5 mM KI (total effective iodine supplied per plant: 507.6 mg I) were used. Both turning red and red fruits were chosen.

The shelf-life experiment was performed by storing the turning red fruits under light at room temperature without any treatment for the following 2 weeks after harvest. The analyses of the iodine content on the stored fruits were carried out 1 and 2 weeks after harvest.

The cooking experiment was performed by boiling red tomato fruits for 30 min in deionized water. Processed fruits were divided into two groups, and boiled, with or without the external peel, respectively.

EXPERIMENT 5: EFFECT OF IODINE ON FRUIT QUALITY

Fruits collected from plants of the Experiment 1 treated for 8 weeks with KI or KIO_3 were used. Both quantitative measures (fruit yield) and qualitative analyses (color, sugar content, total antioxidant power) were carried out.

QUALITATIVE AND QUANTITATIVE ANALYSES OF FRUITS

For the analysis of the iodine content, fruits were harvested waiting at least 1 week after the last iodine treatment. Iodine as I was analyzed by inductively coupled plasma mass Spectrometry (ICP-MS), as previously described (Landini et al., 2011).

For the evaluation of dry weight (DW), fruits and shoots were weighed separately immediately after harvest and then dried in a ventilated oven at 80°C. All the fruits collected from plants at the end of the experiments were weighed for the analysis of fruit yields. Experiments were not continued after the collection of fruits from the first two branches. The calculated yields therefore always refer to the fruits collected from these two trusses, already developed, and those still growing in the third truss.

In order to analyse sugar content, whole fresh fruits were homogenized in a blender. An aliquot of the homogenate was then centrifuged twice for 10 min at 5,000 rpm and some drops of the supernatant were used to determine total soluble solids with a Refractometer (RL3 PZO). The content of sugars was expressed as degrees Brix (°Brix).

The total antioxidant power of fruits was evaluated using the "ferric-reducing/antioxidant power" (FRAP) assay (Benzie and Strain, 1996). Immediately after harvest, each fruit was homogenized in a blender (0.5 g of the flesh extracted in 5 ml of pure methanol) and stored overnight at −20°C. Samples were then centrifuged for 8 min at 5.000 rpm and 100 μl of the supernatant were added to 900 μl of freshly prepared FRAP reagent [1 mM TPTZ + 2 mM $FeCl_3$] and 2 ml of acetate buffer. Absorbance readings at 593 nm were taken after a reaction time of 4 min. The reagents used were: acetate buffer (0.25 M sodium acetate, pH 3.6), TPTZ (2,4,6-tripyridyl-2-triazine 0.01 M in methanol) and $FeCl_3$ (0.01 M in sodium acetate). Standard solutions of known Fe^{2+} concentration (0–50-200-500-1000 μM) were prepared by adding $(NH_4)2Fe(SO_4)_2{\cdot}6\ H_2O$ to the acetate buffer, and were used for the calibration.

STATISTICAL ANALYSIS OF DATA

The experimental design adopted in the Experiments 1, 2, and 3 was completely randomized. The treatments (iodine source and organic matter in the Experiment 2; iodine source and nitrate level in the Experiment 3, respectively) were in factorial combination. Data were subjected to one-way and two-way analysis of variance (ANOVA; Statgraphics Centurion XV program), as described in the Figure legends, and the means were separated using the *F*-test (95% confidence level).

RESULTS

EXPERIMENT 1: EFFECT OF IODINE DOSE AND FORM ON IODINE UPTAKE AND ACCUMULATION

As a starting point, tomato plants, grown with the experimental set-up previously described (**Figure 1**), were root-treated with KI or KIO_3 concentrations ranging from 0 to 60 mM. Although clear damage was never observed in the fruits, plants started to show phytotoxicity symptoms (leaf chlorosis, epinasty, visible wilting) at iodine salt concentrations higher than 10 mM. Moreover, in the presence of 40–60 mM KI or KIO_3, plant development and biomass accumulation were severely compromised, with undeniable consequences on the development of the fruits (data not shown).

These preliminary results prompted us to focus on a narrower and lower range of iodine concentrations, to limit any phytotoxicity symptoms on the plants. KI was thus supplied in concentrations of 1, 2, and 5 mM, while KIO_3 was used at lower concentrations, namely 0.5, 1, and 2 mM, since this salt showed greater phytotoxicity in the preliminary trial. In this experimental set-up, the plants were healthy at the end of the experiment (**Figure 2A**), with the exception of those treated with the highest KI concentration (5 mM) which showed some discoloration and necrotic areas, limited to the basal leaves (**Figure 2B**).

The trial was interrupted when the third truss of fruits was developing and the iodine content was measured in fruits from both the first and the second trusses. **Figures 2C,D** show the iodine content detected in fruits collected from the second branch at the mature green stage. A very regular trend in the increase in fruit iodine content with the increase in its soil administration can be observed. After four treatments with 1, 2, and 5 mM KI, fruits contained an average of 1.5, 4.7, and 10 mg I kg^{-1} fresh weight (FW), respectively (**Figure 2C**). A similar trend can be observed in fruits from the plants treated with potassium iodate, with fruits accumulating 0.3, 0.7, and 1.2 mg I kg^{-1} FW following four applications of 0.5, 1, and 2 mM KIO_3, respectively (**Figure 2D**). Comparable results were obtained in fruits collected from the first truss (data not shown). In the analyses performed, the untreated control fruits showed a small amount of iodine (approximately 0.06 mg I kg^{-1} FW) due to the trace amounts of this element present in both the irrigation water (0.109 mg I l^{-1}) and the soil used (0.084 mg I kg^{-1}); (**Figures 2C,D**).

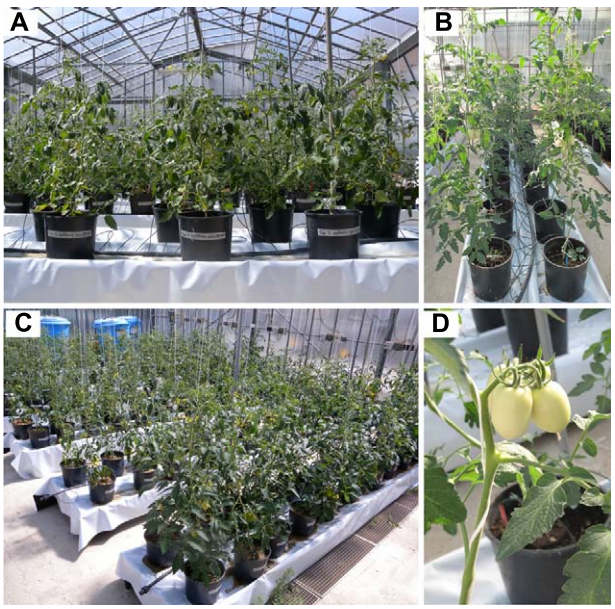

FIGURE 1 | Set-up of tomato plant greenhouse cultivation. Plants were grown in pots **(A)** and organized in rows **(B)**, fertilized with a nutritive solution **(C)**. Iodine treatments started with the set of the first truss of fruits **(D)**.

EXPERIMENT 2: EFFECT OF THE SOIL ORGANIC MATTER ON IODINE UPTAKE AND ACCUMULATION

In this second experiment, plants, grown into low or high organic matter soils, were treated with 10 mM KI or KIO_3, a concentration of iodine higher than those used in the previous trial, chosen to better quantify the possible negative effects of the organic matter on the iodine uptake. Over the 4 weeks of treatments, mild phytotoxicity symptoms appeared on the plants, depending on the form of iodine administered as well as the soil organic matter content. The most affected plants were those grown in the lower organic matter soil and treated with KI. Leaves of this group of plants presented some discolorations and necrotic areas (**Figure 3A**). Similar phytotoxic effects, though less severe, were observed on

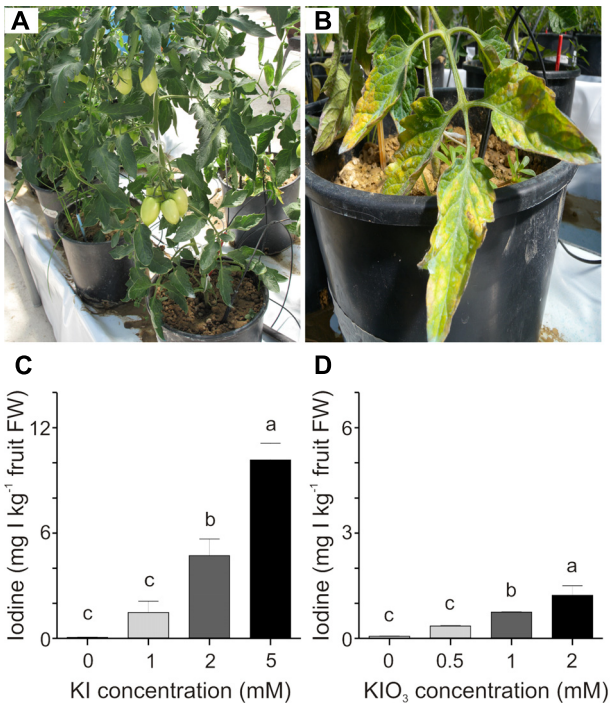

FIGURE 2 | Effect of iodine dose and form on iodine uptake and accumulation. Healthy iodine-treated plants at the end of the experiment **(A)**. 5 mM KI-treated plants showed some phytotoxicity symptoms on the basal leaves **(B)**. Iodine uptake in fruits collected from the second truss of **(C)** KI- and **(D)** KIO_3-treated plants, after four weekly iodine applications. Data were subjected to one-way analysis of variance (ANOVA) and the means were separated using the *F*-test (95% confidence level).

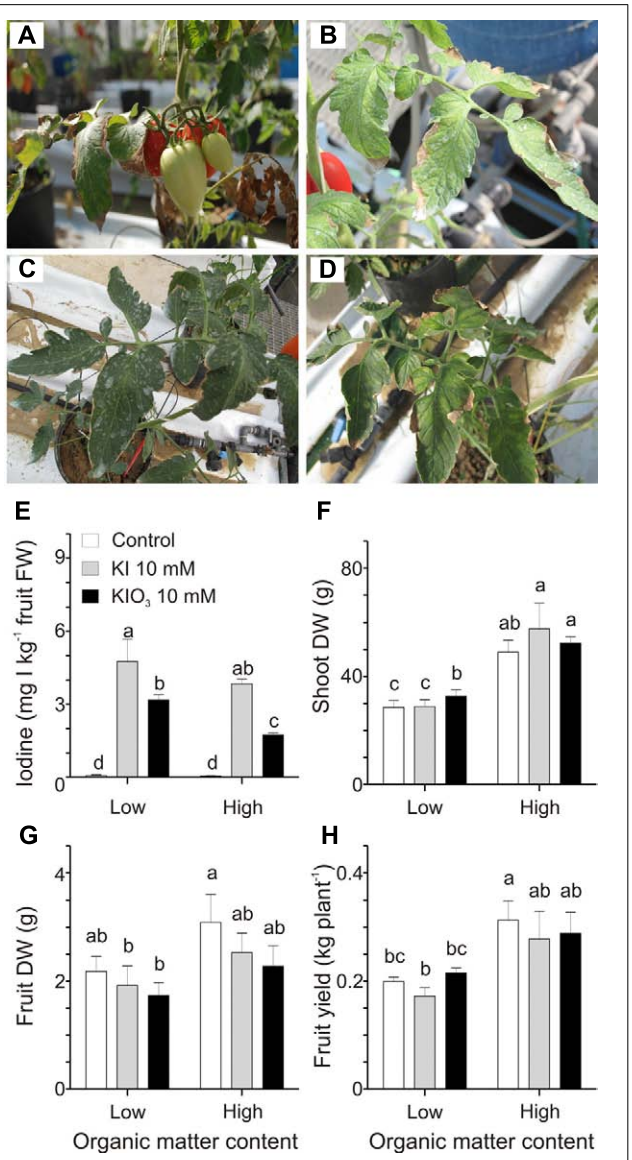

FIGURE 3 | Effect of the soil organic matter on iodine uptake and accumulation. Details of leaves and fruits from plants grown in a soil with 1% organic matter treated with 10 mM KI **(A)** or 10 mM KIO_3 **(B)**, and from plants grown in soil with 10% organic matter treated with 10 mM KI **(C)** or 10 mM KIO_3 **(D)**. Iodine levels in fruits **(E)**, shoot dry weight (DW) **(F)**, fruit DW **(G)**, and fruit yield **(H)** measured in plants grown in low and high organic matter soils and with or without a 10 mM KI or 10 mM KIO_3 treatment. Data were subjected to one-way and two-way analysis of variance (ANOVA) and the means were separated using the *F*-test (95.0% confidence level). Significance of two-way analysis of variance (**P*-value ≤ 0.05; ****P*-value ≤ 0.001; n.s. = not significant): **(E)** organic matter content (a): *; iodine treatment (b): ***; a x b: n.s.. **(F)** organic matter content (a): ***; iodine treatment (b): n.s.; a x b: n.s.. **(G)** organic matter content (a): *; iodine treatment (b): n.s.; a x b: n.s.. **(H)** organic matter content (a): ***; iodine treatment (b): n.s.; a x b: n.s..

plants treated with KIO_3 (**Figure 3B**). In the high organic matter content soil all the plants appeared healthier (**Figures 3C,D**).

Fruits were collected from the first fruit cluster and analyzed for the iodine content (**Figure 3E**). The results obtained show that the increase in the organic matter reduced the iodine accumulation in KIO_3- but not in KI-treated plants.

Some plant growth parameters were analyzed in order to better quantify the effects of the different types of soil in combination with the iodine treatments. Plants grown in organic matter-rich soils showed a strong increase in the dry-matter production of their vegetative organs (**Figure 3F**), which was, on average, 1.5-fold higher than that quantified in the low organic matter soil. This effect was observed irrespectively of the iodine treatment performed. No significant effects were detected in fruit dry weight (**Figure 3G**), while plant yield was positively affected by the organic matter, as, on average, plants grown in the organic matter-enriched soil showed a fruit production 1.5-fold higher than those grown in the organic matter poor soil, but, again, this was observed irrespectively of the iodine treatment performed (**Figure 3H**).

EXPERIMENT 3: EFFECT OF THE NITRATE LEVEL OF THE NUTRITIVE SOLUTION ON IODINE UPTAKE AND ACCUMULATION

The possible interaction between iodine and nitrate contained in the nutritive solution in terms of iodine availability and uptake by tomato plants was examined. Three different nitrate doses (2, 10, and 20 mM) were used to fertilize plants, which were also treated with 10 mM KI or KIO_3. Strong phytotoxicity symptoms on plants treated with KI and grown at the minimal nitrate level

(2 mM) were observed (**Figure 4B**). These plants were strongly reduced in size and biomass production in comparison with plants fertilized with 2 mM nitrate but not treated with KI (**Figure 4A**), and, at the end of the trial, their basal leaves were completely burnt (**Figure 4B**). Leaves of the upper branches still showed chlorosis, necrotic areas, curling of the edges and a reduction in size (**Figures 4B,C**), whereas fruit appearance did not seem to be affected (**Figure 4D**). Similar phytotoxic effects, though less severe, were observed in plants grown at 2 mM nitrate dose and treated with KIO_3 (data not shown). On the other hand, iodine-treated plants fertilized with 10 and 20 mM nitrate did not show significant alterations in their growth, apart from some chlorotic and necrotic areas on the basal leaves of plants treated with KI. Control plants, not treated with iodine, also showed a slight chlorosis when fertigated with 2 mM nitrate (**Figure 4A**). The final amount of iodine in fruits collected from plants treated with the same iodine salt and increasing doses of nitrate was comparable (**Figure 4E**). Only fruits from 10 mM KIO_3-treated plants fertilized with 20 mM nitrate showed a small but significant reduction in the iodine content (**Figure 4E**).

Plant dry weight and yield were measured. A significant reduction in shoot DW was observed only in plants treated with 2 mM nitrate and 10 mM KI (**Figure 4F**), as a likely consequence of the strong iodine phytotoxicity under these conditions (**Figure 4B**). As far as fruit DW is concerned, no significant differences were detected in iodine-treated plants, whereas in control plants, not treated with iodine, a small trend toward a slight increase can be observed comparing, respectively, the 10 and 20 mM nitrate levels (**Figure 4G**). Finally the level of nitrate fertilization did not significantly affect the fruit yield in control plants, whereas the KI treatment reduced fruit yield in all the plants and in particular in those grown at the lowest nitrate concentration (**Figure 4H**), probably due to the phytotoxic effects described above. On the contrary, fruit yield in KIO_3-treated plants slightly increased with the increase in the nitrate level (**Figure 4H**).

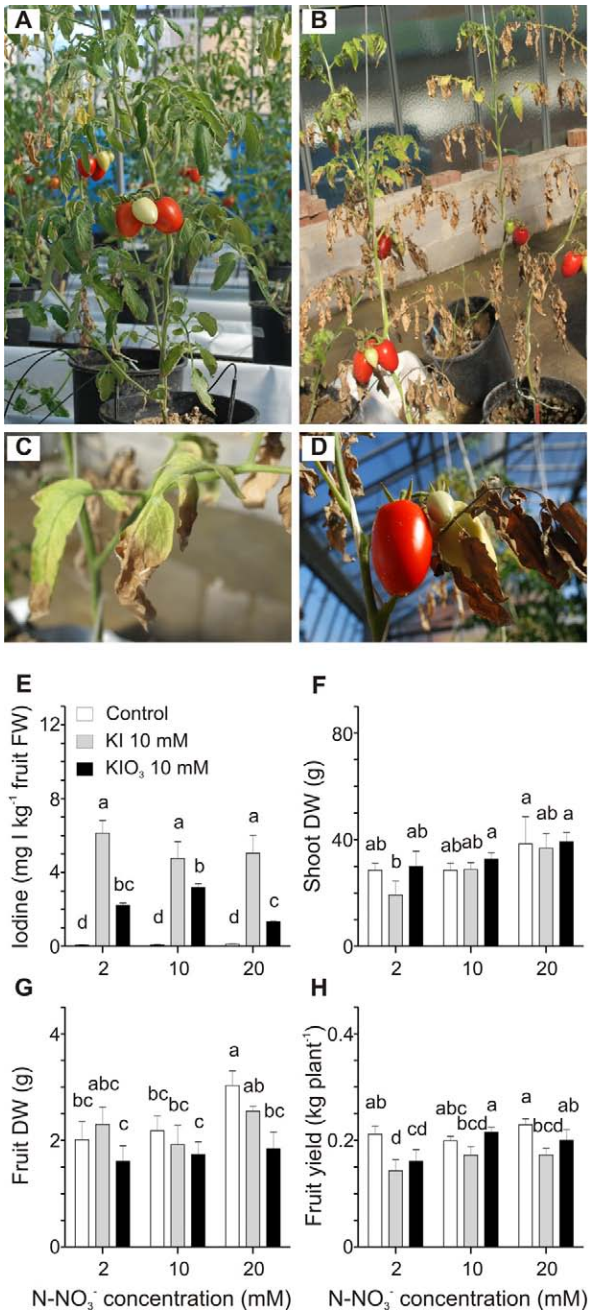

FIGURE 4 | Effect of the nitrate level of the nutritive solution on iodine uptake and accumulation. Plants fertilized with 2 mM nitrate without iodine treatments **(A)**, or treated with 10 mM KI **(B)** are shown. Details of leaves from the upper branches **(C)** and fruits **(D)** from plants fertilized with 2 mM nitrate and treated with 10 mM KI. Iodine levels in fruits **(E)**, shoot dry weight (DW) **(F)**, fruit DW **(G)**, and fruit yield **(H)** measured in plants fertilized with 2, 10 or 20 mM nitrate level and with or without 10 mM KI or 10 mM KIO_3 treatments. Data were subjected to one-way and two-way analysis of variance (ANOVA) and the means were separated using the *F*-test (95.0% confidence level). Significance of two-way analysis of variance (**P*-value $\leq$ 0.05; ****P*-value $\leq$ 0.001; n.s. = not significant): **(E)** nitrate concentration (a): n.s.; iodine treatment (b): ***; a x b: n.s.. **(F)** nitrate concentration (a): *; iodine treatment (*b*): n.s.; a x b: n.s.. **(G)** nitrate concentration (a): n.s.; iodine treatment (b): *; a x b: n.s.. **(H)** nitrate concentration (a): n.s.; iodine treatment (b): *; a x b: n.s..

EXPERIMENT 4: EFFECT OF SHELF-LIFE AND COOKING ON THE IODINE ACCUMULATED IN TOMATO FRUITS

To evaluate the possible effect of storage on the level of iodine accumulated in tomatoes, fruits were collected from 5mM KI-treated plants at the breaker stage (**Figure 5A**). The shelf-life experiment was performed by storing the fruits under light at room temperature without any further treatment for the following 2 weeks, during which fruit ripening continued. The iodine content remained constant in the fruits over time (**Figure 5C**), showing that 2 weeks of storage did not alter their value of biofortified fruits.

To evaluate the possibility of transforming the iodine-enriched tomatoes into processed food, a cooking experiment was performed by boiling red ripened fruits (**Figure 5B**) for 30 min. Both raw and processed fruits were divided into two groups, maintaining or removing the external peel. Iodine was finally measured in intact and peeled fruits and also in the fruit skin. Boiling did not alter the amount of iodine present in fruits, and, irrespectively of the treatment, the content of iodine in fruits without peel was lower than that measured in the same intact

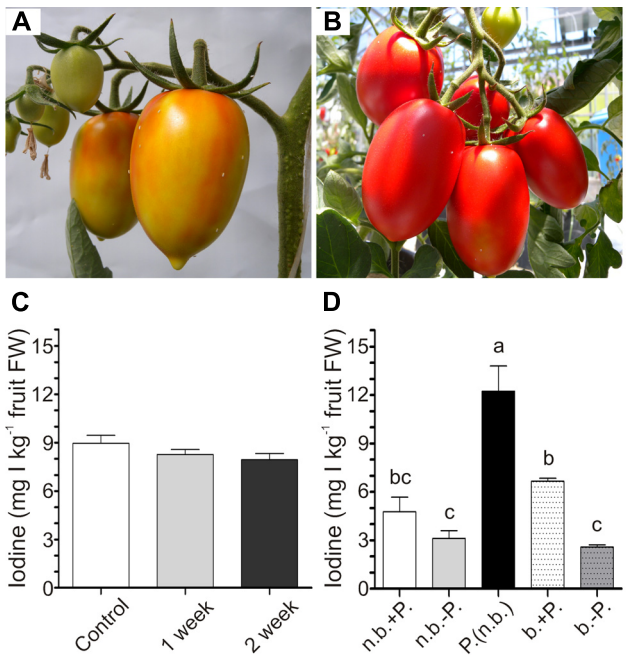

FIGURE 5 | Effects of shelf-life and cooking on iodine content of fruits. Fruits turning red at harvest for the shelf-life experiment **(A)**. Red fruits at harvest for the boiling experiment **(B)**. Iodine content in 5 mM KI-treated fruits at harvest, and after one or 2 weeks of storage at room temperature **(C)**. Iodine content in 5 mM KI-treated fruits at harvest not boiled (n.b.) or boiled (b.) with (+P.) or without (−P.) peel, or in fruit skin not boiled [P. (n.b.)] **(D)**. Data were subjected to one-way analysis of variance (ANOVA) and the means were separated using the *F*-test (95% confidence level).

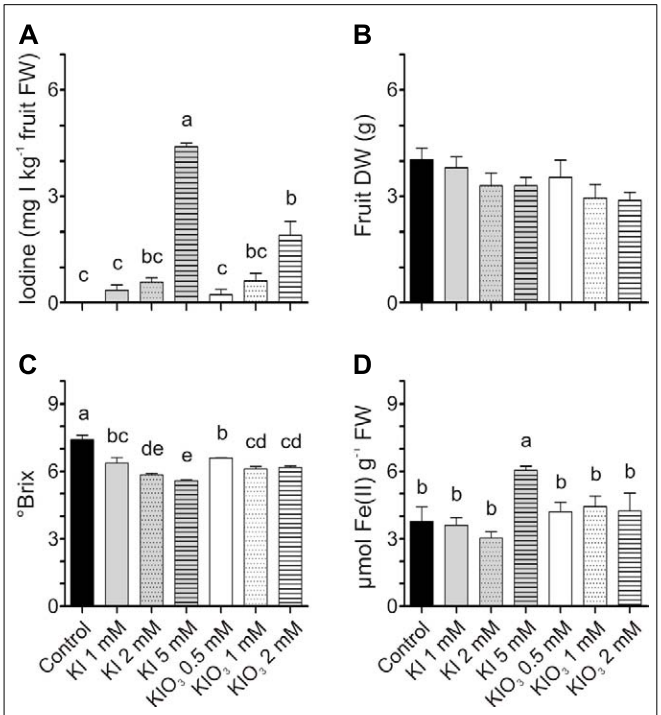

FIGURE 6 | Effect of iodine on fruit quality. Iodine levels **(A)**, dry weight (DW) **(B)**, sugar content **(C)**, and antioxidant capacity **(D)** measured in fruits collected at the red ripening stage from the first truss of plants treated eight times with 0–5 mM KI or 0–2 mM KIO_3. Data were subjected to one-way analysis of variance (ANOVA) and the means were separated using the *F*-test (95% confidence level).

fruits (**Figure 5D**). Indeed, the peel alone contained very high levels of iodine (**Figure 5D**).

EXPERIMENT 5: EFFECT OF IODINE CONTAINED IN FRUITS ON THEIR QUALITY

For the qualitative analyses of fruits, tomatoes were harvested at the red stage of ripening. In this experiment, iodine treatments were thus prolonged, and fruits were collected after eight iodine applications. Iodine accumulated in these fruits (**Figure 6A**) with a trend similar to that previously observed after four administrations (**Figures 2C,D**). The values of the iodine content, with the exception of a few samples, were also comparable. Fruits from KI-treated plants accumulated approximately 0.3–4.5 mg I kg^{-1} FW following treatments with 1–5 mM KI respectively, while fruits from 0.5 to 2 mM KIO_3-treated plants ranged from 0.2 to 1.9 mg I kg^{-1} FW (**Figure 6A**). The DW of fruits was not significantly different (**Figure 6B**).

Fruit quality was evaluated in terms of sugar content and antioxidant power. Treatments with KI and KIO_3 mildly reduced the fruit sugar content, as the °Brix progressively decreased, slightly but significantly, with the increase in potassium iodide or iodate concentrations (**Figure 6C**). On the contrary, no significant differences were detected in the ferric-reducing ability of tomatoes, with the exception of the value measured in fruits from 5 mM KI-treated plants, which was significantly higher than the control (**Figure 6D**). These fruits were those that accumulated the highest level of iodine (**Figure 6A**).

DISCUSSION

In accordance with the previous positive results obtained in hydroponic-grown tomato plants (Landini et al., 2011), the experimental trials presented here clearly indicate that even in soil-grown plants iodine can be accumulated in fruits at very high levels. Both KI and KIO_3 administered to the soil can be efficiently taken up by the roots and the iodine amounts detected in fruits may be adequate for a biofortification program without using iodine doses that are toxic to the plant. On the whole, the most suitable iodine concentrations for a satisfactory biofortification of fruits were the lowest tested in the Experiment 1, corresponding to 0.5–1 mM, of both iodide and iodate (**Figure 2**). In our growth conditions, these doses corresponded to 12.7 and 25.4 mg I per single treatment application, respectively, and with a volume of about 8 dm^3 soil per pot, as in our case, to approximately 1.6–3.2 mg I dm^{-3} soil. Therefore, a weekly fertigation with these doses of KI or KIO_3, starting from the first fruit stage of development, could lead to a final accumulation of iodine in the fruit that would be suitable for a biofortification program.

We started the applications of iodine at the onset of the first fruit cluster. During tomato growth, most of the fruit weight is accumulated by the mature green stage (Ho and Hewitt, 1986; Srivastava and Handa, 2005), and there is recent evidence regarding

the important role of the xylematic system in providing water to tomato trusses (Windt et al., 2009). It is therefore reasonable to assume that iodine can be more easily translocated during the early fast growth of the fruits and that the xylematic system is the main route for iodine translocation within the plant, if iodine is administered to the soil.

The iodine status of a soil is a combination of the supply of iodine and the soil's ability to retain it. It is well-known that one of the most important components for the sorption of iodine in soils is the organic matter (Whitehead, 1974, 1978; Gerzabek et al., 1999; Yamaguchi et al., 2010), which can thus potentially affect the mobility of this element in the soil solution and its availability for plant uptake (Sheppard and Thibault, 1992; Hu et al., 2005; Dai et al., 2009). Results obtained in our Experiment 2 indicate that tomato plants grown in a high organic matter soil accumulated less iodine within the fruits if treated with KIO_3 (**Figure 3E**), thus confirming the possible negative role of the organic matter on the mobility of iodine and also indicating that iodate could be retained stronger than KI by the organic matter fraction of the soil. Due to its direct and indirect effects on the availability of nutrients, organic matter can also interfere with plant development and productivity (Bauer and Black, 1994; Martin-Rueda et al., 2007; Rigane and Medhioub, 2011). In our trial, the soil with high organic matter content, irrespectively of the iodine treatment applications, positively affected the plant growth and productivity (**Figures 3F,H**). In addition, the mild phytotoxicity symptoms, observed almost exclusively on the KI-treated plants, were less severe in the presence of high organic matter in the soil (**Figures 3A,B**). Therefore, in order to select a soil type suitable for iodine biofortification programs, a careful evaluation of all these factors is required.

Evaluating any interactions between iodine and nitrogen (N) is crucial in order to develop optimal agro-techniques for tomato biofortification with iodine. Fertilization of the soil with N can influence the concentration of some microelements in the soil solution, either increasing (e.g., Fe, Cu, Zn, Mn) or reducing (e.g., B, Mo) their solubility (Rutkowska et al., 2009). Furthermore, possible inhibitory effects of nitrate on halide absorption by root plants, likely as a consequence of competition during plant uptake, have been described (Roorda van Eysinga and Spaans, 1985). In our Experiment 3, iodine levels accumulated in tomatoes were generally not influenced by the nitrate dose used in the fertilization of the plants, with only a minor negative effect of high nitrates in fruits from KIO_3-treated plants (**Figure 4E**). However, nitrogen deficiency represented a stressful condition for plant growth and development and KI phytotoxicity symptoms were much more evident on plants grown at the minimal nitrate level (2 mM). Although we cannot rule out that the moderate salinity of the water used for the fertirrigation of the plants increased these effects, such symptoms were not found in plants grown in the same conditions without iodine applications (**Figure 4A**). Furthermore, nitrogen is one of the main nutrients required for plant growth and can also affect plant vigor and fruit quality (Shaahan et al., 1999; Tei et al., 2002). N supply is positively correlated with tomato yields (Guidi et al., 1998; Le Bot et al., 2001; Bernard et al., 2009). We did not detect significant effects on fruit yield production as a result of the nitrate concentration in the nutritive solution (**Figure 4H**). However, if our plants had been cultivated until a higher number of fruit trusses had been formed, there might have been a stronger effect of the low nitrogen supply on fruit yield. Our trials thus indicate that the standard nitrate concentrations (about 10 mM) that are used in tomato cultivation should not negatively affect iodine uptake and accumulation (**Figure 4E**), while the deficiency of nitrogen could have a negative synergistic effect with the phytotoxicity of iodide (Herrett et al., 1962) on plant development and productivity (**Figure 4B**).

Tomatoes are either sold as fresh fruits and therefore consumed after a certain period of storage, or they are processed in order to produce pastes, sauces, or peeled products. The ability of tomato plants to volatilize iodine, described in other plant species (Redeker et al., 2000; Rhew et al., 2003; Itoh et al., 2009), is at present not known. The results obtained in our Experiment 4 indicate that iodine accumulated in tomato fruits is persistent after harvest (**Figure 5C**). A short shelf-life should thus not reduce the biological value of the iodine-rich fresh tomatoes. However, many other factors can affect post-harvest storage of fruits (low temperatures, atmosphere, humidity, packaging), and are therefore worthy of analysis.

We also found that removing the peel from tomato fruits led to a heavy reduction in their iodine content, as the peel appeared to be very rich in this element (**Figure 5D**). On the other hand, boiling of the fruits did not further reduce their iodine content (**Figure 5D**). Therefore, in fruits for industrial processing or simply for cooking, the peel should be maintained in order to preserve a high iodine concentration. Of course, we only measured iodine before and after a single boiling process. We cannot exclude that other cooking methods or cooking at higher temperatures might lead to higher iodine losses.

As a whole, tomato fruits resulted in being able to accumulate high amounts of iodine. Not even when plants were treated with iodine levels exerting strong phytotoxic effects on the vegetative organs, did the fruits appear to be affected, probably due to the lower levels of the element accumulating in fruits compared to in the leaves and stems (Landini et al., 2011). However, a qualitative analysis is necessary to ascertain whether the presence of iodine in tomatoes affects their quality, and in the Experiment 5 we carried out a preliminary evaluation of it. Tomatoes are usually consumed at their stage of maximum organoleptic quality, which occurs when they reach the full red color, but before excessive softening. Our qualitative analyses were thus performed on fruits at the mature red stage of ripening. In concentrations of a few mg I kg^{-1} FW, such as those detected in our biofortified fruits, iodine did not alter the visual appearance of the fruits, which maintained their original size, shape, and color (data not shown), major factors for consumer's choice. As far as nutritional compounds, we observed a small reduction in the content of sugars (**Figure 6C**), which represent the main metabolites, making up over 60% of the dry matter (Davies and Hobson, 1981), and which can affect both the taste and flavor of tomatoes. Iodine may have interfered with the metabolism of the primary compounds within the fruit (Ho, 1996) and this would be worth further evaluation.

Another important qualitative trait of tomato fruits is represented by their antioxidant power. Several studies have established a link between the dietary consumption of tomatoes, representing

a major source of antioxidants, and reduced risk and prevention of important pathologies (Agarwal and Rao, 2000). Interestingly, the antioxidant capacity of tomato fruits was not influenced by the accumulation of low iodine amounts, i.e., those most appropriate for a biofortification program (**Figure 6D**). However, the fruits accumulating higher quantities of the element showed a significant increase in their antioxidant capacity (**Figures 6A,D**), thus suggesting that iodine over a certain threshold could trigger a moderate antioxidant response in the fruit, probably against the mild stress caused by the iodine itself. This is in line with similar effects detected, for example, in lettuce (Blasco et al., 2008, 2011).

Fruit quality is a complex mixture of different traits, related, among others, to color, homogeneity, taste, flavor, size, shape, and content of nutritional compounds (sugars, acids, antioxidants). Although our results did not show major effects of iodine on the quality of the biofortified tomatoes, we analyzed only a few aspects of it. Therefore, further analyses can be performed to go into details and also to characterize other qualitative traits of the fruits.

In conclusion, we believe that the results of our study highlight several positive aspects in using tomato plants as a target for iodine biofortification programs. Plants can efficiently take up and translocate sufficient amounts of this element to the fruits, even if fertilized with low non-toxic doses of both KI and KIO_3. On the whole, it does not seem that these processes are significantly influenced by the organic matter content of the soil or by the level of nitrate used in the fertilization of the plants, two possible factors worth considering when setting up an agronomic protocol. Of the two different iodine forms tested, KIO_3 is preferable in order to avoid the possible, though limited, phytotoxicity problems observed in KI-treated plants. However, in soils rich in organic matter it is likely that KI maintains a higher mobility and availability for the plants. Finally, iodine-biofortified fruits appear to be suitable both for fresh market and for processing, especially if the peel is not removed.

The real efficacy of a biofortification strategy requires the careful evaluation of a series of factors. An effective and significant iodine accumulation in the edible parts of the biofortified plant and the maintenance of sufficient iodine levels when the crop is consumed, as demonstrated in this study, represent only the starting point. In fact, only biofortification protocols combining an effective micronutrient increase with high crop yields (or at least an absence of yield reductions) can be successfully adopted by a significant number of farmers. These productive aspects have been only partially tackled in the present study and certainly require a more extensive evaluation, for example in open field conditions. Finally, a tangible improvement should be demonstrated in the iodine status of those that consume biofortified tomatoes. This means that the iodine accumulated must be sufficiently bioavailable to significantly improve the original malnourished status of the consumer. An iodine bioavailability clinical trial is thus necessary as along with an analysis of the possible effects of iodine intake through tomatoes on thyroid functions.

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Strategies to increase vitamin C in plants: from plant defense perspective to food biofortification

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Vitamin C participates in several physiological processes, among others, immune stimulation, synthesis of collagen, hormones, neurotransmitters, and iron absorption. Severe deficiency leads to scurvy, whereas a limited vitamin C intake causes general symptoms, such as increased susceptibility to infections, fatigue, insomnia, and weight loss. Surprisingly vitamin C deficiencies are spread in both developing and developed countries, with the latter actually trying to overcome this lack through dietary supplements and food fortification. Therefore new strategies aimed to increase vitamin C in food plants would be of interest to improve human health. Interestingly, plants are not only living bioreactors for vitamin C production in optimal growing conditions, but also they can increase their vitamin C content as consequence of stress conditions. An overview of the different approaches aimed at increasing vitamin C level in plant food is given. They include genotype selection by "classical" breeding, bio-engineering and changes of the agronomic conditions, on the basis of the emerging concepts that plant can enhance vitamin C synthesis as part of defense responses.

Keywords: vitamin C, crop, food nutritional value, bio-engineering, QTL analysis

INTRODUCTION

Ascorbate (ASC) is a major soluble redox molecule with pivotal roles in allowing several metabolic pathways to work properly. ASC regenerates other metabolites, among which tocopherols, from oxidative damages and protects the catalytic site of a number of enzymes (e.g., hydroxylases) from irreversible oxidation, possibly caused by reactive oxygen species (ROS) in both animal and plant cells. It can be used as substrate or enzyme cofactor in various biological reactions (Lodge, 2008; De Gara et al., 2010). ASC is synthesized by fungi, protozoa, plants, and animals, even if by means of different biosynthetic pathways (Bleeg and Christensen, 1982; Banhegyi et al., 1997; Wheeler et al., 1998; Logan et al., 2007). For few animal species, among which guinea pig, some birds, humans, and primates in general, ASC is a vitamin (vitamin C), since during their evolution, they have lost the capability to synthesize it. In human this was caused by the loss of functionality of gulono-1,4 γ-lactone oxidase (GuLO), the enzyme catalyzing the last step of animal ASC biosynthesis (Nishikimi et al., 1992, 1994; **Figure 1**). Although ASC is considered essential for aerobic life (De Gara et al., 2010; Gest et al., 2013), it is worth noting that in fungi, protozoa, and animals the last reaction of its biosynthesis also produces hydrogen peroxide (H_2O_2), a putative toxic species (Banhegyi et al., 1997; **Figure 1**). Therefore, in animals consuming foods that ensure sufficient ASC intake, the loss of ASC biosynthetic capability may be done an evolutionary acquisition leading to an ameliorated control of redox homeostasis in the cells of these organisms. It is interesting to notice that plants, which produce a great amount of ASC in almost all their tissues (ASC reaches several tens of millimolar concentrations in green tissues), have evolved a synthesizing pathway with the last step catalyzed by a dehydrogenase (L-Galactono-1,4-γ-lactone dehydrogenase GaLDH) that does not produce H_2O_2 (**Figure 1**).

Plant-derived food are the main dietary source for vitamin C (**Table 1**). Vitamin C is also present in some meats, such as cow liver (liver and kidney are ASC synthesizing organs in animals, Kanfer et al., 1959), but they are irrelevant in supplying vitamin C because of their limited use in human nutrition and the consistent lost of ASC content caused by food processing, mainly due to ASC thermal instability (Munyaka et al., 2010).

Severe vitamin C deficiency causes scurvy, a disease discovered in the sailors of 15th and 16th century that could not consume fresh plant-derived food for months (Baron, 2009). Scurvy has been considered one of the most important disease derived from nutrient deficiency in the history of humanity (Magiorkinis et al., 2011). Scurvy symptoms consists in generalized edema, skin hemorrhages, swollen, bleeding gum and, if prolonged, can cause death (Magiorkinis et al., 2011). It is generally accepted that it is due to an impairment of collagen formation (Peterkofsky, 1991). Indeed ASC participates to collagen cross-linking reactions as cofactor of prolyl hydroxilases. The role of ASC in these reactions is to maintain the iron present into the enzymatic catalytic site in the reduced state and thus converting back the inactivated form of the enzymes into the active one (Gorres and Raines, 2010). Today scurvy is rare in developed as well as developing countries, since it requires a severe and prolonged deficiency in vitamin C in order to become evident; however, recent epidemiological studies underline that even in western populations sub-optimal vitamin C intake is widespread (Troesch et al., 2012).

Recommended dietary allowance (RDA) for vitamin C is a controversial matter, since different countries provide different advice; for example RDAs for adult men are 40 mg/day in UK;

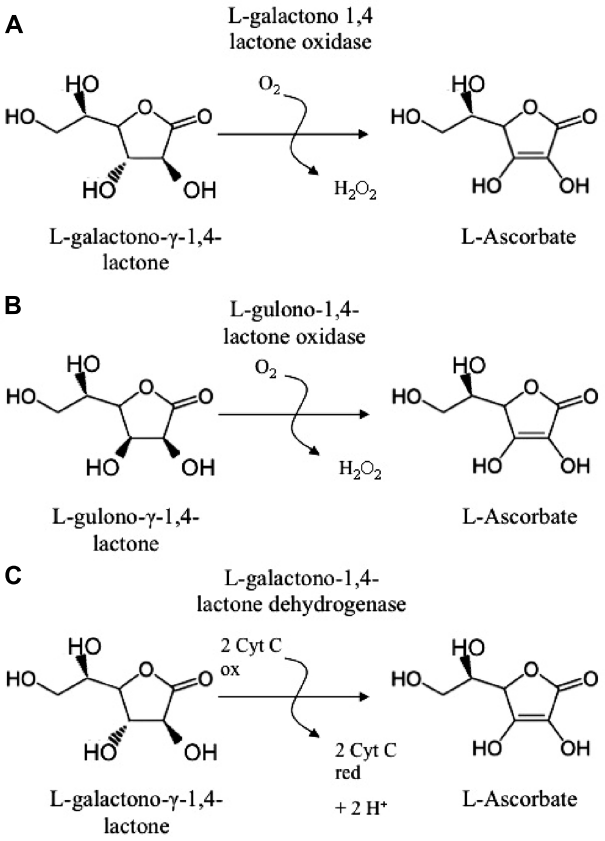

FIGURE 1 | Last enzymes in ASC biosynthetic pathways. (A) Pathway in protists and fungi; **(B)** Animal pathway; **(C)** Main pathway in higher plants.

Table 1 | Vitamin C content in plant edible organs.

Fruit and Vegetables	mg Vit C/100 g FW
Guava	243
Currant	200
Pepper	146
Rocket	110
Turnip Tops	110
Kiwi	85
Brussels Sprouts	81
Broccoli	77
Papaya	60
Cauliflower	59
Strawberry	54
Spinach	54
Clementine	54
Orange	50
Lemon	50
Tangerine	42
Grape Fruit	40
Endive	35
Broad Bean	33
Celery	32
Tomato	23
Melon	22
Radish	18
Lettuce	16
Banana	16
Potatoes	15
Soya Bean Sprout	13
Fennel	12
Apple	8
Carrot	4
Pear	4
Peach	4

A selection of plants of interest for human nutrition has been obtained from a database on food chemical composition (http://www.ieo.it/bda2008/homepage.aspx). Only the vitamin C content of the edible organs has been reported.

90 mg/day in USA; 100 mg/day in Germany; 70 mg/day in Netherland (Troesch et al., 2012). Moreover, in order to enhance health benefits due to vitamin C intake, the scientific community is suggesting to increase its RDA to 200 mg/day (Frei et al., 2012). Epidemiologic studies have actually revealed that ASC intake over the current RDA has a significant impact in reducing the risk of diseases such as respiratory tract infections, cardio-vascular diseases and cancer (Schlueter and Johnston, 2011). With the exception of special population groups, as for example people suffering for kidney stones (whose formation could be promoted by oxalate, a catabolic derivate of ASC in mammals; Linster and Van Schaftingen, 2007), adverse effects caused by vitamin C over-ingestion, such as diarrhea, only occurs when the intake overcomes 2000 mg/day (Schlueter and Johnston, 2011). Moreover, pro-oxidant effects of vitamin C was only reported for daily intake higher than 500 mg (Podmore et al., 1998).

In human, vitamin C uptake is controlled by specific Na-dependent active transporters probably present in all the cells accumulating ASC (Savini et al., 2008). In animal tissues, the oxidized form of vitamin C, dehydroascorbate (DHA), is less efficiently taken up on glucose transporters. Within cells DHA is reduced back to ASC (the active form of the vitamin C) by enzymes using glutathione (GSH) and pyridine nucleotides as electron donor (Rumsey et al., 1997; Linster and Van Schaftingen, 2007; **Figure 2**). According to literature data, 200 mg/day is the intake of vitamin C that leads to the saturation of renal clearance for reabsorption of vitamin C, since a dose-dependent vitamin C release is observed in urine starting from an intake higher that this dose (Levine et al., 1996, 2001). In this perspective all the current RDAs for vitamin C (see above), assessed for avoiding scurvy and mild deficiency, are starting to be considered suboptimal in regards to the potential health benefits triggered by this vitamin (Frei et al., 2012).

In developed countries vitamin C supplementation is largely adopted especially for preventing/reducing cold related diseases.

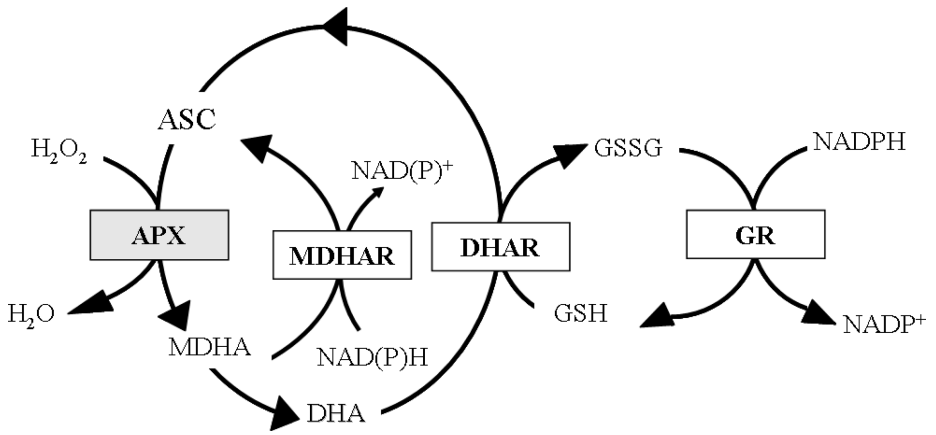

FIGURE 2 | Foyer- Halliwell- Asada cycle. Enzymes and intermediates of the cycle (also known as ASC-GSH cycle) are reported. In white boxes the enzymes active in both animal and plant cells; in gray box the enzyme exclusively presents in plant cells. APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase.

Actually the industrial production of vitamin C represents a low efficient and expensive technology (Hancock and Viola, 2002). Recently, it has also been demonstrated that vitamin C from plant-derived food (i.e., kiwifruit) is more bio-available than the chemically synthesize or purified molecule used in supplementation (Vissers et al., 2011). This could be the consequence of the presence in food matrixes of plant origin of several molecules with antioxidant/redox properties, which can have a synergic effects with ASC or be able to preserve vitamin C in its active reduced state (Villanueva and Kross, 2012). The possibility that other biological molecules could stabilize ASC is supported by DHA/ASC redox potential (estimated around as 90 mV; Noctor, 2006) that makes ASC a good reductant and, at the same time, its oxidized form DHA reducible in cellular metabolic conditions (Szarka et al., 2012).

Another aspect that makes interesting to increase vitamin C level in the edible plant tissues is the fact that this metabolite improves the post-harvesting properties of several vegetables. Indeed, its addition to several food matrix is used for extending their shelf life, as well as for improving specific technological properties, such as the kneading of wheat flour and dough rheological properties (Paradiso et al., 2006).

On these bases, ASC bio-fortification of the plants utilized for food production is becoming an important nutritional claims also for having promising technological implications. The plethora of roles played by ASC in plant metabolism increases the complexity of this goal. Here an overview of the results obtained in vitamin C bio-fortification is given with particular attention to the results obtained on crops and on the reasons why vitamin C bio-fortification still remains an ambitious target.

STRATEGY OF BIOFORTIFICATION

EFFECT OF GENOTYPE AND AGRONOMIC PROCEDURES

It is well known that different crop varieties produce and store different amounts of vitamin C in their tissues. Maize heterotic F1 hybrid (B73xMo17) have higher ASC biosynthetic capability and activities of ASC-GSH cycle enzymes, in comparison with the parental lines B73 and Mo17 (De Gara et al., 2000). In several other crops the effects of genotype on vitamin C levels of edible tissues have been reported (see as few examples Kalt et al., 1999; Kafkas et al., 2006). Variability in ASC content has been deeply studied in *Solanum tuberosum*. In spite of potato tubers storing a moderate amount of vitamin C (8–36 mg/100 g fresh weight) in comparison with other plant-derived foods (**Table 1**), the possibility to improve its level in the tubers is of great interest. Indeed this species is relevant in supplying vitamin C, in particular in the developing countries where potatoes make up a large part of a subsistence diet. The first evidence for the genetic basis of ASC variability as well as the possibility of breeding *S. tuberosum* for increasing its vitamin C content, have been reported more than 30 year ago (Augustin et al., 1978). **Figure 3** reports the contents of vitamin C in 20 cultivars of early potato growth in the same experimental field in Apulia Region (southern Italy). The variation in the content of this nutrient is evident, as well as the deviation from the mean value considered as standard for the same kind of vegetables grown in Italy (indicated as dotted line in **Figure 3**; Buono et al., 2005; Buono et al., 2009). A multi – year study using 75 genotypes from 12 North American potato – breeding programs suggests that most of the tested genotypes produces different amount of vitamin C in response to different growth conditions occurring in the same environment over time. However, few clones with a stable and high capability of storing vitamin C have been selected and suggested as putative genotypes of interest for large scale production of potatoes with enriched levels of vitamin C (Love et al., 2004).

Correlations between ASC contents and environmental conditions have been also taken into consideration both in model and crop plants. The intra-species variability of vitamin C or other antioxidants have often been considered relevant for explaining the differences among cultivars in sensitivity to a plethora of biotic and abiotic environmental stresses. Literature data suggest that tolerance to stress also correlates with the capability of increasing ASC biosynthesis or the activity of ASC – related enzymes, when

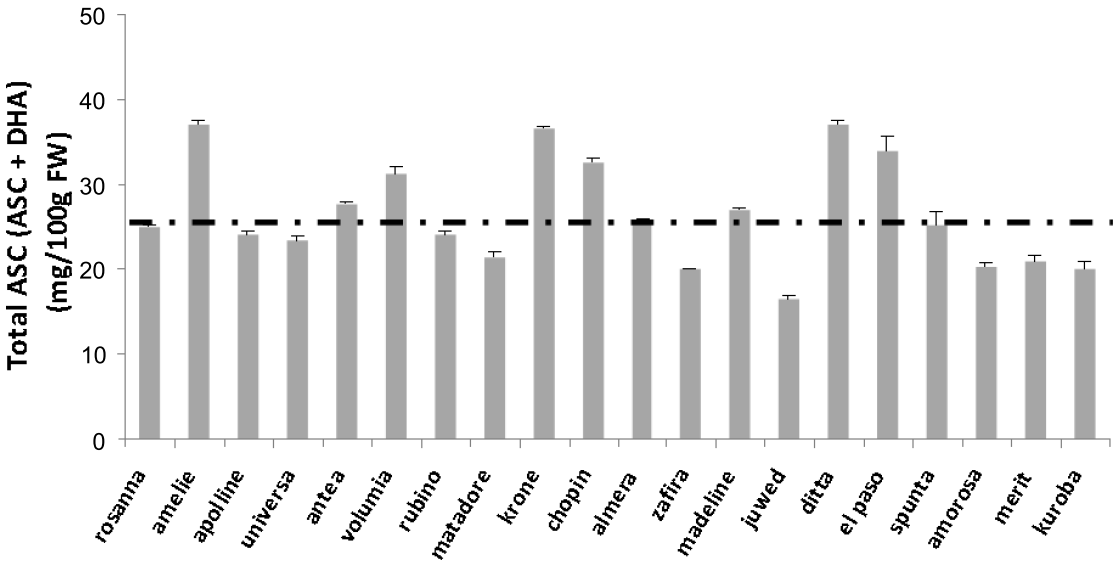

FIGURE 3 | Variability of ASC content in early potato tubers grown in the same agronomic conditions. The content of vitamin C in 20 cultivars of early potato grown in the same experimental field in Apulia Region (southern Italy) is reported. The values are the mean of six different experiments ± standard error. Dotted line represents the value reported in official database as the standard value for early potato tuber cultivated in Italy (http://www.ieo.it/bda2008/homepage.aspx).

plants are exposed to unfavorable environmental conditions (Gill and Tuteja, 2010; Wang et al., 2012). Consistently, mutants with reduced level of vitamin C, i.e., *Arabidopsis* vtc mutants, have been selected for their sensitivity to specific stress conditions (Smirnoff and Wheeler, 2000). An increase in ASC content is also induced by iron deficiency in sugar beet roots, where a 20-fold increase in the activity of root ferric chelate reductase was accompanied by a twofold increase in vitamin C level (Zaharieva and Abadia, 2003).

