Doctoral Thesis

Development of multi-nutrient wheat and rice lines: an approach to simultaneously address different micronutrient deficiencies

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Publication Date:
2016

Permanent Link:
https://doi.org/10.3929/ethz-a-010792954

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DEVELOPMENT OF MULTI-NUTRIENT WHEAT AND RICE LINES: AN APPROACH TO SIMULTANEOUSLY ADDRESS DIFFERENT MICRONUTRIENT DEFICIENCIES

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2016
Acknowledgements

I would like to extend deep sense of gratitude to Dr. Navreet K. Bhullar for giving me a chance to work on this project, her guidance, profound motivation and support. I also gratefully thank Prof. Dr. Wilhelm Gruissem for unconstrained cooperation, valuable guidance and suggestions during the course of my PhD. I express my acknowledgments to Prof. Dr Beat Keller (PhD committee member) for valuable and useful suggestions during the committee meetings, and for collaboration with the wheat transformation and providing the place and the required instruments to do so. I am also very thankful to Prof. Dr Achim Walter (PhD committee member) for suggestions during the committee meetings.

I would like to acknowledge Björn Studer for his guidance on ICP-OES, and Prof. Dr. Schulin Rainer for the access to the ICP-OES. I acknowledge Dr. Christof Sautter for providing the Ferritin plasmid. I would like to thank Gabi and Geri, for their guidance and instructions to perform wheat transformation. I am also happy to acknowledge the efforts of Irene Zurkirchen for taking care of the plants in the greenhouse. I thank Doris Russenberger, Kim Schlegel, Serena, Katharina Holzinger and Daniela Rothe for their help and support during the PhD.

I would like to thank Joel for his help in the german translation of thesis summary.

Many thanks to present and past cereal team members Kulaporn, Jhongwa, Meng, Kumar, Marlen, Jassmine, and Ting-Ying for providing a wonderful atmosphere in the lab. I would thank my student Melanie for her contribution. I also would like to thank Adrian, Ravi, Devang, Joel, Ima, Nathalie, Philip, Sira, Emily, Ezequiel, Julia, Glen, and all the Plant biotechnology group members for helpful suggestions, material & moral support, and wonderful time during the social events.

I would like to acknowledge the support of my father Satpal Singh, mother Balwinder Kaur and all other family members for their motivation and support. Finally, I would like to thank my wife Anita Singh, for her persistence with me during the course of my PhD. I also acknowledge her patience, and support she provided me all the time. All may not be mentioned but none is forgotten.
Summary

The occurrence of micronutrient deficiencies worldwide (termed “hidden hunger”) is often associated with the dependence of affected populations on starchy, often cereal-based, monotonous diets. Two cereals, wheat (*Triticum aestivum*) and rice (*Oryza sativa* L.) make up a major portion of the human diet but are deficient in micronutrients essential for humans. Iron, zinc, and vitamin A deficiency are the most common micronutrient deficiencies, and usually co-exist in poor populations. Deficiencies of these micronutrients negatively impact human health, productivity, mental development, and the global economy. Various interventions ranging from supplementation to diet diversification have been employed with varied amount of success worldwide. Crop biofortification offers the most sustainable method to overcome micronutrient deficiencies by directly enhancing the nutritional content of staple foods. Although conventional breeding strategies to improve micronutrient content in grains have been employed, they have met with very limited success, mainly due to insufficient germplasm variability. In this regard, the utility of genetic engineering technology has already been demonstrated with the development of golden rice (Paine et al. 2005) and iron-biofortified rice (Trijatmiko et al. 2016; Wirth et al. 2009).

In the first part of this thesis, I present wheat lines expressing rice *NICOTIANAMINE SYNTHASE 2* (*OsNAS2*) and bean *FERRITIN* (*PvFERRITIN*) as single transgenic events as well as in combination. NAS catalyzes the biosynthesis of nicotianamine (NA), which is a precursor of deoxymugeneic acid (DMA), a molecule known to chelate iron for long distance translocation. FERRITIN is important for iron storage within plants, owing to its capacity to store up to 4500 iron ions in a single complex. Significant increases in iron and zinc content in wheat grains were observed in plants expressing either *OsNAS2* or *PvFERRITIN* or both. In particular, wheat lines overexpressing *OsNAS2* surpassed the HarvestPlus recommended target level of 30% dietary estimated average requirement (EAR) for iron, and 40% of EAR for zinc with lines containing 93.1 µg/g of iron and 140.6 µg/g of zinc in the grains.

Next, I developed rice lines expressing Arabidopsis *NAS1* (*AtNASI*), *PvFERRITIN*, *Pantoea ananatis CAROTENE DESATURASE* (*CRTI*) and maize *PHYTOENE SYNTHASE* (*ZmPSY*). PSY catalyzes the conversion of geranylgeranyl diphosphate (GGDP) to phytoene, and CRTI performs the function of desaturases required for synthesis of β-carotene from phytoene. A significant increase in the amount of β-carotene, iron and zinc content was observed in plants expressing all the transgenes.
Together, the enrichment of the wheat endosperm with iron and zinc, along with the enrichment of rice endosperms with iron, zinc and β-carotene offers a sustainable and effective intervention strategy. Furthermore, these results indicate the feasibility of combining multiple nutrition traits in a single cultivar to tackle various micronutrient deficiencies in affected populations.
ZUSAMMENFASSUNG


Im ersten Teil meiner Thesis präsentierte ich Weizenlinien, welche das Reis-Gen NICOTIANAMINE SYNTHASE 2 (*OsNAS2*) und das Bohnen - FERRITIN (*PvFERRITIN*) als einzelnes Transgen und in Kombinationen exprimieren. NAS katalysiert die Biosynthese von Nicotianamin (NA), welches das Vorstufenmolekül zur deoxymugenischen Säure (DMA) darstellt und im Langstrecken-transport des Eisens und der Eisen-Chelatisierung involviert ist. FERRITIN dagegen ist wichtig für die Eisen-Speicherung innerhalb einer Pflanze und ist in der Lage bist zu 4500 Eisen Ionen in einem einzelnen Komplex zu speichern. Ein signifikanter Anstieg an Eisen und Zink in Weizenkörner wurde in Pflanzen, welche entweder *OsNAS2* oder *PvFERRITIN* oder beide Gene in Kombination exprimieren, beobachtet. Zudem übertrafen *OsNAS2* exprimierende Weizenlinien die HarvestPlus empfohlene Zielmenge von 30% EAR (dietary estimated average requirement = EAR), und die 40% EAR für Zink mit gemessenen 93.1 µg/g Eisen und 140.6 µg/g Zink in transgenen Weizenkörner.
Als nächstes entwickelte ich transgene Reislinien, welche das Arabidopsis NASI (AtNASI),
PvFERRITIN, Pantoea ananatis CAROTENE DESATURASE (CRTI) und die Mais
PHYTOENE SYNTHASE (ZmPSY) überexprimieren. PSY katalysiert die Umwandlung von
Geranyl-diphosphat (GGDP) zu Phytoen und CRTI bildet eine Desaturase zur Gewinnung
von β-Carotin aus Phytoen. Dabei wurde eine signifikante Erhöhung des β-Carotin-, Eisen-
und Zink - Gehalts in den transgenen Linien beobachtet.
Die Anreicherung des Weizenendosperms mit Eisen und Zink und die Anreicherung des
Reisendosperms mit Eisen, Zink und β-Carotin bietet eine nachhaltige und effektive
Interventionsstrategie Mangelernährung einzuschränken. Diese Studie zeigt auch die
Machbarkeit mehrere ernährungsrelevante Eigenschaften in einer einzelnen Kultursorte zu
vereinen und damit gezielt Spurenelementmangel zu bekämpfen.
LIST OF ABBREVIATIONS

ADSC  AMINODEOXYCHORISMATE SYNTHASE
AHA   Arabidopsis H⁺ATPase
CRTI  CAROTENE DESATURASE
CGIAR Consultative Group for International Agricultural Research
DAF   Days after flowering
DIG   Digoxigenin
DMA   Deoxymugineic acid
DMAS  DEOXYMUGINEIC ACID SYNTHASE
DNA   Deoxyribonucleic acid
EAR   Estimated average requirement
FBP   FOLATE BINDING PROTEIN
FER   FERRITIN
FRD   FERRIC REDUCTASE DEFECTIVE
FRO   FERRIC REDUCTASE OXIDASE
FPN   FERROPORTIN
GGDP  Geranylgeranyl diphosphate
GSLG  Glucosinolate glucoraphanin
GTPCHI GTP CYCLOHYDROLASE I
HMA   HEAVY METAL TRANSPORTING ATPASE
HPT   HYGROMYCIN PHOSPHOTRANSFERASE
ICP-OES Inductively coupled plasma-optical emission spectroscopy
IDA   Iron deficiency anemia
IDS   IRON DEFICIENCY-SPECIFIC CLONE
IREG  IRON REGULATED
IRT   IRON-REGULATED TRANSPORTER
LCY   LYCOPENE β-CYCLASE
MA    Mugineic acid
MATE  Multidrug and toxin efflux
mha   Million hectares
MT    Metric tonnes
MTP   METAL TOLERANCE PROTEIN
NA    Nicotianamine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>NAAT</td>
<td>NICOTIANAMINE AMINOTRANSFERASE</td>
</tr>
<tr>
<td>NAS</td>
<td>NICOTIANAMINE SYNTHASE</td>
</tr>
<tr>
<td>NRAMP</td>
<td>NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN</td>
</tr>
<tr>
<td>OSP</td>
<td>Orange sweet potato</td>
</tr>
<tr>
<td>OPT</td>
<td>OLIGOPEPTIDE TRANSPORTER</td>
</tr>
<tr>
<td>PCA</td>
<td>Protocatechuic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDS</td>
<td>PHYTOENE DESATURASE</td>
</tr>
<tr>
<td>PEZ</td>
<td>PHENOLICS EFFLUX ZERO</td>
</tr>
<tr>
<td>PMI</td>
<td>PHOSPHOMANNOSE ISOMERASE</td>
</tr>
<tr>
<td>PS</td>
<td>Phytosiderophores</td>
</tr>
<tr>
<td>PSY</td>
<td>PHYTOENE SYNTHASE</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNI</td>
<td>Required nutrient intake</td>
</tr>
<tr>
<td>SUT</td>
<td>SUCROSE TRANSPORTER</td>
</tr>
<tr>
<td>TOM</td>
<td>TRANSPORTER OF MUGINEIC ACID</td>
</tr>
<tr>
<td>VAD</td>
<td>Vitamin A deficiency</td>
</tr>
<tr>
<td>VIT</td>
<td>VACUOLAR IRON TRANSPORTER</td>
</tr>
<tr>
<td>YS</td>
<td>YELLOW STRIPE</td>
</tr>
<tr>
<td>YSL</td>
<td>YELLOW STRIPE 1-LIKE</td>
</tr>
<tr>
<td>ZDS</td>
<td>ζ-CAROTENE DESATURASE</td>
</tr>
<tr>
<td>ZIF</td>
<td>ZINC-INDUCED FACILITATOR</td>
</tr>
<tr>
<td>ZIP</td>
<td>ZRT/IRT-RELATED PROTEINS</td>
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1 Introduction

1.1 Prevalence of micronutrient deficiencies

Micronutrients such as vitamins and minerals, are a key component of human nutrition in order to sustain a healthy life. These micronutrients are essential for various metabolic activities, and micronutrient deficiencies often negatively impact human health, productivity, and mental development. The chronic deficiency of essential vitamins and minerals is often referred to as “hidden hunger” and affects people globally, thus having long-term irreversible negative effects on health and economic development (Muthayya et al. 2013). Deficiencies of iron, zinc, vitamin A, vitamin B-12, riboflavin, vitamin D, and vitamin E are the most common and usually exist together (Allen et al. 2009). Iron and zinc deficiencies can cause decreased cognitive performance, reduced physical growth of children, increased infection rate due to reduced immunity, and decreased work performance in all age groups (Prasad 2014; Stevens et al. 2013). Iron deficiency can lead to development of anemia, and worldwide affects 43%, 38%, and 29% of children, pregnant women, and non-pregnant women, respectively (Stevens et al. 2013). Occurrence of iron deficiency anemia (IDA) during pregnancy may be associated with decreased birth weight, and enhanced risk of maternal and perinatal mortality (WHO 2015b). While, zinc deficiency is associated with various diseases such as diarrhea, pneumonia and malaria (WHO 2002). Like iron and zinc, vitamin A deficiency (VAD) is also of major concern among the populations of developing world. Vitamin A is essential for the visual system, growth, epithelial integrity, red blood cell production, immunity and reproduction. VAD can lead to various disorders such as xerophthalmia, the leading cause of preventable blindness in children. It also contributes to morbidity and mortality from infections in children and
pregnant women (WHO 2009). About 250,000 to 500,000 children become blind every year, with half of them dying within 12 months of losing their sight (WHO 2016a). Additionally, various epidemiological studies have shown that the prevalence of vitamin deficiency in a population also increases the risk of anemia (Semba and Bloem 2002). Iron, vitamin A, and zinc deficiency alike are associated with low micronutrient content in diets, poor absorption from food, and the dependence of affected populations on monotonous diets of milled cereal grains.

1.2 Treatment and preventive measures

Dietary diversification, supplementation, and post-harvest fortification are the major intervention strategies used to tackle hidden hunger. Dietary diversification requires the consumption of micronutrient rich food like fish, meat, fresh vegetables and fruits. This approach is impractical in many developing countries due to the unavailability of a wide range of foods, insufficient earnings, poor governance, and poor food distribution networks (Gomez-Galera et al. 2010). Micronutrient supplementation has been implemented in different parts of the world, but is not always successful. For example, a nutritional anemia control program, implemented in India in 1970, only had limited impact, mainly because of mismanagement, logistical problems, poor compliance, and lack of sufficient funds (Vijayaraghavan 2002). Post-harvest food fortification programs depend on industrially processed food items, which are generally unaffordable for the world’s poor, half of whom are living on less than $2 per day, and another 30% of whom live on less than $1 per day (Mayer et al. 2008). In addition, since food fortification involves enrichment of food with micronutrients, public acceptance is a challenge. For example, iron fortification with FeSO$_4$ can cause food to have a generally unacceptable flavor and color, making it unpalatable.
Biofortification of staple crops, i.e., intrinsically enhancing the nutritional content of the edible parts, offers the most sustainable method to overcome micronutrient deficiencies. Biofortification can be accomplished by both conventional plant breeding and/or gene technology approaches. The HarvestPlus program aimed at biofortification is active in different regions around the world, targeting food crops like rice, wheat, maize, beans, sweet potato, cassava and pearl millet (Bouis et al. 2011). Increasing iron and zinc levels in wheat, especially in endosperm, has not been possible through conventional breeding approaches particularly due to insufficient natural genetic variation and a negative correlation between grain yield and micronutrient content (Amiri et al. 2015; Zhao et al. 2009). Similarly, in rice, breeding efforts over several years have failed to achieve the recommended grain iron content of 14 µg/g (Bhullar and Gruissem 2013). Similarly, the screening of rice germplasm for the identification of varieties with high β-carotene content in the grains has not been successful, owing to low genetic variability (Beyer 2010). Biofortification through gene technology offers a multi-dimensional approach for enriching the endosperm of wheat and rice with the required micronutrients. However, this requires detailed knowledge of the various molecular mechanisms controlling the synthesis, uptake and storage of these nutrients.

