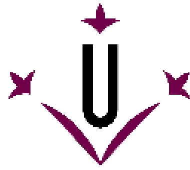

Mechanisms controlling the selective iron and zinc biofortification of rice

Raviraj Banakar

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Mechanisms controlling the selective iron and zinc biofortification of rice

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DOCTORAL DISSERTATION

Lleida, February 2016

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ಅರ್ಪಣೆ

ಪ್ರೀತಿಯ ತಾಯಿ ದಿವಂಗತ ಸುಮಿತ್ರಾ ಬಣಕಾರ್ ಅವರಿಗೆ ಹೃದಯ ಪೂರ್ವ ಸಮರ್ಪಣೆ

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To my mother late Sumitra Banakar

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SUMMARY

Rice is a staple crop for more than 3 billion people. However, rice exhibits a very limited genetic variation for essential minerals such as iron (Fe) and zinc (Zn); therefore, the world's poorest people who depend on rice for most of their nutritional calories suffer from iron deficiency anaemia (IDA) and zinc deficiency (ZnD). Malnutrition is a significant global challenge, particularly in the developing world where measures that are commonplace in industrialized countries such as a varied diet, fortification programs, and dietary supplements are largely absent. Consequently, biofortification of rice at source is an important and sustainable strategy to increase the levels of these two critical micronutrients. Rice is also a major contributor of toxic cadmium (Cd) intake through the diet. Therefore, Fe and Zn biofortification strategies must also minimize Cd levels at source.

I generated a transgenic rice population expressing different combinations of four genes involved in the uptake, transport, accumulation and bioavailability of Fe and Zn. My overall aim was to engineer rice grains containing high levels of these two minerals, simultaneously limiting the accumulation of toxic metals such as Cd. This population was utilized to identify mechanisms controlling mineral accumulation in the plant. I established that even though Fe and Zn levels can be increased in the endosperm to some extent, homeostatic mechanisms operate to establish an upper ceiling in terms of their absolute amounts. I used a number of lines expressing specific transgenes or transgene combinations to understand molecular mechanisms contributing towards the Fe and Zn ceiling in seeds. My results indicate that homeostasis mechanisms operating in vegetative organs such as roots and leaves restricted Fe and Zn seed loading.

The promiscuous nature of metal transporters and metal chelating ligands provide a mechanism for toxic metals such as Cd to accumulate in seeds, together with Fe and Zn. I established that lines able to accumulate high levels of Fe and Zn in seed endosperm also exhibited higher uptake and translocation of Cd in vegetative tissues. However, Fe and Zn

phloem loading and their increased accumulation displaced Cd from the endosperm resulting in lines with substantially reduced amounts of Cd. These lines are thus very promising candidates for mineral biofortification programs because not only they provide germplasm with enhanced amounts of Fe and Zn in the endosperm but importantly, they also exhibit much reduced levels of Cd. Regulatory barriers and political interference need to be overcome in order for this technology to reach the people that need it the most, impoverished and food insecure people in the developing world.

RESUM

L'arròs forma part de la dieta bàsica de més de 3 billones de persones. La planta de l'arròs presenta una carència genètica que resulta en una limitació en la acumulació de minerals essencials com el ferro (Fe) i el zinc (Zn); com a conseqüència las poblacions pobres que depenen majoritàriament de l'arròs com a font essencial de calories per a la seva nutrició sofreixen d'anèmia por falta de ferro (IDA) i de deficiència de zinc (ZnD). El resoldre el problema de la desnutrició es un repte important, especialment en els països en vies de desenvolupament que manquen de les mesures complementaries que son comuns en els països industrialitzats (dieta variada, programes de fortificació i suplements dietètics). Una estratègia sostenible e important per a augmentar els nivells d'aquest dos micronutrients en l'arròs seria bio-enriquir directament la planta originaria. Per un altra part, una de las causes que contribueixen a la toxicitat por metalls pesats como el cadmi (Cd) és el consum d'arròs contaminat, por lo que l'estratègia de bio-enriquiment de Fe i Zn tindria que contribuir a la reducció de l'acumulació de Cd en la planta d'arròs.

Amb l'objectiu de produir grans d'arròs amb un contingut més elevat de Fe i Zn, he enginyat plantes mitjançant la transformació combinatòria amb quatre gens implicats en l'absorció, transport, acumulació i biodisponibilitat d'aquest dos minerals. Al mateix temps he intentat limitar en aquestes plantes l'acumulació de metalls tòxics com el Cd. Aquesta població combinatòria m'ha servit per a identificar els mecanismes antagònics i sinèrgics que son essencials per a controlar l'acumulació de Fe i Zn. He demostrat que utilitzant mecanismes de sinergia podem augmentar las concentracions de Fe i Zn en l'endosperma fins a un cert nivell, però queda clar que hi ha un llindar que no es pot superar en l'acumulació d'aquest minerals. He utilitzat las línees amb acumulació màxima de Fe i Zn per a estudiar els mecanismes moleculars que regulen el llindar no sobrepassable en les

llavors. Els meus resultats també suggereixen que els mecanismes homeostàtics que funcionen en els òrgans vegetals como arrels i fulles limiten l'acumulació Fe i Zn en las llavors.

Los transportadors i lligands de Fe i Zn no son específics d'aquest metalls, la qual cosa fa possible que s'acumulin en les llavors, juntament amb ells, metalls pesats com el Cd. He observat que les plantes que son capaces d'acumular concentracions elevades de Fe i Zn en l'endosperma de les llavors també presenten una major absorció i translocació de Cd en el teixit vegetatiu. No obstant, la carga de Fe i Zn en el floema i l'increment de la seva acumulació provoca en un desplaçament del Cd de l'endosperma, resultant en plantes amb una quantitat reduïda de Cd. Aquestes plantes son candidates a ser integrades als programes de bio-enriquiment mineral per que, no nomes aporten un germoplasma amb quantitats elevades de Fe i Zn en l'endosperma, si no que mes important, presenten nivells molt mes reduïts de Cd. Las barreres regulatòries i las interferències polítiques tenen que ser eliminades per tal que aquestes plantes puguin ser utilitzades per finalitats humanitàries. L'ús d'aquestes línees transgèniques podria ajudar a resoldre alguns dels problemes greus derivats de la desnutrició.

RESUMEN

El arroz forma parte de la dieta básica de más de 3 billones de personas. La planta del arroz presenta una carencia genética que resulta en una limitación en la acumulación de minerales esenciales como el hierro (Fe) y el zinc (Zn); como consecuencia las poblaciones pobres que dependen mayoritariamente del arroz como fuente esencial de calorías para su nutrición sufren de anemia por falta de hierro (IDA) y de deficiencia de zinc (ZnD). La desnutrición es un reto importante, especialmente en los países en vías de desarrollo donde están ausentes las medidas complementarias que son comunes en los países industrializados (dieta variada, programas de fortificación y suplementos dietéticos). Una estrategia sostenible e importante para aumentar los niveles de estos dos micronutrientes en el arroz seria bio-enriquecer directamente la planta originaria. Por otra parte, una de las mayores causas que contribuyen a la toxicidad por metales pesados como el cadmio (Cd) es el consumo de arroz contaminado, por lo que la estrategia de bio-enriquecimiento de Fe y Zn tendría que contribuir a la reducción de la acumulación de Cd en la planta de arroz.

Con el objetivo de producir granos de arroz con un contenido más elevado de Fe y Zn, he obtenido plantas transgénicas mediante transformación combinatoria con cuatro genes implicados en la absorción, transporte, acumulación y biodisponibilidad de estos dos minerales. Al miso tiempo he intentado limitar la acumulación de metales tóxicos como el Cd en estas plantas. Esta población combinatoria me ha servido para identificar los mecanismos antagónicos y sinérgicos que son esenciales para controlar la acumulación de Fe y Zn. He demostrado que utilizando mecanismos de sinergia podemos aumentar las concentraciones de Fe y Zn en endospermo hasta cierto nivel, pero esta claro que hay un umbral que no se puede superar en la acumulación de estos minerales. He utilizado las líneas con máxima acumulación de Fe y Zn para estudiar los mecanismos moleculares que

regulan el umbral no sobrepasable en las semillas. Mis resultados también sugieren que los mecanismos homeostáticos que funcionan en los órganos vegetales como raíces y hojas limitan la acumulación Fe y Zn en las semillas.

Los transportadores y ligandos de Fe y Zn no son específicos de estos metales lo cual hace posible que se acumulen en las semillas, junto con el Fe y Zn, metales pesados como el Cd. He observado que las plantas que son capaces de acumular Fe y Zn en niveles elevados en el endospermo de las semillas también presentan mayor absorción y translocación de Cd en el tejido vegetativo. No obstante, la carga de Fe y Zn en el floema y el incremento de su acumulación resultan en un desplazamiento del Cd del endospermo, resultando en plantas con una cantidad inferior de Cd. Estas plantas son candidatas prometedoras para los programa de bio-enriquecimiento mineral porque, no solo proveen de un germoplasma con cantidades elevadas de Fe y Zn en el endospermo, si no que mas importante, presentan niveles mucho mas reducidos de Cd. Las barreras regulatorias y las interferencias políticas tienen que ser superadas para que estas plantas puedan ser utilizadas para fines humanitarios. El uso de estas líneas transgénicas podría ayudar a resolver algunos de los problemas derivados de la desnutrición.

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LIST OF ABBREVIATIONS

µg	Micrograms
µl	Micro litre
µM	Micro Molar
2.4.D	2,4-Dichlorophenoxy acetic acid
Af	Aspargillus fumigates
BAP	6-Benzyl amino purine
bp	Base pairs
Cd	Cadmium
cDNA	Complementory DNA
Cu	Copper
Cv.	Cultivar
DMA	Deoxy mugenic acid
DMAS	Deoxy mugenic acid synthase
DW	Dry weight
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FAO	Food and Agriculture Organisation
Fe	Iron
FL	Flag leaf
FRDL	Citrate efflux transporter
FW	Fresh weight
g	Grams
GE	Genetic engineering
Gm	*Glycine Max*
H20	Water
H_2O_2	Hydrogen peroxide
HCl	Hydro chloric acid
HD	High DMA
HMA	Heavy metal ATPase
HN	High NA and High DMA

HNO_3	Nitric acid
HPLC-ESI-MS(TOF)	High performance liquid chromotography-Electro spray inisation-Time of flight-mass spectrometry
HPT	Hygromycin phospho transferase
Hv	*Hordeum Vulgare*
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IDA	Iron deficiency anemia
IRT	Iron regulated transporter
kDa	Kilo Dalton
KOH	Potassium hydroxide
kV	Kilo Volts
l	Litre
M	Molar
MES	2-(N-morpholino)ethanesulfonic acid
mg	Milli grams
min	Minute
ml	Milli litre
ML	Middle leaf
mM	Milli molar
Mn	Manganese
mRNA	Messenger RNA
MS	Murashige and Skoog
Mx	*Malus xiaojinensis*
n	Number
N_2	Nitrogen
NA	Nicotianamine
NA	Not analysed
NAA	1-Naphthalene acetic acid
NAAT	Nicotianamine amino transferase
NAS	Nicotianamine synthase
ND	Not determined
ng	Nano grams

NL	Nicotyl lycine
ºC	Degree celcius
Os	*Oryza Sativa*
PCR	Polymerase Chain Reaction
PVDF	polyvinylidene fluoride
qRT-PCR	Quantitative Reverse transcriptase-polymerase chain reaction
R	Roots
RDI	Recommended dietary intake
rRNA	Ribosomal RNA
RT	Reverse Transcriptase
s	Seconds
SAM	S-adenosyl methionine
SE	Standard Error
TOM	Transporters of mugenic acid family phytosiderophores
V	Volts
WHO	World Health Organisation
WT	Wild type
YSL	Yellow stripe like transporter
ZIP	ZRT IRT like proteins
Zn	Zinc

Chapter 1

1 General introduction

1.1 Global rice consumption and the importance of Fe and Zn in human nutrition

Rice is grown in more than one hundred countries worldwide, and more than 3.5 billion people depend upon rice for 27-70% of their daily calorie intake (Dipti et al 2012; Bhullar and Gruissem 2013). Out of more than one billion people living in poverty, 70% are in Asia, where 90% of the global rice production is consumed (Khush 2005; Khush 2013). Iron (Fe) plays an important role in human nutrition; it is a component of the oxygen transporting protein hemoglobin and a number of metabolic enzymes such as catalase, cytochrome P450s, ribonucleotide reductase (Hunt et al 2002). Zinc (Zn) is a co factor for approximately 100 enzymes and immune and nerve cells secrete Zn as a signaling molecule (WHO 1998; Cakmak et al 2008).

1.2 Fe and Zn deficiency in rice consuming countries

Iron deficiency anemia (IDA) is one of the major global health challenge affecting over 2 billion people (Gomez-Galera et al 2010). Approximately 50% of anemia is due to poor intake of Fe from the diet (Stein 2010). Rice endosperm is a very poor source of Fe and there is very little genetic variation for this mineral in rice germplasm worldwide [ranging from 2-5 μg/g dry weight (DW)]. Thus, IDA is severe in rice consuming developing countries (Sperotto et al 2012). Zinc levels in rice endosperm are also low (ranging from 16-25 μg/g DW) (Sperotto et al 2012). Therefore, reliance on rice based diets can induce Zn deficiency (ZnD), leading to impairments in physical development, immune system and brain function (Cakmak 2008).

1.3 Strategies to address Fe and Zn deficiency

Various strategies have been proposed to deal with Fe and Zn deficiencies, including food-based approaches, the provision of supplements, the fortification of processed food, the biofortification of crops at source with mineral-rich fertilizers and the implementation of breeding programs and genetic engineering strategies (GE) to generate Fe and Zn rich rice (White and Broadley 2005; Gómez-Galera et al 2010).

1.3.1 Dietary diversification

A balanced diet would provide all bioavailable Fe and Zn required to cover nutritional requirements for healthy individuals. Therefore, the optimal intervention to alleviate Fe and Zn deficiency is through the consumption of a varied diet, including fresh fruit, vegetables, fish and meat. This, however, is not possible in many countries since such foods are not widely available or if they are, they are unaffordable for those in developing countries with a limited per capita income (Sheikholeslam 2004). Even where fresh food is available, lack of infrastructure such as good transportation, storage and preservation systems as well as political instability act as constraints (Gomez-Galera et al 2010).

1.3.2 Supplementation

Supplementation consists of periodic administration of nutrients as capsules, pills, tablets, nutrient solutions or injections for immediate consumption in order to alleviate deficiencies (WHO/FAO 1998). Supplementation is considered to be the most effective short-term intervention and is recommended in developing countries to alleviate acute nutritional deficiencies; however, it is unsustainable for large populations and should be replaced by fortification at the earliest opportunity (Nantel and Tontisirin 2002; Shrimpton and Schultnik 2002). Even though supplementation of Fe and Zn is cost effective and successful (Hunt 2002; Shrimpton and Schultnik 2002), periodic delivery and consumption

of supplements by target populations must be ensured (Hotz and Brown 2004). Since, overdose of Fe and Zn can have detrimental effects on health, care should be taken in employing supplementation as a means to alleviate Fe and Zn deficiency (Ruel 2001; Clemens 2014). Logistical and economic constraints currently limit the provision of supplements to target populations and fail to reach people in remote rural areas (Allen 2002).

1.3.3 Food fortification

Food fortification is one of the most cost-effective long-term intervention methods for micronutrient nutrition. It consists of the addition of the target nutrient to a food item during processing. Although rice can be fortified with Fe (Hotz and Gibson 2007) and Zn (Pinkaew et al 2013), choice of technology and suitable chemical form of Fe and Zn determines the success of fortification. However, fortification often leads to price increases that prevent the most impoverished people from enjoying the benefits of this intervention (Gomez-Galera et al. 2010).

1.3.4 Biofortification

Although dietary diversity, supplementation and fortification have been described as adequate means to tackle malnutrition in certain cases, they are often impractical in developing countries. An alternative approach is to tackle the problem of Fe and Zn deficiency through enhanced level of bioavailable Fe and Zn in the endosperm of seeds, through biofortification, which should translate into an improved diet (Sperotto et al 2012). The main advantage of nutritionally enhanced GE crops is that target populations do not need to change their dietary habits, i.e. there is no compliance issue (Graham et al 2000; Yang et al 2007). Several different strategies for biofortification have been adopted using

agronomy, conventional breeding or GE approaches, or even a combination (Broadley et al 2006; Naqvi et al 2010; Zhu et al 2009).

1.3.4.1 Agronomic biofortification of rice

Agronomic intervention might be the simplest strategy to increase the mineral content of plants by adding the appropriate mineral as an inorganic compound to the fertilizer. Although, grain Fe level can be improved through the application of Fe as a fertilizer and similarly the Zn content of rice can be improved through application of Zn as fertiliser, such improvements are not adequate to help people to overcome Fe and Zn deficiencies (Fang et al 2008). Even more importantly, agronomic biofortification cannot influence the bioavailability of Fe and Zn (Frosard et al 2000). In addition, the mineral must be applied regularly due to precipitation of Fe and Zn fertilizers in the soil (Sprorotto et al 2012) and this necessitates continuous investment, and increases the negative impact of these minerals on the environment (Frossard et al 2000; Graham 2003; Sors et al 2005).

1.3.4.2 Breeding Fe and Zn rich rice

Plant breeding and GE strategies can enhance the micronutrient content as well as bioavailability in the plant, and require an investment limited to the research and development phase, being entirely sustainable thereafter (Cakmak 2008). Therefore, cost-effectiveness of biofortification through plant breeding and GE would not exclude the most impoverished people (Welch and Graham 2005).

Conventional breeding, sometimes in combination with mutagenesis, is used for improving the nutrient content and bioavailability of staple crops exploring natural variation (Welch and Graham 2005). The identification of mineral-dense cereal varieties and the use of marker-assisted selection to introgress such traits into widely cultivated adapted germplasm have been reviewed (White and Broadley 2005). The natural variation among

rice cultivars for endosperm Fe and Zn is 2-5 µg/g DW and 16-25 µg/g DW, respectively (Sperotto et al 2012). This narrow genetic variation constrains severely efforts to breed Fe and Zn rich rice. A further major limitation with conventional breeding interventions is the time taken to identify useful traits, which must be genes within the genetic pool of sexually compatible species, and breeding them into elite cultivars. This limitation is even more important when several micronutrients must be enhanced simultaneously in the same crop; thus, so far no high-micronutrient rice varieties produced by this method have been introduced to the market (Gómez-Galera et al 2010).

1.3.4.3 Genetic engineering of rice for improved Fe and Zn content

Transgenic strategies in general offer many advantages compared to conventional plant breeding (Zhu et al 2007). While the latter is limited to genes within the genetic pool of sexually compatible species, GE has at its disposal an unrestricted genetic pool to select the most suitable genes for a specific trait, and even synthetic genes can be used. The possibility to design specific genetic constructs permits to target the expression of the desired trait to the edible part of the plant. GE allows a relatively rapid generation of improved elite varieties with the desired traits, in contrast to conventional plant breeding, particularly if the targeted trait(s) needs to be introgressed from a wild relative (Zhu et al 2007). In addition, due to advances in multiple gene transfer technology different nutritional traits can be engineered simultaneously in the same plant, thus avoiding the need for complex breeding programmes (Naqvi et al 2009). Therefore, GE provides the most straightforward way to produce nutritionally complete crops. Regulatory issues and political interference, however, compromise the deployment of such GE plants (Pérez-Massot et al 2013; Berman et al 2013; Dubock 2014).

1.4 Fe and Zn homeostasis in rice

In rice, Fe and Zn homeostasis involves many genes. There are two mechanisms through which Fe is taken up by rice plants, Strategy I and Strategy II. In Strategy I, Fe^{2+} ions are taken up into the root epidermis by plasma membrane bound Fe regulated transporters OsIRT1/OsIRT2 (Vert et al 2002; Lee and An, 2009). Upon transport, Fe^{2+} forms complexes with nicotianamine (NA) and the complex is then transported by the OsYSL2 internal transporter involved in long distance transport (Ishimaru et al 2010). Although the specifics of seed Fe^{2+} loading in rice are yet to be determined, it has been demonstrated that nicotianmine (NA) helps in seed loading of Fe (Schuler et al 2012) and OsYSL2 transporter might play a key role in transporting Fe^{2+}-NA into the seeds (Takahashi et al 2003; Ishimaru et al 2010). Considering that Fe and Zn share a common mechanism for uptake, Fe regulated transporters *OsIRT1*/*OsIRT2* and Zn regulated iron regulated transporters, *OsZIP1*/*OsZIP2*/*OsZIP3*/*OsZIP4* are involved in the uptake of Zn^{2+} ions (Ishimaru et al 2005). Heavy metal transporters *OsHMA2* and *OsHMA4* can transport Zn in the root plasma membrane (Yamaji et al 2013). Transporter *OsHMA2* is involved in loading of Zn to the roots, shoots and developing tissues (Yamaji et al 2013).