The effects of water or salt stress have been studied in several crops (Mittova et al., 2004; Hakeem et al., 2012; Turan and Tripathy, 2013). An increase in salt (NaCl) from 3 to 6 dSm in soilless systems induces a significant rise in the level of ASC, α-tocopherol and dry matter of "cherry" tomato fruits (Serio et al., 2004). Interestingly, irrigation with saline water is commonly used in Southern Italy with the aim at increasing flavor of tomato fruits and other vegetables, NaCl is also supplied for the production of early tomatoes in soilless systems in Northern Europe (Adams, 1991; Raffo et al., 2002).

Treatments with molecules involved in the stress signaling pathways, such as jasmonates, are able to induce a twofold increase in ASC content by altering the expression of genes coding for several enzymes involved in its metabolism (Sasaki-Sekimoto et al., 2005). A relevant increase in ASC content has also been induced both in model and crop plants by exogenous supply of L-galactono 1,4-γ-lactone (GaL), the last precursor of ASC biosynthesis (**Figure 1**). In *Lupinus albus* seedlings a dose – dependent increase of ASC contents in the vegetative tissues is induced by supplying GaL in hydroponic growth medium. This increase also correlates with increase in seedling rate growth due to the ASC-dependent stimulation of both cell division and cell elongation (Arrigoni et al., 1997). GaL exogenous treatments also increase ASC content in wheat leaves and kernels. Interestingly, this ASC increase induces a delay in the activation of programmed cell death, a process typically occurring at the end of the storing process in cereal endosperm cells. The ASC-enriched kernels also have an increased weight and protein content, probably due to the extension of the filling phase (Paradiso et al., 2012). However, although GaL supply could be an efficient strategy for vitamin C biofortification, its feasibility on large scale is discouraged by the high cost of the treatments.

BIO-ENGINEERING OF ASC BIOSYNTHESIS

As virtually for all metabolites, ASC accumulation can be achieved in plants by manipulating its metabolism at biosynthetic, catabolic or recycling level. Indeed transgenic crops with increased ASC level have been obtained by increasing the expression of its biosynthetic or recycle enzymes. It is generally accepted that ASC *de novo* synthesis occurs in higher plants mainly through the Smirnoff–Wheeler pathway (S-W; Wheeler et al., 1998; **Figure 4**). This is supported by the fact that all the identified *Arabidopsis thaliana* mutants, which are partially deficient in ASC (vtc1, 2, 3, 4, 5), are impaired in the expression of enzymes involved in this pathway. Moreover, mutants completely lacking the ability to produce ASC through this pathway are lethal (Conklin et al., 2000; Dowdle et al., 2007). The S-W route uses mannose and galactose as main intermediates and it shares these metabolites with pathways leading to the synthesis of glycoproteins and cell wall polysaccharides (Lukowitz et al., 2001; Reuhs et al., 2004). Therefore, alterations in the metabolic fluxes toward one of these pathways might also affect the availability of intermediates for the other correlated pathways, with relevant consequences for plant development and fitness. As previously mentioned, the direct precursor of ASC in S-W pathway is GaL which is converted to ASC by a dehydrogenase localized

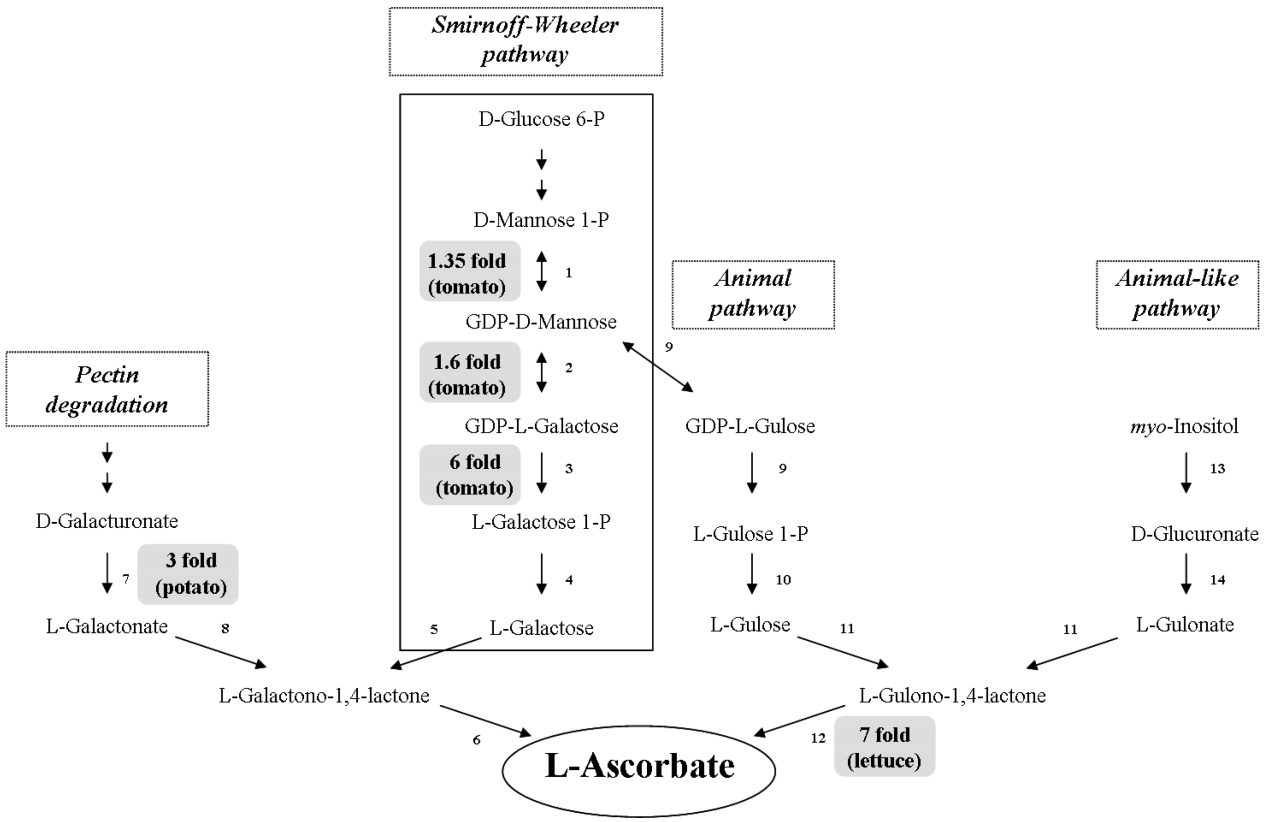

FIGURE 4 | ASC biosynthetic routes and ASC increases in bio-engineered crops. The ASC biosynthetic pathways active in plant tissues are reported. In gray boxes the highest increases obtained by overexpressing the corresponding enzyme are indicated. Enzymes: 1 GDP-Mannose pyrophosphorylase (GMPase), 2 GDP-Mannose epimerase (GME), 3 GDP-Galactose phosphorylase (GGP), 4 L-Galactose 1P phosphatase (GPP), 5 L-Galactose dehydrogenase, 6 L-Galactono-1,4-γ-lactone dehydrogenase (GaLDH), 7 Galacturonate reductase (GalUR), 8 aldono-lactonase, 9 phosphodiesterase, 10 sugar phosphatase, 11 L-gulose dehydrogenase, 12 L-Gulono-1,4-γ-lactone oxidase (GuLO), 13 Myo-inositol oxygenase (MIOX), 14 D-Glucuronate reductase.

in the inner mitochondrial membrane (GaLDH, step number 6 in **Figure 4**). GaLDH seems to be part of the respiratory complex I and requires oxidized cytochrome C as electron acceptor (Bartoli et al., 2000; Millar et al., 2003). This tight link between ASC biosynthesis and respiratory electron chain makes ASC synthesis in plant cells strongly sensitive to certain stress conditions that cause impairment in electron flux through respiratory complexes (Bell et al., 1971; Millar et al., 2003; Vacca et al., 2004). A strict correlation between mitochondrial electron flow and ASC biosynthesis seems also to occur in some climacteric fruits. In tomato the increase in ASC level, occurring during fruit ripening after breaker stage, might be correlated with an increase in respiration rate (Ioannidi et al., 2009). Ethylene itself seems to control ASC biosynthesis. In tomato fruits ethylene treatment stimulates the expression of L-Galactose 1P phosphatase (GPP; step number 4 in **Figure 4**); while in *Arabidopsis* the overexpression of the ethylene responsive transcription factor ERF98 increases ASC biosynthesis probably through the ERF98 interaction with the promoter of GDP-mannose pyrophosphorylase (GMPase, step number 1 in **Figure 4**; Ioannidi et al., 2009; Zhang et al., 2012). On the other hand, in climacteric fruits ASC is also responsive for ethylene production, being co-factor of 1-aminocyclopropane-1carboxylic acid oxidase, the last enzyme of ethylene biosynthesis (Ververidis et al., 1992; Liu et al., 1999). In kiwifruit, another climacteric fruits, the highest ASC level occurs in an early stage of development and thus seems to be independent on ethylene production (Li et al., 2010a). These findings underline the complexity of a network of events that has different peculiarities depending on the species.

The overexpression of enzymes involved in S-W pathway have increased vitamin C level from 1.2- up to 6-fold in the edible parts of plants, such as tomato, potato, and strawberry (**Figure 4**). The highest increase has been obtained by Bulley et al. (2012) in transgenic tomato overexpressing GDP-galactose phosphorylase (GGP, step number 3 in **Figure 4**). These transgenic plants also have smaller fruits which were seedless or provided with nonviable seeds. In tomato and strawberry fruits GGP overexpression induces polyphenol levels higher than in wild-type (2–0.5-fold, respectively; Bulley et al., 2012). The overexpression of genes responsible for the biosynthesis of specific phenolic compounds has often been reported to induce seedless fruit production (Ingrosso et al., 2011 and references wherein). Moreover, it is well known that changes in ASC content, redox state and related redox enzymes characterize the different phases of seed

maturation (Arrigoni et al., 1992; De Gara et al., 2003). Therefore, alterations in both polyphenol and ASC levels might contribute to the observed impairment in seed development and viability. Interestingly, ASC bioavailability seems to be increased by high polyphenol concentration in plant-derived food; this feature further increases the putative nutritional value of these engineered crops (Vissers et al., 2013).

Tomato plants overexpressing GDP mannose epimerase (GME, step number 2 in **Figure 4**) or GDP-GMPase, (step number 1 in **Figure 4**) have been also obtained with a modest increase in ASC accumulation in ripe fruits (up to 1.6–1.35-fold compared to wild-type plants, respectively; Zhang et al., 2010; Cronje et al., 2012). GME mutants also show enhanced tolerance to oxidative stress (Zhang et al., 2010).

Another success in term of vitamin C bio-fortification has been obtained by expressing rat GuLO (step number 12 in **Figure 4**) in lettuce, where up to a sevenfold increase of ASC level has been obtained (Jain and Nessler, 2000). The high increase in ASC biosynthesis observed in this plant could also be due to the very low level of ASC present in wild-type leaves (about 4 mg/100 g FW).

Surprisingly, no positive evidence of increasing ASC content by overexpressing GaLDH in crops have been reported in literature, at least to our knowledge. GaLDH overexpression only gives a positive effect on ASC biosynthesis in cultured tobacco cells (Tokunaga et al., 2005). The failure in obtaining an increase in ASC level by overexpressing the last enzyme of its biosynthetic pathway could be correlated by the presence of feedback control of ASC toward its *de novo* synthesis (Mieda et al., 2004; Mellidou et al., 2012a). When ASC reaches a threshold value a feedback control is activated by inhibiting one of the initial steps of the pathway. Therefore the catalytic activity of the last enzyme strongly depends on the availability of its substrate (De Gara et al., 1989; Mieda et al., 2004; Mellidou et al., 2012a).

Intermediates not present in the S-W pathway, such as D-galacturonate and myo-inositol, have been also identified as ASC precursors in plants; thus suggesting that alternative pathways can be utilized for the vitamin production. Indeed cell wall degradation leads to galacturonate release, thus providing this intermediate for ASC biosynthesis. This minor route has been suggested to become relevant during tomato ripening, when pectine degradation, responsible for fruit softening, increases the availability of alternative ASC precursor (Badejo et al., 2012). The increase in pectine degradation by pectinesterase or polygalacturonase overexpression probably does not represent a feasible strategy to increase ASC level, since it might decrease fruit shelf life. In this perspective the extension of the shelf life of tomato fruits achieved by the down-regulation of polygalacturonases (Smith et al., 1988) could also decrease the ASC accumulation in the mature fruits of the engineered plants. On the other hand, it has been reported that ASC itself acts on fruit softening by promoting pectine breakdown through a non-enzymatic mechanism: in the presence of Cu^{2+} and H_2O_2 in cell wall, ASC induces hydroxyl radical formation responsible for direct polysaccharides scission (Fry et al., 2001; Dumville and Fry, 2003). Therefore the relation between ASC levels and fruits firmness is another complex aspect that merits to be better investigated even in order to select the best strategies for obtaining fruits enriched in vitamin C by means of bio-engineering approaches.

Galacturonate reductase (GalUR step number 7 in **Figure 4**) expression positively correlated with ASC content in strawberry (Agius et al., 2003). GalUR overexpression has been performed leading to an increase in ASC accumulation up to threefold in potato tubers (Hemavathi et al., 2009). Myo-inositol, a compound involved in the biosynthesis of signaling molecules, can also generate D- glucuronate, which is then transformed into gulono-1,4-γ-lactone as direct ASC precursor, thus suggesting the existence of an animal-like pathway for ASC production also in plant (Lorence et al., 2004). It has been also hypothesized that the enzyme of S-W route GPP catalyses also myo-inositol production by de-phosphorylation of myo-inositol phosphates feeding ASC production in plants through both pathways (Torabinejad et al., 2009). Myo-inositol oxygenase (MIOX; step 13 in **Figure 4**) overexpression has successfully been tested for increasing ASC in the model plant *Arabidopsis thaliana* (up to threefold), but not in tomato (Lorence et al., 2004; Cronje et al., 2012). This could be due to a species – specific capability to use alternative routes to synthesize ASC and further underlines the peculiarity of each plant species in ASC accumulation.

BIO-ENGINEERING FOR INCREASING ASC RECYCLE

As in animals, also in plants the stable oxidized form of ASC is DHA even if ASC undergoes to an univalent oxidation giving radical monodehydroascorbate (MDHA). Therefore spontaneous MDHA dismutation produces DHA. MDHA and DHA regenerate ASC through the recycling reactions catalyzed by MDHAR and DHAR in the Foyer-Halliwell-Asada cycle (Foyer and Halliwell, 1976; Foyer and Noctor, 2011; **Figure 2**). This cycle is ubiquitous in plants and different isoforms of its enzymes are present in almost all subcellular compartments (Locato et al., 2009). A high efficiency in the ASC regeneration from its oxidized forms has been proposed as the mechanism allowing the mature fruits of tomato cultivar Santorini to store higher levels of ASC compared to those present in other cultivars (Mellidou et al., 2012a). However, DHAR overexpression induces a modest increase in ASC accumulation in maize kernels and potato tubers (Chen et al., 2003; Qin et al., 2011). On the other hand, data on the effects caused by MDHAR overexpression are contradictory. In tobacco the expression of an *Arabidopsis* peroxisomal isoform of MDHAR targeted to cytosol induces a 2.2-fold increase in ASC level (Eltayeb et al., 2007); while tomato transgenic lines, obtained by overexpressing the tomato MDHAR3 targeted to cytosol and peroxisomes, have shown unchanged or even decreased ASC accumulation in fruits and leaves, respectively. In the same study, transgenic lines with silenced MDHAR3 showed significant ASC increase in both fruits and leaves (Gest et al., 2012). The differences in the capability to accumulate ASC between the two kinds of transgenic plants were enhanced by high light exposure (Gest et al., 2012). It is known that light exposure increased ASC production in plants (Smirnoff, 2000; Tabata et al., 2002). Recently, it has been reported that a light-dependent increase in ASC occurs in both fruits and leaves being stronger in the latter (Li et al., 2010b; Massot et al., 2012). This is consistent with the pivotal role of ASC in the chloroplastic photo-protecting mechanisms: it is cofactor of

violaxanthine de-epoxidase, an enzymes involved in the xanthophyll cycle, and the major player of the water–water cycle, two pathways preserving photosynthetic components by the photo-oxidation due to high irradiance-dependent ROS release (Eskling et al., 1997; Asada, 2000). ASC also acts as electron donor of PSII when the oxygen evolving system is lost (Tóth et al., 2009, 2011). The involvement of ASC in redox reaction aimed at protecting photosynthetic functionality may explain why the overexpression of the ASC recycling enzymes generally induces an increase in plant tolerance toward a number of stresses, such as chilling, salt, ozone even when it does not substantially affect ASC levels in plant tissues (Eltayeb et al., 2007; Stevens et al., 2008; Li et al., 2010c).

Interestingly, the enhancement of ASC level by overexpressing its recycle enzymes has been suggested as a good strategy for extending shelf life of edible plants that can be stored at low temperature as in the case of apple fruits. The increased capability of ASC recycle makes these fruits more tolerant to cold stress, since ASC oxidation to DHA has been suggested to be responsible for the flesh browning during the long storage period (6 months; Mellidou et al., 2012b).

When DHA is not converted back to ASC an irreversible loss of the vitamin occurs. It has been reported that in plant cells DHA catabolism irreversibly converts this molecule to oxalate and threarate (Green and Fry, 2005). Even if the involvement of enzymes in DHA catabolism has been hypothesized, oxalate and threarate production from DHA catabolism also occurs spontaneously *in vitro*. This makes the control of this process by bio-engineering not viable (Parsons et al., 2011).

ASC OXIDATION, A NECESSARY LOST FOR PLANT METABOLISM

Enzymatic ASC oxidation mainly occurs in plants through the reactions catalyzed by ascorbate oxidase (AOX) and ASC peroxidase (APX), two typical plant enzymes. AOX is an apoplastic enzyme involved in cell elongation (Takahama and Oniki, 1994). The down-regulation of this enzyme causes a shift in the apoplastic ASC pool toward its reduced state; it also increases plant yield during water deficit, through a carbon flux re-allocation, but does not determine a significant ASC increase in the investigated tissues (Garchery et al., 2013).

APX down-regulation is not a feasibly strategy for preserving ASC in plant, since this enzyme is a key player in many plant defense responses. A decrease in its activity might enhance plant susceptibility to stress (Örvar and Ellis, 1997; de Pinto et al., 2006). Indeed, the appearance of various APX isoforms during evolution can be considered a specific acquisition for promoting survival of these sessile organisms by using a molecule that plants can produce by themselves (Ishikawa and Shigeoka, 2008). APX uses ASC as electron donor to scavenge H_2O_2 normally produced in aerobic metabolism and over-produced during abiotic and biotic stresses (Karpinski et al., 1997; Mittler et al., 1999; Paradiso et al., 2008; De Gara et al., 2010; **Figure 2**). It has been suggested that transcriptional and post-transcriptional regulation of APX is a signaling strategy able to finely regulate the H_2O_2 level into the cell, switching the role of this molecule from toxic compound to signaling molecule (de Pinto et al., 2012). Actually, being the only ROS able to cross cell membranes, H_2O_2 can work as messenger in the transduction pathway activated as consequence of different stimuli (Foyer and Noctor, 2005; Bienert et al., 2007). Indeed H_2O_2 has been supposed to regulate gene expression during plant defense response playing a major role in tolerance acquisition against stress (Miller et al., 2008). For example, in thermal acclimation, H_2O_2 is reported to regulate the expression of heat shock proteins and of a thermostable APX isoenzyme (Banzet et al., 1998; Lee et al., 2000; Suzuki and Mittler, 2006; Volkov et al., 2006).

A general enhancement of antioxidant systems are involved in plant acclimation to stress. This has relevant implications in post-harvest procedures, since post-harvest controlled stress, such as moderate temperature, are able to increase antioxidant shield in plant tissues thus improving food quality during storage (Cisneros-Zevallos, 2003).

A SYSTEMIC APPROACH

As emerging from data reported above, ASC is involved in a wide net of metabolic reactions controlling growth and development as well as stress responses of plants (as reviewed by Foyer and Noctor, 2011). Indeed a plethora of different reactions depend on ASC and affect its level (from ROS removal to the synthesis of secondary metabolites and phyto-hormones, or to prolyl hydroxylation). This is probably why bio-engineering of a single gene involved in ASC biosynthesis or recycle often led to unsatisfactory results (see above). Actually, ASC level in plant organs and tissues can be considered a quantitative complex trait.

For this reason, in order to obtain plant-derived foods with a consistent enrichment in vitamin C, systemic approaches have been recently used. At this purpose a quantitative trait loci (QTL) analysis have been implemented in order to identify polygenic traits able to enhance ASC in edible crops. It is expected that these findings can ameliorate breeding strategies for increasing nutritional value of plant-derived foods.

Tomato has been mainly investigated, as a model crop (Stevens et al., 2007). A number of tomato wild accessions accumulating higher amounts of ASC (up to fivefold) than the cultivated lines have been identified (Di Matteo et al., 2010). An introgression line (IL) has been obtained by using the QTL identified in a wild progenitor and correlated to high ASC accumulation in mature tomato fruits. Di Matteo et al. (2010) demonstrated that the rise of ASC occurring during ripening in this IL depends on an increased flux of ASC precursors not involved in the S-W pathway. In particular, pectine degradation seems to be pivotal for feeding ASC *de novo* synthesis during tomato fruit ripening.

It is known that domestication often caused the loss of characters able to promote plant fitness and competitiveness in natural environment. In this perspective, it is possible that human selection on plants has caused a reduction in the synthesis/storage of precious metabolites. This could be occurred with vitamin C during tomato domestication. Indeed tomato varieties have been selected in the past for high yield and it probably caused ASC loss by oxidation. As already discussed, ASC oxidation promotes plant cell elongation (Takahama and Oniki, 1994; Fry et al., 2001). Moreover, the selection of tomato cultivars having prolonged fruit shelf life, and therefore prolonged flesh firmness, might lead to the selection of lines having low ASC as a consequence of a

reduced or delayed pectine degradation which is responsible for fruit softening (Dumville and Fry, 2003).

However, different species can require different strategies for optimizing the post-harvest properties of their edible organs. In apples and pears the ASC level has been reported to be correlated with post-harvest quality (Veltman et al., 2000; Davey et al., 2007). In apple fruits, ASC level depends on harvest time and, as a consequence, it affects susceptibility to phytopathogens during post-harvest storage (Davey and Keulemans, 2004; Davey et al., 2007). Co-localized QTL for wound-related flesh browning and DHA content were recently identified in apple (Davey et al., 2006). ASC oxidation occurring during storage conditions has been also hypothesized to be mainly involved in post-harvest diseases of pear (Cascia et al., 2013). QTL analysis have recently suggested that candidate genes regulating ASC level and post-harvest quality in apple flesh are a paralog of GGP and MDHAR3, respectively (Mellidou et al., 2012b). An allele of MDHAR has been also proposed as major candidate gene for high ASC level in tomato fruit (Stevens et al., 2008); whereas in strawberry candidate genes for stable QLT correlated to high ASC have identified in alternative biosynthesis pathways, such as GalUR e MIOX (Zorrilla-Fontanesi et al., 2011). All these findings are supported by the role of high levels of ASC and related redox enzymes in protecting plant from stress conditions. Indeed, ASC production is enhanced by several injuries. It is in fact reported that jasmonate and its derivates produced in plant as consequence of wounding, promptly induces ASC biosynthesis (Suza et al., 2010). Consistently *Arabidopsis* vtc mutants, containing about 30% of ASC compared to wt, were identified for their increased susceptibility to ozone and then characterized for their slow growth phenotype (Conklin et al., 1996; Veljovic-Jovanovic et al., 2001). Moreover, pathogenesis related genes were up-regulated in vtc1, thus suggesting an impairment in the defense responses of this ASC-deficient mutant (Pastori et al., 2003; Pavet et al., 2005).

Another omic approach that has recently given information on metabolic networks responsive for ASC accumulation in fruit is trascriptomic analysis. Such analysis has been carried on in tomato IL showing reduced fruit ASC accumulation in comparison with its cultivated parental line. The main differences between the two lines have been identified in the steady state of mRNA related to oxidative and antioxidant pathways. In particular, this tomato IL showed an accelerated oxidative metabolism and decreased antioxidant systems compared to the parental line (Di Matteo et al., 2012). The accelerated oxidative metabolism could explain the low ASC amount in tomato IL by a reduction of sugar flux toward ASC biosynthesis. It has been suggested that ASC plays a protective role in climacteric fruits contrasting ROS rise during ripening (Jimenez et al., 2002). Therefore in the mentioned tomato IL the increased oxidative metabolism probably increases ROS, thus causing a further ASC consumption.

CONCLUSION

As emerging above, so far scientific studies have failed in identifying a single master regulator responsible for ASC accumulation in plant -derived foods. This makes vitamin C bio-fortification a real challenge of plant science research. It is clear that ASC level of plant edible tissues depends on several cross-talking factors acting at different physiological levels. Within cells, competition for hexose fluxes between vitamin C biosynthesis and other metabolic pathways, as well as the balance between ASC consuming and recycling reactions are pivotal for ASC storing. This is further complicated by the fact that ASC is used in almost all cellular compartments which are characterized by the presence of diversely regulated isoenzymes of the Foyer-Halliwell-Asada cycle and of different ASC-utilizing enzymes. At organ level, the fluxes, between source and sink tissues, of ASC precursors and ASC itself could be a critical aspect for increasing ASC level in certain tissues or organs. These fluxes are developmentally regulated but they can also be altered by specific stresses or environmental conditions that diversely affect various organs of the plant. Therefore the strategies adopted to increase ASC in plant edible tissues or organs have to take into account all these considerations in order to obtain plants with an increased nutritional value and with the opportune productivity and resistance against adverse environmental conditions.

Since fruits can be considered the best dietary sources for vitamin C being consumed raw, they are the main target of vitamin C bio-fortification. In this perspective, the identification of the most efficient strategy for increasing vitamin C in fruits is further complicated by the variety of ASC accumulation trends showed during ripening of fruits from different species (**Figure 5**). In particular, depending on the specie, ASC level is reported to decrease during fruit ripening (i.e., peach; Imai et al., 2009), to remain almost constant during fruit ripening after reaching a maximum level during early fruit development (i.e., kiwifruit; Li et al., 2010a) or increasing during ripening (i.e., tomato, Ioannidi et al., 2009). These trends can show further intra-species variations at least in terms of the timing of different development and ripening stages as well as they can be affected by agronomic and environmental conditions. All these aspects make clear why ASC manipulation of biosynthesis or recycle level not always has led to a consistent vitamin C bio-fortification (Zhang et al., 2010; Cronje et al., 2012; Gest et al., 2012).

A novel systemic approach is promising in skipping difficulties possibly derived by single gene bio-engineering. Indeed it is emerging the concept that specific allelic forms of genes directly involved in ASC metabolism, or positively correlated with ASC storage, can differently affect ASC level in plant edible tissues. In particular, the identification of the QTL common or positively correlated to both ASC level and plant defense responses are opening new perspectives. In this context the comparison of wild progenitors and the derived cultivated lines seems to be very useful for identifying strategies adopted by plants during evolution for increasing their fitness. In a near future, these strategies could be implemented in crop by introgression through "classical" breeding techniques or multiple genes bio-engineering. In this perspective, the discovery of the main genetic mechanisms controlling ASC level in different plant species is a prerequisite that can allow scientists to identify successful strategies for vitamin C bio-fortification.

It is also necessary to move toward a novel and more efficient concept of biofortification, that at the same time takes into account the increase in nutritional value and plant stress tolerance as a unique goal of the improving strategy. Indeed,

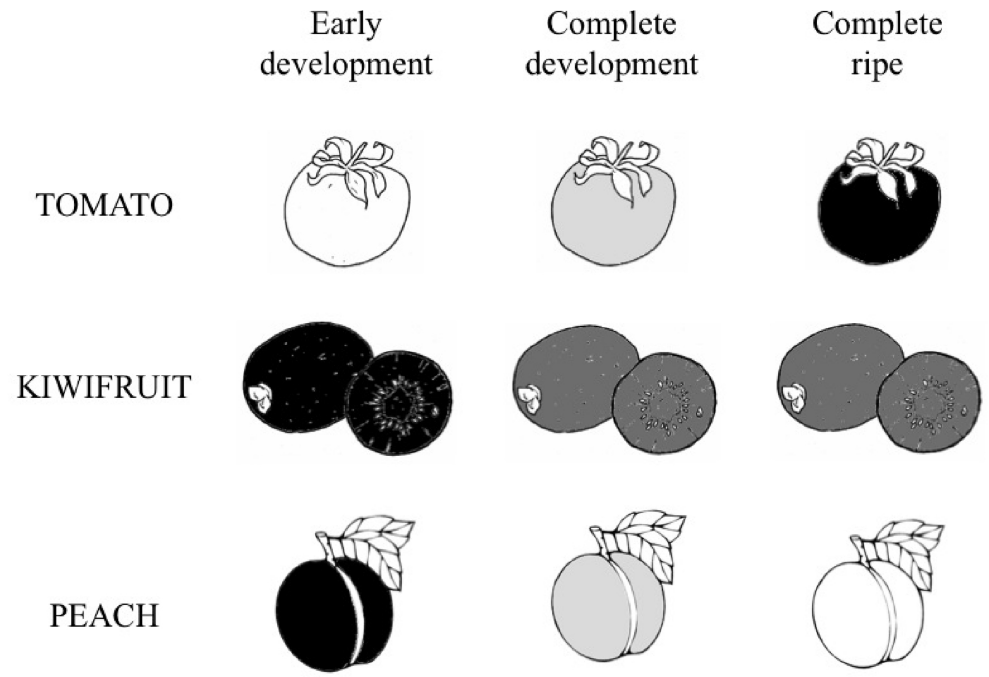

FIGURE 5 | Three models of ASC accumulation trend in different climacteric fruits. Three illustrative development and ripening phases of different fruits. White, gray and black colors correspond to the lowest, intermediate and the highest ASC content, respectively. Since the timing of the diverse stages, as well as the vitamin C levels, are quite different, depending on several factors (see text for more details), a simplification of ASC changes over fruit maturation is given on the basis of data reported in Ioannidi et al., 2009 for tomato; Imai et al., 2009 for peach, Li et al., 2010a for kiwifruit.

improvement of plant tolerance to adverse environmental conditions has also a direct positive effect on human health, as an example by possibly reducing the use of pesticides during field grown and post-harvest storage with an expectable effect on both environmental pollution and human health. Even if the level of pesticides are strictly controlled by law, a number of studies reports carcinogenic effects at high doses that can be also reaches for a low but prolonged exposure of toxic molecule (Alavanja and Bonner, 2012). Moreover, producing "fortified" crops in this novel perspective can also increase the availability of plant foods for the increasing world population. This is particularly challenging for reducing harvest and post-harvest crop losses and consequently food costs in an era of climatic changes increasing the geographic areas subjected to water and thermal stresses.

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Iron-biofortification in rice by the introduction of three barley genes participated in mugineic acid biosynthesis with soybean *ferritin* gene

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Iron deficiency is a serious problem around the world, especially in developing countries. The production of iron-biofortified rice will help ameliorate this problem. Previously, expression of the iron storage protein, ferritin, in rice using an endosperm-specific promoter resulted in a two-fold increase in iron concentration in the resultant transgenic seeds. However, further over expression of ferritin did not produce an additional increase in the seed iron concentration, and symptoms of iron deficiency were noted in the leaves of the transgenic plants. In the present study, we aimed to further increase the iron concentration in rice seeds without increasing the sensitivity to iron deficiency by enhancing the uptake and transport of iron via a ferric iron chelator, mugineic acid. To this end, we introduced the soybean *ferritin* gene (*SoyferH2*) driven by two endosperm-specific promoters, along with the barley nicotianamine synthase gene (*HvNAS1*), two nicotianamine aminotransferase genes (*HvNAAT-A* and *-B*), and a mugineic acid synthase gene (*IDS3*) to enhance mugineic acid production in rice plants. A marker-free vector was utilized as a means of increasing public acceptance. Representative lines were selected from 102 transformants based on the iron concentration in polished seeds and ferritin accumulation in the seeds. These lines were grown in both commercially supplied soil (iron-sufficient conditions) and calcareous soil (iron-deficient conditions). Lines expressing both ferritin and mugineic acid biosynthetic genes showed signs of iron-deficiency tolerance in calcareous soil. The iron concentration in polished T_3 seeds was increased by 4 and 2.5 times, as compared to that in non-transgenic lines grown in normal and calcareous soil, respectively. These results indicate that the concomitant introduction of the *ferritin* gene and mugineic acid biosynthetic genes effectively increased the seed iron level without causing iron sensitivity under iron-limited conditions.

Keywords: anemia, iron, zinc, rice, mugineic acid, biofortification, ferritin, IDS3

INTRODUCTION

Iron (Fe) is an essential micronutrient for most organisms, including all plants and animals. Fe deficiency is one of the most prevalent micronutrient deficiencies in the world, affecting an estimated two billion people (Stoltzfus and Dreyfuss, 1998) and causing 0.8 million deaths per year worldwide. Fe deficiency is ranked sixth among the risk factors for death and disability in developing countries (WHO, 2002).

There are three basic ways to solve micronutrient deficiencies: micronutrient supplementation, food fortification, and biofortification. Among these options, biofortification does not require specific processing after harvest or a special infrastructure (Grusak and DellaPenna, 1999; Mayer et al., 2008). Therefore, it is beneficial for people who may find it difficult to change their dietary habits because of financial, cultural, regional, or religious restrictions. Rice is a particularly suitable target for biofortification because Fe-deficiency anemia is a serious problem in developing countries where rice is a major staple crop (Juliano, 1993; WHO, 2002). Rice endosperm accumulates a high concentration of starch and becomes the edible part of the seed after milling, at which point the seeds are known as polished or white seeds (Juliano, 1993). Rice seeds, and especially endosperm, contain low levels of most minerals, including micronutrient metals (Grusak and Cakmak, 2005); therefore, it is important to improve the Fe concentration in polished seeds.

There are many trials to improve mineral nutrition in rice seeds by traditional breeding or transgenic methods. IR68144 was produced using traditional breeding by International Rice Research Institute (IRRI). This variety contains over two times higher Fe concentration in seeds than local varieties in Philippine (Gregorio et al., 2000). IR68144 improved the Fe states of

Philippine women better than a local rice variety (Haas et al., 2005).

Nowadays, transgenic approach can be used for the production of micronutrient-fortified rice varieties. The first transgenic approach to increase Fe concentration in rice seeds is the enhancement of Fe accumulation in rice seeds by *ferritin* gene expression under the control of endosperm-specific promoters. Goto et al. (1999) generated transgenic rice plants that expressed the soybean *ferritin* gene, *SoyferH1*, in endosperm using the endosperm-specific, 1.3-kb *OsGluB1* rice promoter; the transformants showed increased Fe accumulation in brown seeds. A few reports have described the production of Fe-biofortified rice through the endosperm-specific expression of ferritin (Lucca et al., 2002; Vasconcelos et al., 2003). Furthermore, Qu et al. (2005) expressed *SoyferH1* under the control of both the *OsGlb* promoter and 1.3-kb *OsGluB1* promoter to further increase the seed Fe concentration. However, increasing the level of ferritin expression in rice seeds did not significantly increase the Fe concentration; moreover, it caused symptoms of iron deficiency in the leaves of the transgenic plants. Thus, the enhancement of ferritin expression may not be sufficient to further increase the Fe concentration in rice grains. Qu et al. (2005) proposed that in addition to increased Fe storage in seeds, enhanced Fe uptake from the soil and enhanced translocation within the plant body are required to further improve the Fe biofortification of rice seeds.

Fe uptake, translocation, and homeostasis in rice are beginning to be understood at the molecular level (Grusak et al., 1999; Bashir et al., 2010). Graminaceous plants synthesize and secrete mugineic acid family phytosiderophores (MAs), which are natural Fe(III) chelators that take up Fe from the rhizosphere (Figure S1; Takagi, 1976; Mihashi and Mori, 1989). Nicotianamine (NA) is biosynthesized from *S*-adenosyl methionine via NAS (Higuchi et al., 1999). In graminaceous plants, including rice, deoxymugineic acid (DMA) is synthesized from NA by NA aminotransferase (NAAT) and DMA synthase (DMAS) (Takahashi et al., 1999; Bashir et al., 2006; Inoue et al., 2009). In barley and other graminaceous plants, other types of MAs are synthesized from DMA by Fe deficiency-specific clone no. 2 (IDS2) and no. 3 (IDS3: also known as mugineic acid synthase) (Nakanishi et al., 2000; Kobayashi et al., 2001). Among graminaceous plants, barley is highly tolerant to Fe deficiency and possesses a series of biosynthetic genes for MAs, including *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, *HvDMAS1*, *IDS2*, and *IDS3*, which are up-regulated in Fe-deficient barley roots (Higuchi et al., 1999; Takahashi et al., 1999; Nakanishi et al., 2000; Bashir et al., 2006). In contrast, rice lacks *IDS2* and *IDS3* and secretes only DMA. This is thought to be one of the reasons why barley has greater tolerance to Fe deficiency than rice (Kobayashi et al., 2001). In rice, Fe(III)-DMA complexes are thought to be absorbed through the transporter OsYSL15 (Inoue et al., 2009; Lee et al., 2009a). In addition to its function in Fe uptake, Fe(III)-DMA is transported into rice seeds more efficiently, as compared to Fe(III) through the rice plant body (Tsukamoto et al., 2009). Based on our knowledge of the mechanism of Fe uptake and transport by MAs in graminaceous plants, transgenic rice lines with increased tolerance to Fe deficiency were produced. Suzuki et al. (2008) cultivated three types of transgenic rice lines carrying the barley genes responsible for MAs biosynthesis (*HvNAS1*, *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3*) in a field with calcareous soil. Rice lines expressing *HvNAS1* or *IDS3* showed Fe-deficiency tolerance, possibly because of improved Fe uptake and translocation caused by the enhancement of DMA and MA biosynthesis. In addition to DMA, the introduction of *IDS3* conferred MA secretion in rice (Kobayashi et al., 2001). Because MA have greater Fe(III)-complex stability than DMA at a slightly acidic pH (von Wirén et al., 2000), the production of MA via *IDS3* might be advantageous for Fe translocation in rice. Furthermore, because these transformants contained introduced barley genome fragments, expression of the genes responsible for MAs biosynthesis was regulated by their own promoters. In rice, these promoters induced expression in response to Fe deficiency in roots and leaves (Higuchi et al., 2001; Kobayashi et al., 2001). Thus, these genes are expected to be expressed when and where the requirement for Fe is elevated.

The Fe concentration in seeds of rice lines transformed with *HvNAS1*, *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3* was analyzed after cultivation in the field in Fe-sufficient (Andosol) or Fe-deficient (calcareous) soil (Masuda et al., 2008; Suzuki et al., 2008). The *IDS3* rice line showed an increased Fe concentration in polished seeds up to 1.25–1.4 times that in non-transgenic (NT) rice following cultivation in Andosol and calcareous soil (Masuda et al., 2008; Suzuki et al., 2008).

In the present report, we produced Fe biofortified rice by the concomitant introduction of soybean *ferritin* gene (*SoyferH2*) under the control of the *OsGluB1* and *OsGlb* promoters and barley genes encoding enzymes for MAs biosynthesis (genome fragments of *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3*). The transformants exhibited Fe-deficiency tolerance in calcareous soil. The Fe concentration in T_3 polished seeds was increased 4 and 2.5 times, as compared to that in NT plants grown in commercially supplied soil and calcareous soil, respectively. We found that Fe biofortification through the concomitant introduction of genes encoding ferritin and biosynthetic enzymes for MAs effectively increased the seed Fe level and improved Fe sensitivity under Fe limitation, which is caused in case of single introduction of *ferritin*.

MATERIALS AND METHODS

PLANT MATERIALS

The *Japonica* rice (*Oryza sativa* L.) cultivar Tsukinohikari was used as the NT control and for transformation.

VECTOR CONSTRUCTION, CONFIRMATION OF VECTOR CONSTRUCT AND RICE TRANSFORMATION

pBIMFN (marker-free vector), which was produced by Nishizawa et al. (2006), was used as the backbone of the binary vector for rice transformation. Using this vector, the Fer-NAS-NAAT-IDS3 and Fer rice transformation vectors were constructed according to the scheme shown in Figures S2, S3, respectively. The constructed vectors were verified by PCR, as shown in Figure S4. For Fer-NAS-NAAT-IDS3 vector, Glbp 5′R primer 5′-ACC AGA TAC AAC GGG TCC CTC-3′ and NAAT 5′R primer 5′-GGT ATC GCC ATT CGC CAA GCC AGT-3′ were used to confirm

the gene connection of gene cassette Os*Glb promoter-SoyferH2* and *HvNAAT-A, -B*. NAAT 3′F primer 5′-GTC ACT CGC TCT ATC TTG GTC ATT G-3′ and NAS 5′R primer 5′-GTT GAG GAT ACA CTA TTG CTC ATG C-3′ were used to confirm the gene connection of *HvNAAT-A, -B* genome and *HvNAS1* genome. NAS 3′F primer 5′-GAC TAA GCG TCG TCA TGA ACC TGT G-3′ and tNos 3′F primer 5′-GAA TCC TGT TGC CGG TCT TGC G-3′ were used to confirm the gene connection of *HvNAS1* genome and Os*GluB1 promoter-SoyferH2* gene construct. GluBp 5′R primer 5′-TGA ACA GTC GTG CTC ACG GTC-3′ and IDS3g 5′R primer 5′-AAC ACA GTA TAG ACG CAA GTG TTC A-3′ were used to confirm the gene connection of *OsGluB1 promoter-SoyferH2* gene construct and *IDS3* genome. For Fer vector, Glbp 5′R primer and GluBp 5′R primer were used to confirm the gene connection of gene cassette Os*Glb promoter-SoyferH2* and *OsGluB1 promoter-SoyferH2*. Sequence of PCR product was checked by ABI PRISM 310(ABI) and compared to the expected sequence from the data.

Agrobacterium tumefaciens (strain C58) was used to introduce the constructs into *O. sativa* L. cv. Tsukinohikari using the method outlined in Hiei et al. (1994). Transgenic calli were serially selected by 10, 20, and 30 mg/L concentrations of hygromycin. 30 mg/L concentration of hygromycin was also included in regeneration medium and root elongation medium.

GREENHOUSE CULTIVATION

T_0 regenerate plants as well as T_1 Fer-NAS-NAAT-IDS3 lines, Fer lines, and NT plants were germinated on Murashige and Skoog (MS) medium and cultivated in 3.5 CL pots (1,000-ml volume; Kaneya, Aichi, Japan) containing a 2:1 mixture of Bonsol-ichigou (commercially supplied soil used for rice cultivation in Japanese nurseries; Sumitomo Chemicals, Tokyo, Japan) and vermiculite (Green Tec, Tochigi, Japan). The soil was evenly fertilized with 3.5 g of Long Total-70 and Long Total-140 (slow-release fertilizers; JCAM AGRI Co. Ltd., Tokyo, Japan; N:P:K, 13:11:13 and 2% Mg, 0.1% Mn, 0.06% B, 0.20% Fe, 0.050% Cu, 0.015% Zn, and 0.020% Mo as micronutrients) per plant. The plants were grown in a greenhouse under natural light conditions, with 14 h of light at 30°C and 10 h of dark at 25°C. Six plants each of the T_2 Fer-NAS-NAAT-IDS3 lines (1-12, 22-4, and 34-11), Fer line 13-6, and NT plants were cultivated in commercially supplied soil and vermiculite as described above under Fe-sufficient conditions. The T_2 plants were also cultivated in calcareous soil (pH = 8.9) obtained from Takaoka City, Toyama, Japan (Nihonkai Kougyo, Toyama, Japan) in 3.5 CL pots with 3.5 g of Long Total-70 and -140 under Fe-deficient conditions. The seeds obtained from greenhouse cultivation were used for metal concentration analysis.

For Northern blot analysis, T_2 Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11 were germinated on MS medium. After 3 weeks, six seedlings from each line were transferred to nutrient solution [2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 0.1 mM Fe(III)-EDTA, 10 mM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, and 0.01 μM $(NH_4)_6Mo_7O_{25}$] and grown in a greenhouse under the conditions described above. The pH of the culture solution was adjusted daily to between 5.5 and 5.8 with 1 N HCl. After 8 days, the plants were transplanted to fresh nutrient solution without Fe(III)-EDTA and cultivated for 1 week. Next, the leaf chlorophyll level was measured using a SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan), and leaves and roots were harvested for Northern blot analysis.

GENOMIC PCR

For each line, leaf samples were harvested and DNA was extracted using an automated genomic DNA isolation system (NA-2000; Kurabo, Osaka, Japan). Next, genomic PCR was performed using the following primers. The *OsGluB1* promoter *SoyferH2* construct was detected using the *OsGluB1* promoter FW primer (5′-CAG CTC TCC GTG GTC AGA TGT G-3′) and *SoyferH2* RV primer (5′-GCC ACA CAC CAT GAC CCT TTC CAA C-3′). The *OsGlb* promoter *SoyferH2* construct was detected using the *OsGlb* promoter FW primer (5′-CCA ACC GAT CCA TGT CAC CCT CAA GC-3′) and *SoyferH2* RV primer. *IDS3* genome insertion was detected using the *IDS3* FW primer (5′-AAG CTT ACT GGT TGG ACG GTA TTT CA-3′) and *IDS3* RV primer (5′-GGA TCC ACG GGC CAC ATG ATC CA-3′). *HvNAAT-A* genome insertion was detected using the *HvNAAT-A* FW primer (5′-GTG TTG CAT GTC AAA TGA CCG G-3′) and *HvNAAT-A* RV primer (5′-CTA CTC CCT CTG TCC CAA AAT AAC TG-3′). *HvNAAT-B* genome insertion was detected using the *HvNAAT-B* FW primer (5′-CCG AAA ATG CAT CCA ACA TAA TTA C-3′) and *HvNAAT-B* RV primer (5′-GCC AAT GTA ACT TCA CTA ACA TAA C-3′). *HvNAS1* genome insertion was detected using the *HvNAS1* FW primer (5′-CGG TGG AGG TAA TAG CCC TAC GTC-3′) and *HvNAS1* RV primer (5′- GGA GGC AGT CCT GTT GTG GCA TTC-3′).

NORTHERN BLOT ANALYSIS

The ORFs for *HvNAS1* (AB010086), *HvNAAT-A* (D88273), *HvNAAT-B* (AB005788), and *IDS3* (AB024058) were used to synthesize *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3* probes using the primers described in Suzuki et al. (2006). This fragment was labeled with [a-^{32}P]-dATP using the random labeling method; the labeled DNA was purified using a ProbeQuant G-50 Micro Column (Pharmacia, Uppsala, Sweden). Total RNA from the roots and shoots was extracted using the sodium dodecyl sulfate (SDS)-phenol method. Aliquots of the total RNA (20 μg per lane) were separated on 1.4% (w/v) agarose gels. Blotting, hybridization, and radioactive detection were performed as described previously (Ogo et al., 2006; Suzuki et al., 2006).

WESTERN BLOT ANALYSIS

Six T_2 or six T_3 mature seeds of the Fer-NAS-NAAT-IDS3, Fer, and NT lines were harvested and examined for ferritin expression by Western blotting. The seeds were homogenized with a mortar and pestle, soaked in extraction buffer (4% SDS, 5% 2-mercaptethanol, 20% glycerol, 20 mM Tris-HCl, 8 M urea, and 0.1% bromophenol blue, pH 6.8), and shaken for 30 min. The resulting extracts were centrifuged at 13,000 rpm for 20 min and supernatant fractions were collected. Protein separation by SDS-polyacrylamide gel electrophoresis, transfer to polyvinylidene fluoride membranes, and detection with antibodies were performed as described in Goto et al. (1999).

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS OF *SoyferH2* IN FE DEFICIENT LEAVES

Plants were germinated on MS medium. After 3 weeks, three plants from each line were cultivated in nutrient solution with Fe(III)-EDTA for 2 weeks and then in nutrient solution without Fe(III)-EDTA for 13 days. Total RNA was extracted from the second newest leaf of each plant by using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan). Real-time RT-PCR was carried out using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Tokyo, Japan) with SYBR Premix Ex Taq II (Takara, Shiga, Japan). *SoyferH2* forward (5′-GCT TTT ATC TCT CGC CCG TTG-3′) and *SoyferH2* reverse (5′-CAT TGT GTG CAA TCG GAA CAG C-3′) primers were used. Transcript levels were normalized to the expression levels of alpha-*Tubulin*, as determined using the primers alpha-*Tubulin* forward (5′-TCT TCC ACC CTG AGC AGC TC-3′) and alpha-*Tubulin* reverse (5′-AAC CTT GGA GAC CAG TGC AG-3′). The sizes of the amplified fragments were confirmed by agarose gel electrophoresis.

METAL CONCENTRATION ANALYSIS

A seed metal concentration analysis was performed according to the method of Masuda et al. (2008); Masuda et al. (2009). Brown seeds were collected randomly from the ear of the main tiller (the tiller in the center or the largest among all tillers in one plant). Ten seeds from each plant were dried overnight at 80°C in a heat drying machine. After determining the dry weight of each sample, the seeds were digested in 1 ml of 13 M HNO_3 and 1 ml of 8.8 M H_2O_2 (Wako, Osaka, Japan) at 200°C for 20 min with MARS Xpress (CEM, Matthews, NC, USA). After digestion, the samples were diluted to a volume of 5 ml and analyzed via inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko, Tokyo, Japan).

To obtain polished seeds, 30 brown seeds from the ear of the main tiller were placed into a 2-ml tube and shaken vigorously for 150 s at 2500 rpm for four cycles using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Ten well polished seeds from each line were selected and dried overnight, weighed, digested, and then metal concentration was analyzed as described above.

Rice husks (approximately 100 mg) were also dried overnight, weighed, digested, and subjected to metal concentration analysis as described above.

STATISTICS

Student's *t*-test was used for each sample set to compare the data for the significant differences. The level of significance was set at $P < 0.05$.