1.3 Molecular mechanisms involved in iron uptake and its translocation in plants

1.3.1 Importance of iron in plant growth

Iron is involved in the key physiological and metabolic activities of plants. It is an essential cofactor of many cellular redox reactions involved in photosynthesis, respiration and other metabolic pathways. Iron undergoes cyclic oxidation and reduction, hence generating free
radicals and other oxidizing species capable of wide range of biological injury (Winterbourn 1995). Consequently, plants have developed tightly regulated homeostatic mechanisms regulating iron acquisition from soil, and its movement between various plant parts (Grusak et al. 1999).

1.3.2 Iron uptake from the soil

Iron bioavailability to plants, rather than its abundance in the soil is the bottleneck in iron uptake. Plants have developed two distinct strategies to overcome poor availability of iron: a reduction based strategy called Strategy I, and a chelation based, Strategy II (Marschner and Romheld 1994). Strategy I comprises of the use of three coordinately induced steps. The first step involves the release of proton by-proton ATPases into the soil to acidify the rhizosphere, thus increasing the solubility of Fe$^{3+}$. Arabidopsis $H^+\text{ATPase} \, 2$ ($AHA2$), belonging to the $AHA$ gene family, is known to regulate the acidification of soil in response to iron deficiency (Santi and Schmidt 2009). In the second step, NADPH-dependent ferric chelate reductases reduce Fe$^{3+}$ to Fe$^{2+}$. This has been demonstrated in Arabidopsis where FERRIC REDUCTASE OXIDASE 2 (FRO2), a member of the FRO protein family serves this function (Robinson et al. 1999). In the final step, the ZINC-REGULATED TRANSPORTER/IRON-REGULATED TRANSPORTER-RELATED PROTEIN (ZIP) family transporters transfer Fe$^{2+}$ across the epidermal plasma membrane. This has been demonstrated in Arabidopsis with the functioning of the IRON-REGULATED TRANSPORTER 1 (IRT1) protein (Eide et al. 1996; Eng et al. 1998). In addition, proton ATPase activity allows the induction of negative membrane potential, thus driving cation uptake (Palmgren 2001). All the components of the reduction-based strategy are upregulated under iron deficient conditions (Santi and Schmidt 2009).
Strategy II, used by graminaceous plants including rice and wheat, is a chelation-based strategy for iron uptake. Plants are able to directly take up the Fe\(^{3+}\) form of iron by releasing mugineic acid (MA) family phytosiderophores (PS) in the rhizosphere. These PS are hexadentate metal chelators having a high affinity for Fe\(^{3+}\), forming a PS-Fe\(^{3+}\) complex, which is then transported by specific transporters into the root cells (Takagi et al. 1984; Takagi 1976). PS are synthesized by condensation of three S-adenosyl-L-methionine molecules to form the precursor, nicotianamine (NA), in a reaction catalyzed by NICOTIANAMINE SYNTHASE (NAS). NA is then converted to 2'-deoxymugineic acid (DMA) through a reaction catalyzed by NICOTIANAMINE AMINOTRANSFERASE (NAAT) and DEOXYMUGINEIC ACID SYNTHASE (DMAS) (Kobayashi and Nishizawa 2012). In grasses like barley, further hydroxylation of DMA is carried out by two dioxygenases, i.e., IRON DEFICIENCY-SPECIFIC CLONE 2 (IDS2) and 3 (IDS3), to form 3-epihydroxyMA and 3-hydroxyMA (Nakanishi et al. 2000). The release of PS from the root epidermis is carried out by efflux transporters like TRANSPORTER OF MUGINEIC ACID 1 (TOM1) in rice, and HvTOM1 in barley (Nozoye et al. 2011). These PS-Fe\(^{3+}\) complexes are transported into root cells by YELLOW STRIPE 1 (YS1) and YELLOW STRIPE 1-LIKE (YSL) transporters (Curie et al. 2001; Inoue et al. 2009; Lee et al. 2009a). In addition, there are reports of chelation of zinc and cadmium by MAs as well, but they have higher affinity for Fe\(^{3+}\) (Meda et al. 2007). Both NA and PS are also important for the translocation of metal nutrients within the plants (Kobayashi and Nishizawa 2012).

### 1.3.3 Iron translocation within the plant

Iron translocation involves both long and short distance transport. Short distance transport constitutes transport across the root tissue; xylem loading, transport, and unloading; xylem to
phloem transfer. Long distance transport consists of phloem loading, transport, and unloading; symplastic movement toward the sink; and retranslocation from source or senescing tissue (Kim and Guerinot 2007). In roots, iron movement in the root symplast also requires chelation with NA, several organic acids and amino acids so as to avoid precipitation and generation of oxygen radicals (Hell and Stephan 2003). Symplast loading of xylem vessels requires an iron efflux transporter, FERROPORTIN (FPN). FPN was initially identified in mammalian systems and functions both for iron absorption in the intestine and iron recycling in macrophages (Muckenthaler et al. 2008). Subsequently, a homolog in Arabidopsis, FPN1 / IRON REGULATED1 (IREG1) was identified and shown to regulate iron transport (Morrissey et al. 2009). In addition, a tri-Fe$^{3+}$ tri-citrate complex has been discovered to be involved in chelating and trafficking iron into the xylem sap in tomato (Rellan-Alvarez et al. 2008). A transporter from the multidrug and toxin efflux (MATE) family, FERRIC REDUCTASE DEFECTIVE 3 (FRD3), was identified in Arabidopsis for facilitating the citrate efflux in to the xylem (Durrett et al. 2007; Rogers and Guerinot 2002). Furthermore TOM1, IRT, and FRO have also been suggested to be involved in translocation of iron in the xylem (Ishimaru et al. 2006; Mukherjee et al. 2006; Nozoye et al. 2011). In addition, Fe-NA complexes transported by YSL2 are also known to transport iron from the xylem to neighboring cells (Schaaf et al. 2005). Increasing the level of solubility in the stele of the plant also plays an important role in iron translocation. Ishimaru et al. (2011) discovered a novel effluxer named after its mutant phenotype PHENOLICS EFFLUX ZERO 1 (PEZ1), which releases protocatechuic acid (PCA) and caffeic acid in the xylem sap, facilitating the uptake of precipitated apoplastic iron.

For long distance translocation of iron to sink tissues, transport from the xylem to phloem is required. This transport can occur in the roots as well as basal part of the shoots during remobilization. Oligopeptide TRANSPORTER 3 (OPT3) has been shown to facilitate
this transport from xylem to phloem in Arabidopsis (Zhai et al. 2014). Moreover, for long distance transport, NA, DMA, and YSL family transporters may also play a role in iron transport in the phloem (von Wiren et al. 1999). In fact, YSL2 is reported to be involved in the lateral movement of metals in the vascular tissue (DiDonato et al. 2004).

1.3.4 Intracellular iron transport and storage

Plants regulate iron homeostasis at the cellular level to tackle iron deficient and toxic conditions. The over accumulation of iron is managed by transporting excessive iron to suitable organelles for its utilization in various cellular functions. The vacuole is the key organelle involved in the sequestration of iron and other metals. In Arabidopsis seeds, VACUOLAR IRON TRANSPORTER 1 (VIT1) has been demonstrated to be involved in the import of iron from the cytosol into the vacuole (Kim et al. 2006). ZINC-INDUCED FACILITATOR 1 (ZIF1), a member of the MATE family, and IREG2/FPN2 also enable the transfer of iron to vacuole (Haydon et al. 2012; Morrissey et al. 2009). Since the vacuole acts as a storage unit for various metals, transporters capable of transporting metals back into the cytosol are predicted. For example, members of the NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN (NRAMP) family are known iron transporters located in the vacuoler membrane. In Arabidopsis, AtNRAMP3 and AtNRAMP4 are known to facilitate retrieval of iron from vacuole into the cytosol (Lanquar et al. 2005).

Iron can catalyze the formation of oxygen free radicals when present in free form and in excess through the Fenton reaction (Winterbourn 1995). Thus, for iron storage in a non-toxic and bioavailable form, the iron storage protein FERRITIN is required in plants. The ferritin complex has a molecular weight of 480 kDa, and is capable of storing up to 4,500 ferric molecules. Each ferritin complex is made up of 24 individual FERRITIN protein subunits
arranged to form a hollow structure (Harrison and Arosio 1996; Liu and Theil 2005). Two FERRITIN isoforms have been reported in rice and wheat: FER1, FER2 (Borg et al. 2012; Stein et al. 2009). It has also been shown that iron from plant ferritin is as bioavailable to mammals as that obtained from animal ferritin. Another study with human subjects showed that iron from purified soybean ferritin was as well absorbed as ferrous iron (Lonnerdal 2007).

1.4 Molecular mechanisms involved in the uptake and transport of zinc in plants

1.4.1 Importance of zinc in plant growth

Zinc, an essential micronutrient, exists in the Zn$^{+2}$ oxidation state and is redox-stable under physiological conditions. It is an essential component of several enzymes and has been found in each of the six classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Auld 2001; Barak and Helmke 1993). Structural, catalytic, and cocatalytic sites are the three primary Zn$^{2+}$-ligand binding sites. Structural zinc sites ensure appropriate protein folding, while in catalytic sites, zinc is directly involved in the catalytic function of the enzyme. Zinc in cocatalytic sites can be used for catalytic, regulatory and structural functions (Auld 2001). Protein, membrane lipids and DNA/RNA molecules are also known to contain zinc-binding sites. Zinc finger domain containing proteins are the largest class of zinc binding proteins and help in the regulation of transcription by modifying DNA/RNA binding, site specific modification and the regulation of chromatin structure (Englbrecht et al. 2004). Since both deficient and excessive zinc conditions also affect plant metabolism, plants have a tightly controlled metal homeostasis network for adjusting to zinc fluctuations.
1.4.2 Zinc uptake, transport and intracellular translocation

Primary availability of zinc in soil is from the chemical and physical weathering of parent rocks. Transport of bioavailable zinc across the plasma membrane is the preliminary step for its uptake and accumulation in plants. Plants acquire zinc from the soil, primarily as Zn$^{2+}$ and/or with organic ligands (Broadley et al. 2007). One of the processes involved in cation uptake is negative membrane potential, which is maintained by H$^+$-ATPase (Palmgren 2001). In addition, metal transporters of the ZIP family are thought to play a role in the transfer of zinc into the root (Grotz et al. 1998; Guerinot 2000). For example, IRT1 and IRT2 are two ZIP family transporters contributing in the uptake of Zn$^{2+}$ and Cd$^{2+}$ by plants (Korshunova et al. 1999; Vert et al. 2001; Vert et al. 2002). In graminaceous plants, YSL transporters play a key role in the transport of zinc bound with MAs and NA. (Schaaf et al. 2005; vonWiren et al. 1996). MAs are also involved in the chelation and uptake of zinc through the formation of MAs-Zn$^{2+}$ complexes (Suzuki et al. 2006; Suzuki et al. 2008).

Metals present in excess in the cytosol are sequestered in the vacuoles of plant cells. METAL TOLERANCE PROTEIN 1 (MTP1) and MTP3 transport excess zinc from the cytosol into the vacuole, thus inhibiting its transfer to the shoot in Arabidopsis (Arrivault et al. 2006; Desbrosses-Fonrouge et al. 2005; Kobae et al. 2004). Another vacuolar influx transporter identified in Arabidopsis, ZINC INDUCED FACILITATOR 1 (ZIF1) is involved in the transport of zinc ligands or a zinc ligand complex into the vacuole (Haydon and Cobbett 2007a). Moreover, in Arabidopsis HEAVY METAL TRANSPORTING ATPASE 2 (HMA2) and HMA4, translocate zinc across the plasma membrane of root vascular cells into the xylem for transport to the shoot (Hussain et al. 2004).
1.5 Carotenoid biosynthesis

Carotenoids are pigments synthesized in all photosynthetic organisms, and geranylgeranyl diphosphate (GGDP) is the precursor molecule required for the synthesis of β-carotene. PHYTOENE SYNTTHASE (PSY) condenses two molecules of GGDP to produce phytoene. Further, the conversion of phytoene to β-carotene requires three more enzymatic steps in plants involving PHYTOENE DESATURASE (PDS), ζ-CAROTENE DESATURASE (ZDS) and Lycopene β-CYCLASE (LCY) (Burkhardt et al. 1997). While endosperm of rice grains does not synthesize β-carotene, the immature rice endosperm is known to produce GGDP. In fact, this is attributed to a very low expression of endogenous genes encoding for PSY, PDS, ZDS, and LCY (Figure 1)(Schaub et al. 2005).

![Carotenoid biosynthetic pathway in plants](image)

**Figure 1 The carotenoid biosynthetic pathway in plants.** Geranylgeranyl diphosphate is the precursor molecule that is synthesized in the rice grain immature endosperm. Phytoene synthase, phytoene desaturase and ζ-CAROTENE DESATURASE are the additional enzymes required for the synthesis of β-carotene.
1.6 Iron biofortification using gene technology in cereals

One strategy to biofortify plants with iron is to engineer iron storage in the endosperm. *FERRITIN*, under the control of an endosperm-specific promoter increased iron content in polished rice by 2- to 3.7-fold (Goto et al. 1999; Lucca et al. 2001b; Oliva et al. 2014; Qu et al. 2005; Vasconcelos et al. 2003). Similarly, constitutive expression of soybean *FERRITIN* (*GmFERRITIN*) increased iron content in the leaves of wheat (Drakakaki et al. 2000). Borg et al. (2012) overexpressed endogenous *FERRITIN* (*TaFERRITIN1-A*) under the control of it’s native endosperm specific promoter and reported an increase in iron content of 50-80% in wheat grains. Previous reports also showed that endosperm-specific expression of *FERRITIN*, led to increased zinc content in the grains of both wheat and rice (Borg et al. 2012; Vasconcelos et al. 2003).

Increasing iron and zinc uptake from the soil is another important strategy to biofortify plants. The over-expression of the iron transporter *OsIRT1* in rice increased the iron and zinc concentration in shoots, roots and mature seeds (Lee and An 2009). The over-expression of OsNAS3, a key enzyme in the synthesis of PS, increased iron and zinc content by 2.9-fold and 2.2-fold, respectively in activation-tagged mutant rice lines (Lee et al. 2009b). Similarly, constitutive overexpression of the *HvNAS1* gene in rice led to a 3-fold increase in the amount of iron in the rice endosperm (Masuda et al. 2009). Likewise, constitutive overexpression of *OsNAS2* increased the iron content in polished rice by 2- to 4-fold (Johnson et al. 2011; Lee et al. 2012). In addition, over-expression *OsYSL2* (a metal-NA transporter) under the regulation of the *SUCROSE TRANSPORTER 1* (*OsSUT1*) promoter increased iron content by 4.4-fold in polished rice grains (Ishimaru et al. 2010). With the over-expression of *HvYS1*, no change was observed in the iron content of seeds of transgenic rice plants, but increased plant growth and iron accumulation in leaves was reported, when grown on alkaline soils.
OsVIT1 and OsVIT2 knockdown in rice showed an increase in iron content of up to 1.8-fold in the polished grains (Bashir et al. 2013; Zhang et al. 2012). As summarized above, single gene strategies have shown promising results but have failed to increase endosperm iron content to the required dietary level. In rice, a combination of both FERRITIN and NAS over-expression has been deployed that led to synergistic increases in grain iron content. Endosperm-specific expression of bean PvfERRITIN and constitutive expression of Arabidopsis AtNAS1 from a single construct increased iron content in the rice endosperm by 6-fold and zinc content by 1.3-fold (Wirth et al. 2009). Later on, various other studies focused on the use of combinations of genes responsible for iron storage, iron uptake and translocation. In one study, endosperm-specific overexpression of soybean FERRITIN (GmFERRITIN), HvNAS, and OsYSL2 increased iron content by 6-fold in greenhouse grown rice plants and by 4.4-fold in the field grown plants (Masuda et al. 2012). The combination of GmFERRITIN, NAS, as well as NAAT and IDS3 from barley, increased iron content by 3.6-fold in the polished rice grains (Masuda et al. 2013). A recent study showed that the co-expression of AtIRT1, AtNAS1 and PvfFERRITIN synergistically increased iron in both polished and unpolished rice grains (Boonyaves et al. 2016). More recently, rice lines expressing GmFERRITIN and OsNAS2 are reported to contain iron levels sufficient to meet 30% of the estimated average requirement (EAR) as recommended by HarvestPlus (Trijatmiko et al. 2016).