In strategy II chelating phytosiderophores (deoxy mugenic acid) are used by the plant to fix Fe^{3+} by the formation of Fe^{3+}-DMA complexes (Ma et al 1999). Deoxy mugenic acid is synthesized from L-methionine. L-methionine adenylation by S-adenosylmethionine synthatase leads to the production of S-adenosyl methionine (SAM) (Mori and Nishizawa 1987). Trimerisation of SAM by nicotinamine synthase (NAS) produces nicotianamine (Higuchi et al 1999). An amino transfer from nicotianamine by nicotianamine aminotransferase (NAAT) leads to a 3''-keto intermediate, and deoxymugenic acid synthase (DMAS) caries out the reduction of a 3'' carbon leading to 2''-deoxy mugenic acid (DMA) (Suzuki et al 2008). DMA is extruded into the rhizosphere by plasma membrane

bound *OsTOM1* transporter (Nozoye et al 2011) leading to the formation of Fe^{3+}-DMA complexes (Nozoye et al 2011). Fe^{3+}-DMA complexes are transported into the roots by a plasma membrane bound Fe^{3+}-DMA complex transporter, *OsYSL15* (Inoue et al 2009). Once inside the plant Fe^{3+}-DMA can be transported in the form of Fe^{3+}-DMA and Fe^{3+}-citrate through the phloem and xylem with the help of *OsYSL18* and *OsFRDL1,* respectively (Ayoma et al 2009; Yokosho et al 2009). DMA is also able to bind Zn. In rice Zn-DMA is preferentially transported over Zn^{2+} and DMA plays a key role in the long distance transport of Zn (Suzuki et al 2008).

1.5 Transgenic approaches to improve Fe and Zn accumulation in rice grains

Transgenic strategies to increase the Fe and Zn content of rice have used five different approaches: (i) increasing Fe storage capacity in seeds; (ii) increasing the amount of bioavailable Fe along with improved Fe storage capacity; (iii) increasing the efficiency of Fe and Zn uptake; (iv) increasing the internal transport of Fe and Zn to seeds; (v) combination of increasing internal transport followed by improved storage capacity and bioavailability.

To increase the iron storage capacity, the Fe storage protein ferritin has been expressed in rice seeds. Overexpression of soybean ferritin in rice, under the control of an endosperm-specific promoter has been reported (Goto et al 1999; Vasconcelos et al 2003). Resulting grains accumulated 3 (Goto et al 1999) and 4.4 (Vasconcelos et al 2003) times the Fe levels measured in wild type plants, respectively. Iron concentration was measured in polished grains because minerals are lost during polishing, and the levels of Fe (and Zn) were still higher than in non-polished grains of wild-type plants (Vasconcelos et al 2003). Although the total amount of Fe and other minerals in plants is an important determinant of nutritional quality, what really matters is the amount of bioavailable Fe and how well it is

absorbed by the human gut. Phytic acid (also known as phytate) is an antinutritional compound that chelates minerals and reduces their bioavailability in the gut. Therefore, a strategy was developed that involves the expression of both ferritin and phytase (a fungal enzyme that breaks down phytate), resulting in rice grains accumulating twice the amount of Fe usually present in wild type (Lucca et al. 2002).

Similarly, to increase Fe and Zn uptake efficiency, rice was engineered with the plasma membrane bound Fe (III)-DMA transporter *OsYSL15* (Lee et al 2009) and the Fe (II) regulated transporter *OsIRT1* (Lee and An 2009). The resulting transgenic lines showed increased uptake of Fe and Zn, followed by a slight but significant increase in seed Fe and Zn levels compared to wild type. Nicotianamine and deoxymugenic acid have the ability to promote uptake and transport of Fe and Zn through vascular tissues (Takahashi et al 2001; Lee et al 2009). The expression of barley *NAATa* and *NAATb*, encoding nicotianamine aminotransferases (involved in phytosiderophore biosynthesis) in rice under the control of *NAATa/NAATb* promoters resulted in increased NA to DMA conversion followed by increased Fe/Zn uptake (Takahashi et al 2001; Takahashi et al 2003). Rice engineered with nicotianamine synthase from barley (*HvNAS1*) and rice (*OsNAS1*) (Masuda et al 2009; Johnson et al 2011) had increased levels of NA and DMA leading up to 4 fold increase in Fe and 2 fold increase in Zn in transgenic lines over wild type, respectively (Johnson et al 2011). Surprisingly a combination of enhanced synthesis of NA and DMA through over expression of nicotianamine synthase coupled with increased iron storage capacity through over expression of ferritin was shown to increase Fe by 6-fold and Zn by 2-fold in transgenic rice lines compared to wild type (Wirth et al 2009; Masuda et al 2012). Along with the positive role NA plays in Fe and Zn uptake and translocation, enhanced level of NA in transgenic rice increases the bioavailability of Fe and Zn (Zheng et al 2007; Lee et al 2011; Lee et al 2012). Even though, combined use of multiple strategies for Fe

fortification appear to translate to high levels of bioavailable Fe and Zn (refer to Tables 1.1 and 1.2 for transgenic rice enhanced for Fe and Zn content) the levels reported thus far are far from delivering the recommend daily intake for Fe (14 ppm/day/adult) and Zn (14 ppm/day/adult) (Sperotto et al 2012).

Table 1.1 Examples of transgenic rice enhanced for Fe concentration in seeds

Gene and Source	**Seed**	**Fe concentration (µg/g DW)**	**Fold increase over wild type**	**Reference**
HvNAS1+OsYSL2+Gmferritin	Polished	6	6	Masuda et al 2012
OsNAS2	Polished	14	4.2	Johnson et al 2011
Gmferritin+Af phytase+*OsNAS1*	Polished	7	6	Wirth et al 2009
Activation tagging *OsNAS3*	Dehusked	32	2.9	Lee et al 2009
Gmferritin	Dehusked	20	-	Qu et al 2005
Gmferritin	Polished	37	4.4	Vasconcelos et al 2003
Gmferritin+Afphytase	Dehusked	22	2	Lucca et al 2002
Gmferritin	Dehusked	35	3	Goto et al 1999

Hv=Hordeum Vulgare, Os=Oryza sativa Gm=Glycine Max, Af=Aspergillus fumigatus DW=Dry weight

Table 1.2 Examples of transgenic rice enhanced for Zn concentration in seeds

Gene and source	Seed	Zn concentration (µg/g DW)	Fold increase over wild type	Reference
Activation tagging of *OsNAS2*	Polished	40-45	2.9	Lee et al 2011
OsNAS2	Polished	52-76	2.2	Johnson et al 2011
GmferritinGmferritin+Afphytase+OsNAS1	Polished	35	1.6	Wirth et al 2009
Activation tagging of *OsNAS3*	Dehusked	42	2.2	Lee et al 2009

Hv=*Hordeum Vulgare*, Os=*Oryza sativa* Gm=*Glycine Max*, Af=*Aspergillus fumigatus* DW=Dry weight

Thus, considering the complexity of Fe and Zn homeostasis, it appears that there is a mechanism which limits Fe and Zn levels in endosperm by 6-and 3-fold, respectively (Slamet-Loedin et al 2015). Therefore, it will be important to follow a different approach in order to identify key mechanisms which would allow surpassing the ceiling for Fe and Zn accumulation in rice endosperm. Although such an approach has not been employed in rice for Fe and Zn biofortification, it was utilized in corn through combinatorial genetic transformation which resulted in library of transgenic plants stably expressing different combinations of transgenes representing different steps in the carotenoid pathway (Zhu et al 2008). This approach identified as yet unknown bottlenecks limiting the accumulation carotenoids and permitted the development and implementation of more refined interventions which resulted in transgenic corn lines accumulating extraordinary levels of carotenoids in the endosperm (Zhu et al 2008). Thus, a similar approach might be helpful in unravelling and ultimately overcoming constraints imposed by Fe and Zn homeostasis genes.

1.6 The problem of strictly toxic heavy metal Cd and possibility of decreasing Cd levels in Fe and Zn biofortified rice through genetic engineering

Cadmium (Cd) is a highly toxic heavy metal for human health. Adverse effects of Cd include cancer of prostate, lungs, kidney tubule damage, rhinitis, emphysema, osteomalacia, and bone fractures (Järup 2003; Ueno et al 2010). Food is the major source of Cd to humans. Industrial emissions, mining, application of sewage sludge, use of fertilizers and pesticides, increases Cd pollution of farm land (Sebastian and Prasad 2013), leading to increased uptake of Cd by crop plants (Järup, 2003). This exacerbates the intake of Cd through the diet, followed by detrimental effects on human health. For example "Itai-Itai" (renal osteomalacia) disease in Japan was caused by the consumption of rice grown on Cd contaminated soil (Ogawa et al 2004).

The majority of metal transporters are promiscuous in nature and are also able to transport Cd along with Fe and Zn (Clemens et al 2013). Plasma membrane bound iron regulated transporters (IRT) e.g. OsIRT1 or OsIRT2 can take up Fe (II), Zn (II), Mn (II), as well as Cd (II) (Lee and An 2009). Similarly, natural resistance associated macrophase protein-OsNRAMP5 is also a major transporter of Cd (II), Fe (II), Mn(II) (Ishikawa et al 2012). Presence of Cd can alter the homeostasis of other metals, leading to drastic effects on several physiological processes including photosynthesis, electron transport chain reaction, etc (Sebastian and Prasad 2013). Vacuolar sequestration of Cd in rice roots is employed as a detoxification mechanism by plants (Ueno et al 2010). A member of the heavy metal ATPase (HMA) family, OsHMA3 is a vacuolar tonoplast localized transporter, shown to carry out vacuolar sequestration of Cd (II) and Zn (II) (Ueno et al 2010; Sasaki et al 2012). Xylem loading followed by root to shoot translocation of Cd is a factor determining the amount of Cd in seeds (Sasaki et al 2012) and transporters OsHMA2 and OsNRAMP1 carry out Cd (II)/Zn (II) and Cd (II)/Fe (II) xylem loading and root to shoot translocation,

respectively (Takahasi et al 2011a; 2011b; 2012). Transfer of Cd from xylem to phloem, followed by transfer of Cd to seeds are a critical steps in determining the level of Cd in seeds (Fujimaki et al 2010). Low affinity cation transporter OsLCT1 with the ability to transport Cd (II), Fe (II), Mn (II) regulates this process (Uraguchi et al 2011). Along with the promiscuous nature of transporters, metal ligands such as NA and DMA play a key role in regulating metal homeostasis (Cheng et al 2007; Lee et al 2009). Although the role of DMA in Cd uptake and translocation has not been elucidated it has been reported that NA is able to promote Cd uptake and translocation (Cheng et al 2007).

Among transgenic approaches used thus far to engineer rice with metal transporters, *OsIRT1* (Lee and An 2009) and *MxIRT1* (Tan et al 2015) improved Fe and Zn uptake, but also higher uptake of Cd as well. Rice engineered for higher levels of nicotianamine had higher level of Cd uptake and mobilization into leaves (Cheng et al 2007). Therefore, considering the detrimental effect of Cd on plant and animal health it will be important to choose selective Fe and Zn transporters and also to understand the role of NA/DMA in the homeostasis of Cd along with Fe and Zn (Clemens et al 2013).

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AIMS AND OBJECTIVES

Aims

The major aim of my research dissertation was to generate a library of transgenic rice plants through combinatorial nuclear genetic transformation with different combination of transgenes for Fe and Zn enhancement which will eventually help to understand the mechanistic basis and bottlenecks in mineral uptake, transport and accumulation, leading to the identification of a lead transgenic event accumulating high levels of iron and zinc. A further aim was to characterize Fe and Zn accumulating transgenic lines for the uptake, translocation and accumulation of the strictly toxic heavy metal cadmium.

Objectives

- Combinatorial nuclear genetic transformation of rice with genes for iron and zinc, enhancement
- Select and regenerate putative transgenic plants
- Analysis of putative transgenic plants at mRNA level
- Determination of iron and zinc in roots, leaves and seeds of transgenic plants
- Characterization of high mineral accumulating transgenic plants for heavy metal cadmium uptake and accumulation
- Bioavailability assessment of high mineral accumulating transgenic rice lines

Chapter 2

2 Combinatorial genetic transformation of rice with genes involved in Fe and Zn homeostasis

2.1 Abstract

Iron and zinc biofortification of rice through genetic engineering offers a long term and sustainable solution for increasing the level of these important micronutrients in the endosperm. Combinatorial nuclear genetic transformation can create a diverse population of transgenic lines expressing different combination of transgenes. Since all input transgenes are integrated in one genetic locus there is no segregation of the transgenes in subsequent generations. The main aim in the work described in this chapter was to establish a population of transgenic lines expressing genes representing mobilization, uptake, translocation and accumulation of Fe and Zn. I recovered 450 independent transgenic lines. I used this population to elucidate underlying molecular mechanisms controlling the accumulation of iron and zinc in storage tissues. Here I describe the generation and molecular characterization of this population. I also report the mineral content of select lines. Outputs from these experiments set the stage for more in depth investigations focusing on particular genes and/or gene combinations critical in mineral accumulation in the rice plant.

2.2 *Aims*

The major aim of the work described in this chapter was to generate a population of transgenic plants constitutively expressing genes for iron and zinc homeostasis. My specific objectives were:

i) To transform rice embryos with four transgenes and to recover a combinatorial population of putative transgenic plants

ii) To analyse putative transgenic lines for the expression of input transgenes at the mRNA level and to identify sub-populations with useful expression patterns of the relevant input transgenes

iii) To analyse polished seeds of transgenic lines for accumulation of iron and zinc in the endosperm

iv) To analyse seeds for the accumulation of the phytosiderophores nicotianamine and deoxy mugenic acid in lines expressing *OsNAS1* and *HvNAATb*

iv) To analyse the bioavailability of iron in high-iron accumulating transgenic lines

2.3 *Materials and Methods*

2.3.1 Gene cloning and transformation vectors

The *OsNAS1* cDNA (GenBank ID: AB021746.2) and *OsIRT1* cDNA (AB:070226.1) were cloned from the roots of 2-week-old rice (*Oryza Sativa* L cv EYI 105) plants grown in vitro, on MS medium without iron (Murashige and Skoog 1962). *HvNAATb* cDNA (GenBank ID:AB005788.1), *HvYS1* cDNA (GenBank ID:AB214183.1) were cloned from the roots of 2-week-old barley (*Hordeum vulgare* L. cv. Ordalie) plants grown in vitro on MS medium lacking iron (Murashige and Skoog 1962). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and 2 µg of total RNA was reverse

transcribed using the Omniscript RT Kit (Qiagen, Hilden, Germany). The full size *OsNAS1* (999 bp), *OsIRT1*(1125 bp), *Hvnaatb* (1656 bp), *HvYS1* (2037 bp) cDNA were amplified by PCR using, forward primer *OsNAS1*-BamHI-FOR (5'-AGG ATC CAT GGA GGC TCA GAA CCA AGA GGT CG-3') and reverse primer *OsNAS1*-HindIII-REV (5'-AAA GCT TCA TAA TAT AGT GCG CCT TTC GAT CGT CCG GCTGT-3') for *OsNAS1*, forward primer *OsIRT1*-BamHI-FOR (5'AGG ATC CAT GGC GAC GCC GCG GAC ACT GGT GCC CAT TCT G-3') and reverse primer *OsIRT1-HindIII-Rev* (5'-AAA GCT TTC ACG CCC ACT TTG GCC ATG ACG GAC-3') for *OsIRT1*, forward primer *HvNAATb*-BamHI-FOR (5'-AGG ATC CAT GGC CAC CGT ACG GCC AGA GAG CGA CG-3') and reverse primer *HvNAATb* -HindIII-REV (5'- AAA GCT TCT AGC AAT CAT CGC TCG CTC GAA TTT CTC -3') for *HvNAATb*, forward primer *HvYS1*-BamHI-FOR (5'-AGGATC CAT GGA CAT CGT CGC CCC GGA CCG CA-3') and reverse primer *HvYS1*-HindIII-REV (5'-AAA GCT TTT AGG CAG CAG GTA GAA ACTTCA TG-3') for *HvYS1* gene. The products were transferred to the pGEM®-T Easy vector (Promega, Madison, WI, USA) for sequencing and verification. The *OsNAS1, OsIRT1, HvNAATb* and *HvYS1* cDNAs were then sub cloned using the BamHI and HindIII sites and inserted into the plant transformation vector pAL76 (Christensen and Quail 1996) which contains the maize Ubi-1 promoter and first intron, and an *Agrobacterium tumefaciens* nos transcriptional terminator. The constructs for *OsNAS1*, *HvNAATb*, *OsIRT1*, *HvYS1* were generated by Sonia Gómez-Galera in 2009.

2.3.2 Rice combinatorial transformation

Mature rice embryos (*Oryza sativa* L. cv EYI 105) were excised and cultured as previously described (Sudhakar et al 1998; Valdez et al 1998). After 7 days, the embryos were bombarded with gold particles carrying the transgenes (*OsIRT1, OsNAS1, HvYS1, HvNAATb*) and the *HPT* selectable marker gene (Christou et al 1991). The rice embryos

were incubated on high-osmoticum medium (0.2 M mannitol, 0.2 M sorbitol) for 4 h prior to bombardment. Bombarded embryos were selected on medium supplemented with 30 mg/l hygromycin and callus pieces were transferred sequentially to shooting and rooting media containing hygromycin. Regenerated plantlets were transferred to pots containing soil (Traysubstract; Klasmann-Deilmann GmbH, Geeste, Germany) and were cultivated under flooded conditions in a growth chamber at 26±2° C, with a 12-h photoperiod (900 mmohn m^{-2} s^{-1} photosynthetically-active radiation) and 80% relative humidity. Plants were irrigated with a soluble iron solution (Sequestrene 138 Fe G-100; Syngenta Agro SA, Madrid, Spain) until seed maturity (Figure 2.1; Table 2.1).

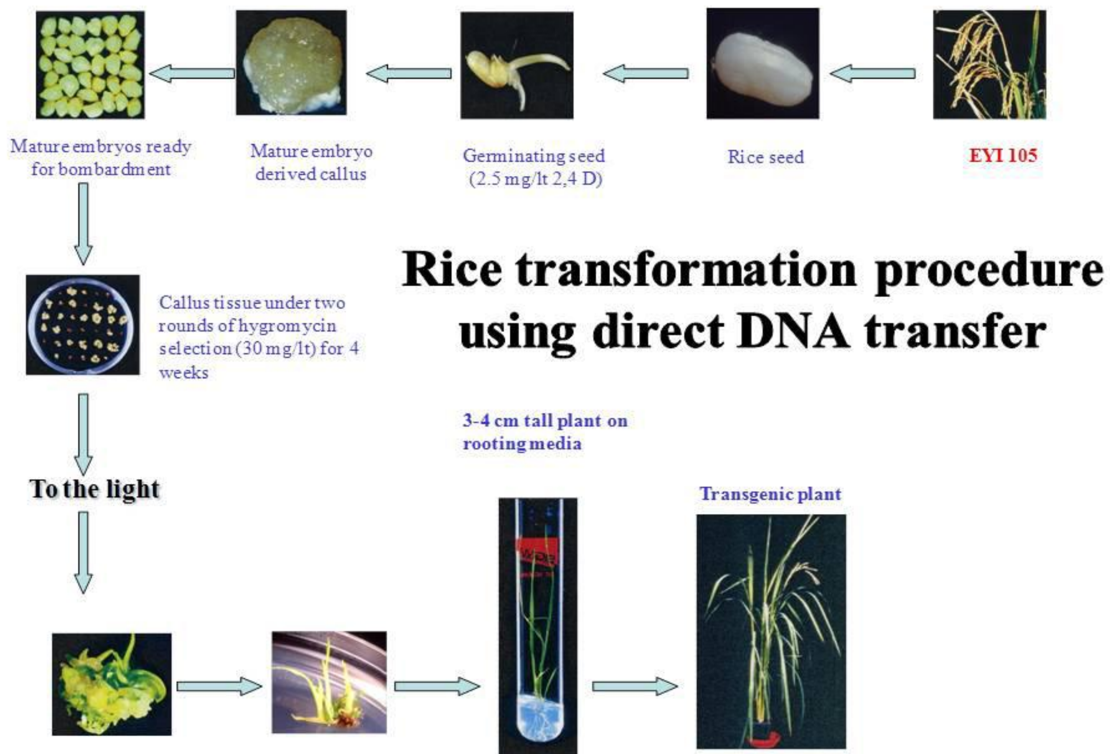

Figure 2.1 Illustration of the rice transformation process showing preparation of embryos prior to bombardment, selection of transformed tissues and regeneration of putative transgenic plants.

Table 2.1 Media composition (1 l) for rice transformation and regeneration

	Callus induction	**Osmoticum medium**	**Selection medium**	**Shoot induction medium**	**Root induction medium**
Approx. time in culture	5 to 7 days	24h	Four weeks (two week subcultures)	21 to 25 days	2 to 3 weeks
MS powder *	4.4 g	4.4 g	4.4 g	4.4 g	2.2 g
Casein hydrolysate	300 mg	300 mg	300 mg	100 mg	--
Proline	500 mg	500 mg	500 mg	--	--
Sucrose	30 g	30 g	30 g	--	10 g
Maltose	--	--	--	30 g	--
Mannitol	--	72.8 g	--	--	--
2,4-D (5mg/ml)	500 µl	500 µl	500 µl	--	--
Adjust pH to 5.8 using KOH					
Phytagel (Sigma)	5 g	3 g	5 g	4 g	3 g + 2 g agar
Autoclave at 121ºC for 20 min					
BAP (Sigma, 1 mg/ml)	--	--	--	3 mg (3 ml)	--
NAA (Sigma, 1 mg/ml)	--	--	--	0.5 mg (0.5 ml)	--
Hygromycin (50 mg/ml)	--	--	600 µl	600 µl	600 µl
Vitamin B5 (200x)**	--	--	--	--	2.5 ml

*MS Medium with Gamborg's B5 vitamins (Duchefa Biochemie, Haarlem, The Netherlands)

**B5 Vit (200x stock): dissolve 1 g myo-inositol, 10 mg nicotinic acid, 10 mg pyridoxine HCl and 100 mg thiamine HCl in 50 ml sterile water

2.3.3 Expression analysis

Total RNA was isolated from leaves by using the RNeasy Plant Mini Kit (Qiagen, Paisley, Scotland, UK), and 20 microgram aliquots were fractionated on a denaturing 1.2% (wt/vol) agarose gel containing formaldehyde before blotting. The membranes were probed with digoxigenin-labeled partial cDNA of *OsIRT1, HvYS1, OsNAS1, HvNAATb* at 50°C overnight by using DIG Easy Hyb (Roche Diagnostics, Mannheim, Germany). After washing and immunological detection with anti-DIG-AP (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, CSPD chemiluminescence (Roche Diagnostics, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma-Aldrich. St Louis, USA).