RESULTS

PRODUCTION OF Fer-NAS-NAAT-IDS3 TRANSGENIC LINES

To produce transgenic rice lines that concomitantly expressed soybean ferritin and barley enzymes for MAs biosynthesis, the transformation vector Fer-NAS-NAAT-IDS3 was produced (**Figure 1A**). This vector contained the *OsGluB1* promoter-*SoyferH2*, *OsGlb* promoter-*SoyferH2*, a 5-kb *HvNAS1* genome fragment, an 11-kb *HvNAAT-A,-B* genome fragment, and a 5-kb *IDS3* genome fragment in the marker-free vector pBIMFN (Nishizawa et al., 2006). This vector was used for rice

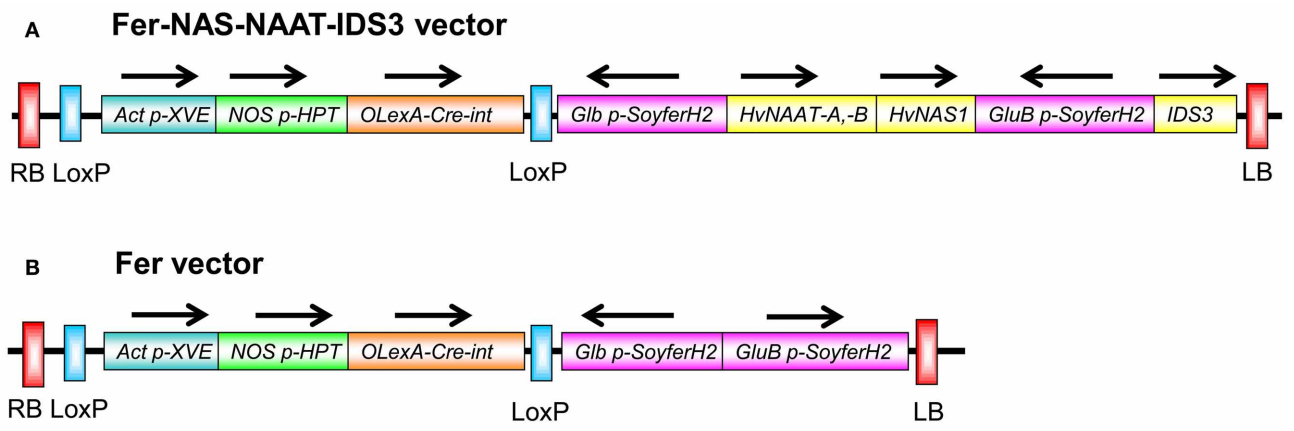


FIGURE 1 | T-DNA regions of the constructs introduced into rice. **(A)** Fer-NAS-NAAT-IDS3 vector. **(B)** Fer vector. Arrows show the direction of transcription. RB, right border; LB, left border; *Act p*, promoter region of rice *OsActin1* (Os03g0718100); *XVE*, estradiol receptor-based transcription factor gene (Zuo et al., 2001); *NOS p*, nopaline synthase (NOS) gene promoter (Zuo et al., 2001); *HPT*, hygromycin phosphotransferase gene (K01193); *OLexA*, eight copies of the *LexA* operator sequence fused to the −46 CaMV *35S* promoter (Zuo et al., 2001); *Cre-int*, the coding sequence of Cre recombinase interrupted by an intron (Zuo et al., 2001); *Glb p*, promoter region of the 26-kDa *OsGlb1* gene (AY427575) (Qu and Takaiwa, 2004); *SoyferH2*, soybean *ferritin* gene (AB062754) (Masuda et al., 2001); *HvNAAT-A,-B*, barley genome fragment containing the nicotianamine aminotransferase genes *HvNAAT-A* and *HvNAAT-B* (AB024006); *HvNAS1*, barley genome fragment containing a nicotianamine synthesis gene (Higuchi et al., 2001); *GluB p*, 2.3-kb promoter region of *OsGluB1* (AY427569) (Qu and Takaiwa, 2004); *IDS3* genome, barley genome fragment containing a mugineic acid synthesis gene (AB024007). Each gene expression cassette, except the *HvNAS1* genome, *HvNAAT-A,-B* genome, and *IDS3* genome fragments, possessed the *A. tumefaciens* NOS gene terminator (AF485783) *Tnos* at the 3′ end of the *ORF*. The T-DNA region between two *LoxP* sites can be removed from the vector by estradiol treatment (Cre/loxP system; Zuo et al., 2001; Figure S8). The vector backbone was derived from pBIMFN (Nishizawa et al., 2006).

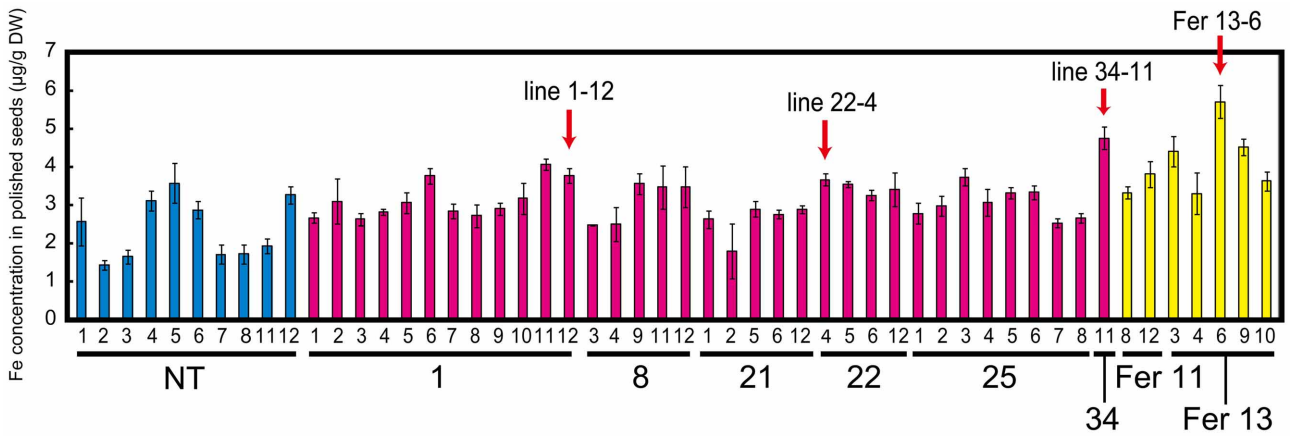


FIGURE 2 | Fe concentration in polished T_2 seeds. NT, non-transgenic line (blue bars); 1, 8, 21, 22, 25, and 34, Fer-NAS-NAAT-IDS3 lines (red bars); Fer 11 and Fer 13, Fer lines (yellow bars). Small numerals under each bar indicate the number of sub-lines. The lines indicated with arrows were selected for further analysis. Bars represent the means ± standard errors of three independent analyses ($n = 3$).

transformation and 102 lines were produced (referred to as Fer-NAS-NAAT-IDS3 lines).

To determine the advantage of introducing biosynthetic genes for MAs together with the *ferritin* gene, another rice transformation vector, Fer vector, which included the *OsGluB1* promoter-*SoyferH2* and *OsGlb* promoter-*SoyferH2* but no genes for MAs biosynthesis, was also produced (**Figure 1B**). Using this vector, 14 lines were obtained (referred to as Fer lines). The efficiency of transgenic plant production was lower in the Fer-NAS-NAAT-IDS3 lines than in the Fer lines and other transgenic lines of Tsukinohikari (data not shown). Among the transformants obtained, we screened desirable lines on the basis of the Fe concentration level in polished seeds rather than the transgene expression.

OsGluB1 promoter-*SoyferH2* and *IDS3* genome insertion in the Fer-NAS-NAAT-IDS3 lines was detected by genomic PCR. Among the 102 lines, insertion of the *OsGluB1* promoter-*SoyferH2* and *IDS3* was confirmed in Fer-NAS-NAAT-IDS3 lines 1, 4, 12, 13, 14, 16, 18, 19, 21, 25, 27, 30, and 34 (data not shown). Among the 14 Fer lines, insertion of the *OsGluB1* promoter-*SoyferH2* was confirmed in lines 2, 11, 13, and 14 (data not shown). Next, the transgenic lines were cultivated in a greenhouse to obtain seeds.

FE CONCENTRATION MEASUREMENT AND FERRITIN ACCUMULATION IN T_1 SEEDS

Among the transformants obtained, we screened desirable lines on the basis of Fe concentration level in polished seeds rather than transgene expression. Therefore, after harvest, the Fe concentration in polished T_1 seeds was measured. The Fe concentration was around two to three times higher in Fer-NAS-NAAT-IDS3 lines 8, 14, 21, 22, 25, and 34, as compared to that in the NT line (Figure S5). These Fer-NAS-NAAT-IDS3 lines and Fer lines 11 and 13 were cultivated in a greenhouse and the Fe concentration in polished T_2 seeds was analyzed (**Figure 2**). Among the Fer-NAS-NAAT-IDS3 lines, 1-12, 22-4, and 34-11 showed especially high Fe concentrations. These lines also exhibited normal yields (data not shown) and were therefore selected for further cultivation and detailed analysis. Among the Fer lines, 13-6 had a higher Fe concentration than the other Fer sub-lines. This line was also selected for further analysis. SoyferH2 accumulation in brown seeds of the selected lines (Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11, and Fer line 13-6) was confirmed (**Figure 3**).

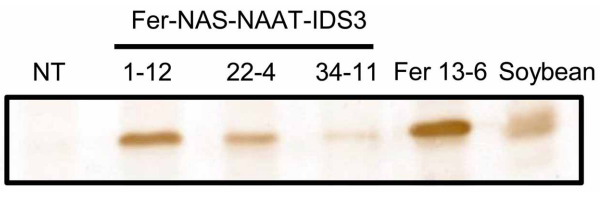


FIGURE 3 | Ferritin accumulation in T_2 seeds as determined by Western blot analysis. NT, non-transgenic seeds; 1-12, 22-4, and 34-11, T_2 seeds of the Fer-NAS-NAAT-IDS3 lines; Fer 13-6, T_2 seeds of Fer line 13-6; Soybean, protein extracted from soybean seeds used as a positive control for soybean ferritin.

HYDROPONIC CULTURE UNDER FE-DEFICIENT CONDITIONS AND GENE EXPRESSION PATTERNS OF *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, AND *IDS3*

To confirm the expression of *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3* in the Fer-NAS-NAAT-IDS3 lines, plants were grown in hydroponic culture under both Fe-sufficient and -deficient conditions. After 1 week of Fe-deficient cultivation, the leaf color in Fer-NAS-NAAT-IDS3 lines 22-4 and 34-11 remained greener than that in the NT line (**Figure 4A**), as confirmed by the higher SPAD value (leaf chlorophyll index; **Figure 4B**). In contrast, the leaves of Fer line 13-6 were yellow and the SPAD value was lower than in the NT line and Fer-NAS-NAAT-IDS3 lines (**Figures 4A,B**).

After Fe-deficiency treatment, total RNA was extracted from the roots and shoots, and the expression of biosynthetic genes for

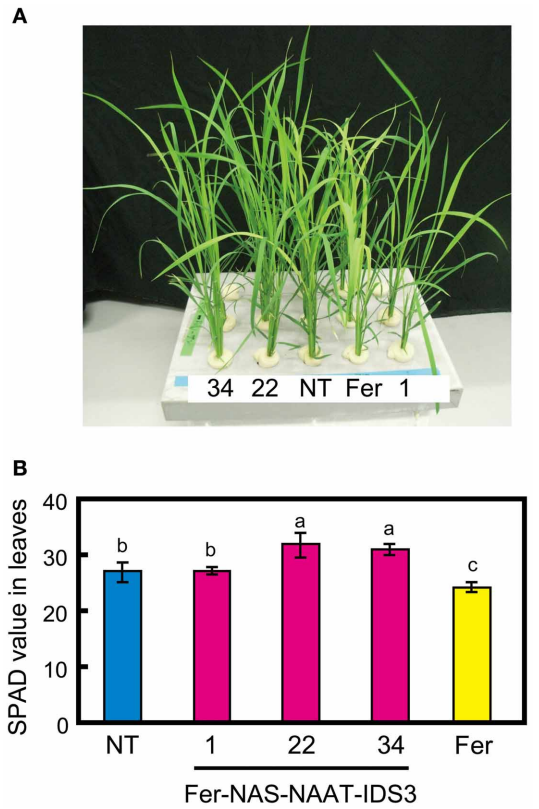


FIGURE 4 | Fe-deficiency symptoms in hydroponically grown transgenic lines. (A) Appearance of T_2 plants cultivated for 7 days in hydroponic culture solution without Fe. **(B)** The SPAD value of T_2 leaves under Fe-deficient conditions. NT, non-transgenic line; 1, 22, and 34, Fer-NAS-NAAT-IDS3 T_2 lines 1-12, 22-4, and 34-11, respectively; Fer, Fer line 13-6. Bars represent the means ± standard errors of six independent plants ($n = 6$). Different letters above the bars indicate significant differences ($P < 0.05$) by Student's *t*-test for each line.

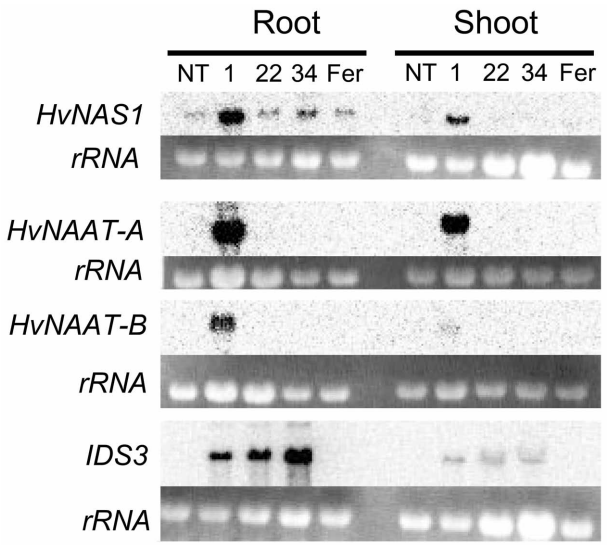


FIGURE 5 | Expression analysis of mugineic acid synthetic genes by Northern blotting. Total RNA was extracted from the roots and shoots of T_2 plants grown for 8 days under Fe-deficient hydroponic culture. NT, non-transgenic line; 1, Fer-NAS-NAAT-IDS3 line 1-12; 22, Fer-NAS-NAAT-IDS3 line 22-4; 34, Fer-NAS-NAAT-IDS3 line 34-11; Fer, Fer line 13-6; rRNA, rRNA band detected by ethidium bromide staining as a loading control.

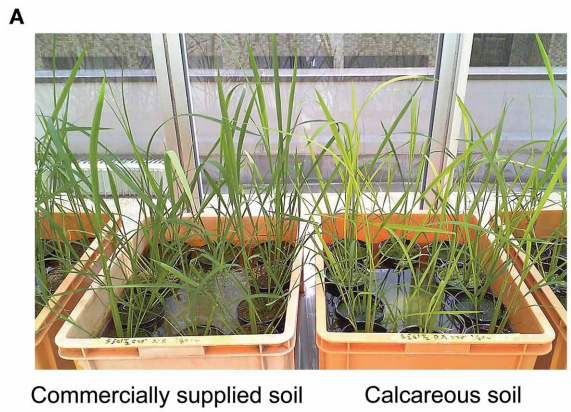
MAs (*HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3*) was analyzed by Northern blot analysis (**Figure 5**). In Fer-NAS-NAAT-IDS3 line 1-12, all of the introduced genes were expressed strongly in the roots, and they were also expressed in the shoots. In Fer-NAS-NAAT-IDS3 lines 22-4 and 34-11, *IDS3* expression was detected as same as line 1-12. On the other hand, *HvNAAT-A* and *-B* expression was not detected in lines 22-4 and 34-11. In these lines, weak bands were detected by *HvNAS1* hybridization, but the band strength was similar to that in the NT line and Fer line 13-6, in which *HvNAS1* was not introduced. These weak bands might be derived from endogenous *OsNAS1* expression, which could be hybridized to the *HvNAS1* probe because of the strong similarity between *HvNAS1* and *OsNAS1* (84% identity). To confirm the reason for the lack of strong *HvNAS1*, *HvNAAT-A*, and *HvNAAT-B* expression in lines 22-4 and 34-11, insertion of these genes was verified by genomic PCR (Figure S6). The insertion of all of these genes was detected in Fer-NAS-NAAT-IDS3 line 1, but not in lines 22-4 and 34-11, or in the NT line and Fer line 13-6.

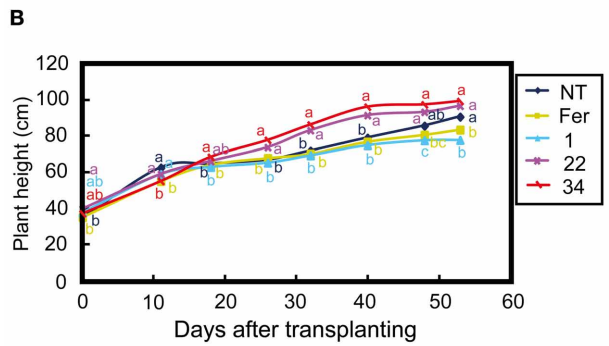
GROWTH IN CALCAREOUS SOIL

Suzuki et al. (2008) showed that rice lines with introduced barley *HvNAS1* or the *IDS3* genome fragment grew better than NT plants in calcareous soil. Therefore, we expected that Fer-NAS-NAAT-IDS3 rice would also exhibit Fe-deficiency tolerance, which might be distinguishable in calcareous soil. Therefore, NT plants and T_2 plants of Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, 34-11 and Fer line 13-6 were grown in both commercially supplied soil (Fe-sufficient conditions) and calcareous soil (Fe-deficient conditions) in a greenhouse (**Figure 6A**). The shoot height was higher in Fer-NAS-NAAT-IDS3 lines 22-4 and 34-11 than in the NT line and Fer line 13-6 at 30 days after transplanting (DAT) in calcareous soil (**Figure 6B**). At around 60 DAT, the shoots of Fer-NAS-NAAT-IDS3 lines 22-4 and 34-11 were 15-20 cm higher than those of the NT line and Fer line 13-6. The SPAD values were also higher in Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11 than in the NT line and Fer line 13-6 at around 10-30 DAT. The SPAD value was lowest in Fer line 13-6 among all of the lines after 26 DAT (**Figure 6C**).

FERRITIN ACCUMULATION AND FE CONCENTRATION IN T_3 SEEDS

Ferritin accumulation was observed in T_3 seeds of Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11, and in Fer line 13-6 seeds by Western blot analysis (**Figure 7**). Next, the metal concentrations in T_3 seeds were analyzed (**Figures 8**, **9**). After calcareous soil cultivation, the average Fe concentrations in Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11, and in Fer line 13-6 were 4.0, 4.0, 4.9, and 3.3 μg/g, respectively, which is higher than that in the NT line (2.0 μg/g) (**Figure 8A**). After commercially supplied soil cultivation, the average Fe concentrations in Fer-NAS-NAAT-IDS3





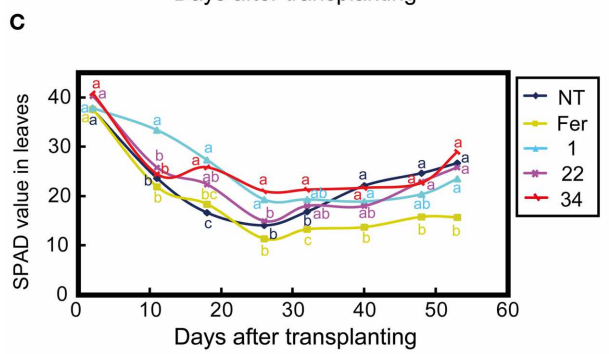


FIGURE 6 | Calcareous soil cultivation of T_2 transgenic lines. **(A)** Appearance of plants grown in commercially supplied soil and calcareous soil (47 days after germination and 11 days after transplanting to soil). **(B)** Shoot height during calcareous soil cultivation. **(C)** SPAD value of leaves during calcareous soil cultivation. NT, non-transgenic line; Fer, Fer line 13-6; 1, 22, and 34, Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11, respectively. The data shown are the average of six plants ($n = 6$). Different letters beside the graph of each line indicate significant differences ($P < 0.05$) by Student's *t*-test for each line.

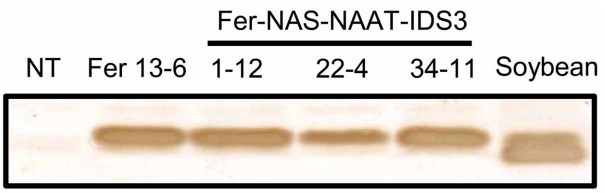


FIGURE 7 | Ferritin accumulation in T_3 seeds. Total protein was extracted from the seeds of six independent T_3 plants for each line cultivated in calcareous soil, and ferritin was detected by Western blot analysis. NT, non-transgenic seeds; 1-12, 22-4, and 34-11, T_3 seeds of the corresponding Fer-NAS-NAAT-IDS3 lines; Fer 13-6, T_3 seeds of Fer line 13-6; Soybean, protein extracted from soybean seeds as a positive control for soybean ferritin.

lines 1-12, 22-4, and 34-11, and in Fer line 13-6 were 2.5, 3.4, 4.0, and 3.3 μg/g, respectively, which is also higher than that in the NT line (1.1 μg/g) (**Figure 8B**). The Fe concentrations in brown seeds of Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11 were also higher than that in the NT line following cultivation in either calcareous soil or commercially supplied soil (**Figures 8C,D**). There was no difference in the Fe concentration in brown seeds between the Fer and NT lines. The Zn concentrations in polished and brown seeds were up to 30% higher in Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11, as compared to the NT and Fer lines following cultivation in calcareous soil or commercially supplied soil (**Figures 9A–D**). There was no remarkable difference in the husk Fe or Zn concentration between the transgenic and NT lines (**Figures 8E,F** and **9E,F**).

In addition to Fe and Zn concentrations, the Fe and Zn contents in endosperm per seed were also higher in Fer-NAS-NAAT-IDS3 lines than in Fer or NT lines following cultivation in both calcareous and commercially supplied soil (Figure S7). Fe and Zn content in bran also tended to increase in Fer-NAS-NAAT-IDS3 lines, with line 34-11 showing significant increase compared to NT lines.

DISCUSSION

GENERATION OF Fer-NAS-NAAT-IDS3 VECTOR

To generate new Fe-fortified rice with improved growth under conditions of low Fe availability, we produced transgenic rice lines in which the soybean *ferritin* gene and barley genes responsible for MAs biosynthesis were introduced (**Figure 1A**). We designed the expression vector Fer-NAS-NAAT-IDS3 with the following issues in mind.

Soybean seeds possess two types of ferritin proteins: SoyferH1 (26.5 kDa) and SoyferH2 (28 kDa) (Masuda et al., 2001). *SoyferH1* can be digested by proteases, which may alter its structure, allowing Fe release, while *SoyferH2* is more resistant to protease digestion than *SoyferH1* (Masuda et al., 2001). Thus, we assumed that the stable ferritin, *SoyferH2*, would be more suitable for Fe accumulation in rice seeds. Therefore, we used *SoyferH2* instead of *SoyferH1* for expression in rice endosperm, which is similar to our previous report (Masuda et al., 2012). In fact, two ferritin bands appeared in soybean seed protein by Western blot analysis, among which the upper band (28 kDa) matched the ferritin bands in our Fer-NAS-NAAT-IDS3 and Fer lines (**Figures 3**, **7**).

Qu et al. (2005) reported that transgenic seeds with introduced *ferritin* under the control of both the 1.3-kb *OsGluB1* promoter and *OsGlb* promoter showed increased accumulation of ferritin compared to those with *ferritin* expressed under the control of either the 1.3-kb *OsGluB1* promoter or the *OsGlb* promoter. In the present study, *SoyferH2* was expressed under the control of both the 2.3-kb *OsGluB1* promoter and the *OsGlb*

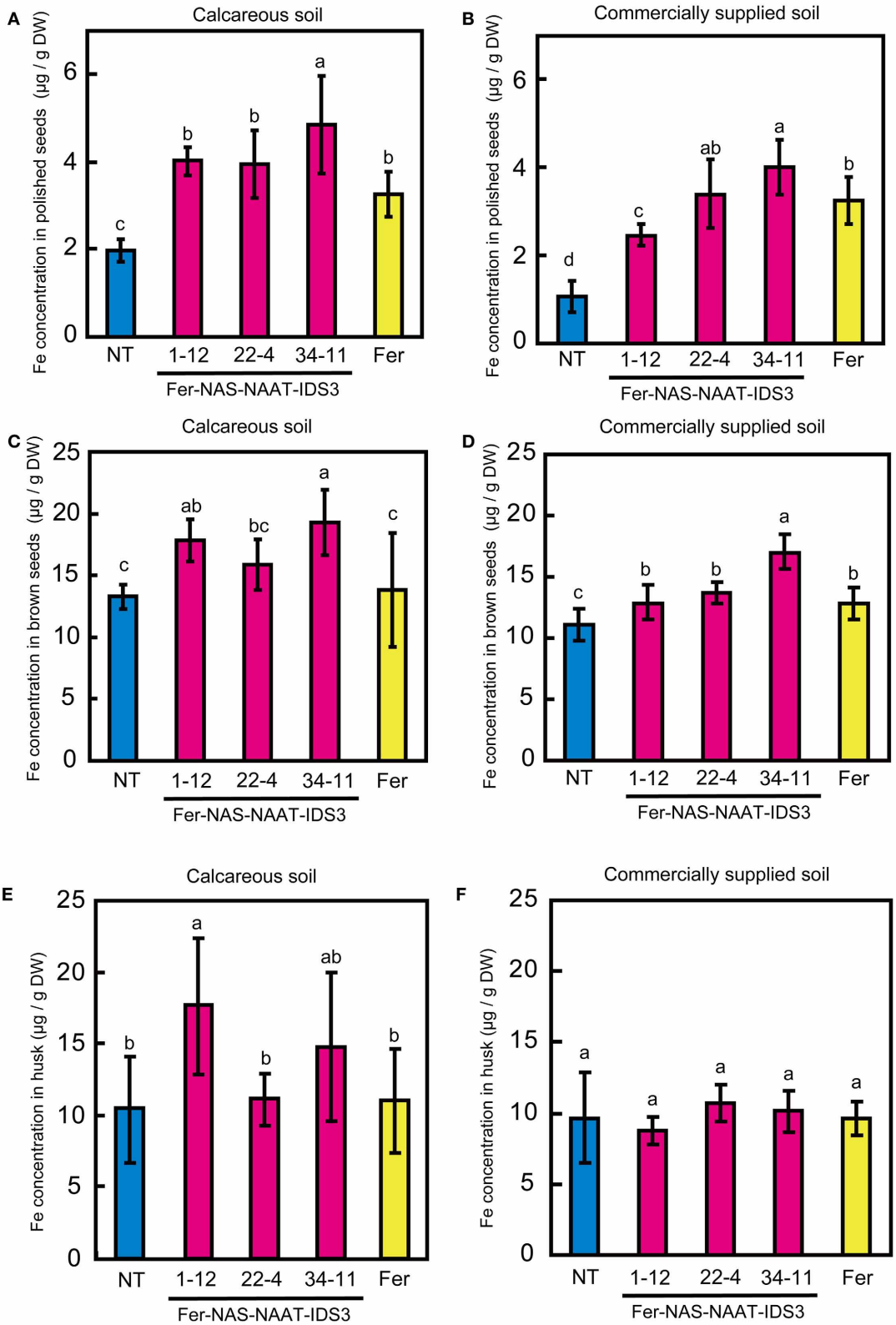


FIGURE 8 | Fe concentration in T_3 seeds. (A,B) Polished seeds. **(C,D)** Brown seeds. **(E,F)** Husk. Plants were cultivated in calcareous soil **(A**, **C**, and **E)** or commercially supplied soil **(B**, **D**, and **F)**. Bars represent the means ± standard errors of six independent plants ($n = 6$). NT, non-transgenic line; 1-12, 22-4, and 34-11, Fer-NAS-NAAT-IDS3 T_3 lines; Fer, Fer T_3 line 13-6. Different letters above the bars indicate significant differences ($P < 0.05$) by Student's *t*-test for each line.

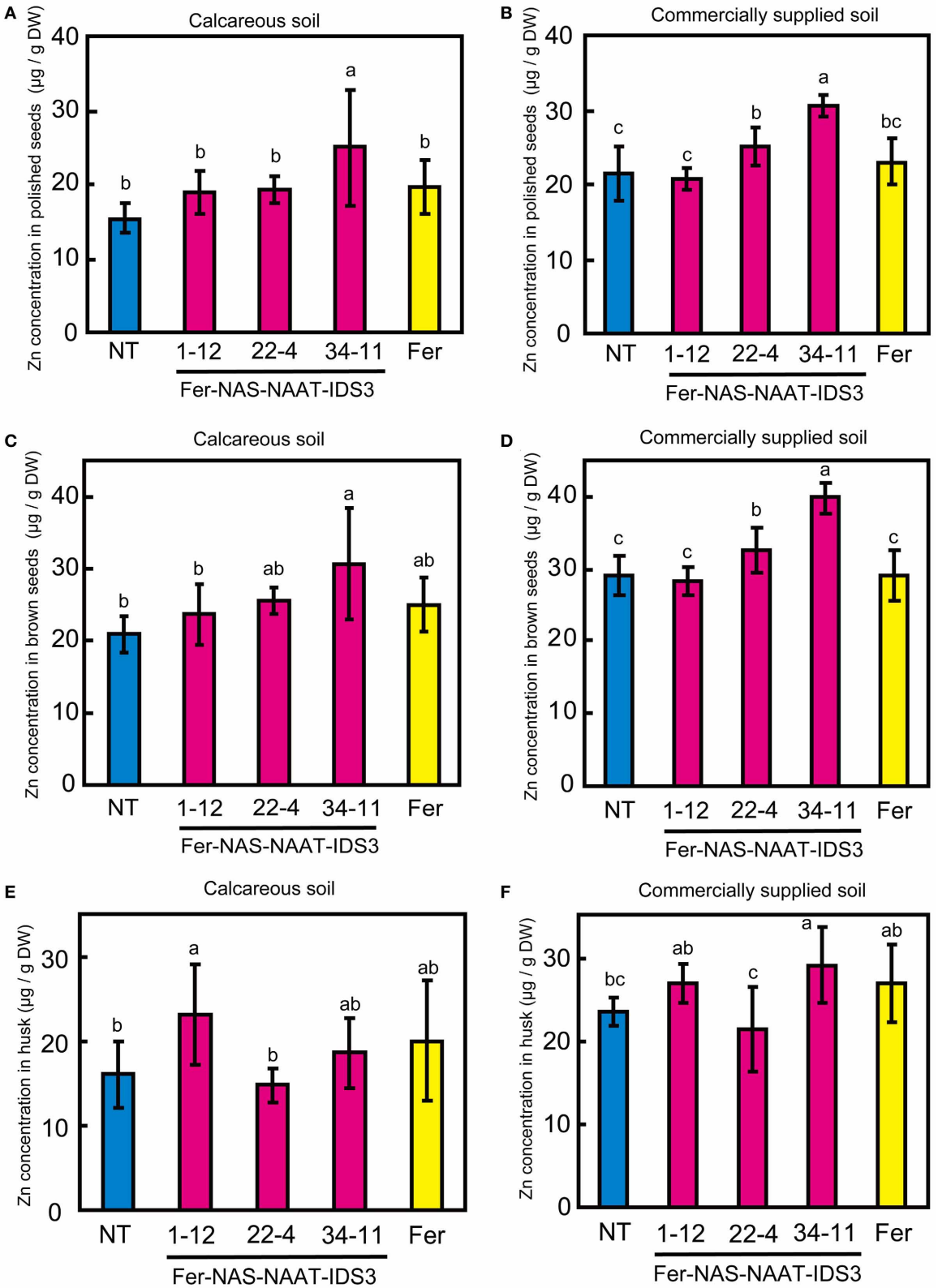


FIGURE 9 | Zn concentration in T_3 seeds. (A,B) Polished seeds. **(C,D)** Brown seeds. **(E,F)** Husk. Plants were cultivated in calcareous soil **(A**, **C**, and **E)** or commercially supplied soil **(B**, **D**, and **F)**. Bars represent the means ± standard errors of six independent plants ($n = 6$). NT, non-transgenic line; 1-12, 22-4, and 34-11, Fer-NAS-NAAT-IDS3 T_3 lines; Fer, Fer T_3 line 11-6. Different letters above the bars indicate significant differences ($P < 0.05$) by Student's *t*-test for each line.

promoter (**Figure 1**) in order to achieve high ferritin accumulation in seeds, which is similar to our previous report (Masuda et al., 2012).

For the expression of genes for MAs biosynthesis in rice, the introduction of barley genomic fragments containing the corresponding genes and their promoters has been proven to be highly effective (Higuchi et al., 2001; Kobayashi et al., 2001). However, the introduction of multiple genomic fragments requires a large T-DNA insert, which causes difficulty in transformation. To solve this problem, pBIGRZ1 was used as a suitable binary vector because it allows the introduction of large insertions into the rice genome (Kawasaki, 2003). Using this vector as a backbone, pBIMFN (marker-free vector) was produced (Nishizawa et al., 2006). This vector, which utilizes the Cre/loxP system, allows unnecessary portions of the T-DNA to be removed at any time from Fer-NAS-NAAT-IDS3 lines through estradiol treatment (Zuo et al., 2001) (Figure S8). Usuda et al. (2009) produced transgenic rice lines using this marker-free vector, and successfully removed the marker region from selected transgenic rice lines through estradiol treatment of the seeds. After removal of the two *loxP* regions from Fer-NAS-NAAT-IDS3 lines, all of the remaining transgenes would be derived from rice, soybean, and barley. This should make crops produced using this method easier for the public to accept, as compared to those created using the original binary vector, which includes bacterial selection marker genes such as that encoding hygromycin phosphotransferase or neomycin phosphotransferase II.

CONFIRMATION OF INSERTED GENES

Expression of all transgenes, including *SoyferH2*, *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3*, was observed only in line 1-12 (**Figures 3**, **5**, and **7**). Lines 22-4 and 34-11 did not possess the *HvNAS1*, *HvNAAT-A*, and *HvNAAT-B* transgenes, although *IDS3* expression and ferritin accumulation were observed (Figure S6). In the present study, the Fer-NAS-NAAT-IDS3 construct introduced into rice was large: about 35 kb between the right and left borders (Figure S8). Nakano et al. (2005) introduced a 92-kb wheat genome fragment into rice by *Agrobacterium*-mediated transformation, but none of the four transgenic rice lines possessed the entire sequence; instead, fragments had been inserted. In addition to the 5′ or 3′ sides of the transgene that were missing, the central parts of the transgenes were also found to be missing. Similarly, the insertion of fragments is thought to have occurred in our Fer-NAS-NAAT-IDS3 transgenic lines. The *IDS3* transcripts and ferritin proteins expressed in lines 1-12, 22-4, and 34-11 were similar in size, suggesting a lack of deletion in the expression cassettes for these transgenes (**Figures 3**, **5**, and **7**). Lines 22-4 and 34-11 may have lost the central part of the Fer-NAS-NAAT-IDS3 vector, between the *HvNAS1* genome fragment and *HvNAAT-A,-B* genome fragment. Nevertheless, these lines, as well as line 1-12, showed effective Fe accumulation in polished seeds both under Fe-sufficient and -deficient conditions (**Figures 8A,B**), along with improved tolerance to Fe deficiency (**Figure 6**), providing promising candidates for future applications as Fe-fortified crops that can tolerate Fe-limiting environments.

THE SINGLE INTRODUCTION OF *ferritin* CAUSED SENSITIVITY TO FE DEFICIENCY BUT COULD BE OVERCOME BY THE CONCOMITANT INTRODUCTION OF BIOSYNTHETIC GENES FOR MAS

Under both hydroponic culture and calcareous soil cultivation, Fer-NAS-NAAT-IDS3 lines 1-12, 22-4, and 34-11 showed Fe-deficiency tolerance (**Figures 4**, **6**). Interestingly, Fer line 13 showed the opposite phenotype: sensitivity to Fe deficiency (**Figures 4**, **6**). Wuytswinkel et al. (1999) reported that the overexpression of *ferritin* in tobacco caused abnormal Fe localization and symptoms of Fe deficiency. In our transgenic lines, *SoyferH2* were expressed in Fe-deficient leaves of FC and Fer-NAS-NAAT-IDS3 lines (Figure S9). Data produced using a 44K rice microarray also showed that *OsGluB1* (Os02g0249900) and *OsGlb* (Os05g0499100) were expressed weakly in the leaves of plants grown under Fe-sufficient and -deficient conditions (data not shown). Thus, ectopic *ferritin* expression in the leaves of the Fer line under conditions of Fe deficiency might cause the accumulation and sequestration of Fe, which is needed for growth, leading to an Fe deficiency-sensitive phenotype. This adverse effect was successfully complemented by the concomitant introduction of biosynthetic genes for MAs, as shown in the Fer-NAS-NAAT-IDS3 lines and also in previous reports. Lee et al. (2009b) reported that rice lines with enhanced *OsNAS3* expression showed Fe-deficiency tolerance in addition to an increased Fe concentration in seeds. The overexpression of *NAS* genes together with *ferritin* may also help to avoid Fe deficiency sensitivity caused by introduction of the *ferritin* gene.

In the Fer-NAS-NAAT-IDS3 lines, introduction of the *IDS3* genome fragment is thought to be responsible for the avoidance of sensitivity and further tolerance to Fe deficiency, because lines 22-4 and 34-11 expressed only *IDS3* among the biosynthetic genes for MAs introduced. Suzuki et al. (2008) reported that introduction of the *IDS3* genome fragment into rice conferred Fe-deficiency tolerance in field cultivation. This effect may be attributed to both enhanced production of MAs (DMA plus MA) and increased stability of Fe(III)-MA, as compared to Fe(III)-DMA under some conditions (von Wirén et al., 2000; Kobayashi et al., 2001).

THE COMBINED INTRODUCTION OF *ferritin* AND *IDS3* ENABLES EFFECTIVE FE AND ZN ACCUMULATION IN SEEDS

In Fer-NAS-NAAT-IDS3 lines 22-4 and 34-11, even though the expression of *HvNAS1*, *HvNAAT-A*, and *HvNAAT-B* could not be detected, the Fe concentration in the seeds was the same or higher than that in line 1-12, which expressed all of the introduced genes for MAs biosynthesis (*HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and *IDS3*) (**Figures 5** and **8A,B**). In previous field experiments, transgenic rice lines with introduced *HvNAS1* or *HvNAS1* plus *HvNAAT-A* and *HvNAAT-B* did not significantly increase the Fe concentration in seeds (Masuda et al., 2008; Suzuki et al., 2008). On the other hand, rice lines with the introduced *IDS3* genome fragment showed an increased Fe concentration in polished seeds up to 1.25–1.4 times, as compared to that in the NT line in both Andosol soil, which has a normal pH, and calcareous soil (Masuda et al., 2008; Suzuki et al., 2008). Therefore, *IDS3* is thought to be one of the most effective biosynthetic genes for MAs for the Fe biofortification of rice seeds. Rice possesses three *HvNAS1* homologs (*OsNAS1-3*) and six *HvNAAT-A* and

HvNAAT-B homologs (*OsNAAT1-6*) (Inoue et al., 2003, 2008). In contrast, rice lacks *IDS3* homologs, which synthesize MA from DMA (Nakanishi et al., 2000; Kobayashi et al., 2001). The introduction of *IDS3* to rice confers the ability to produce MA (Kobayashi et al., 2001), which could be advantageous for efficient Fe translocation within plants and might result in increased Fe accumulation in seeds, in addition to Fe-deficiency tolerance. Thus, the increased Fe concentration detected in Fer-NAS-NAAT-IDS3 lines 22-4 and 34-11, as compared to line 1–12, might have been caused by differences in the expression level or pattern of the *IDS3* transgene (**Figure 5**).

The Fe concentration in brown seeds was increased by up to 30% in the Fer-NAS-NAAT-IDS3 lines but not in the Fer lines, as compared to the NT line under both calcareous soil and commercially supplied soil cultivation (**Figures 8C,D**). Because *ferritin* was expressed predominantly in the endosperm under the control of the *OsGlb* and *OsGluB1* promoters, the contribution of *ferritin* expression to Fe accumulation is thought to be more obvious in polished seeds than in brown seeds. The concomitant introduction of *IDS3* is thought to have contributed to Fe accumulation in brown seeds by enhancing Fe translocation.

The seed Fe concentration was higher in NT plants grown in calcareous soil, as compared to NT plants grown in commercially supplied soil (**Figure 8**). This may be because the yield in calcareous soil was low (data not shown) and Fe accumulated well in a limited number of seeds.

The seeds of the Fer-NAS-NAAT-IDS3 lines accumulated both Fe and Zn. In contrast, Fer line 13-6 did not contain higher levels of Zn in its seeds, as compared to the NT line (**Figure 9**). Masuda et al. (2008) showed that the insertion of *IDS3* into the rice genome increased the Zn concentration by 35% in polished seeds and by 29% in brown seeds in an Andosol field experiment. Suzuki et al. (2008) also showed that the Zn concentration was increased by 37% in brown seeds in a calcareous soil field experiment. Therefore, the increased Zn concentration in the Fer-NAS-NAAT-IDS3 lines may have been caused by the introduction of *IDS3*.

Traditional breeding has also produced high Fe rice such as IR68144. Therefore, for further improvement of Fe concentration in seeds, it will be more efficient to generate higher Fe biofortified rice by transgenic method using a high Fe variety which has already been produced by traditional breeding.

In conclusion, transgenic rice expressing both *ferritin* and the barley MA synthase gene *IDS3* showed increased Fe concentration when the plants were cultivated in both commercially supplied soil and calcareous soil. Fe-deficiency tolerance was also noted under calcareous soil cultivation. These results indicate that the concomitant introduction of *ferritin* and *IDS3* is an effective way to biofortify seeds with Fe without causing Fe-deficiency symptoms under Fe-limited conditions. This method will be especially advantageous for use in Fe-limited environments, including those with a high soil pH and upland cultivation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Physiology/10.3389/fpls.2013.00132/abstract

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Pyrophosphate levels strongly influence ascorbate and starch content in tomato fruit

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Ascorbate (vitamin C) deficiency leads to low immunity, scurvy, and other human diseases and is therefore a global health problem. Given that plants are major ascorbate sources for humans, biofortification of this vitamin in our foodstuffs is of considerable importance. Ascorbate is synthetized by a number of alternative pathways: (i) from the glycolytic intermediates D-glucose-6P (the key intermediates are GDP-D-mannose and L-galactose), (ii) from the breakdown of the cell wall polymer pectin which uses the methyl ester of D-galacturonic acid as precursor, and (iii) from *myo*-inositol as precursor via *myo*-inositol oxygenase. We report here the engineering of fruit-specific overexpression of a bacterial pyrophosphatase, which hydrolyzes the inorganic pyrophosphate (PPi) to orthophosphate (Pi). This strategy resulted in increased vitamin C levels up to 2.5-fold in ripe fruit as well as increasing in the major sugars, sucrose, and glucose, yet decreasing the level of starch. When considered together, these finding indicate an intimate linkage between ascorbate and sugar biosynthesis in plants. Moreover, the combined data reveal the importance of PPi metabolism in tomato fruit metabolism and development.

Keywords: tomato fruit, ripening, ascorbate, sugars, pyrophosphatase

INTRODUCTION

Nutrition is, by definition, aimed at maintaining human cell and organ homeostasis (Goodacre, 2007). In this context, a balance diet should be considered not just to include carbohydrates, proteins, and lipids, but also other physiologically active components such as certain amino acids and vitamins. Plants are the main dietary source in almost all trophic chains. Therefore, human nutritional health is ultimately dependent on the intake of mayor and minor nutrients from plants, especially given that humans are unable to synthesize certain organic compounds such as vitamins (Fitzpatrick et al., 2012).

L-ascorbate (AsA) is commonly called "vitamin C." In plants, it acts as a scavenger of the free radicals generated by photosynthesis, cellular respiration, and abiotic stresses such as ozone and UV radiation (Conklin et al., 1996; Noctor and Foyer, 1998; Smirnoff and Wheeler, 2000). AsA has additionally been described to play an important role as an enzyme cofactor while participating in defense, cellular elongation, division and fruit ripening (Arrigoni and De Tullio, 2002; Pastori et al., 2003; Green and Fry, 2005). In humans, AsA has an integral role as cofactor of some dioxygenases enzymes which are involved in biosynthesis of carnitine and collagen (Padayatty et al., 2003). Therefore, its deficiency is associated with conditions such as scurvy and low immunity, which is mainly a consequence of the inactivation of these dioxygenases (De Tullio, 2012). AsA has also been associated to molecular events such as oxygen sensing, redox homeostasis, and carcinogenesis (Valko et al., 2006). In addition, different epidemiological studies have established a positive link between AsA content in food and health benefits such as the prevention of cardiovascular disease, cancer, and other inflammatory diseases (Blot et al., 1993; Steinmetz and Potter, 1996). Current approaches to relieve micronutrient deficiencies include the promotion of balanced diets, supplementation and food fortification, such as iodination of salt or fluoride fortification of toothpaste and tap water (Fletcher et al., 2004; Poletti et al., 2004). However, AsA is a difficult micronutrient for food fortification since it is oxidized very easily. AsA biofortification through metabolic engineering therefore represents an attractive alternative strategy to increase the intake of natural AsA in rich and poor countries alike (Muller and Krawinkel, 2005).

In plants, the biosynthetic pathway of AsA occurs by four different pathways, D-mannose/L-galactose (D-Man/L-Gal) or Smirnoff–Wheeler pathway, the major AsA biosynthetic route in plants, which involves GDP-D-mannose in the initial step (Wheeler et al., 1998). An alternative pathway with L-galacturonic acid as intermediate has been reported in strawberry, which proceeds via D-galacturonic acid to L-galactono-1,4-lactone (Agius et al., 2003) that serves as the linkage with the D-Man/L-Gal pathway. There are also alternative pathway of synthesizing AsA through the intermediates of L-gulose (Wolucka and Van Montagu, 2003) and *myo*-inositol (Lorence et al., 2004). However, the *myo*-inositol pathway remains controversial due to the lack

of strong evidence. The AsA concentration remains the same in wild type and *MiOX* overexpression lines (Endres and Tenhaken, 2009).

Formation of GDP-D-mannose is the initial step in the D-Man/L-Gal pathway, which is synthesized from D-mannose-1P via GDP-mannose pyrophosphatase (Conklin et al., 1999) (**Figure A1**). This reaction generates inorganic pyrophosphate (PPi) as by-product. In plants, PPi plays a central role not only as by-product of activation and polymerization steps (Sonnewald, 1992; Geigenberger et al., 1998; Rojas-Beltran et al., 1999; Farre et al., 2001), if not as an energy donor *per se* (Stitt, 1998; Lopez-Marques et al., 2004). PPi is generally removed by inorganic pyrophosphatases, which hydrolyze PPi to orthophosphate (Pi). Heterologous expression of the *Escherichia coli* pyrophosphatase in an untargeted manner, conferring cytosolic localization of the encoded protein, showed an important role in the partitioning between sucrose (Suc) and starch (Sonnewald, 1992; Farre et al., 2001; Lee et al., 2005). In contrast, expression of the *E. coli* pyrophosphatase targeted to the plastid displayed only minor changes in metabolites levels (Farre et al., 2006). A transient down-regulation of plastid-targeted soluble pyrophosphatase in *Nicotiana benthamiana*, revealed an important role in photosynthesis as well as in the regulation of water exchanges under mild drought stress (George et al., 2010).

In this study, we generated *Solanum lycopersicum* cv MoneyMarker lines, which overexpress the gene encoding the inorganic pyrophosphatase from *E. coli* in an untargeted manner under the control of a fruit specific promoter. This strategy resulted in increased vitamin C levels up to 2.5-fold in ripe fruit as well as increasing in the major sugars, sucrose, and glucose, yet decreasing the level of starch. When considered together, these finding indicate an intimate linkage between ascorbate and sugar biosynthesis in plants.

RESULTS

GENERATION OF B33-PPi OVEREXPRESSION TOMATO LINES

To assess the effect of over expression of a pyrophosphatase from *E. coli* (Sonnewald, 1992) in tomato fruit we introduced this gene in the sense orientation under the control of the patatin B33 promoter (Jelitto et al., 1992). This promoter has been shown to be ripening-specific promoter in tomato fruit (Frommer et al., 1994; Centeno et al., 2011). An initial screening was carried out on the basis of pyrophosphatase activity of ripe tomato fruits (data not shown). This screen allowed the identification of four lines displaying considerably elevated activity (L9, L28, L29, and L39), which were taken to the next generation. Eight T2 plants per line were grown in the greenhouse and young leaves (3 weeks old plants) as well as fruits at green (35 days after pollination; DAP) and red (60 DAP) stages were harvested. Assay of alkaline pyrophosphatase activity revealed that the selected lines displayed considerable increase in activity in red fruit (**Figure 1**). To ensure that this increase of target enzyme activity was restricted to fruits, the activity of the enzyme was additionally tested in young leaves where they were unaltered (10.8 ± 0.2; 11.1 ± 0.5; 11.4 ± 0.4; 10.4 ± 0.3; $10.5 \pm 0.5\,\mu mol\ min^{-1}\ g^{-1}$ FW in wild type, L9, L28, L29, and L39, respectively; values are mean $\pm$ SE).

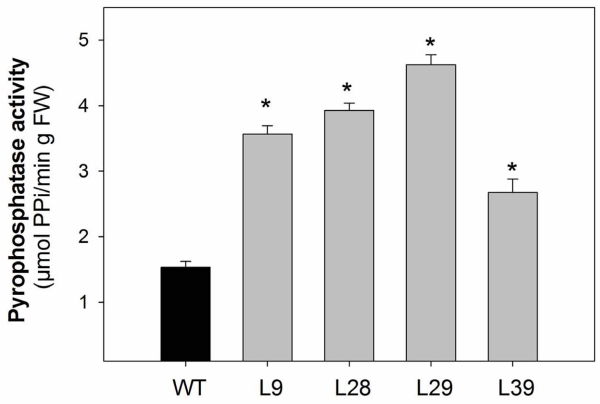

FIGURE 1 | Pyrophosphatase activity of B33-PPi tomato lines. Pyrophosphatase activity in red tomato fruit (60 DAP). An asterisk indicates the values that were determined by the *t*-test to be significantly different ($P < 0.05$) from wild type.

FRUIT SIZE AND YIELD

Fruit size and weight per fruit were determined in red fruit (60 DAP). All four lines exhibited a significantly lower weight per fruit (**Figure 2A**) as well as smaller fruit size in three of the four lines (L9, L28, and L29; **Figure 2B**). The total fruit number was, however, essentially unaltered (**Figure 2C**).

PYROPHOSPHATE AND INORGANIC PHOSPHATE LEVELS

Having determined that the transformants displayed the desired alteration in enzyme activity, we next evaluated pyrophosphate levels themselves. For this purpose, pericarp tissues of red fruit at 60 DAP and young leaves (3 weeks old plants) were harvested and pyrophosphate levels were determined taking care to observe all control procedures required to minimize the influence of contaminants (Farre et al., 2001). These analyses revealed significant decreases in pyrophosphate levels in all lines ranking from 25 for L39 to 55% for L29 in red tomato fruit (**Table 1**). However, as anticipated both from the specificity of expression of the transgene and the lack of change in the activity no changes in pyrophosphate levels were observed in leaves (**Table 1**). Relatively consistent changes were also seen in the level of inorganic phosphate in red fruit. Inorganic phosphate level increased in three transgenic lines (L9, L28, and L39; **Table 1**).