1.7 Vitamin A biofortification of the rice endosperm

β-carotene synthesis requires PSY and three additional enzymes in plants: PHYTOENE DESATURASE, ζ-CAROTENE DESATURASE and LYCOPENE β-CYCLASE (Burkhardt et al. 1997). However, it was found that CAROTENE DESATURASE (CRTI) of Pantoea
ananatis (formerly *Erwinina uredovora*) is able to perform the function of both desturases (Ye et al. 2000). The engineering of the β-carotene biosynthetic pathway in the rice endosperm was demonstrated by Ingo Potrykus and colleagues with development of Golden Rice. Endosperm-specific overexpression of *PSY* from daffodil, and the constitutive expression of *PaCRTI* removed the barriers for carotenoid synthesis in the rice endosperm (Ye et al. 2000). This established the proof of concept for the synthesis of β-carotene, and Golden Rice has since been improved further. Paine et al. (2005) overexpressed maize *PSY* (*ZmPSY*), and *PaCRTI*, and reported 23-fold increase of carotene in rice endosperm as compared with the previous iteration. The biofortification of the rice endosperm with β-carotene was a major milestone in combating vitamin A deficiency.

1.8 Aim of the PhD thesis

My thesis is aimed at biofortification of two important cereals, wheat and rice. There are two main objectives, 1) biofortification of wheat, and 2) production of multi trait rice. In rice, combination of both *FERRITIN* and *NAS* has been deployed to synergistically increase iron content in rice endosperm (Wirth et al. 2009). Therefore, for iron biofortification of wheat we deployed *OsNAS2* and *PvFERRITIN* individually, as well as in combination. Our second aim was to develop a rice cultivar for improved iron content and β-carotene content. Infact, both high iron trait and vitamin A traits have been shown to work individually in other studies (Paine et al. 2005; Wirth et al. 2009). Therefore, we expressed combination of *AtNAS1*, *PvFERRITIN*, *ZmPSY*, and *PaCRTI* to increase iron content and install β-carotene in rice endosperm. Hence, the PhD study was conducted with following objectives:

1. To test if the iron content in wheat grains can be increased by over-expression of *PvFERRITIN* and *OsNAS2* individually, or in combination.
2. To test if the combination of *AtNAS1*, *PvFERRITIN*, *ZmPSY*, and *PaCRT1* can increase iron content and install β-carotene pathway in rice endosperm, and also to determine if these traits can express synergistically in transformed rice.
Rice *NICOTIANAMINE SYNTHASE 2* overexpression improves dietary iron and zinc levels in wheat

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Published *Theoretical and Applied Genetics* 2016

(doi: 10.1007/s00122-016-2808-x)
2.1 Abstract

Micronutrient deficiencies, including iron and zinc deficiencies, have negative impacts on human health globally. Iron-deficiency anemia affects nearly two billion people worldwide and is the cause of reduced cognitive development, fatigue and overall low productivity. Similarly, zinc deficiency causes stunted growth, decreased immunity and increased risk of respiratory infections. Biofortification of staple crops is a sustainable and effective approach to reduce the burden of health problems associated with micronutrient deficiencies. Here, we developed wheat lines expressing rice NICOTIANAMINE SYNTHASE 2 (OsNAS2) and bean FERRITIN (PvFERRITIN) as single genes as well as in combination. NAS catalyzes the biosynthesis of nicotianamine (NA), which is a precursor of the iron chelator deoxymugeneic acid (DMA) required for long distance iron translocation. FERRITIN is important for iron storage in plants because it can store up to 4500 iron ions. We obtained significant increases of iron and zinc content in wheat grains of plants expressing either OsNAS2 or PvFERRITIN, or both genes. In particular, wheat lines expressing OsNAS2 greatly surpass the HarvestPlus recommended target level of 30% dietary estimated average requirement (EAR) for iron, and 40% of EAR for zinc, with lines containing 93.1 µg/g of iron and 140.6 µg/g of zinc in the grains. These wheat lines with dietary significant levels of iron and zinc represent useful germplasm for breeding new wheat varieties that can reduce micronutrient deficiencies in affected populations.
2.2 Introduction

Micronutrients are essential in the human diet because they are required for key metabolic reactions and biological functions. A large fraction of the global human population suffers from micronutrient deficiencies, which has a negative impact on well-being and economic development (Muthayya et al. 2013). The most common deficiencies include iron, zinc, iodine, vitamin A, vitamin B-12, riboflavin, vitamin D, and vitamin E deficiencies (Allen et al. 2009). Iron and zinc deficiencies are among the most widespread micronutrient deficiencies. Iron deficiency is the leading cause of iron-deficiency anemia (IDA), which is prevalent worldwide in both developed and developing countries (WHO 2016b). IDA affects cognitive development, decreases immune function, and causes higher mortality of mothers and children at birth. Children as well as pregnant and non-pregnant women are at a higher risk with approximately 43%, 38% and 29% of the population, respectively, affected with IDA (Stevens et al. 2013). Zinc deficiency is associated with diseases such as diarrhea, pneumonia and malaria (WHO 2002). The main clinical symptoms include growth retardation, cell-mediated immune dysfunction and cognitive impairment (Prasad 2014). Iron and zinc deficiencies are often associated with low micronutrient content in staple foods, poor micronutrient absorption from food, and dependence of the affected populations on simple and monotonous diets.

The three recommended approaches to overcome micronutrient deficiencies include supplementation, food fortification and biofortification. Micronutrient supplementation, which has been implemented in different parts of the world, is not always successful. For example, a nutritional anemia control program was implemented in India in 1970 but had only limited impact mainly because of logistical problems, poor compliance, and lack of sufficient funds (Mayer et al. 2008). This suggests that supplementation is not a sustainable
preventive solution, but rather a short-term curative measure. Food fortification with iron is difficult because iron compounds such as FeSO₄ cause food to have a generally unacceptable flavor and color, thus making it unpalatable (Abbaspour et al. 2014; Hurrell 2002). Biofortification of staple crops, i.e., enhancing the nutritional content of the edible parts, offers the most sustainable method to overcome micronutrient deficiencies. Wheat (*Triticum aestivum*) is grown on 219 million hectares (*mha*) with a production of over 715 metric tonnes (MT) and is one of the most widely grown and consumed cereals globally (FAOSTAT 2013). Several billion people around the world depend on wheat for protein, starch, fiber, and various essential micronutrients (Shewry 2009). However, most of the bread wheat varieties do not contain sufficient iron and zinc in the grains to meet the daily dietary recommendations for these nutrients (Zhao et al. 2009). Therefore, biofortification of wheat for iron and zinc can greatly benefit global human health. According to the HarvestPlus program initiated by the Consultative Group for International Agricultural Research (CGIAR), 59 µg/g (dry weight) of iron and 38 µg/g (dry weight) of zinc is required in the wheat grains in order to meet 30% and 40% of the estimated average requirement (EAR) of an adult diet, respectively (Bouis et al. 2011). To date, conventional breeding has achieved little or no success in enhancing the iron content of wheat. Screening of wheat germplasm showed a negative correlation between grain yield and micronutrient content (Amiri et al. 2015; Zhao et al. 2009), which might be the consequence of plant breeding focusing mainly on agronomic yield than nutritional quality in the past. Therefore, increasing grain iron and zinc content in wheat while maintaining yield has been difficult to achieve via conventional breeding. Biofortification utilizing gene technology offers a multi-dimensional approach to enrich required micronutrients specifically in the grains without altering overall yield (Bhullar and Gruissem 2013).
Iron is important for both plants and animals. However, excessive iron accumulation is toxic to cells, and iron homeostasis is tightly regulated in animals and plants. Thus, a careful choice of strategies is required in order to increase iron content in cereal grains (Grusak et al. 1999). Cereals, including rice and wheat, use a chelation-based strategy (also known as Strategy II) for iron uptake (Marschner and Romheld 1994). Mugineic acid family phytosiderophores (PS) are released into the soil where they chelate the ferric form of iron, forming a PS-Fe$^{3+}$ complex, which is subsequently transported into the roots by specific transporters. The PS are synthesized from S-adenosyl-L-methionine via a conserved pathway of reactions catalyzed by nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS) (Kobayashi and Nishizawa 2012).

Based on this knowledge various single gene and multigene strategies have been developed in rice to improve iron content in the endosperm (Bhullar and Gruissem 2013). Masuda et al. (2009) showed that constitutive overexpression of the HvNAS1 gene in rice resulted in a 3-fold increase of iron in the rice endosperm. Similarly, constitutive overexpression of OsNAS2 increased iron content in polished rice by 2- to 4-fold (Johnson et al. 2011; Lee et al. 2012). In addition to iron, the zinc content was also increased in polished grains of NAS overexpressing lines (Johnson et al. 2011; Wirth et al. 2009). PS can also bind Zn$^{2+}$ and form PS-Zn$^{2+}$ complexes, thus explaining the increased zinc content that is associated with increased production of PS by overexpression of NAS (Schaaf et al. 2004). Transformation of cereals with FERRITIN to increase the iron content has also been reported (Borg et al. 2012; Vasconcelos et al. 2003). FERRITIN is a complex of 24 protein subunits arranged to form a hollow structure that can store up to 4,500 ferric molecules (Harrison and Arosio 1996). In rice, FERRITIN expressed under the control of an endosperm-specific promoter increased iron content in polished rice grains by 2- to 3.7-fold (Goto et al. 1999; Lucca et al. 2001b; Oliva et al. 2014; Qu et al. 2005; Vasconcelos et al. 2003). Moreover, constitutive expression
of soybean FERRITIN (GmFERRITIN) increased iron content in the leaves of wheat (Drakakaki et al. 2000). Borg et al. (2012) overexpressed endogenous wheat FERRITIN (TaFERRITIN1-A) under the control of the native endosperm-specific promoter of TaFERRITIN1-A, and reported an increase of 50-80% of iron content in wheat grains. In rice, expression of both FERRITIN and NAS synergistically increases the grain iron content further. Endosperm-specific expression of bean PvFERRITIN and constitutive expression of Arabidopsis AtNAS1 from a single construct increased iron content in rice endosperm by 6-fold (Wirth et al. 2009).

To achieve the recommended level of iron in wheat grains for healthy human diets (i.e minimum 59 µg/g iron/dry weight; Bouis et al. 2011) it is necessary increase iron content by 100% in the major wheat cultivars. We have engineered bread wheat with a bean FERRITIN (PvFERRITIN) gene expressed under the control of the endosperm-specific rice GLOBULIN (OsGLOBULIN) promoter and the rice NAS2 (OsNAS2) gene expressed under the control of constitutive-promoter maize UBIQUITIN (ZmUBIQUITIN) as single gene constructs and as a combination of both genes. Expression of PvFERRITIN or OsNAS2 alone as well as in combination significantly increased iron and zinc levels in wheat grains, with several lines surpassing the recommended target levels for both iron and zinc content.

2.3 Materials and methods

2.3.1 Transformation vectors

PvFERRITIN (X58274) under the control of the rice endosperm-specific OsGLOBULIN promoter (Wirth et al. 2009) was excised from the parent plasmid using XbaI and PstI and was used for transformation of wheat. For generation of the construct combining both PvFERRITIN and OsNAS2, we incorporated KpnI restriction site upstream of the
OsGLOBULIN promoter and SmaI restriction site at 3’ of nopaline synthase (NOS) gene terminator (nosT) in the PvFERRITIN construct, and cloned it to the Pjet1.2 vector, generating PvFERRITIN-Pjet1.2 plasmid. The rice OsNAS2 (LOC_Os03g19420.2) gene was commercially synthesized along with nosT from GenScript® (http://www.genscript.com). SpeI, HindIII, and BamHI restriction sites were inserted upstream of OsNAS2, and SmaI was inserted at 3’end, and cloned into the puc57 vector, generating the OsNAS2-puc57 construct. The ZmUBIQUITIN promoter from pAHC17 (Christensen and Quail 1996) was excised using HindIII and BamHI, and was inserted into the OsNAS2-puc57 construct. The entire OsNAS2 cassette was then excised using SpeI and SmaI restriction sites, and was inserted to the PBSKII(-) vector, generating OsNAS2-PbskII(-). Similarly, the PvFERRITIN cassette was excised from the PvFERRITIN-Pjet1.2 construct using KpnI and SmaI, and cloned into the OsNAS2-PbskII(-) construct, finally generating PvFERRITIN-OsNAS2. For transformation, vector backbone-free gene cassettes were co-transformed along with the PHOSPHOMANNOSE ISOMERASE (PMI) selectable marker gene expressed under the control of the ZmUBIQUITIN promoter (Fig. 1) (Brunner et al. 2011).
2.3.2 Wheat transformation, plant growth and characterization

Hexaploid spring wheat cultivar Bobwhite SH 98 26 was transformed using particle gun bombardment (Brunner et al. 2011; Pellegrineschi et al. 2002). Overall, approximately 6500 immature embryos were co-transformed with PMI and PvFERRITIN, PMI and OsNAS2, or PMI and PvFERRITIN-OsNAS2 generating transformed lines named PvFERRITIN-Bobwhite (hereafter Fer-BW), OsNAS2-Bobwhite (hereafter Nas-BW), and PvFERRITIN-OsNAS2-Bobwhite (hereafter FerNas-BW), respectively. Selection of the transformed plants was performed on culture media containing mannose during the regeneration phase (Wright et al. 2001). Plants were later grown in commercial soil (Klasmann-Deilmann GmbH, Germany) in the greenhouse in 16 h light/22°C and 8 h dark/18°C, and a humidity of 60%. Genomic
DNA was isolated from three-week-old seedlings in the T0-, T1-, T2- and T3-generation (Stein et al. 2001; Vasudevan et al. 2014). PCR was used to confirm the presence of the transgenes using gene specific primers (Table. S1). PCR-positive lines were further selected based on the iron content in the subsequent generations. Southern blot analysis was performed using a P$^{32}$-labelled probe to select lines with a single copy insert in T0-generation plants (Green and Sambrook 2012). The probes for *PvFERRITIN*, *OsNAS2*, and *PMI* were generated using gene specific primers (Table. S1). For phenotypic characterization of transformed wheat lines, parameters including days to flowering (DTF), plant height, 1000 grain weight (1000 GW), and tiller number were recorded.