2.3.4 Determination of mineral levels

Dehusked seeds of wild type and transgenic lines were polished using a non metal contaminating polisher (Kett Electric laboratory, Tokyo, Japan). Polished seeds were ground using a pestle and mortar previously treated with 6.5% HNO_3. Samples were then dried at 70°C for 2 days (as described in Gomez-Galera et al 2012) and 300 mg portions were wet-ashed with 4.4M HNO_3, 6.5M H_2O_2 and double deionised H_2O (3:2:2) for 20 min at using a MLS 1200 Mega high performance microwave oven (Milestone, Sorisole, Italy).The selected digestion program started with 250W for 1 min, followed by 0 W for 3 min, 250W for 4 min, 400 W for 4 min and finally 600 W for 3 min. Following dilutions to 25 ml with double de-ionized water (metal free), metal concentrations were determined by inductively coupled plasma mass spectrometry (Agilent 7700X ICP-MS, Agilent Technologies, Santa Clara, USA).

2.3.5 Measurements of Nicotianamine and Deoxy mugenic Acid

Nicotianamine (NA) (98%, Hasegawa, Kawasaki, Japan), 2'-deoxy mugineic acid (DMA) (98%, Toronto Research Chemicals Inc, Toronto, Canada) and the internal standard nicotyl-lysine (Wada et al 2007) were used in the assays. All standards were dissolved in water to a concentration of 1–10 mM and stored in darkness at –80°C. Working standard solutions were prepared by diluting the stock solutions with water. Before injecting into the HPLC/ESI-MS(TOF), 5 µl of standard solutions were diluted with 5 µl of 50 mM EDTA, 5 µl of nicotyl-lysine and 30 µl of a solution of 10 mM ammonium acetate/acetonitrile (10/90) at pH 7.3 and filtered through polyvinylidene fluoride (Durapore® PVDF) 0.45 µm ultrafree-MC centrifugal filter devices (Merck KGaA, Darmstadt, Germany).

For NA and DMA analysis, seeds were first ground to a fine powder in liquid N_2. For each sample, the seed powder was extracted three times (Wada et al 2007, with modifications). Briefly, NA and DMA were extracted from 15 mg seed powder with 300 µl double-deionized water and 18 µl of 1000 µM nicotyl-lysine. The supernatant was recovered by centrifugation (15,000 g, 4ºC and 15 min) and stored at –20°C, and the pellet was re-suspended twice in 300 µl water, homogenized and the supernatant was recovered. The three supernatant fractions were pooled and the total extract was passed through a 3-kDa centrifugal filter (regenerated cellulose Amicon® Ultra filter units, Merck). The filtered solution obtained by centrifugation at 15,000 g for 30 min was concentrated under vacuum until dry. The residue was dissolved in 5 µl water, diluted with 10 µl 50 mM EDTA, 15 µl water and 30 µl 10 mM ammonium acetate/acetonitrile (10/90) at pH 7.3 and filtered through polyvinylidene fluoride 0.45 µm ultrafree-MC centrifugal filter devices (Durapore, Merck) before injecting into the HPLC/ESI-MS (TOF).

Nicotianamine and DMA were determined in the seed extracts using a modified version of the method developed by Xuan et al (2006). Separations were performed with an Alliance 2795 HPLC system (Waters, Mildford, MA, USA) using a micro LC column (SeQuant ZIC®-HILIC, 15 cm x 1 mm i.d., 5 µm, 200 Å, Merck KGaA), with a mobile phase consisting of a gradient obtained using 10 mM ammonium acetate/acetonitrile (10/90) at pH 7.3 (solvent A) and 30 mM ammonium acetate/acetonitrile (80/20) at pH 7.3 (solvent B) at a flow rate of 0.15 ml min^{-1}. The gradient program started at 100% of A for 3 min, and then decreased linearly to 30% A for 7 min. These conditions were maintained for 7 min and then returned to the initial conditions in 8 min. The column was then allowed to stabilize for 10 min at the initial conditions before proceeding to the next injection. The injection volume was 10 µl and the autosampler and column temperatures were 6 and 30ºC, respectively. The HPLC was coupled to a time-of-flight mass spectrometer (MicrOTOF, Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) source. The ESI-MS (TOF) operating conditions were optimized by direct injection of 100 µM solutions of NA and DMA standards at a flow rate of 180 µl h^{-1}. Mass spectra were acquired in negative ion mode in the range of 150-700 mass-to-charge ratio (*m/z*) units. The mass axis was calibrated externally and internally using Li–formate adducts [10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol]. Bruker Daltonik software packages micrOTOF Control v.2.2, HyStar v.3.2 and Data Analysis v.4.0 were used to control the ESI-MS (TOF) apparatus, interface the HPLC with the MS system and process data, respectively. Concentrations of NA and DMA were always quantified by external calibration with internal standardization using nicotyl-lysine as internal standard.

2.3.6 Fe bioavailability

Seeds of the transgenic line which accumulated the highest levels of iron in the endosperm and wild type rice seeds were polished using a non metal contaminating polisher (Kett, North Yorkshire, United Kingdom). Five hundred mg of polished seeds were washed twice with double deionised water. Seeds were then cooked at 121ºC for 15 minutes and samples were homogenized using a polytron homogenizer. Caco2 cell culture and in vitro digestion assay was performed according to Glahn et al 1998. Total protein from cells was measured by the Bradford method and the concentration of the iron bioavailability marker ferritin was measured by an ELISA kit (Abcam, Cambridge, United Kingdom). In order to confirm the amount of transferable iron in the cells, transferrin protein concentration was also measured by ELISA (Abcam, Cambridge, United Kingdom).

2.3.7 Statistical analysis

Differences between transgenic and wild type plants were tested by comparison of means using the *t* test ($P<0.05$).

2.4 Results

2.4.1 Recovery of transgenic plants

Particle bombardment was used to co-transform mature seed-derived-callus with *OsIRT1* (iron regulated transporter), *OsNAS1* (nicotianamine synthase), *HvYS1* (Fe-III-DMA transporter), *HvNAATb* (nicotianamine amino transferase) genes together with the hygromycin phosphotransferase (*HPT*) selectable marker gene. I recovered ca: 450 independent hygromycin resistant transgenic lines. A total of 176 lines were fertile.

2.4.2 Expression analysis and identification of combinatorial population of transgenic lines expressing different transgene complements

Expression analysis for *OsIRT1, HvYS1, OsNAS1* and *HvNAATb* was carried out in all the fertile transgenic lines I recovered using ribosomal RNA isolated from leaf tissue of the T_0 generation (Figure 2.2). mRNA blot analysis using probes specific for *OsIRT1, HvYS1, OsNAS1, HvNAATb*, respectively, resulted in the identification of 146 transgenic plants expressing 13 different combinations of the input transgenes (Table 2.2). Although 30 transgenic lines out of 176 transgenic lines did not express any of the genes of interest (accounting for 17% of plants), considering the stringency of hygromycin selection they were assumed to be positive for the expression of hygromycin phosphotransferase alone. This is the normal frequency of recovering transgenic plants containing only the selectable marker gene in combinatorial transformation experiments (P. Christou, personal communication). Among the 146 transgenic lines expressing different combinations of transgenes, 68 lines expressed only single transgenes (*OsIRT1*, *HvYS1*, *OsNAS1*, *HvNAATb*); 57 lines co-expressed two transgenes: (*OsIRT1+HvYS1*, *OsIRT1+HvNAATb*, *OsIRT1+OsNAS1*, *HvYS1+OsNAS1*, *OsNAS1+HvNAATb*); 17 lines co-expressed 3 transgenes: (*OsIRT1+OsNAS1+HvNAATb*, *HvYS1+OsNAS1+HvNAATb*, *OsIRT1+HvYS1+OsNAS1*) and only 4 lines co-expressed the full complement of four genes: (*OsIRT1+HvYS1+OsNAS1+HvNAATb*. Seeds from these lines were advanced to the next generation for seed multiplication to perform further in depth analyses as described in subsequent chapters of the thesis.

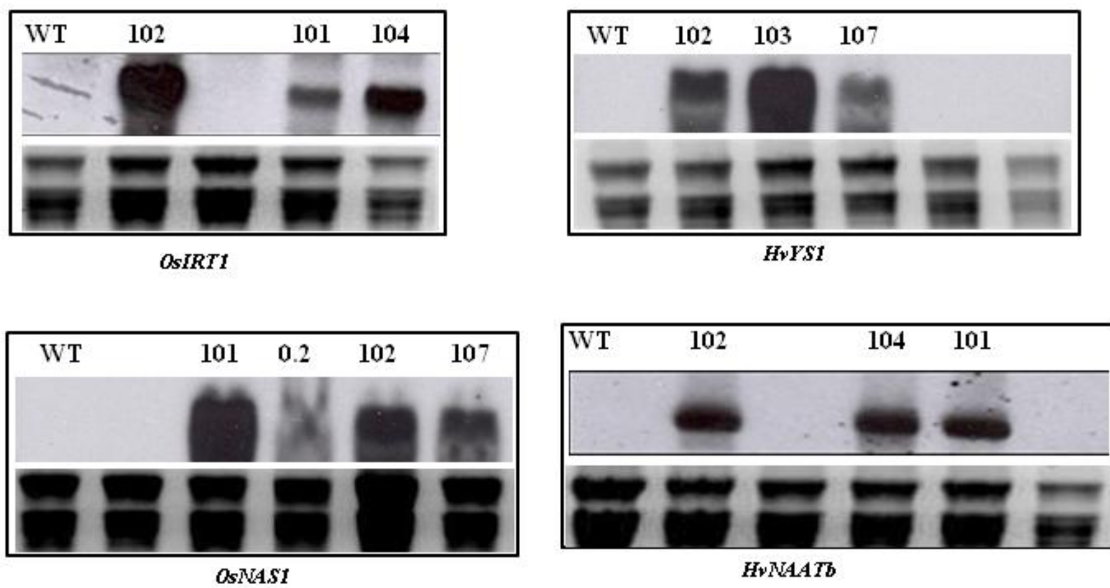

Figure 2.2 Representative mRNA blots showing expression of *OsIRT1*, *HvYS1*, *OsNAS1*, *and HvNAATb* in transgenic lines. The gaps on the membranes represent no expression of input genes of interest in particular lines.

Table 2.2 Population of transgenic plants expressing different combinations of input transgene

<table>
<tr><th>Expressing transgenes</th><th>Number of independent fertile lines</th><th>Total number of lines expressing one, two, three or four input transgenes</th></tr>
<tr><td>OsNAS1</td><td>23</td><td rowspan="4">68</td></tr>
<tr><td>HvNAATb</td><td>21</td></tr>
<tr><td>OsIRT1</td><td>12</td></tr>
<tr><td>HvYS1</td><td>12</td></tr>
<tr><td>OsNAS1+HvNAATb</td><td>35</td><td rowspan="5">57</td></tr>
<tr><td>OsIRT1+HvNAATb</td><td>4</td></tr>
<tr><td>OsIRT1+OsNAS1</td><td>10</td></tr>
<tr><td>OsIRT1+HVYS1</td><td>3</td></tr>
<tr><td>HvYS1+OsNAS1</td><td>5</td></tr>
<tr><td>OsIRT1+OsNAS1+HvNAATb</td><td>6</td><td rowspan="3">17</td></tr>
<tr><td>HvYS1+OsNAS1+HvNAATb</td><td>9</td></tr>
<tr><td>OsIRT1+HvYS1+OsNAS1</td><td>2</td></tr>
<tr><td>OsIRT1+OsNAS1+HvNAATb+HvYS1</td><td>4</td><td>4</td></tr>
<tr><td>Selectable marker alone</td><td>30</td><td>-</td></tr>
</table>

2.4.3 Fe and Zn accumulation in polished seeds of transgenic lines

2.4.3.1 Fe accumulation in polished seeds

I quantified Fe levels in polished seeds (T_2 generation) of the transgenic population (see Table 2.2). Transgenic lines expressing the transporters *OsIRT1* or *HvYS1* individually had a 2.6 and 2.5-fold increase in Fe levels compared to wild type, respectively (Table 2.3). Co-expression of *OsIRT1* and *HvYS1* improved the levels of Fe further up to 3.5-fold compared to the wild type (Table 2.3). These results suggest that expression of transporters only leads to a slight increase in Fe levels in endosperm.

Similarly to the effect of individual transporters on the amount of Fe accumulation in the polished seeds, expression of the phytosiderophore-encoding *HvNAATb* gene resulted in 3.2-fold increase in Fe levels over wild type. Co-expression of *HvNAATb* and the *OsIRT1* transporter resulted in a 3.6-fold increase in Fe levels over wild type (Table 2.4). This suggests that expression of *HvNAATb* alone or in combination with an Fe transporter only leads to a marginal increase in Fe levels in the polished seeds of the transgenic lines.

Transgenic lines expressing a second phytosiderophore-encoding transgene, *OsNAS1* had much higher levels of Fe accumulation in the endosperm, i.e. 6.3-fold over wild type (Table 2.5). In contrast, co-expression of *OsNAS1* with *OsIRT1* or *HvYS1* did not result in further increase in Fe levels. Co-expression of *OsNAS1* and *OsIRT1* resulted in 6.5-fold increase in Fe levels over wild type and co-expression of *OsNAS1* and *HvYS1* resulted in a 6 fold-increase (Table 2.5). When *OsNAS1* co-expressed with *OsIRT1* and *HvYS1* levels of Fe accumulation increased to 7.4-fold higher over the wild type in the triple transgenic lines (Table 2.5).

Among all transgenic lines, lines co-expressing *OsNAS1* and *HvNAATb* had the highest amount of Fe in polished seeds compared to lines expressing other combinations of transgenes (Table 2.6). Lines co-expressing *OsNAS1*and *HvNAATb* had a 12-fold increase in Fe endosperm content over wild type (Table 2.6), suggesting that co-expression of these two transgenes resulted in a synergistic effect that enhanced Fe endosperm content further. Such increases were not measured in lines which co-expressed *OsNAS1* and *HvNAATb* with any of the metal transport encoding transgenes. Thus lines co-expressing *OsNAS1+HvNAATb+OsIRT1* had a 5.6-fold increase in endosperm Fe over wild type (Table 2.6); lines co-expressing *OsNAS1+HvNAATb+OsHvYS1* had an 8.3-fold increase in Fe content (Table 2.6), and lines co-expressing *OsNAS1+HvNAATb+OsIRT1 +HvYS1* only had a 5.2-fold increase in endosperm Fe over wild type (Table 2.6). These results are consistent with an antagonistic relationship between *OsNAS1+HvNAATb* and the transporters in terms of Fe accumulation in the endosperm. Thus, the amount of Fe accumulation in the endosperm of the transgenic lines does not necessarily depends upon the number of input transgenes; rather synergistic mechanisms involving particular transgenes seem to determine Fe levels in the rice endosperm.

2.4.3.2 Zn accumulation in polished seeds

To further understand whether transgenic lines accumulate higher level of Zn in addition to Fe I measured the levels of Zn in polished seeds of the transgenic lines. In transgenic lines expressing the *OsIRT1* transporter Zn levels were 1.7-fold higher over the wild type (Table 2.3). These results suggest that *OsIRT1* can co-transport Zn along with Fe, but *OsIRT1* expression can only contribute to marginal improvement in Zn levels. However, lines expressing the Fe (III)-DMA transporter *HvYS1* did not have any increased levels of Zn. This is not surprising as *HvYS1* is known to be selective for Fe (Table 2.3). Lines

expressing *OsIRT1* had a higher level of Zn (1.7-fold higher over wild type); however, when *OsIRT1* was co-expressed with *HvYS1* in the same transgenic lines, there was no difference in the accumulation of Zn in the transgenic lines compared to wild type (Table 2.3). This suggest that Zinc levels in transgenic lines expressing Fe (III)-DMA transporter *HvYS1* either alone or in combination with *OsIRT1* did not influence Zn levels in the seeds.

Similarly to transgenic lines expressing *HvNAATb* alone or in combination with *OsIRT1* which only exhibited a slight but statistically significant increase in Fe levels, Zn levels in lines expressing *HvNAATb* were 1.9-fold higher compared to the wild type (Table 2.4). In lines co-expressing *HvNAATb* and *OsIRT1* had a 1.5 fold increase in Zn over wild type (Table 2.4). Thus, Zn levels in lines expressing *HvNAATb* alone or in combination with *OsIRT1* were not much higher compared to the wild type.

Transgenic lines expressing *OsNAS1* alone and in combination with the metal transporters *OsIRT1* and *HvYS1,* had higher levels of Zn (Table 2.5). Transgenic lines expressing *OsNAS1* contained 3.3-fold more Zn over wild type (Table 2.5). The data indicate that among the four transgenes used in the combinatorial experiments, *OsNAS1* appears to be key in terms of influencing positively to increase Zn levels in seed endosperm. Transgenic lines co-expressing *OsNAS1* and *HvYS1* accumulated similar level of Zn compared to lines only expressing *OsNAS1* due to the high selectivity of *HvYS1* for Fe (Table 2.5). Lines co-expressing *OsNAS1* and *OsIRT1* or *OsIRT1+HvYS1* accumulated slightly higher levels of Zn (4.2-fold and 4.1-fold, respectively, over the wild type).

Similarly to the situation with Fe transgenic lines co-expressing *OsNAS1* and *HvNAATb* had highest levels of Zn over wild type (Table 2.6). Transgenic lines expressing this combination had 5.3 fold higher Zn levels over the over wild type. This is the highest level of Zn measured in all the transgenic lines. In contrast lines which expressed the

transporters *OsIRT1* or *HvYS1* individually or in combination with *OsNAS1+HvNAATb* had even lower levels of Zn in the seed endosperm compared to lines co-expressing *OsNAS1* and *HvNAATB* (Table 2.6). Transgenic lines co-expressing *HvYS1* and *OsNAS1+HvNAATb* had 3.1-fold higher levels of Zn compared to the wild type (Table 2.6). Lines co-expressing *OsIRT1* and *OsNAS1+HvNAATb* had a 3-fold increase in Zn over wild type (Table 2.6). Zn levels in lines co-expressing *OsIRT1, HvYS1, OsNAS1,* and *HvNAATb* were 4.5-fold higher over wild type (Table 2.6).

2.4.4 NA and DMA level in lines expressing nicotianamine synthase and nicotianamine aminotransferase

Quantification of Fe and Zn levels in polished seeds of the combinatorial transgenic rice population shows that phytosiderophore biosynthetic genes (*OsNAS1* and *HvNAATb*) (Figure 2.3) appear to exert the most influence in terms of enhancing levels of Fe and Zn in the endosperm. I quantified the corresponding metabolites, NA and DMA in transgenic lines expressing *OsNAS1* and *HvNAATb* either individually or in combination. I also measured NA and DMA levels in lines which co-expressed *OsNAS1* and/or *HvNAATb* in combination with metal transporters.

In transgenic lines expressing *HvNAATb* alone or *HvNAATb* with *OsIRT1,* DMA accumulated in polished seeds (Table 2.4) most likely because of the conversion of NA to DMA. When expressed alone *HvNAATb* resulted in a 10-fold lower accumulation of NA and a 4-fold higher level of DMA over wild type in polished seeds (Table 2.4). Lines co-expressing *HvNAATb* and *OsIRT1* exhibited a similar trend for NA and DMA accumulation, resulting in 18-fold lower NA and 3-fold higher DMA levels in polished seeds (Table 2.4). Thus, our results suggest that in lines expressing only *HvNAATb* there is

a rather limited pool of NA to be converted to DMA, resulting in a very limited increase in DMA levels in these lines.

In contrast transgenic lines expressing *OsNAS1* alone or in combination with metal transporters there was a simultaneous increase in NA and DMA levels (Table 2.5). Lines expressing *OsNAS1* alone had a 456-fold and a 38-fold increase in NA and DMA over wild type, respectively (Table 2.5). In lines in which *OsNAS1* was co-expressed with *OsIRT1*, NA and DMA level were 145-fold and 48-fold higher in the transgenic lines over wild type, respectively (Table 2.5). In lines co-expressing *OsNAS1* and *HvYS1* NA and DMA levels were 166-fold 29-fold higher over wild type (Table 2.5). Similarly in transgenic lines co-expressing *OsNAS1* with *OsIRT1* and *HvYS1,* NA and DMA levels increased 378-fold and 15-fold, respectively (Table 2.5). These results suggest that conversion of S-adenosyl methionine to NA catalysed by *OsNAS1* is very important for the efficient synthesis of the important phytosiderophores NA and DMA.