METABOLITE PROFILING OF GREEN AND RED FRUITS OF THE B33-PPi LINES

In order to further characterize the effects of the reduction of pyrophosphate content, we next applied and established gas chromatography (GC)-MS-based metabolite profiling method (Osorio et al., 2012) to pericarp tissue derived from green and red fruits. Surprisingly, at the green stage the metabolite profiles of the transgenic lines were remarkable similar to those of the WT (**Figure 3**). However, similar analysis in red stage revealed important changes in the levels of several few metabolites (**Figure 4**). In all four lines Suc was significantly increased in red tomato fruit by up to 2.5-fold in comparison to WT (**Figure 4**). This increase in Suc was accompanied by increases in Glc in three lines but

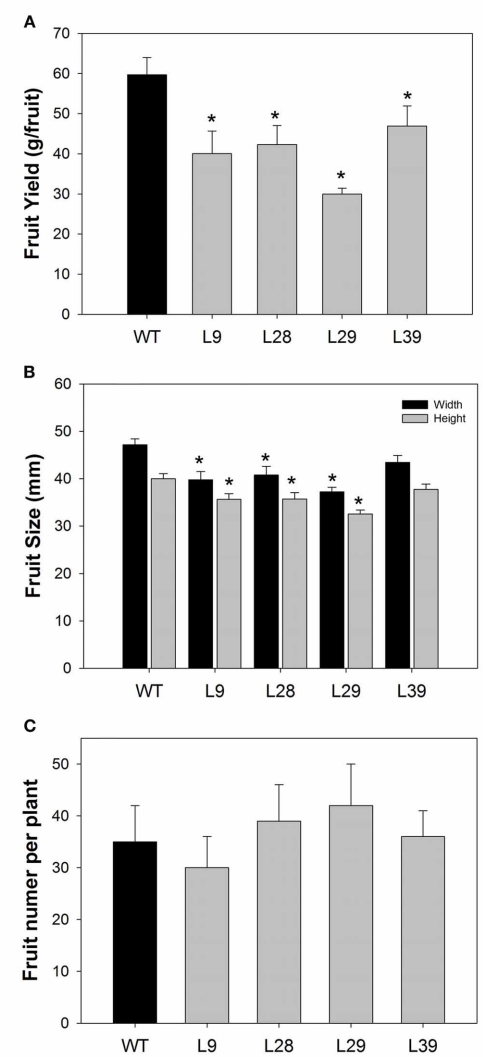

FIGURE 2 | Characterization of B33-PPi tomato lines. (A) Fruit yield (g per fruit), **(B)** fruit size, and **(C)** total fruit number of B33-PPi lines. All measurements were done in red stage (60 DAP). For all parameters, values are presented as the mean ± SE of eight biological replicates (one biological replicate is represented by one individual plant). An asterisk indicates the values that were determined by the *t*-test to be significantly different ($P < 0.05$) from wild type.

Table 1 | Pyrophosphate and inorganic phosphate concentration in the B33-PPi red tomato fruits (60 DAP) and leaves (3 weeks old plants).

	WT	L9	L28	L29	L39
	PPi content (nmol g^{-1} FW^{-1})				
Fruit	4.3 ± 0.8	**2.5 ± 0.7**	**2.3 ± 0.4**	**1.8 ± 0.6**	**3.2 ± 0.5**
Leaves	12.3 ± 2.5	10.8 ± 2.4	11.5 ± 2.2	13.4 ± 3.5	12.9 ± 2.4
	Pi content (μ mol g^{-1} FW^{-1})				
Fruit	3.5 ± 0.5	**7.1 ± 0.7**	**6.9 ± 0.5**	**8.5 ± 0.7**	4.5 ± 0.5

Values are presented as the mean ± SE of 6 biological determinations. The values that are significantly different by t-test from the wild type are set in bold type ($P < 0.05$).

no significant changes in Fru (only L9 showed slight decrease). The changes in these sugars were coupled to a decrease in starch content (**Table 2**) as well as an increase in the total soluble solids content (Brix) in all transgenic red fruit (6.2 ± 0.3; 7.4 ± 0.2; 8.4 ± 0.4; 6.9 ± 0.2 for the wild type, L9, L28, L29, and L39, respectively). To better understand these metabolic alterations, we next measured the AGPase in the red B33-PPi fruits. We observed a decrease in this activity in all transformants with the exception of the L39. However, the activation stage of this enzyme was invariant (**Table 3**).

Interestingly, a strong increase in metabolites related to ascorbate biosynthesis, such as dehydroascorbic acid (all four lines), *myo*-inositol (all four lines), galacturonic acid (lines L28 and L39) (**Figure 4**) as well as a substantial increase of ascorbate (approximately between 2 and 3-fold) were observed (**Figure 5**).

Additionally, the transformants revealed an increase in two amino acids Ala (L28 and L39), and Asp (L28, L29, and L39) as well as a reduction in putrescine (L9, L28, L39) (**Figure 4**).

EXPRESSION OF *E. Coli* PYROPHOSPHATASE IN TOMATO FRUITS LEADS TO ALTERATION IN ASCORBIC ACID BIOSYNTHESIS

Since some related ascorbate biosynthesis metabolites as well as ascorbate were modified in the red B33-PPi tomato fruits, we next evaluated if ascorbate biosynthesis and/or recycling were altered in these fruits. For this purpose we examined the different AsA biosynthetic pathways. First, we analyzed the transcript levels of some genes in the D-Man/L-Gal pathway. The expression of the two *GDP-L-galactose phosphorylase* (*GGP*) genes, a key point for the control of ascorbate pathway (Dowdle et al., 2007; Laing et al., 2007; Bulley et al., 2012) was up-regulated in all transgenic lines as well as the *L-galactono-1,4-lactone dehydrogenase* (*GaLDH*) gene, while the L-galactose dehydrogenase (*GDH*) gene showed a significant decrease only in one line (L9) (**Figure 6**). The higher activity of the last enzyme in this pathway, GaLDH, corroborated that D-Man/L-Gal pathway was up-regulated in red ripened B33-PPi fruits (**Table 3**). Second, related with the D-galacturonic acid pathway, we observed an increase in the level of its precursor, galacturonic acid, in two lines (Loewus and Kelly, 1961; Agius et al., 2003). However, the enzyme activities of the last two enzymes of this pathway, D-galacturonate reductase (GalUR) and aldonolactonase, were unaltered in these fruits (**Table 3**). Third, we observed that the *myo*-inositol level was altered in all transformants and considering that *myo*-inositol has been proposed as a precursor of ascorbate (Lorence et al., 2004), we determined the total *myo*-inositol oxygenase activity in red fruits (**Table 3**). Intriguingly, a significant decrease in the total *myo*-inositol oxygenase activity was observed in all transgenic lines (**Table 3**).

Additionally, we also determined the gene expression of three monodehydroascorbate reductase (*MDHAR*) and two dehydroascorbate reductase genes (*DHAR*) found in tomato. Both are involved in ascorbate recycling pathway. Interestingly, all transformats displayed a significant increase in *MDHAR1, MDHAR2,*

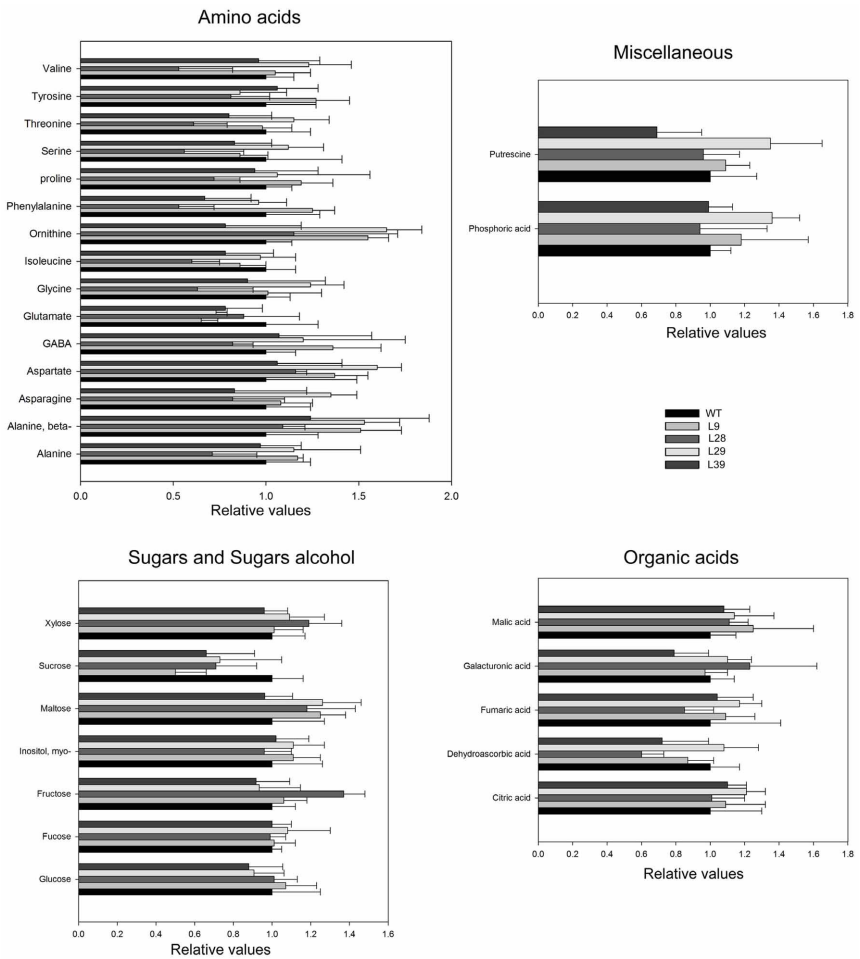

FIGURE 3 | Primary metabolite levels in the receptacle of WT and B33-PPi lines at green stage. Data are normalized to the mean value of WT at the G stage. Values are means SE of six replicates. Asterisks indicate significant differences by *t*-test ($P < 0.01$) of the transgenic lines compared with WT at green stage.

and *MDHAR3* transcript abundances (**Figure 7**). This result was in agreement with an increase in the MDHAR activity (**Table 3**). In contrast, we did not observed changes in the expression of the *DHAR1* and *DHAR2* genes (**Figure 7**).

DISCUSSION

Until now, the breeding of tomato has been dominated by a focus on traits that benefit the grower, such as yield, plant and fruit size, and storage characteristics (Schuch, 1994; Giovannoni, 2006; Cong et al., 2008). As a result, there has been a loss of consumer quality traits such as flavor and nutritional value, and this has focused recent interest on the molecular genetics of such traits (Giovannoni, 2001; Causse et al., 2002, 2004; Fraser et al., 2009; Mounet et al., 2009; Enfissi et al., 2010; Centeno et al., 2011; Morgan et al., 2013). The accumulation of a range of soluble metabolites is important for both flavor and nutrition. In this paper, we characterized the consequences of over-expressing an *E. coli* pyrophosphatase gene under fruit-specific promoter. This manipulation had a broad impact on fruit development and ripening, emphasizing both the important role of pyrophosphatase in ascorbate and starch biosynthesis.

EFFECT OF INCREASING PYROPHOSPHATASE ACTIVITY ON STARCH AND SUGARS METABOLISM

Detailed analysis of sugars level revealed that starch content decreased while the major sugars, Suc and Glc increased in red ripe B33-PPi fruit. These data support the contention that active starch accumulation is an important contributory factor in determining the soluble solids content of mature fruit (Schaffer and Petreikov, 1997; Baxter et al., 2005). Here, we demonstrated that alterations in PPi metabolism have a strong effect on sugars metabolism and, hence, influence agronomic yield. Intriguingly, the data presented here are analogous to those previously described for transgenic potato plants in which higher PPi levels increased starch accumulation and decrease the level of Suc (Fernie et al., 2001a; Geigenberger et al., 2001), and decreased levels have been associated with lower starch biosynthetic rates (Geigenberger et al., 2001).

Different studies concerning starch metabolism in potato and tomato have suggested that AGPase activity plays an important role in its regulation (Geigenberger et al., 1999; Sweetlove et al., 1999). Regulation of the AGPase reaction has been very well characterized for several years. This enzyme is sensitive to allosteric

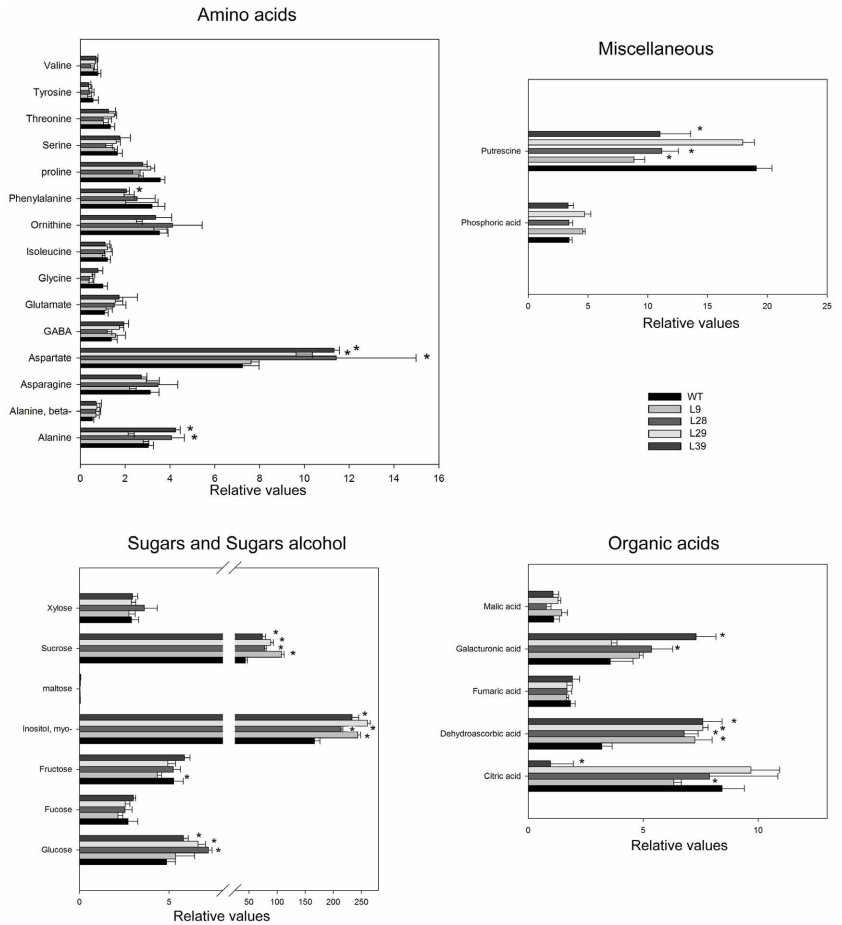

FIGURE 4 | Primary metabolite levels in the receptacle of WT and B33-PPi lines at red stage. Data are normalized to the mean value of WT at the G stage. Values are means SE of six replicates. Asterisks indicate significant differences by *t*-test ($P < 0.01$) of the transgenic lines compared with WT at red stage.

Table 2 | Starch content in the B33-PPi red tomato fruits (60 DAP).

	WT	L9	L28	L29	L39
			Starch (nmol g^{-1} FW^{-1})		
Fruit	438.3 ± 9.5	**354.2 ± 13.1**	**316.4 ± 16.9**	**275.5 ± 20.5**	384.6 ± 27.7

Values are presented as the mean ± SE of 6 biological determinations. The values that are significantly different by t-test from the wild type are set in bold type ($P < 0.05$).

regulation, being inhibited by inorganic phosphate and activated by 3PGA (3-phosphoglycerate) (Sowokinos, 1981; Sowokinos and Preiss, 1982). Additionally, it has been demonstrated to be transcriptionally regulated by sugars, nitrate, phosphate and trehalose-6-phosphate (Muller-Rober et al., 1990; Nielsen et al., 1998; Kolbe et al., 2005; Michalska et al., 2009). Moreover, it has been described that AGPase is also redox regulated (Tiessen et al., 2002; Centeno et al., 2011; Osorio et al., 2013) with malic acid potentially being a key component in this process at least in photosynthetically active tissues (Szecowka et al., 2012).

In this study, a strong correlation was found between starch concentration and AGPase activity in red ripe B33-PPi stage. Additionally, we observed that redox-state of AGPase was not altered in these transgenic fruit. This observation described here have lead us to propose that the activity of this enzyme was modified due to either a change in the rate of sugar influx into the tomato fruit and/or in the lower PPi levels found in these transgenic fruits.

INCREASED ACTIVITY OF PYROPHOSPHATASE CORRELATES WITH INCREASED ASCORBATE CONTENT IN TOMATO FRUIT

There is a large potential for improving ascorbate content in food products by means of both genetic engineering and breeding. The exploitation of the large natural variation in ascorbate content in many fruit crops gives the opportunity of improving their nutritional value by classical breeding. The generation of linkage maps and the conduction of quantitative trait loci (QTL) analysis allow the identification of genomic regions associated with ascorbate content (Davey et al., 2006; Stevens et al., 2007; Zorrilla-Fontanesi

Table 3 | Enzyme activities in the B33-PPi red tomato fruits (60 DAP).

Enzymatic activities	WT	L9	L28	L29	L39
			nmol min^{-1} g^{-1} FW^{-1}		
AGPase	6.65 ± 0.75	**3.98 ± 0.73**	**4.65 ± 0.42**	**3.97 ± 0.89**	6.38 ± 1.02
AGPase activation state (Vsel/Vred)	0.67 ± 0.08	0.58 ± 0.09	0.73 ± 1.04	0.63 ± 0.76	0.76 ± 0.06
MDHAR	6.35 ± 0.31	**12.34 ± 0.21**	**15.22 ± 0.46**	**11.10 ± 0.36**	**9.43 ± 0.42**
GalUR	15.65 ± 0.86	14.89 ± 0.75	16.24 ± 0.65	15.23 ± 0.54	16.33 ± 0.76
Aldonolactonase	11.23 ± 1.34	10.34 ± 0.78	12.36 ± 1.06	13.23 ± 1.03	12.54 ± 0.87
			μmol min^{-1} g^{-1} FW^{-1}		
MYOX	12.43 ± 0.93	**8.24 ± 0.85**	**7.77 ± 0.74**	**9.26 ± 0.82**	**10.52 ± 0.69**
GaLDH	0.35 ± 0.09	**0.78 ± 0.07**	**0.97 ± 0.04**	**1.12 ± 0.07**	**0.67 ± 0.05**

Values are presented as the mean ± SE of 6 biological determinations. The values that are significantly different by t-test from the wild type are set in bold type (P < 0.05).

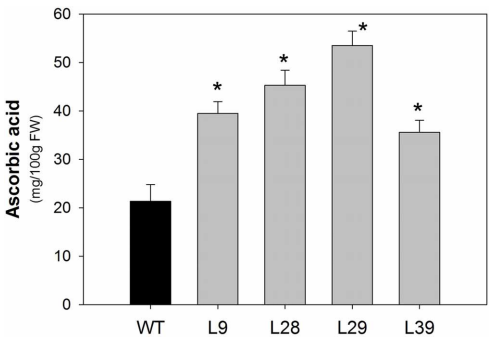

FIGURE 5 | Ascorbate of B33-PPi tomato lines. Ascorbic acid was determined in red tomato fruit (60 DAP). The values are presented as the mean ± SE of six biological replicates. An asterisk indicates the values that were determined by the *t*-test to be significantly different ($P < 0.05$) from wild type.

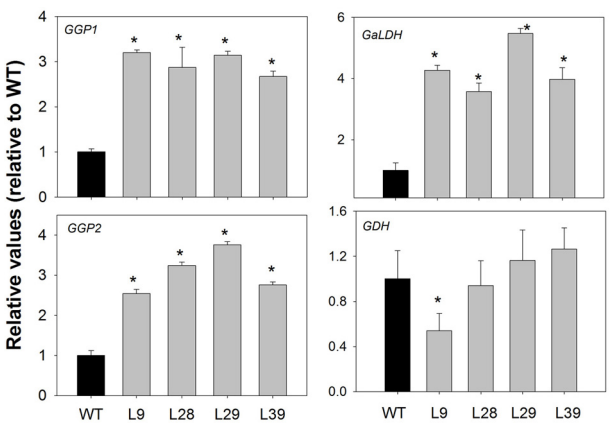

FIGURE 6 | Expression of *GGP, GaLDH,* and *GDH* genes in red B33-PPi fruits. The abundance of GGP1(acc. number Solyc06g073320), GGP2 (acc. number Solyc02g091510), GaLDH (acc. number Solyc10g079470), and GDH (acc. number Solyc01g106450) mRNAs were measured by quantitative RT-PCR, respectively. An asterisk indicates the values that were determined by the *t*-test to be significantly different ($P < 0.05$) from wild type.

et al., 2011). Such QTL analyses therefore increase our knowledge of the molecular mechanism by which ascorbate is regulated in plants.

The strategy to improve the amount of ascorbate by genetic engineering has been based on the up-regulation of genes encoding for enzymes of the biosynthetic or recovery pathways. In general, plants transformed with genes from different pathways have shown variable increases in ascorbate content in different plant tissues (Agius et al., 2003; Chen et al., 2003; Tokunaga et al., 2005; Eltayeb et al., 2007; Badejo et al., 2008, 2009; Bulley et al., 2009, 2012; Hemavathi et al., 2009; Qin et al., 2011; Zhang et al., 2011; Cronje et al., 2012). Mean increases in ascorbate content were usually two- to three-fold i.e., similar to those reported here. Other approaches have used genes that do not encode enzymes of the ascorbate biosynthetic pathway in plants. Thus, the ectopic expression of a rat *L-gulonolactone oxidase*, a gene involved in the synthesis of ascorbate in animals, produced an increase of about 7-fold in lettuce (Jain and Nessler, 2000). Similar levels were observed in potato plants ectopically expressing a bacterial pyrophosphorylase or a yeast invertase (Farre et al., 2008).

In this study, a strong correlation was displayed between the cellular PPi and ascorbate levels (up to 2.5-fold increase in red ripe transgenic fruits) and it was demonstrated that this was mechanistically linked to pyrophosphatase activity as previously was observed in potato tuber overexpressing a bacteria pyrophosphatase with a plastid targeting sequence (Farre et al., 2006). Additionally, an increase in some intermediates of ascorbate biosynthesis such as dehydroascorbate, galacturonate, and *myo*-inositol were also observed.

Formation of GDP-D-mannose is the initial step in the D-Man/L-Gal pathway of ascorbate biosynthesis, which is synthetized from D-mannose-1 phosphate via GDP-mannose pyrophosphatase (Conklin et al., 1999; Keller et al., 1999)

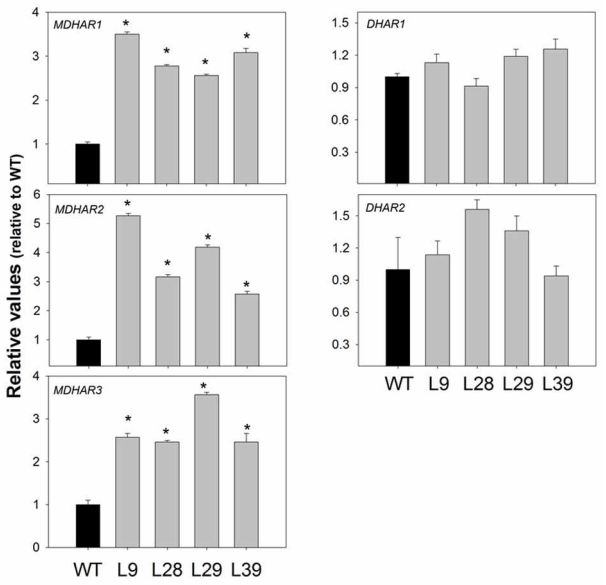

FIGURE 7 | Expression of *MDHAR,* and *DHAR* genes in red B33-PPi fruits. The abundance of MDHAR1 (acc. number Solyc09g009390), MDHAR2 (acc. number Solyc02g086710), MDHAR3 (acc. number Solyc08g081530), DHAR1 (acc. number Solyc05g054760), and DHAR2 (acc. number Solyc11g011250). mRNAs were measured by quantitative RT-PCR, respectively. An asterisk indicates the values that were determined by the *t*-test to be significantly different ($P < 0.05$) from wild type.

(**Figure A1**). This reaction in the direction of ascorbate biosynthesis produces PPi as by-product. It is thus conceivable that the removal of PPi is favorable for ascorbate synthesis. Furthermore, the observed higher expression of *GGP1, GGP2* genes, a key point for the control of ascorbate pathway (Dowdle et al., 2007; Laing et al., 2007; Bulley et al., 2012), and *GalDH,* the last gene in the AsA biosynthesis pathway, corroborates that D-Man/L-Gal pathway is activated in the B33-PPi fruits in comparison to WT fruits.

We also evaluated if other alternative pathways of AsA biosynthesis were altered in these fruits. When looked at the D-galacturonic acid pathway, an increase in the level of its precursor, galacturonic acid, was observed. However, the enzyme activities that catalyze the two last steps in this pathway, GalUR and aldonolactonase, were not altered. Increasing *myo*-inositol production has also shown varied results, with both increased (Lorence et al., 2004) and unaffected (Endres and Tenhaken, 2009) leaf AsA being reported. Together with an increase in *myo*-inositol levels, we observed a decrease in the *myo*-inositol oxygenase activity in the B33-PPi red fruits, suggesting that *myo*-inositol can act as a precursor for AsA biosynthesis as suggested by Lorence et al. (2004). Although, a previous study suggests that the L-galactose-1-phosphate phosphatase enzyme from the D-Man/L-Gal pathway, has a dual function that impacts both *myo*-inositol and AsA biosynthesis pathways (Torabinejad et al., 2009), further investigation are required to understand the impact of *myo*-inositol on AsA biosynthesis.

Also, our results revealed that ascorbate recycling pathway was altered in the B33-PPi red fruits since we found higher dehydroascorbic acid content and expression of the three tomato *MDHAR* genes.

INCREASED ACTIVITY OF PYROPHOSPHATASE ALSO AFFECTS OTHER METABOLIC CHANGES

When other areas of metabolism are considered some interesting observations are apparent. Interestingly, the total level of organic acids and amino acids were largely invariant in the B33-PPi lines in comparison with WT. The exception to this was that we observed an accumulation in two amino acids, namely Ala and Asp. The accumulation of Asp can be explained because it acts as precursor in the synthesis of Asn via asparagine synthase. This reaction produces PPi as by-product that can be removed via pyrophosphatase. Therefore, we expect a shift in the reaction equilibrium to favor the Asn synthesis direction. Although significant differences were not found in the levels of Asn, this may be due to a co-ordinate up regulation of its metabolism. The reason for the increase in alanine is less clear but may merely reflect the additional availability of glucose for glycolytic reactions.

MORPHOLOGICAL EFFECTS ON B33-PPI FRUITS

In addition to the increase in soluble solids content, the fruit of the transgenic lines were compromised in size. This observation was also largely to be expected, since several direct genetic studies (Zrenner et al., 1996; Sonnewald et al., 1997; Sturm and Tang, 1999) have implicated Suc mobilization as a key determinant of sink strength in a broad range of species. As much as 10% (w/v) Suc has been reported in the phloem of plants (Hayashi and Chino, 1990), and the presence of AsA in the phloem sap was confirmed by radiolabeling (Franceschi and Tarlyn, 2002). It was also reported that the presence of sugar within the plant acts as potent signal that promotes AsA biosynthetic gene expression (Nishikawa et al., 2005). Within tomato fruit itself, a positive correlation between Suc feeding and the expression level in some genes of the D-Man/L-Gal pathway was described (Badejo et al., 2012), supporting the view that transportation of sugars from source tissues affect the AsA content in sink tissues through the up-regulation of AsA biosynthesis pathway genes. Despite the wide changes in morphological parameters, metabolic changes in the transgenic fruit were, by and large, confined to sugar and AsA metabolisms. We believed that fruit growth is largely dependent on the relationship between import of photoassimilate and AsA intake and/or biosynthesis.

In summary, the results presented in this study provide direct evidence that the reduction in PPi content had strong effects on metabolism of sugar and ascorbate contents. Detailed analysis of starch metabolism revealed that this phenomenon was due to alteration in AGPase activity, caused by either a change in the rate of sugar influx into the tomato fruit and/or in the lower PPi levels found in these transgenic fruits. During ripening, the lack of accumulation of transitory starch was reflected by a decrease of soluble sugars. Moreover, we demonstrated that alterations in the level of PPi resulted in dramatic effect on ascorbate metabolism. These lines displaying low PPi content showed and increased flux to, and accumulation of, ascorbate. This occurred in spite

of increases the ascorbate level via D-mannose-1P and via GDP-mannose pyrophosphatase. Further investigation is required to define this control, especially in fruit such as tomato, where it may contribute to taste (sugars and organic acids) and nutritional value (ascorbate) of the fruit which are important in determining fruit quality.

MATERIALS AND METHODS

PLANT MATERIAL

The gene encoding a pyrophosphatase from *E. coli* (Sonnewald, 1992) was introduced in the sense orientation into the vector pBinAR between the patatin B33 promotor (Rocha-Sosa et al., 1989) and the octopine synthase polyadenylation signal. This construct was introduced into tomato (*Solanum lycopesicum,* L.) cv Moneymaker plants by an Agrobacterium-mediated transformation protocol, and plants were selected and maintained as described in the literature (Tauberger et al., 2000). An initial screening was carried out on the basis of pyrophosphatase activity. This screen allowed the identification of four lines, which were taken to the next generation.

METABOLITE DETERMINATIONS

PPi and Pi determination

PPi was extracted from tomato fruit by TCA/ether method (Jelitto et al., 1992). PPi was determined using the colorimetric PiPer Pyrophosphate assay kit (Invitrogen) according to the manufacturer's specifications. All glassware was pretreated overnight with 0.1 M HCl to remove residual phosphate. PPi levels were determined by a sample blank with or without sPPase, and total Pi was calculated by comparison at 595 nm with a linear Pi standard curve.

Pi was determined in the TCA extracts with a colorimetric assay as described by Taussky and Shorr (1953).

Primary metabolic profiling

Metabolite extraction derivatization, standard addition, and sample injection for GC-MS were performed according (Osorio et al., 2012). Both chromatograms and mass spectra were evaluated using TAGFINDER (Luedemann et al., 2008).

Ascorbic acid determination

Ascorbic acid extraction and analysis were performed as described (Lima-Silva et al., 2012). Ascorbic acid content was determined by comparison with a linear ascorbic acid standard curve.

Starch determination

The level of starch in the tissues were determined exactly as described previously (Fernie et al., 2001b).

ENZYME ACTIVITIES

Alkaline pyrophosphatase activity

The protein extraction and enzyme activity were analyzed as described Farre et al. (2001).

AGPase

AGPase activity was measured in the pyrophosphorolysis direction with a spectrophotometric assay, as described Tiessen et al. (2002, 2003). Frozen tissues were homogenized in liquid N_2 and approx. 100 mg was extracted rapidly (1 min) with 1 ml of extraction buffer (50 mM Hepes-KOH, pH 7.8, and 5 mM $MgCl_2$) at 4°C. After centrifugation (30 s at 13,000 g at 4°C), 10 μl of the supernatant was used for the AGPase assay. The reaction was performed in a total volume of 200 μl containing 50 mM Hepes-KOH, pH 7.8, 5 mM $MgCl_2$, 10 μM Glc-1,6-bisP, 0.6 mM $NADP^+$, 2.5 mM Na-PPi, 1 unit/ml phosphoglucomutase, 2.5 units/ml Glc-6-P dehydrogenase, and a range of concentrations of ADP-Glc (0.4 − 1 mM) in the absence of Pi, with or without DTT (10 mM) for activation assay. Reactions were followed on line at 340 nm and were linear up to 30 min. The activation state of AGPase is defined as the ratio of Vsel (−DTT) to Vred (+DTT).

Myo-inositol oxygenase

Two-hundred mg of tissue was incubated for 30 min at 30°C in a buffer containing 100 mM KPO_4 (pH 7.2), 2 mM L-cysteine, 1 mM ammonium ferrous sulfate hexahydrate, and 60 mM *myo*-inositol. The reaction was stopped by boiling for 10 min and denatured protein removed by centrifugation (20,000 g, 15 min). Glucuronic acid was determined at 540 nm before and after samples developed a pink color with addition of a 3-hydroxybiphenylphenol color reagent (van den Hoogen et al., 1998).

D-galacturonate reductase

One gram of samples were homogenized in liquid nitrogen and extracted with 50 mM sodium phosphate buffer, pH 7.2, containing 2 mM EDTA, 2 mM dithiothreitol, 20% glycerol and PVPP. GalUR activity was measured by the decrease in absorbance at 340 nm at 25°C after the addition of 100 μl of crude enzyme extract to the assay mixture (1 ml) consisted of 50 mM phosphate buffer (pH 7.2), 2 mM EDTA, 0.1 mM NADPH, 30 mM D-galacturonic acid and 2 mM dithiothreitol. The GalUR activity in the crude enzyme extract was recorded as nmol of NADPH oxidized min-1 mg-1 protein (Agius et al., 2003).

Aldonolactonase

The activity was measured by the change in absorbance of *p-nitrophenol* through acidification at 405 nm essentially as described Ishikawa and Shigeoka (2008).

L-galactono-1,4-lactone dehydrogenase

Samples were prepared as described by Mieda et al. (2004) and assayed at 340 nm by measuring the reduction of NAD^+ in a reaction mixture containing 0.5 mM NAD^+, 1 mM L-Gal, and the enzyme extract. L-Galactono-1,4-lactone dehydrogenase activity was assayed by the reduction of cytochrome *c* resulting in an increase in absorbance at 550 nm in a reaction mixture containing 50 mM TRIS-HCl, pH 8.5, 1 mM sodium azide, 42 mM L-Gal, 0.1% Triton X-100, 1.05 mg^{-1} ml cytochrome *c*, and the extract in a final volume of 1 ml as described by Yabuta et al. (2000).

Monodehydroascorbate reductase

The activity was measured according to the method of Hossain and Asada (1984).

MEASUREMENT OF FRUIT BRIX

Ripe fruit tissue was homogenized with a razor blade, and the soluble solids (Brix) content of the resulting juice measured on a portable refractometer (Digitales Refrktometer DR6000; Krüss Optronic GmbH, Hamburg, Germany).

ANALYSIS OF GENE EXPRESSION BY QRT-PCR

Total RNA was extracted according to Bugos et al. (1995) with minor modifications. Integrity of the extracted RNA was checked by electrophoresis under denaturing conditions after treating the RNA with RNase-free DNaseI (Roche). First-strand cDNA synthesis of 1 mg of RNA in a final volume of 20 mL was performed with Moloney murine leukemia virus reverse transcriptase, Point Mutant RNase H Minus (Promega), according to the supplier's protocol using oligo(dT) T19 primer.

Expression of the monodehydroascorbate reductase (*MDHAR*), dehydroascorbate reductase (*DHAR*), L-galactono-1,4-lactone dehydrogenase (*GaLDH*), and L-galactose dehydrogenase (*GDH*) genes was analyzed by real-time qRT-PCR using the fluorescent intercalating dye SYBR Green in an iCycler detection system (Bio-Rad; http://www.bio-rad.com/). Relative quantification of the target expression level was performed using the comparative Ct method. The following primers were used: for analysis of *MDHAR1* transcript levels (GenBank accession no. Solyc09g009390), forward, 5′-TCTACGGTGATAATGTGGGTGA-3′, reverse, 5′-ATTGCCTTGTTCTCTTCAGGTG-3′; for *MDHAR2* (GenBank accession no. Solyc02g086710), forward, 5′-TTGAGTGATAAACCAGAGCCATC-3′, reverse, 5′-TTCTACGCCTCCTACCATACCA-3′; for *MDHAR3* (GenBank accession no. Solyc08g081530), forward, 5′-ATTTCAAGGGTTTCGGTTCCT-3′, reverse, 5′-CATTTCCTCCTCCAACTACCAC-3′; for *DHAR1* (GenBank accession no. Solyc05g054760), forward, 5′-TTTCCTACCTTCGTCTCATTTCTG-3′, reverse, 5′- GAACAAACATTCTGCCCATTGA-3′; for *DHAR2* (GenBank accession no. Solyc11g011250), forward, 5′-GCTTCATTTGCGACTTCTATCAA-3′, reverse, 5′-AAAACCTCTTCTGGGTGCTCTG-3′; for *GaLDH* (GenBank accession no. Solyc10g079470), forward, 5′-GCTATTTCGGTATGCTCCGTTG-3′, reverse, 5′-CCTCACATTCGCTTCTTTCACT-3′; for *GDH* (GenBank accession no. Solyc01g106450), forward, 5′-TGTTTGTCAGTTCAACGAGGTC-3′, reverse, 5′-TTGTTTTAGATGTCCAAGTGCAA-3′ (Gilbert et al., 2009); for *GGP1* (GenBank accession no.Solyc06g073320) forward, 5′-AGGGTGCAACTGAGGCAAATGC-3′, reverse, 5′-ATGGGCTGTGGAGGTGTGACA-3′; for *GGP2* (GenBank accession no.Solyc02g091510) forward, 5′-GTCTTGGTTGGAGGTTGTAAT-3′, reverse, 5′-TGCACAAAAGTTGCTAGTCCT-3′. To normalize gene expression for differences in the efficiency of cDNA synthesis, transcript levels of the constitutively expressed elongation factor 1a of tomato (GenBank accession no. X14449) were measured using the following primers: forward, 5′-ACCACGAAGCTCTCCAGGAG-3′, reverse, 5′-CATTGAACCCAACATTGTCACC-3′ (Zanor et al., 2009).

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APPENDIX

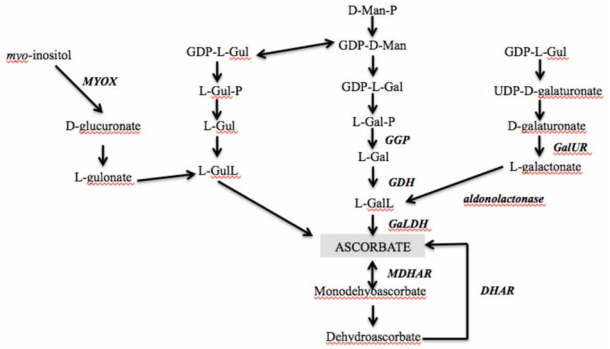

FIGURE A1 | The proposed biosynthetic pathways for ascorbate in plants. L-Gal, L-galactose; L-GalL, l-galactono-1,4-lactone; -LGul, L-gulose; L-GuL, L-gulono-1,4-lactone; D-Man, d-mannose; UDP, uridine diphosphate; *GGP*, GDP-L-galactose phosphorylase; *GDH*, L-galactose dehydrogenase; *GaLDH*, L-galactono-1, 4-lactone dehydrogenase; *GalUR*, D-galacturonate reductase *MYOX*, *myo*-inositol oxidase; *MDHAR*, monodehydroascorbate reductase ; *DHAR*, dehydroascorbate reductase.

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Examining strategies to facilitate vitamin B_1 biofortification of plants by genetic engineering

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Thiamin (vitamin B_1) is made by plants and microorganisms but is an essential micronutrient in the human diet. All organisms require it as a cofactor in its form as thiamin pyrophosphate (TPP) for the activity of key enzymes of central metabolism. In humans, deficiency is widespread particularly in populations where polished rice is a major component of the diet. Considerable progress has been made on the elucidation of the biosynthesis pathway within the last few years enabling concrete strategies for biofortification purposes to be devised, with a particular focus here on genetic engineering. Furthermore, the vitamin has been shown to play a role in both abiotic and biotic stress responses. The precursors for *de novo* biosynthesis of thiamin differ between microorganisms and plants. Bacteria use intermediates derived from purine and isoprenoid biosynthesis, whereas the pathway in yeast involves the use of compounds from the vitamin B_3 and B_6 groups. Plants on the other hand use a combination of the bacterial and yeast pathways and there is subcellular partitioning of the biosynthesis steps. Specifically, thiamin biosynthesis occurs in the chloroplast of plants through the separate formation of the pyrimidine and thiazole moieties, which are then coupled to form thiamin monophosphate (TMP). Phosphorylation of thiamin to form TPP occurs in the cytosol. Therefore, thiamin (or TMP) must be exported from the chloroplast to the cytosol for the latter step to be executed. The regulation of biosynthesis is mediated through riboswitches, where binding of the product TPP to the pre-mRNA of a biosynthetic gene modulates expression. Here we examine and hypothesize on genetic engineering approaches attempting to increase the thiamin content employing knowledge gained with the model plant *Arabidopsis thaliana*. We will discuss the regulatory steps that need to be taken into consideration and can be used a prerequisite for devising such strategies in crop plants.

Keywords: thiamin, riboswitch, HMP, HET, biofortification, genetic engineering

INTRODUCTION

Vitamin B_1, also known as thiamin, was the first B vitamin to be identified (Funk, 1912). In cells, it exists as three predominant vitamers, i.e., free thiamin, thiamin monophosphate (TMP), and thiamin pyrophosphate (TPP), although adenylated and triphosphorylated forms also exist (Bettendorff et al., 2007; Gangolf et al., 2010). The cofactor form of the vitamin is TPP, which is essential for the activity of enzymes involved in acetyl-CoA and amino acid biosynthesis, as well as in the Krebs and Calvin cycles. Acute deficiency in vitamin B_1 in humans leads to a disease called beriberi, which can result in fatal neurological and cardiovascular disorders. Therefore, vitamin B_1 as an essential micronutrient for humans must be taken in the diet. Free thiamin is the predominant B_1 vitamer used and can be taken up *via* specific carriers in epithelial cells (Komai et al., 1974; Said et al., 1999). Paradoxically, although the main source of vitamin B_1 in the diet is plants, the edible portions of some of the most abundant crops used in human nutrition have a natural thiamin content below the recommended dietary allowance (RDA, 1.3 mg/day), e.g., rice (*Oryza sativa*, 18% RDA), wheat (*Triticum aestivum*, 25% RDA), and maize (*Zea mays*, 33% RDA; Fitzpatrick et al., 2012). In addition to a low content in thiamin, crop postharvest processing can aggravate this shortfall. Moreover, thiamin is unevenly distributed in cereal grains. The aleurone layer and germ, which are removed during the refining process, are much richer than the endosperm. Therefore, most of the thiamin is lost in the production of white flour and polished rice. To overcome this problem, wheat flour and rice are often supplemented with chemically synthesized thiamin in developed countries. However, although being common and/or compulsory (in the case of enriched wheat flour in certain developed countries; Harper et al., 1998), such postharvest supplementation remains expensive and inaccessible to people in developing countries, where crops represent the most important dietary source of vitamin B_1. An alternative approach to tackle the problem of malnutrition is to improve crop nutritional qualities through biofortification using genetic engineering or conventional breeding.

With a particular focus on genetic engineering, it is clearly imperative to know how thiamin is biosynthesized. Significant progress has been made in this area in plants over the last few years (for comprehensive reviews, see Goyer, 2010; Rapala-Kozik, 2011). Thiamin is formed by the condensation of two separately biosynthesized moieties, hydroxyethylthiazole phosphate (HET-P) and hydroxymethylpyrimidine pyrophosphate (HMP-PP) to form

TMP (**Figure 1**). The HET-P biosynthesis pathway in plants is assumed to be similar to that of yeast, in which HET-P synthase (THI4p) catalyzes the formation of the thiazole moiety from NAD^+, glycine, and a sulfur from a backbone cysteine in THI4p itself (Chatterjee et al., 2011). Homologs of *THI4*, called *THI1* in plants, have been characterized at the genetic level in maize (Belanger et al., 1995), *Arabidopsis* (Machado et al., 1996), and rice (Wang et al., 2006). The pyrimidine moiety is biosynthesized *via* an identical pathway to bacteria in which phosphomethylpyrimidine synthase (THIC) (Raschke et al., 2007) converts aminoimidazole ribonucleotide (AIR) to hydroxymethylpyrimidine phosphate (HMP-P). The latter is then phosphorylated to HMP-PP by a bifunctional protein characterized in maize as THI3 and in *Arabidopsis* as TH1 (Ajjawi et al., 2007a; Rapala-Kozik et al., 2007). The same protein catalyzes the condensation step between HET-P and HMP-PP to produce TMP (Ajjawi et al., 2007a; Rapala-Kozik et al., 2007). All of these steps have been shown to occur in the chloroplast (Chabregas et al., 2001; Ajjawi et al., 2007a; Raschke et al., 2007). As TMP is not directly phosphorylated to TPP, it is assumed to be subsequently dephosphorylated to thiamin by broad specificity phosphatases (**Figure 1**). Indeed, a broad substrate acid phosphatase has been isolated from maize and biochemically characterized showing it can dephosphorylate TMP (Rapala-Kozik et al., 2009). However, the same phosphatase showed relatively higher specificity toward TPP (Rapala-Kozik et al., 2009). Thus, it has been suggested that TPP can also be successively dephosphorylated to thiamin. On the other hand, thiamin can be pyrophosphorylated to make the active cofactor TPP by thiamin pyrophosphokinase (TPK) (Mitsuda et al., 1979; Molin and Fites, 1980; Ajjawi et al., 2007b; Rapala-Kozik et al., 2009; **Figure 1**). It has been suggested that the phosphatase and kinase activities are regulated such that TPP is only provided when the cell needs it (Rapala-Kozik et al., 2009). To date, the subcellular localization of the phosphatase(s) that acts on TMP (or TPP) is unknown. However, the exclusive cytosolic localization of the *Arabidopsis* TPK suggests that the pyrophosphorylation of thiamin into TPP takes place in the cytosol (Ajjawi et al., 2007b).

In prokaryotes, algae, plants, and certain fungi, *de novo* biosynthesis of thiamin is regulated *via* RNA sequences called riboswitches (Winkler et al., 2002; Sudarsan et al., 2003). This mode of regulation is mediated through the direct binding of the product TPP to the pre-mRNA of particular thiamin biosynthetic genes without the need for intermediary proteins. The binding causes a change in RNA secondary structure that interferes with gene expression. A TPP riboswitch sequence has been found in the 3′-UTR of the *THIC* gene of species across the plant kingdom (Sudarsan et al., 2003; Bocobza et al., 2007; Wachter et al., 2007) and in the 3′-UTR of the *THI1* gene in ancient plant *taxa* (Bocobza et al., 2007). In prokaryotes, the change in mRNA secondary structure, induced by metabolite binding, alters transcription termination or translation, whereas in eukaryotes the pre-mRNA is affected by splicing (Breaker, 2011). In contrast, no TPP riboswitch has been found to date in yeast where thiamin biosynthesis is instead regulated through the coordinate action of transcriptional regulators (for review, see Nosaka, 2006; Kowalska and Kozik, 2008).

In this report, we use the current knowledge on thiamin biosynthesis and its regulation in plants to hypothesize on approaches and pitfalls for efficient biofortification of thiamin in crop plants. The hypotheses presented are corroborated by simple feeding studies in *Arabidopsis*, which in turn reveal certain bottlenecks that should be taken into account for biofortification studies.

IS BYPASSING RIBOSWITCH CONTROL A FAVORABLE WAY TO INCREASE VITAMIN B_1 CONTENT?

As mentioned above, plants have a TPP riboswitch located in the 3′-UTR of *THIC* that acts as a negative regulator of the thiamin biosynthesis pathway (Bocobza et al., 2007; Raschke et al., 2007; Wachter et al., 2007). The mechanism behind this riboswitch has been studied in *Arabidopsis*. Specifically, supplementation experiments with TPP (1 μM) or thiamin (100 μM) indicates that under such conditions the pre-mRNA of *THIC* undergoes a conformational change that exposes a splice site in the 3′-UTR of this gene (Bocobza et al., 2007; Wachter et al., 2007). As a consequence, there is splicing of intron 2 within the 3′-UTR of *THIC* eliminating the consensus polyadenylation signal and resulting in a transcript that is unstable. On the other hand, in the absence of supplementation there is strong accumulation of an intron retained variant (as the splice site is not exposed). The latter transcript has a single consensus polyadenylation signal that is more stable (Bocobza et al., 2007; Wachter et al., 2007). It is therefore assumed that when TPP reaches high enough levels, regulation of expression of the *THIC* gene occurs through this alternative splicing mechanism, i.e., high TPP levels lead to an unstable mRNA whereas the gene is stably expressed when TPP levels are low. However, the actual levels of TPP required in the cell to induce this splicing event remain to be determined. *In vitro* studies indicate a tight binding constant for TPP to the *Arabidopsis THIC* riboswitch (500 nM; Bocobza et al., 2007). As an equilibrium between the different pools of *THIC* transcript would be expected and as TPP levels can reach high micromolar quantities at the cellular level (Ajjawi et al., 2007b; Bocobza et al., 2013), we hypothesize that the TPP binding constant for the riboswitch is likely to be much larger *in vivo*.

As THIC is thought to be one of the key enzymes of the biosynthesis pathway, modulating this regulation would seem to be a tangible approach to increase thiamin content in plants. Indeed, very recently, Bocobza et al. (2013) have studied the impact of the *THIC* promoter as well as its riboswitch on vitamin B_1 levels in *Arabidopsis*. Interestingly, they could show that *THIC* is regulated in a circadian fashion through its promoter region and that there is an oscillation of the B_1 vitamer, TMP. Specifically, the *THIC* transcript is highest at the end of the light period and is lowest at the end of the dark period. In addition, the authors introduced a construct harboring the *THIC* promoter, gene and a mutated version of the riboswitch (A515G in the 3′-UTR) that can no longer bind TPP tightly (Bocobza et al., 2007) into a *THIC* knockdown mutant line (Kong et al., 2008). The expectation was that intron 2 of the 3′-UTR would be retained leading to a more stable *THIC* transcript and thereby enhancement of the vitamin B_1 content. The approach led to an up to threefold accumulation of TMP in leaves. On the other hand, the levels of the cofactor form, TPP, did not show a

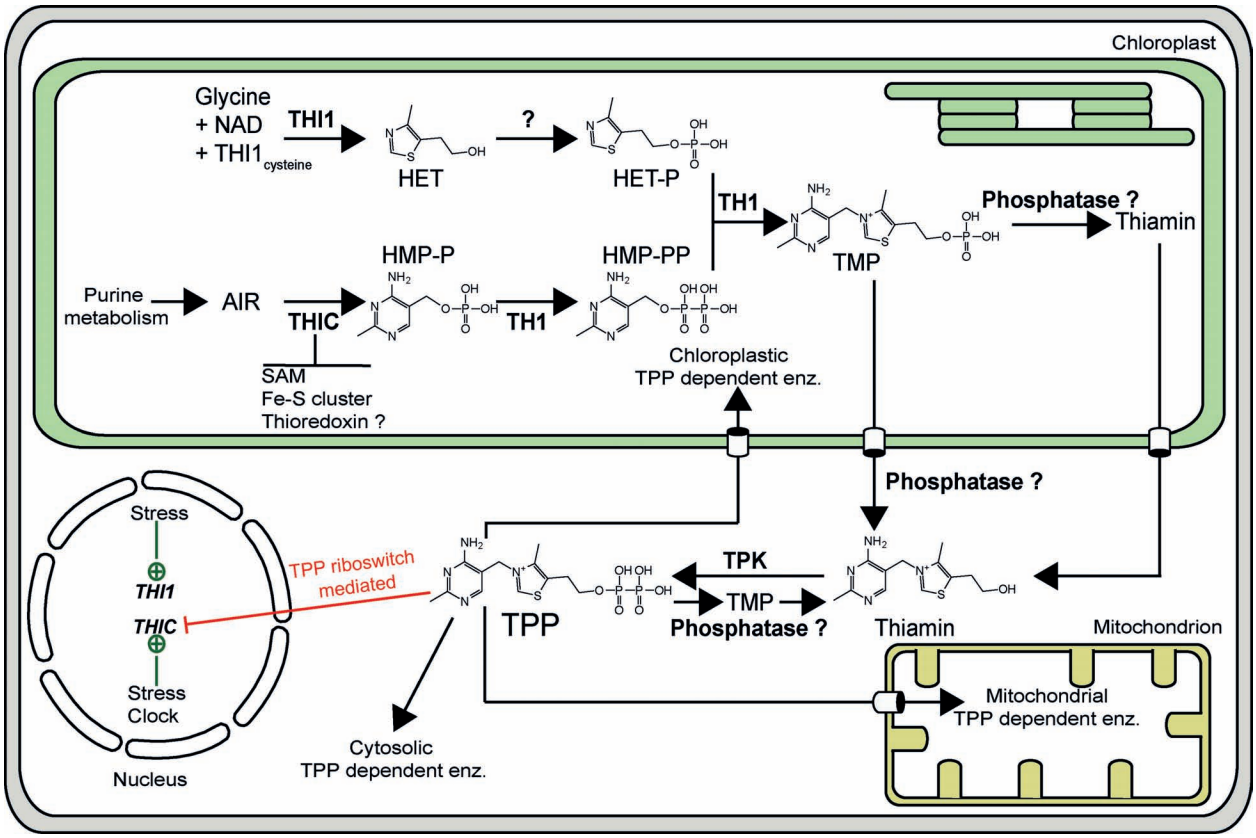

FIGURE 1 | The thiamin biosynthesis pathway of *Arabidopsis thaliana*. As in all organisms, TMP is generated from the condensation of HET-P and HMP-PP by the action of TMP synthase (TH1). THI1 catalyzes the formation of the thiazole moiety from NAD^+, glycine, and the sulfur of a backbone cysteine residue. The phosphorylated pyrimidine moiety is biosynthesized from aminoimidazole ribonucleotide (AIR) via the action of THIC and TH1. THIC contains an iron–sulfur cluster and uses *S*-adenosyl methionine (SAM) in a radical mechanism for catalytic activity. There is evidence for interaction with thioredoxin. To generate the active cofactor, TPP, TMP is first dephosphorylated by a phosphatase (it is not known if this takes place in the plastid or cytosol), and then subsequently pyrophosphorylated by thiamin pyrophosphokinase (TPK). TPP can also be successively dephosphorylated to thiamin by a phosphatase. Expression of the *THIC* gene is regulated through a TPP riboswitch present in its 3′-UTR (depicted as a red line). Abiotic stresses as well as the circadian clock regulate *THIC* and *THI1* gene expression (depicted as green lines). Specific transporter sites across the plastid and mitochondrial membranes are depicted as open barrels.

considerable change and surprisingly, the thiamin vitamer could not be detected. In seeds, an approximate 20% increase in thiamin content was observed in the same lines but no increase in TMP or TPP was noted (Bocobza et al., 2013). However, these transgenic lines exhibited severe chlorosis, growth retardation, and delayed flowering, especially under short day conditions (10 h of light). In another approach, the authors constitutively overexpressed *THIC* in wild-type plants under the control of the *UBIQUITIN1* promoter and terminator. While *THIC* was indeed overexpressed (up to 25-fold) in some of the transgenic lines and a moderate increase in the levels of TMP and TPP was observed (up to 1.5-fold) in leaf material, the corresponding lines were chlorotic (Bocobza et al., 2013). Based on a further comprehensive series of metabolomic and physiological experiments, the authors concluded that the alteration of riboswitch function in this way, while leading to a moderate increase in TPP levels, leads to enhanced carbohydrate oxidation as a result of higher activity of the associated TPP requiring enzymes. Clearly, this has an undesirable impact on the plant manifested as augmented flux through central metabolism and as a consequence negatively perturbing metabolic homeostasis. Therefore, this strategy appears to be unsuitable for vitamin B_1 biofortification of plants.