2.3.3 Metal ion measurements

Plants were harvested six weeks after flowering and spikes were dried at 37°C for 3 days. Grain samples were de-husked and grounded for metal ion measurements. Additionally, ground grain samples were fractioned using a 250 μm nylon sieve to obtain sieved flour (Borg et al. 2012), referred to as ‘flour’ hereafter. Two hundred mg of sample was boiled in 15 ml of 65% v/v HNO$_3$ solution at 120°C for 90 min. Three ml of 30% v/v H$_2$O$_2$ was added and boiled at 120°C for 90 min. Metal concentrations were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Varian Vista-MPX CCD Simultaneous ICP-OES). The wavelength used for iron, zinc, copper, manganese, and magnesium was 238.204, 213.857, 324.754, 257.610, and 285.213, respectively. The iron concentrations were recorded in T2, T3 and T4 grains, and the seeds from plants with highest iron concentration were used to grow the next generation of plants (Fig. S2). Data were analyzed using the Student’s t-test to determine statistically significant differences among the transformed lines and their respective controls.
2.3.4 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to assess the expression of the transgenes in the transgenic lines. Total RNA was extracted from leaves and grains collected 18 days after flowering (DAF) in the T3 generation plants. Leaf RNA was extracted using Trizol® reagent (Invitrogen, U.S.A.), and the RNA was treated with DNase I (Thermo Fisher Scientific, Inc., U.S.A.) to remove genomic DNA contamination. cDNA synthesis was done using the RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., U.S.A.). qRT-PCR was performed on the 7500 FAST Real Time PCR system (Applied Biosystems, Inc., U.S.A.). The total reaction volume of 20 µl included 1 µl cDNA, 0.4 µl forward primer, 0.4 µl reverse primer, 10 µl Sybrgreen Mastermix (Applied Biosystems, Ltd., U.S.A.), and 8.2 µl H2O. Primers were designed using a CLC Genomics Workbench (Table S1). For data normalization, Ta.22845 was used as reference gene that encodes for ATP-dependent 26S proteasome regulatory subunit (Paolacci et al. 2009), and it expresses both in the flag leaves as well as the grains of wheat (own unpublished data). The Ct value was obtained from 7500 Fast System Software. The data normalization was done as described by Liu et al. (2011).

2.4 Results

2.4.1 Seed specific expression of PvFERRITIN and constitutive expression of OsNAS2 in wheat

Single insertion Fer-BW, Nas-BW, and FerNas-BW transgenic lines (Fig. 1, Fig. S1) were analysed for transgene expression in grains and leaves. PvFERRITIN was specifically expressed in the grains of all Fer-BW and FerNas-BW lines, except for two lines, Fer-BW 51 and FerNas-BW 30, which also showed expression in leaves (Fig. 2a and 2e, Fig. S3). These
results confirm the seed-specific expression of *PvFERRITIN* under the control of the rice *OsGLOBULIN* promoter in most wheat lines. *OsNAS2*-specific primers (Table S1) were used to analyse the expression of the transgene in Nas-BW leaves and both leaves and grains of FerNas-BW lines (Fig. 2b and 2d). All Nas-BW lines showed *OsNAS2* expression in the leaves. In FerNas-BW lines *OsNAS2* expression was also detected in grains, except for FerNas-BW 17. However, *OsNAS2* expression was manyfold higher in leaves than grains, indicating that the *ZmUBIQUITIN* promoter is not very active in wheat endosperm.
Fig. 2 Relative expression of transgenes *PvFERRITIN* and *OsNAS2* at 18 d after flowering (DAF) in T3-generations. a) Relative expression of *PvFERRITIN* in grains and leaves of Fer-BW lines. b) Relative expression of *OsNAS2* in leaves of Nas-BW lines. c) Relative expression of *PvFERRITIN* in FerNas-BW lines. d) Relative expression of *OsNAS2* in FerNas-BW lines. No expression of *PvFERRITIN* and *OsNAS2* was observed in Bobwhite (BW) and non transgenic sibling (NTS) controls. The data were normalized to the endogenous expression of *Ta.22845*. Values are the average of three biological replicates (±SD).
2.4.2 Expression of *PvFERRITIN* increases iron and zinc content in both whole grains and sieved flour

The single insertion Fer-BW lines 18, 47, 51, 62, and 69 were analysed for their metal content. Iron content was significantly increased in all lines except Fer-BW 69. Iron content in the T4 grains of these lines ranged between 42.0 and 61.9 µg/g DW, as compared to 38.5 µg/g DW in Bobwhite (Fig. 3). Fer-BW 51 had the highest iron content (61.9 µg/g) with an increase of 1.6-fold as compared to the control grains. The iron content in the flour (the endosperm of the grains) of Fer-BW transgenic lines ranged from 20.2 to 33.9 µg/g DW compared to 20.4 µg/g DW in Bobwhite. Flour from grains of line 47 had the highest iron content, with a 1.7-fold increase (33.6 µg/g) as compared to the control (Fig. 3). In addition, whole grains of lines 18, 47, 51 and 62 had significantly increased zinc content ranging from 59.1 to 72.6 µg/g DW as compared to 43.1 µg/g DW in Bobwhite (Fig. 3, Fig. S4). Zinc content in the flour ranged from 30.7 to 35.3 µg/g DW in the transformed lines as compared to 23.0 µg/g DW in the control. Line Fer-BW 47, which contained the highest iron content in the flour, also contained the highest zinc content in whole grains (72.6 µg/g) as well as in flour (35.3 µg/g), representing 1.7-fold and 1.5-fold increases, respectively, as compared to the control. In addition, copper, manganese, and magnesium levels were also measured in whole grains and flour. Copper was increased in grains of four of the Fer-BW lines, except for line 69 (Fig. S5), and ranged from 6.9 to 8.3 µg/g DW compared to 6.5 µg/g DW in the control line. A similar trend was observed in the copper content of flour which ranged from 4.8 to 5.7 µg/g DW in the transformed lines, compared to 3.91 µg/g DW in Bobwhite. With few exceptions, manganese and magnesium content was not altered in the transformed lines (Fig. S5). Phenotypic greenhouse performance of the Fer-BW lines were similar to Bobwhite.
for plant height and 1000 GW, while they showed some variation for days to flowering (DTF) and tiller number (Table. S2).

Fig. 3 Iron (Fe) and zinc (Zn) content in T4 grains and flour of lines expressing *PvFERRITIN* (Fer-BW). Values are the mean of three biological replicates (±SD). Black asterisks above the bars indicate statistically significant values calculated using Student’s T test, in comparison to the control line Bobwhite (BW) (*P < 0.05; **P < 0.01). NTS, non-transgenic sibling.
2.4.3 OsNAS2 overexpression increases iron and zinc content to dietary significant levels

Ten single insertion Nas-BW lines expressing rice OsNAS2 had significant increases in iron and zinc content in comparison to Bobwhite (Fig. 4). The iron content in T4 whole grains ranged from 59.6 to 93.1 µg/g DW, as compared to 42.7 µg/g DW in the control. Moreover, iron content in the flour of these lines ranged from 30.1 to 53.3 µg/g DW, as compared to 21.4 µg/g DW in the control. Line 65 had the greatest increase in iron (2.1-fold) in whole grains and a 2.5-fold increase in the flour as compared to Bobwhite. The Nas-BW lines had relatively higher iron content in comparison to Fer-BW lines and all of these lines also surpass the suggested target levels of 59 µg/g DW iron in whole grains to meet the 30% estimated average requirement of human diets. The iron increases in the NAS-BW lines correlated well with the transgene expression (Fig. 2) in these plants, with line 65 having the highest OsNAS2 expression in leaves.

The zinc content in whole grains of the transformed lines ranged from 54.2 to 140.6 µg/g DW and in the flour from 31.9 to 94.1 µg/g DW, as compared to 37.9 µg/g DW and 22.4 µg/g DW in Bobwhite, respectively (Fig. 4). Line 40 had the greatest zinc levels with a 3.7-fold increase (140.6 µg/g) in whole grains and a 4.2-fold increase (94.1 µg/g) in flour, as compared to control. Increases in copper, magnesium, and manganese content were also measured in most of the transformed lines (Fig. S6). All lines had significantly higher copper content in whole grains as well as flour. The manganese content also increased significantly in whole grains and flour in most of the lines except in whole grains of lines 38, 53, 66 and 81 and flour of line 66. As an exception, line 91 showed significantly lower manganese content in both whole grains and flour. The magnesium content in whole grains as well as flour was significant increased except in whole grains of lines 42 and 66 and flour of line 91.
Importantly, most of the Nas-BW lines were phenotypically indistinguishable from Bobwhite for DTF, plant height, 1000 GW, and tiller number (Table 1). Lines 40 and 91 flowered earlier, and 1000 GW of lines 40 and 42 was significantly increased but decreased in line 81 as compared with Bobwhite.

**Fig. 4** Iron (Fe) and zinc (Zn) content in T4 grains and flour of lines expressing OsNAS2 (Nas-BW). Values are the mean of three biological replicates (±SD). Black asterisks above the bars indicate statistically significant values calculated using Student’s T test, in comparison to the control line Bobwhite (BW) (*P < 0.05; **P < 0.01). NTS, non-transgenic sibling
Table 1  Phenotypic performance of greenhouse-grown T3 generation Nas-BW transgenic lines

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Days to flowering</th>
<th>Height (cm)</th>
<th>1000 GW(g)</th>
<th>Tiller</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>99.3±2.9</td>
<td>53.7±4</td>
<td>30.1±3</td>
<td>15.7±1.5</td>
</tr>
<tr>
<td>38</td>
<td>105.3±6.4</td>
<td>58.5±4.9</td>
<td>23.3±3.2</td>
<td>17±1.7</td>
</tr>
<tr>
<td>40</td>
<td>92±3.5,**</td>
<td>36.3±2.8</td>
<td>34.6±1.7,*</td>
<td>20.7±3.1</td>
</tr>
<tr>
<td>42</td>
<td>97.7±3.5</td>
<td>49.1±1.6</td>
<td>37.8±1.8,**</td>
<td>20.7±6.1</td>
</tr>
<tr>
<td>49</td>
<td>107.3±5</td>
<td>54.8±1.5</td>
<td>29.6±3.7</td>
<td>20.3±1.2</td>
</tr>
<tr>
<td>53</td>
<td>106.3±4.7</td>
<td>58.9±10.3</td>
<td>24.8±4.1</td>
<td>18.7±3.5</td>
</tr>
<tr>
<td>65</td>
<td>98.7±5.7</td>
<td>58.5±1.7</td>
<td>27.7±6.7</td>
<td>16±2.6</td>
</tr>
<tr>
<td>66</td>
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<td>55.8±5.3</td>
<td>29±5.9</td>
<td>17±3.5</td>
</tr>
<tr>
<td>81</td>
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<td>52.8±4.4</td>
<td>18.8±3,**</td>
<td>16±2.6</td>
</tr>
<tr>
<td>91</td>
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<td>47.2±2.3</td>
<td>29.9±3.9</td>
<td>15.3±4</td>
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<td>50.3±2.7</td>
<td>31.5±0.5</td>
<td>14.7±5.5</td>
</tr>
<tr>
<td>BW</td>
<td>101.7±0.5</td>
<td>49.6±1.9</td>
<td>29.2±1.9</td>
<td>16±3</td>
</tr>
</tbody>
</table>

Values are the average of three biological replicates (± standard deviation). Transgenic plants were compared to Bobwhite (BW). Black and red asterisks indicate statistically higher and lower significant values calculated using Student’s T test, respectively (*P < 0.05; **P < 0.01)

2.4.4  Iron and zinc levels in lines expressing both *PvFERRITIN* and *OsNAS2*

Wheat lines expressing *PvFERRITIN* and *OsNAS2* from a single construct (FerNas-BW lines) were analysed to determine if the transgenes act synergistically leading to higher iron and zinc increases in comparison to plants expressing single genes (Fer-BW and Nas-BW lines). The iron content in whole grains of five single insertion FerNas-BW lines ranged from 35.6 to 60.3 µg/g DW, as compared to 42.7 µg/g DW in Bobwhite (Fig. 5). The flour of these transgenic lines had iron content in the range of 24.9 to 45.3 µg/g DW, compared to 21.4 µg/g DW in Bobwhite. FerNas-BW lines had a maximum 1.7-fold increased of iron (60.3 µg/g in line 15) in whole grains and of 1.8-fold increased iron in the flour (45.3 µg/g in line 3), as compared to Bobwhite. Among the FerNas-BW lines, line 3 had highest zinc content in whole grains (82.4 µg/g DW) as well as in flour (55.7 µg/g DW) (Fig. 5).
However, in contrary to the expectations, these increases for iron and zinc content are smaller than those observed in plants expressing either \textit{PvFERRITIN} or \textit{OsNAS2} alone. Copper, manganese and magnesium contents were variable among the different FerNas-BW lines (Fig. S7). Lines 3, 15, and 42 showed significantly increased copper and magnesium content in both whole grains as well as the flour. In contrast, lines 17 and 30 had reduced copper content in comparison to the control. Manganese content was also significantly higher in lines 3 and 15 for both the whole grains and the flour, as compared with Bobwhite. FerNas-BW lines were phenotypically similar to Bobwhite for DTF, 1000 GW and tiller number as well as plant height, except for line 42 which was significantly taller (Table S3).
Fig. 5 Iron (Fe) and zinc (Zn) content in T4 grains and flour of lines expressing *PvFERRITIN-OsNAS2* (FerNas-BW). Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant values calculated using Student’s T test, respectively, in comparison to the control line Bobwhite (BW) (*P < 0.05; **P < 0.01). NTS, non-transgenic sibling.

2.5 Discussion

The development of high yielding farmer-preferred wheat cultivars with increased micronutrient content in the grains can improve the well-being of millions of malnourished humans around the globe. While increases for some micronutrients could be achieved via conventional breeding, the improvements for many other micronutrients including iron,
vitamin A and folate are difficult to achieve (Bhullar and Gruissem 2013; Mayer et al. 2008; Slamet-Loedin et al. 2015). In 2015, zinc biofortified wheat varieties were released in India and Pakistan (HarvestPlus 2015a; HarvestPlus 2015b). Zinc fertilizer application has also been suggested as a useful strategy for zinc biofortification of wheat (Cakmak 2008). Similar agronomic practices as well as conventional breeding approaches, however, have not been successful in achieving the recommended increase of iron content in wheat grains. Furthermore, it is difficult to implement targeted fertilizer application strategies, particularly in the developing countries, because it is impractical and expensive. Thus, engineering of wheat lines with high-iron and -zinc grains is a more economical and sustainable solution for micronutrient improvement and human health. As we show here, iron biofortification of wheat using gene technology is a robust method for approaching or achieving the required target levels. So far, only few genetic engineering attempts have been made to biofortify wheat for increased iron content in the grains (Drakakaki et al. 2000; Borg et al. 2012; Sui et al. 2012). We focused on two different strategies using constitutive overexpression of NAS and endosperm-specific expression of FERRITIN either alone or combined. The results we obtained for the Fer-BW lines expressing PvFERRITIN (Fig. 3) are similar to those previously reported (Borg et al. 2012; Sui et al. 2012). The increased zinc content in the Fer-BW lines (Fig. 3) is consistent with previous reports of expressing FERRITIN in both wheat and rice (Borg et al. 2012; Trijatmiko et al. 2016; Vasconcelos et al. 2003; Wirth et al. 2009). In contrast to the Fer-BW lines, the lines overexpressing OsNAS2 had higher iron and zinc increases in the grains, with selected lines surpassing the suggested iron target of 59 µg/g to meet the recommended 30% EAR (Fig. 4). These lines also have higher zinc contents. Most importantly, the high whole grain iron and zinc increases are retained in the flour (Fig. 4), which usually is the part of the grain that is consumed. Overexpression of NAS increases the production of nicotianamine (NA) and deoxymugineic acid (DMA), ultimately facilitating
the uptake and transport of PS-Fe$^{3+}$ chelates (Wang et al. 2013; Wirth et al. 2009). NAS overexpression in rice also increases the expression of genes encoding the zinc transporters OsZIP1 and OsZIP4 (Wang et al. 2013), which can explain the increased zinc content of the transgenic lines. NA is also involved in long-distance iron transport in the phloem and translocation to the grains (Puig et al. 2007). Constitutive expression of OsNAS2 also increased iron content in polished rice (Johnson et al. 2011; Lee et al. 2012). Moreover, mugineic acid (MA) and NA function in the uptake of other essential micronutrients, including zinc, copper, and manganese (Haydon and Cobbett 2007b). Collectively, the significant increases in copper and manganese content that we find in nearly all of the Nas-BW lines in whole grains and as well as flour is consistent with the reported data (Fig. S6).