Further NA and DMA analysis indicated that lines co-expressing *OsNAS1* and *HvNAATb* had the highest levels of NA and DMA (Table 2.6). In these lines NA levels were 2,031-fold higher and DMA levels were 142-fold higher compared to the wild type in polished seeds (Table 2.6). The levels of NA and DMA in lines co-expressing *OsNAS1* and *HvNAATb,* compared with lines that express individually *OsNAS1* or *HvNAATb* suggest that co-expression of the two genes results in a synergistic effect on the synthesis and accumulation of NA and DMA in seeds. In transgenic lines co-expressing *OsNAS1+HvNAATb* with *OsIRT1*, NA and DMA levels were 133-and 26-fold higher over wild type in polished seeds, respectively (Table 2.6). Lines co-expressing *OsNAS1+HvNAATb* with *HvYS1* had 122-fold higher NA and 36-fold higher DMA levels in seeds (Table 2.6). When both transporters (*OsIRT1* and *HvYS1*) co-expressed in the

same lines with *OsNAS1* and *HvNAATb*, the amounts of NA and DMA in the polished seeds were 304 and 35-fold higher over wild type, respectively (Table 2.6). These results suggest that a negative feedback mechanism might be in operation when metal transporters are co-expressed with *OsNAS1* and *HvNAATb*. This mechanism appears to negatively impact the overall accumulation of NA and DMA in the polished seeds of the transgenic lines.

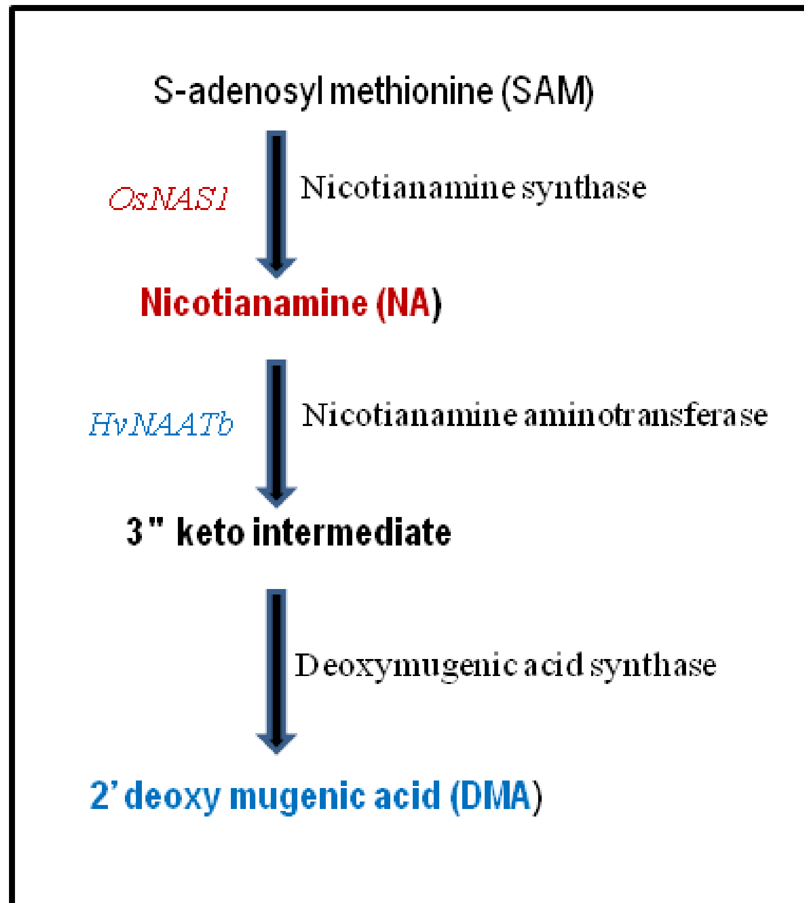

Figure 2.3 Endogenous phytosiderophore synthesis pathway in rice and genetic engineering for higher level of NA and DMA. Phytosiderophores; nicotianamine (NA) and deoxy mugenic acid (DMA) are synthesized from S-adenosyl methionine (SAM). Trimerization of SAM by nicotianamine synthase leads to nicotianamine (NA), which is subsequently converted to a 3" keto intermediate through an amino group transfer by nicotianamine aminotransferase. Subsequent reduction of the 3" keto intermediate by deoxy mugenic acid synthase leads to deoxy mugenic acid (DMA). Transgenic over expression of *OsNAS1* and *HvNAATb* boosts the production of NA and DMA in seeds of transgenic lines. This in turn translates into higher levels of Fe and Zn in seeds of the transgenic lines compared to the wild type.

Table 2.3 Concentration of Fe and Zn (µg/g DW) in the polished seeds of wild type and lines expressing metal transporters, *OsIRT1* and *HvYS1* either alone or in combination

Expressing transporter	Line	Iron (µg/g DW)	Zinc (µg/g DW)
-	WT	1.91±0.27	17.95±2.2
OsIRT1	RB-EYI-32	4.92±1.34*	31.06±2.04*
	RB-EYI-2	4.26±0.93	31.24±0.33*
	RB-EYI-41	4.01±0.11*	27.51±0.18*
	RB-EYI-13	3.88±1.22*	25.16±0.43*
	RB-EYI-81	3.33±0.85*	26.52±1.08*
HvYS1	RB-EYI-19	4.72 ± 0.52*	22.56 ± 0.42
	RB-EYI-3	4.66±0.45*	23.387±1.08
	RB-EYI-179	4.31±0.04*	17.25±0.91
	RB-EYI-16	3.86±0.686*	16.84±1.54
	RB-EYI-161	3.40 ± 0.50*	23.13± 0.05
OsIRT1+ HvYS1	RB-EYI-11	6.88±0.51*	22.23±0.33
	RB-EYI-14	5.73±0.37*	16.66±0.12
	RB-EYI-15	5.26±0.32*	14.62±0.31

The data represents means±standard errors. The symbol asterisks indicate a statistically significant difference between wild type and transgenic lines determined by Student's t test ($p<0.05$), n=6. DW: dry weight, WT-wild type

Table 2.4 Concentration of Fe, Zn, NA and DMA in the polished seeds of wild type and lines expressing *HvNAATb* and*OsIRT1+HvNAATb*

Expressing transgene	Line	Iron (µg/g DW)	Zinc (µg/g DW)	NA (µg/g FW)	DMA (µg/g FW)
-	WT	1.91±0.27	17.95±2.2	2.17±0.33	5.57±0.15
HvNAATb	RB-EYI-198	6.22±1.9*	29.56±0.44*	0.23±0.07*	23.88±4.30*
	RB-EYI-72	6.16±0.67*	32.39±4.6*	0.24±0.05*	16.06±0.05*
	RB-EYI-63	5.47±2.1*	27.06±8.4*	0.23±0.02*	11.70±1.26*
	RB-HNN-96	6.00±1.14*	32.28±0.02*	0.42±0.018*	12.38±2.08*
	RB-HNN-49	6.02±1.15*	22.9±0.24*	NA	NA
	RB-HNN-0.4	5.93±0.19*	22.65±0.68*	NA	NA
	RB-HNN-85	5.55±1.1*	35.50±0.87*	NA	NA
	RB-HNN-67	4.89±0.66*	33.24±0.32*	NA	NA
	RB-EYI-59	4.09±0.68*	26.13±0.12*	NA	NA
OsIRT1+HvNAATb	RB-EYI-33	7.05±2.07*	27.11±0.16*	0.25±0.07*	16.15±1.55*
	RB-EYI-84	5.994±1.45*	24.42±2.5*	0.56±0.01*	10.5±0.49*
	RB-EYI-104	6.43±1.09*	28.58±1.33*	0.11±0.01*	6.11±0.74*
	RB-EYI-46	5.11±1.05*	23.63±1.37*	NA	NA

The data represents means±standard errors.The symbol asterisks indicates a statistically significant difference between wild type and transgenic lines determined by Student's t test ($p<0.05$), n=6. DW: dry weight, WT-wild type

Table 2.5 Concentration of Fe, Zn, NA and DMA in polished seeds of wild type and lines expressing *OsNAS1*, *OsNAS1+OsIRT1*, *OsNAS1+HvYS1*, and *OsNAS1+OsIRT1+HvYS1*

Expressing transgene	Line	Iron (µg/gDW)	Zinc (µg/gDW)	NA (µg/g FW)	DMA (µg/g FW)
-	WT	1.91±0.27	17.95±2.2*	2.17±0.33	5.57±0.15
OsNAS1	RB-EYI-267	12.05±2.5*	60.40±4.8*	987.7±5.42*	210±6.4*
	RB-HNN-56	10.78±0.67*	64.25±2.2*	832.1±9*	94.3±1.2*
	RB-HNN-60	10.89±1.23*	49.54±0.60*	323±5.7*	71.1±2.1*
	RB-EYI-234	10.32±0.46*	53.23±0.47*	249±1.7	41.3±1.5*
	RB-EYI-38	8.47±1.13*	40.12±0.47*	134±2.4*	33.8±1.92*
	RB-HNN-0.2	7.41±0.92*	40.29±0.58*	109±1.05*	27.7±1.76*
	RB-EYI-216	6.13±0.66*	54.46±2.04*	NA	NA
	RB-HNN-89	6.7±1.08*	57.67±0.2*	NA	NA
	RB-HNN-0.9	6.45±0.76*	50.74±1.0*	NA	NA
	RB-HNN-0.7	5.99±0.82*	41.70±2.63*	NA	NA
OsIRT1+OsNAS1	RB-HINZ-39	12.35±1.41*	77.03±0.58*	313±14.8*	268±4.62*
	RB-EYI-274	8.39±1.63*	66.61±0.44*	161.45±2.76*	109±2.89*
	RB-HINZ-3	8.01±1.25*	61.84±1.63*	71.31±2.76*	60.95±0.45*
	RB-HINZ-59	7.18±0.37*	57.45±1.52*	NA	NA
	RB-ZN-3	7.22±1.44*	25.33±0.54*	NA	NA
	RB-HINZ-55	4.56±0.32*	34.97±0.68*	NA	NA
HvYS1+OsNAS1	RB-HINZ-57	12.76±1.43*	40.35±0.79*	359±7.5*	162±13.4*
	RB-HINZ-58	11.33±0.84*	57.45±1.51*	215±7.76*	110±25.4*
	RB-HINZ-56	8.20±0.57*	51.70±1.32*	117±6.21*	68.7±15.9*
OsIRT1+HvYS1+ OsNAS1	RB-HINZ-4	14.24±0.5*	74.03±0.58*	818.48±16*	85.59±3.52*
	RB-HINZ-52	8.20±0.57*	66.01±2.45*	691±48*	69.42±9.4*
	RB-HINZ-11	7.9±0.8*	58.77±2.01*	NA	NA

The data represents means±standard errors. The symbol asterisks indicates a statistically significant difference between wild type and transgenic lines determined by Student's t test ($p<0.05$), n=6. DW: dry weight, WT-wild type

Table 2.6 Concentration of Fe, Zn, NA and DMA in polished seeds of wild type (WT) and lines expressing *OsNAS1+HvNAATb*, *OsNAS1+HvNAATb+OsIRT1*, *OsNAS1+HvNAATb+HvYS1*, and *OsNAS1+HvNAATb+OsIRT1+HvYS1*

Expressing transgene	Line	Iron (µg/g DW)	Zinc (µg/gDW)	NA (µg/g FW)	DMA (µg/g FW)
-	WT	1.91±0.27	17.95±2.2	2.17±0.33	5.57±0.15
OsNAS1+HvNAATb	RB-HNN-0.1	23.78±1.03*	96.49±6.99*	2199±155*	795.93±39.65*
	RB-HNN-0.6	20±0.78*	80.67±2.23*	2063±52.2*	360.41±47.92*
	RB-HNN-23	18.1 ± 0.33*	74.9±7.2*	1681±228*	275.98±22.72*
	RB-HNN-0.5	14.1±2.13*	72.9±2.1*	1171±53*	260.55±7.52*
	RB-HNN-92	14.28 ± 1.21*	68.4±0.3*	NA	NA
	RB-HNN-10	14.68±3.40*	45.43±0.39*	NA	NA
	RB-HNN-13	9.26±1.22*	49.52 ± 0.75*	NA	NA
	RB-HNN-4	8.6±2.5*	53±1.59*	NA	NA
	RB-HNN-18	7.92±0.15*	36.48±0.8*	NA	NA
	RB-HNN-3.1	5.96±1.19*	45.85±0.88*	NA	NA
	RB-HNN-2.1	5.83±1.29*	46.34±2.36*	NA	NA
	RB-HNN-4	5.87±0.65*	24.76±0.97*	NA	NA
	RB-HNN-0.8	5.53±1.72*	15.94±1.28	NA	NA
	RB-HNN-0.9	6.4±0.28*	20.79±0.76	NA	NA
	RB-HNN-21	5.64±1.7*	22.52±3.6	NA	NA
OsIRT1+OsNAS1+ HvNAATb	RB-EYI-33	10.8±0.3*	54.5±4.37*	265.37±15.52*	143.73±1.98*
	RB-EYI-36	10.2±0.1*	56.50 ± 0.54*	226.37±16.93*	119.75±1.79*
	RB-EYI-51	7.5±0.2*	44.4±1.2*	133.89±0.18*	68.92±0.56*
HvYS1+OsNAS1+ HvNAATb	RB-HNN-29	15.8±0.2*	57.8±0.7*	248.83±2.62*	201.20±7.94*
	RB-HNN-64	13.6±0.1*	54.3±0.4*	188.32±8.31*	37.28±1.43*
	RB-HNN-31	7.6±0.1*	48.5±0.7*	37.63±1.7*	16.90±0.98*
OsNAS1+OsIRT1+ HvNAATb+HvYS1	RB-EYI-102	10.3±0.1*	63.2±0.59*	608.94±53*	191.90±7.9*
	RB-EYI-156	9.56±1.58*	56.42±0.49*	454.7±8.03*	29.30±0.52*
	RB-EYI-112	8.6±0.69*	59±0.96*	NA	NA

The data represents means±standard errors. The symbol asterisks indicate a statistically significant difference between wild type and transgenic lines determined by Student's t test ($p<0.05$), n=6. DW: dry weight, WT-wild type

2.4.5 Fe bioavailability

Analysis of transgenic lines for Fe and Zn content and measurements of NA and DMA levels indicated that co-expression of *OsNAS1* and *HvNAATb* is important in order to increase the levels of Fe and Zn in rice seeds. To determine whether increased level of Fe in transgenic lines leads to increased bioavailability I measured the bioavailability of Fe in the highest accumulating transgenic line using Caco-2 cells (Glahn et al 1998). To estimate the amount of bioavailable and transferable Fe levels in transgenic lines, the corresponding marker proteins, ferritin and transferrin, were measured. In comparison with Caco-2 cells fed with MES media (experimental control) and extracts from wild type, Caco-2 cells fed with seed extracts from the transgenic lines accumulated significantly higher levels of ferritin and transferrin. Caco-2 cells treated with MES formed 10 ng ferritin/mg protein (Figure 2.6).When the cells were treated with seed extract from wild type ferritin levels were 16 ng/mg protein (Figure 2.6). Whereas, ferritin levels in Caco-2 cells treated with seed extract from transgenic lines were 33-34 ng/mg protein (Figure 2.6), which is 2-fold higher compared to Caco-2 cells treated with seed extract from wild type. Thus, our results indicate that Fe in the transgenic lines is in a bioavailable form.

Transferrin levels in Caco-2 cells treated with MES were 67 ng/mg protein. Levels of transferrin in Caco-2 cells treated with seed extract from wild type seeds were 77 ng /mg protein (Figure 2.6), whereas transferrin levels in Caco-2 cells treated with seed eaxtract from transgenic lines were 96-102 ng/mg protein (Figure 2.6) suggesting that Fe in the transgenic lines is transferable, in addition to being bioavailable. This is important because transferability is a measure of Fe transport and distribution.

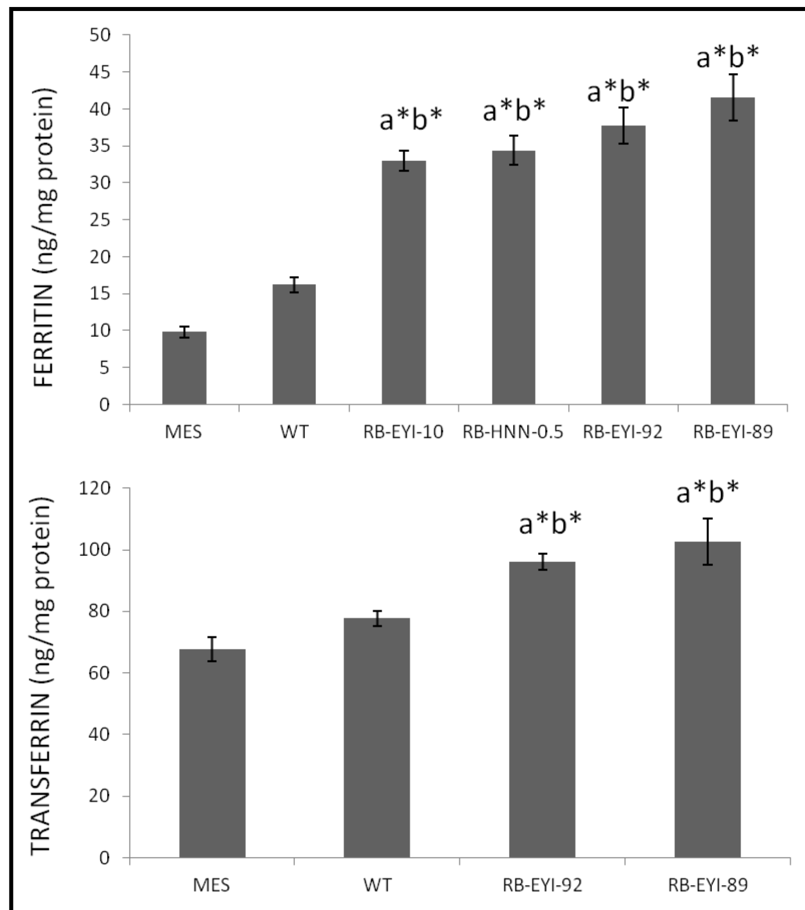

Figure 2.4 Concentration of ferritin and transferrin (ng/mg protein) in Caco-2 cells treated with MES media control, and seed extracts from wild type and transgenic lines as measured by ELISA. Caco-2 cells treated with seed extract from transgenic lines accumulated significantly higher level of ferritin and transferrin compared to Caco-2 cells treated with seed extract from the wild type. The data represents means±standard errors. a* statistically significant difference between MES control and transgenic lines as determined by Student's t test ($p<0.05$), n=6; b* statistically significant difference between wild type and transgenic lines as determined by Student's t test ($p<0.05$), n=6; MES: 2-(*N*-morpholino) ethanesulfonic acid.

2.5 Discussion

Rice varieties grown across the world contain 2-5 µg Fe /g DW and 16-25 µg Zn/g DW in the endosperm (Sperotto et al 2012). The RDI for Fe and Zn is 14 ppm per day/ per adult. In places where rice is the major staple crop prevalence of Fe deficiency anaemia (IDA) and Zn deficiency (ZnD) is therefore severe (Stein et al 2010; Gómez-Galera et al 2010). Biofortification of rice with Fe and Zn can offer a sustainable, long term solution to alleviate IDA and ZnD (Gómez-Galera et al 2010; Masuda et al 2012). Genetic engineering is an effective approach to increase the level of Fe and Zn in rice endosperm.

Since, Fe and Zn cannot be synthesized *de novo*, they have to be taken up from the soil (Vert et al 2002; Lee and An 2009), transported internally (Lee et al 2009; Suzuki et al 2008) and accumulated in seeds (Masuda et al 2012). This process involves a large number of genes and transcription factors (Sperotto et al 2012). When present at toxic levels Fe and Zn are detrimental to plant as well as human health. Homeostasis mechanisms regulating the levels of Fe and Zn are tightly regulated (Kobayashi et al 2010). For this reason, thus far efforts to enhance the levels of Fe and Zn in rice endosperm resulted in a maximum of 4 to 6-fold increase in Fe over wild type (Wirth et al 2009; Johnson et al 2011; Masuda et al 2012) and up to 2 to 3-fold in Zn over wild type (Johnson et al 2011; Lee et al 2011). These values translate to 14 and 76 µg/g DW, for Fe and Zn, respectively. Considering the complexity of Fe and Zn homeostasis and the number of genes involved in the process, there seems to be a ceiling in terms of the levels of the two micronutrients which prevents the accumulation of the two metals beyond the levels reported in seed endosperm (Sperotto et al 2012).

In order to understand the mechanisms that control Fe and Zn homeostasis in rice which limit their accumulation in the endosperm, I embarked on experiments in which I used

combinatorial genetic transformation to simultaneously introduce multiple transgenes involved in Fe and Zn homeostasis. The transgenes represent genes for Fe and Zn mobilisation in the soil through increased synthesis of phytosiderophores (*OsNAS1* and *HvNAATb*); transport of Fe into the plant by Fe (III)-DMA and Fe (II) regulated transporters; internal mobilisation of Fe and Zn through increased synthesis and seed loading of nicotianamine (*OsNAS1*) and deoxy mugenic acid (*HvNAATb*). I then analysed the expression of the introduced transgenes at the mRNA level and correlated this to metal levels in polished seeds.