ARE BOTH BRANCHES OF THE THIAMIN BIOSYNTHESIS PATHWAY REQUIRED FOR VITAMIN B_1 BIOFORTIFICATION?

Several studies have demonstrated that thiamin is accumulated in plants during responses to abiotic and biotic stress conditions as well as oxidative stress (Ahn et al., 2005; Ribeiro et al., 2005; Rapala-Kozik et al., 2008; Tunc-Ozdemir et al., 2009). However, few studies have looked at the response of the entire core thiamin biosynthetic genes until recently. Rapala-Kozik et al. (2012) examined the response of *THIC*, *THI1*, *TH1*, and *TPK* (**Figure 1**) under several types of abiotic stress in *Arabidopsis*. We found it interesting that under conditions where upregulation of the expression of the genes was observed, e.g., salt stress, both *THIC* and *THI1* were upregulated to similar relative levels, at least during the initial phases of the response. TPK was also upregulated under the same conditions but to a lesser extent (Rapala-Kozik et al.,

2012). Notably in this study, while TPP levels did not show considerable alterations, there were significant changes in the level of the thiamin vitamer, in particular. As the key genes of both the pyrimidine and thiazole branches (i.e., *THIC* and *THI1*) were upregulated concomitant with an increase in thiamin in this study, this prompted us to raise the question whether both branches of the thiamin biosynthesis pathway need to be manipulated in order to increase vitamin B_1 levels for biofortification purposes in plants. To test this hypothesis, we grew *Arabidopsis* seedlings in the presence of the thiamin precursors from the pyrimidine branch (i.e., HMP) and/or the thiazole branch (i.e., HET). It has previously been demonstrated that both of these compounds are taken up and can restore growth in the corresponding *Arabidopsis* thiamin requiring mutants (Feenstra, 1964). However, as we had the *Arabidopsis thiC* knock-out mutant (affected in the pyrimidine branch) in hand (Raschke et al., 2007), we re-confirmed this for HMP. The *thiC* mutant is severely chlorotic and developmentally impaired when grown on basal salt medium (**Figure 2A**, top panels). Supplementation with HMP, HMP + HET, or thiamin in the medium fully rescues the chlorotic and developmental phenotype (**Figure 2A**, top panels). Notably, HMP supplementation alone rescues the phenotype, thus reaffirming its uptake, while HET does not, as would be expected. Neither HMP nor HET supplementation affected wild-type seedling development (**Figure 2A**, bottom panels).

We next quantified the individual B_1 vitamer contents (i.e., thiamin, TMP, TPP) by high-performance liquid chromatography (HPLC). Similar to other studies on *Arabidopsis* plant seedlings (Rapala-Kozik et al., 2012; Bocobza et al., 2013), TPP was found to be the most abundant vitamer in wild-type seedlings (~6151 pmol g^{-1} FW) compared to TMP and thiamin (~207 and 127 pmol g^{-1} FW, respectively; **Figures 2B–D**). As expected, we observed that the TPP content increased in the *thiC* mutant when HMP (1 μM) was included in the medium, although the level was not completely restored to that of the wild-type (~3220 pmol g^{-1} FW) under these conditions (**Figure 2B**). Interestingly, we observed an additive effect upon supplementation of both HMP and HET to the *thiC* mutant with the TPP levels approaching those of wild-type seedlings under the same conditions (**Figure 2B**). This suggests that HET is indeed taken up, metabolized and converted into TPP by the seedlings. Supplementation with thiamin (1 μM) restores the TPP level in *thiC* to wild-type levels (**Figure 2B**). In the wild-type seedlings on the other hand, supplementation with either HMP or HET alone did not significantly change the B_1 vitamer content (**Figures 2B–D**). However, when both HMP and HET were added together to the medium, the level of thiamin, in particular, was found to be substantially higher (eightfold increase) from 127 to 973 pmol g^{-1} FW (**Figure 2D**). On the one hand, these results suggest that if one precursor is available in excess the other becomes limiting and further implies that bypassing the *THIC* riboswitch alone will not induce B_1 vitamer accumulation sufficiently. A similar increase in the thiamin vitamer content (sevenfold increase) was observed when both HMP and HET were added to the *thiC* mutant, i.e., from 39 to 272 pmol g^{-1} FW compared to HMP alone (**Figure 2D**). These observations provide strong evidence that the first two steps of the thiamin biosynthesis pathway (i.e., those catalyzed by THIC

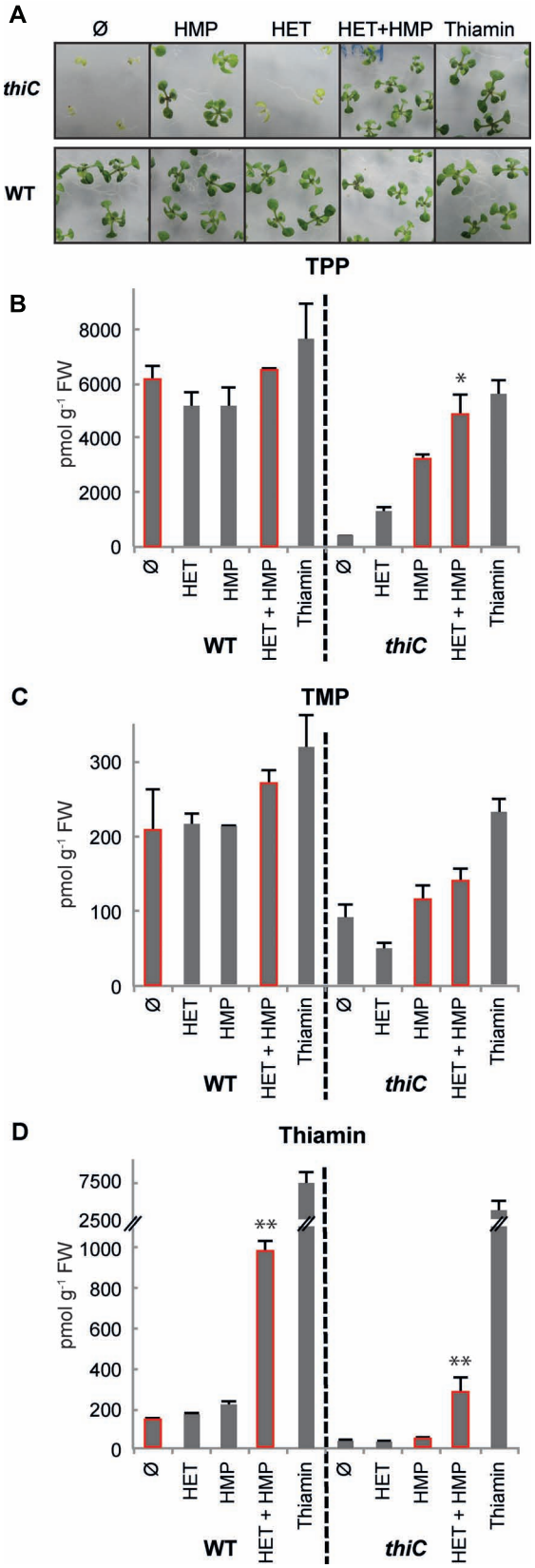

FIGURE 2 | Continued

FIGURE 2 | Continued
Effect of feeding vitamin B_1 precursors on *Arabidopsis*.
(A) Eleven-day-old wild-type (WT) and *thiC* seedlings (SAIL_793_H10) were grown on half-strength basal salt medium (Murashige and Skoog, 1962) containing 0.5% agar, with or without HMP, HET, HMP and HET, or thiamin (1 μM each, where present). The seeds were stratified at 4°C for 3 days before transfer to a growth chamber maintained under long-day conditions (16 h of light at 150 μmol photons m^{-2} s^{-1}, 22°C; 8 h dark at 18°C).
(B–D) HPLC analysis of the B_1 vitamer content (TPP, TMP, thiamin, respectively) in wild-type (WT) or *thiC* after addition of HMP, HET, HMP and HET, or thiamin (1 μM each, where present). In each case, 30 mg of fresh seedling tissue was used. The results shown are the average of three biological replicates. The asterisks denote significant differences between the values of the bars highlighted in red as determined from a two-sided *t*-test, $^{**}p < 0.05$ and $^{*}p < 0.1$.

and THI1) are critical in order to increase the thiamin content of plants.

EXAMINING THE BALANCE BETWEEN THE PHOSPHATASE AND KINASE ENZYMES IN RELATION TO ENHANCING THIAMIN CONTENT

It is interesting that while the thiamin vitamer content was considerably increased by the dual supplementation of HET and HMP, TPP and TMP vitamer contents were not considerably perturbed in wild-type *Arabidopsis* (**Figures 2B–D**). On the one hand, this could suggest that the protein machinery that converts the products of THIC and THI1 into thiamin (i.e., TH1 and a phosphatase) are not limiting for its enhanced production, whereas, the steps necessary for the conversion of thiamin into TPP are. However, as mentioned above, it has been suggested from a study in maize that there is a dynamic equilibrium between phosphatase and kinase actions that interconvert thiamin and its esters depending on the cellular requirement for TPP-dependent enzymes (Rapala-Kozik et al., 2009). To date, it is not known whether the product of the TH1 condensation reaction, TMP, is dephosphorylated into thiamin in the chloroplast, or in the cytosol (**Figure 1**). However, it is known that thiamin is pyrophosphorylated to TPP in the cytosol by TPK of *Arabidopsis* (Ajjawi et al., 2007b). Therefore, we hypothesize that steps restricting the production of the TPP vitamer in particular, could be either the export of thiamin (or TMP) from the chloroplast to the cytosol, the availability of the cytosolic kinase TPK, or dephosphorylation of TPP by a phosphatase. As the transporter(s) for thiamin or its esters out of the chloroplast have not yet been identified, we fed wild-type *Arabidopsis* seedlings with thiamin and followed the production of TPP to perform preliminary tests on the former hypothesis. Significantly, we assume that exogenous thiamin accumulates in the cytosol. Firstly, we could confirm that exogenous thiamin was indeed taken up by wild-type seedlings as shown in **Figure 2D**. Even when the thiamin content was increased 50-fold in wild-type by supplementation (**Figure 2D**), the TPP content only increased by 10% compared to either no supplementation or both HMP and HET supplementation (**Figure 2B**). In a parallel fashion, thiamin addition to *thiC* led to a 92-fold increase of the vitamer (**Figure 2D**), while the level of TPP was similar to that observed under both HMP + HET supplementation (**Figure 2B**). This strongly suggests that a bottleneck for accumulation of the TPP vitamer is either a limitation of the cytosolic TPK enzyme itself or the enhanced action of a TPP phosphatase. It remains to be determined in future studies if TPK activity is a function of its dynamic equilibrium with the associated phosphatase and the requirement of the cell for TPP. Notwithstanding, our feeding studies reveal a fortuitous outcome in the context of biofortification. Dietary vitamin B_1 is most available to animals in its non-phosphorylated form (Komai et al., 1974; Said et al., 1999). Therefore, specifically increasing levels of TPP in the context of biofortification does not appear to be necessary. Furthermore, in contrast to the findings of Bocobza et al. (2013), where an accumulation of TMP (threefold) and to a lesser extent TPP (1.5-fold) was observed with the strategy undertaken, we do not see a significant increase in either of these vitamers and there is no observable negative effect on the plants under any of the supplementation conditions employed (**Figure 2A**). However, although Bocobza et al. (2013) did not measure thiamin, we do not know if this vitamer was also increased in their study but it is noteworthy that TPK expression was significantly increased with the strategy that was employed. Thus, whether the accumulation of thiamin is due to a limitation in TPK activity or enhancement of a TPP phosphatase, it is a fortuitous act of nature in the context of biofortification. However, any inadvertent increase in the levels of the phosphorylated vitamers through genetic engineering strategies will need to be carefully monitored.

EXAMINING HOW STRAIGHTFORWARD IT WILL BE TO INDUCE THE PYRIMIDINE AND THIAZOLE BRANCHES

To date, increasing vitamin B_1 in the context of biofortification has never been overcome in the model plant *Arabidopsis* or in any crop plant. Based on several recent studies, some of which have been discussed above, it is now becoming clear that several regulatory steps will need to be taken into account in order to enhance the vitamin B_1 content. At its most simplistic level and as shown in this report through feeding studies, genetically engineering the overproduction of the HMP and HET precursors *in planta* should led to an overaccumulation of thiamin. Logically, this could be achieved by overexpressing the THIC and THI1 proteins, respectively. The riboswitch regulation of the expression of *THIC* can be bypassed by employing a transgene construct (i.e., promoter, gene, terminator) harboring a non-functional version of the riboswitch in the 3′-UTR (e.g., A515G) or indeed by employing an alternative terminator as has recently been shown (Bocobza et al., 2013). To counteract differential expression levels, both *THIC* and *THI1* transgenes could be inserted in tandem through a binary vector, under the control of a strong promoter, as has been shown to be successful in the β-carotene (vitamin A) biofortification of rice endosperm (Ye et al., 2000). Moreover, options to use tissue-specific promoters, thus minimizing the energy drain on the whole plant, could be exploited to enhance the process. Clearly, the chosen organ/tissue would need to express the other downstream enzymes of the biosynthetic pathway (i.e., TH1, phosphatases) and the precursors for HMP and HET biosynthesis would need to be available in sufficient amounts in the same tissue. In the context of vitamin B_1 biofortification in a crop such as rice, the endosperm would be favored but whether the aforementioned requirements

are fulfilled in order to engineer such a "minipathway" therein is not yet known.

However, while we surmise that increasing the expression of *THIC* and *THI1* in plants can be achieved, it is important to remember that these are complex enzymes both of which require other key cellular components to fulfill their functions. THIC contains an iron–sulfur cluster that must be coupled with a reductant in order to produce a 5′-deoxyadenosyl radical from *S*-adenosyl methionine that is necessary for its catalytic activity (Raschke et al., 2007; Chatterjee et al., 2008; Martinez-Gomez and Downs, 2008). Moreover, affinity chromatography and proteomic approaches have revealed that THIC could be a target of the thioredoxin/ferredoxin system of oxygenic photosynthesis (Balmer et al., 2003, 2006) and therefore is likely to be also regulated by light (Raschke et al., 2007). On the other hand, while the involvement of THI1 in HET biosynthesis has been deduced from genetic studies (Belanger et al., 1995; Machado et al., 1996), the molecular nature of the substrates required for its biochemical activity in plants remain unclarified. The first study on the substrates of this enzyme claimed that deoxyxylulose 5-phosphate (DXP) is used as one of the precursors for the biosynthesis of HET (Juillard and Douce, 1991), as is the case in microorganisms (Hazra et al., 2009). However, the homolog of THI1 from *Saccharomyces cerevisiae* (called THI4p) has very recently been shown to be a suicidal enzyme (i.e., it can only perform a single turnover upon which it becomes non-catalytic). In this reaction, the thiazole is assembled from sulfur of a backbone cysteine (Cys205) of THI4p itself in addition to glycine and NAD^+ (Chatterjee et al., 2011). Several key residues including this cysteine are conserved in the plant THI1 proteins. Therefore, a similar mechanism can be anticipated and would represent yet another regulatory checkpoint *via* this enzyme. Furthermore, taken together, the involvement of NADH, the ferredoxin/thioredoxin system and the necessity of active site cysteines additionally make thiamin production highly dependent on the redox state of the cell. Therefore, engineering thiamin production in plants may not be so straightforward with several regulatory checkpoints needing to be surmounted. In this context, any genetic engineering strategy that proposes to enhance production of thiamin will need to be balanced with the physiological impact on the plant. While the branches of the biosynthetic pathway will need to be balanced, the flux through the pathway and its possible draining of other resources necessary for plant health will need to be monitored. These latter statements are corroborated by the undesirable physiology observed in *Arabidopsis* by overexpression of *THIC* alone, as a result of a negative impact on metabolic homeostasis (Bocobza et al., 2013). While the moderate increase in the pool of thiamin esters resulting from the latter strategy may negatively perturb carbohydrate metabolism as suggested by the authors (Bocobza et al., 2013), possible debilitating effects from the overuse of the complex biochemistry associated with THIC also need to be taken into account.

While the thiamin biosynthetic pathway in plants is close to being completely unraveled and could be exploited for biofortification purposes, we must also draw attention to thiamin binding proteins, specific variants of the major seed storage globulins (see Rapala-Kozik, 2011 for review). These proteins have been found and characterized in several types of seeds, e.g., rice, buckwheat, sunflower, and sesame (Golda et al., 2004). They appear to be found in the peripheral cells of the seed, accumulate during seed maturation and are degraded during seed germination (Watanabe et al., 2004). Notably, it is the thiamin vitamer that accumulates during seed maturation and serves as a reserve utilized upon seed germination (Golda et al., 2004). A closer examination of these phenomena could reveal novel strategies for thiamin biofortification of seeds in particular. For example, targeting of a thiamin binding protein to the rice endosperm is a strategy to be explored.

Although it is not the focused topic of this report, exploitation of natural variation is also likely to be a worthwhile approach that should be considered. Recently, Goyer and Sweek (2011) have reported on the identification of wild potato species and primitive cultivars that accumulate high amounts of thiamin compared to a modern potato variety (Russet Burbank). They propose to integrate these materials into breeding programs in order to enhance the nutritional value of potato. A closer investigation of the molecular basis behind the increased thiamin accumulation in these varieties may help genetic engineering strategies in other crop plants.

CONCLUSION

To date, there is no evidence of toxicity upon supplementation and accumulation of thiamin in plants. This is supported by the feeding studies provided herein. Rather, thiamin has been demonstrated to have beneficial effects enhancing tolerance to both abiotic and biotic stress (Ahn et al., 2005; Ribeiro et al., 2005; Rapala-Kozik et al., 2008; Tunc-Ozdemir et al., 2009). On the other hand, genetic engineering strategies that lead to overaccumulation of thiamin esters must proceed with caution as they may have a negative impact on metabolic homeostasis as indicated by the recent study of Bocobza et al. (2013). A simple engineering of the riboswitch associated with THIC has been discussed at the outset as a likely effective strategy to manipulate thiamin levels in plants, particularly in the context of biofortification. It now appears that the challenge of enhancing thiamin levels in crop plants for biofortification purposes will not be so straightforward. We hypothesize in this study that a balanced provision of the two key precursors (HMP and HET) will lead to the accumulation of the most bioavailable vitamer, thiamin. It will have the added benefit of not being toxic to the plant and could be anticipated to even enhance tolerance to stress. However, genetic engineering of the biosynthesis of the precursor compounds in plants will come with inherent challenges of its own. As discussed above, THI1 and THIC, in particular, are highly complex enzymes and are regulated in a highly complicated fashion, the entire mechanisms of which have not been unraveled yet.

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kNACking on heaven's door: how important are NAC transcription factors for leaf senescence and Fe/Zn remobilization to seeds?

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Senescence is a coordinated process where a plant, or a part of it, engages in programmed cell death to salvage nutrients by remobilizing them to younger tissues or to developing seeds. As Fe and Zn deficiency are the two major nutritional disorders in humans, increased concentration of these nutrients through biofortification in cereal grains is a long-sought goal. Recent evidences point to a link between the onset of leaf senescence and increased Fe and Zn remobilization. In wheat, one member of the NAC (NAM, ATAF, and CUC) transcription factor (TF) family (NAM-B1) has a major role in the process, probably regulating key genes for the early onset of senescence, which results in higher Fe and Zn concentrations in grains. In rice, the most important staple food for nearly half of the world population, the NAM-B1 ortholog does not have the same function. However, other NAC proteins are related to senescence, and could be playing roles on the same remobilization pathway. Thus, these genes are potential tools for biofortification strategies in rice. Here we review the current knowledge on the relationship between senescence, Fe and Zn remobilization and the role of NAC TFs, with special attention to rice. We also propose a working model for OsNAC5, which would act on the regulation of nicotianamine (NA) synthesis and metal–NA remobilization.

Keywords: biofortification, iron, NAC transcription factor, nutrient remobilization, senescence, zinc

Fe AND Zn BIOFORTIFICATION

Iron (Fe) and zinc (Zn) are essential micronutrients for almost all living organisms and are two of the most versatile metals in biology. Fe participates as a catalytic cofactor in multiple metabolic pathways (photosynthesis, respiration, hormone synthesis, nitrogen fixation, DNA synthesis and repair) due to the ability to participate on reversible redox reactions as Fe^{2+} (ferrous) and Fe^{3+} (ferric) ions (Puig et al., 2007). Zn does not participate directly on redox reactions, since it occurs in a single oxidation state, but is a key structural component of around 300 enzymes and 2,000 transcription factors (TFs; Palmgren et al., 2008; Prasad, 2012). Both Fe and Zn are present in low quantities in most plant staple foods, leading to Fe and Zn deficiency in humans. Malnutrition of these micronutrients are leading risk factors for disability and death worldwide, especially to children eating cereal-based diets, with low intake of micronutrient-rich foods as meat, poultry, fish, fruits, legumes, and vegetables.

Strategies to alleviate micronutrient malnutrition include fortification (addition during food processing) and supplementation (ingestion of pills or sachets). Although somewhat successful, these approaches are not widely accessible due to logistic and economic issues. A very cost-effective alternative is biofortification, the increase of bioavailable concentrations of an element in edible portions of crops before harvesting (for comprehensive reviews see White and Broadley, 2005; Sperotto et al., 2012a; Carvalho and Vasconcelos, 2013).

Biofortification includes different approaches like soil fertilization or foliar application, conventional breeding and/or transgenic strategies. Mineral fertilization is an effective method to increase seed mineral concentrations, but can be problematic due to continuous cost and environmental carryover. Conventional breeding has been used for decades. Although there is genetic diversity available within existing germplasm collections, rice seems to have the narrowest range, making substantial increases in Fe and Zn concentrations more difficult compared to maize and wheat (Kennedy and Burlingame, 2003; Gómez-Galera et al., 2010; Sperotto et al., 2012a). Thus, it seems imperative that transgenic approaches be used to enable significant increases in Fe and Zn content and bioavailability.

Single or multiple-transgene insertions into the rice genome have successfully increased Fe concentration in grains. Independent over-expression of *OsNAS* genes produced the most promising results so far (Johnson et al., 2011), while other multi-transgene approaches also increased grain Fe concentration (Wirth et al., 2009; Masuda et al., 2012). However, except in the study from Johnson et al. (2011), the levels are still not effective enough to impact human nutrition. An unexplored avenue would be the controlled expression of regulatory genes involved in key processes to Fe and Zn seed allocation. This approach has already

been performed to generate rice plants more resistant to Fe deficiency (Kobayashi et al., 2007; Ogo et al., 2011). In order to do that for mineral concentrations in grains, we still need to identify the molecular players relevant to their transport within the plant and during remobilization.

SENESCENCE PROCESSES: WHAT, WHEN, AND HOW?

Senescence represents endogenously degenerative processes which ultimately lead to organ death. However, it is not a passive process (Yoshida, 2003), but rather a series of coordinated and controlled events. Decline of photosynthesis, chloroplast and chlorophyll degradation, dismantling of biomolecules and decrease in cellular metabolic activities take place, which result in available nutrients and metabolites that can be transported from source (the tissue that supplies nutrients, most commonly green tissues) to sink (the net importer of nutrients, younger or reproductive organs) through the vascular system (Thomas, 2013). Part of leaf senescence seems to be regulated by sugar levels (Rolland et al., 2006; Sperotto et al., 2007), since a senescence-related loss of chlorophyll or protein can be induced by increased sugar contents (Wingler et al., 1998). Hormones and nutrients also contribute to regulation of senescence in source tissues, especially cytokinins, which have a senescence-delaying effect (Sperotto et al., 2009; Davies and Gan, 2012).

The source–sink signaling is not fully understood, and it depends on the species and circumstances. It is already known that senescence in source leaves can be delayed by removal of strong sinks (Zavaleta-Mancera et al., 1999). Elevated levels of N alter sugar signaling in source leaves (Thomas, 2013) and have a significant impact on Fe and Zn acquisition and grain allocation in wheat (Kutman et al., 2011). According to Shi et al. (2012), a sufficient N supply inhibits Fe export from source leaves, but N deficiency enhances Fe pools in source leaves and stimulates Fe export from senescing leaves to sink tissues in barley, corroborating previous findings that high protein concentrations in N-fertilized leaves tend to immobilize Fe and delay senescence (Marschner, 1995).

Nitrogen remobilization from source tissues to seeds (Hortensteiner and Feller, 2002) has received much more attention than metal remobilization over the last decades, since grain yield has been treated as the most important trait to be improved. Part of the seed N is acquired by the roots, but N remobilization from almost all vegetative organs also contributes to the seed N loading, especially during senescence processes (Burstin et al., 2007). In this way, the senescence process partly satisfies grain N concentration, as well with other minerals (Himelblau and Amasino, 2001). However, it is already known that application of fertilizer N generally decreases whole-plant remobilization efficiency (Bahrani et al., 2011).

Previous work has shown that although seed minerals are partially supplied by continuous uptake and translocation during reproductive growth, remobilization of previous stored minerals in green source tissues is also important (Jiang et al., 2007; Waters and Grusak, 2008; Sperotto et al., 2012b), as commonly seen for N. In rice, it was shown that Fe remobilization is dependent on Fe status: under low or sufficient Fe supply, flag leaf Fe remobilization is observed; under high but non-toxic Fe concentrations, there is no Fe remobilization, presumably because of continuous root uptake (Sperotto et al., 2012b). It is also known that mineral remobilization from leaves to seeds can be enhanced by senescence (Zhang et al., 1995; Uauy et al., 2006; Distelfeld et al., 2007; Shi et al., 2012). As several proteins include Fe and Zn ions, a substantial level of metals can be released during leaf senescence due to the high level of protein degradation (Waters et al., 2009).

Recent molecular studies have shown that senescence processes are driven by TF networks that regulate the expression of several senescence-related genes (Guo et al., 2004; Lin and Wu, 2004). One of the most important families of genes described as associated with senescence, and also with nutrient remobilization from source organs to developing seeds, is the NAC (NAM, ATAF, and CUC) family of TFs (Guo et al., 2004; Uauy et al., 2006).

NAC TRANSCRIPTION FACTORS

Proteins of the NAC family are one of the largest classes of plant-specific TFs. Roles of many NAC TFs have been demonstrated in diverse plant developmental processes. The earliest reports include the NAM (non-apical meristem) protein from petunia (*Petunia hybrida*); *nam* mutants lack the shoot apical meristem (SAM) and die at the seedling stage (Souer et al., 1996). The CUC1/CUC2 (cup-shaped cotyledon) TF from *Arabidopsis*, which participates in the development of embryos and flowers (Aida et al., 1997), defines the boundary domain around organs in the meristem (Nikovics et al., 2006). Later, NAC proteins have been related to diverse processes such as auxin and ethylene signaling (He et al., 2005; Park et al., 2011), cell wall formation (Wang et al., 2011), biotic and abiotic stresses (Puranik et al., 2012), and senescence (Kou et al., 2012).

NAC proteins contain a highly conserved N-terminal domain known as the NAC domain, which has been implicated in DNA binding (Duval et al., 2002; Ernst et al., 2004) as well as protein–protein interactions, forming homodimers or heterodimers with TFs from the NAC family (Ernst et al., 2004; Jeong et al., 2009) or other families (Xie et al., 2000; Greve et al., 2003). The NAC domain reveals a fold consisting of a twisted beta-sheet surrounded by a few helical elements (Ernst et al., 2004). On the other hand, C-terminal regions of NAC proteins are highly divergent (Olsen et al., 2005; Fang et al., 2008), and are related to transcriptional regulation (Xie et al., 2000; Duval et al., 2002).

Many NAC genes have been involved in responses to various environmental stresses like drought, cold, salinity, pathogen attack, and wounding. For recent reviews on NAC TFs in stress response, see Nakashima et al. (2012) and Puranik et al. (2012). In rice, the stress-responsive NAC group (SNAC) includes some already characterized members. *OsNAC5*, *OsNAC6*, and *OsNAC10* are induced by abiotic stresses, abscisic acid (ABA), and methyl jasmonic acid, a plant hormone that activates defense responses against herbivores and pathogens (Ohnishi et al., 2005; Sperotto et al., 2009; Jeong et al., 2010; Takasaki et al., 2010; Song et al., 2011); *OsNAC6* is also induced by biotic stresses (such as wounding and blast disease; Nakashima et al., 2007). Rice plants over-expressing either *OsNAC5*, *OsNAC9*, or *OsNAC10* under the control of the root-specific RCc3 promoter improved tolerance to abiotic stresses during the vegetative stage of growth and, most importantly, at the reproductive stage, with a concomitant increase in grain

yield (Jeong et al., 2010, 2013; Redillas et al., 2012). Another characterized SNAC from rice, OsNAC4, was proposed to lead to hypersensitive response in plants after an appropriate pathogen recognition signal is encountered (Kaneda et al., 2009). In *Arabidopsis*, the SNAC genes *ANAC019*, *ANAC055*, and *ANAC072* are induced by pathogen attack and wounding, and transgenic plants over-expressing either one showed a significant increase in drought tolerance (Tran et al., 2004). Considering that these proteins group with the rice paralogs SNAC1, OsNAC3, OsNAC4, OsNAC5, and OsNAC6, their function seems to be conserved.

Several members of the NAC family have been functionally characterized as playing a prominent role in leaf senescence. In *Arabidopsis*, almost one-fifth of the predicted 109 NAC members are in the database of senescence leaf expression sequence tags (dbEST; Guo et al., 2004). Characterization of NAC TFs involved in senescence processes is also available for other plant species, like rice *OsNAC5* (Sperotto et al., 2009); wheat *NAM-B1* (Uauy et al., 2006); and bamboo *BeNAC1* (Chen et al., 2011). The relation between NAC TFs, senescence and nutrient remobilization will be discussed in the next section.

SENESCENCE AND METAL REMOBILIZATION: ARE NAC TRANSCRIPTION FACTORS BRIDGING THE GAP?

In monocarpic plants such as wheat and rice, whole-plant senescence is a coordinated process where catabolic activity provides nutrients that are exported and remobilized to developing grains (Matile et al., 1996; Hortensteiner and Feller, 2002). The *Gpc-B1* quantitative trait loci (QTL) from wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) was first described as conferring high grain protein content in wheat across diverse environments (Chee et al., 2001; Olmos et al., 2003), an important trait for improving bread and pasta quality. Although already mapped and used in breeding programs, *Gpc-B1* locus was cloned later by Uauy et al. (2006), which found the causal gene to be a NAC TF, NAM-B1. *NAM-B1* expression is up-regulated after anthesis in flag leaves and accelerates senescence. In modern wheat varieties, a 1-bp frame-shift insertion at the *NAM-B1* coding sequence results in a truncated version of the protein, while the wild relative has an intact, fully functional NAM-B1. Silencing of NAM-B1 and other NAM paralogs mimicked the insertion effect, resulting in delayed senescence, decreased grain protein, and lower Fe and Zn concentrations due to reduced nutrient remobilization from vegetative tissues (Uauy et al., 2006; Waters et al., 2009). Moreover, a transcriptomic study showed enrichment of sequences related to transport in wild-type (WT) compared to NAM-B1 RNA interference (RNAi) lines during senescence (Cantu et al., 2011). Taken together, these results established NAM-B1 as a positive regulator of senescence and nutrient remobilization during grain maturation, suggesting that an early senescence onset could lead to increased Fe and Zn concentrations in grains (Uauy et al., 2006).

To describe genes with similar functions in other crops, an obvious avenue would be looking at orthologous proteins. The closest homolog of NAM-B1 in the rice genome, named ONAC010 (LOC_Os07g3792; Uauy et al., 2006), has a function in flower development but not in senescence, as neither ONAC010 loss-of-function nor over-expression have the expected effects on senescence timing (Distelfeld et al., 2012). Thus, another paralog with lower sequence similarity could play the NAM-B1 role in rice plants. In this context, *OsNAC5* was demonstrated to be a senescence associated gene that is up-regulated during grain maturation in rice flag leaves (Sperotto et al., 2009). *OsNAC5* is regulated by ABA, a hormone with a known central role in senescence processes (Lim et al., 2007). A comparison of diverse cultivars showed a positive correlation of *OsNAC5* expression in flag leaves before and during anthesis with final Fe, Zn, and protein concentrations in mature grains (Sperotto et al., 2009, 2010). These results showed that *OsNAC5* expression pattern resembles that of *NAM-B1*, and suggested that OsNAC5 could act during senescence-associated nutrient remobilization to rice grains, probably downstream on the senescence onset pathway.

Transgenic plants bearing constructs with *OsNAC5* under the control of a constitutive or a root-specific promoter were generated in a recent work. When OsNAC5 was expressed only in roots, plants increased root diameter and improved recovery after drought stress (Jeong et al., 2013). Interestingly, transcriptomic analysis of roots from both transgenic lines showed commonly up-regulated genes, indicating potential targets of OsNAC5, but not necessarily linked to changes in root morphology (Jeong et al., 2013). Among them, *Nicotianamine Synthase 2* (*OsNAS2*) and *Yellow Stripe-Like 2* (*OsYSL2*), two genes related to metal homeostasis, were up-regulated. OsNAS2 is a key enzyme in nicotianamine (NA) synthesis, a low-molecular weight compound that chelates metals and a precursor for phytosiderophore synthesis (Higuchi et al., 1999), while OsYSL2 is an Fe–NA transporter expressed in phloem cells (Koike et al., 2004). Both NA and OsYSL2 are involved in Fe seed loading and metal long distance transport through the phloem (Koike et al., 2004; Curie et al., 2009; Klatte et al., 2009; Ishimaru et al., 2010; Schuler et al., 2012).

Different studies point that regulation of NA synthesis and metal–NA complex transporters could be involved in remobilization. Constitutive over-expression or activation-tagging of *OsNAS1*, *OsNAS2*, or *OsNAS3* genes in rice increased concentrations of Fe, Zn, or both in grains (Lee et al., 2009, 2011, 2012; Johnson et al., 2011). Concomitant insertion of constructs driving *Hordeum vulgare NAS1* constitutive expression, *OsYSL2* expression in phloem cells and endosperm and *Ferritin* in endosperm, led to increased Fe concentrations (and Zn to a lower extent) in grains (Masuda et al., 2012). In *H. vulgare*, both dark and N deficiency-induced senescence up-regulated *HvNAS2* expression in leaves, resulting in increased phytosiderophore concentration rather than NA (Shi et al., 2012). In *A. thaliana*, loss-of-function of *OsYSL2* homologs, *AtYSL1* and *AtYSL3*, resulted in reduced remobilization of metals to seeds during senescence (Waters et al., 2006).

Therefore, we speculate that OsNAC5 has a role in senescence and metal movement to grains by controlling, either directly or indirectly, the biosynthesis of NA and metal transport through the phloem. Our proposed model, based on data from the literature, is shown in **Figure 1**: (A) a senescence signal is sensed by the cell, activating signaling molecules that regulate the onset of senescence; (B) *OsNAC5* transcription is up-regulated as part of the senescence-induced nutrient remobilization process; (C) OsNAC5 protein up-regulates, either directly or indirectly, *OsNAS2* and *OsYSL2* transcription, as well as other targets (not necessarily

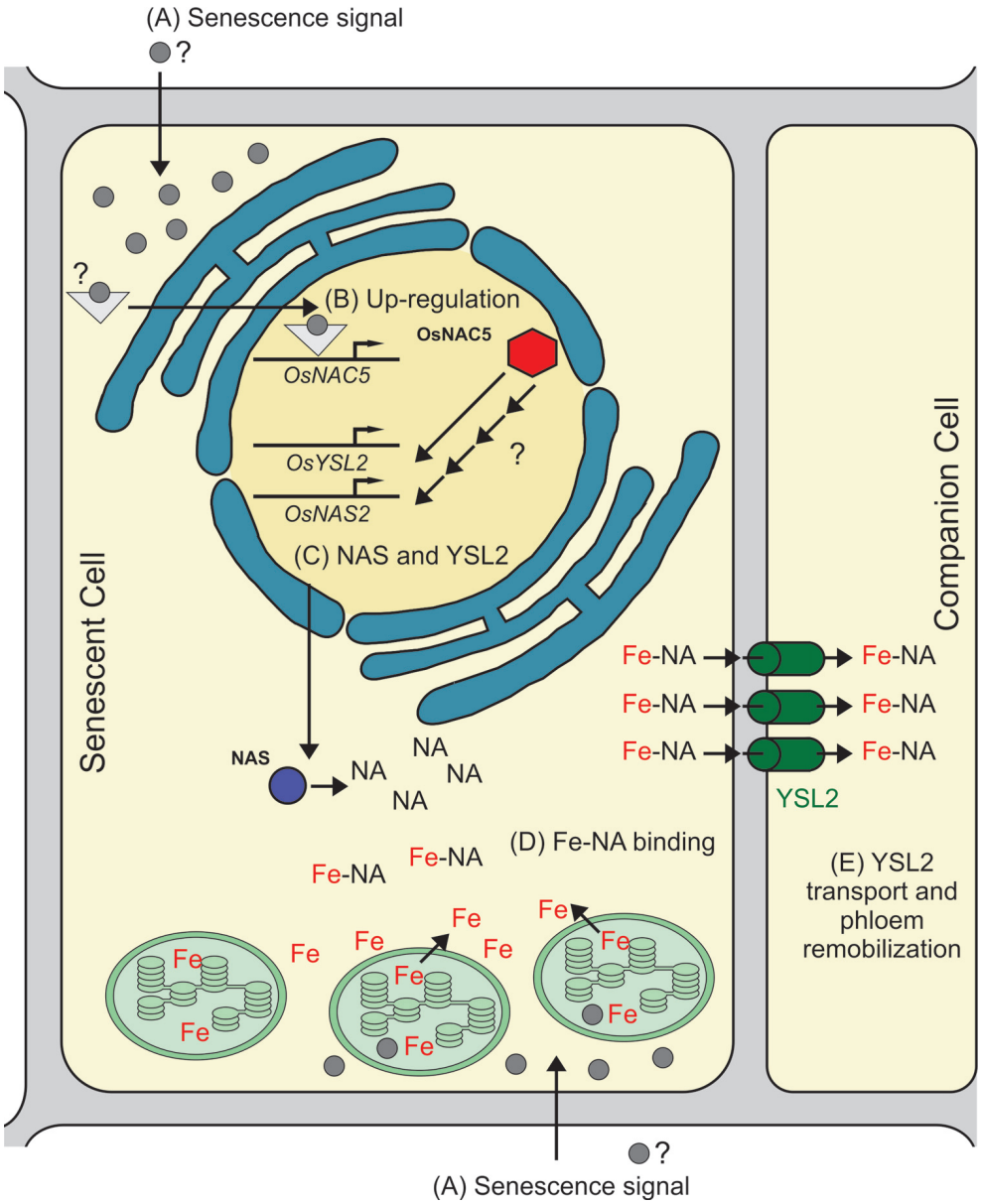

FIGURE 1 | Proposed model for OsNAC5 role in senescence and metal remobilization. The model is based on indirect evidence provided by several studies, especially on Sperotto et al. (2009) and Jeong et al. (2013), and shows only Fe remobilization, although a similar pathway is likely to be involved in Zn and other metals remobilization. **(A)** A signal is sensed by the cell, triggering the senescence-associated cellular components degradation, including chloroplasts, the main site of Fe concentration in the cell. **(B)** *OsNAC5* transcription is up-regulated by the senescence downstream signaling pathway. **(C)** OsNAC5 protein is produced and triggers the up-regulation of *OsNAS2* and *OsYSL2* transcription (based on microarray data presented in the work performed by Jeong et al., 2013). The increased transcription observed is either directly or indirectly regulated by OsNAC5. **(D)** OsNAS2 protein increases intracellular NA concentration, which in turn chelates free Fe coming from chloroplast and other cellular components degradation. **(E)** The Fe–NA complex is transported across the plasma membrane of the senescent cell, and then transported into phloem by OsYSL2, which allows Fe–NA complex long distance translocation. It is important to point the possibility that Fe and NA are exported independently from the cell, interacting in the apoplast and then transported into the phloem by OsYSL2.

related to metal remobilization); (D) OsNAS2 increases NA production, which binds free Fe coming from cellular degradation; (E) after efflux from the cell, OsYSL2 acquires the Fe–NA complex into phloem cells for long distance transport. It is important to note that, while OsYSL2 was not demonstrated to transport Zn–NA complexes, NA is able to bind Zn^{2+}, and Zn–NA complexes are the major Zn form found in the rice phloem sap (Nishiyama et al., 2012). Thus, other transporters could be playing a similar role to OsYSL2 to load Zn–NA into the phloem.

However, work analyzing distinct *OsNAC5* over-expressing lines did not show up-regulation of *OsNAS2* or *OsYSL2* (Takasaki et al., 2010). We should consider that the increased

expression observed by Jeong et al. (2013) was a result from roots transcriptomic analyses, which is not the tissue where remobilization takes place. Moreover, up-regulation of these genes could be an indirect effect rather than a result of OsNAC5 binding to *OsNAS2* or *OsYSL2* promoters.

Transcriptomic analysis of *NAM-B1* RNAi wheat lines did not reveal down-regulation of homologous sequences to *NAS2* or *YSL2* compared to WT, but rather putative *ZIP* (zinc-regulated/iron-regulated transporter) and *NRAMP* (natural resistance associated macrophage protein) metal transporters (Cantu et al., 2011), indicating that NAS and YSL homologs are not regulated by NAM-B1. This could be due to the fact that NAM-B1 and OsNAC5 are not necessarily regulating the same set of genes. NAM-B1 silencing leads to late senescence, while the functional version from wheat wild relative accelerates it. On the other hand, OsNAC5 overexpressing plants were not reported as senescing earlier than WT (Takasaki et al., 2010; Song et al., 2011; Jeong et al., 2013), indicating that OsNAC5 increased expression cannot trigger senescence alone. Silencing of *OsNAC5* did not lead to late senescence as well (Song et al., 2011). OsNAC5 is known to homo- and heterodimerize with other TFs (Jeong et al., 2009), which could be necessary for OsNAC5-mediated response. Thus, it seems that OsNAC5 is acting more downstream on the senescence pathway than NAM-B1, or even in a distinct parallel regulatory network, regulating a different set of senescence-related processes.

Although we should be cautious in the analysis of the available evidence, our model seems to be holding as the first attempt to point out the mechanism of NAC proteins in mineral remobilization in crops. Further work will be necessary to clearly elucidate the role of OsNAC5 in senescence and metal remobilization, as well to functionally demonstrate which genes are controlled by this TF. If true, the regulation of metal remobilization by OsNAC5 could be an interesting avenue for biofortification strategies.

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Roles of plant metal tolerance proteins (MTP) in metal storage and potential use in biofortification strategies

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Zinc (Zn) is an essential micronutrient for plants, playing catalytic or structural roles in enzymes, transcription factors, ribosomes, and membranes. In humans, Zn deficiency is the second most common mineral nutritional disorder, affecting around 30% of the world's population. People living in poverty usually have diets based on milled cereals, which contain low Zn concentrations. Biofortification of crops is an attractive cost-effective solution for low mineral dietary intake. In order to increase the amounts of bioavailable Zn in crop edible portions, it is necessary to understand how plants take up, distribute, and store Zn within their tissues, as well as to characterize potential candidate genes for biotechnological manipulation. The metal tolerance proteins (MTP) were described as metal efflux transporters from the cytoplasm, transporting mainly Zn^{2+} but also Mn^{2+}, Fe^{2+}, Cd^{2+}, Co^{2+}, and Ni^{2+}. Substrate specificity appears to be conserved in phylogenetically related proteins. MTPs characterized so far in plants have a role in general Zn homeostasis and tolerance to Zn excess; in tolerance to excess Mn and also in the response to iron (Fe) deficiency. More recently, the first MTPs in crop species have been functionally characterized. In Zn hyperaccumulator plants, the MTP1 protein is related to hypertolerance to elevated Zn concentrations. Here, we review the current knowledge on this protein family, as well as biochemical functions and physiological roles of MTP transporters in Zn hyperaccumulators and non-accumulators. The potential applications of MTP transporters in biofortification efforts are discussed.

Keywords: biofortification, iron, metal storage, manganese, MTP proteins, zinc

INTRODUCTION

Metal micronutrients such as zinc (Zn), manganese (Mn), and iron (Fe) are essential for plant metabolism and development. These micronutrients serve as substrates for a range of membrane transporters in plants, including the metal tolerance proteins (MTPs). Zn is a cofactor for more than 300 enzymes and 2000 transcription factors, being associated with auxin metabolism, maintenance of membrane integrity and reproduction (Marschner, 1995; Barker and Pilbeam, 2007; Prasad, 2012). Mn activates enzymes involved in DNA synthesis, antioxidant defense, sugar metabolism, and protein modification, and is important for photosynthesis by catalyzing water oxidation in photosystem II (Marschner, 1995; Crowley et al., 2000; Merchant and Sawaya, 2005; Williams and Pittman, 2010). Fe is necessary for redox reactions in chloroplast and mitochondria, chlorophyll synthesis, nitrogen fixation, and DNA replication (Marschner, 1995; Briat et al., 2007). As deficiency of these metals is limiting for growth, plants have developed specific acquisition and transport mechanisms for each of them (for reviews see Puig and Peñarrubia, 2009; Williams and Pittman, 2010; Conte and Walker, 2011; Hindt and Guerinot, 2012; Sinclair and Krämer, 2012).

However, metals can be harmful when in excess. Mn and Fe toxicity are two of the main limiting factors for agriculture in acid soils, which cover 30% of the planet, whereas Zn toxicity is observed in contaminated soils around mining sites (von Uexküll and Mutert, 1995; Krämer, 2010). Excess of both Zn and Mn impairs growth and leads to chlorosis, competing with other ions for binding sites (e.g., leading to Fe deficiency). Fe participates in Fenton chemistry, generating toxic levels of reactive oxygen species when in excess (Marschner, 1995; Clemens, 2001; Schutzendubel and Polle, 2002; Briat et al., 2007; Williams and Pittman, 2010; Shanmugam et al., 2011). Moreover, the Fe transporter IRT1 non-specifically transports Mn and Zn into the root symplast, as well as non-essential but toxic cations such as cadmium (Cd) and nickel (Ni), increasing their concentrations in the plant during the Fe-deficiency response (Eide et al., 1996; Korshunova et al., 1999; Baxter et al., 2008). To avoid cellular damage, heavy metals are generally chelated by low molecular weight compounds, sequestered into organelles or expelled to the extracellular space by specific transporters. One class of metal transporters involved in these functions is the Cation Diffusion Facilitator (CDF) family, also known as the Metal Tolerance Proteins (MTPs) in plants.

CDF transporters are phylogenetically ubiquitous, spanning the Archaea, Eubacteria, and Eukaryote kingdoms (Nies and Silver, 1995). CDF proteins seem to act as Metal^{2+} (Me^{2+})/H^+ antiporters, and contain amino and carboxy cytoplasmic termini, as well as six transmembrane domains (TMD), with a few exceptions (Guffanti et al., 2002; Chao and Fu, 2004; Grass et al., 2005; Kawachi et al., 2008). The three dimensional structure of the CDF protein from *Escherichia coli* YiiP is known (Lu and Fu, 2007). The protein structure, together with other pieces of evidence, suggests that CDF transporters act as homodimers (Blaudez et al., 2003; Wei and Fu, 2006; Lu and Fu, 2007). Transported substrates include a wide range of divalent cations, such as cobalt (Co^{2+}), Ni^{2+}, Mn^{2+}, Cd^{2+}, Fe^{2+}, and Zn^{2+}, the latter being the most commonly transported ion (Anton et al., 1999; Persans et al., 2001; Delhaize et al., 2003; Munkelt et al., 2004; Grass et al., 2005; Montanini et al., 2007). CDF transporters are found in different membranes, such as the bacterial cell membrane, Golgi apparatus of mammals and plants, and vacuolar membranes of yeast and plants, acting in metal efflux from the cytoplasm, either to the extracellular space or into organelles (Haney et al., 2005; Peiter et al., 2007). Only one case of a CDF protein that transports Zn into the cytoplasm was described (Cragg et al., 2002).

Comprehensive phylogenetic analyses of the CDF family in several species, including bacteria, fungi, plants, and mammals, have grouped its members into three major clusters, named Zn-CDFs, Fe/Zn-CDFs, and Mn-CDFs, based on the hypothesized or confirmed transported substrate of a few members (Montanini et al., 2007). A CDF signature was found in a region that encompasses TMD II, TMD III, and the cytoplasmic loop between them. Based on multiple protein alignments and putative substrate specificity, it was also proposed that two amino acid motifs located at TMD II and TMD V are responsible for metal selectivity (Montanini et al., 2007).

In plants, the CDFs are referred to as MTP, mainly due to their role in sequestration of excessive Zn in the vacuoles of both metal non-hyperaccumulators (e.g. *Arabidopsis thaliana*) and hyperaccumulators (e.g. *Arabidopsis halleri* and *Noccaea caerulescens*). Plant-focused phylogenetic analysis confirmed the three specificity-based clusters previously proposed (Montanini et al., 2007; Migeon et al., 2010; Gustin et al., 2011). The plant MTPs divide further phylogenetically into seven groups, and group naming follows nomenclature of MTP sequences from *A. thaliana* (Gustin et al., 2011). Sequences of all seven groups are found in each plant genome analyzed, indicating that expansion of the MTP family predates the emergence of land plants. Groups 1, 12, and 5 are part of the Zn-CDFs; groups 6 and 7, of the Fe/Zn-CDF; and groups 8 and 9, of the Mn-CDF (Gustin et al., 2011). To date, the only characterized proteins in plants are members of group 1, which includes the Zn vacuolar transporters AtMTP1 and AtMTP3 (Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006); and from group 9, which includes the trans-Golgi/pre-vacuolar compartment-localized AtMTP11 (Delhaize et al., 2007; Peiter et al., 2007). The most updated and comprehensive phylogenetic tree of plant MTP proteins can be seen in Gustin et al. (2011). A simplified version of that tree (with only MTP sequences from *A. thaliana*, which are used to name MTP groups, plus sequences from proteins mentioned in this review) is shown in **Figure 1**, to help the reader comprehend the relationships among the different groups mentioned in the text.