Unexpectedly, however, the expression of both PvFERRITIN and OsNAS2 in wheat does not result in higher iron levels when compared to lines expressing OsNAS2 alone. Thus, unlike rice in which the expression of FERRITIN and NAS increased iron content by 6-fold in the polished rice grains (Trijatmiko et al. 2016; Wirth et al. 2009), the two genes do not seem to function synergistically in wheat. Although the reasons for this are unclear, it is plausible that iron homeostasis is controlled differently in hexaploid wheat as compared to rice and that iron accumulation is tightly regulated. Furthermore, we did not find a correlation between PvFERRITIN expression and iron content in both Fer-BW and FerNas-BW transgenic lines (Fig. 2) while OsNAS2 expression in leaves correlated with the iron content of Nas-BW and FerNas-BW lines.

Together, we found that constitutive expression of OsNAS2 alone is the preferred strategy for nutritionally relevant iron biofortification in wheat. Our data suggest that unsuccessful breeding efforts to increase iron in wheat grains could be the consequence of regulated iron uptake and transport, which can be overcome by constitutive expression of OsNAS2. Further detailed molecular analysis of the transgenic lines is required to understand the mechanism
of metal homeostasis in wheat and how this is changed in the transgenic lines to increase grain iron content. Understanding the consequences of constitutive OsNAS2 expression on endogenous wheat metal homeostasis-related genes will help to expand our knowledge of their interactions and will assist in improving iron in other preferred crops as well. The high iron and zinc wheat lines that we have developed are a promising breeding material after further agronomic performance testing to confirm that the iron and zinc increases observed in the greenhouse conditions are maintained in the field. The high iron and zinc traits could be easily bred into farmer-preferred and commercial cultivars, thereby reducing iron and zinc micronutrient deficiencies in affected populations.

2.6 Acknowledgments

This research was supported by ETH Zurich to W.G. and N.K.B., and from the State Secretariat for Education, Research, and Innovation (SERI) to N.K.B. We thank Gabriele Buchmann and Gerhard Herren for their support in performing the wheat transformation. We acknowledge Prof. Rainer Schulin (Soil Protection Group, ETH Zurich) for extending access to ICP-OES, and Björn Studer for guidance while performing the metal measurements. We also acknowledge the help of Irene Zurkirchen and Ting-Ying Wu for technical support in the greenhouse and the laboratory. We thank Dr. Christof Sautter for providing the PvfERRITIN expressing construct.

2.7 Author contributions

N.K.B. conceived and designed the experiments. S.S. carried out the experiments. S.S. and N.K.B. analyzed the data. S.S., N.K.B., B.K and W.G. discussed the data. S.S. and N.K.B. wrote the manuscript. W.G. and N.K.B. edited the manuscript. All authors have read the
manuscript and agree with its content.
2.8 Supplementary material

Fig. S1 Example of Southern hybridization analysis of Fer-BW transgenic lines. Genomic DNA was digested by BglII. Lines 18, 47, 51, 62 and 69 each contain a single copy of the transgene. Lines marked with a box were chosen for further analysis. The plasmid containing \(PvFERRITIN\) was used as a positive control. Bobwhite (BW) is the negative control and shows no signal for the \(PvFERRITIN\) specific probe.
Fig. S2 Iron concentration in T3 grains of lines expressing a) OsNAS2 (Nas-BW). b) PvFERRITIN (Fer-BW). c) PvFERRITIN-OsNAS2 (FerNas-BW). Each bar represent individual plant for independent single insert lines. Seeds from plant represented by the black bar were used to grow the next generation (T3) to harvest T4 grains (data presented in the manuscript). BW(Bobwhite); NTS (non-transgenic sibling)
**Fig. S3 Semi-quantitative RT PCR for detection of FERRITIN expression.** Semi-quantitative RT PCR was done on samples 3, 30 and 42 among the FerNas-BW lines using primers listed in Table S1. The results are captured after 21, 25, 30 and 35 cycles.
Fig. S4 Zinc concentration in T3 grains of lines expressing a) OsNAS2 (Nas-BW). b) PvFERRITIN (Fer-BW). c) PvFERRITIN-OsNAS2 (FerNas-BW). Each bar represent individual plant for independent single insert lines. BW(Bobwhite); NTS (non-transgenic sibling)
Fig. S5 Metal content in T4 grains and flour of lines expressing *PvFERRITIN* (Fer-BW). Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant values calculated using Student’s T test, respectively, in comparison to the control line Bobwhite (BW) (*P < 0.05; **P < 0.01). NTS, non-transgenic sibling. Cu, copper, Mn, manganese, Mg, magnesium.
Fig. S6 Metal content in T4 grains and flour of lines expressing OsNAS2 (Nas-BW). Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant values calculated using Student’s T test, respectively, in comparison to the control line Bobwhite (BW) (*P < 0.05; **P < 0.01). NTS, non-transgenic sibling.
Fig. S7 Metal content in T4 grains and flour of lines expressing \textit{PvFERRITIN-OsNAS2} (FerNas-BW). Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant values calculated using Student’s T test, respectively, in comparison to the control line Bobwhite (BW) (*P < 0.05; **P < 0.01). NTS, non-transgenic sibling.
Table S1 Primers used for PCR screening, generation of probe for Southern hybridization, and for quantitative gene expression analysis (qRT-PCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
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<td>PCR screening and Southern hybridisation probe</td>
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<tr>
<td><em>PvFERRITIN</em></td>
<td>CTCTTTTTCCAACCGATCCA</td>
<td>AAGCTTTTCAGCGTGCTCTC</td>
</tr>
<tr>
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<td>CAAGTGCTGCAAGATGGA</td>
<td>GCCGGACTCTAATCATAAAA</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td></td>
<td></td>
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<tr>
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<td>AGGGTACATTCTTGTGATCG</td>
</tr>
<tr>
<td><em>OsNAS2</em></td>
<td>GCAAGTGCTGCAAGATGGA</td>
<td>CCGGATCAGACGGATAGC</td>
</tr>
<tr>
<td>Ta.22845</td>
<td>AAGAAGCAAGAGCTCGGATTT</td>
<td>TGCACCCTTGAAATCATCA</td>
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</table>

Table S2 Phenotypic performance of greenhouse-grown T3 generation Fer-BW transgenic lines

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Days to flowering</th>
<th>Height (cm)</th>
<th>1000 GW (g)</th>
<th>Tiller nr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>96 ± 3**</td>
<td>71.8 ± 7.9</td>
<td>33.8 ± 0.8</td>
<td>10 ± 1**</td>
</tr>
<tr>
<td>47</td>
<td>91.7 ± 4.6</td>
<td>55.9 ± 6</td>
<td>34.6 ± 1.9</td>
<td>7.7 ± 0.6**</td>
</tr>
<tr>
<td>51</td>
<td>89 ± 7.5</td>
<td>65.4 ± 2.1</td>
<td>34.6 ± 3.3</td>
<td>12.7 ± 1.5</td>
</tr>
<tr>
<td>62</td>
<td>87.7 ± 7.4</td>
<td>58.8 ± 4.1</td>
<td>37 ± 1.3</td>
<td>10 ± 2.6**</td>
</tr>
<tr>
<td>69</td>
<td>99.3 ± 3.5**</td>
<td>70 ± 3.2</td>
<td>37.5 ± 0.3</td>
<td>10.3 ± 0.6**</td>
</tr>
<tr>
<td>NTS</td>
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<td>71 ± 4.7</td>
<td>36.2 ± 0.6</td>
<td>15.7 ± 1.2</td>
</tr>
<tr>
<td>BW</td>
<td>83 ± 3.5</td>
<td>64.8 ± 5.2</td>
<td>36.4 ± 1.6</td>
<td>15 ± 1</td>
</tr>
</tbody>
</table>

Values are the average of three biological replicates (± standard deviation). Transgenic plants were compared to Bobwhite (BW). Black and red asterisks indicate statistically higher and lower significant values calculated using Student’s T test, respectively (*P < 0.05; **P < 0.01)
**Table S3** Phenotypic performance of greenhouse-grown T3 generation FerNas-BW transgenic lines

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Days to flowering</th>
<th>Height (cm)</th>
<th>1000 GW (g)</th>
<th>Tiller nr.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>94±4</td>
<td>48.6±1.5</td>
<td>29.7±2.8</td>
<td>20±5.6</td>
</tr>
<tr>
<td>17</td>
<td>102±5.6</td>
<td>53.9±3.3</td>
<td>30.5±0.4</td>
<td>16.7±3.2</td>
</tr>
<tr>
<td>30</td>
<td>103.7±3.8</td>
<td>54.5±8.1</td>
<td>26.2±6.7</td>
<td>13.3±3.1</td>
</tr>
<tr>
<td>42</td>
<td>100.3±9.3</td>
<td>56.9±1,*</td>
<td>33.4±1.8</td>
<td>19.7±2.3</td>
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<tr>
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<td>46.7±5.3</td>
<td>32.5±1</td>
<td>20±3.6</td>
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<tr>
<td>BW</td>
<td>101.7±0.5</td>
<td>49.6±1.9</td>
<td>29.2±1.9</td>
<td>16±3</td>
</tr>
</tbody>
</table>

Values are the average of three biological replicates (± standard deviation). Transgenic plants were compared to Bobwhite (BW). Black asterisks indicate statistically higher significant values calculated using Student’s T test, (*P < 0.05; **P < 0.01)
3 Single genetic locus improvement of iron, zinc and β-carotene content in rice grains

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Submitted for scientific publication
3.1 Abstract

Nearly half of the world’s population obtains its daily calories from rice grains, which lack or have insufficient levels of essential micronutrients. The deficiency of micronutrients vital for normal growth is a global problem and in affected populations often the result of monotonous diets. Iron, zinc and vitamin A deficiencies are most prevalent among micronutrient deficiencies. Iron deficiency anemia (IDA) and zinc deficiency can cause reduced physical growth in children, decreased cognitive performance, increased infections because of reduced immunity, and impaired work ability in all age groups. Vitamin A deficiency (VAD) is associated with night blindness and xerophthalmia, and is the leading cause of preventable childhood blindness. We developed rice lines expressing Arabidopsis NICOTIANAMINE SYNTASE 1 (AtNAS1), bean FERRITIN (PvFERRITIN), bacterial CAROTENE DESATURASE (CRTI) and maize PHYTOENE SYNTHASE (ZmPSY) in a single genetic locus in order to increase iron, zinc and β-carotene content in the rice endosperm. NAS catalyzes the synthesis of nicotianamine (NA), which is a precursor of deoxymugeneic acid (DMA) iron and zinc chelators, and also chelate iron and zinc for long distance transport. FERRITIN provides efficient storage of up to 4500 iron ions. PSY catalyzes the conversion of GGDP to phytoene, and CRTI performs the function of desaturases required for the synthesis of β-carotene from phytoene. All transgenic rice lines have significantly increased β-carotene, iron, and zinc content in the polished rice grains. Our results establish a proof-of-concept for multi-nutrient enrichment of rice grains from a single genetic locus, thus offering a sustainable and effective approach to address different micronutrient deficiencies at once.
3.2 Introduction

Deficiencies of minerals and essential vitamins, collectively termed “hidden hunger”, are prevalent in human populations all over the world. Iron deficiency anemia (IDA), zinc deficiency and vitamin A deficiency (VAD) are among the most common forms of micronutrient deficiencies, which are especially widespread in the developing world. Around two billion people are affected by iron deficiency worldwide, in both developed and developing countries (WHO 2016b). IDA leads to retarded mental development, decreased immunity, and higher maternal and perinatal mortality. Maternal and neonatal mortality due to IDA are significant contributors to global mortality rate, especially in the developing world alone (WHO 2015b). Globally, IDA affects 43% children as well as 38% pregnant and 29% non-pregnant women (Stevens et al. 2013). Similarly, zinc deficiency is also prevalent globally, and based on severity it is categorized into severe, moderate, and mild forms. The most common symptoms of zinc deficiency include male hypogonadism, retarded growth, cell-mediated immune dysfunction and abnormal neurosensory changes (Prasad 2013). VAD is also a major health concern and causes night blindness and xerophthalmia. It is the main cause of preventable childhood blindness and a major contributor to morbidity and mortality from infections in children and pregnant women (WHO 2009). Around 250 million preschool children suffer vitamin A deficiency and are at a higher risk of death as a result of measles, diarrhea and malaria. On a yearly basis, between 250,000 to 500,000 vitamin A-deficient children become blind, with half of them dying within a year of losing their vision (WHO 2016a).

Various epidemiological surveys conducted in developing countries showed that the prevalence of iron deficiency anemia is higher in populations affected by vitamin A deficiency (Semba and Bloem 2002). The occurrence of micronutrient deficiencies has also
been associated with the monotonous, cereal-based diets of the affected populations (Hefferon 2015). Various intervention strategies such as diet diversification, supplementation, and fortification are being utilized to combat micronutrient deficiencies in these populations. Addressing the deficiencies through diet diversification requires the consumption of micronutrient-rich food, which still is difficult in developing countries due to poverty and thus the lack of access to diverse foods. Similarly, providing vitamin A and iron supplements to an affected population is a useful short-term intervention, but often it is not sustainable because it requires stable government policies, appropriate social infrastructure, and continuous investment (White and Broadley 2005). Thus, the direct improvement of nutritional quality of staple foods by biofortification offers an effective solution to address micronutrient deficiencies (Lucca et al. 2006). Rice (*Oryza sativa* L.) is a major contributor to the human diet, with half of the world’s population depending on it to meet their daily caloric requirements (Meng et al. 2005). However, this staple cereal lacks β-carotene (provitamin A) and has insufficient iron, zinc and other nutrients in the endosperm (polished grains) to meet dietary requirements. Biofortification of rice endosperm for essential nutrients can have a significant positive impact on global human health. In order to provide 30% of dietary estimated average requirement (EAR) of iron, 15 µg/g DW iron should be present in polished rice grains, while 28 µg/g DW of zinc can provide 40% of the EAR (Bouis et al. 2011). Around 17 µg/g DW β-carotene in polished rice grains are required to provide 50% of the EAR (Bouis et al. 2011), while 4 µg/g β-carotene could maintain appropriate blood levels of vitamin A, provided that a reasonable contribution of provitamin A is available from other foods besides rice (Golden Rice Project). The breeding efforts over the past several years have failed to achieve the recommended iron content in the rice endosperm, mainly due to a lack of genetic diversity (Bhullar and Gruissem 2013). Similarly, rice cultivars accumulating β-carotene in the endosperm could not be identified in
germplasm screenings (Beyer 2010). Genetic engineering, on the other hand, has been successfully used to enrich the rice endosperm with iron, provitamin A, and folate (Blancquaert et al. 2015; Paine et al. 2005; Trijatmiko et al. 2016; Wirth et al. 2009).