Although, combinatorial nuclear genetic transformation has not been reported for Fe and Zn enhancement, it was utilized earlier to engineer corn with a number of carotenogenic genes (Zhu et al 2008; Naqvi et al 2010). Generation of a library of transgenic plants expressing transgenes in different combinations helped to understand key steps limiting carotenoid biosynthesis. This in turn, led to the identification of bottlenecks in carotenoids biosynthesis and accumulation, eventually leading to the development of lead transgenic events accumulating extraordinary levels of β carotene and other nutritionally important carotenoids (Zhu et al 2008; Naqvi et al 2010).

Through combinatorial nuclear genetic transformation with *OsIRT1*, *HvYS1*, *OsNAS1* and *HvNAATb* I recovered 176 fertile transgenic lines. When screened at the mRNA level 146 lines expressed input genes either individually, or in combinations of two, three and all four trangenes. This analysis allowed the identification of 13 different transgene combinations (Table 2.2): lines expressing input transgenes individually: *OsIRT1*, *HvYS1*, *OsNAS1*, *HvNAATb*; lines co-expressing two of the input transgenes: *OsIRT1+HvYS1*, *OsIRT1+HvNAATb*, *OsIRT1+OsNAS1*, *HvYS1+OsNAS1*, *OsNAS1+HvNAATb;* lines co-expressing three of the input transgenes: *OsIRT1+OsNAS1+HvNAATb*;

HvYS1+OsNAS1+HvNAATb; *OsIRT1+HvYS1+OsNAS1;* and lines co-expressing all input transgenes (*OsIRT1+HvYS1+OsNAS1+HvNAATb).*

Previous efforts to engineer rice with Fe and Zn homeostasis genes have been reported, but those efforts focussed either on the expression of metal transporters alone: *OsIRT1* (Lee and An et al 2009) or *OsYSL15* (Lee et al 2009) or genes involved in the internal mobilisation of metals, such as *OsYSL2* (Ishimaru et al 2010), *AtNAS1* (Wirth et al 2009), *HvNAS1* (Masuda et al 2009*), OsNAS1, OsNAS2, OsNAS3* (Johnson et al 2011), *HvNAS1+OsYSL2* (Masuda et al 2012). None of these reports achieved levels of Fe and Zn beyond the 4 and 3-fold ceiling, respectively, in the endosperm. Thus it appears as though there might still be unknown genes or mechanisms controlling this ceiling. My experiments, therefore, were designed to circumvent the laborious generation of transgenic rice plants with pre-determined transgene combinations by taking advantage of the ability of combinatorial transformation to generate a large populations of rice plants with diverse transgene combinations in a single experiment.

This combinatorial population helped to identify bottlenecks in Fe and Zn accumulation. Lines expressing the transporters *OsIRT1* and *HvYS1* individually or in combination had 2.5 to 3.5-fold increased levels of Fe in polished seeds over wild type (Table 2.3). Among these lines, only lines expressing *OsIRT1* alone had 1.7-fold higher levels of Zn in polished seeds over wild type (Table 2.3). Thus the behavior of *OsIRT1*-expressing lines is consistent with the ability of *OsIRT1* to transport both Fe and Zn (Vert et al 2002; Lee and An et al 2009). When over expressed in yeast, *HvYS1* was shown to be selective for Fe (III)-DMA but not Zn transport (Murata et al 2006 and 2008). Similarly, in our case expression of *HvYS1* did not result in Zn increases in polished seeds (Table 2.3). Our results confirm the selective Fe transport ability of HvYS1.

The phytosiderophore deoxy mugenic acid is important in the mobilisation, internal transport and accumulation of Fe and Zn in seeds (Ma et al 1999; Lee et al 2009; Masuda et al 2012). *HvNAATb* expression lead to increases in both Fe and Zn in lines expressing this transgene alone, or in combination with *OsIRT1* (Table 2.4). Although expression of *HvNAATb* under its native promoter was reported in rice, Fe and Zn levels were not enhanced in seeds of the resulting transgenic plants (Takahashi et al 2001). The difference between the earlier reports and our experiments might be due to the use of the strong constitutive maize ubiquitin1 promoter in our transgenic lines, which indeed had higher levels of Fe (3.2-fold over wild type) and Zn (1.9-fold over wild type) in the endosperm. Though expression of: *OsIRT1* increased levels of Fe and Zn (2.6 and 1.7-fold over wild type, respectively), *HvYS1* increased levels of Fe alone (2.5-fold over wild type), *OsIRT1+HvYS1* increased Fe alone (3.5-fold over wild type), *HvNAATb* expression increased levels of Fe and Zn (3.2 and 1.9-fold over wild type) and *HvNAATb* in combination with *OsIRT1* increased Fe and Zn levels (3.6 and 1.5-fold over wild type) in the endosperm, but the fold increase in Fe was still lower than previous reports in which Fe and Zn levels were improved by 6-fold and 3-fold respectively (Wirth et al 2009; Lee et al 2011).Therefore, the ability of metal transporters *OsIRT1*, *HvYS1* alone or in combination with *HvNAATb* which convert NA into DMA; is limited in terms of enhancing Fe and Zn levels over levels reported already.

Among the combinatorial transgenic population, lines expressing *OsNAS1* either alone or in combination with the transporters *OsIRT1* and *HvYS1* (alone or in combination) had 6.3- to 7.5-fold increase in Fe, and 3.3- to 4.4-fold increase in Zn over wild type (Table 2.5). These lines had higher levels of Fe and Zn compared to lines expressing metal transporters (*OsIRT1*, *HvYS1*, *OsIRT1+HvNAATb*) or lines expressing *HvNAATb* alone or

HvNAATb+OsIRT1. Hence, *OsNAS1* appears to be important in terms of improving Fe and Zn levels. Similar results were reported through expression of nicotianamine synthase from arabidopsis (Wirth et al 2009), rice (Johnson et al 2011; Lee et al 2011) and barley (Masuda et al 2012). In these reports, it was shown that over expression of nicotianamine synthase gives rise to higher levels of both NA and DMA, leading to higher levels of Fe and Zn in seeds. This was particularly due to availability of a greater pool of S-adenosyl methionine (SAM) which is a substrate for nicotianamine synthase for its conversion to NA. Although *OsNAS1*-expressing lines from the combinatorial population had higher levels of Fe, the levels were similar to the already reported 4 to 6-fold increases in the levels for Fe (Johnson et al 2011; Wirth et al 2009; Masuda et al 2012). The situation for Zn was different in these lines, Zn levels in lines co-expressing *OsNAS1* with *OsIRT1* or *OsIRT1* and *HvYS1* were 4.2 fold higher over wild type in the endosperm. The levels were higher than 3-fold increase in Zn levels in lines expressing *OsNAS1* alone in our case and 3-fold increase in Zn levels reported through the over expression of nicotianamine synthase from rice and barley (Johnson et al 2011; Lee et al 2011; Masuda et al 2012). *HvYS1* is a selective Fe transporter (Murata et al 2006; 2008). In contrast *OsIRT1* can transport both Fe and Zn (Lee et al 2009; Connolly et al 2002).Thus, enhanced level of Zn in the endosperm of lines co-expressing *OsNAS1* with *OsIRT1* or *OsIRT1+HvYS1* can be attributed to Zn transport by *OsIRT1* in these lines. Interestingly, within the combinatorial population lines co-expressing *OsNAS1* and *HvNAATb* had up to 12-fold increases in Fe and 5.3-fold increases in Zn levels in the endosperm (Table 2.6). When expressed alone *HvNAATb* resulted in a 3-fold increase in Fe and a 1.6-fold increase in Zn endosperm levels. Lines expressing *OsNAS1* alone had 6.3 and 3.3-fold increase in Fe and Zn levels, respectively. The increases in Fe and Zn accumulation in lines co-expressing *OsNAS1* and *HvNAATb* can be attributed to a possible synergistic effect of the two transgenes. Although

such synergism has not been reported for *OsNAS1* and *HvNAATb*, it has been reported that co-expression of *HvNAS1* with *OsYSL2* and *Gmferritin* (Masuda et al 2012) and co-expression of *AtNAS1* with *Pvferritin* (Wirth et al 2009) had a synergistic effect on Fe accumulation in rice endosperm. In our lines co-expressing the two phytosiderophore biosynthetic genes, the level of Fe was 23 µg/g DW and that of Zn 96 µg/g DW ie 12 and 5.3-fold increase in Fe and Zn levels, respectively. These levels are the highest reported to date and they compare favourably with the 14 µg/g dry weight for Fe and 76 µg/g DW for Zn, reported by Johnson et al 2011. Thus, lines co-expressing *OsNAS1+HvNAATb* accumulated the highest levels of the two metals compared to what has been reported thus far for any transgene or transgene combination. Despite the ability of *OsNAS1* and *HvNAATb* to enhance levels of Fe and Zn to high levels in rice endosperm when co-expressed, when combined with transporters *OsIRT1* and *HvYS1* (individually or in combination) level of Fe and Zn in the resulting transgenic plants co-expressing three or four transgenes were lower than in lines co expressing *OsNAS1* and *HvNAATb*. It has been reported that over expression of *HvNAS1* results in enhanced levels of NA and DMA which modulated the expression of endogenous metal transporters responsible for Fe and Zn uptake, translocation in vegetative tissues, and accumulation in seeds (Masuda et al 2012). Therefore, it appears that expression of *OsIRT1* and *HvYS1* along with *OsNAS1+HvNAATb* possibly disturb the modulation of endogenous metal homeostasis genes. Hence, expression of *OsIRT1* and *HvYS1* along with *OsNAS1+HvNAATb* acts antagonistically for Fe and Zn accumulation in seeds. Our results confirm that Fe and Zn increases in seeds is independent of the number of input transgenes but it can be due to combination of transgenes which act synergistically for Fe and Zn accumulation. The phytosiderophores: NA and DMA are synthesized from the precursor molecule SAM. This involves three enzymes: nicotianamine synthase, nicotianamine aminotransferase and

deoxy mugenic acid synthase (Ma et al 1999). In our combinatorial transgenic population, transgenic lines expressing *HvNAATb* and *OsNAS1* individually or in combination exhibited changes in NA and DMA levels in polished seeds (Table 2.4; Table 2.5; Table 2.6). In lines in which *HvNAATb* expressed alone, NA level was 4 to 18-fold lower and DMA was increased to a modest 3 to 4-fold. Analysis of wild type rice seeds in our experiments as well as results reported by Takahashi et al 2001 and 2003 indicate that the available NA pool for *HvNAATb* to act upon is limited as a result there is a modest increase in DMA in lines expressing *HvNAATb*. Thus *HvNAATb*-expressing lines only had a limited increase in Fe and Zn levels.

Within the combinatorial population, in transgenic lines in which *OsNAS1* was expressed either alone or in combination with metal transporters, NA and DMA levels were up to 456- and 48-fold higher in seeds, respectively. Similarly, over expression of *AtNAS1*, *HvNAS1* and *OsNAS2* resulted in 20 and 5-fold increase in NA and DMA levels in seeds, respectively (Wirth et al 2009; Johnson et al 2011; Masuda et al 2012). Therefore, *OsNAS1* expression not only increases NA but also DMA levels, simultaneously. Thus conversion of SAM into NA by nicotianamine synthase appears to be key in terms of increasing NA and DMA levels in seeds. Therefore the increased levels of Fe and Zn in *OsNAS1*-expressing lines appear to be due to the substantially increased levels of NA and DMA. These results are consistent with results reported by Wirth et al 2009; Lee et al 2009; Masuda et al 2009; Lee et al 2011; Johnson et al 2011; Masuda et al 2012. Although *OsNAS1* expression resulted in 456- and 48-fold higher levels of NA and DMA in the seeds, respectively.When *OsNAS1* expressed in combination with the transporter *OsIRT1* or *HvYS1* (individually or in combination), the levels of these two metabolites were lower than in lines expressing *OsNAS1* alone. Lines co-expressing *OsNAS1* and *OsIRT1*

accumulated 145- and 48-fold higher level of NA and DMA in seeds, respectively. In lines which co-expressed *OsNAS1* and *HvYS1*, the level of NA and DMA was 166 and 29-fold higher in seeds, respectively. Similarly, co-expression of *OsNAS1* with *OsIRT1+HvYS1* resulted in 378- and 15-fold higher levels of NA and DMA in seeds, respectively. It was reported that NA and DMA synthesis in rice is increased under Fe and Zn deficiency (Suzuki et al 2008). In contrast, Fe and Zn sufficiency reduces NA and DMA synthesis to limit Fe and Zn uptake and translocation (Kobayashi et al 2004). Therefore, it appears that transport of Fe (*HvYS1*) as well as Fe and Zn (*OsIRT1*) by these transporters results in iron sufficiency condition which negatively influences NA and DMA levels, limiting the amounts of Fe and Zn accumulation in seeds.

In addition to substantially increased levels of Fe and Zn, lines co-expressing *OsNAS1* and *HvNAATb* had extra-ordinary levels of NA and DMA. These lines accumulated 2031- and 142-fold higher NA and DMA in seeds, respectively. It appears that *OsNAS1* and *HvNAATb* act synergistically in these lines to produce much higher levels of NA and DMA. Although nicotianamine synthase and nicotianamine amino transferase were expressed in rice independently previously (Takahasi et al 2001; Wirth et al 2009; Lee et al 2009; Masuda et al 2009; Lee et al 2011; Johnson et al 2011; Masuda et al 2012) the co-expression of the two genes had not been investigated. Our results confirm that the higher level of Fe and Zn in these lines compared to lines expressing other transgene combination might be attributed to the combined ability of *OsNAS1* and *HvNAATb* lines to synthesize extraordinary levels of NA and DMA. Johnson et al 2011 and Masuda et al 2012 reported a positive correlation between NA and DMA levels, and levels of Fe and Zn in rice endosperm. Our results extend the findings reported earlier, but importantly we provide a mechanistic basis for these increases. Similarly to the situation discussed earlier where co-

expression of *OsNAS1* with metal transporters negatively influenced the levels of NA and DMA, lines co expressing *OsNAS1+HvNAATb* with metal transporters also had lower levels of NA and DMA compared to lines expressing only *OsNAS1* and *HvNAATb*. In lines co-expressing *OsNAS1+HvNAATb* and *OsIRT1*, NA and DMA levels in seeds were 132- and 26-fold higher, respectively. Lines co-expressing *OsNAS1+HvNAATb* and *OsIRT1* had 122- and 36-fold higher NA and DMA levels in seeds, respectively. Similarly, lines co-expressing *OsNAS1+HvNAATb* with *OsIRT1* and *HvYS1* had 304- and 35-fold higher NA and DMA in seeds, respectively. These results confirm the negative influence of co-expression of metal transporters and phytosiderophore genes on NA and DMA accumulation. Overall, our results confirm the pivotal role of NA and DMA in controlling levels of Fe and Zn in rice endosperm.

Four of the highest Fe accumulating lines co-expressing *OsNAS1* and *HvNAATb* were analysed for Fe bioavailability using Caco-2 cells. Intracellular ferritin formation in Caco-2 cells fed with extract from seeds of transgenic lines was 2-fold higher compared to cells fed with extract from seeds of the wild type. Similarly, intracellular transferrin formation in Caco-2 cells treated with extract from seeds of transgenic lines was significantly higher than transferrin formation in Caco-2 cells treated with extract from seeds of the wild type. This implies that Caco-2 cells treated with transgenic lines absorb more Fe and as a result ferritin synthesis is increased. Increased synthesis of transferrin in Caco-2 cells fed with extracts from transgenic lines is an indication of higher transferability of Fe. It was reported that NA and DMA promote Fe bioavailability (Zheng et al 2010; Eagling et al 2014).Therefore, transgenic lines co-expressing *OsNAS1* and *HvNAATb* not only had higher Fe levels but importantly the Fe was bioavailable. This is important because rice endosperm contains anti nutritional factors such as phytic acid and polyphenols which

sequester Fe into a non-bioavailable form. Thus increasing Fe concentration in seeds does not necessarily lead to increased Fe bio-availability (Drakakaki et al 2005). Hence, increasing endosperm Fe concentration in a bioavailable form is important. Thus, availability of higher amount of bioavailable Fe in *OsNAS1* and *HvNAATb* lines is very important.

2.6 Conclusions and future prospects

I have generated a diverse population of transgenic plants expressing different combinations of four transgenes involved in the uptake, translocation, and accumulation of Fe in rice endosperm. This combinatorial population allowed the identification of key synergistic and antagonistic mechanisms controlling Fe and Zn accumulation in rice endosperm. Co-expression of genes encoding *OsNAS1* and *HvNAATb* from the phytosiderophore synthesis pathway results in a synergistic effect on the synthesis of phytosiderophores NA and DMA leading to extraordinary levels in the endosperm. This, in turn leads to transgenic lines containing higher levels of Fe and Zn in the endosperm to what had been previously reported. When metal transporters are over expressed in *OsNAS1* and *HvNAATb* co-expressing lines, metal transporters act antagonistically on NA and DMA synthesis leading to lower levels of both NA/DMA and Fe/Zn. Further in depth analysis at transcript and metabolite levels in high Fe and Zn accumulating lines can help to develop a more in depth understanding of the mechanistic basis of this synergistic effect in these lines. Conducting field trials of high Fe and Zn accumulating lines would help to determine their agronomic performance prior to their deployment.

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Chapter 3

3 Enhanced accumulation of phytosiderophores reveals a threshold for iron accumulation in rice endosperm but allows toxic Cd to be replaced with Fe and Zn

3.1 Abstract

Rice endosperm shows little natural genetic variation for the levels of total iron (Fe) and zinc (Zn), and thus conventional breeding has proven unsuitable as a strategy to enhance the accumulation of these minerals. Metabolic engineering to boost the synthesis of the phytosiderophores nicotianamine (NA) and 2'-deoxymugenic acid (DMA) could offer an alternative approach to promote mineral accumulation in the endosperm by facilitating Fe and Zn uptake, as well as internal mobilization and seed loading of both metals. I therefore generated transgenic lines co-expressing rice nicotianamine synthase (*OsNAS1*) and barley nicotianamine amino transferase (*HvNAATb*) and recovered transgenic plants producing 165-fold more NA and 29-fold more DMA than the wild type. These plants also accumulated four-fold higher Fe and Zn levels in the endosperm. Feeding the plants with different amounts of Fe [100μM (standard), 200μM and 300μM] did not have a significant effect on Fe or Zn accumulation in wild type or transgenic lines, suggesting an intrinsic upper limit for metal accumulation in polished grains, reflecting strict Fe/Zn homeostasis in vegetative tissues and seeds. When grown in the presence of cadmium (Cd), transgenic plants accumulated less Cd in the endosperm than the wild type. Our data suggest that although Fe and Zn homeostasis limits the over-accumulation of Fe (up to 22μg g^{-1} DW) and Zn (up to 84μg g^{-1} DW) in endosperm, enhancement of seed loading with Fe and Zn also displaces Cd and thus increases the nutritional value of polished rice.

3.2 Aims

The aims of this study were to generate *OsNAS1* and *HvNAATb* co-expressing lines accumulating high level of Fe and Zn and to investigate: i) Fe accumulation in seeds in response to external levels of Fe; ii) impact of different levels of external Fe supply on Zn accumulation; and iii) Cd levels in seeds with high Fe and Zn content. Our data provide an insight into the existence of a ceiling for Fe and Zn accumulation in rice endosperm and the exclusion of Cd in transgenic lines with enhanced level of Fe and Zn.

3.3 Materials and methods

3.3.1 Rice transformation

Mature rice seed derived embryos (*Oryza sativa* L. cv EYI 105) were transformed with *OsNAS1*, *HvNAATb* and the *hpt* selectable marker by particle bombardment as described in chapter 2.

3.3.2 mRNA blot analysis

Expression analysis for *OsNAS1* and *HvNAATb* was conducted using 20 µg of total leaf RNA as described in chapter 2.

3.3.3 Fe uptake studies

T_2 seeds from transgenic rice lines EYI-9, EYI-89 and EYI-98 were germinated on ½ MS medium supplemented with 50 mg l^{-1} hygromycin and WT seeds were germinated on ½ MS medium without hygromycin. After 5 days, seedlings were transferred to floating trays and watered for 3 weeks with nutrient solution (Kobayashi et al 2005) containing 100 µM $FeCl_3$. Ten uniform seedlings from each transgenic line and WT were transferred to nutrient solution containing 100 µM (standard - $FeCl_3$), 200 µM (2X- $FeCl_3$) or 300 µM (3X- $FeCl_3$). Fresh nutrient solution was applied every 2 days and the pH of the solution

was adjusted to 5.3 with 0.1N KOH. Every week the nutrient solution was completely replaced. Plants were maintained as described in chapter 2 until seed maturity. Samples were collected from 10 plants per group and pooled as roots, culm, middle leaf, flag leaf and seeds for analysis of metal concentrations by ICP-MS.

3.3.4 Cd uptake studies

T_2 seeds from transgenic lines EYI-9, EYI-89 and EYI-98 were germinated on ½ MS medium supplemented with 50 mg l^{-1} hygromycin and WT seeds were germinated on ½ MS medium without hygromycin. After 5 days, seedlings were transferred to floating trays and watered for 3 weeks with nutrient solution (Kobayashi et al 2005) containing 100 µM $FeCl_3$. Fifteen uniform-sized seedlings from WT and transgenic lines were transferred to nutrient solution containing 10µM $CdCl_2$ or an equivalent control as described in the Fe uptake studies section. The pH of the solution was adjusted to 5.3 with 0.1 N KOH and the plants were maintained as described in chapter 2 until seed maturity. Seeds were harvested from all plants and metal concentrations were quantified by ICP-MS.