Here, we review the current knowledge on MTP proteins and their roles in plant metal homeostasis, as well as studies on molecular determinants of specificity in the MTP family. We also discuss the function that MTPs play in metal hypertolerant/hyperaccumulator species. As MTP proteins have the ability to isolate metals inside cells of specific plant organs in a safe way, we discuss the potential for using these proteins in biofortification applications. The current information available on characterized plant MTP proteins as well as mutations (including single and multiple residue deletions and substitutions) that could change substrate specificity are presented in **Tables 1** and **2**, respectively.

MTPs FROM GROUP 1 OF THE Zn-CDF CLUSTER

GROUP 1 MTP PROTEINS ARE VACUOLAR Zn TRANSPORTERS

Group 1 of MTPs went through some duplication events during evolution, and include *MTP1*, *MTP2* (*MTP1* and *MTP2* cluster is referred as *MTP1/2*), *MTP3*, and *MTP4* sequences (Gustin et al., 2011). The *Sorghum bicolor* and *Oryza sativa* genomes lack MTP4 sequences, suggesting that the monocot lineage may have lost this gene. Phylogenetic analysis indicates that MTP1/2 and MTP3 sequences share a common ancestor; at the time of duplication, after the monocot/eudicot divergence, MTP1/2 and MTP3 most likely shared identical redundant function (Gustin et al., 2011). Although MTP1/2 and MTP3 functions diverged in *A. thaliana* (**Figure 1**), *Sorghum bicolor* and *Oryza sativa* MTP1 may retain the full function of the ancestral gene, as these species do not show *MTP2* and *MTP3* sequence in their genomes (Gustin et al., 2011). Transcriptional evidence suggests that genes from Group 1 are largely expressed in a variety of plants and algae, indicating the group's general importance in the photosynthetic lineage (Gustin et al., 2011).

MTP1 and *MTP3* DNA sequences from *A. thaliana* share 68% sequence identity, and proteins have similar predicted secondary structure (van der Zaal et al., 1999; Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006). Several studies have demonstrated that AtMTP1 has Zn transport activity (**Table 1**). When heterologously expressed in *E. coli* and reconstituted into proteoliposomes, uptake of Zn did not require a proton gradient across the liposomal membrane. More Zn accumulates in *Xenopus laevis* oocytes expressing *AtMTP1* than in water-injected oocytes. The Zn-hypersensitive yeast mutant *zrc1cot1* expressing AtMTP1 is tolerant to excessive Zn (**Table 1**). Similarly, AtMTP3 is able to transport Zn and Co when heterologously expressed in the *zrc1cot1* yeast mutant (**Table 1**). Both proteins have a function in metal detoxification by transport into the vacuole (**Figure 2**; Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006; Kawachi et al., 2009).

Silencing of *AtMTP3* or *AtMTP1* and the disruption of *AtMTP1* by T-DNA insertion (Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Kawachi et al., 2009) led to Zn sensitivity, which suggested that the silenced or mutant plants cannot compensate the lack of one gene by expressing the other one, and therefore *AtMTP3* and *AtMTP1* have non-redundant functions in Zn tolerance (Arrivault et al., 2006). Promoter activity of *AtMTP3* and *AtMTP1* partially overlaps in roots: the former has

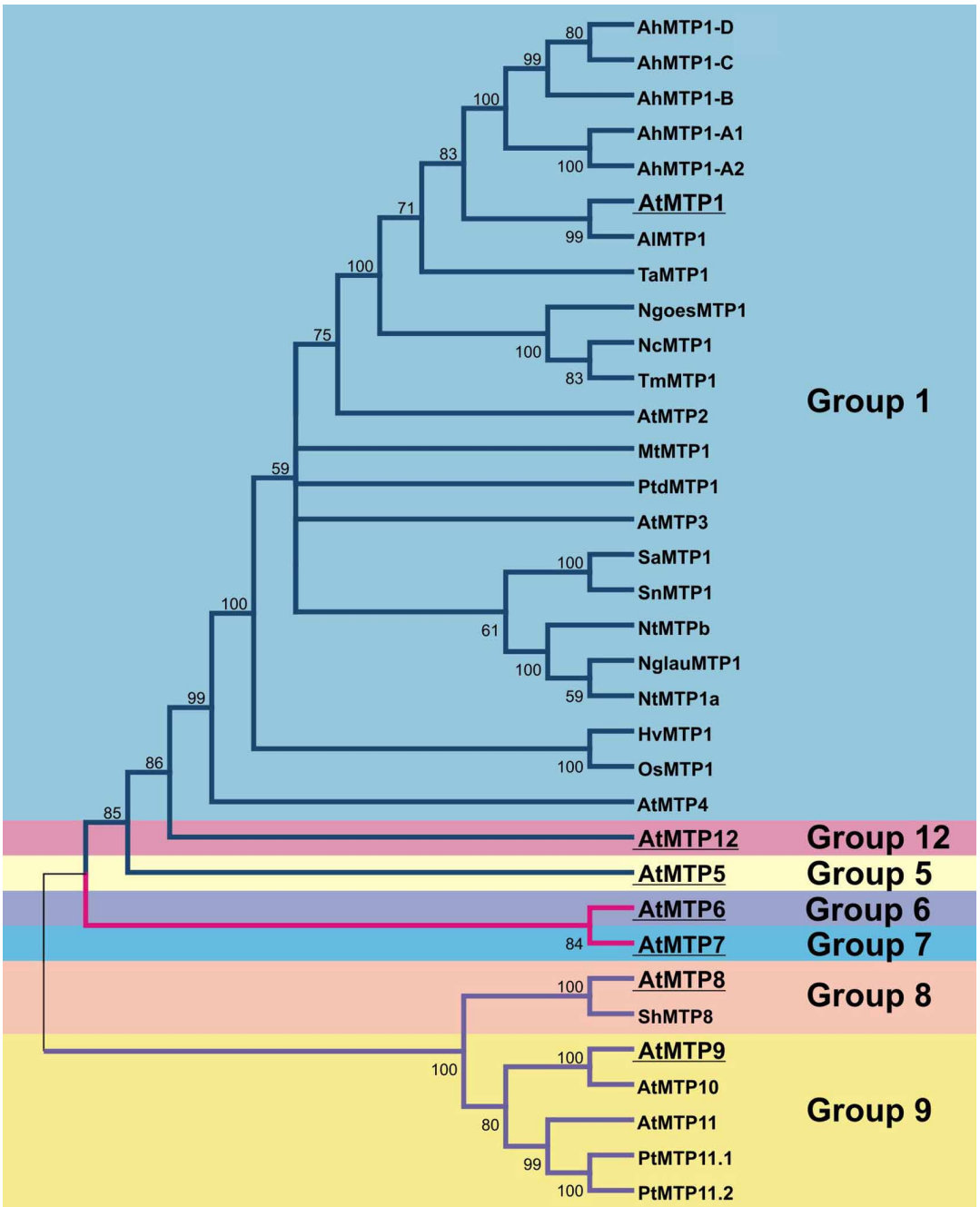

FIGURE 1 | Guide tree showing the phylogenetic groups of MTP proteins in plants. The tree was constructed using all sequences cited in **Table 1** plus all MTP proteins from *A. thaliana*, as each group of MTP was named after its first *A. thaliana* member. Founding members of each group are highlighted. Colors of branches are in blue for Zn-CDF group proteins; pink for Fe/Zn-CDF proteins; and purple for Mn-CDF proteins. The purpose of the tree is to help understand the discussion of MTP proteins presented in this review. For evolutionary relatedness analyses, we refer to the more complete datasets and trees showed by Gustin et al. (2011). For clarity, both branched and group colors are similar to those used by Gustin et al. (2011). Our phylogenetic analysis was performed using protein sequences aligned by ClustalW; the tree constructed using Neighbor-Joining algorithm, with the following parameters: pairwise deletion, Poisson correction, and 1000 replications for bootstrap confidence level estimation. Accession numbers are: from Phytozome (http://www.phytozome.org): AtMTP1(At2g46800), AtMTP2(At3g61940), AtMTP3(At3g58810), AtMTP4(At2g29410), AtMTP5 (At3g12100), AtMTP6(At2g47830), AtMTP7(At1g51610), AtMTP8 (At3g58060), AtMTP9(At1g79520), AtMTP10(At1g16310), AtMTP11 (At2g39450), AtMTP12(At2g04620), AlMTP1(483845), OsMTP1 (LOC_Os05g03780); from Genbank (http://www.ncbi.nlm.nih.gov/genbank/): AhMTP1-A2(AJ556183), AhMTP1-B(FN386317), AhMTP1-C(FN386316), AhMTP1-D(FN386315), HvMTP1(AM286795), MtMTP1(FJ389717), NcMTP1 (AF275750), NglauMTP1(AB201239), NtMTP1a(AB201240), NtMTPb (AB201241), NgoesMTP1(AY044452), PtMTP11.1(EF453693), PtMTP11.2 (EF453694), PtdMTP1(AY450453), SaMTP1(JF794551), ShMTP8 (AY181256), SnMTP1(JF794552), TaMTP1(AY483145), TmMTP1(AY483144). Gene names are according to **Table 1**.

Table 1 | Functionally characterized plant MTP proteins.

Protein	Species	Substrate	Substrate test	Transcriptional regulation	Subcellular localization	References
AhMTP1-A1	*Arabidopsis halleri*	Zn	*zrc1cot1*	++Zn (↑roots)[b]	Tonoplast	Dräger et al., 2004; Shahzad et al., 2010
AhMTP1-A2	*Arabidopsis halleri*	Zn	*zrc1cot1*	++Zn (↑roots)[b]	–	Shahzad et al., 2010
AhMTP1-B1	*Arabidopsis halleri*	Zn	*zrc1cot1*	–	–	Shahzad et al., 2010
AhMTP1-C	*Arabidopsis halleri*	Zn	*zrc1cot1*	++Zn (↓shoots, ↓roots)	–	Shahzad et al., 2010
AhMTP1-D	*Arabidopsis halleri*	Zn	*zrc1cot1*	++Zn (↓shoots, ↓roots)	–	Shahzad et al., 2010
AlMTP1	Arabidopsis lyrata	Zn	*zrc1cot1*	–	–	Kim et al., 2004
AtMTP1	*Arabidopsis thaliana*	Zn	*zrc1cot1, E. coli* proteoliposomes, *X. laevis* oocytes	–	Tonoplast	Bloss et al., 2002; Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Kawachi et al., 2008
AtMTP3	*Arabidopsis thaliana*	Zn, Co	*zrc1cot1*	−Fe, ++Zn, ++Co, ++Mn (↑roots)	Tonoplast	Arrivault et al., 2006
AtMTP11	*Arabidopsis thaliana*	Mn, Cu	INVSc2, *pmr1*	–	Pre-vacuolar compartment/trans-Golgi	Delhaize et al., 2007; Peiter et al., 2007
HvMTP1	*Hordeum vulgare*	Zn, Co	*zrc1cot1, zrc1, cot1*	–	Tonoplast	Podar et al., 2012
MtMTP1	*Medicago truncatula*	Zn	*zrc1, cot1*	++Zn	Tonoplast	Chen et al., 2009
NcMTP1	*Noccaea caerulescens*	Zn	*zrc1cot1*	–	–	Assunção et al., 2001; Kim et al., 2004
NgoesMTP1[a]	*Noccaea goesingense*	Zn, Cd, Co and Ni	*zrc1cot1, cot1, zcr1, pep5*	–	Tonoplast[c]	Persans et al., 2001; Kim et al., 2004; Gustin et al., 2009
NglauMTP1	*Nicotiana glauca*	Zn, Co	*zrc1, cot1*	–	Tonoplast[d]	Shingu et al., 2005
NtMTP1-A	*Nicotiana tabacum*	Zn, Co	*zrc1, cot1*	–	Tonoplast[d]	Shingu et al., 2005
NtMTP1-B	*Nicotiana tabacum*	Zn, Co	*zrc1, cot1*	–	Tonoplast[d]	Shingu et al., 2005
OsMTP1	*Oryza sativa*	Zn, Co, Ni, Cd, and Fe	*cot1, zrc1 cot1, smf1,* DY1455, *ycf1, ccc1, pmr1*	++Zn, ++Cd, ++Fe, ++Cu (↑shoots, ↑roots)	plasma membrane, Tonoplast[c,e]	Lan et al., 2012; Yuan et al., 2012; Menguer et al., 2013
PtMTP11.1	*Populus trichocarpa*	Mn	*pmr1*	–	trans-Golgi	Peiter et al., 2007
PtMTP11.2	*Populus trichocarpa*	Mn	*pmr1*	–	trans-Golgi	Peiter et al., 2007
PtdMTP1	*Populus trichocarpa* × *Populus deltoides*	Zn	*zrc1, cot1*	–	Tonoplast	Blaudez et al., 2003; Montanini et al., 2007
SaMTP1	*Sedum alfredii*	Zn	*zrc1*	–	Tonoplast	Zhang et al., 2011
ShMTP8	*Stylosanthes hamata*	Mn, Cu	INVSc2, *cnb1, pmr1*	–	Tonoplast, Endoplasmic Reticulum	Delhaize et al., 2003, 2007
SnMTP1	*Sedum aldredii*	Zn	*zrc1*	–	–	Zhang et al., 2011
TaMTP1	*Thlaspi arvense*	Zn	*zrc1cot1*	–	–	Kim et al., 2004
TmMTP1	*Thlaspi montanum*	Zn	*zrc1cot1*	–	–	Kim et al., 2004

Table shows protein transported substrate, used method for substrate determination, transcript regulation (up ↑ or down ↓) by metal deficiency (–) or excess (++) and tested subcellular localization.

[a] *All three allelic variants/distinct loci (Kim et al., 2004).*

[b] *Not distinguishable by PCR, same pair of primers.*

[c] *Subcellular localization in yeast.*

[d] *First shown to be localized also in the plasma membrane by Kim et al., 2004.*

[e] *Subcellular localization in Arabidopsis thaliana.*

Table 2 | MTP1 mutations already published are reported with the corresponding topological position and yeast complementation assays performed for different metals.

Protein	Species	Amino acid changes	Location	Zn	Co	Cd	Fe	Mn	References
HvMTP1	*Hordeum vulgare*	WT		+	+	−	−	−	Podar et al., 2012
		Substitution Hv/At-His-loop	His-loop	+	−	×	×	×	‖
		Substitution Hv/At-N-His-loop	His-loop	+	−	×	×	×	‖
		Substitution Hv/At-C-His-loop	His-loop	+	+	×	×	×	‖
		Deletion VTVTT	His-loop	+	−	×	×	×	‖
		Substitution VTVTT/NESDD	His-loop	+	+	×	×	×	‖
		N206V	His-loop	+	+	×	×	×	‖
		S207T	His-loop	+	+	×	×	×	‖
		E208V	His-loop	+	+	×	×	×	‖
		D209T	His-loop	+	+	×	×	×	‖
		D210T	His-loop	+	+	×	×	×	‖
AtMTP1	*Arabidopsis thaliana*	WT		+	−	−	−	−	Kawachi et al., 2008
		Deletion His-loop	His-loop	+	+	−	×	×	‖
		Substitution At/Hv-His-loop	His-loop	+	+	×	×	×	Podar et al., 2012
		Substitution At/Hv-N-His-loop	His-loop	+	+	×	×	×	‖
		Substitution At/Hv-C-His-loop	His-loop	+	−	×	×	×	‖
		Deletion NSEDD	His-loop	+	+	×	×	×	‖
		Substitution NESDD/VTVTT	His-loop	+	+	×	×	×	‖
		V205N	His-loop	+	+	×	×	×	‖
		T206S	His-loop	+	−	×	×	×	‖
		V207E	His-loop	+	+	×	×	×	‖
		T208D	His-loop	+	+	×	×	×	‖
		T209D	His-loop	+	+	×	×	×	‖
		H196Q	His-loop	+	+	×	×	×	‖
		H201L	His-loop	+	+	×	×	×	‖
		H206A	His-loop	+	+	×	×	×	‖
		T208A	His-loop	+	+	×	×	×	‖
		H212N	His-loop	+	+	×	×	×	‖
		H212L	His-loop	+	+	×	×	×	‖
		C31A	N-terminal	±	−	×	×	×	Kawachi et al., 2012
		C31D	N-terminal	±	−	×	×	×	‖
		C31E	N-terminal	±	−	×	×	×	‖
		C31S	N-terminal	±	−	×	×	×	‖
		C36A	N-terminal	±	−	×	×	×	‖
		C36D	N-terminal	+	−	×	×	×	‖
		C36E	N-terminal	±	−	×	×	×	‖
		C36M	N-terminal	±	−	×	×	×	‖
		Deletion 2-12	N-terminal	+	+	+	×	×	‖
		Deletion 2–28	N-terminal	+	+	+	×	×	‖
		Deletion 2–55	N-terminal	−	−	−	×	×	‖
		C59A	TMD I	+	−	×	×	×	‖
		C65A	TMD I	+	−	×	×	×	‖
		E72A	TMD I	±	−	×	×	×	‖
		S81A	EL1	±	−	×	×	×	‖
		T86A	EL1	+	+	+	×	×	‖
		D87A	EL1	±	−	×	×	×	‖
		H90A	TMD II	−	−	×	×	×	‖
		L91M	TMD II	+	+	−	×	×	‖
		S93A	TMD II	+	−	×	×	×	Podar et al., 2012
		S93T	TMD II	+	−	×	×	×	‖
		D94A	TMD II	−	−	×	×	×	Kawachi et al., 2012

(Continued)

Table 2| Continued

Protein	Species	Amino acid changes	Location	Zn	Co	Cd	Fe	Mn	References
		A99T	TMD II	+	+	×	×	×	Podar et al., 2012
		A99V	TMD II	+	+	×	×	×	‖
		S101A	TMD II	+	+	+	×	×	Kawachi et al., 2012
		S104A	TMD II	+	−	×	×	×	‖
		T113A	IL1	±	−	×	×	×	‖
		T117C	IL1	−	−	×	×	×	‖
		F120A	IL1	+	−	×	×	×	‖
		R122A	TMD III	−	−	×	×	×	‖
		E124A	TMD III	−	−	×	×	×	‖
		V130A	TMD III	+	+	×	×	+	Podar et al., 2012
		I135F	TMD III	±	+	×	×	+	‖
		I135V	TMD III	+	−	×	×	−	‖
		I135G	TMD III	+	+	×	×	−	‖
		I135L	TMD III	+	+	×	×	+	‖
		I135Y	TMD III	±	+	×	×	+	‖
		I135Q	TMD III	+	+	×	×	+	‖
		I135N	TMD III	±	+	×	×	+	‖
		I135E	TMD III	±	+	×	×	+	Podar et al., 2012
		G140A	TMD III	+	+	×	×	+	‖
		Y144C	TMD III	+	+	+	×	×	Kawachi et al., 2012
		E145G	TMD III	+	+	×	×	+	Podar et al., 2012
		E145N	TMD III	+	+	×	×	+	‖
		E145A	TMD III	+	+	+	×	×	Kawachi et al., 2012
		R149C	EL2	+	+	+	×	×	‖
		E153A	EL2	+	−	×	×	×	‖
		E156A	EL2	+	−	×	×	×	‖
		N158A	EL2	±	−	×	×	×	‖
		N173A	TMD IV	±	−	×	×	×	‖
		N258A	TMD V	+	+	+	×	×	‖
		H265A	TMD V	±	−	×	×	×	‖
		D269C	TMD V	±	−	×	×	×	‖
		E288A	EL3	+	−	×	×	×	‖
		W289A	EL3	+	−	×	×	×	‖
		D289A	EL3	−	−	×	×	×	‖
		C296A	TMD VI	+	−	×	×	×	‖
		L298A	TMD VI	+	+	+	×	×	‖
		L305A	TMD VI	±	+	−	×	×	‖
		I312A	TMD VI	+	+	+	×	×	‖
		L319A	TMD VI	±	−	−	×	×	‖
		L298A/L305A	TMD VI	±	+	+	×	×	‖
		I312A/L319A	TMD VI	±	−	−	×	×	‖
		L298A/L305A/I312A	TMD VI	±	−	+	×	×	‖
		L298A/L305A/I312A/L319A	TMD VI	±	+	−	×	×	‖
		T323C	C-terminal	+	+	+	×	×	‖
		H346A	C-terminal	−	−	×	×	×	‖
		E347A	C-terminal	−	−	×	×	×	‖
		C362A	C-terminal	+	−	×	×	×	‖
		D373A	C-terminal	+	−	×	×	×	‖
		H391C	C-terminal	±	−	×	×	×	‖
		Q395A	C-terminal	−	−	×	×	×	‖

(Continued)

Table 2| Continued

Protein	Species	Amino acid changes	Location	Zn	Co	Cd	Fe	Mn	References
		E397A	C–terminal	±	−	×	×	×	‖
PtdMTP1	*Populus trichocarpa* × *Populus deltoides*	WT		+	−	−	×	−	Blaudez et al., 2003
		C30S	N-terminal	±	×	×	×	×	Montanini et al., 2007
		C35S	N-terminal	±	×	×	×	×	‖
		C64S	TMD I	±	×	×	×	×	‖
		D86A	TMD II	−	×	×	×	×	Blaudez et al., 2003; Montanini et al., 2007
		H89A	TMD II	−	×	×	×	×	Blaudez et al., 2003
		H89K	TMD II	−	×	×	×	×	‖
		D93A	TMD II	−	×	×	×	×	Blaudez et al., 2003; Montanini et al., 2007
		H260D	TMD V	−	×	×	×	×	Montanini et al., 2007
		D264A	TMD V	−	×	×	×	×	‖
		D264E	TMD V	−	×	×	×	×	‖
		D288A	TMD VI	−	×	×	×	×	‖
		D288E	TMD VI	−	×	×	×	×	‖
		L293A	TMD VI	+	×	×	×	×	Blaudez et al., 2003
		L300A	TMD VI	±	×	×	×	×	‖
		L307A	TMD VI	−	×	×	×	×	‖
		L314A	TMD VI	−	×	×	×	×	‖
		L293A/L300A	TMD VI	+	×	×	×	×	‖
		L293A/L307A	TMD VI	±	×	×	×	×	‖
		L293A/L314A	TMD VI	−	×	×	×	×	‖
		L300A/L307A	TMD VI	−	×	×	×	×	‖
		L300A/L314A	TMD VI	−	×	×	×	×	‖
		L307A/L314A	TMD VI	−	×	×	×	×	‖
OsMTP1	*Oryza sativa*	WT		+	+	+	+	−	Menguer et al., 2013
		L82F	EL1	±	+	+	+	+	‖
		L82S	EL1	±	±	+	±	−	‖
		H90D	TMD II	−	+	+	+	−	‖
		G127S	TMD III	±	+	+	+	−	‖
		E145G	TMD III	±	+	+	+	−	‖
		R149G	TMD III	+	+	+	+	−	‖
		L317A	TMD VI	±	+	+	+	−	‖

Mutations presented are substitutions, deletions or site-directed mutations.

WT, wild type; TMD, transmembrane domain; His-loop, histidine loop; IL, intracytosolic loop; EL, extracytosolic loop; +, growth in yeast complementation assay; −, no growth in yeast complementation assay; ±, partial growth in yeast complementation assay; X, not tested; ‖, same reference.

low but inducible expression, mainly localized to the epidermis and cortex of the root hair zone, while the later has constitutive expression, localized at the meristematic and elongation zones. *AtMTP3* expression is undetectable in shoots, whereas *AtMTP1* is expressed in young leaves (Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006). *AtMTP1* transcripts are not regulated by Zn supply, whereas AtMTP3 is up-regulated by Zn, Mn, and Co excess, as well as Fe deficiency (Bloss et al., 2002; Kobae et al., 2004; Desbrosses-Fonrouge et al., 2005; Arrivault et al., 2006; Kawachi et al., 2008). It was proposed that the AtMTP1 functions in sequestering Zn in sensitive, dividing and expanding tissues, and generating Zn stores in specific tissues of the shoot, while AtMTP3 would be involved in removing Zn from the root-to-shoot translocation pathway under high Zn influx (Sinclair and Krämer, 2012).

The MTP1 protein from the legume model plant *Medicago truncatula* can complement the Zn-susceptible *zrc1cot1* yeast double mutant (**Table 1**). The expression of *MtMTP1* is detected in all vegetative organs with the highest level of expression observed in leaves. Zn supplementation reduces *MtMTP1* expression in roots and increases it in stems (**Table 1**), while no obvious changes are detected in leaves (Chen et al., 2009). However, the subcellular localization of MtMTP1 is not known. *PtdMTP1* from a hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) is constitutively expressed, complements both yeast single mutants *zrc1* and *cot1* and is localized at the vacuole (**Table 1**). When over-expressed in *Arabidopsis*, *PtdMTP1* confers enhanced Zn tolerance (Blaudez et al., 2003). Taken together, these results indicate that MtMTP1 and PtdMTP1 have a similar role to AtMTP1/AtMTP3 from *A. thaliana*.

The rice *OsMTP1* gene was recently characterized. Located on chromosome 5, it is most highly expressed in mature leaves and stems. *OsMTP1* expression is significantly induced by exposure to metals such as Zn, Cd, Cu, and Fe (**Table 1**; Lan et al., 2012; Yuan

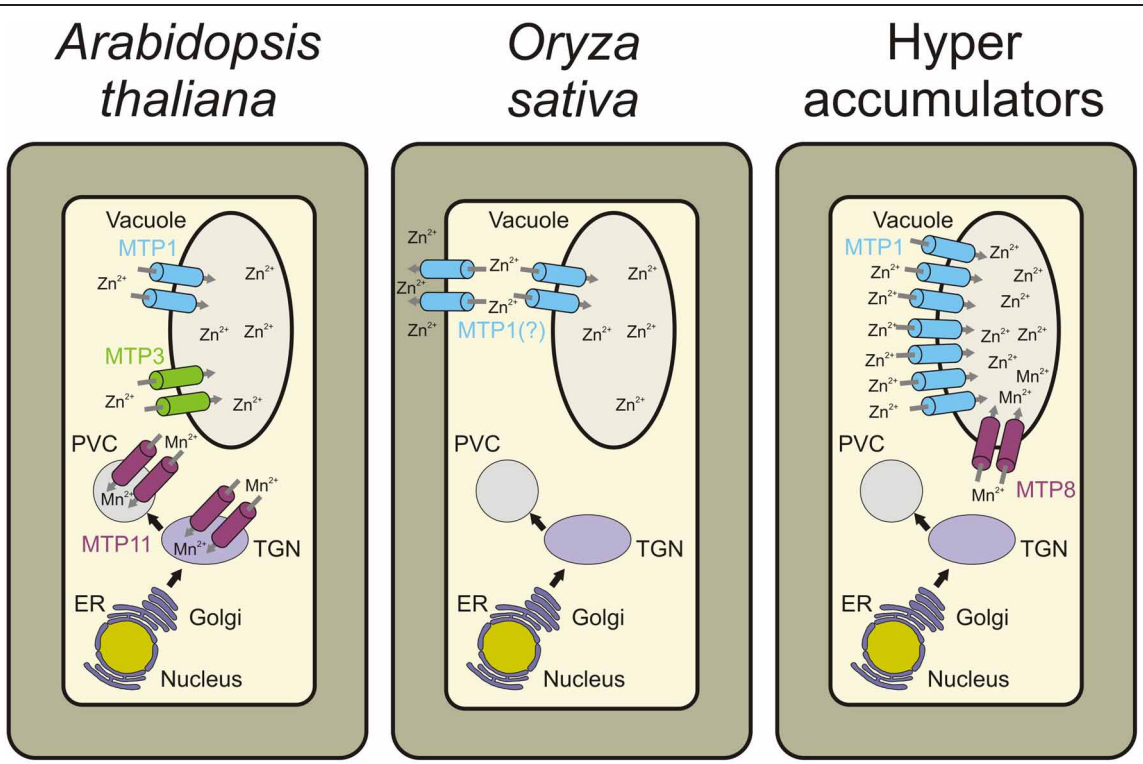

FIGURE 2 | Schematic representation of a cell and the different MTP roles in cellular metal homeostasis in each plant species. In *A. thaliana*, MTP1 and MTP3 are vacuolar Zn transporters. Whereas MTP1 is more widely expressed in the plant, MTP3 expression is restricted to root epidermis and cortex. MTP11 transports Mn into the trans-Golgi network (TGN) and/or the prevacuolar compartment (PVC; Delhaize et al., 2007; Peiter et al., 2007). In *Oryza sativa*, MTP1 is described as a plasma membrane Zn transporter based on onion epidermal cell transient expression or as tonoplast-localized when heterologously expressed in yeast and Arabidopsis (Menguer et al., 2013). In Zn hyperaccumulators such as *A. halleri*, *N. caerulescens*, and *N. goesingense*, the MTP1 protein is highly expressed, being necessary for hypertolerance to high metal concentrations. In the Mn hyperaccumulator *S. hamata*, MTP8 transports Mn into the vacuole. Contrary to MTP1, the role of MTP8 in hyperaccumulation/hypertolerance seems to be more related to higher transport efficiency than to increased copy number (Delhaize et al., 2007).

et al., 2012). Both overexpression and RNAi-mediated silencing suggest a role for the transporter in Zn, Cd, and Ni movement. *OsMTP1* expression increased tolerance to Zn, Cd, and Ni in *cot1*, *ycf1*, and *smf1* yeast mutants, respectively, during the exponential growth phase, but was not able to complement the *pmr1* mutant yeast strain hypersensitivity to Mn (**Table 1**; Yuan et al., 2012). More recently, heterologous expression of *OsMTP1* in the yeast strain *zrc1cot1* complemented the Zn-hypersensitivity of this mutant. OsMTP1 could also alleviate to some extent the Co sensitivity of *zrc1cot1* and rescued Fe and Cd hypersensitivity in *ccc1* and *ycf1* mutants, respectively, when tested at low concentrations of corresponding metals (Menguer et al., 2013). OsMTP1 did not complement the *pmr1* mutant for Mn transport, as reported previously. Overall, the results suggest that OsMTP1 transports Zn but also Co, Fe, Cd, and Ni, possibly with lower affinity (Menguer et al., 2013). Based on QTLs localization, *OsMTP1* was identified as a high priority candidate gene for enhancement of Fe and Zn concentrations in seeds (Anuradha et al., 2012).

There is controversy over OsMTP1 localization, reported at the plasma membrane when expressed in onion epidermal cells (Yuan et al., 2012) or at the vacuole when expressed in *Saccharomyces cerevisiae* and in the *A. thaliana* mutant *mtp1-1* (**Table 1**; Lan et al., 2012; Menguer et al., 2013). The barley ortholog HvMTP1 exhibits selectivity for both Zn and Co, which was demonstrated by its ability to suppress hypersensitivity to both metals in yeast mutants, and localizes at the vacuolar membrane (**Table 1**). *HvMTP1* transcripts are ubiquitously present in barley organs including roots, shoots, and seeds (Podar et al., 2012). *OsMTP1* and *HvMTP1* have high identity (84%) and similarity (90%) at the amino acid level. Based on conservation and phylogenetic relatedness, we may expect a similar function and subcellular localization. Therefore, OsMTP1 is more likely located at the tonoplast.

KEY RESIDUES DETERMINE METAL SELECTIVITY OF GROUP 1 Zn-CDF MTP TRANSPORTERS

Key positions at structural sites, including single and multiple residue stretches at cytosolic and TMD, contribute to cation selectivity of MTP proteins. Mutations affecting just one of these structural sites can significantly broaden cation specificity. This suggests that the cation is selected throughout the process of ion translocation, possibly with considerable cooperativity (Kawachi et al., 2012).

Group 1 MTP proteins contain key polar and charged residues conserved within TMDs I, II, V, and VI, which are likely involved

in metal transport of group 1 proteins (Gaither and Eide, 2001; Haney et al., 2005). Besides, a histidine-rich cytoplasmic loop between TMD IV and V (**Figure 3**) is thought to be vital for transporter specificity, which might act as a chaperone determining the identity of metal ions to be transported and/or as a sensor of cytoplasmic Zn levels (Shingu et al., 2005; Kawachi et al., 2008; Podar et al., 2012). A summary of all MTP1 mutations described in the literature is presented in **Table 2**. This is the first effort to assemble all information about MTP1 substrate specificity in relation to the presence or absence of key residues.

Removal of the His loop from the NcMTP1 protein from the hyperaccumulator *Noccaea* (formerly *Thlaspi*) *caerulescens* results in a non-functional MTP protein (Kim et al., 2004). However, deletion of the 32 terminal residues from the loop of AtMTP1 confers the ability to complement Co-sensitive phenotypes, while still transporting Zn in yeast (**Table 2**; Kawachi et al., 2008). MTP proteins from *Nicotiana tabacum* and *N. glauca* complement both Zn and Co-sensitive yeast mutants (**Table 1**), and the His loop of both species show more His residues than AtMTP1 (Shingu et al., 2005). By swapping the His loops of AtMTP1 and HvMTP1, HvMTP1 lost Co transport activity, and a five-residue (VTVTT) region of the loop was shown to confer specificity to Zn transport when present (**Table 2**; Podar et al., 2012).

These proteins also contain a Leu-zipper (LZ) motif within TMD VI, a repeating pattern of leucine residues at every seventh position that forms a functional alpha helix (Blaudez et al., 2003). The presence of a LZ motif in MTP proteins was first described in PtdMTP1, from poplar. The authors had discovered that the PtdMTP1 protein forms multimers, possibly stabilized by S–S bridges. Looking for other features possibly involved in protein-protein interactions, they identified a LZ motif in the C-terminal region of the protein (Blaudez et al., 2003). Further experiments demonstrated that the LZ motif is not necessary for oligomer formation, but Leu residues within this motif are required for PtdMTP1 functional activity, possibly mediating subtle interactions that are required for the function of the multimeric complex. Leucine residues within the motif were serially substituted for alanine (**Table 2**), maintaining the non-polar environment but changing the side-chain. These mutations had increasing negative effects on the Zn-transport ability of PtdMTP1 as they were located closer to the protein's C terminal end (Blaudez et al., 2003). It was suggested that the last Leu residue (Leu-314) of the LZ motif is the most critical one for the function of PtdMTP1 (Blaudez et al., 2003). The LZ motif is conserved in a range of CDF proteins from several species, although some interruptions are common within the motif. The final Leu residue of the LZ was found to be highly conserved among plant CDFs, being probably the most important one for function. Recent works demonstrated that mutations falling at the beginning of the LZ motif have little or no effect on Zn tolerance, although possibly impacting in transport of other metals: AtMTP1 mutation in the residue Leu298 conferred gain of function of Co and Cd transport in yeast experiments and OsMTP1 mutation in the residue L317 enhanced Co tolerance (**Table 2**; Kawachi et al., 2012; Menguer et al., 2013). In contrast, the last Leu residue of the LZ motif in AtMTP1 (Leu319) is essential for protein function and is predicted to form dimerization contact between two protomers, in analogy to the corresponding residue Leu205 of the broad-specificity divalent cation transporter EcYiiP from *E. coli*, which had its crystal structure previously determined (**Table 2**; Kawachi et al., 2012).

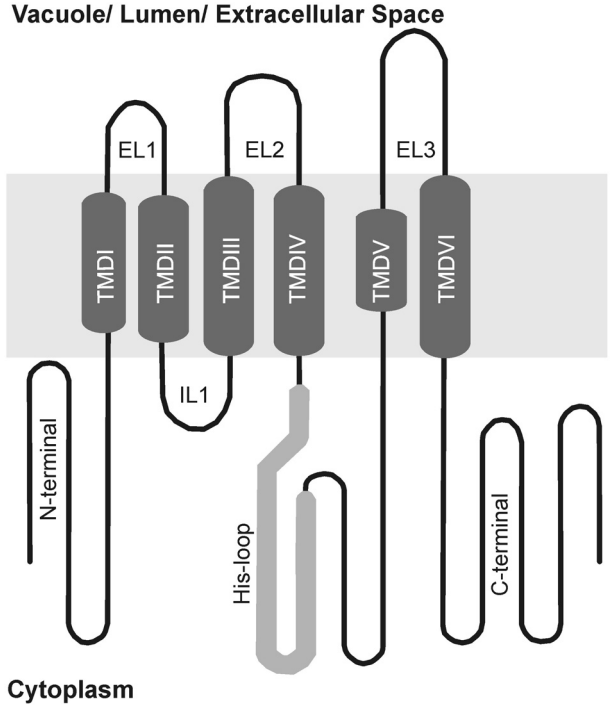

FIGURE 3 | Schematic membrane topology model of group 1 MTP proteins. Predicted transmembrane domains (TMDs) in the center (I to VI), cytoplasmic loops below and vacuolar loops above. His-loop, histidine rich loop; IL, intracytosolic loop; EL, extracytosolic loop.

The signature sequence proposed by Paulsen and Saier (1997) and modified by Montanini et al. (2007) enables predictions regarding uncharacterized MTP family members, as this signature is highly conserved between species and seems to be important for metal transport and specificity. Within the CDF signature, mutations in PtdMTP1 (H89K and H89A; **Table 2**; Blaudez et al., 2003), AtMTP1 (H90A; **Table 2**; Kawachi et al., 2012) and OsMTP1 (H90D; **Table 2**; Menguer et al., 2013) abolish Zn transport, but the effect of the mutation on transport of other metals besides Zn was only tested for OsMTP1, which had no effect on Co transport while enhanced Fe tolerance. In the mammalian metal transporters ZnT5 and ZnT8, mutation in the homologous residues (H451D and H106D, respectively; Hoch et al., 2012) abolished metal selectivity allowing Zn and Cd transport. It was concluded that this motif was important in discriminating between Zn and Cd, and that metal selectivity is tuned by a coordination-based mechanism that raises the thermodynamic barrier to Cd binding (Hoch et al., 2012). Mutations in the residues Thr86 and Leu91 of AtMTP1 conferred tolerance to high levels of Co, whereas changes in Thr86 yielded some level of Cd tolerance, while still transporting Zn (**Table 2**; Kawachi et al., 2012). In OsMTP1, the mutation L82F appears to confer a gain-of-function: the protein can transport low levels of Zn, with

enhanced affinity for Fe, Co, and Mn (Menguer et al., 2013). As AtMTP1 and OsMTP1 can still transport Zn, it appears that these residues contribute to Zn selectivity over other metals.

The AtMTP1 protein contains a total of six cysteine (Cys) residues, all distinct from related microbial proteins but mostly present in plant MTP1 sequences. The mutations C59A or C65A did not confer any substantial modification in yeast tolerance to Zn, whereas mutations C296A and C362A increased it (**Table 2**). However, mutation of either of the two Cys residues C31A or C36A, which are located within the N-terminal domain, strongly reduced the ability of AtMTP1 to confer Zn tolerance (**Table 2**). Interestingly, compared to wild type AtMTP1, a mutant carrying an N-terminal deletion of residues 2–12 (Δ2–12) conferred higher Zn tolerance to yeast, but the mutant Δ2–55, which also lacks the Cys residues required for the transport function, did not confer any Zn tolerance at all (**Table 2**; Kawachi et al., 2012). These results indicate that all or a subset of the 12 N-terminal amino acids of AtMTP1 act negatively on the Zn transport capability, and confirm that the part of the N-terminal domain that includes Cys31 and Cys36 is essential for protein function (Kawachi et al., 2012).

Homology modeling of AtMTP1 based on the 3D structure of *E. coli* YiiP, combined with site-directed mutagenesis and yeast complementation assays, strongly suggests that the active Zn-binding site (site A) of AtMTP1 is formed by His90 and Asp94 in TMD II and His265 and Asp269 in TMD V, with conserved positions of Zn-binding residues (**Table 2**). These key residues of the Zn-binding site are highly conserved in numerous other CDF family proteins (Kawachi et al., 2012). The corresponding aspartate residue in *S. cerevisiae* Zrc1, when mutated to alanine, results in loss of Zn transport activity, suggesting functional conservation of the corresponding residues. Consistent with this finding, it was shown that mutations in four critical residues in TMD II (Leu33, Phe40, Asn44, and Ala52) affected metal specificity in Zrc1 (Lin et al., 2009).

Some other residues such as Glu72, Asp87, Glu124, Asn173, and Asp293 are also important for the Zn transport function of AtMTP1 (**Table 2**). Based on 3-D AtMTP1 modeling, it was suggested that Glu72, Asp87, and Asn173 are located close to the active Zn-binding site; Asp293 is located in the opening of the cavity to the vacuole; and Glu124 is located at the entrance of the cavity facing the cytosol (Kawachi et al., 2012). Although these residues are not necessary for Zn binding, they may be involved in translocation of Zn and/or protons through the membrane (Kawachi et al., 2012).

MTPs FROM GROUP 8 AND 9 OF THE Mn-CDF CLUSTER

According to phylogenetic analyses, groups 8 and 9 of the CDF superfamily have an ancient origin, containing both prokaryote and eukaryote sequences. Products of a possible duplication event, these two distinct groups have been functionally characterized as Mn transporters (Gustin et al., 2011). The protein ShMTP8 (originally ShMTP1, name changed to maintain consistency with *Arabidopsis* nomenclature), encoded by the tropical Mn hyperaccumulating legume *Stylosanthes hamata*, was the first characterized transporter from the Mn-CDF group in plants. Through the expression of a cDNA library prepared from *S. hamata* in the yeast *S. cerevisiae*, Delhaize et al. (2003) identified four cDNAs encoding membrane-bound proteins of the CDF superfamily which confer Mn tolerance to yeast. One of these cDNAs, *ShMTP8*, confers Mn tolerance in yeast by sequestration of Mn^{2+} to an internal organelle, instead of causing Mn^{2+} efflux to the media. Such Mn transport must function as an H^+:Mn^{2+} antiporter, due to the requirement of an active V-type H^+-ATPase for effective Mn tolerance. In yeast, the protein fused with GFP appeared to localize at the endoplasmic reticulum, but such localization is inconclusive due to the lack of an ER marker. ShMTP8 over-expression in plants (*Arabidopsis* and tobacco) also conferred Mn^{2+} tolerance; however, it was localized specifically at the tonoplast (**Figure 2**), strongly suggesting that Mn^{2+} sequestration to the vacuole is the mechanism conferring Mn^{2+} tolerance (Delhaize et al., 2003).

The high similarity of ShMTP8 with AtMTP8 to 11 suggests that these proteins may have similar functions. Of the four tested genes, *AtMTP11* showed the highest expression levels in whole seedlings of *Arabidopsis* grown under different Mn^{2+} supplies. As expected, when expressed in yeast cells, AtMTP11 conferred enhanced tolerance to Mn^{2+} (and to a lesser extent to Cu^{2+}), but no increased tolerance to a range of other metals (Zn^{2+}, Co^{2+}, or Ni^{2+}) was detected. Using yeast microsomal membrane vesicles, it was shown that AtMTP11 also confers Mn^{2+}-dependent proton-transport activity (Delhaize et al., 2007). In contrast to ShMTP8, AtMTP11 was not located at the vacuolar membrane, but was found in pre-vacuolar compartments or trans-Golgi (**Figure 2**; Delhaize et al., 2007; Peiter et al., 2007). It was suggested that the Mn tolerance conferred by AtMTP11 probably relies on vesicular trafficking and exocytosis of excess Mn^{2+} (Peiter et al., 2007), which is corroborated by the increased accumulation of Mn in leaves of *Arabidopsis* mutant plants with disrupted *AtMTP11* gene (*mtp11*). The same *Arabidopsis* mutant plants are hypersensitive to elevated levels of Mn, whereas over-expressing plants are hypertolerant (Delhaize et al., 2007; Peiter et al., 2007). Interestingly, two homologous genes from poplar, PtMTP11.1 and PtMTP11.2, are also targeted to a Golgi-like compartment and are able to complement *mtp11 Arabidopsis* mutant plants, suggesting that poplar and *Arabidopsis* MTP11 proteins have similar functions (Peiter et al., 2007). As previously stated by Gustin et al. (2011), it is clear that at least some of the CDF members from MTP groups 8 and 9 are important to Mn homeostasis, and the early bifurcation and further expansion of these groups represent the adaptive strategies that each plant species has developed to maintain Mn homeostasis and to deal with Mn toxicity.

OTHER MTPs FROM THE CDF SUPERFAMILY (GROUPS 5, 6, 7, AND 12)

Similarly to other groups from the CDF superfamily, groups 5, 6, 7, and 12 have an ancient origin, including CDF sequences from diverse prokaryote and eukaryote organisms (Gustin et al., 2011). However, in contrast to groups 1, 8 and 9, these four groups have maintained only one copy of each sequence within genomes of the analyzed plants. Although the existence of most of the members in these groups is supported by cDNAs or ESTs sequences, the only functional evidence for these groups

comes from a high throughput ionomic study which investigated several *A. thaliana* accessions and mutant lines (Baxter et al., 2007). Two mutant lines screened (*mtp5* and *mtp6*, members of groups 5 and 6, respectively) showed alterations in the ionome of mutant leaves. Repeatable alterations in multiple ions in the *mtp5* mutant leaves included reduced levels of Mo, Mn, and Mg and increased levels of K and Zn. The *mtp6* mutant showed consistent diverse alterations in the ionome with reduced levels of Mg, Mo, and Ca and increased levels of Na, K, Mn, and Cd. Therefore, it was suggested that both AtMTP5 and AtMTP6 have important roles in regulating ion concentrations in *A. thaliana* under normal conditions (Gustin et al., 2011). Although members of the group 6 have been included in the Zn/Fe-CDF group (Montanini et al., 2007), no functional data is available regarding the substrate specificity of these transport proteins in plants. If functional characterization of group 6 members confirms the hypothesis of Zn/Fe transport, these sequences could be used as putative targets in plant biofortification strategies.

MTP PROTEINS IN METAL HYPERACCUMULATOR SPECIES

ROLE OF MTP1 IN THE HYPERTOLERANCE TRAIT OF HYPERACCUMULATORS

Metal hypertolerant/hyperaccumulator plants are able to accumulate from one to four orders of magnitude higher metal concentrations in their above-ground biomass compared to other plants growing in the same environment. Metals accumulated include Zn, Mn, Cd, Co, Ni, Cu, selenium (Se), arsenic (As), lead (Pb), antimony (Sb), and thallium (Tl) (Baker and Brooks, 1989; Reeves and Baker, 2000; Krämer, 2010). These traits were reported in ∼500 taxa so far, and are especially common in the Brassicaceae family (Krämer, 2010). The study of hypertolerance/hyperaccumulation traits is attractive in Brassicaceae because of the high similarity of "model" hyperaccumulators *Arabidopsis* (former *Cardaminopsis*) *halleri* and *Noccaea* (former *Thlaspi*) *caerulescens* with the model species *A. thaliana*, allowing use of the large number of tools available to the *Arabidopsis* community in cross-species comparisons. Due to that, we focused on the role of MTP proteins in hypertolerance/hyperaccumulation in these species.

A. halleri is a Zn and Cd hypertolerant/hyperaccumulator (Krämer, 2010) that shares 94% nucleotide identity within coding regions with *A. thaliana* (Koch et al., 1999, 2000; Clauss and Koch, 2006). *N. caerulescens* has diverged earlier, sharing 88% nucleotide identity (Peer et al., 2003, 2006). *A. halleri* and *N. caerulescens* are constitutively Zn-tolerant and Zn hyperaccumulators, with intraspecific variation for Cd and/or Ni (Milner and Kochian, 2008; Krämer, 2010). Although metal accumulation evolved independently in *A. halleri* and *N. caerulescens* (Krämer, 2010), they share a common set of alterations, indicating that a small number of constraints need to be changed for metal hyperaccumulation/hypertolerance traits to emerge (Hanikenne and Nouet, 2011).

Hyperaccumulators change metal partitioning between roots and shoots. In non-hyperaccumulators, metal shoot-to-root ratio is below unity, whereas in hyperaccumulators it is generally above (Baker et al., 1994; Talke et al., 2006; Krämer, 2010). Both hyperaccumulation and hypertolerance result from changes in physiological processes, namely: (1) root metal uptake; (2) enhanced xylem loading for root-to-shoot translocation; and (3) enhanced metal sequestration and detoxification in shoots (Hanikenne and Nouet, 2011). Studies in *A. halleri* and *N. caerulescens* indicate that ZIP influx transporters are probably involved in metal uptake (Talke et al., 2006; van de Mortel et al., 2006; Weber et al., 2006) whereas the presence of multiple copies of HMA4 efflux transporter genes in both hyperaccumulators is responsible for increased xylem loading and subsequent root-to-shoot translocation (Hanikenne et al., 2008; O'Lochlainn et al., 2011). In shoots, a key protein for metal sequestration and detoxification is the vacuolar protein MTP1 (**Figure 2**; Dräger et al., 2004; Gustin et al., 2009).

Root and shoot transcriptomic comparisons indicated that *AhMTP1* transcripts are constitutively higher in *A. halleri* than in *A. thaliana*, especially in leaves (Becher et al., 2004; Dräger et al., 2004; Talke et al., 2006). AhMTP1 is a vacuolar protein that is able to complement the Zn-sensitive phenotype of the *zrc1cot1* mutant yeast (**Table 1**; Dräger et al., 2004). Surprisingly, three copies of the *AhMTP1 loci* were first identified, explaining the observed high transcript levels. Segregant populations of *A. halleri* × *A. lyrata* crosses showed that only two out of the three identified *A. halleri MTP1 loci* co-segregate with Zn tolerance (Dräger et al., 2004). This was later confirmed by another study showing that the *A. halleri* genome has 4–5 *MTP1* paralogs, located at four *loci*, with one copy not fixed in the population analyzed (*AhMTP1-D*, see below; Shahzad et al., 2010). *AhMTP1-A1* and *AhMTP1-A2* are duplicated *in tandem* and thus linked, whereas *AhMTP1-B*, *AhMTP1-C,* and *AhMTP1-D* are segmentally duplicated (Shahzad et al., 2010). *AhMTP1-A1*/*AhMTP1-A2* and *AhMTP1-B loci* are the copies associated with high accumulation of MTP1 transcripts in shoots, up-regulation upon high Zn concentrations in roots, and Zn hypertolerance (Dräger et al., 2004; Shahzad et al., 2010). Interestingly, although all five copies were able to confer Zn-tolerance to *zrc1cot1* mutant yeast, AhMTP1-B was less competent than AhMTP1-C and AhMTP1-D (**Table 1**; Shahzad et al., 2010). These results indicated that although duplication of MTP1 loci could be the basis of Zn tolerance in *A. halleri*, the five MTP genes seem to be undergoing distinct evolutionary fates (Shahzad et al., 2010).

An *N. caerulescens* MTP1 protein was cloned by homology with *AtMTP1* (van der Zaal et al., 1999) and named *NcZTP1* (previously *TcZTP1*, Zn Transporter; Assunção et al., 2001). *NcZTP1*, renamed *NcMTP1*, was shown to be highly expressed in leaves compared to homologous genes in its closest non-hyperaccumulator relative, *Thlaspi arvense*, and in *A. thaliana* (Assunção et al., 2001). *NgMTP1*, an ortholog from another Zn hyperaccumulator, *Noccaea* (former *Thlaspi*) *goesingense*, was also highly expressed in shoots compared to non-hyperaccumulators *A. thaliana*, *T. arvense*, and *Brassica juncea* (Persans et al., 2001). NgMTP1 was shown to complement Zn, Cd, and Co-sensitivity in yeast (**Table 1**; Persans et al., 2001; Kim et al., 2004). At first, transient expression of a GFP-tagged version of NgMTP1 in *A. thaliana* protoplasts showed plasma membrane localization, which is unusual for MTP1 homologs (Kim et al., 2004). In yeast, NgMTP1 was localized at both the plasma membrane and the vacuolar membrane, acting in Zn efflux from the cell as

well as in vacuolar storage (Kim et al., 2004). However, a later work using NgMTP1-specific antibody and membrane fractionation demonstrated that NgMTP1 is vacuolar. Both the native protein in *N. goesingense* and the protein heterologously expressed in *A. thaliana* were localized at the tonoplast, in agreement with the localization of other MTP1 homologs (Gustin et al., 2009; **Table 1**).