Iron is required for key physiological and metabolic activities of plants, and hence biofortification for iron requires a careful choice of strategy (Grusak et al. 1999). So far, successful iron biofortification has involved a combination of two approaches: iron uptake and translocation, and iron storage. The first approach is the enhancement of iron uptake from the soil, and then its translocation within the plant. Rice, a graminaceous plant, also utilizes a chelation-based strategy (Strategy II) for iron acquisition, in addition to direct uptake of Fe$^{2+}$. In strategy II, plants release mugineic acid (MA) family phytosiderophores (PS) into the rhizosphere that bind Fe$^{3+}$ in form of strong hexadentate chelates, and these chelates are transported into the roots by special transporters (Takagi et al. 1984; Takagi 1976). One such PS is deoxymugineic acid (DMA), which is synthesized from S-adenosyl-L-methionine in a reaction catalyzed by NICOTIANAMINE SYNTHASE (NAS), NICOTIANAMINE AMINOTRANSFERASE (NAAT), and DEOXYMUGINEIC ACID SYNTHASE (DMAS) (Kobayashi and Nishizawa 2012). In this pathway, NAS is the key enzyme for synthesis of nicotianamine (NA), the precursor of DMA. Constitutive overexpression of rice OsNAS2 increased the iron content in polished rice grains of transgenic plants by 2- to 4-fold (Johnson et al. 2011; Lee et al. 2012; Masuda et al. 2009).

The second aspect of increasing endosperm iron content involves FERRITIN, an iron storage complex of 24 protein subunits arranged to form a hollow structure. A single ferritin complex is capable of storing up to 4,500 ferric molecules in a bioavailable form (Harrison and Arosio 1996). The endosperm-specific expression of FERRITIN increased the iron content in polished rice by 2- to 3.7-fold (Goto et al. 1999; Lucca et al. 2001a; Oliva et al. 2014; Qu et al. 2005; Vasconcelos et al. 2003). The combined expression of bean FERRITIN
(PvFERRITIN) and Arabidopsis NAS1 (AtNAS1) increased iron content in the rice endosperm by 6-fold and zinc content by 1.3-fold (Wirth et al. 2009). More recently, rice lines expressing a combination of soybean FERRITIN and rice NAS2 were reported to have a 7.5-fold increase in iron content and 3.3-fold increase in zinc content in the polished grains (Trijatmiko et al. 2016). In addition to the increased iron content, the overexpression of NAS often results in beneficial increases of the zinc content, which are likely due to the increased production of PS because zinc also complexes with PS (Schaaf et al. 2004).

The rice endosperm does not produce β-carotene, which is the precursor of vitamin A. However, the immature rice endosperm can synthesize geranylgeranyl diphosphate (GGDP), the precursor molecule required for the synthesis of β-carotene. Production of β-carotene in plants requires PHYTOENE SYNTHASE (PSY) and three additional enzymes: PHYTOENE DESATURASE, ζ-CAROTENE DESATURASE and LYCOPENE β-CYCLASE. However, CAROTENE DESATURASE (CRTI) of Pantoea ananatis (formerly Erwinina uredovora) performs the function of both the desaturases and cyclase (Ye et al. 2000). Thus, the expression of PSY from daffodil and PaCRTI in rice endosperm resulted in the synthesis of β-carotene (‘Golden rice’; (Ye et al. 2000). Paine et al. (2005) later reported that endosperm-specific expression of maize PSY (ZmPSY) and PaCRTI increased β-carotene in the rice endosperm by 23-fold compared to the golden rice prototype. Since the concepts of rice endosperm biofortification with high β-carotene as well as iron and zinc content are now well established independently, the next challenge is to combine these traits as a single locus to facilitate breeding of multi-nutrient rice varieties.

In this study, we transformed rice with Arabidopsis NAS1 (AtNAS1) expressed under the control of the constitutive CaMV 35S promoter, bean FERRITIN (PvFERRITIN) expressed under the control of the endosperm-specific rice GLOBULIN (OsGLOBULIN) promoter, and PaCRTI and ZmPSY under the expression of the endosperm-specific rice GLUTELIN1
(OsGLUTELIN1) promoter in a single construct. We also transformed the high iron NFP rice (Wirth et al. 2009) with a construct carrying ZmPSY and PaCRTI. In either case, iron, zinc and β-carotene levels are significantly increased in the rice endosperm. This establishes a proof of concept for adapting the biofortification approaches to simultaneously address several important micronutrient deficiencies.

3.3 Material and methods

3.3.1 Transformation vectors

The construct expressing PvFERRITIN under the control of the OsGLOBULIN endosperm-specific promoter and AtNAS1 under the control of constitutive CaMV 35S promoter was previously described (Wirth et al. 2009). The KpnI restriction site in the construct (located outside of the coding region) was mutated in order to use KpnI for further cloning. The PvFERRITIN and AtNAS1 containing fragment was excised using BamHI and PstI. The pCAMBIA-1300 binary vector was the final destination vector used for transformation. The HYGROMYCIN PHOSPHOTRANSFERASE (HPT) selection marker in pCAMBIA-1300 was replaced with PHOSPHOMANNOSE ISOMERASE (PMI) using the restriction site XhoI, thus generating pCAMBIA-1300PMI. The PvFERRITIN and AtNAS fragment was cloned into pCAMBIA-1300PMI using the BamHI and PstI sites, generating pCAMBIA-1300PMI-NASFER. The ZmPSY (NM_001114652) under the control of the OsGLUTELIN endosperm-specific promoter (D00584 1568–2406) along with the nopaline synthase gene terminator (nosT) was synthesized using GenScript® (http://www.genscript.com) in the pUC57 plasmid, generating pUC57-GtPsy. Similarly, the DNA sequence for the pea RUBISCO SSU transit peptide (X00806) combined with PaCRTI and nosT was synthesized with GenScript® (http://www.genscript.com) in pUC57, named as pUC57-ssuCrt. Afterwards, the
OsGLUTELIN promoter was inserted upstream of pUC57-ssuCrt using the restriction sites KpnI and XbaI, generating pUC57-GtssuCrt. Later, both constructs were combined in the pBluescript II SK(-) vector using the restriction site KpnI flanking the 5’ and 3’ end of the gene cassette, generating the pBluescript II SK(-) PsyCrt construct. In the last step, ZmPSY and CRTI were cloned into the pCAMBIA-1300PMI-NASFER and pCAMBIA-1300 binary vectors to generate the pCAMBIA-1300PMI-NASFER-CRTPSY and pCAMBIA-1300-CRTPSY constructs, respectively.

3.3.2 Rice transformation, genotypic characterization, and greenhouse conditions

The Oryza sativa (rice) cultivar Nipponbare (japonica type) was transformed with pCAMBIA-1300PMI-NASFER-CRTPSY construct generating transgenic lines, hereafter named NFCP. The high-iron NFP rice (Wirth et al. (2009) expressing AtNASI under the control of the CaMV 35S promoter and PvFERRITIN and Aspergillus fumigates PHYTASE (AfPHYTASE) genes under the control of the OsGLUTELIN promoter, was transformed with pCAMBIA-1300-CRTPSY construct generating transgenic lines, hereafter named CP. The Agrobacterium tumefaciens strain EHA105 was used for rice transformation. Transformation, and regeneration of NFCP lines were performed as described by Nishimura et al. (2006), while selection for PMI was done on mannose-containing media as described by Lucca et al. (2001b). For generation of CP lines, transformation, selection and plant regeneration was performed as described by Nishimura et al. (2006) and HPT selection was done on hygromycin-containing media. The plant growth chamber conditions were maintained at 16h light/8h dark, 28°C and 60% humidity. Genomic deoxyribonucleic acid (DNA) isolation from three-week-old seedlings in the T0, T1, and T2 generations was performed as previously described (Vasudevan et al. 2014). PCR was used to confirm full-length integration of the constructs and subsequent selection of transgenic lines was done in
T0, T1 and T2 generations with transgene specific primers (Supplementary table S3). Southern blot hybridization using digoxigenin (DIG) labeling was performed on transgenic lines, with *PmlI* digested genomic DNA for NFCP, and *HindIII* digested genomic DNA for CP, to select for transformants with a single insertions of the constructs. A *PMI* specific probe was used for NFCP lines and a *HPT* specific probe was used for CP lines. Selected transgenic lines were grown in commercial soil (Klasmann-Deilmann GmbH, Germany) in the greenhouse with conditions set at 12h light, 30°C, 80% humidity and 12h dark, 22°C, 60% humidity.

### 3.3.3 Metal ion measurements

Plants were harvested at maturity, and spikes were dried at 37°C for three days. Grain samples were de-husked to obtain rice grains and polished with a grain polisher (Kett grain polisher ‘Pearlerst’, Kett Electric Laboratory, Tokyo, Japan) for one minute. For metal quantification in the shoots and the roots, 18 days old seedlings (T3 generation) were grown in hydroponic solution containing 0.70 mM K2SO4, 2 mM Ca(NO3)2, 0.1 mM KH2PO4, 0.5 mM MgSO4, 0.1 mM KCl, 10 µM H3BO3, 0.5 µM MnSO4, 0.2 µM CuSO4, 0.01 µM (NH4)6Mo7O24, 0.5µM ZnSO4, 100 µM Fe-EDTA and pH of 5.5. Both shoot and root samples were dried at 60°C for five days. 200 mg of sample for polished grains and 20 mg of sample for shoots and roots was boiled in 15 ml of 65% v/v HNO3 solution at 120°C for 90 min. Three ml of 30% v/v H2O2 was added and boiled at 120°C for 90 min. Metal concentrations were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Varian Vista-MPX CCD Simultaneous ICP-OES). The wavelength used for iron, zinc, copper, manganese, and magnesium was 238.204, 213.857, 324.754, 257.610, and 285.213, respectively. The data were analyzed using the Student’s t-test to determine statistically significant differences among the tested lines.
3.3.4 β-carotene measurement

Polished grains were homogenized to a fine powder and a one-gram sample was dissolved in two ml of distilled water by sonication and vortexing. In order to evaluate the efficacy of extraction, a control sample with known amount of β-carotene was prepared. Rehydrated samples were incubated at 60°C for 10 min, followed by centrifugation for 10 min at 3300 rpm, and the supernatant was collected in a tube. Extraction was repeated three times with one ml of acetone, each time collecting the supernatant for final pooling. Carotenoids extraction was done twice from the pooled sample with 2ml of 2:1 v/v PE:DE (petroleum ether:diethylether). The samples were mixed by inverting and vortexing, followed by centrifugation for five min at 3300 rpm, and the supernatant was collected. Later, the collected supernatant was dried with a stream of nitrogen gas and then was re-dissolved in 1.5 ml of acetone. β-carotene standards with concentration of 1 µg/ml, 2 µg/ml, 3 µg/ml, 4 µg/ml, 6 µg/ml, and 8 µg/ml were prepared for the calibration curve. Measurement of β-carotene was done using a VARIAN UV-VISIBLE Spectrophotometer at 454.9 nm (n=3). β-carotene content in the test samples was calculated based on the calibration curve in comparison to the known standard concentration (Karnjanawipagul et al. 2010).

3.3.5 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed to analyze the expression of the transgenes. Total RNA was extracted from grains and leaves collected 18 days after flowering from T2-generation plants. Grain RNA was extracted as described by Singh et al. (2003) and leaf RNA was extracted using Trizol® reagent (Invitrogen, U.S.A.). RNA was treated with DNase I (Thermo Fisher Scientific, Inc., U.S.A.) to eliminate genomic DNA. The RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., U.S.A.)
was used for cDNA synthesis. qRT-PCR was performed on the LightCycler® 480 Instrument (Roche, Switzerland). The total reaction volume of 10 µl included 1 µl cDNA, 0.2 µl forward primer, 0.2 µl reverse primer, 5 µl Sybregreen Mastermix (Applied Biosystems, Ltd., U.S.A.), and 3.6 µl H2O. Primers were designed using a CLC genomics workbench (Supplementary table S3). The Ct value was obtained from LightCycler® 480 Instrument (Roche, Switzerland). The data normalization was done as described by Liu et al. (2011).

3.4 Results

3.4.1 β-carotene enrichment of high iron and zinc containing endosperm of NFP rice grains

In order to assess if increased iron and β-carotene synthesis can be combined in rice endosperm, the high-iron NFP rice (Wirth et al. 2009) expressing AtNAS1 and PvFERRITIN was super-transformed with a construct containing PaCRTI and ZmPSY (CP lines). Eight CP lines with single insertions of the construct (Supplementary Fig. S1) were analyzed for transgene expression and for metal as well as β-carotene content in the endosperm. As expected, PvFERRITIN, PaCRTI, and ZmPSY transgene expression was restricted to the grains of these lines, and no expression was found in the leaves (Fig. 1). AtNAS1 was expressed in both grains and leaves of the CP lines, similar to that in the control line NFP. All transformed lines produced β-carotene in the endosperm. The β-carotene content in polished T3 grains ranged from 1.57 to 2.69 µg/g DW with highest content in CP97, while polished grains of control NFP produced no β-carotene (Fig. 2). Furthermore, the iron content in the T3 polished grains ranged from 6.1 to 9.1 µg/g DW, as compared to 5.9 µg/g DW in the NFP control line (Fig. 2). Five lines CP22, CP87, CP97, CP101, and CP105
showed further increases of 1.2- to 1.5-fold in the endosperm iron content as compared to NFP, with highest iron levels in CP87. Zinc content was only increased in the polished grains of CP22 (36.7 µg/g) and CP87 (38.7 µg/g), representing a change of 1.1- and 1.2-fold as compared to the NFP control (32 µg/g), respectively. Overall, line CP87 had superior iron, zinc and β-carotene levels in the polished grains, while CP105 still had significantly improved levels of iron and β-carotene. Additionally, the copper content in most of the lines was significantly increased, except in lines CP17, CP101, and CP105 (Supplementary Fig. S2). Manganese content decreased in the polished grains of lines CP22, CP87, CP97, and CP101, while magnesium content in lines CP22, CP87, CP97, and CP105 increased by 1.3 to 1.5-fold (Supplementary Fig. S2). Iron, zinc, copper, manganese, and magnesium contents in the shoots and the roots of the transformed lines were not altered, except for few differences as compared to the control (Fig. 3 and Supplementary Fig. S3). Phenotypic characterization of T2-generation transgenic plants grown in soil showed some variability for growth parameters including days to flowering, plant height, 1000 grain weight, while tiller number did not differ as compared to the control (Supplementary table S1).
Figure 1. Relative expression of transgenes *PvFERRITIN*, *AtNAS1*, *PaCRTI*, and *ZmPSY* at 18 d after flowering (DAF) in T2-generation. a) Relative expression of *PvFERRITIN*. b) Relative expression of *AtNAS1*. c) Relative expression of *ZmPSY*. d) Relative expression of *PaCRTI*. The data was normalized with the endogenous expression of *Os16530*. NTS is the segregating NFP sibling that does not contain the *PaCRTI-ZmPSY* construct. Values are the average of three biological replicates (±SD) for all except NTS, for which two biological replicates used.
Figure 2. β-carotene, iron (Fe) and zinc (Zn) content in T3 polished grains of CP lines. Values are the mean of three biological replicates (±SD). Black asterisks above the bars indicate statistically significant differences calculated using Student’s T test in comparison to the control line NFP (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.
Figure 3. Iron (Fe) and zinc (Zn) in the shoots and the roots of 18 d T\textsubscript{3} seedlings of CP lines. Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant differences calculated using Student’s T test, respectively, in comparison to the control line NFP (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.