3.3.5 Measurement of metal levels

For the collection of roots and leaves, plastic containers were previously treated with 6.5 % HNO_3 to avoid external contamination of minerals. To remove metals from surface of samples, harvested roots and leaves were washed three times with double de-ionised water followed by 100 µM-Na_2EDTA and to remove EDTA used for washing a final two time washing was performed with double deionised water. Similarly, to avoid metal contamination during polishing, dehusked seeds of wild type and transgenic lines were polished using a non metal contaminating polisher (Kett Electric laboratory, Tokyo, Japan). Roots, leaves and seeds were ground using pestle and mortar previously treated with 6.5% HNO_3. Samples were then dried at 70°C for 2 days and 300 mg portions were

wet-ashed with 4.4M HNO_3, 6.5M H_2O_2 and double deionised H_2O (3:2:2) for 20 min at using a MLS 1200 Mega high performance microwave oven (Milestone, Sorisole, Italy)).The selected digestion program started with 250W for 1 min, followed by 0 W for 3 min, 250W for 4 min, 400 W for 4 min and finally 600 W for 3 min. Following dilutions to 25 ml with double de-ionized water metal concentrations were determined by inductively coupled plasma mass spectrometry (Agilent 7700X ICP-MS, Agilent Technologies, Santa Clara, USA).

3.3.6 NA and DMA measurements

Nicotianamine (98%) and 2'-deoxymugineic acid (98%) were purchased from T. Hasegawa Co. Ltd. (Kawasaki, Japan) and Toronto Research Chemicals Inc. (Toronto, Canada), respectively. Nicotyl-lysine was synthesized by following the procedure described by Wada et al (2007). All standards were dissolved in water to a concentration of 1–10 mM and stored in darkness at –80°C. Working standard solutions were prepared by diluting the stock solutions with water. Before injecting into the HPLC-ESI-MS (TOF), 5 µl of standard solutions were diluted with 10 µl of 50 mM EDTA, 5 µl of 1mM nicotyl-lysine, 10µl double de-ionised water and 30 µl of a solution of 10 mM ammonium acetate/acetonitrile (10/90) at pH 7.3 and filtered through polyvinylidene fluoride (Durapore® PVDF) 0.45 µm ultrafree-MC centrifugal filter devices (Merck KGaA, Darmstadt, Germany).

For NA and DMA analysis, seeds were first ground to a fine powder in liquid N_2. For each sample, the seed powder was extracted three times (Wada et al 2007, with modifications). Briefly, NA and DMA were extracted from 50mg seed powder with 300µl double de-ionised water and 18 µl of 1000 µM nicotyl-lysine. The supernatant was recovered by centrifugation (15,000g, 4°C and 15 min) and stored at –20°C, and the pellet was

resuspended twice in 300µl double de-ionised water, homogenized and the supernatant was recovered. The three supernatant fractions were pooled and the total extract was passed through a 3-kDa centrifugal filter (regenerated cellulose Amicon® Ultra filter units, Merck). The filtered solution obtained by centrifugation at 15,000g rpm for 30 min was concentrated under vacuum until dry. The residue was dissolved in 10µl double de-ionised water, an aliquot of 5µl extract was diluted with 10 µl of 50 mM EDTA, 15 µl of double de-ionised water and 30 µl of 10 mM ammonium acetate/acetonitrile (10/90) at pH 7.3 and filtered through polyvinylidene fluoride 0.45 µm ultrafree-MC centrifugal filter devices (Durapore, Merck KGaA) before injecting into the HPLC-ESI-MS (TOF).

Nicotianamine and DMA were determined in the seed extracts using a modified version of the method developed by Xuan et al (2006). Separations were performed with an Alliance 2795 HPLC system (Waters, Mildford, MA, USA) using a micro LC column (SeQuant ZIC®-HILIC, 15 cm x 1 mm i.d., 5 µm, 200Å, Merck KGaA), with a mobile phase consisting of a gradient obtained using 10mM ammonium acetate/acetonitrile (10/90) at pH 7.3 (solvent A) and 30 mM ammonium acetate/acetonitrile (80/20) at pH 7.3 (solvent B) at a flow rate of 0.15 ml min^{-1}. The gradient program started at 100% of A for 3 min, and then decreased linearly to 30% A for 7 min, and maintained for 7 min at 30% A and then returned to the initial conditions in 8 min. The column was then allowed to stabilize for 10 min at the initial conditions before proceeding to the next injection. The injection volume was 10 µl and the autosampler and column temperatures were 6 and 30ºC, respectively. The HPLC was coupled to a time-of-flight mass spectrometer (MicrOTOF, Bruker Daltonics, Bremen, Germany) equipped with an electrospray (ESI) source. The ESI-MS (TOF) operating conditions were optimized by direct injection of 100 µM solutions of NA and DMA standards at a flow rate of 180 µl h^{-1}. Mass spectra were

acquired in negative ion mode (settings are described in Table 1) in the range of 150-700 mass-to-charge ratio (*m/z*) units. The mass axis was calibrated externally and internally using Li–formate adducts [10mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol]. Bruker Daltonik software package micrOTOF Control v.2.2, HyStar v.3.2 and Data Analysis v.4.0 were used to control the ESI-MS (TOF) apparatus, interface the HPLC with the MS system and process the data, respectively. Concentrations of NA and DMA were always quantified by external calibration with internal standardization using nicotyl-lysine as internal standard.

Table 3.1 Operating conditions of the time-of-flight (TOF) mass spectrometer (MS) used for NA and DMA determinations

Source	Electrospray
Polarity	Negative
Endplate voltage	-0.5 kV
Spray tip voltage	3.0 kV
Orifice voltage	107 V
Nebulizer gas	N_2
Nebulizer gas pressure	2.0 bar
Drying gas	N_2
Drying gas (N_2) flow rate	8.5 l min^{-1}
Drying gas temperature	180ºC

3.3.7 qRT-PCR for endogenous metal homeostasis genes

qRT PCR was carried out to determine the levels of expression for the endogenous genes involved in metal homeostasis listed in Table 3.2 using mRNA from roots, flag leaf and immature seeds on a BioRad CFX96^{TM}system using 25 µl mixtures containing 10 ng of

synthesized cDNA, 1x iQSYBR Green supermix (BioRad, Hercules, CA, USA) and 0.2 µM forward and reverse primer (primer sequences for qRT-PCR are described in Table 3.2). Relative expression levels were calculated on the basis of serial dilutions of cDNA (125–0.2 ng) and used to generate standard curves for genes listed in Table 3.2. PCR was performed in triplicate using 96-well optical reaction plates. Cycling conditions consisted of a single incubation step at 95°C for 5 min followed by 39 cycles of 95°C for 10s, 59.4°C for 35 s and 72°C for 15 s. Specificity was confirmed by product melt curve analysis over the temperature range 50–90°C with fluorescence acquired after every 0.5°C increase. The fluorescence threshold value and gene expression data were calculated with BioRad CFX96^{TM} software. Values represent the mean of three RT-PCR replicates ± SE. Amplification efficiencies were compared by plotting the DCt values of different primer combinations of serial dilutions against the log of starting template concentrations using the CFX96^{TM} software.

Table 3.2 Details of endogenous genes involved in metal homeostasis and primers used for quantitative real time RT-PCR

Gene	Gene Bank ID	Amplicon (bp)	Forward primer	Reverese primer
OsYSL15	AB190923.1	78	CTGGTGCTGTTTGCTTGGA	ATGAGCCCAGACGCAACAGC
OsIRT1	BAB85123.1	139	GCATCATGCAATTCGCTGC	CCTGAACAACCACGCTACAA
OsZIP1	AY324148.1	90	CGATGGTCCTCTTCTTCTGC	GTCGGGCTGCTCTCGTTGTA
OsZIP4	AB126089.1	107	TCCACCAGTTCTTTGAAGGC	CGGAGCAGTGAGGGAGAAGA
OsVIT1	NM_001059545.1	107	CCACAGCACAGAACGCCAT	GCTGAGGAATGGACGGTTT
OsYSL2	AB164646	169	GGTGGAGAGAGTTGTGGGTT	TCATTCCCGCACCAACATA
OsFERRITIN1	AF519570.1	130	GCTTGAATGGAGGAGACTGTG	CTCCATTGCTACTGCGTGCT
OsACTIN1	AB047313.1	120	TCA TGT CCC TCA CAA TTT CC	GAC TCT GGT GAT GGT GTC AGC

3.3.8 Statistical analysis

Differences between transgenic and wild type plants were tested by comparison of means using the *t* test ($p<0.05$).

3.4 Results

3.4.1 Recovery of transgenic plants co-expressing *OsNAS1 and HvNAATb*

Mature seed-derived zygotic rice embryos were bombarded with constructs encoding rice NAS (*OsNAS1*), barley NAAT (*HvNAATb*) and the selectable marker *hpt*. Genes of interest, *OsNAS1* and *HvNAATb* were driven by the constitutive maize ubiquitin-1 promoter, and selectable marker *hpt* was driven by the constitutive CaMV 35S promoter. mRNA blot analysis carried out using total RNA isolated from leaf tissue identified 19 transgenic lines co-expressing *OsNAS1* and *HvNAATb* (Figure 3.1). T_1 seeds were harvested from 10 of these lines selected on the basis of first to flower and set seeds and these lines were advanced to the T2 generation for further analysis.

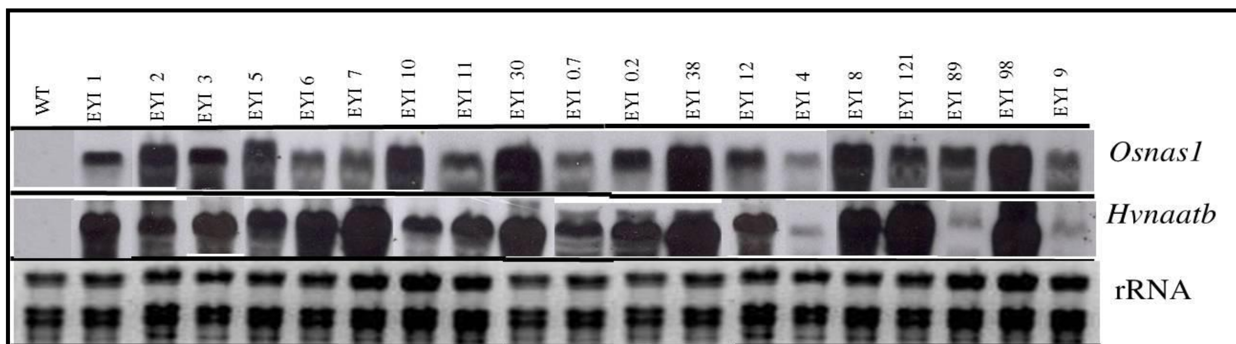

Figure 3.1 mRNA blot analysis showing transgene expression in leaf tissue of wild type (WT) and T_1 transgenic lines. rRNA: ribosomal RNA; *OsNAS1*: rice nicotianamine synthase; *HvNAATb*: barley nicotianamine amino transferase.

3.4.2 Fe and Zn concentration in seeds

In order to understand the influence of *OsNAS1 and HvNAATb* co-expression on Fe and Zn levels, I carried out Fe and Zn quantification in unpolished seeds. In comparison with wild type all the transgenic lines accumulated higher level of Fe and Zn (Table 3.3). The concentration range of Fe in unpolished seeds was 22–57µg g^{-1} DW, 1.4–3.7 times higher than that in the WT seeds, and the concentration range of Zn was 22–78µg g^{-1} DW, 1.2–4.2 fold higher than that in the WT seeds. These results suggest that *OsNAS1+HvNAATb* expression resulted in improved Fe and Zn seed loading.

Seeds from the three best-performing lines (EYI-9, EYI-89 and EYI-98) were polished to determine the accumulation of Fe and Zn in the endosperm. Transgenic lines EYI-9, EYI-89, EYI-98 had 8, 16 and 10µg Fe g^{-1} DW respectively (i.e., up to 4-fold over the WT values, that were 4µg Fe g^{-1} DW), and 36, 42 and 65µg Zn g^{-1} DW, respectively (i.e., up to 4-fold over the WT values, that were 16 µg Zng^{-1} DW). Thus, co-expression of *OsNAS1 and HvNAATb* improves total Fe and Zn seed loading and also the retention of Fe and Zn in the endosperm.

Table 3.3 Concentration of Fe and Zn in unpolished seeds of wild type and transgenic rice seeds (T_2 generation)

Line	Fe in unpolished seeds (µg/g DW)	Zn in unpolished seeds (µg/g DW)
EYI-9	26.35 ±0.31*	47.49±1.19*
EYI-89	56.68±1.37*	77.80±0.68*
EYI-98	34.47±0.63*	58.7±2.21*
EYI-0.7	33±0.41*	43±0.1*
EYI-0.2	30±1.37*	42±0.65*
EYI-38	25±0.61*	37±0.22*
EYI-4	26.29±0.54*	28.02±0.39*
EYI-8	22.96±1.1*	21.57±1.19
EYI-12	23.42±0.84*	23.24±1.65
EYI-121	21.92±0.35*	22.77±0.4
WT	15.33±0.38	18.51±0.1

The data represents means±standard errors. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test ($p < 0.05$). Number of replications n=6, DW-dry weight; WT- wild type.

3.4.3 NA and DMA levels in seeds

Similarly, when measured for NA and DMA levels the unpolished seeds of the three lines (EYI-9, EYI-89 and EYI-98) accumulated both phytosiderophores at higher levels than WT seeds (Figure 3.2). Transgenic lines had 7- to 165-fold increase in NA over WT and 11- to 29-fold increase in DMA over WT. The three lines had different NA/DMA ratios. EYI-9 and EYI-89 accumulated more NA than DMA and EYI-98 exhibited the opposite behaviour.

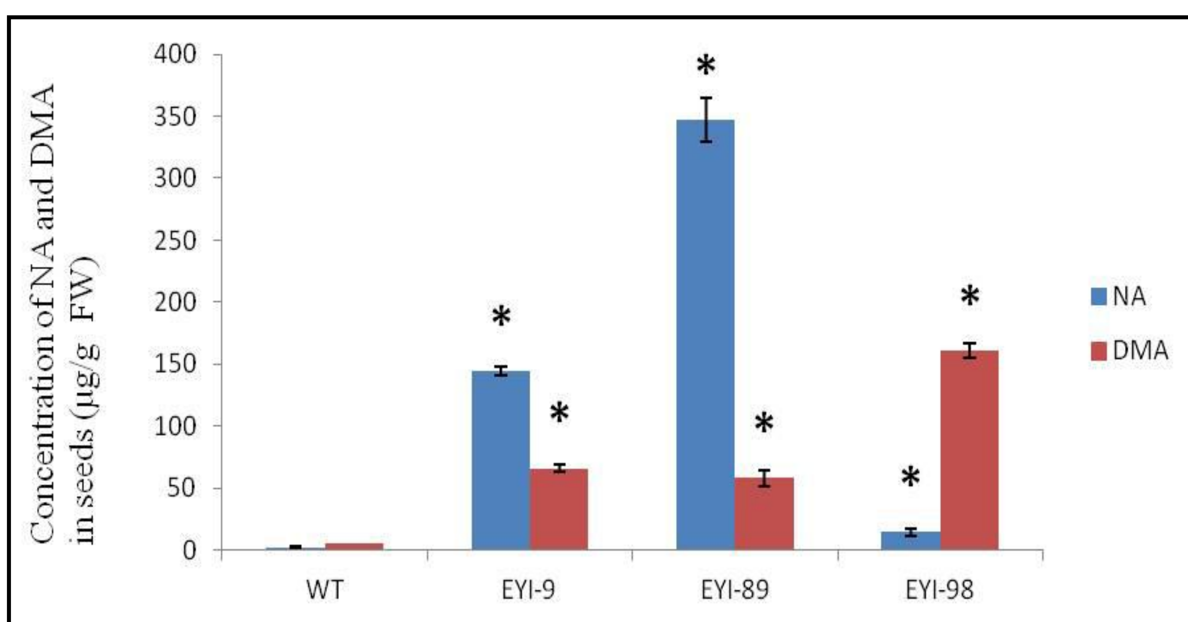

Figure 3.2 Concentrations of nicotianamine (NA) and 2' deoxymugenic acid (DMA) (in µg g^{-1} FW) in unpolished seeds of wild type (WT) and three different T_2 generation transgenic lines expressing *OsNAS1+HvNAATb* (EYI-9, EYI-89 and EYI-98). The data represents means±standard errors. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3). FW: fresh weight.

3.4.4 Impact of external Fe feeding to Fe and Zn uptake, translocation and endosperm accumulation

3.4.4.1 Fe concentration in roots, culm, middle leaf, flag leaf and endosperm

In order to determine whether Fe seed loading can be further improved in the three selected lines by boosting the external availability of metal ions and to investigate the mechanism of Fe homeostasis, I grew T_2 plants from the three lines and a WT under three different external Fe feeding regimes: standard (100 µM Fe), double (200 µM Fe) and triple (300 µM Fe) the standard levels of Fe. I then measured the Fe concentration in the roots, culm, middle leaf, flag leaf and polished seeds of all lines.

Under standard external Fe supply no differences in root Fe concentrations were found between the transgenic lines and the WT (Figure 3). However, the roots of the transgenic lines fed with 200 µM Fe contained significantly higher levels of Fe than WT plants under the same conditions, whereas the roots of transgenic lines fed with 300 µM Fe contained significantly less Fe than WT plants under the same conditions, suggesting that excess Fe above a certain threshold triggers a compensatory mechanism that restricts Fe uptake into the roots (Figure 3.3). The culms of the transgenic lines generally accumulated more Fe than the WT under all three treatments, indicating that Fe root-to-shoot translocation was enhanced in the transgenic plants (Figure 3.3). In the middle leaf the transgenic lines accumulated more Fe under all three treatments, with the plants fed with 200 µM Fe containing the highest concentrations of Fe (Figure 3.3). However, although the flag leaves of the transgenic lines accumulated more Fe than the corresponding WT plants (Figure 3.3), there were no significant differences among the three treatments.

Under standard conditions (100 µM Fe) the polished seeds of the T_3 transgenic plants accumulated 2.6– to 4.3-fold more Fe in the endosperm compared to WT plants (Figure

3.4). In the plants fed with 200 and 300 µM Fe the polished seed Fe concentrations were 2.2– to 4.5-fold and 1.9– to 4.6-fold higher than the WT values, and in both cases there was no statistically significant change when compared to plants fed with 100 µM Fe (Figure 3.4). These data strongly support the presence of a strict homeostatic mechanism that limits the accumulation of Fe in the endosperm to a maximum (22.5µg g^{-1} DW, in our case) regardless of the excess external Fe supply. Interestingly, the highest Fe concentration in the seeds was found in the line with a highest DMA/NA ratio.

3.4.4.2 Zn concentration in root, middle leaf, flag leaf, culm and endosperm

The promiscuity of ligands and transporters that mobilize Fe and Zn makes it likely that the homeostatic mechanisms controlling the distribution of Fe in the plant may also affect Zn. Therefore, I analyzed the same tissues described in the previous section for the concentration of Zn under the three different Fe feeding regimes. The concentration of Zn in different plant tissues differed according to the Fe supply, but the profile was distinct to that observed for the distribution of Fe (Figure 3.3), suggesting there was competition between the two metals. The roots of the transgenic lines contained higher levels of Zn than WT plants under the standard and 200µM Fe treatments but significantly less Zn in the presence of 300µM Fe, which was similar to the profile observed for Fe (Figure 3.3). This suggests that Zn accumulation may be restricted by the same feedback mechanism that limits the uptake of Fe and may therefore involve the same transporters. In contrast, the profile of Fe and Zn in the culms was different. The transgenic lines showed more active root-to-shoot translocation of Zn under the control treatment so that higher levels of Zn were present in the culms of transgenic plants than WT. However, in both the 200 and 300µM Fe treatment groups root to shoot translocation of Zn was reduced drastically in transgenic plants, and as a result transgenic lines did not differ much from the WT (Figure

3.3). Therefore, the external supply of Fe suppresses the accumulation of Zn suggesting competition between the two metals for root-to-shoot transporters, with Fe displacing Zn. The profile in the culm was not replicated exactly in the middle leaf but there was a similar trend: the transgenic lines accumulated more Zn in the middle leaf than WT plants when fed with the control treatment and (to a lesser extent) when fed with 300 µM Fe, whereas there was no significant difference in Zn levels in the middle leaves of transgenic and WT plants when fed with 200 µM Fe (Figure 3.3). Again, these data suggest a competition between Fe and Zn for transporters, which is most prevalent at 200 µM Fe, when the greatest amount of Fe accumulates in the middle leaves. The amount of Zn in the flag leaves was generally higher in the transgenic lines under all three treatments than in WT plants, but the lowest level of Zn accumulated in plants treated with 300µM Fe (Figure 3.3). This result is intriguing because the similar levels of Fe in the flag leaves under all three treatments suggested an upper threshold for accumulation, but clearly there is still competition between Fe and Zn that differs according to the external supply even when the internal levels of Fe are similar, suggesting that Zn accumulation in the flag leaf is regulated by signals emanating from other tissues.

Even so, the accumulation of Zn in the endosperm, like that of Fe, appeared to be governed by an intrinsic threshold that was independent of the external Fe supply. The endosperm accumulated 1.2– to 2.2-fold the WT level of Zn in polished seeds of T_3 transgenic lines with a normal Fe supply, and this barely changed as the amount of external Fe increased to 200 µM (1.5– to 2-fold higher Zn) and 300 µM (1.2– to 2-fold higher Zn) (Figure 3.4). These data indicate that the external supply of Fe does not alter the threshold level of Zn in the endosperm, which was approximately 84 µg g^{-1} DW in our case.