NgMTP1 expression under control of the 35S promoter in *A. thaliana* led to increased Zn concentration in roots, but decreased in shoots (Gustin et al., 2009). Reciprocal grafting of wild type and 35S::*NgMTP1 A. thaliana* lines showed that shoot-specific NgMTP1 expression leads to Zn accumulation in leaves, as well as up-regulation of *AtZIP4* and *AtZIP5* in shoots and *AtZIP3* and *AtZIP9* in roots, characteristic of Zn-deficiency response (Grotz et al., 1998; Wintz et al., 2002, 2003; van de Mortel et al., 2006; Talke et al., 2006; Gustin et al., 2009). Importantly, it was demonstrated that metal tolerance is dependent on NgMTP1 expression in shoots (Gustin et al., 2009). These results were confirmed in reciprocal grafting experiments of *N. caerulescens* with the non-hyperaccumulator *Noccaea perfoliatum*, which demonstrated that hyperaccumulation is a root-driven phenotype, whereas hypertolerance is shoot-driven (de Guimarães et al., 2009). Considering that *NgMTP1* transcripts are more abundant in shoots of *N. goesingense* than in non-hyperaccumulators (Persans et al., 2001), it is clearly established that shoot MTP1 expression has a pivotal role in the Zn hypertolerance trait.

MTP1 genes from the Zn/Cd hyperaccumulating and non-hyperaccumulating ecotypes of *Sedum alfredii* were both able to suppress Zn hypersensitivity in the *zrc1* yeast mutant (**Table 1**), induced Zn accumulation and were localized at the tonoplasts of onion cells (Zhang et al., 2011). As described for *A. halleri* (Dräger et al., 2004), the amount of *MTP1* transcripts of the hyperaccumulating ecotype is higher in shoots than in roots, a difference not observed in the non-hyperaccumulator ecotype. Upon Zn or Cd excess treatment, *MTP1* transcripts accumulate in shoots of the hyperaccumulator and in roots of the non-hyperaccumulator ecotype (Zhang et al., 2011). These results indicate that, as in other hyperaccumulator species, *S. alfredii* hypertolerance probably relies on increased expression of MTP1 transporters in shoots.

The mesophyll cells are the major site of metal accumulation in *A. halleri* (Küpper et al., 2000; Sarret et al., 2009), whereas the epidermal cells show higher Zn concentrations in *N. caerulescens* (Küpper et al., 1999; Schneider et al., 2012). Unexpectedly, *in situ* hybridization and proteomics approaches showed that *N. caerulescens* MTP1 is not differentially expressed, but rather equally distributed in epidermal and mesophyll cells (Schneider et al., 2012). Protein fragments homologous to plasma membrane influx transporters from the ZIP family were enriched in the epidermal fraction, which would explain the differential accumulation observed (Küpper and Kochian, 2010; Schneider et al., 2012). Therefore, it is suggested cell type-specific expression of MTP1 is not driving differential accumulation of metals, which would be based on other proteins such as influx transporters.

A model explaining the role of MTP1 in the hyperaccumulation phenotype has been proposed. High root-to-shoot translocation of Zn is achieved by HMA4, whereas metal detoxification occurs at the vacuoles of leaf cells by MTP1 transporter proteins (Gustin et al., 2009; Hanikenne and Nouet, 2011). In this model, HMA4 would be necessary for hyperaccumulation, whereas MTP1 would be necessary for hypertolerance. In agreement with the model, *A. thaliana* plants expressing *AhHMA4p*::*AhHMA4* constructs hyperaccumulated Zn in shoots, but were not tolerant to high Zn concentrations (Hanikenne et al., 2008). Plants expressing *NgMTP1* in shoots were hypertolerant to high Zn but did not accumulate metals to the same extent as hyperaccumulators (Gustin et al., 2009). It was also shown that a Zn accumulation QTL co-localized with *HMA4* (Frérot et al., 2010), and two Zn tolerance QTLs were co-localized with the *MTP1 loci* and another one with *HMA4* in *A. halleri* (Willems et al., 2007). Still, it is possible that MTP1-dependent increase of sink strength in shoots might have a minor, secondary effect on hyperaccumulation, depleting cytoplasmic pools of Zn in leaves, and increasing Zn demand (Becher et al., 2004; Gustin et al., 2009), as suggested for HMA4 root-to-shoot metal translocation activity (Talke et al., 2006; Hanikenne et al., 2008; Hanikenne and Nouet, 2011). The full understanding of molecular mechanisms involved in hyperaccumulation/hypertolerance will be directly applicable to phytomining and phytoremediation, as well as crop nutrient use efficiency and biofortification (Clemens et al., 2002).

MTP PROTEINS AS TOOLS FOR BIOFORTIFICATION

The only source of metal micronutrients for humans is the diet, and in developing countries plants are the main source of these metals. Consequently, the presence of minerals and the maintenance of their homeostasis within the edible tissues of plants are of great importance for human nutrition. Biofortification of grains has been considered a promising strategy to improve human nutrition, and expression of genes encoding metal transporters, alone or in combination with other genes, has been suggested as a useful approach in this direction (Palmgren et al., 2008; Sperotto et al., 2012).

During the grain-filling stage, plants remobilize and transport nutrients distributed throughout the vegetative source organs into seeds [reviewed by Waters and Sankaran (2011)]. Micronutrients such as Fe, Zn, Cu, and Mn are mainly localized in the seed aleurone layer, where phytic acid (main form of Pi storage in seeds) acts as a strong chelator of metal cations and bind them to form phytate, a salt of inositol phosphate (Raboy, 2009). In developing rice grain, Zn stays near the aleurone layer and moves more deeply into the inner endosperm than other micronutrients (Iwai et al., 2012). In barley, *MTP1* is expressed in all tissues of the grain (transfer cells, aleurone, endosperm, and embryo) with the lowest expression in transfer cells and the highest in aleurone and embryo, where Zn accumulates at higher levels (Tauris et al., 2009). The biotechnological potential of *MTP1* for Zn biofortification purposes has already been highlighted (Palmgren et al., 2008; Anuradha et al., 2012; Podar et al., 2012). In future experiments, an interesting approach would be endosperm-specific overexpression of *MTP1*, which could lead to enhanced accumulation of Zn in edible tissues. In a complementary strategy, specific silencing of MTP proteins

responsible for metal accumulation in leaf vacuoles could increase the amount of available minerals to be transported to grains.

Mutations in amino acid residues within the MTP1 protein may also be important for altering the selectivity of transport for biofortification purposes. Indeed, one of the major challenges in biofortification efforts is to avoid increasing concentrations of undesirable toxic metals in edible plant organs. Micronutrient transporters often have broad substrate specificity: an example is the Arabidopsis Fe^{2+} transporter AtIRT1, which is also able to transport Mn^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, and Ni^{+2} (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002; Nishida et al., 2011). Because of this, plants over-expressing AtIRT1 have increased accumulation of Fe, but also of other toxic metals, and efforts have been made to alter protein selectivity (Rogers et al., 2000; Barberon et al., 2011). Thus, the determination of amino acid residues that are important for metal specificity is useful in two ways: to generate transporters that would select only beneficial ions such as Fe and Zn, which would be used to increase the concentrations of these metals in edible parts of plants; and to generate transporters that specifically transport toxic ions such as Cd, trapping them inside the vacuoles or other compartments of non-edible plant organs. Any of these approaches would need careful choice of specific promoters.

Recent studies with the Arabidopsis MTP1 protein failed to discover a single substitution that entirely shifts the substrate specificity of AtMTP1 from Zn (Kawachi et al., 2012; Podar et al., 2012), suggesting that higher organisms regulate transport tightly by holding more than one residue responsible for substrate specificity (Podar et al., 2012). However, the H90D mutation in rice OsMTP1 abolishes Zn transport and enhances Fe tolerance (Menguer et al., 2013). Fortunately, some mutations have the capacity to allow MTP1 to transport Fe along with Zn, making it a good candidate for biofortification approaches (Kawachi et al., 2012; Podar et al., 2012). Fe and Zn deficiency are the most common mineral nutritional disorders in humans, and simultaneous increases in Fe and Zn concentrations would be highly beneficial.

Since studies with MTP proteins from hyperaccumulators demonstrated that high copy number and enhanced expression are more likely explanations for hypertolerant/hyperaccumulator phenotypes (Dräger et al., 2004; Shahzad et al., 2010), it would seem that genes from metal hyperaccumulator species have no special features to distinguish them from those of ordinary plants, and that the biotechnological approach aiming at biofortified crops would be similar when using genes obtained from either group of plants. However, the ShMTP8 protein seems to be more efficient in Mn^{2+} transport than AtMTP11 (Delhaize et al., 2003, 2007). This improved efficiency in a protein from a hyperaccumulator species indicates that hyperaccumulators are potential sources of useful new genes for biofortification.

CONCLUSIONS AND FURTHER DIRECTIONS

There seems to be enough variation within the MTP family of transporters to provide a wide range of candidate genes to be used in specific biotechnological applications in plants, including biofortification, phytoremediation, and improved efficiency in the use of nutrients (avoiding deficiency and toxicity). Successful strategies will depend on the amount of information available about the specific function of each of these proteins, as well as on the appropriate combinations of genes and promoters. As an example, Masuda et al. (2012) recently showed that basic knowledge on Fe homeostasis-related proteins, such as the Fe(II)-NA transporter OsYSL2, the NA synthase OsNAS2 and the Fe-binding protein Ferritin, combined with the right choice of promoters, can lead to increased Fe concentrations in the rice endosperm.

Most certainly, the exploitation of specific promoters should be pursued in strategies for improving genotypes for particular biotechnological purposes. Although useful in basic studies of gene function, constitutive over-expression changes metal homeostasis in all organs, and often affects uptake and distribution of more than one element. Knowledge about the expression patterns of diverse MTP genes may also provide useful promoters for expression of other transporters, with specific substrate preferences, in a tissue- or cell layer-targeted fashion.

Manipulation of CDF substrate specificity may also be a promising avenue. The description of the 3D structure of a bacterial CDF transporter, YiiP (Lu and Fu, 2007), presents an opportunity to apply molecular modeling to spatially understand why certain residues are determinants of metal selectivity in other members of the family. This approach was successfully applied to AtMTP1 (Kawachi et al., 2012) and could be used more widely in the future as the basis for tailoring the substrate specificity of CDFs for targeting particular metals. Although MTP proteins are able to transport metals other than Zn, site-directed mutagenesis can be employed to change specificity into a broader or narrower range, depending on the desired outcome.

Most MTP proteins have not yet been functionally characterized, and the subcellular localization of some of those characterized is still a matter of controversy. Therefore, further work is required before the roles of all family members are fully understood. Substrate specificity, sub-cellular localization, and expression patterns (in different tissues, developmental stages, and in response to a wide range of treatments) are crucial information to be sought. Even in Arabidopsis, there are MTP proteins which are not functionally characterized. In this species, and in others that have similar resources, characterization of insertion mutants, and over-expressing lines should help clarify the physiological relevance of MTP genes. Additionally, ionomic studies used with these plants will provide the opportunity of a wider understanding of the role of MTPs and their influence on the relationship between different elements at different stages of growth and development and under a variety of environmental conditions (Williams and Salt, 2009). The more we learn about MTP proteins, the greater the chances of designing successful biofortification programs that make use of their appropriate and rational manipulation.

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Strategies for vitamin B6 biofortification of plants: a dual role as a micronutrient and a stress protectant

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Vitamin B6 has an essential role in cells as a cofactor for several metabolic enzymes. It has also been shown to function as a potent antioxidant molecule. The recent elucidation of the vitamin B6 biosynthesis pathways in plants provides opportunities for characterizing their importance during developmental processes and exposure to stress. Humans and animals must acquire vitamin B6 with their diet, with plants being a major source, because they cannot biosynthesize it *de novo*. However, the abundance of the vitamin in the edible portions of the most commonly consumed plants is not sufficient to meet daily requirements. Genetic engineering has proven successful in increasing the vitamin B6 content in the model plant *Arabidopsis*. The added benefits associated with the enhanced vitamin B6 content, such as higher biomass and resistance to abiotic stress, suggest that increasing this essential micronutrient could be a valuable option to improve the nutritional quality and stress tolerance of crop plants. This review summarizes current achievements in vitamin B6 biofortification and considers strategies for increasing vitamin B6 levels in crop plants for human health and nutrition.

Keywords: vitamin B6, oxidative stress, biofortification, genetic engineering, *PDX* genes, crop biotechnology

INTRODUCTION

The term vitamin B6 refers to a group of six water soluble vitamers (**Figure 1**), namely pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their phosphorylated derivatives. Pyridoxal-5′-phosphate (PLP) is the vitamer of central importance because it is required as a cofactor for over 140 chemical reactions in the cell (Hellmann and Mooney, 2010). In particular, it is involved in amino acid, sugar, and fatty acid metabolism (Percudani and Peracchi, 2003). Only plants, fungi, and bacteria can biosynthesize vitamin B6 *de novo*, therefore animals and humans must obtain it from their diet. Various factors such as inadequate food intake, limited dietary diversity, or diseases can cause vitamin B6 deficiency (Di Salvo et al., 2012). Based on the ability of plants to biosynthesize and accumulate vitamin B6 (Chen and Xiong, 2009; Raschke et al., 2011), increasing levels of the vitamin in crops can be a direct way to provide enriched food to the population and reduce the corresponding deficiencies (Martin et al., 2011; Fitzpatrick et al., 2012; Bhullar and Gruissem, 2013). During the last decade, the function and regulation of key genes involved in vitamin B6 biosynthesis have been characterized in the model plant *Arabidopsis*, revealing the importance of the vitamin in plant development, photosynthesis and responses to stress (reviewed in Mooney and Hellmann, 2010). Therefore, the current molecular understanding of vitamin B6 metabolism provides the opportunity of developing crop plants with an increased content of this vitamin beneficial for both human health and improved agronomic performance.

VITAMIN B6: BENEFITS FOR HUMANS

A vitamin is defined as an organic compound required in limited amounts by an organism that cannot produce it and thus needs to take it up with the diet. The importance of vitamin B6 for human health has been widely reported. Studies have shown that vitamin B6 intake reduces the incidence of important human diseases such as cardiovascular disease, hypertension, epilepsy, diabetes, kidney disease, neurological disorders, and pellagra (Hellmann and Mooney, 2010; **Figure 1**). Furthermore, the cofactor form of vitamin B6 (PLP) may have positive effects on various forms of cancer by strengthening the immune system and delaying tumor progression (Galluzzi et al., 2013). Other studies investigating the impact of vitamin B6 deficiency on health, using the concentration of PLP in the blood as a biomarker, suggest that a low plasma level correlates with multifactorial neurological pathologies such as depression, Alzheimer's disease, autism, schizophrenia, epilepsy, and Parkinson's disease (Di Salvo et al., 2012). On the other hand, the beneficial impact of the vitamin on human health is illustrated by reduced mortality and improved health of the elderly with an adequate PLP plasma concentration (O'Leary et al., 2011; Huang et al., 2012). More recently, vitamin B6 deficiency was suggested as the cause of "nodding syndrome," a disease affecting a growing number of children in Uganda and Southern Sudan (Wadman, 2011; Vogel, 2012). Importantly, vitamin B6 is also a potent antioxidant (Ehrenshaft et al., 1999; Osmani et al., 1999; Bilski et al., 2000). Several studies focusing on the effect of PN on cell cultures indicate that vitamin B6 functions as a protectant against reactive oxygen species produced in the

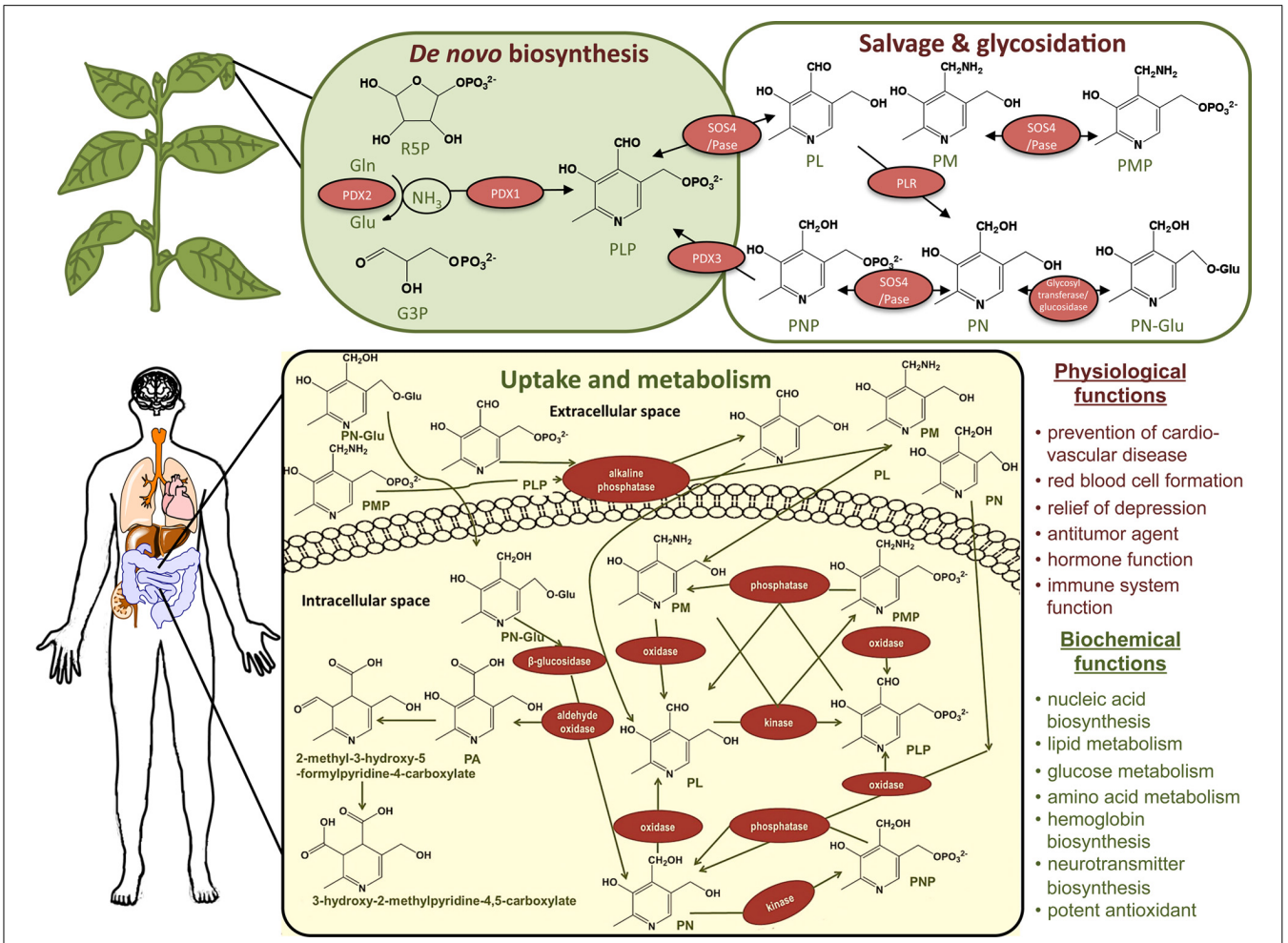


FIGURE 1 | Plants as a source of vitamin B6 and its uptake by humans. Plants can biosynthesize vitamin B6 *de novo*. It is predominantly produced in the metabolically active parts of the plant like the leaves but can be found in all organs. The *de novo* pathway (upper left corner) is operating in the cytosol involving only two enzymes: a synthase, PDX1, and a glutaminase, PDX2. The abbreviations used are Glu (glutamate), Gln (glutamine), G3P (glyceraldehyde 3-phosphate), R5P (ribose 5-phosphate), PA (pyridoxic acid), PN (pyridoxine), PN-Glu (PN-glycoside), PL (pyridoxal), PM (pyridoxamine), PNP (pyridoxine 5′-phosphate), PLP (pyridoxal 5′-phosphate), PMP (pyridoxamine 5′-phosphate). The cofactor form of the vitamin, PLP, is produced directly from R5P, G3P, and Gln. A salvage pathway also operates in plants (upper right corner) through which the vitamers can be interconverted. PDX3 is a PNP/PMP oxidase (only the reaction for PNP is depicted), SOS4 is a PN/PL/PM kinase and PLR is a PL reductase. Plants can also convert PN to PN-Glu, a form that is only partially bioavailable to humans, using a glycosyl transferase. In the human body, vitamin B6 is taken up in the jejunum (the second part of the small intestine; lower panel) where the free forms PN, PL, and PM can be taken up directly. The phosphorylated vitamers are dephosphorylated prior to uptake by a membrane bound alkaline phosphatase. In both cases uptake is driven by diffusion, which necessitates the phosphorylation of the vitamers immediately after their internalization to keep a low concentration of the non-phosphorylated forms inside the cells. The PN-Glu is taken up in its glycosylated form and processed by a cytosolic β-glucosidase to release PN. Its low bioavailability is not determined by the uptake itself but by the limiting activity of a β-glucosidase. The uptake is followed by further conversion inside the cells (salvage pathway) yielding the active cofactor form PLP involved in numerous biochemical and physiological processes in the human body (right lower panel). The catabolism of the vitamin involves conversion to PA (as well as more oxidized derivatives as depicted). The latter are excreted in the urine.

human body (Jain and Lim, 2001; Kannan and Jain, 2004; Mahfouz et al., 2009). Among other factors, malnutrition, pregnancy, diabetes, HIV, alcoholism, and the use of oral contraceptives increase the risk of vitamin B6 deficiency (Merrill and Henderson, 1987; Di Salvo et al., 2012; Fitzpatrick et al., 2012). Limited dietary diversity can accentuate micronutrient deficiencies (Acham et al., 2012) and is the major cause of a vitamin B6 deficit for inhabitants of developing countries. However, inadequate vitamin B6 status has recently been reported in the U.S.A. as well (Morris et al., 2008).

MAIN SOURCES

Vitamin B6 is present in many food products including meat and vegetables. The major sources of vitamin B6 in Western diets are cereals, poultry, beef, and potatoes (O'Neil et al., 2012). Among the five most important staple crops, potatoes have by far the highest vitamin B6 content (Fitzpatrick et al., 2012). The content is much lower in other staple crops and should be increased several fold (e.g., wheat 12.2-, rice 3.2-, maize 3.5-, and cassava 2.3-fold, respectively) in order to reach the recommended daily allowance (RDA) of vitamin B6 (Fitzpatrick et al., 2012). The bioavailability

of vitamin B6 derived from plant food sources is also an important factor influencing adequate intake (**Figure 1**). Plants contain multiple B6 vitamers and a substantial fraction of vitamin B6 is present as pyridoxine-5′-β-D-glucoside (PN-glucoside; Gregory and Ink, 1987; Ollilainen, 1999; **Figure 1**). While PN, PL, and PM and their phosphorylated derivatives are fully bioavailable from plant food, PN-glucoside is only 50% bioavailable (Gregory, 2012; **Figure 1**). On the other hand, PN and its glycosylated form are more stable than PL and PLP (the main forms of the vitamin available in animal tissues; Mehansho et al., 1979) and are less susceptible to thermal degradation.

VITAMIN B6: BENEFITS FOR PLANTS

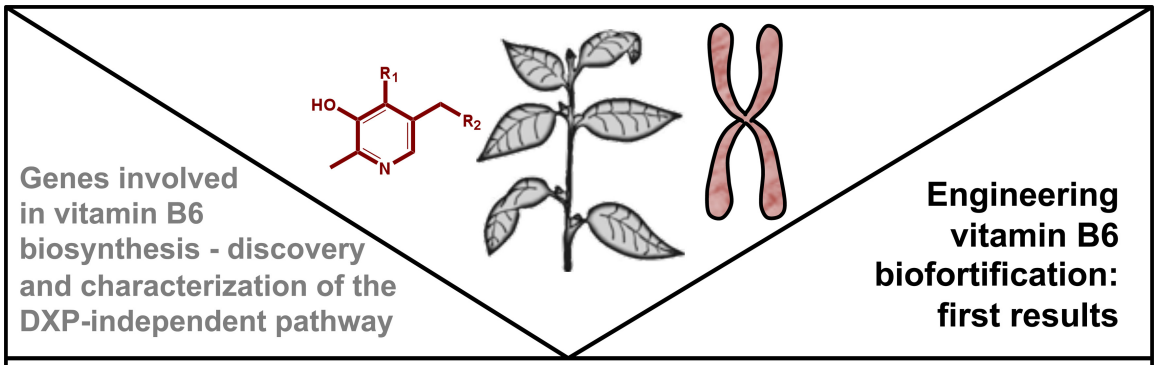
Unlike animals, plants can biosynthesize their own vitamins with the exception of vitamin B12. In the plant context, therefore, the term vitamin does not correspond to its definition but has been widely used. In plants, as in animals, vitamin B6 is required as a cofactor. There are an estimated 177 PLP-dependent enzymes in *Arabidopsis* (Percudani and Peracchi, 2009; Mooney and Hellmann, 2010). These enzymes have a central role because they participate in universal (e.g., biosynthesis and catabolism of amino acids) as well as plant specific pathways (e.g., starch metabolism, glucosinolate biosynthesis, ethylene and auxin biosynthesis and degradation; Mooney and Hellmann, 2010). To date, the importance of the B6 vitamers *in planta* has mostly been demonstrated from the analysis of mutant plants deficient in vitamin B6 biosynthesis. For example, an *Arabidopsis* mutant, *rsr4-1*, with reduced *de novo* biosynthesis displays altered levels of metabolites such as amino acids and organic acids (Wagner et al., 2006). Moreover, *Arabidopsis* mutants that accumulate lower vitamin B6 levels, i.e., *rsr4-1, pdx1.1, pdx1.3*, are phenotypically distinct from wild-type plants because of impaired seed and seedling development, delayed flowering and reduced plant growth (Chen and Xiong, 2005; Tambasco-Studart et al., 2005, 2007; Titiz et al., 2006; Wagner et al., 2006). Complete biosynthesis knockout mutants (i.e., *pdx2, pdx1.1, pdx1.3*) are embryo lethal (Tambasco-Studart et al., 2005; Titiz et al., 2006). The increased sensitivity of the mutants with reduced levels of vitamin B6 to high concentrations of sucrose, salt, and mannose, as well as high light, UV-B and oxidative stress (Chen and Xiong, 2005; Titiz et al., 2006; Havaux et al., 2009; Ristilä et al., 2011) indicates that vitamin B6 has an important function in plant stress responses as well. Indeed, *Arabidopsis* plants accumulate vitamin B6 when exposed to UV-B (Ristilä et al., 2011) and increased levels of the vitamin in transgenic *Arabidopsis* confer tolerance to oxidative stress (Raschke et al., 2011). The potent *in vitro* antioxidant activities of some B6 vitamers also corroborate this particular function of the vitamin (Ehrenshaft et al., 1999; Bilski et al., 2000; Denslow et al., 2005). In addition, metabolite profiling has revealed increased lipid peroxidation in the *Arabidopsis rsr4-1* mutant deficient in vitamin B6 biosynthesis *de novo* (Lytovchenko et al., 2009), consistent with its role as an antioxidant. Furthermore, the hypersensitive response triggered in tobacco upon incompatible bacterial infection is delayed following leaf infiltration with PN (Denslow et al., 2005). Supplementation of *Arabidopsis* growth medium with the same vitamer reduces singlet oxygen-mediated cell death in the conditional *flu* mutant (Danon et al., 2005). Notably, the phenotype of the latter mutant was also shown to be attenuated in plants with enhanced levels of vitamin B6 (Raschke et al., 2011). Similarly, PN supplementation was demonstrated to restore accumulation of the D1 protein in the *pdx1.3* mutant of *Arabidopsis* (Titiz et al., 2006). As the degradation of this protein is known to be triggered by singlet oxygen generated in the reaction center of photosystem II, its steady state level reflects the oxidative state of the chloroplast. Application of exogenous vitamin B6 on leaf disks also reduces singlet oxygen accumulation caused by high light exposure in wild-type and *pdx1.3* mutant plants (Havaux et al., 2009).

Although vitamin B6 in plants is biosynthesized *de novo* (**Figure 1**), the supplementation experiments discussed above and a similar study using PL, PM, and PN (Huang et al., 2011) suggest that plants can take up exogenously supplied non-phosphorylated B6 vitamers. This is corroborated by the rescue of the root and leaf developmental phenotypes associated with the *Arabidopsis pdx1.1* and *pdx1.3* mutants (Titiz et al., 2006; Wagner et al., 2006) and rescue of the arrest of embryo development at the globular stage in the *pdx2* mutant of *Arabidopsis* by direct application of PN (Tambasco-Studart et al., 2007). The latter study indicates that some transfer of the vitamin occurs between the embryo and the maternal tissue. Very recently a purine permease (PUP1) has been shown to function in recycling of vitamin B6 during guttation (Szydlowski et al., 2013) but no other transporters for this vitamin have been described so far in plants. Since *de novo* vitamin B6 biosynthesis occurs in the cytosol (Tambasco-Studart et al., 2005), diffusion or active transport of vitamin B6 across organelle envelopes is required to support the activity of organellar enzymes dependent on the vitamin as a cofactor (Gerdes et al., 2012). The relatively high polarity of the phosphorylated derivatives of the vitamin does not favor a passive diffusion mechanism (Mooney and Hellmann, 2010) and would require the existence of transporters.

GENETIC ENGINEERING STRATEGIES TO INCREASE VITAMIN B6 IN CROPS

From a genetic engineering perspective, it is attractive that *de novo* vitamin B6 biosynthesis in plants involves only two enzymes, PDX1 and PDX2 (Ehrenshaft et al., 1999; Ehrenshaft and Daub, 2001; Tambasco-Studart et al., 2005; **Figure 1**). This pathway is also referred to as deoxyxylulose 5-phosphate (DXP)-independent (Tambasco-Studart et al., 2005) to distinguish it from the seven enzyme DXP-dependent pathway first unraveled in *E. coli* (reviewed in Fitzpatrick et al., 2007) and only found in a small subset of bacteria (Ehrenshaft et al., 1999; Mittenhuber, 2001). Interestingly, *Arabidopsis* has three PDX1 homologs, only two of which catalyze vitamin B6 biosynthesis PDX1.1 and PDX1.3 with the function of the third homolog PDX1.2 remaining to be unraveled, and only one PDX2 homolog (Tambasco-Studart et al., 2005). Analyses of the rice and cassava genomes (Ouyang et al., 2007; Prochnik et al., 2012) have revealed an identical number of homologs for both genes.

Efforts to increase vitamin B6 accumulation in plants have so far been focused on genes from the DXP-independent pathway. In a first attempt, the *PDX1* and *PDX2* genes from *Cercospora nicotianae* were heterologously expressed in tobacco resulting in a 1.2-fold increase in total vitamin B6 content (**Figure 2**), with



Cercospora nicotianae

* Identification of the *SOR1 (PDX1;* Ehrenshaft et al. 1999) and the *PDX2* gene (Ehrenshaft & Daub, 2001)

Arabidopsis thaliana

** B6 is produced *de novo* by two enzymes PDX1 and PDX2 (Tambasco-Studart et al., 2005)

*** Identification of the 'salvage pathway': a kinase (SOS4; Shi et al., 2002; Shi & Zhu, 2002; Leasure et al., 2011), a PN-oxidase (PDX3; Sang et al., 2011), and a PL-reductase (Herrero et al., 2011).

Tobacco

* **Expressing the *Cercospora PDX* genes resulted in a 1.2-fold increase (Herrero & Daub, 2007)**

Arabidopsis thaliana

** **Using the endogenous *PDX1.3* and *PDX2* led to 1.2-fold and 3-fold higher levels in seeds (Chen & Xiong, 2009)**

*** **Overexpressing the *PDX1.1* gene alone or with *PDX2* brought about an increase of 4 and 5-fold, respectively (Raschke et al., 2011)**

Perspectives

* **Engineering of crop plants employing the endogenous *PDX* genes or the *Arabidopsis* homologs.**

** **Better knowledge of the salvage pathway genes allowing overproduction of specific vitamers.**

*** **Understanding the regulation of vitamin B6 biosynthesis, metabolism and degradation in order to design future biofortification strategies.**

FIGURE 2 | Summary of the genetic approaches used in unraveling the deoxyxylulose 5-phosphate (DXP)-independent pathway for vitamin B6 biosynthesis *de novo* and first proof-of-principle strategies for vitamin B6 biofortification in *Arabidopsis thaliana* and tobacco. The lower panel provides an overview of the possible new engineering strategies for vitamin B6 biofortification.

some of the transgenic lines displaying delayed seed germination and plant growth (Herrero and Daub, 2007). Moreover, the expression of the endogenous *PDX* genes was altered in these lines, and a tight regulation of vitamin B6 homeostasis in plants was suggested. In another approach, *PDX1.3* and/or *PDX2* were expressed in *Arabidopsis* under the control of the constitutive 35S cauliflower mosaic virus (CaMV) promoter (Chen and Xiong, 2009; **Figure 2**). A significant increase in total vitamin B6 content was observed only in seeds (1.2-fold) and the transgenic lines overexpressing *PDX2* alone apparently had a higher total vitamin B6 content than the transgenic *PDX1* lines. In this study, gene expression was only assessed at the transcript level. However, the protein level also needs to be analyzed to better understand the mechanism behind these observations. On the other hand, the employment of a seed-specific promoter for the expression of both *PDX1.3* and *PDX2* resulted in a threefold increase of total vitamin B6 in transgenic seeds (Chen and Xiong, 2009; **Figure 2**). In contrast to the work from Herrero and Daub performed with *Cercospora* genes in tobacco, the latter study reported that ectopic expression of the endogenous *PDX* genes in *Arabidopsis* did not affect plant growth and development. However, the choice of *PDX1* gene appears to be particularly important considering a more recent report, which describes an at least fourfold increase in total vitamin B6 content in shoots and seeds, respectively using the 35S CaMV promoter (Raschke et al., 2011; **Figure 2**). In this study, it was shown that plants overexpressing *PDX1.1* at the transcript level also had an increase of the corresponding protein, while *PDX1.3* transcript overexpression did not result in an increase at the protein level, suggesting a tighter regulation of this paralog. Indeed, PDX1.3 is known to be an ubiquitination target for protein degradation by the proteasome (Manzano et al., 2008). Interestingly, the increase in PDX1.1 protein content was associated with an increased expression of PDX2 and accordingly the vitamin B6 level was increased (Raschke et al., 2011). However, in contrast to the study of Chen and Xiong (2009), overexpression of PDX2 alone did not lead to enhanced levels of vitamin B6 (Raschke et al., 2011). Overexpression of both *PDX1.1* and *PDX2* in *Arabidopsis* led to even further increases in the vitamin B6 content and therefore, appears to be the best strategy for vitamin B6 enrichment in crop plants. Intriguingly, vitamin hyper-accumulator lines also have larger aerial organs as well as increased seed size through embryo enlargement (Raschke et al., 2011). Consistent with the previously reported antioxidant properties of vitamin B6, its accumulation in *Arabidopsis* also correlated with improved tolerance to oxidative stress (Raschke et al., 2011). It is important to mention that the enzymatic activities of PDX1

and PDX2 lead directly to the production of the cofactor form, PLP (Raschle et al., 2005; Tambasco-Studart et al., 2005). However, the enhanced vitamin B6 content in the aforementioned *Arabidopsis* lines was distributed across PN, PM as well as PLP (Raschke et al., 2011). In this context, it should be noted that in addition to *de novo* biosynthesis, plants also have a so called "salvage pathway" that interconverts the different B6 vitamers. The salvage pathway is common to all living organisms (Tanaka et al., 2005; **Figure 1**). In plants, enzymes that are known to be involved include the oxidase PDX3, which transforms PNP or PMP to PLP (Sang et al., 2011); a kinase SOS4, which can phosphorylate PL, PN, or PM (Shi and Zhu, 2002; Shi et al., 2002; Leasure et al., 2011); a PL reductase (Herrero et al., 2011) and as yet unknown, perhaps unspecific phosphatases, which can dephosphorylate the phosphorylated vitamers (**Figure 1**). Therefore, it is likely that there is cross-talk between *de novo* and salvage pathways of biosynthesis, which should be taken into account in any biofortification strategy. Notwithstanding, the vitamin increase reported by Raschke et al. (2011) would be sufficient to meet the RDA in most staple crops (Fitzpatrick et al., 2012). Furthermore, the reduced bioavailability of glycosylated forms must be taken into account in genetic engineering strategies. Future work should also determine whether the fractions of free, phosphorylated and glycosylated B6 derivatives can be modulated. Such approaches will be instrumental in determining whether particular vitamers are associated with improved agronomic traits.

In order to exploit the benefits of an increased vitamin B6 content, the successful strategy used in *Arabidopsis* remains to be demonstrated in a crop plant. This also requires a good understanding of the regulatory mechanisms that control genes involved in vitamin B6 biosynthesis and metabolism, regardless of whether a biofortification strategy employs heterologous or homologous expression. A better knowledge of the natural diversity of vitamin B6 content and availability in crop species could also be instrumental in selecting the *PDX* orthologs providing the highest increase. Comprehensive studies of provitamin A biosynthesis have revealed intra-species genetic mutations linked to differential accumulation of vitamin A precursors that can be exploited to increase vitamin content in crops (Bang et al., 2007; Harjes et al., 2008; Welsch et al., 2010; Yan et al., 2010). Very limited data is currently available on the inter- and intra-species diversity of vitamin B6 accumulation. A recent survey of various wheat genotypes cultivated under different conditions has shown low variation in vitamin B6 content (Shewry et al., 2011) but additional genotypes and species should be investigated to assess vitamin B6 accumulation especially in crop plants. Such evaluation should be performed under controlled conditions given the regulation of vitamin B6 biosynthesis and accumulation by various factors (Denslow et al., 2007; Ristilä et al., 2011).

ALTERNATIVE CROP PLANTS OF POTENTIAL INTEREST

In order to select appropriate food crops for vitamin B6 biofortification strategies, two criteria should ideally be taken into account: (1) the crop has to be widely used and of economic importance; (2) vitamin B6 accumulation in the consumed part of the crop plant should not be constrained by physiological or developmental limitations. Genetic engineering using *Arabidopsis* suggests that a comparable increase of vitamin B6 can be reached in shoots and seeds (i.e., four to fivefold; Raschke et al., 2011). However, it remains to be demonstrated that similar increases can be reached in rice seeds or potato tubers for example. If successful, the combination of the existing nutritional qualities with a higher level of vitamin B6 would provide additional value to already important staple foods. Based on the findings of Raschke et al. (2011) using *Arabidopsis*, an improvement of plant growth and survival could also be expected but remains to be confirmed for other species. In developing countries, much of the population is highly dependent on mainly one staple crop and therefore particularly at risk of inadequate micronutrient intake (Ruel, 2003; Stupak et al., 2006). Nutritional quality improvement, including fortification of vitamin B6, of these crops could help diminish the occurrence of malnutrition and micronutrient deficiency (Welch and Graham, 2004; Stupak et al., 2006). The generation of crops combining enhanced levels of bioavailable B6 vitamers and better generic traits such as tolerance to oxidative stresses would represent a valuable tool to improve nutrition and food security.

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Nicotianamine synthase overexpression positively modulates iron homeostasis-related genes in high iron rice

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Nearly one-third of the world population, mostly women and children, suffer from iron malnutrition and its consequences, such as anemia or impaired mental development. Biofortification of rice, which is a staple crop for nearly half of the world's population, can significantly contribute in alleviating iron deficiency. NFP rice (transgenic rice expressing nicotianamine synthase, ferritin and phytase genes) has a more than six-fold increase in iron content in polished rice grains, resulting from the synergistic action of nicotianamine synthase (*NAS*) and ferritin transgenes. We investigated iron homeostasis in NFP plants by analyzing the expression of 28 endogenous rice genes known to be involved in the homeostasis of iron and other metals, in iron-deficient and iron-sufficient conditions. RNA was collected from different tissues (roots, flag leaves, grains) and at three developmental stages during grain filling. NFP plants showed increased sensitivity to iron-deficiency conditions and changes in the expression of endogenous genes involved in nicotianamine (NA) metabolism, in comparison to their non-transgenic siblings (NTS). Elevated transcript levels were detected in NFP plants for several iron transporters. In contrast, expression of *OsYSL2*, which encodes a member of yellow stripe like protein family, and a transporter of the NA-Fe(II) complex was reduced in NFP plants under low iron conditions, indicating that expression of *OsYSL2* is regulated by the endogenous iron status. Expression of the transgenes did not significantly affect overall iron homeostasis in NFP plants, which establishes the engineered push-pull mechanism as a suitable strategy to increase rice endosperm iron content.

Keywords: iron, homeostasis, NFP rice, biofortification, expression profiling

INTRODUCTION

Iron deficiency anemia (IDA) is the most severe degree of iron deficiency and a global problem that affects an estimated one-third of the world's population in both developing and developed countries. IDA has major consequences for human health as well as social and economic progress (WHO, 2013). Human IDA could be relieved by iron supplementation or food fortification. However, iron supplementation is difficult to achieve due to transportation and economic circumstances, especially in rural areas of developing countries. Iron fortification of food is also technically difficult and often results in unacceptable color and flavor of fortified products (Hurrell and Egli, 2010). In the recent years, bio-fortification has emerged as a possible solution to combat iron deficiency anemia through an economical and natural way.

Rice is the second largest produced cereal in the world and the most important grain with regard to human nutrition and caloric intake. It provides more than one fifth of the calories consumed worldwide. Around 3 billion people, mostly in Asia, depend on rice for 35–59% of their caloric intake. However, rice is a poor source of micronutrients, including iron. Most commercial rice varieties have only around 2 μg/g iron in the endosperm. Therefore, rice cannot provide daily iron needs of humans i.e., at least 8 mg/day for males and 18 mg/day for females, with pregnant women requirements rising up to 27 mg/day (Institute of Medicine, 2013). Considering these facts, enrichment of rice endosperm with bioavailable iron has the potential to decrease iron malnutrition worldwide. However, iron biofortification of rice strongly relies on information on the genes that control iron homeostasis in plants.

Iron translocation and homeostasis in rice has been well-studied. Several genes, most of which are transcriptionally regulated in response to iron availability, are known to coordinate iron uptake, translocation and storage in various tissues/compartments of the plant (Kobayashi and Nishizawa, 2012). However, the contribution of each type of transporter(s) and the precise iron flux still need to be clarified for each step involved in iron translocation. The transcription factors *OsIDEF1* and *OsIDEF2* regulate iron homeostasis-related genes in rice during Fe deficiency (Ogo et al., 2008; Kobayashi et al., 2009, 2010a). It has been suggested that *OsIDEF1* senses the cellular iron status by binding directly to the metal ions (Kobayashi et al., 2012). To cope with Fe starvation, rice roots release phytosiderophores (PS), which are molecules of the mugineic acid (MAs) family that form strong hexadentate chelates with Fe(III)to solubilize and transport it to the plant (Walker and Connolly, 2008; Palmer and Guerinot, 2009). The resulting Fe(III)-PS complexes are transported into root cells via transporters of the Yellow stripe like (YSL) family of proteins (Inoue et al., 2009; Lee et al., 2009a). Nicotianamine (NA), which is synthesized by

nicotianamine synthase (NAS) from S-adenosyl-L-methionine, is a ubiquitous metal chelator in plants and regulates iron translocation within and between cells and transports it to veins, flowers, and seeds (Takahashi et al., 2003). NA also serves as a substrate for nicotianamine aminotransferase (NAAT) to produce a 3″-oxo intermediate and subsequently, DMA is synthesized by deoxymugineic acid synthase (DMAS) (Haydon and Cobbett, 2007; Kim and Guerinot, 2007). Six members of *OsNAAT* family have been identified in rice plants, however, only *OsNAAT1* is regulated by plant iron status (Inoue et al., 2008). *OsDMAS1* is also up-regulated in both root and shoots under iron-deficient condition (Bashir et al., 2006; Bashir and Nishizawa, 2006). In addition to the iron uptake using phytosiderophores/DMA, rice also possesses an Fe(II) uptake system. *OsIRT1* and *OsIRT2*, the homologs of iron-regulated transporter *IRT1* in Arabidopsis, are specifically up-regulated in roots of iron-deficient rice plants (Ishimaru et al., 2006).

Once iron is loaded into the xylem, the chelators such as citrate, NA, and DMA are required for further transport in the plant (Jeong and Guerinot, 2009). In rice, a ferric reductase defective (*FRD*) *3*-like gene, *OsFRDL1*, is involved in iron-citrate translocation from rice roots to shoots (Yokosho et al., 2009). Transporters that are encoded by the YSL family of genes, *OsYSL2*, *OsYSL15*, *OsYSL16*, *OsYSL18*, are also involved in long distance transport of DMA-Fe(III) and/or NA-Fe(II) complexes (Aoyama et al., 2009; Inoue et al., 2009; Ishimaru et al., 2010; Kakei et al., 2012; Zheng et al., 2012). However, much still remains to be unraveled about intracellular metal transport involving vacuoles, chloroplasts, and mitochondria, although some transporters have been identified with specific iron translocation roles for these compartments. An iron deficiency-inducible mitochondrial iron-regulated gene (*OsMIR*; Ishimaru et al., 2009) and mitochondrial iron transporter (*OsMIT*), whose expression increases under excessive iron condition, were identified in rice (Bashir et al., 2011). Permease in chloroplasts 1 (*OsPIC1*) is also associated with chloroplast iron transport, while the vacuolar membrane localized *OsVIT1* and *OsVIT2* (Vacuolar iron transporter 1 and 2) mediate sequestration of Fe(II), Zn(II), and Mn(II) into vacuoles, with *OsVIT2* being very responsive to Fe treatments (Zhang et al., 2012). Conversely, transporters of the Natural Resistance Associated Macrophage Protein (NRAMP) family appear to have important roles in mobilizing export of vacuolar Fe stores (Lanquar et al., 2005). Despite these advancements, the coordinated function of different transporters that have a role in iron homeostasis is not fully understood.

Strategies to improve iron content in rice grains were mostly targeted at effective iron (Fe) uptake from the soil and translocation in the plant, in addition to directing Fe into the rice endosperm. Most of the strategies used NAS and ferritin, a protein that stores iron in a bioavailable form (Lonnerdal et al., 2006; Jin et al., 2009). Endosperm-specific expression of ferritin or the constitutive expression of NAS mostly achieved around 2- to 3-fold increases of iron in the endosperm (Goto et al., 1999; Lucca et al., 2001; Vasconcelos et al., 2003; Qu et al., 2005; Lee et al., 2009b, 2012). A 4.2-fold increase in Fe content was reported in plants over-expressing *OsNAS2* under the control of the CaMV35S promoter (Johnson et al., 2011). The possibility of using other transporters for improving endosperm iron content has also been explored recently. Specific expression of *OsYSL2* in the vascular tissue and around the endosperm lead to a 4.4-fold increase of iron concentration in the polished rice grains (Ishimaru et al., 2010). Over-expression of *OsIRT1* under the control of the maize ubiquitin promoter also increased Fe concentration to 113% compared to wild type grains (Lee and An, 2009). Alternatively, a few studies focused on the endosperm-specific expression of *Phytases* (Lucca et al., 2001). These enzymes can degrade phytate, a chelating agent that binds iron as well as other metals and store them in a non-bioavailable form for human consumption within the grain (Brinch-Pedersen et al., 2002).

The overexpression of multiple genes through a single construct, i.e., barley NAS expressed under rice actin promoter, soybean ferritin duplicated and expressed under two different endosperm specific promoters as well as rice *OsYSL2* duplicated and expressed under endosperm specific and sucrose transporter promoters, resulted in 4.4-fold increase of iron in polished grains of field grown T3 rice plants (Masuda et al., 2012). In another approach, Wirth and collaborators reported a more than 6-fold increase in endosperm of rice plants constitutively expressing *A. thaliana NAS* (*AtNAS*), together with endosperm-specific expression of *Phaseolus vulgaris* ferritin and *Aspergillus fumigates* phytase as a single construct (NFP rice; Wirth et al., 2009). The effect of NAS and ferritin genes was synergistic in these plants, indicating that none of the iron uptake, transport, or storage systems in the engineered rice plants were saturated.

Here, we investigated the molecular impact of the transgenes on the expression of endogenous iron homeostasis-related genes in the engineered NFP rice plants. We performed targeted expression profiling of 28 genes involved in iron homeostasis. Our data suggests that the transgenes did not interfere with endogenous iron homeostasis at large, but modulated the expression of a few genes to facilitate iron uptake, translocation, and storage. The results provide new insights into coordinated role of different genes, particularly those involved in phytosiderophore synthesis and iron translocation, in maintaining iron homeostasis within the NFP plants while transporting more iron to the grains in these plants.

RESULTS

The relative expression levels of 28 endogenous rice genes related to iron (or metal) homeostasis (**Table 1**) were analyzed in transgenic NFP plants and non-transgenic control plants (NTS). The genes studied included those involved in NA and DMA synthesis, the YSL transporters, the iron-regulated transporters, genes from zinc-regulated transporter IRT-like proteins (ZIP) family, transcription factors, as well as the inter- and intra-cellular transporters. The plants were subjected to sufficient and deficient iron availability conditions and the expression levels of selected genes were studied in flag leaf, root, and grain samples collected at three grain development stages, i.e., milky, dough, and mature. In order to select for the reference genes which could be used for all the different sample types and growth stages, a preliminary test with at least 13 genes selected from the gene expression database, Genevestigator™ (Zimmermann et al., 2005) as well as

Table 1 | List of genes tested for their expression pattern in the NFP plants in comparison to their non-transgenic siblings.

Genes tested	Root	Leaf	Grain	References
PHYTOSIDEROPHORES SYNTHESIS RELATED GENES				
S-adenosyl methionine synthetase 2 (*OsSAMS2*)	X	X	X	Lee et al., 1997
Nicotianamine synthase 1 (*OsNAS1*)	X	X		Inoue et al., 2003; Kobayashi et al., 2005
Nicotianamine synthase 2 (*OsNAS2*)	X	X	X	Inoue et al., 2003; Kobayashi et al., 2005
Nicotianamine synthase 3 (*OsNAS3*)	X	X		Inoue et al., 2003
Nicotianamine aminotransferase (*OsNAAT1*)	X	X	X	Inoue et al., 2008
Deoxymugineic acid synthase (*OsDMAS1*)	X	X	X	Bashir et al., 2006
INTER- AND INTRA-CELLULAR METAL TRANSPORTERS AND OTHER IRON RESPONSIVE GENES				
Iron-regulated transporter 1 (*OsIRT1*)	X	X	X	Ishimaru et al., 2006
Iron-regulated transporter 2 (*OsIRT2*)	X	X		Ishimaru et al., 2006
Yellow stripe like 2 (*OsYSL2*)	X	X	X	Koike et al., 2004
Yellow stripe like 5 (*OsYSL5*)	X	X		Narayanan et al., 2007
Yellow stripe like 6 (*OsYSL6*)	X	X	X	Narayanan et al., 2007
Yellow stripe like 9 (*OsYSL9*)	X	X		Aoyama et al., 2009
Yellow stripe like 13 (*OsYSL13*)	X	X		Nozoye et al., 2011
Yellow stripe like 15 (*OsYSL15*)	X			Inoue et al., 2009
Zinc-regulated transporter, Iron-regulated transporter-like 1 (*OsZIP1*)	X	X		Ramesh et al., 2003
Zinc-regulated transporter, Iron-regulated transporter-like 3 (*OsZIP3*)	X	X		Ramesh et al., 2003
Zinc-regulated transporter, Iron-regulated transporter-like 4 (*OsZIP4*)	X	X	X	Ishimaru et al., 2005
Zinc-regulated transporter, Iron-regulated transporter-like 8 (*OsZIP8*)	X			Narayanan et al., 2007
Heavy metal ATPase 2 (*OsHMA2*)	X	X		Takahashi et al., 2012
Ferric reductase defective 3 like (*OsFRDL1*)	X			Yokosho et al., 2009
Natural resistance associated macrophage protein 4 (*OsNRAMP4*)	X	X		Narayanan et al., 2007
Mitochondrial iron-regulated gene (*OsMIR*)	X	X	X	Ishimaru et al., 2009
Metal tolerance protein 1 (*OsMTP1*)	X	X		Yuan et al., 2012
Vacuolar iron transporter 2 (*OsVIT2*)	X	X	X	Zhang et al., 2012
Permease in chloroplasts1 (*OsPIC1*)		X	X	Duy et al., 2007
IRON STORAGE PROTEIN FERRITIN AND TRANSCRIPTION FACTORS				
Ferritin 1 (*OsFER1*)	X	X	X	Stein et al., 2009
IDEF1	X	X	X	Kobayashi et al., 2009, 2012
IDEF2	X	X	X	Ogo et al., 2008

The symbol "X" marks the tissue (root, flag leaf, or grain) in which the corresponding gene was tested.

literature were tested. The genes with medium to high expression in all rice tissues were chosen from Genevestigator™ for the pilot qRT-PCR test. Among these tested genes, IWS1 C-terminus family protein (LOC_Os01g05420) and ATP binding protein (LOC_Os11g43970.1) ranked among the best five genes identified in our analysis and were therefore used in the experiment. The data from LOC_Os01g05420 expression was used for normalization of real-time quantitative expression of the test genes. The observed changes in the expression patterns of the tested genes in the NFP plants in comparison to their non-transgenic siblings (NTS plants) are summarized below.