3.4.2 Simultaneous enrichment of rice endosperm with β-carotene, iron and zinc by expression of \textit{PvFERRITIN}, \textit{AtNAS1}, \textit{ZmPSY} and \textit{PaCRTI} from a single construct

Ten T2 NFCP lines expressing \textit{PvFERRITIN}, \textit{AtNAS1}, \textit{ZmPSY} and \textit{PaCRTI} in \textit{O. sativa japonica} cv. Nipponbare from a single construct and with a single insertion of the construct...
(Supplementary Fig.S4) were selected for advanced analysis. All of the lines showed expression of *PvFERRITIN*, *PaCRTI*, and *ZmPSY* in the grains, while no significant expression was detected in the leaves (Supplementary table S3; Fig. 4). Expression of *AtNAS1* was found in both grains and leaves of the transgenic lines as expected. In comparison to the non-transgenic sibling and Nipponbare controls, all transformed lines showed significantly higher iron content in the polished grains and with exception of three lines, the zinc content was also increased. Additionally, β-carotene was produced in the polished grains of all lines (Fig. 5). The endosperm iron content of the transgenic lines ranged from 2.6 to 6.02 µg/g DW as compared to 1.82 µg/g DW in Nipponbare. Among all lines, NFCP18 had the highest iron content (6.02 µg/g), which is an increase of 3.3-fold as compared to the control grains. NFCP22 and NFCP169 also showed an iron increase of more than 2.5-fold in the polished grains. The β-carotene content in the polished grains (Fig. 5) ranged between 1.9 and 3.4 µg/g DW, while no β-carotene was detected in the polished grains of the control lines. The β-carotene content was highest in line NFCP6 with 3.4 µg/g DW, and NFCP18 (line with highest iron content) had 2.1 µg/g DW of β-carotene in its endosperm. The zinc content of the transgenic lines ranged from 24.9 to 29.7 µg/g DW, which represents an increase of 1.07- to 1.28-fold as compared to the Nipponbare control (23.2 µg/g DW). Overall, lines NFCP18 and NFCP22 have the most favorable combination of increased iron, zinc and β-carotene content in the endosperm. The divalent metals including copper, magnesium, and manganese were also increased in most of the lines (Supplementary Fig. S5). Significantly higher copper content, with 1.06- to 1.41-fold increases, was found in most of the lines, except NFCP111, NFCP169, and NFCP185. NFCP78 was an exception, and showed a decrease in copper content (3.5 µg/g) as compared to the control line (4.6 µg/g DW). The manganese content ranged from 4.9 to 8 µg/g DW and thus increased by 1.2- to 1.8-fold as compared to the control, with the exception of lines
NFCP78 and NFCP185. Similarly, magnesium content was increased in most of the transgenic lines as compared to the control, except for lines NFCP78 and NFCP185 (Supplementary Fig. S5).

Figure 4. Relative expression of transgenes *PvFERRITIN*, *AtNAS1*, *PaCRTI*, and *ZmPSY* in the NFCP lines at 18 d after flowering in T2-generation. a) Relative expression of *PvFERRITIN*. b) Relative expression of *AtNAS1*. c) Relative expression of *ZmPSY*. d) Relative expression of *PaCRTI*. No expression was observed in Nipponbare (NPBR) control. The data was normalized with the endogenous expression of Os16530. Values are the average of three biological replicates (±SD).

With some exceptions, the metal profiles in the shoots and the roots of the NFCP lines were comparable to the control lines (Fig. 6 and Supplementary Fig. S6). The iron content in the
shoots and roots of the NFCP lines was comparable to the Nipponbare control, except for slightly increased iron in the shoots of lines NFCP36 and NFCP111, and a decrease in root iron levels in NFCP6 and NFCP111 (Fig. 6). Although zinc content in the NFCP shoots was not different from the control (except NFCP111), the zinc content in the roots was significantly higher in most NFCP lines, except NFCP6 and NFCP185 (Fig. 6). The concentration of copper, manganese, and magnesium was not changed in shoots and roots of most of the transformed lines, indicating that with the exception of root zinc levels the overall metal homeostasis of the NFCP plants is not altered (Supplementary Fig. S6). Phenotypic characterization of T2-generation transgenic plants grown in soil showed some variability for measured parameters such as days to flowering, plant height, tiller, 1000 grain weight (Supplementary table S2).
**Figure 5.** β-carotene, iron (Fe) and zinc (Zn) content in T_3 polished grains of *PvFERRITIN, AtNAS1, PaCRTI,* and *ZmPSY* expressing NFCP lines. Values are the mean of three biological replicates (±SD). Asterisks above the bars indicate statistically significant differences calculated using Student’s T test, in comparison to the control line Nipponbare (NPBR) (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.
Figure 6. Iron (Fe) and zinc (Zn) in the shoots and the roots of 18 d T$_3$ seedlings of *PvFERRITIN*, *AtNAS1*, *PaCRTI*, and *ZmPSY* expressing NFCP lines. Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant differences calculated using Student’s T test, respectively, in comparison to the control line Nipponbare (NPBR) (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.

3.5 Discussion

In developing countries, hidden hunger is often associated with the dependence of affected populations on monotonous cereal-based diets. The causes for this over-reliance on starchy cereals, especially in developing countries, are numerous and complex. Low incomes, a lack of access, poor healthcare, and cultural traditions all contribute to a lack of balanced diets. In
this regard, increasing one or more micronutrients in staple food crops have substantial potential in addressing micronutrient deficiencies. The biofortification of rice with micronutrients such as iron, zinc, vitamin A and folate has been successful in recent years, demonstrating that this approach is feasible. For example, the development of Golden Rice (first in 2000 and improved in 2005) proved that it is possible to successfully engineer the β-carotene pathway in rice endosperm (Paine et al. 2005; Ye et al. 2000). Several studies reported similar success in developing rice lines with high iron content in the endosperm (Johnson et al. 2011; Masuda et al. 2012; Oliva et al. 2014; Trijatmiko et al. 2016; Wirth et al. 2009). However, these improvements of individual micronutrients only address a single deficiency at a time. The combination of several nutritional traits into a single variety could offer significant benefits to human health. For example, rice lines with improved iron and β-carotene levels in the endosperm could address two most detrimental nutritional deficiencies, especially since the prevalence of IDA is higher in populations suffering from VAD (Semba and Bloem 2002).

We demonstrate that rice can be engineered with genes facilitating an increase in iron and zinc content as well as the production of β-carotene in the endosperm, either as two traits expressed from independent loci or as two traits combined in a single genetic locus. Since the NFCP lines express AtNAS1, PvFERRITIN, PaCRTI and ZmPSY from a single locus, increased uptake and translocation of iron and zinc as well as the targeted storage of iron and production of β-carotene in the endosperm could be expected but needed to be validated to demonstrate synergistic function of the four genes. The NFCP lines have up to 3.3-fold iron and 1.28-fold zinc increases in the endosperm, respectively, and also produce β-carotene at significantly higher levels compared to the control plants. Similar β-carotene levels were obtained in the NFP lines transformed with PaCRTI and ZmPSY, while the iron content in these lines (CP) was even higher than in the control NFP lines. The iron and zinc increases
are consistent with the activity of NAS in increasing the production of nicotianamine (NA) and deoxymugineic acid (DMA), which facilitate uptake and transport of iron and zinc (Haydon and Cobbett 2007b; Takagi et al. 1984; Takagi 1976). The endosperm of wild type rice plants does not produce β-carotene, but synthesizes the GGDP precursor. The production of β-carotene in the NFCP rice endosperm is therefore solely due to the activity of PSY (which converts GGDP to phytoene) and CRTI (which synthesizes β-carotene from phytoene) and consistent with the results first obtained for Golden Rice (Ye et al. 2000).

The amount of β-carotene produced in NFCP lines as compared to the improved Golden Rice version 2 (Paine et al. 2005) is, however, lower, with a maximum of 3.4 µg/g DW. Several factors, including the different genetic background of the rice cultivar and the small number of transgenic lines we used in our proof-of-concept study, could account for the difference. Several hundred independent transgenic lines were generated during the production of Golden Rice 2 that contained between 9 and 37 µg/g of carotenoids, with 31 µg/g of β-carotene content in the line with highest levels of carotenoids. Nevertheless, our work demonstrates the feasibility of combining β-carotene, high-iron and zinc traits in a single cultivar. Provided that a moderate intake of β-carotene from other food sources besides rice is available, around 4 µg/g of β-carotene provides 50% of the required nutrient intake (RNI) (Golden Rice Project). Although the recommended daily dietary intake levels are higher, a persistent supply of 50% RNI of β-carotene is sufficient to maintain adequate levels of vitamin A in blood (Golden Rice Project). Thus, line NFCP18 could provide moderate β-carotene supplementation as well as 40% of the recommended increase of iron content and 100% of the zinc requirements. IDA and VAD co-occur within populations, and β-carotene has been suggested to facilitate iron mobilization and transport, and enhance overall iron absorption (Donald and Kraemer 2012; West et al. 2007). We therefore expect that combining high-iron with β-carotene in rice endosperm could benefit iron absorption in the
human gut, but this needs to be tested in nutritional studies. Furthermore, the increased iron, zinc and β-carotene levels in the NFCP lines address three micronutrient deficiencies at once. The effectiveness of combined supplementation of iron, vitamin A, and zinc in micronutrient-deficient populations has been well documented in different studies (Donald and Kraemer 2012). Combining different nutritional traits as a single genetic locus in a major staple crop is a significant step towards realizing combined supplementation for health benefits, although further improvements and optimization of the combined traits are still possible. Selected lines must now be tested in the field to demonstrate trait stability. If successful, the multi-nutrient trait locus can be transferred to farmer-preferred rice cultivars or rice mega-varieties, either by breeding or direct transformation. Combining several nutrient traits into a single genetic locus allows breeders to easily introduce these into breeding lines. Moreover, engineering a multi-nutrient trait using proven genes in single and simple inserts may be favorable from a regulatory point of view. Together, we demonstrate that it is possible to combine nutritional traits in a staple crop, which is a promising approach to greatly improve the nutritional quality of agricultural crops.

3.6 Acknowledgments

This research was supported by ETH Zurich internal grant to W.G. and to N.K.B. We acknowledge Prof. Rainer Schulin (Soil Protection Group, ETH Zurich) for providing facility access for metal and β-carotene measurements, and Björn Studer for guidance while performing the measurements. We also acknowledge the help of Irene Zurkirchen for technical support in the greenhouse. We thank Prof. Uta Paszkowski, University of Lausanne, Switzerland for providing seeds of rice cultivar Nipponbare and Dr. Christof Sautter for providing the construct containing PvFERRITIN and AtNAS1.
3.7 Author contributions

N.K.B. conceived and designed the experiments. S.S. carried out the experiments and analyzed the data. S.S., N.K.B. and W.G. discussed and interpreted the data. S.S. wrote the draft manuscript. W.G. and N.K.B. edited the manuscript. All authors have read the manuscript and agree with its content.
Supplementary Figure S1. Example of Southern hybridization analysis of CP lines. Genomic DNA was digested by *HindIII*. Line CP17, CP22, CP87, CP89, CP97, CP101, CP105, and CP107 were detected to contain single copy of transgene insertion and were chosen for further analysis. NFP is the negative control and showed no signal for the *HPT* specific probe.
Supplementary Figure S2. Metal content in T3 polished grains of CP lines. Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant differences calculated using Student’s T test, respectively, in comparison to the control line NFP (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.
Supplementary Figure S3. Metal content in the shoots and the roots of 18 d T₃ seedlings of CP lines. Values are the mean of three biological replicates (±SD). Black asterisks above the bars indicate statistically significant differences calculated using Student’s T test, respectively, in comparison to the control line NFP (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.
Supplementary Figure S4. Example of Southern hybridization analysis of NFCP lines. Genomic DNA was digested by *PmlI*. Line NFCP1, NFCP6, NFCP18, NFCP22, NFCP36, NFCP72, NFCP78, NFCP111, NFCP169, and NFCP 185 were detected to contain single copy of transgene insertion and were chosen for further analysis. NPBR (Nipponbare) is the negative control and showed no signal for the *PMI* specific probe.
Supplementary Figure S5. Metal content in T$_3$ polished grains of *PvFERRITIN*, *AtNAS1, PaCRTI*, and *ZmPSY* expressing NFCP lines. Values are the mean of three biological replicates (±SD). Black and red asterisks above the bars indicate statistically higher and lower significant differences calculated using Student’s T test, respectively, in comparison to the control line Nipponbare (NPBR) (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.
Supplementary Figure S6. Metal content in the shoots and the roots of 18 d T3 seedlings of *PvFERRITIN*, *AtNAS1*, *PaCRTI*, and *ZmPSY* expressing NFCP lines. Values are the mean of three biological replicates (±SD). Asterisks above the bars indicate statistically higher significant values calculated using Student’s T test, in comparison to the control line Nipponbare (NPBR) (*P < 0.05; **P < 0.01). NTS is the non-transgenic sibling.
Supplementary Figure S7. Example of the phenotype of T3 seed of NFCP lines. NFCP18 line shows the characteristic yellow colored endosperm, while Nipponbare devoid of it.
**Supplementary Table S1.** Phenotypic performance of T₂ generation CP transgenic lines in the greenhouse. Values are the average of three biological replicates (± standard deviation). Transgenic plants were compared to Nipponbare. NTS is non transgenic sibling. Black and red asterisks indicate statistically higher and lower significant values calculated using Student’s T test, respectively (*P < 0.05; **P < 0.01).

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<tr>
<th>Plant line</th>
<th>Days to flowering</th>
<th>Height(cm)</th>
<th>1000GW(g)</th>
<th>Tiller nr.</th>
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<td>CP17</td>
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<td>CP101</td>
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<td>20.3 ± 0.8,*</td>
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**Supplementary Table S2.** Phenotypic performance of generation NFCP transgenic lines in the greenhouse. Values are the average of three biological replicates (± standard deviation). Transgenic plants were compared to Nipponbare. NTS is non transgenic sibling. Black and red asterisks indicate statistically higher and lower significant values calculated using Student’s T test, respectively (*P < 0.05; **P < 0.01).