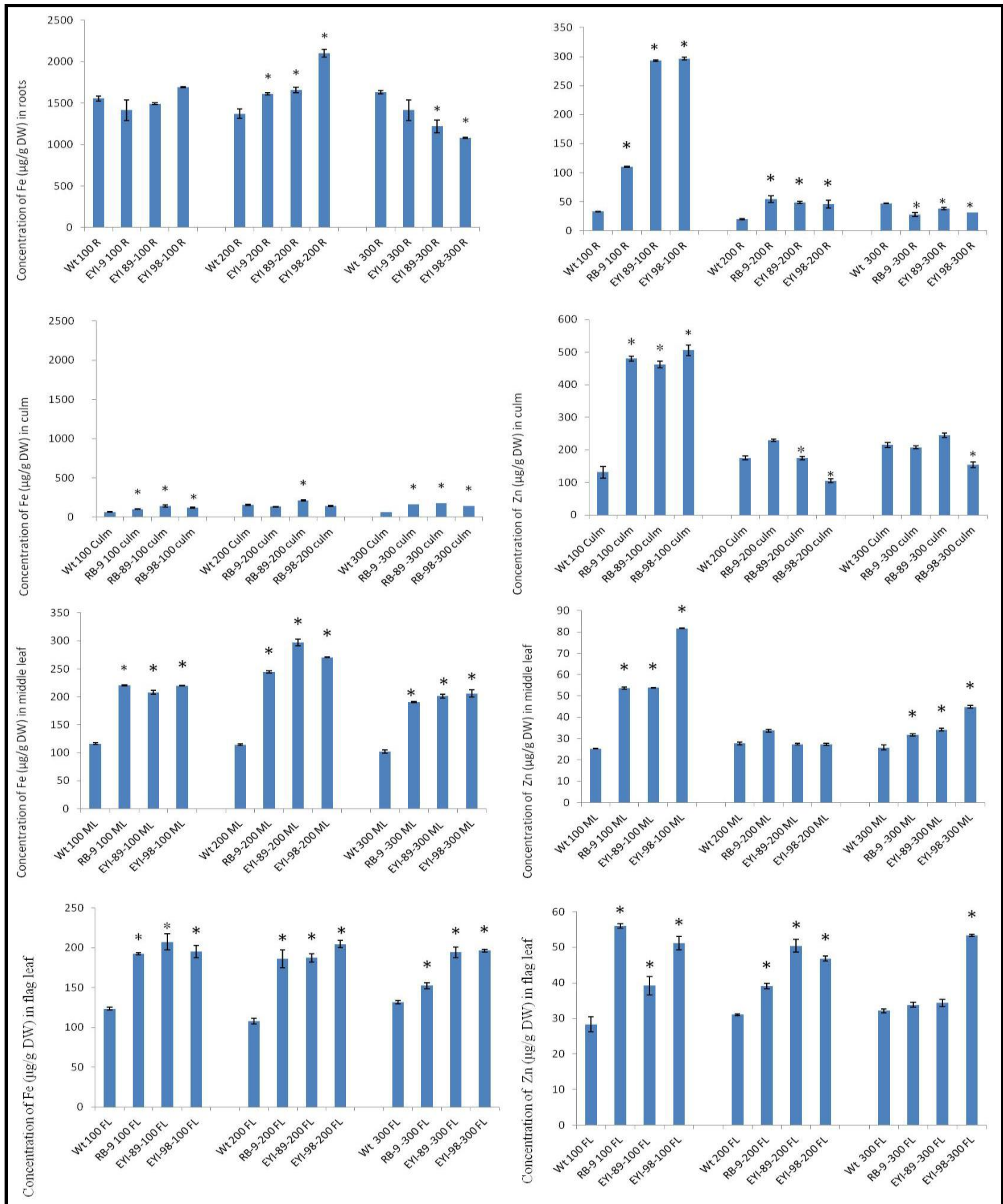

Figure 3.3 Concentrations of Fe and Zn (in µg g^{-1} DW) in roots, culm, middle leaf and flag leaf in wild type (WT) and three different T_2 generation transgenic *OsNAS1+HvNAATb* lines (EYI-9, EYI-89 and EYI-98) under 100 (standard), 200 and 300 µM Fe treatments. The data represents means±standard errors. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3). DW: dry weight; R: roots; ML: middle leaf; FL: flag leaf.

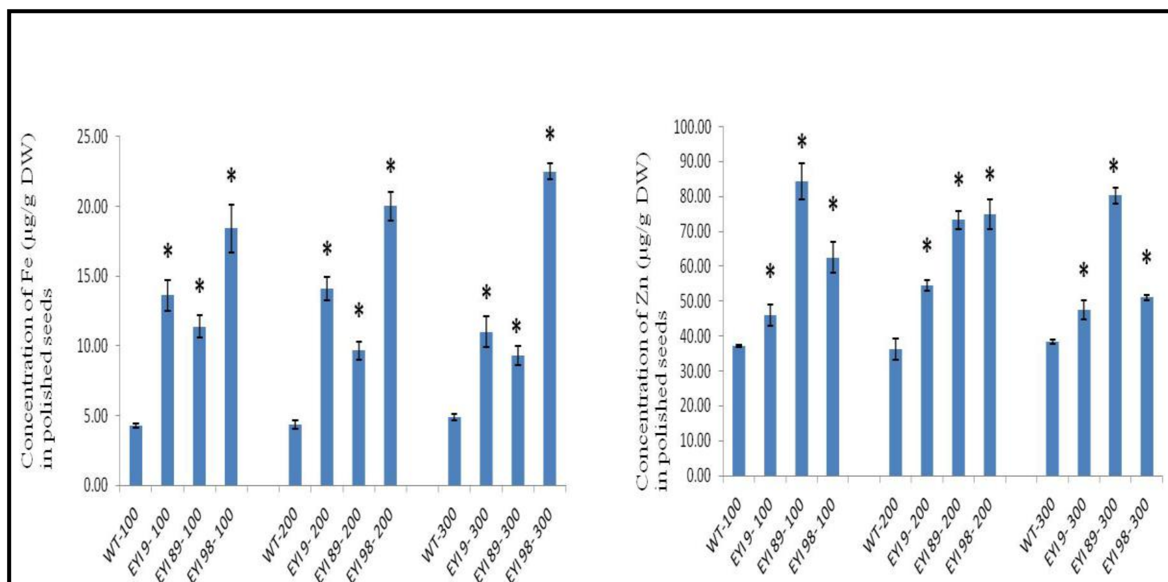

Figure 3.4 Concentrations of Fe and Zn (in μg g^{-1} DW) in polished seeds of wild type (WT) and three different T_3 generation transgenic *OsNAS1+HvNAATb* lines (EYI-9, EYI-89 and EYI-98) under 100 (standard), 200 and 300 μM Fe treatments. The data represents means±standard errors. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6). DW: dry weight.

3.4.4.3 Expression of endogenous genes involved in metal uptake and translocation

One potential explanation for the competition between Fe and Zn under different feeding regimes is that the external supply of Fe regulates the expression of genes encoding metal transporters, resulting in the preferential mobilization of Fe. I therefore investigated the expression of three metal transporters in roots (in T_3 EYI9, EYI89 and the WT): *OsYSL15*, which encodes the Fe^{3+}-DMA transporter YSL15, *OsIRT1*, which encodes the IRT1 transporter that mobilizes Fe and Zn, and finally *OsZIP1*, a Zn-transporter. *OsYSL15* was expressed at higher levels in the roots of the transgenic lines than in WT plants under the control and 200 μM Fe treatments but it was expressed at lower levels than WT plants in the presence of 300 μM Fe (Figure 3.5A). The expression of *OsIRT1* was downregulated in the transgenic lines compared to WT plants under all three treatments, with the expression

level being lowest under the 100 µM Fe standard treatment (Figure 3.5A). Compared to WT plants, *OsZIP1* was expressed at a lower level in the transgenic lines under the standard treatment, there was no significant difference in the presence of 200 µM Fe, and it was expressed at higher levels in the transgenic plants fed with 300µM Fe (Figure 3.5A). These data suggest that the genes for all three transporters are modulated by the increase in phytosiderophore synthesis and by the external Fe supply, although the phytosiderophores may have an indirect effect by increasing the internal concentration of Fe. *OsYSL15* appears to be regulated in response to a feedback mechanism that suppresses Fe transport when there is an excess external supply, whereas *OsIRT1* and *OsZIP1* appear to be suppressed by the higher internal levels of Fe in the transgenic plants but this effect is partially (*OsIRT1*) or fully (*OsZIP1*) alleviated as the external Fe supply increases.

Transgenic plants generally accumulated higher levels of Fe and Zn in the flag leaf over WT. It appears that most of the Fe and Zn is sequestered into the vacuoles and as a result availability of the free metals is limited for seed loading. Therefore, I investigated the expression of a number of metal transporters to gain further insights into vacuolar sequestration and flag leaf accumulation of metals. These included the Fe and Zn vacuolar transporter *OsVIT1*, the Fe-NA/Mn-NA phloem transporter *OsYSL2*, the Fe-regulated ZIP family transporters *OsZIP1* and *OsZIP4* (transport Zn), and the Fe storage protein-ferritin, *OsFERRITIN1*. In comparison to the WT, *OsVIT1* expression was up-regulated in transgenic lines under all treatments. Interestingly, *OsVIT1* transcript levels increased when the external Fe supply increased (Figure 3.5B). The expression of *OsYSL2* in transgenic lines was no different to the WT under all treatment conditions, but among the treatments expression was highest at 200 µM Fe (Figure 3.5B). Compared to WT, *OsFERRITIN1* was up-regulated in transgenic lines in all treatments (Figure 3.5B). *OsZIP4*

expression was higher in the transgenic lines in all treatments. In transgenic lines *OsZIP1* expression was at the highest levels in the standard and the 300 µM Fe treatments, but there was no change between the WT and transgenic lines in the 200µM Fe treatment (Figure 3.5B). These data suggest that higher levels of Fe and Zn in the flag leaf of transgenic lines modulate expression of transporters to control excess transport of Fe and Zn towards seeds by means of vacuolar sequestration. Thus, whereas *OsVIT1* expression is regulated in response to the external supply of Fe and the amounts of Fe and Zn in the flag leaf, *OsYSL2* appears to serve as a molecular control mechanism operating to limit the phloem transport of Fe. *OsZIP1/OsZIP4* expression patterns suggest a higher capacity for Zn transport over Fe for seed loading.

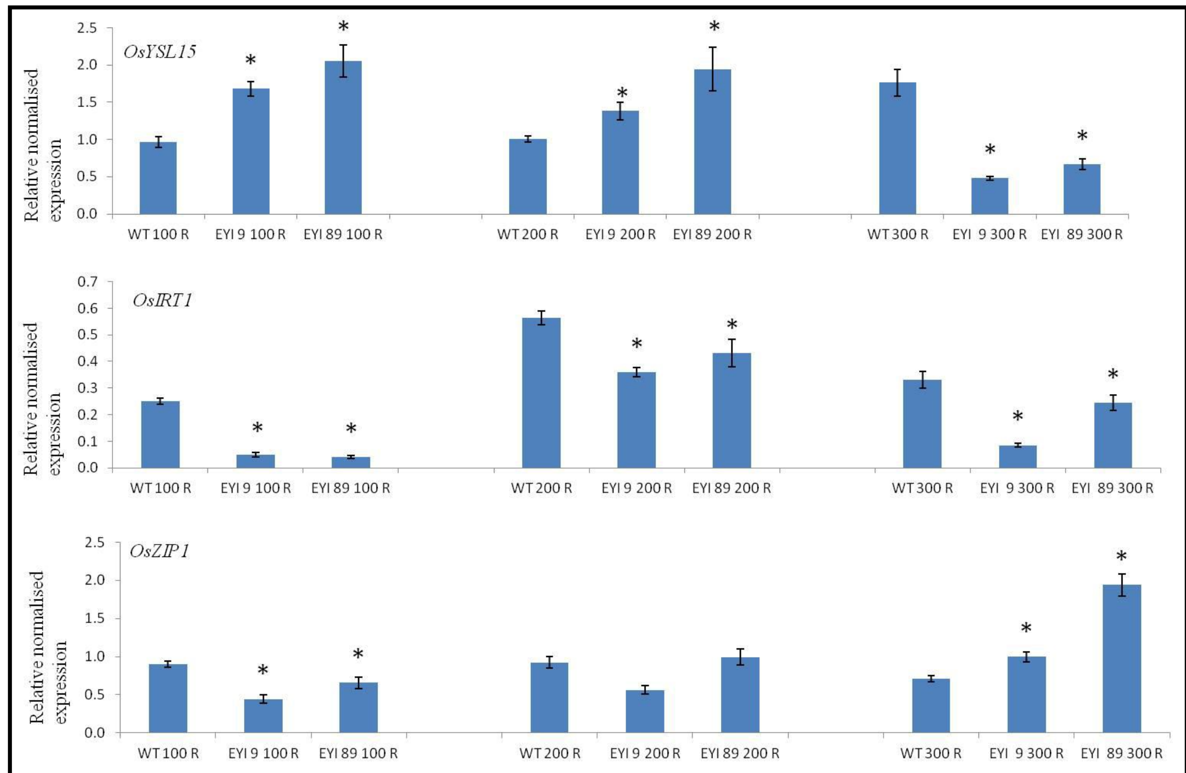

Figure 3.5A qReal-time PCR analysis of *OsYSL15, OsIRT1, OsZIP1* in roots (R) under 100 (standard), 200 and 300 µM external Fe supply at grain filling stage in wild type and transgenic T_3 lines expressing *OsNAS1+HvNAATb* (EYI-9, EYI-89). Each value is the average of three independent experiments. Transcript levels are represented by the ratio between mRNA levels of *OsYSL5, OsIRT1, OsZIP1* and those of Os*ACTIN1*. The data represents means±standard errors. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3).

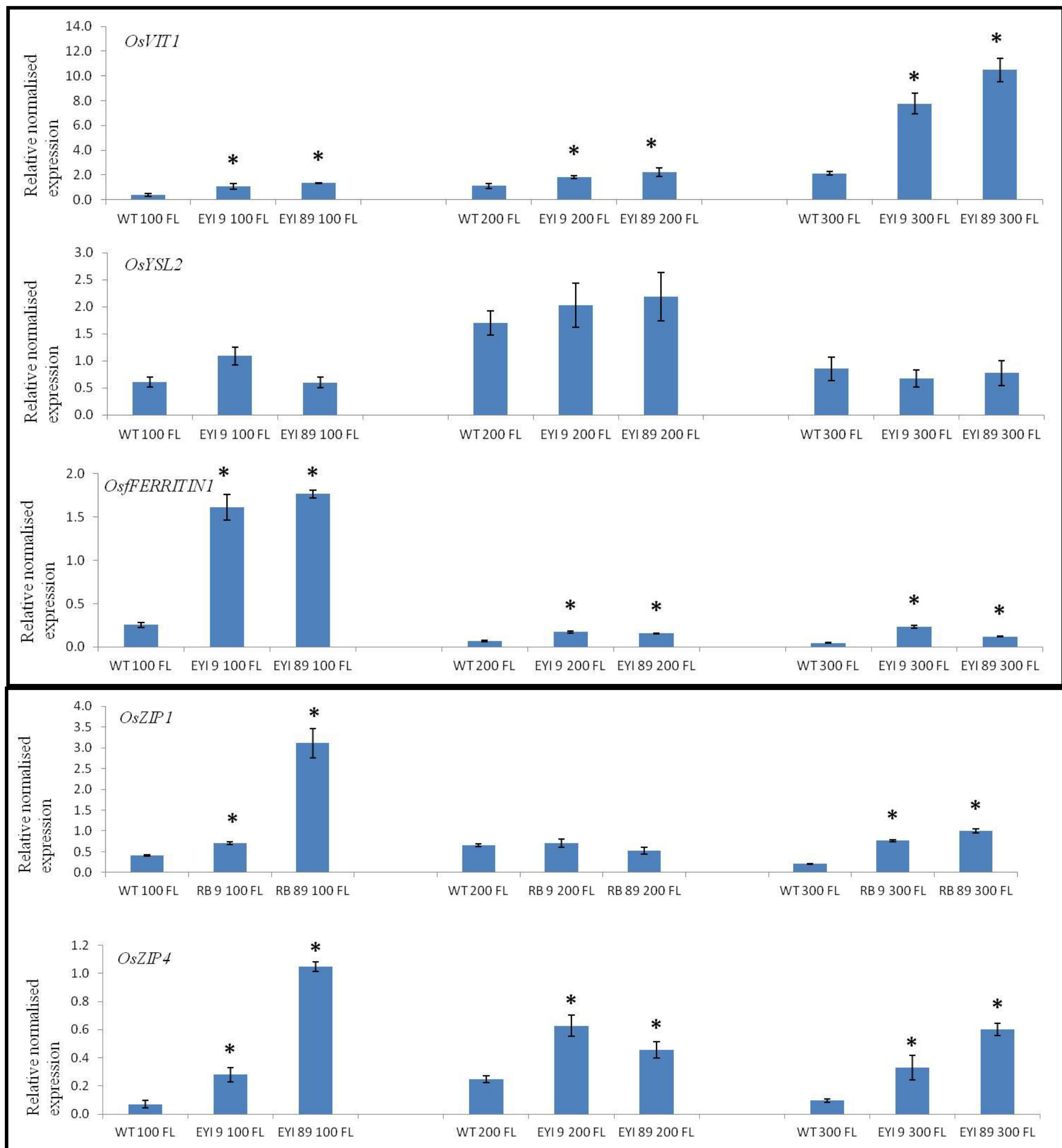

Figure 3.5B qReal-time PCR analysis of *OsYSL2, OsVIT1, OsFERRITIN1, OsZIP1, OsZIP4* in flag leaf (FL) under 100 (standard), 200 and 300 µM external Fe supply at grain filling stage in wild type and transgenic T_3 lines expressing *OsNAS1+HvNAATb* (EYI-9, EYI-89). Each value is the average of three independent experiments. Transcript levels are represented by the ratio between mRNA levels of *OsYSL2, OsVIT1, OsFERRITIN1, OsZIP1, OsZIP4* and those of Os*ACTIN1*. The data represents means±standard errors. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3).

3.4.5 Cadmium accumulation in seeds

There was no significant difference between WT and transgenic lines for Cd accumulation in unpolished seeds (Figure 3.6). Polishing WT seeds, however, significantly reduced Cd levels by ~9%, suggesting that >90% of the Cd in rice seeds accumulates in the endosperm. Interestingly, polishing the transgenic seeds significantly reduced the Cd levels by 14–18%, which suggests that the endosperm of the transgenic lines accumulates less Cd than WT seeds, possibly because Cd is displaced by the high Fe and Zn levels in the endosperm (Figure 3.6). The concentrations of Fe and Zn in polished seeds of transgenic plants were similar when grown in the presence (Figure 3.6) and absence of Cd (Figure 3.4), whereas in the case of the WT the concentration of Zn decreased 20% in the presence of Cd and that of Fe did not change (Figures 3.4 and 3.6).

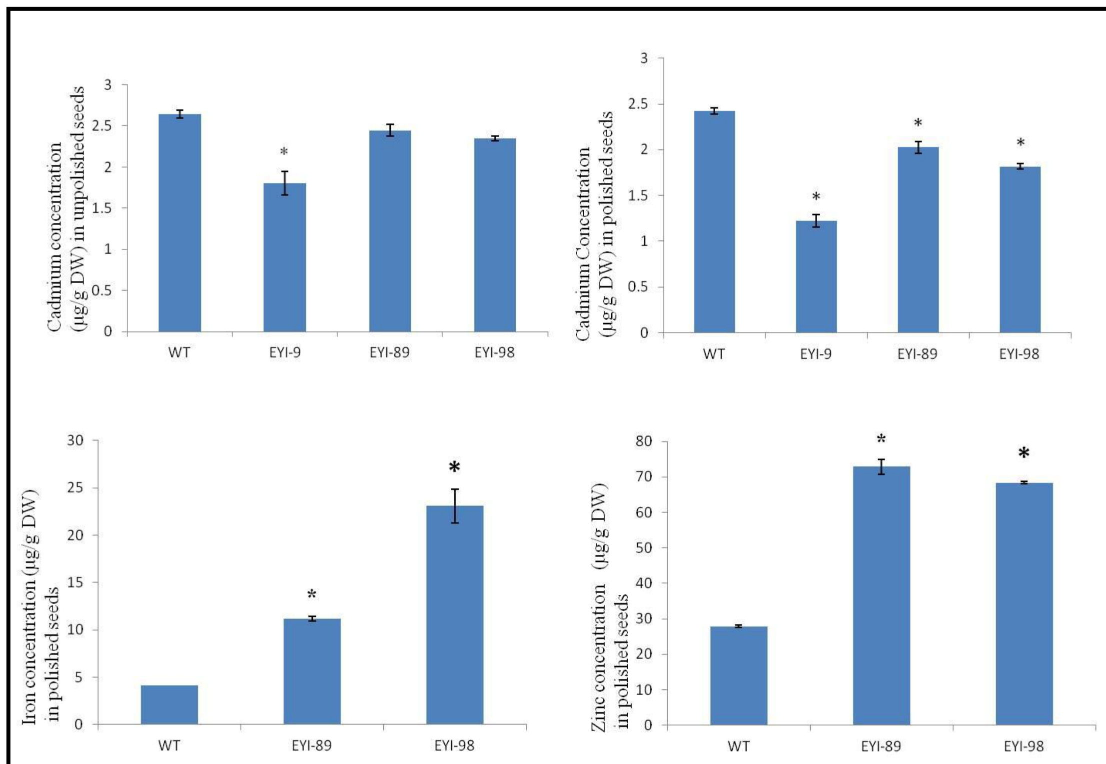

Figure 3.6 Concentrations of Cd (unpolished and polished seeds), Fe and Zn (polished seeds) (in µg g^{-1} DW of Cd) in wild type (WT) and T_3 generation transgenic *OsNAS11+HvNAATb* lines (EYI-9, EYI-89 and EYI-98) grown with 100µM $FeCl_3$ and 10µM $CdCl_2$. The data represents means±standard errors. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6). The Cd concentrations in unpolished and polished seeds were significantly different both in WT and transgenic lines. Note: As transgenic line EYI-9 did not produce enough seeds, quantification of Fe and Zn was not performed.