EXPRESSION PROFILES OF GENES INVOLVED IN PHYTOSIDEROPHORE SYNTHESIS AND IRON UPTAKE IN ROOTS

Six rice genes encoding enzymes in phytosiderophore synthesis, and thus involved in iron uptake as well as in iron translocation, were studied. The genes included S-adenosyl-l-methionine synthetase 2 (*OsSAMS2*), the NAS family members *OsNAS1*, *OsNAS2* and *OsNAS3*, *OsNAAT1*, as well as *OsDMAS1*. The genes (except *OsNAS3*) are predominantly expressed in roots and induced by low Fe availability as a part of the Fe deficiency response that serves to increase Fe acquisition (**Figures 1–3**, **A1**). As could be expected, over-expression of *AtNAS* in NFP plants resulted in higher expression of *OsSAMS2*, *OsNAS1*, *OsNAS3,* and *OsDMAS1* as compared to the NTS plants, primarily under iron-deficient conditions (**Figure A1**).

OsSAMS2 was expressed at higher levels during Fe deficiency (mainly in the early development stages) in both NFP and NTS plants, but at the milky stage of grain filling the NFP roots showed further 1.7-fold higher expression of *OsSAMS2* (**Figure A1**, **Table 2**) than NTS roots. At other stages and in other tissues tested, the NFP and NTS plants did not differ significantly for *OsSAMS2* expression. Among the *NAS* genes, *OsNAS3* was overall expressed at low levels compared to *OsNAS1* and *OsNAS2*, but its expression in NFP roots was significantly increased at mature stage under high iron condition and at the milky stage

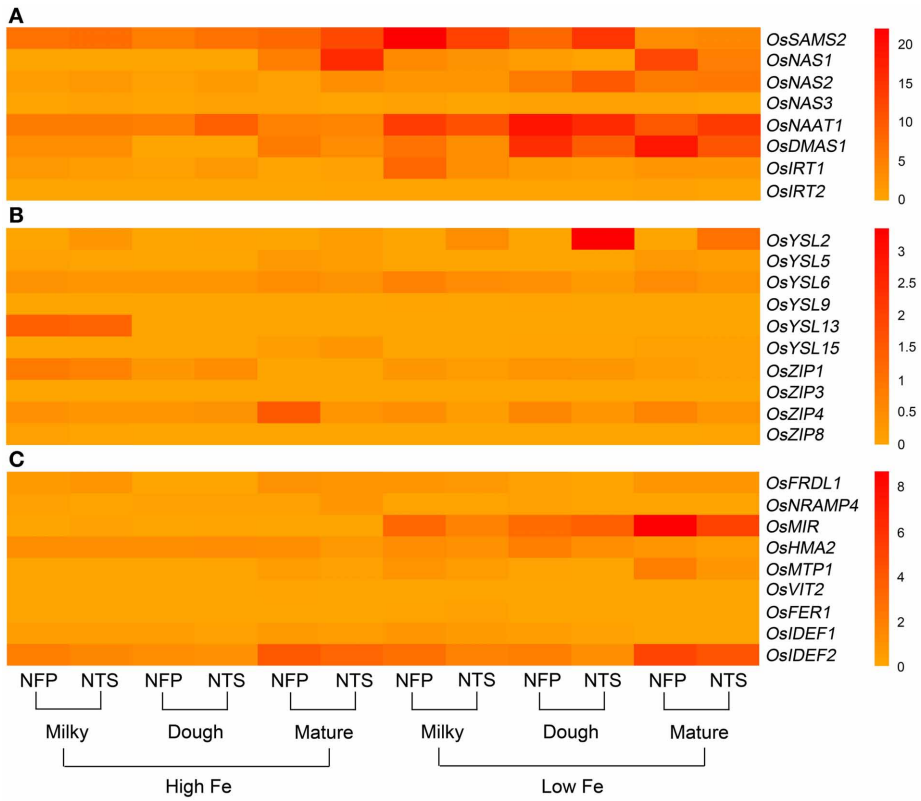

FIGURE 1 | Overall view of genes that are differentially expressed in NFP roots as compared to NTS roots over different plant development stages (corresponding to grain filling stages—milky, dough, and mature) and upon different iron supplies (high and low Fe). (A) Expression levels of genes involved in nicotianamine and deoxymugeneic acid synthesis as well as the iron-regulated transporters. **(B)** Expression levels of genes belonging to yellow stripe like family of transporters and the transporters belonging to zinc-regulated transporter IRT-like proteins (ZIP) family. **(C)** Expression levels of transcription factors *OsIDEF1* and *OsIDEF2* regulating iron homeostasis as well as other inter- and intra-cellular metal transporters. Data represents the mean of three biological replicates. Note the differences in scale between panels **(A), (B),** and **(C)** for relative expression levels of tested genes.

in the plants grown in iron-deficient conditions (**Figure A1**). In the mature stage of grain filling, *OsNAS1* was up-regulated in NFP roots (2.3-fold) as compared to NTS roots. *OsDMAS1* was generally up-regulated in iron-deficient conditions in both the genotypes, but at the milky and mature stages of grain filling, its expression was 2-fold and 1.8-fold higher in the NFP roots than NTS roots, respectively (**Figure A1**, **Table 2**). A significant increase of *OsDMAS1* expression could also be detected in NFP grains at the milky stage in iron-deficient conditions. These results suggest that the genes involved in NA and DMA synthesis are coordinately regulated in NFP plants, which contributed to increased iron uptake and facilitated translocation within these plants.

OTHER GENES INVOLVED IN IRON UPTAKE AND IRON TRANSLOCATION WITHIN THE PLANT

The two iron-regulated transporters *OsIRT1* and *OsIRT2*, as well as several members of the YSL and zinc-regulated transporter IRT-like proteins (ZIP) family were studied, including *OsYSL2*, *OsYSL5*, *OsYSL6*, *OsYSL9*, *OsYSL13*, *OsYSL15* among YSLs and *ZIP1*, *ZIP3*, *ZIP4*, and *ZIP8* from the ZIP family (**Figures 1–3**).

Expression of *OsIRT1* and *OsIRT2* were mainly induced during iron deficiency and particularly in the roots, with *OsIRT1* expressed at higher levels than *OsIRT2* as was previously reported (Ishimaru et al., 2006). Further, *OsIRT1* was expressed 2.6-fold higher in NFP roots than NTS roots at the milky stage of grain filling (**Figure A2**, **Table 2**), while *OsIRT2* expression was not significantly different in NFP and NTS plants. *OsIRT1* was also significantly up-regulated in the grains (1.5-fold) and leaves (3.5-fold) of NFP plants growing under sufficient iron conditions, both at milky and mature stages of grain filling, respectively, but expression levels were generally low in these tissues.

Among the *YSL* genes, significant transcript level differences were detected for *OsYSL2* and *OsYSL6*, while *OsYSL5*, *OsYSL9*, *OsYSL13*, and *OsYSL15* showed no or negligible expression differences between NFP and the NTS plants (**Figures 1–3**). *OsYSL2*, a transporter of the NA-Fe(II) complex, is induced during iron deficiency (Ishimaru et al., 2010), which is also the case in NTS plants where *OsYSL2* is significantly up-regulated in leaves at the milky stage and in the roots at the dough stage of grain filling (**Figure A3**, **Table 2**). However, the *OsYSL2* expression remained unchanged in NFP plants grown in low iron conditions. In contrast, a significant overexpression of *OsYSL6* was observed in

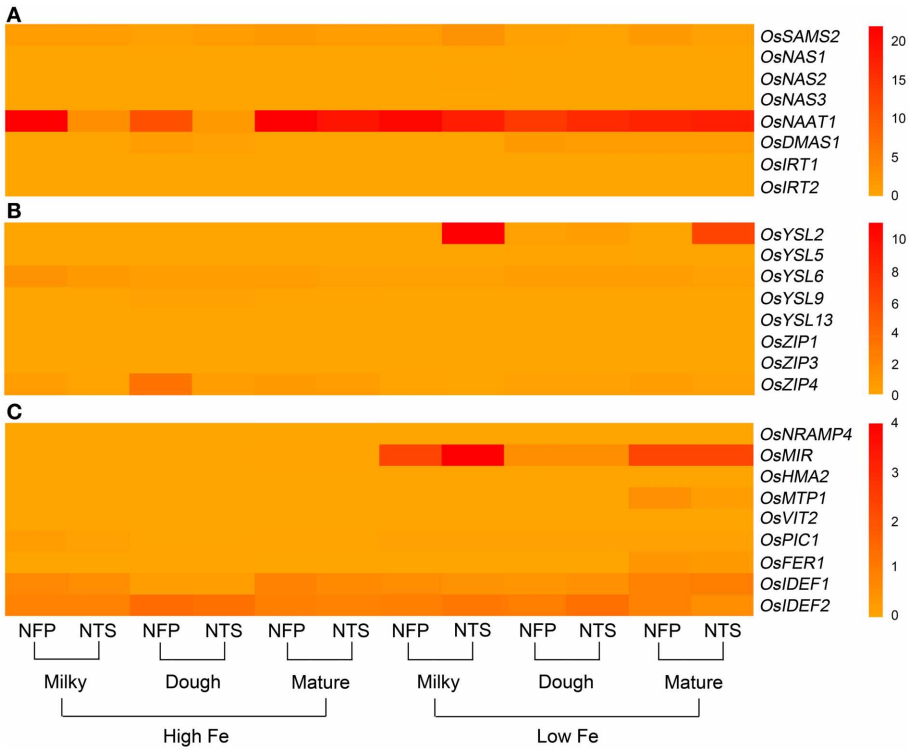

FIGURE 2 | Overall view of genes that are differentially expressed in NFP flag leaves as compared to NTS flag leaves over different plant development stages (corresponding to grain filling stages—milky, dough, and mature) and upon different iron supplies (high and low Fe). (A) Expression levels of genes involved in nicotianamine and deoxymugeneic acid synthesis as well as the iron-regulated transporters. **(B)** Expression levels of genes belonging to yellow stripe like family of transporters and the transporters belonging to zinc-regulated transporter IRT-like proteins (ZIP) family. **(C)** Expression levels of transcription factors *OsIDEF1* and *OsIDEF2* regulating iron homeostasis as well as other inter- and intra-cellular metal transporters. Data represents the mean of three biological replicates. Note the differences in scale between panels **(A), (B),** and **(C)** for relative expression levels of tested genes.

NFP grains at maturity and a slightly increased expression in the roots at the dough stage of grain filling in iron-deficient conditions, as compared to NTS plants. Also, *OsYSL6* was up-regulated in NFP flag leaves at the milky stage of grain filling as compared to NTS leaves in iron-sufficient conditions. *OsYSL6* has been suggested as a Mn-NA transporter and also to play a role in detoxification of high Mn in roots and shoots (Sasaki et al., 2011). NFP and NTS plants contain similar Mn concentrations, except for some increase in polished and brown grains in iron-deficient conditions (Fe 20 μM; Wirth et al., 2009). It is possible that the induced expression of *OsYSL6* in NFP plants contributes to the small increase of Mn in NFP grains when iron availability is low.

OsZIP4 was up-regulated in NFP leaves at dough stage of grain filling (6.3-fold) and in roots at grain maturity (4.2-fold) in iron-sufficient conditions. In iron-deficient conditions, NFP grains at maturity and NFP roots at the dough stage of grain filling had 3.3-fold and 2.1-fold higher expression of *OsZIP4* as compared to NTS plants, respectively (**Figure A3**, **Table 2**). *OsZIP1* was also up-regulated in low iron conditions in NFP roots at the dough stage (1.3-fold) and leaves at the milky stage of grain filling (2.5-fold). In addition, an approximate 6-fold higher expression was detected at the milky and mature stages of grain filling in the leaves of NFP plants grown with sufficient iron supply. However, it should be noted that *OsZIP1* is expressed only weakly in the leaves. *OsZIP3* and *OsZIP8* were expressed at even lower levels than *OsZIP1*, and their expression profiles were not significantly different between the genotypes, except for up-regulated *OsZIP3* expression in NFP roots at the dough stage of grain filling and down regulation in leaves at the milky stage of grain filling in low Fe conditions, as compared to NTS plants (**Table 2**). The upregulation of *OsZIP1* and *OsZIP4* under high iron conditions suggests that NFP plants signaled zinc deficiency when external iron concentration was high. Nevertheless, NFP plants perform better than the NTS plants in terms of zinc content in leaves and grains (Wirth et al., 2009) at both low and high iron conditions.

TRANSCRIPTION FACTORS AND OTHER INTER- AND INTRA-CELLULAR TRANSPORTERS

The transcription factors encoded by *OsIDEF1* and *OsIDEF2* are known to be constitutively expressed and not affected by Fe deficiency (Kobayashi et al., 2007; Ogo et al., 2008). Similar expression patterns were obtained in our experiments, with an exception of *OsIDEF2*, which responded to iron deficiency in NFP plants. *OsIDEF2* expression was increased by 1.5- and 1.8-fold

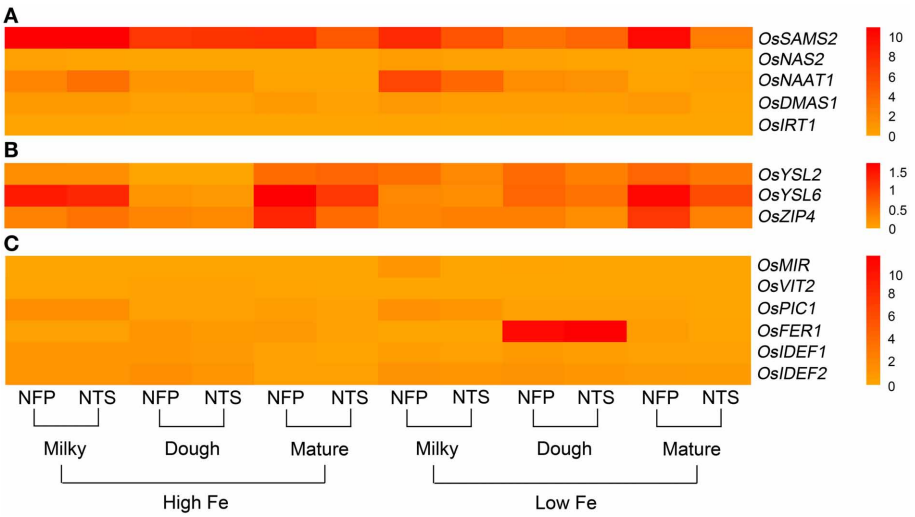

FIGURE 3 | Overall view of genes that are differentially expressed in NFP grains as compared to NTS grains over different plant development stages (corresponding to grain filling stages—milky, dough, and mature) and upon different iron supplies (high and low Fe). (A) Expression levels of genes involved in nicotianamine and deoxymugeneic acid synthesis as well as the iron-regulated transporters. **(B)** Expression levels of genes belonging to yellow stripe like family of transporters and the transporters belonging to zinc-regulated transporter IRT-like proteins (ZIP) family. **(C)** Expression levels of transcription factors *OsIDEF1* and *OsIDEF2* as well as other inter- and intra-cellular metal transporters. Data represents the mean of three biological replicates. Note the differences in scale between panels **(A), (B),** and **(C)** for relative expression levels of tested genes.

in NFP roots at milky and dough stages of grain filling as compared to NTS roots (**Figure A2**, **Table 2**). This up-regulation of *OsIDEF2* in NFP roots perhaps reinforced the Fe deficiency signal and thus led to up-regulation of genes involved in Fe translocation. *OsIDEF2* is known to be dominantly expressed in vascular bundles in the roots (Kobayashi et al., 2010b).

The mitochondrial iron-regulated (*OsMIR*) gene was mainly expressed upon iron deficiency. In comparison to the NTS plants, NFP grains had elevated expression of *OsMIR* at milky and dough stages of grain filling in iron-deficient conditions and at the milky stage of grain filling with sufficient iron availability. However, *OsMIR* was less induced in NFP leaves than the NTS leaves at the milky stage of grain filling (**Figure A2**, **Table 2**), but no significant expression differences were found at other stages. *OsHMA2*, *OsPIC1*, *OsMTP1*, *OsNRAMP4*, *OsFRDL1*, *OsFER1*, and *OsVIT2* did not show any significant differences between NFP and NTS plants (**Figures 1–3**). Only in iron-deficient conditions, NFP mature grains showed a higher expression of *OsPIC1* and NFP roots had higher *OsHMA2* expression at the dough stage of grain filling when compared to NTS plants (**Table 2**). *OsVIT2* was mostly expressed under sufficient iron conditions, with low expression detected under iron deficiency in roots and leaves. *OsVIT1* does not respond to Fe starvation while *OsVIT2* was found to be down-regulated in rice roots and shoots (Zhang et al., 2012).

The summary of genes that are differentially regulated in the NFP plants as compared to NTS plants, under Fe-deficient conditions is presented in **Figure 4**.

DISCUSSION

The combined overexpression of NAS and endosperm-specific expression of ferritin have a synergistic effect in increasing the iron content in the endosperm of NFP grains (Wirth et al., 2009). In the greenhouse, NFP plants show normal agronomic performance (e.g., plant height, tiller number, grain yield) and perform better under low Fe conditions than NTS plants (Wirth et al., 2009), suggesting that expression of *AtNAS1* and ferritin promoted iron increase in the endosperm without interfering with Fe homeostasis in NFP plants.

NA and DMA levels in plants are regulated by NAS and NAAT genes, and both NA and DMA are involved in Fe distribution in plants (Aoyama et al., 2009; Kakei et al., 2009). DMA/phytosiderophore synthesis involves successive reactions that are catalyzed by SAMS, NAS, NAAT, and DMAS (Bashir et al., 2010). *OsNAS1*, *OsNAS2*, and *OsNAS3* were proposed to perform different physiological functions in response to Fe deficiency (Inoue et al., 2003). Expression of *OsNAS1* and *OsNAS2*, both located on chromosome 3, is induced in rice roots exposed to Fe deficiency. Thus, a main role of these enzymes in NA synthesis appears to be the increased production of phytosiderophores in iron-deficient roots (Inoue et al., 2003). Expression of *OsNAS3*, which is located on chromosome 7, was found confined to pericycle cells close to protoxylem and companion cells, and was suggested to play rather limited role in phytosiderophore secretion from roots. Nevertheless, the OsNAS3 protein was shown to catalyze the trimerization of SAM to form NA (Inoue et al., 2003).

During low iron availability, expression of *OsSAMS2* and *OsNAS3* was significantly up-regulated at the milky stage of grain filling while *OsNAS1* had elevated expression levels at maturity in NFP roots when compared to NTS roots. This suggests that under iron deficiency, *AtNAS1* overexpression together with the enhanced expression of *OsNAS3* and *OsNAS1* resulted in increased NA synthesis at milky and mature stages of grain filling.

Table 2 | Summary of expression differences obtained between NFP and NTS rice plants.

Gene studied	Root						Leaf						Grain					
	High Fe			Low Fe			High Fe			Low Fe			High Fe			Low Fe		
	Milky	Dough	Mature	Milky	Dough	Mature	Milky	Dough	Mature	Milky	Dough	Mature	Milky	Dough	Mature	Milky	Dough	Mature
OsSAMS2	–	–	–	1.7↑↑	–	–	–	–	–	–	–	–	–	–	–	–	–	–
OsNAS1	–	–	–	–	–	2.3↑	–	–	–	–	–	–	x	x	x	x	x	x
OsNAS2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
OsNAS3	–	–	2.1↑	3.5↑	–	–	–	–	–	–	–	–	x	x	x	x	x	x
OsNAAT1	–	–	–	–	–	–	–	–	–	–	–	–	1.7↓↓	–	–	–	–	–
OsDMAS1	–	–	–	2.0↑↑	–	1.8↑	–	–	2.1↑	–	–	–	–	–	–	1.5↑	–	–
OsIRT1	–	–	–	2.6↑	–	–	–	–	3.5↑	–	–	–	1.5↑	–	–	–	–	–
OsYSL2	–	–	–	–	279↓	–	1.6↑	–	–	267↓↓	–	–	–	–	–	–	–	–
OsYSL5	–	–	–	–	–	–	–	–	1.9↑	–	–	–	x	x	x	x	x	x
OsYSL6	–	–	–	–	1.8↑↑	–	1.7↑↑	–	–	–	–	–	–	1.4↑	–	–	–	1.8↑↑
OsYSL9	–	–	–	–	–	–	–	–	–	2.1↑	–	–	x	x	x	x	x	x
OsZIP1	–	–	–	–	1.3↑	–	5.8↑↑	–	6.4↑	2.5↑↑	–	–	x	x	x	x	x	x
OsZIP3	–	–	–	–	2.1↑	–	–	–	–	4.3↓	–	–	x	x	x	x	x	x
OsZIP4	–	–	4.2↑↑	–	2.1↑	–	–	6.3↑↑	–	–	–	–	–	–	–	–	–	3.3↑
OsZIP8	–	–	–	–	–	–	x	x	x	x	x	x	x	x	x	x	x	x
OsFRDL1	–	–	–	–	–	–	x	x	x	x	x	x	x	x	x	x	x	x
OsMIR	–	–	–	–	–	–	–	–	–	1.7↓	–	–	3.2↑↑	–	–	38↑	17↑↑	–
OsHMA2	–	–	–	–	1.6↑	–	–	–	–	–	–	–	x	x	x	x	x	x
OsMTP1	–	–	–	–	–	–	–	–	–	–	–	–	x	x	x	x	x	x
OsPIC1	x	x	x	x	x	x	–	–	–	–	–	–	–	–	–	–	–	3.3↑
OsIDEF1	–	–	1.5↑	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
OsIDEF2	–	–	–	1.5↑	1.8↑↑	–	–	–	–	–	–	–	–	–	–	–	–	–

For a particular gene studied, "–" represents no significant difference among the two genotypes, "x" represent no test made, and the values on the table represent fold changes observed in NFP plants as compared to NTS (statistically significant changes), where red upward arrows depict up-regulation while the downward green arrows depict down-regulation in the NFP plants, for a particular gene. Double arrows indicate significance at $p \leq 0.01$ and single arrows indicate significance at $p \leq 0.05$. The milky, dough, and mature represent respective grain filling stages at which the plant material was collected.

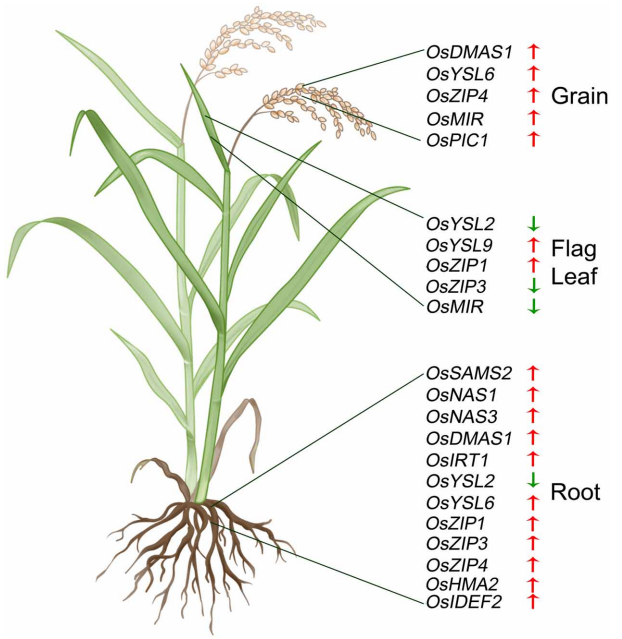

FIGURE 4 | Summary of significant expression changes observed in case of NFP plants vs. NTS plants, when subjected to low iron conditions. Upward red arrows indicate up-regulated expression while downward green arrows indicate down-regulation of a particular gene, in the NFP plants as compared to the NTS plants. The genes are specifically marked according to the parts (roots, leaves, or grains) where the expression difference was observed.

NFP plants produce more NA under iron deficiency (Wirth et al., 2009). Since NA serves as the precursor for DMA, this increase in NA production most likely contributed to increased DMA content in NFP plants. Increased DMA production in NFP plants is further supported by the up-regulation of *OsDMAS1* in NFP roots, both at milky and mature stages of grain filling (**Figure A1**), as well as with increased DMA content in NFP leaves as compared to control plants (unpublished data). *OsNAS3* expression is also higher in NFP roots grown at high iron conditions, particularly at maturity. Together, these results suggest that the increased production of NA and DMA in NFP plants facilitated uptake of iron in roots and improved Fe translocation in the plants. In the roots of iron-deficient plants, *OsNAS1-3*, *OsNAAT1*, and *OsDMAS1* have similar expression patterns, with strong induction in pericycle cells adjacent to protoxylem (Inoue et al., 2003; Bashir et al., 2006; Inoue et al., 2008). Rice plants have increased DMA concentration in the xylem in iron-deficient conditions (Kakei et al., 2009). The specific contribution of *OsNAS3* to iron homeostasis has been previously reported (Lee et al., 2009b), where *OsNAS3* activation resulted in increased Fe and Zn concentration in the rice grains as well as increased tolerance to heavy metals. This increased Fe concentration is also well-correlated with increased NA and DMA content in plants that have increased *OsNAS3* expression (Lee et al., 2009b). Johnson et al. (2011) also showed a positive correlation between increased NA content and increased grain iron content in rice plants overexpressing either of *OsNAS1*, *OsNAS2*, and *OsNAS3*.

Furthermore, the demand for methionine is increased in iron-deficient plants in order to support the increased production of NA and then subsequently DMA. In our experiment, *OsSAMS2* was significantly up-regulated in roots of iron-deficient NFP and NTS plants, with a further expression increase in NFP roots at the milky stage of grain filling as compared to NTS plants (**Figure A1**). This increase of *OsSAMS2* expression would be expected to meet the demand for SAM, which is an immediate precursor for NA synthesis. Increased expression of genes participating in the methionine cycle in the roots of iron-deficient wheat, rice, and barley has been reported earlier (Ma et al., 1995; Negishi et al., 2002; Kobayashi et al., 2005).

In iron-deficient conditions, Fe needs to be effectively transported from roots to the shoots via the xylem, and then between cells. Significant advances have been made in identifying transporters involved in iron translocation. However, our understanding of the exact contribution of each one of these transporters in metal flux is rather limited. To date, the principal chelators known to bind iron include citrate (Rellan-Alvarez et al., 2010), NA and DMA (Takahashi et al., 2003; Aoyama et al., 2009; Kakei et al., 2009). The major role of DMA was initially considered to be in iron uptake from the rhizosphere, but several lines of evidence support a chelating role of DMA in both xylem and phloem (Aoyama et al., 2009; Kakei et al., 2009). Based on the expression of *OsDMAS1* it has been proposed that DMA is synthesized in the phloem companion cells (Inoue et al., 2008). High concentrations of DMA have been detected in phloem sap in independent studies advocating the involvement of DMA in long distance iron transport (Nishiyama et al., 2012). Several transporters belonging to YSL family could be transporting these chelator-bound iron complexes to other parts of the plant. OsYSL15 and OsYSL18 transport Fe(III)-DMA complexes and are involved in internal translocation of iron (Aoyama et al., 2009; Inoue et al., 2009), while OsYSL2 transports Fe(II)-NA and Mn(II)-NA complexes, but not Fe(III)-DMA (Koike et al., 2004; Ishimaru et al., 2010).

OsYSL2 is induced by iron-deficiency and may be actively involved in long distance phloem transport of Fe(II)-NA complexes in the plant and into the grains (Koike et al., 2004; Ishimaru et al., 2010). Rice plants with reduced *OsYSL2* function (RNAi-OsYSL2) have reduced Fe and Mn concentrations in the grains (Ishimaru et al., 2010). Consistent with previous reports (Ishimaru et al., 2010), NTS plants showed induced expression of *OsYSL2* upon iron deficiency, but the gene was not up-regulated in NFP roots and leaves under the same condition (**Figure A3**). In the grains, however, the expression of *OsYSL2* increased in NFP plants as well but was not significantly different than in NTS grains. A plausible explanation for this result could be that expression of *OsYSL2* is regulated by the endogenous iron status of the plants. It has also been suggested that OsIDEF2 directly regulates expression of *OsYSL2* (Ogo et al., 2008). However, the up-regulation of *OsIDEF2* in iron-deficient NFP roots (**Figure A2**) did not lead to increased *OsYSL2* expression. It is possible that OsIDEF2 also senses cellular iron status in order to induce the iron-deficiency responsive genes, as was suggested for OsIDEF1 which binds directly to

divalent metals for sensing cellular metal ion balance (Kobayashi et al., 2012). Importantly, the NFP plants had higher iron content in the grains as compared to NTS plants. It is also possible that NFP plants deployed alternate modes of iron transport to grains than the transfer by OsYSL2 and that the function of OsYSL2 is complemented by another transporter. Our results also reflect effective crosstalk between molecular components involved in Fe homeostasis in different growth conditions and during development to meet the needs for Fe in the plant.

OsMIR, a recently evolved rice-specific mitochondrial gene, is strongly induced under iron deficiency (Ishimaru et al., 2009). NFP leaves had lower expression levels of *OsMIR* as compared to NTS leaves under low iron conditions, but the gene was upregulated in NFP grains (**Figure A2**). Since mitochondrial Fe regulation is poorly understood, it is difficult to predict how and to what extent these expression differences contributed to higher grain iron content in the NFP plants. In addition, NFP leaves also had increased expression of *OsZIP1* and *OsZIP4* under high iron conditions, indicating that NFP plants might signal zinc deficiency when external iron concentration is high. Although *OsZIP4* is regulated by zinc (Ishimaru et al., 2005), there exists a strong crosstalk between Zn and Fe homeostasis in plants. Fe concentrations doubled in Zn-deficient roots (Ishimaru et al., 2005) and plants overexpressing *OsZIP4* had significantly increased Fe in the shoots and roots, in addition to the Zn increases (Ishimaru et al., 2007). This demonstrates the coordination of Fe and Zn homeostasis in plants and also that the expression of *OsZIP1* and *OsZIP4* are affected by the external supply of iron to the plants (as in the case of both NFP and NTS plants) as well as by endogenous Fe nutritional status of plants (further increases in NFP plants). Nevertheless, NFP plants had a similar zinc content in the leaves as compared to the controls over a range of tested external iron concentrations and outperformed NTS plants in terms of zinc content in the grains (Wirth et al., 2009). Therefore, Zn homeostasis is also unaffected in NFP plants.

Together, the increased production of NA and DMA in NFP plants facilitated iron uptake from the rhizosphere as well as effective internal translocation. Expression of several transporter genes appear to be adjusted in the NFP plants in order to utilize overproduced NA and DMA, and the expanded sink for iron storage in the grains via ferritin. However, these adjustments did not interfere with Fe homeostasis in the NFP plants. Further investigations focused on iron speciation in the grain, i.e., Fe(II) or Fe(III), and the relative abundance of two forms together with the information on molecules chelated to these forms, will be required for elucidating the exact mechanisms of iron translocation to the grains in NFP plants.

MATERIALS AND METHODS

PLANT MATERIAL

The NFP plants and their non-transgenic siblings (NTS) were grown under greenhouse conditions, in the hydroponics system. The NFP plants are the *Oryza sativa* ssp. japonica cv. Taipei 309 transformed with *Arabidopsis Nicotianamine Synthase* gene, *Phaseolus vulgaris Ferritin*, and *A. fumigatus Phytase* gene (NFP plants; Wirth et al., 2009). Solutions for the hydroponic system were prepared according to the protocol modified from Kobayashi et al. (2005), using 0.70 mM K_2SO_4, 0.10 mM KCl, 0.10 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.50 mM $MgSO_4$, 10 μM H_3BO_3, 0.50 μM $MnSO_4$, 0.20 μM $CuSO_4$, 0.01 μM $(NH4)_6Mo_7O_{24}$, and 0.5 μM $ZnSO_4$, with different iron concentrations added as Fe(III)-EDTA according to the treatment (high iron condition: 200 μM iron; iron-deficient condition: 10 μM iron). Samples were collected at three different grain filling stages: milky stage, dough stage, and mature stage. At the milky stage grains are starting to fill with a white, milky liquid that can be squeezed by pressing the grain between fingers, while in dough stage the milky portion of grain turns into a soft dough and at maturity, the grain is fully developed and hard (parameters as defined in the Rice Knowledge Bank, IRRI, Philippines). At each developmental stage, roots, flag leaves and grains were collected, with at least three biological replicates.

TOTAL RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted from the root, flag leaf, and grain samples using Trizol® reagent (Invitrogen, USA) and was treated with DNase I (Thermo Fisher Scientific Inc., USA). RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific Inc., USA) was used for cDNA synthesis. All steps were carried out following the manufacturers' instructions.

REAL-TIME QUANTITATIVE PCR

Real-time quantitative PCRs (qRT-PCR) were carried out using Taqman hydrolysis probes (Roche, Switzerland) on 7500 FAST Real Time PCR system (Applied Biosystems, Inc., USA). Total reaction volume of 25 μl was used, comprising of 12.5 μl mastermix (Applied Biosystems Inc., USA), 1 μl cDNA, 2.25 μl forward primer and 2.25 μl reverse primer, 0.25 μl probe (Roche Ltd., Switzerland) and 6.75 μl H_2O.

Primers were designed using Roche primer design website (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). Probe number and primer sequences are presented in the Appendix (**Table A1**). The Ct value was obtained from 7500 Fast System Software (Applied Biosystems, Inc., USA). The primer efficiency was calculated using LinReg PCR (Tuomi et al., 2010). qRT-PCR data normalization was done as described by Schefe et al. (2006). The obtained data were further analyzed by ANOVA and significant differences between the tested plant materials are presented.

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APPENDIX

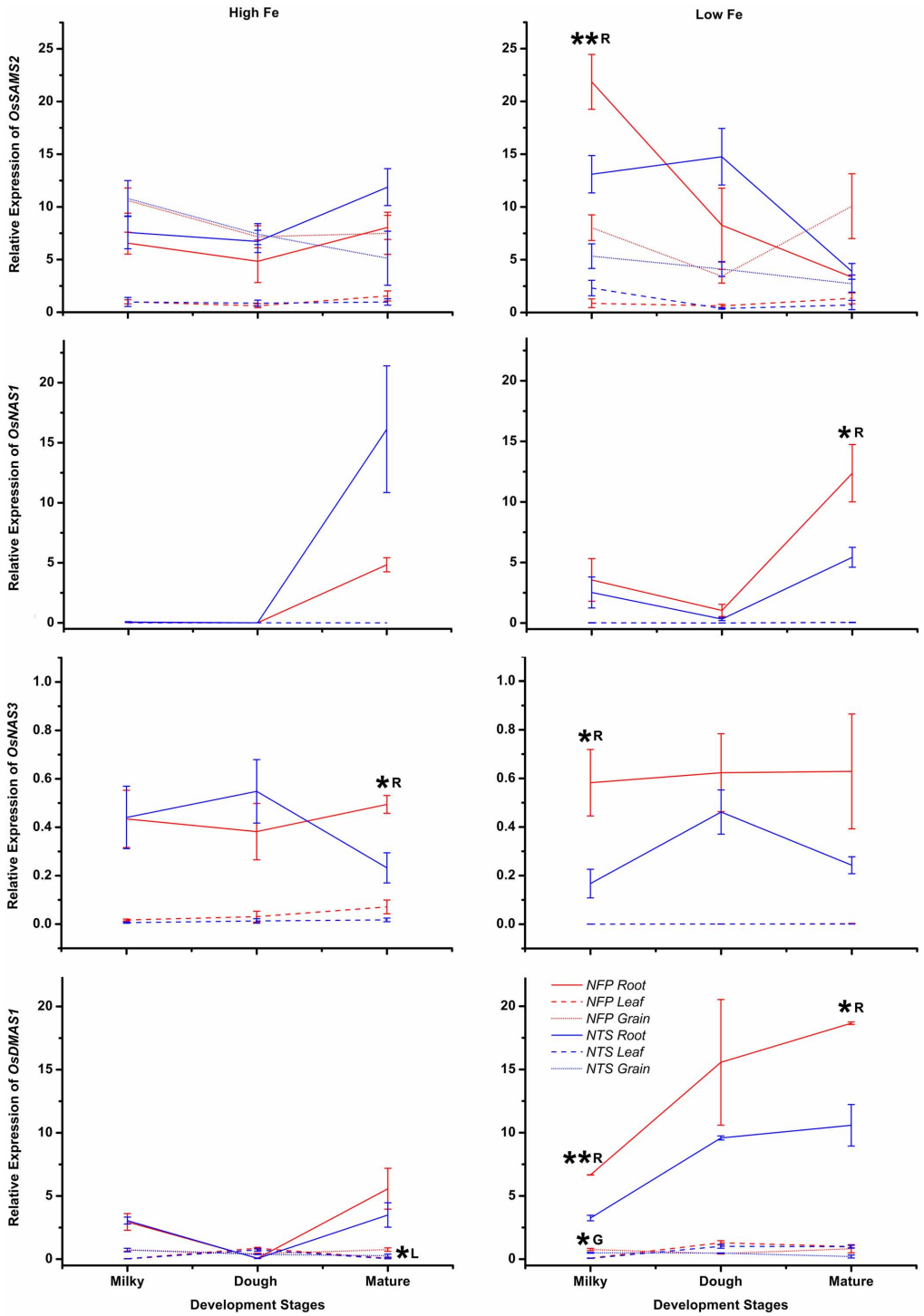

FIGURE A1 | Differences in expression levels for genes involved in nicotianamine and deoxymugeneic acid synthesis (*OsSAMS2, OsNAS1, OsNAS3,* and *OsDMAS1*), in the roots, flag leaves, and grains of NFP vs. NTS plants are presented. Values are mean ± standard error of mean, of the three biological replicates. Significant differences between NFP and NTS tissues (roots, flag leaves, grains) under a particular treatment (high Fe or low Fe) or at a particular development stage (milky, dough, or mature) are marked with single ($*p \leq 0.05$) or double asterisks ($**p \leq 0.01$), respectively. The letters R, L, and G represent root, leaf, and grain, respectively. Note the differences in scale at Y-axis for relative expression levels of tested genes.

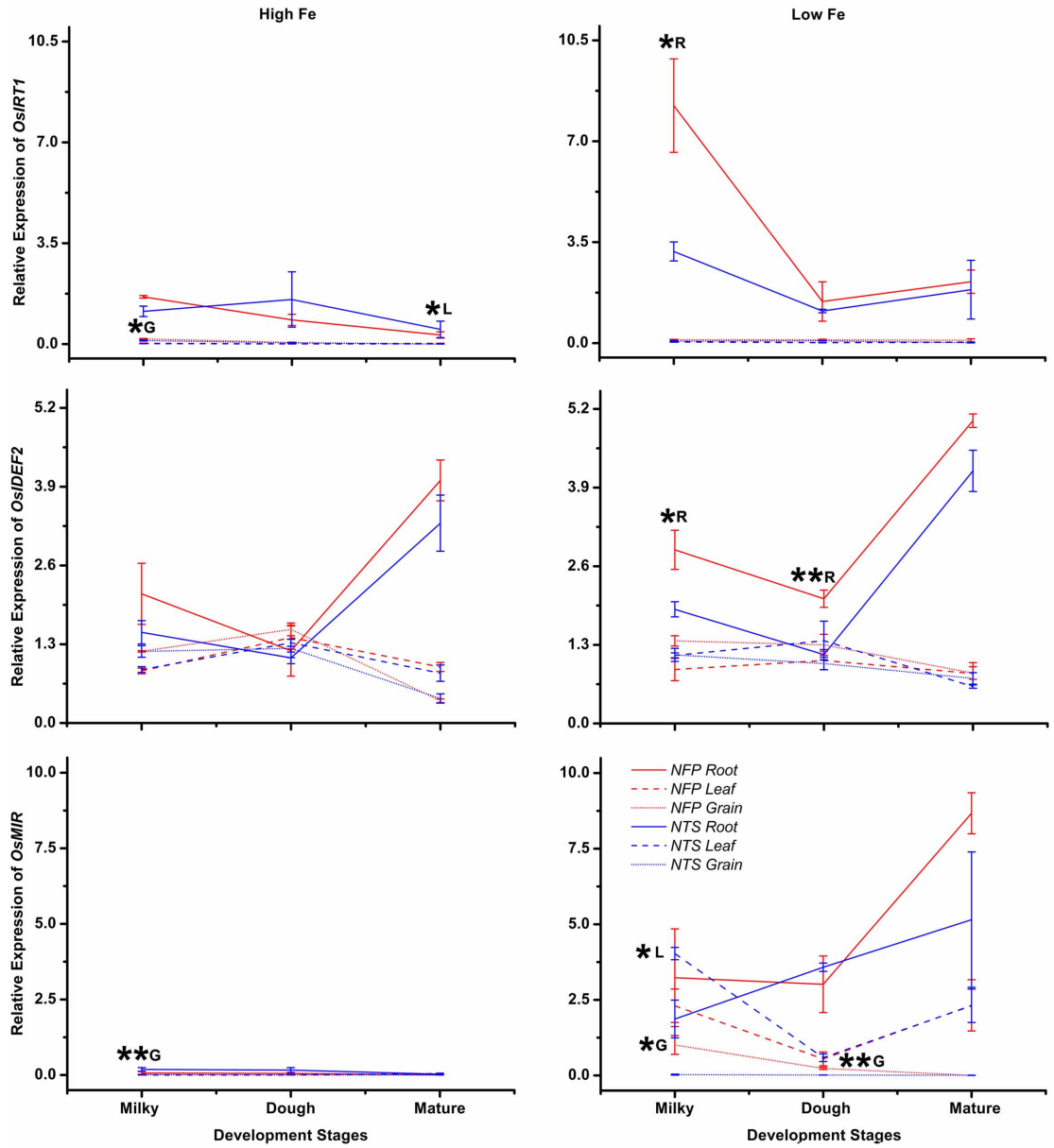

FIGURE A2 | Differences in expression levels of iron-regulated transporter *OsIRT1,* the transcription factor *OsIDEF2,* and the mitochondrial iron regulated gene *OsMIR,* in the roots, flag leaves, and grains of NFP vs. NTS plants are presented. Values are mean ± standard error of mean, of the three biological replicates. Significant differences between NFP and NTS tissues (roots, flag leaves, grains) under a particular treatment (high Fe or low Fe) or at a particular development stage (milky, dough, or mature) are marked with single ($*p \leq 0.05$) or double asterisks ($**p \leq 0.01$), respectively. The letters R, L, and G represent root, leaf, and grain, respectively. Note the differences in scale at Y-axis for relative expression levels of tested genes.

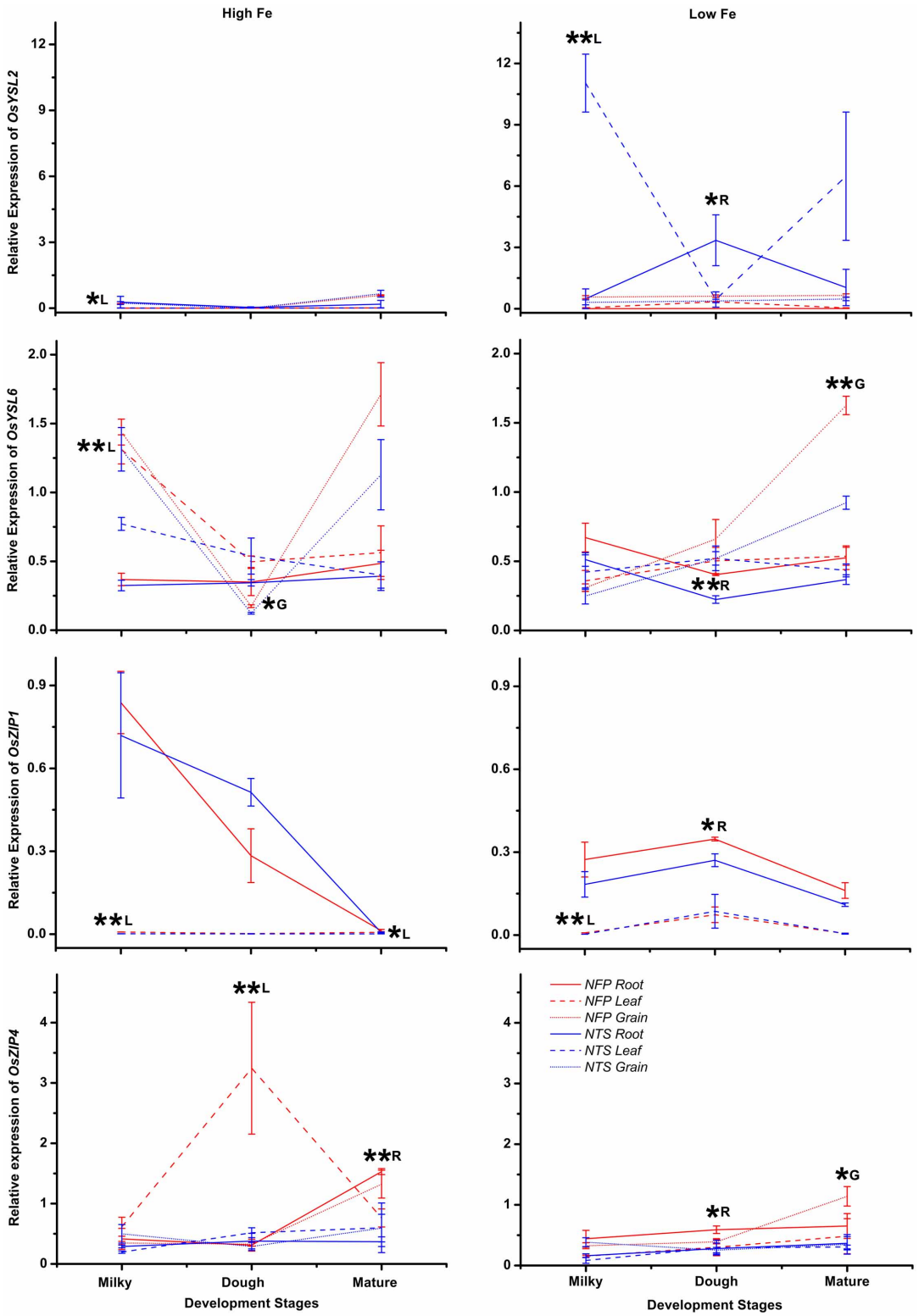

FIGURE A3 | Differences in expression levels of *OsYSL2* and *OsYSL6* (yellow stripe like transporters) as well as *OsZIP* and *OsZIP4* (zinc- and iron-regulated transporters), in the roots, flag leaves, and grains of NFP vs. NTS plants are presented. Values are mean ± standard error of mean, of the three biological replicates. Significant differences between NFP and NTS tissues (roots, flag leaves, grains) under a particular treatment (high Fe or low Fe) or at a particular development stage (milky, dough, or mature) are marked with single ($*p \leq 0.05$) or double asterisks ($**p \leq 0.01$), respectively. The letters R, L, and G represent root, leaf, and grain, respectively. Note the differences in scale at Y-axis for relative expression levels of tested genes.

Table A1 | Primers and probes list for real-time quantitative PCR.

Nr.	Gene	Accession ID	Forward primer	Reverse primer	TaqMan probe
1	*OsSAMS2*	U82833	ccgaataaaggcgagaagc	gactcggaggtgaagaggaa	70
2	*OsNAS1*	AB021746	cggttgagaaggcagaagagt	cgatcgtccggctgttag	17
3	*OsNAS2*	AB023818	cacctcgaggcgcactac	tccagcttgctcaggttga	149
4	*OsNAS3*	AB023819	gaggaggaggtgatcgagaa	atcaccagctccgtgaaca	70
5	*OsNAAT1*	AB206814	ttgccaacttgcgaaagaa	aatgcgaacccaattcttca	61
6	*OsDMAS1*	AB269906	aaaagctcgacaccctgct	tccctcagcttcctctgct	39
7	*OsIRT1*	AB070226	gacactggtgcccattctg	gaggatggggatggagga	63
8	*OsIRT2*	AB126086	tcaggaatcgcgtcattgt	agcccgatcaccactgag	105
9	*OsYSL2*	AB164646	tggagcttcttccagtggtt	gaggctgaaatcaaaatagaacg	22
10	*OsYSL5*	AB190915	agcttgcatggaaaaacagaa	aaagaagctccagccaaaact	4
11	*OsYSL6*	AB190916	gaacaccgtcatccagacct	gttttctgatccattgcaagc	141
12	*OsYSL9*	AB190919	tgcgtggatgactggattc	tcccacttgggtaagttaatttgt	152
13	*OsYSL13*	AK067235	atccagacctgcgtcgtc	catggacaatatgtagttaccaaagc	161
14	*OsYSL15*	AB190923	tcgcctgctacaccatagc	cagctcgtacgtcctcttgtt	107
15	*OsZIP1*	AY302058	gtgatgagccgcaaggag	cctcgaacgacgatgtcc	159
16	*OsZIP3*	AY323915	tgcatctgtgaggccatcta	tgacggttgcccttacctta	31
17	*OsZIP4*	AB126089	gtcaatcaggccactcgtc	gctttcgccttaaaatttgc	31
18	*OsZIP8*	AK070864	atcagttcttcgagggcatc	gcgtcgtagacggaggag	143
19	*OsFRDL1*	NM_001055921	gactccacttcgatccacaaa	ctcgctccgcttatcacg	146
20	*OsNRAMP4*	AK102180	gccattggcttcctagatcc	aagatgacccacagaagctca	78
21	*OsMIR*	AK103636	acatttatcctactcgttgctcct	tgcctaagtgctgagtcacg	43
22	*OsHMA2*	HQ646362	gtcaacatactcatgctgattgc	cccagcctcagaatagtcctt	67
23	*OsMTP1*	AK100735	tcaatctccatcaccatcca	tcactcttgagcaatggttcc	157
24	*OsVIT2*	Os09g0396900	ggcctcggagggtatctg	acagtatgtccgcgatctcc	15
25	*OsPIC1*	XM_464386	accgagcaggacatcgag	ggaaacaactgtggacacca	93
26	*OsFER1*	AF519570	aggggatgccttgtatgct	cggtcagctgtggatcatt	148
27	*OsIDEF1*	AK107456	gtcttcaggctggggatgt	gggatttgttgtctgctgatg	91
28	*OsIDEF2*	AK099540	cagatgttgaactgtataaatttgctc	ttcaagatctctgctctggagac	15