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<th>1000GW(g)</th>
<th>Tiller nr.</th>
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<td>56.9 ± 2.2</td>
<td>17.9 ± 0.4,**</td>
<td>10 ± 1,*</td>
</tr>
<tr>
<td>NFCP72</td>
<td>121 ± 1.2,*</td>
<td>51.9 ± 1.4,**</td>
<td>20.2 ± 0.7</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>NFCP78</td>
<td>117 ± 2.9,*</td>
<td>53.1 ± 1.3,**</td>
<td>19.2 ± 0.3,*</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>NFCP111</td>
<td>120 ± 0.6,*</td>
<td>51.5 ± 1,**</td>
<td>18.5 ± 0.9,*</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>NFCP169</td>
<td>126 ± 1.2,*</td>
<td>48.7 ± 2.5,**</td>
<td>18.6 ± 0.8,*</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>NFCP185</td>
<td>120 ± 1.7,*</td>
<td>52.3 ± 1.2,**</td>
<td>18.2 ± 0.2,**</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Nipponbare</td>
<td>123 ± 0.6</td>
<td>57.8 ± 0.5</td>
<td>20.7 ± 0.5</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>NTS</td>
<td>119 ± 0.6,**</td>
<td>52.6 ± 1.9,**</td>
<td>21.2 ± 0.3</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>
**Supplementary Table S3.** Primers used for PCR screening, generation of probe for Southern hybridization, and for quantitative gene expression analysis (qRT-PCR)

<table>
<thead>
<tr>
<th>Gene cassette</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR and Southern blot probe</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PMI</em></td>
<td>CTGGCTAATGGTGGTTTCT</td>
<td>CGTGATGTGATTTGAGAGT</td>
</tr>
<tr>
<td><em>HPT</em></td>
<td>CAAGCTGCATCATCGAAATTG</td>
<td>TCTGATCGAAAGTTCGACAG</td>
</tr>
<tr>
<td><strong>qRT-PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PvFER**RITIN</em></td>
<td>AAGCAGGAACCTTGTTGT</td>
<td>AAGGCTACATTCTTGATCG</td>
</tr>
<tr>
<td><em>AtNAS1</em></td>
<td>GCAAGTGTGCTGAAGATGG</td>
<td>CCGGATCGACGGAATAGC</td>
</tr>
<tr>
<td><em>PaCRTI</em></td>
<td>ATCTGCGCCAGGCGTTT</td>
<td>GGTGGCGAAGGGATTGC</td>
</tr>
<tr>
<td><em>ZmPSY</em></td>
<td>GCCGAGATCTGTGAGGAGT</td>
<td>TGTTGTAATGTAGTGTCCG</td>
</tr>
<tr>
<td><strong>Fragment Integration PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>LB-PMI</em></td>
<td>GGGGGATCTGATTTTAGT</td>
<td>TGCCAGCTCATTATGA</td>
</tr>
<tr>
<td><em>PMI-PSY</em></td>
<td>TGTTGTGGAATTGTAGAG</td>
<td>AGTGTGAGTGTATTGTT</td>
</tr>
<tr>
<td><em>PSY-CRT</em></td>
<td>GGTAAAAAGGAAATTTG</td>
<td>TTGTAGACGATTGAGCC</td>
</tr>
<tr>
<td><em>CRT-AtNAS</em></td>
<td>AAAACAGCAGAAAACAAGA</td>
<td>GGAATGTAAGGAAAGATAG</td>
</tr>
<tr>
<td><em>AtNAS-FER</em></td>
<td>CCTCTCACATCCATCGTATT</td>
<td>ACCCAGAAAAAGGGGAA</td>
</tr>
<tr>
<td><em>FER-RB</em></td>
<td>AGGGAGTTAAGGAGAGAG</td>
<td>AAGGCGATTAAGTGGGT</td>
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</table>
4 General Discussion

4.1 Role of biofortification in reducing micronutrient deficiencies

Hidden hunger is the term used to address the prevalence of micronutrient deficiencies in human population. This has a negative impact on health and the economic development, particularly in the developing countries. Among the cereals, wheat (*Triticum aestivum*) and rice (*Oryza sativa* L.) constitute a major share of the human diet, however these cereals are deficient in essential micronutrients. Several intervention strategies like diet diversification, supplementation, and post-harvest fortification are employed, but these interventions require a suitable infrastructure and significant financial investment. Biofortification of food crops to enhance its nutritional value by the use of improved agronomic practices, conventional plant breeding and/or biotechnology is another important strategy. This is the appropriate strategy to reach the populations where supplementation and conventional fortification are difficult to implement (WHO 2015a). Biofortification is sustainable, targeted, and cost-effective approach, and it has been characterized in the “highly” cost-effective category of interventions (Meenakshi et al. 2010).

4.2 Conventional plant breeding, and improved agronomic practices for biofortification

HarvestPlus challenge program of Consultative Group for International Agricultural Research (CGIAR) focuses on delivering biofortified nutritious food to the undernourished populations. It employs the use of improved agronomic practices and conventional plant breeding techniques where possible, and use of biotechnology when there is less or no genetic diversity (HarvestPlus 2014). Various nutritious traits in different crops are the target for biofortification, which include vitamin A biofortification in maize, cassava, sweet potato
and banana; zinc biofortification in rice and wheat; and iron biofortification in common bean and pearl millet. The first biofortified crop to be released by HarvestPlus was Orange sweet potato (OSP), having substantial amount of β-carotene. Since then, many other biofortified crops have been developed and have been released, or are in line to be released. Recently, zinc enriched rice variety “BRRI dhan 62” comprising 20 ppm of zinc was released in Bangladesh (HarvestPlus 2014), and zinc biofortified wheat varieties like BHU-3, BHU-6, and Zincol were released in India and Pakistan in 2015 (HarvestPlus 2015a; HarvestPlus 2015b). Likewise, several other biofortified varieties of maize, cassava, and pearl millet, have been developed and released, while some are in trials to be released in future (HarvestPlus 2014). All the mentioned biofortified crops were developed via conventional plant breeding by making use of available genetic diversity. Furthermore, various studies to test the efficacy of the absorption of the micronutrient from the biofortified crops have been completed or are ongoing. Sweet potato is the main staple food in countries like Uganda and Mozambique, thus β-carotene rich variety OSP was provided for cultivation and consumption. A study to determine the effectiveness of biofortified OSP was done, and it showed that increased vitamin A intake was associated with the incorporation of OSP into the diets of women and children (Hotz et al. 2012a; Hotz et al. 2012b). Likewise, a study done in school children in Kenya revealed that consumption of biofortified yellow cassava varieties rich in β-carotene, led to modest increase in the serum retinol concentration and large increase in β-carotene concentration of the children (Talsma et al. 2016). These studies also highlighted the public acceptance to nutritional enriched varieties, and importance of biofortification for combating hidden hunger. Despite these successes, however, for several micronutrients there is very less genetic diversity available in the crop germplasm making it difficult to biofortify crops via conventional breeding. For example, β-carotene enrichment
of rice endosperm; iron biofortification of rice and wheat grains could not be achieved using conventional breeding approaches.

Apart from genetic improvement, use of improved agronomic practices like soil and foliar application has also been recommended for biofortification of minerals like selenium, zinc, and iodine in various crops (Cakmak et al. 2010; Galinha et al. 2015; Kiferle et al. 2013). Another improved agronomic practice includes the co-culturing of bacteria and crop plants, as microbes are known to affect the bioavailability and uptake of selenium (Yasin et al. 2015b). For example, wheat grown with selenium-tolerant strain (YAM2) of bacteria (99% similarity with *Bacillus pichinotyi*) leads to significant increase in the selenium content of wheat stem and grains (Yasin et al. 2015a). Meanwhile, soil and foliar application of minerals aided the biofortification, but it is not sustainable and cost-effective method, as it requires stable, bioavailable, and cheap mineral compound.

### 4.3 Biofortification of vitamins and minerals via biotechnology

Enrichment of various organic antioxidant molecules like β-carotene, folate, ascorbic acid, and tocopherols has been achieved in different crops. One such example is biofortification of vitamin A in rice endosperm, which has been possible only because of advanced gene technology approaches. Overexpression of *Phytoene synthase* (*PSY*) gene from maize (*Zea mays*) and *carotene desaturase* (*CRTI*) from *Pantoea ananatis* (formerly *Erwinina uredovora*) leads to production of β-carotene in rice endosperm (Paine et al. 2005; Ye et al. 2000). Similarly, significant increase in the production of carotene was accomplished in crops like potato and flaxseed by overexpression of *CRTB* gene encoding PHYTOENE SYNTHASE (Ducreux et al. 2005; Fujisawa and Misawa 2010). Likewise, biofortification of folate in tomato and rice has been achieved by overexpression of *GTP CYCLOHYDROLASE I* (*GTPCHI*) in combination with *AMINODEOXYCHORISMATE SYNTHASE* (*ADSC*) (de La
Garza et al. 2007; Storozhenko et al. 2007). First generation folate-biofortified lines had significantly higher folate content, but the concentrations dropped to 40% of original level after eight months of storage (Blancquaert et al. 2015). So in order to develop second generation of folate-biofortified rice having stable folate content, combination of GTPCHI, ADSC, and FOLATE BINDING PROTEIN (FBP) was deployed, which lead to significant increase in folate content and was more stable if stored longer than four months (Blancquaert et al. 2015). The combination of folate and folate-binding protein enhances the folate stability and thus enabling its long-term storage. Apart from vitamin biofortification of crops, enrichment of the crop plants with anti-cancer compounds is being accomplished as well. Enrichment of Glucosinolate glucoraphanin (GSLG), precursor molecule of anti-cancer compound sulphoraphane, was done in Brassica juncea by constitutive silencing of the GSL-ALK homolog gene family encoding for the enzyme 2-oxoglutaratedependent dioxygenases or GSL-ALK enzyme responsible for degradation of GSLG.

Likewise, biofortification of food crops via biotechnology with essential minerals like iodine, iron, and zinc, is in progress as well. Enrichment of iodine in arabidopsis has been demonstrated as a proof of concept, by overexpression human SODIUM-IODIDE SYMPORTER (NIS) gene (Landini et al. 2012). Similarly, iron biofortification of rice has achieved noteworthy success so far by overexpression of combination of genes including FERRITIN, nicotianamine synthase (NAS), and/or iron transporters (Boonyaves et al. 2016). The increases in iron content were often accompanied by increase in the amount of zinc, copper, and manganese as well, because NAS is also known to play a key role in their uptake and translocation (Haydon and Cobbett 2007b). This study has demonstrated that constitutive overexpression of rice NAS2 in wheat facilitated achieving the required level of iron and zinc in wheat grains and sieved flour (Chapter 2).
4.4 Expression of rice NAS alone is sufficient for biofortification of wheat

For iron biofortification of cereals, various combination strategies have been suggested. Previously, endosperm specific expression of ferritin along with the constitutive expression NAS increased the iron content by more than 6-fold in the polished rice (Trijatmiko et al. 2016; Wirth et al. 2009). In this case increased iron uptake and its transport within the plant and iron storage in endosperm was attributed as the main reason. Similarly in wheat project, we achieved iron increases in the whole grains and flour by endosperm specific overexpression of \textit{PvFERRITIN}. Additionally, constitutive overexpression of \textit{OsNAS2} leads to 2-fold increase in amount of iron in the whole grains and the flour of transgenic wheat. However, transgenic lines expressing both \textit{PvFERRITIN} and \textit{OsNAS2} did not show higher iron content as compared to plants expressing only one of these genes. The difference in the result of iron increases in wheat and rice could be attributed to genetic and physiological differences of wheat and rice. These results suggest iron uptake and transport is likely the bottleneck in the iron biofortification of wheat (Chapter 2).

4.5 Stacking of multiple nutritious traits in single cultivar

Significance of biotechnological approaches for biofortification of micronutrients in food crops is now well established. Production of beta-carotene as well as increased iron content in the rice endosperm has been achieved separately (Paine et al. 2005; Trijatmiko et al. 2016; Wirth et al. 2009). Increased iron and zinc content is attributed to the synergistic action of \textit{FERRITIN} and \textit{NAS} as shown by Wirth et al. (2009). The next step leads to development of food crops with multiple nutritious traits stacked in single locus of cultivar, and this could offer multi directional approach to tackle various micronutrient deficiencies. This approach has been demonstrated to work in maize (\textit{Zea mays}), where multivitamin corn having
enhanced levels of β-carotene, ascorbate, and folate was generated (Naqvi et al. 2009). This study demonstrates the first successful effort to develop multi nutrient rice, with increased levels of beta-carotene, iron and zinc in a single cultivar (Chapter 3).

With the recent advancement in the genome editing tools, development of multiple traits has become more practical and achievable. Further studies to understand the occurrence and pattern of related micronutrients deficiencies in human populations will enable us to develop smart crops required for specific geographic area to provide micronutrient enriched diet. Combination of biotechnology, conventional breeding, and improved agronomic practices should be employed to develop more sustainable and cost-effective strategies for combating hidden hunger.

4.6 Concluding remarks

World’s population by 2050 will touch the 9 billion mark, and providing nutritious food to the ever-increasing world’s population will be a challenging proposition. New scientific approaches offer opportunities to produce nutritionally enhanced food with minimal resources (DeFries et al. 2015). Many genetically engineered crops like, herbicide resistant, pest resistant, and with enhanced storage qualities have been released worldwide, and have received mixed response for their acceptance in the public. Likewise, several genetically engineered crops for enhanced nutritional traits are in the developing and testing phase. The genetically engineered crops are subjected to a strict regulation testing to evaluate the prevalent concerns in the public domain. Golden rice is one such example and is in advanced phase of regulatory evaluation. Although, there is opposition for the genetically engineered crops in the public domain, but keeping in mind the benefits of these nutritionally enriched crops, release and acceptance of these crops is expected in future. In this study, we developed iron and zinc biofortified wheat, paving way for breeding farmer preferred
cultivars improved for iron and zinc content in the grains. Also with the development of multi-trait rice, we have shown that different nutritious traits can be combined and can exist together, offering a multi dimensional approach to tackle micronutrient malnutrition.
5 References


HarvestPlus (2014) Biofortification Progress Briefs. Washington DC


Accessed 6 April 2016


Hurrell R (2002) How to ensure adequate iron absorption from iron-fortified food Nutr Rev 60:S7-S15
Inoue H et al. (2009) Rice OsYSL15 is an iron-regulated iron(III)-deoxymugineic acid transporter expressed in the roots and is essential for iron uptake in early growth of the seedlings J Biol Chem 284:3470-3479 doi:10.1074/jbc.M806042200
Ishimaru Y et al. (2011) A rice phenolic efflux transporter is essential for solubilizing precipitated apoplasmic iron in the plant stele J Biol Chem 286:24649-24655 doi:10.1074/jbc.M111.221168
Ishimaru Y et al. (2006) Rice plants take up iron as an Fe3+-phytosaliphore and as Fe2+ Plant J 45:335-346 doi:10.1111/j.1365-313X.2005.02624.x


Lanquar V et al. (2005) Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron Embo J 24:4041-4051 doi:10.1038/sj.emboj.7600864


Masuda H et al. (2012) Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. Sci Rep-Uk 2 doi:Artn 543Doi 10.1038/Srep00543


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Paine JA et al. (2005) Improving the nutritional value of golden cice through increased provitamin A content Nat Biotechnol 23:482-487 doi:Doi 10.1038/Nbt1082


Trijatmiko KR et al. (2016) Biofortified indica rice attains iron and zinc nutrition dietary targets in the field Sci Rep-Uk 6 doi:ARTN 1979210.1038/srep19792


Wright M et al. (2001) Efficient biolistic transformation of maize (Zea mays L.) and wheat (Triticum aestivum L.) using the phosphomannose isomerase gene, pmi, as the selectable marker Plant Cell Rep 20:429-436


Zhai ZY et al. (2014) OPT3 is a phloem-specific iron transporter that is essential for systemic iron signaling and redistribution of iron and cadmium in Arabidopsis Plant Cell 26:2249-2264 doi:10.1105/tpc.114.123737