3.5 Discussion

The uptake and distribution of metal ions in rice is controlled by transporters that recognize either free metal ions or metal complexes with phytosiderophores such as nicotianamine (NA) and deoxymugenic acid (DMA). One strategy to enhance the accumulation of metals in plants is therefore to increase the abundance of phytosiderophores providing a greater capacity for metal mobilization. However, metal accumulation in plants is controlled by strict homeostatic mechanisms that limit toxicity. Several key metal transporters are promiscuous so that the overall impact of boosting phytosiderophore synthesis is unclear, particularly in the context of variable external supplies of metal ions.

Nicontianamine and DMA are synthesized from the precursor S-adenosylmethionine (SAM) in three steps involving the enzymes nicotianamine synthase (NAS), nicotianamine amino transferase (NAAT) and DMA synthase (DMAS) (Ma et al 1999). I generated transgenic plants expressing two key enzymes in the phytosiderophore biosynthesis pathway (NAS and NAAT) to investigate in detail the impact of an enhanced level of phytosiderophores on metal uptake, mobilization, accumulation and the homeostasis mechanism limiting accumulation of metals beyond certain level to avoid toxicity. In transgenic plants expressing *OsNAS1* (NAS) and *HvNAATb* (NAAT), the T_2 seeds from the best-performing lines contained up to 165-fold more NA and 29-fold more DMA than WT seeds. Previous studies reported 6 to 20-fold increases in NA (Cheng et al 2007; Lee et al 2009; Masuda et al 2008; Masuda et al 2009; Johnson et al 2011; Lee et al 2011; Masuda et al 2012) and 3- to 5- fold increases in DMA (Lee et al 2009; Masuda et al 2009; Lee et al 2011; Masuda et al 2012), but in all cases the plants expressed either NAS (Lee et al 2009, 2011; Johnson et al 2011; Masuda et al 2008; 2009; 2012; 2013; Wirth et al 2009) or NAAT alone (Takahashi et al 2001). This suggests that the much higher levels of NA and

DMA in our transgenic lines reflect the co-expression of both enzymes. The seeds of the best-performing T_2 transgenic lines accumulated up to 57 μg Fe g^{-1} DW (3.7-fold higher than WT) and up to 78 μg Zn g^{-1} DW (4.2-fold higher than WT) in the unpolished grain, similar to the levels reported for rice plants expressing *HvNAS1* (Masuda et al 2009) and *OsNAS1–3* (Johnson et al 2011). Therefore, phytosiderophore levels appear to be a limiting factor in the accumulation of Fe and Zn in grains as previously proposed by Masuda et al (2012) and Johnson et al (2011). After polishing, the transgenic lines accumulated up to 16 μg Fe g^{-1} DW and 65 μg Zn g^{-1} DW in the endosperm, in each case a four-fold increase over the WT, suggesting improved loading of the endosperm. Similarly, the expression of *AtNAS1* (Wirth et al 2009), *HvNAS1* (Masuda et al 2009), and *OsNAS1–3* (Johnson et al 2011) resulted in the enhanced loading of Fe and Zn into the endosperm, suggesting that the modulation of NA and DMA synthesis is a useful strategy to enhance the mineral content of the endosperm. Both in wild type and in lines co-expressing *OsNAS1 and HvNAATb*after polishing, Zn retention was higher than Fe retention, suggesting that rice prefers Zn over Fe for endosperm deposition and our results are consistent with previous reports by Masuda et al 2009; Johnson et al 2011; Lee et al 2011.

Having observed increasing the availability of NA and DMA can increase the capacity of rice plants to mobilize metals and store them in the endosperm, I investigated the impact of different external concentrations of Fe –as inorganic salt ($FeCl_3$)- and how this might interact with the mechanisms of metal homeostasis that prevent metal toxicity. Transgenic plants supplied with 200 μM Fe took up more Fe into the roots than WT plants, suggesting an increased uptake capacity, whereas the advantage was abolished in the presence of 300μM Fe where WT plants performed better. It is possible that the high external concentration of Fe could have increased the concentration of Fe immediately above the

root epidermis, resulting in the precipitation of Fe^{3+} generated by the release of O_2 from the roots (Chen et al 2006).

The remobilization of Fe from the roots to the aboveground vegetative organs (culm and leaves) is necessary for seed loading during grain filling (Sperotto et al 2012a, 2012b; Sperotto 2013). The culms and middle leaves of the transgenic plants generally accumulated more Fe than their WT counterparts under all three treatments, suggesting an effective root to shoot translocation of Fe under all treatments. Interestingly, the flag leaves of the transgenic plants generally contained more Fe than those of WT plants but there was not much difference among the three treatments. The remobilization of Fe from middle and flag leaves is an important determinant of Fe levels in seeds (Jiang et al 2007; Fang et al 2008). In *OsVIT1/2* knockdown mutants, the impaired vacuolar sequestration of Fe reduced the level of Fe in the flag leaves and higher levels accumulated in the seeds, suggesting that the flag leaf acts as a buffer to prevent the seed being overloaded (Zhang et al 2012). The higher level of Fe in the middle and flag leaves of our transgenic plants suggests that a large part of the metal may have been sequestered into the vacuole making it unavailable for remobilization, as observed during foliar applications of Fe in rice (Jin et al 2008). Accordingly, accumulation of Fe in the endosperm of our transgenic plants fell within a similar range regardless of the external supply, and never exceeded 4.6-fold the WT levels. These data support the existence of a homeostasis mechanism to prevent Fe overloading in the developing seeds. This is in line with the limited genetic diversity in terms of Fe levels in rice, which remains at 2–5 μg g^{-1} DW in the polished seeds in all varieties (Yang et al 1998; Sperotto et al 2012). The threshold of accumulation was a maximum of 6 μg Fe g^{-1} DW in polished seeds in plants expressing *AtNAS1* (Wirth et al

2009), 14 μg Fe g^{-1} DW in plants expressing *OsNAS2* (Johnson et al 2011) and 22.5 μg Fe g^{-1} DW in our T_3 plants expressing *OsNAS1+HvNAATb*.

One of the key questions posed by the existence of a ceiling for Fe accumulation in rice endosperm is whether increasing the accumulation of Fe towards this ceiling has an inhibitory effect on the accumulation of other metals that share the same finite storage space as well as some of the same transporters and phytosiderophores, and that are therefore presumably subject to the same regulatory mechanisms. Accordingly, amount of Zn taken up by the roots declined as the amount of Fe increased, suggesting there is competition for uptake with Fe being preferentially mobilized (Zhang et al 1998; Sperotto et al 2012). As well as sharing some of the same transporters, which would result in the statistical exclusion of Zn by Fe even if both ions were equally competitive, the precipitation of Fe to form plaques on the root surface would also exclude Zn from large areas of the root surface. The amount of Zn in the culms and middle leaves of the transgenic plants was inversely related to the amount of Fe, suggesting a knock-down effect from the inhibition of uptake into the roots that was strongest in the presence of 200 μM Fe in parallel to the greatest internal concentration of Fe. In the flag leaf, generally Zn accumulated to higher levels in the transgenic plants than in the WT plants under all three treatments. This suggests that unlike in roots, culm and middle leaf the Zn levels are unaffected in the flag leaf. It is also possible that Zn levels in the flag leaf could be regulated as a buffer system to limit seed Zn levels. A comparison of different tissues in the transgenic plants suggests that most of the Zn is held up in the culm, acting as a rate limiting step in Zn mobilization possibly due to the sequestration of Zn into vacuoles, e.g., by vacuolar Zn transporters such as MTP1 (Menguer et al 2013) and OZT1 (Lan et al 2013). Even though transgenic lines were able to accumulate up to 84μg Zn g^{-1} DW, there

was no difference in the levels of Zn in the endosperm of our transgenic plants regardless of the external Fe supply, suggesting that the buffering effect of the flag leaf on Fe levels may also act on Zn.

The competition between Fe and Zn in roots of plants grown under different external Fe feeding regimes may partly reflect the availability of metal transporters with different preferences for the two metals. I therefore investigated the expression of three metal transporters in roots: YSL15, which is specific for Fe^{3+}-DMA complexes (Inoue et al 2009; Lee et al 2009), IRT1, which transports Fe and Zn (Lee and An et al 2009), and ZIP1, which is specific for Zn (Chen et al 2008). Compared to WT plants, the *OsYSL15* gene was induced in the roots of our transgenic lines in response to 100 and 200 μM Fe but was suppressed in the presence of 300 μM Fe and this mirrors the level of Fe in roots. *OsIRT1* was expressed at lower levels in the transgenic than in WT plants at all external Fe concentrations, but the gap between transgenic and WT plants closed as the Fe concentration increased. The IRT1 protein transports Fe^{2+} whereas I supplied Fe^{3+} in our experiments so the availability of Fe^{2+} remained low. The relative increase in expression at higher concentrations of Fe may reflect the displacement of Zn and the corresponding Zn deficiency. *OsZIP1* was also induced at higher Fe levels, and Zn deficiency may also explain this trend.

Remobilization of Fe and Zn from vegetative tissues to seeds is important during grain filling, and this process is tightly regulated by metal transporters (Sperotto 2012). I thus investigated the expression of the vacuolar Fe transporter VIT1 (Zhang et al 2012), Fe-NA/Mn-NA transporter YSL2 (Ishimaru et al 2010), and the Fe-regulated ZIP family transporters, ZIP1 and ZIP4 (Ishimaru et al 2005) in flag leaves. *OsVIT1* expression was increased compared to WT under all treatments in the transgenic lines and this mirrored the

levels of Fe and Zn in the flag leaf. Expression of *OsYSL2* did not differ between WT and transgenic lines under all treatments, which indicates the limitations in Fe remobilization from the flag leaf. Since *OsZIP4* expression increased over WT in transgenic lines under all treatments, this reflects the lower level of Zn over Fe in flag leaf and points out to a preference for Zn over Fe in endosperm loading in the transgenic lines.

As well as the competition between Fe and Zn, both of which are essential micronutrients, the metal transport mechanisms in plants are also shared by strictly toxic heavy metals and in this case the competition could be advantageous by providing a strategy to exclude such metals from the endosperm. NA and DMA are involved in the homeostasis of Fe, Zn, Mn and Cu in rice leading to their accumulation in polished seeds (Masuda et al 2012). Since the endosperm is a finite space, enhancing the phytosiderophore-facilitated transport of these micronutrients may also affect the accumulation of other heavy metal ions with low affinity for this ligand such as Cd^{2+}, which is detrimental to plants and the animals that consume them (Meda et al 2007; Ueno et al 2010; Uraguchi et al 2011; Uraguchi et al 2012; Clemens et al 2013; Slamet-Loedin 2015). When grown on medium supplemented with Cd, the transgenic lines and WT controls accumulated similar levels of Cd in the seeds, but in the WT lines most of the metal accumulated in the endosperm whereas in the transgenic lines ~18% was in the bran, presumably because it was displaced by the increased retention of Fe and Zn in the endosperm. Cadmium and Zn accumulation behaviour in wild type and transgenic lines from our experiment, and Zn accumulation reported by previous reports by Masuda et al 2009, Johnson et al 2011, Lee et al 2011, suggest endosperm is the preferred site for Cd and Zn deposition. It was reported in wheat plants grown with high dose of Zn fertilizer accumulated lower level of Cd in seeds (Oliver et al 1993; Sarwar et al 2015), and similarly in rice application of higher level of Zn and Fe

negatively influences the Cd uptake and accumulation (Hassan et al 2005; Shao et al 2007). Therefore, the lower level of Cd in our transgenic lines can be attributed to higher level of Fe and Zn in endosperm enacting a competitive inhibition of Cd.

3.6 Conclusions and future prospects

In conclusion, I have shown that the coexpression of *OsNAS1* and *HvNAATb* in rice increases the levels of NA and DMA and leads to accumulation of higher levels of Fe and Zn in the endosperm. The phytosiderophore metabolic engineering strategy therefore achieved the enhanced retention of Fe and Zn in the endosperm. Increasing the external supply of Fe affected the uptake of Fe and Zn into roots and their mobilization in the above-ground organs, but compensatory mechanisms presumably involving the vacuolar sequestration of metals in the flag leaf have a buffering effect and impose strict limits on the accumulation of metal micronutrients in the endosperm: 22.5 and 84 $\mu g\ g^{-1}$ DW for Fe and Zn, respectively. Furthermore, the preferential retention of Fe and Zn in the endosperm led to the competitive exclusion of Cd, and this can provide a useful strategy to increase the concentration of metal nutrients in polished rice seeds while ensuring that toxic metals are exported to the bran. Such strategies could help to address simultaneously micronutrient deficiency and heavy metal toxicity in communities that rely predominantly on cereal-based diets.

3.7 References

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Chapter 4

4 The ratio of the phytosiderophores nicotianamine and 2'-deoxymugenic acid influences organ specific Cd uptake and translocation in rice, excludes Cd from the endosperm and thus facilitates the preferential accumulation of Fe and Zn

4.1 Abstract

Metal transporters and ligands in rice are promiscuous, so the endosperm not only accumulates iron (Fe) and zinc (Zn) but also the strictly toxic heavy metal cadmium (Cd), affecting the health of those relying upon rice as a staple crop. Metabolic engineering in rice can increase the production of 2'-deoxymugenic acid (DMA) and nicotianamine (NA) and this can help to determine the mechanistic basis of Cd uptake, translocation and seed loading. I generated two metabolic phenotypes, one accumulating more DMA but less NA than wild-type rice (phenotype HD) and another accumulating higher levels of both phytosiderophores (phenotype HN). The plants were grown in nutrient solution supplemented with 10 µM Cd. Compared to wild-type plants, both phenotypes showed an increase in Cd uptake and root-to-shoot translocation, but Cd remobilization from the middle leaf to the flag leaf and Cd seed loading were both suppressed, resulting in lower Cd levels in the endosperm. Interestingly, the HD and HN phenotypes also showed increases in Zn uptake, Zn root-to-shoot translocation, Fe remobilization from the middle leaf to the flag leaf, and accumulation of Fe and Zn in the endosperm. These results suggest that although DMA and NA promote Cd uptake and root-to-shoot translocation, the preference for Fe and Zn during phloem loading and seed deposition prevents Cd enrichment in the aerial organs and seeds.

4.2 Aims

The aims of the work described in this chapter were to generate two transgenic genotypes differing in the amounts of accumulated phytosiderophores, and to study three aspects of the transgenic plants: (i) Cd uptake, translocation and seed loading; (ii) Fe and Zn uptake, translocation and seed loading in the presence of externally supplied Cd; and (iii) molecular mechanisms governing the uptake, translocation and seed loading of Fe, Zn and Cd.

4.3 Materials and methods

4.3.1 Rice transformation

Mature rice seed-derived embryos (*Oryza sativa* L. cv EYI 105) were transformed with the *OsNAS1* and *HvNAATb* cDNAs and the selectable marker *hpt* by particle bombardment as described in Chapter 2.

4.3.2 mRNA blot analysis

OsNAS1 and *HvNAATb* expression was analyzed using 20 μg of total leaf RNA as described in Chapter 2.

4.3.3 Cd uptake studies

Seeds from transgenic rice lines HD-1, HD-2, HN-1 and HN-2 were germinated on half-strength MS medium supplemented with 50 mg L^{-1} hygromycin and wild-type seeds were germinated on half-strength MS medium without hygromycin. After 7 days, 15 uniform-sized seedlings from wild-type and transgenic lines were transferred to nutrient solution (Kobayashi et al 2005) containing 10 μM $CdCl_2$. The pH of the solution was adjusted to 5.3 with 0.1 N KOH and the plants were maintained until maturity as described in Chapter

2. Samples of roots, middle leaves, flag leaves and seeds were harvested from all plants to determine metal concentrations and to analyze endogenous gene expression.

4.3.4 Measurement of metals

Fe, Zn and Cd levels in different plant tissues were measured as described in Chapter 3.

4.3.5 Extraction and quantitation of NA and DMA from roots, leaves and seeds

Roots and leaves were collected and stored immediately in liquid nitrogen at –80°C. NA and DMA were extracted as previously described (Schimdt et al 2011) with modifications. Briefly, 200 mg of plant tissue was suspended in 200 µL (roots) or 400 µL (leaves) of double deionized water and mixed with 36 µL 1 mM nicotyl-lysine. The homogenate was vortexed for 30 s and sonicated for 5 min before centrifuging at 15,000 g for 10 min. The supernatant was passed through 3-kDa centrifugal filter (regenerated cellulose Amicon® Ultra filter units, Merck KGaA) at 15,000 g for 30 min and the filtered solution was concentrated under vacuum until dry. The residue was dissolved with 20 µL double deionized water and a 5-µL aliquot was diluted with 10 µL 50 mM EDTA, 15 µL of double deionized water and 30 µL of a 1:9 mixture of 10 mM ammonium acetate/acetonitrile at pH 7.3. The mixture was filtered through polyvinylidene fluoride (Durapore® PVDF) 0.45-µm ultrafree-MC centrifugal filter devices (Ultrafree® MC HV, Merck KGaA) before separation by HPLC/ESI-TOF-MS as described in Chapter 2. NA and DMA were extracted from seeds and measured as described in Chapter 2.

4.3.6 Quantitative real-time RT-PCR for endogenous metal homeostasis genes

Quantitative real-time RT-PCR was carried out for the endogenous genes listed in Table 4.1 as described in Chapter 3.

Table 4.1 Details of genes and primers used for quantitative real time RT-PCR

Gene family	Gene	Role of gene	GenBank ID	Forward primer	Reverse primer
Plasma membrane metal transporters	*OsYSL15*	Fe (III)-DMA	AB190923.1	CTGGTGCTGTTTGCTTGGA	ATGAGCCCAGACGCAACAGC
	OsIRT1	Fe (II), Zn (II), Mn (II)	BAB85123.1	GCATCATGCAATTCGCTGC	CCTGAACAACCACGCTACAA
	OsNRAMP5	Fe (II), Mn (II), Cd (II)	AB690551.1	GCTGCCGTTTGCTCTCATCC	CCGATGATGAGCAGACCCAGGA
	OsZIP1	Zn (II)	AY324148.1	CGATGGTCCTCTTCTTCTGC	GTCGGGCTGCTCTCGTTGTA
	OsZIP4	Zn (II)	AB126089.1	TCCACCAGTTCTTTGAAGGC	CGGAGCAGTGAGGGAGAAGA
Vacuolar tonoplast membrane transporters	*OsHMA3*	Zn (II), Cd(II)	AB559519.1	GCTCAATCCAAATCCATCCA	GTTGACTGAGGTGATGACGC
	OsVIT1	Fe(II), Zn (II)	NM_001059545.1	CCACAGCACAGAACGCCAT	GCTGAGGAATGGACGGTTT
Long distance transporters	*OsNRAMP1*	Fe (II), Cd(II)	DQ431468.1	CGGTCGTCTCATCGTCATTGC	ACCTATCTTGTTGCTGCTGCTGC
	OsHMA2	Zn (II), Cd (II)	AB697186.1	CGAGGAATACACCCGCATCAGC	CAAGCAGAGCCACGAAAAGAGC
	OsLCT1	Cd(II), Mn (II)	AB905364.1	GCTCAAGTGGATGGCGGACA	GCCACCATCTTGTCCTTGCTT
	OsYSL2	Fe (II)-NA, Mn(II)-NA	AB164646	GGTGGAGAGAGTTGTGGGTT	TCATTCCCGCACCAACATA
	OsYSL16	Fe(II)-NA, Cu (II)-NA	AB673449.1	GGCTCTACTGGACTGCTTTCG	GCTGCGACAAAGAAGACCG
	OsFRDL1	Fe (III)-citrate	NM_001055921.1	GCATTCCTTTTGTCGCTGG	GGAGATAGCAGCCACACCA
	OsYSL18	Fe (III)-DMA	NM001051227.1	CGGAGTTCGGTCGGATGATG	TTGATGAGGTGAGCCGTCGC
Iron storage protein	*OsFERRITIN1*	Fe	AF519570.1	GCTTGAATGGAGGAGACTGTG	CTCCATTGCTACTGCGTGCT
Phytosiderophore synthesis pathway	*OsSAMS2*	S-adenosyl methionine synthase2	U82833.1	GTCCTCATCTCCACCCAGCA	GAGGGGTTGAGGTGGAAGAT
	OsNAS2	Nicotianamine synthase2	NM_001056429.1	GTCCTCATCTCCACCCAGCA	GAGGGGTTGAGGTGGAAGAT
	OsNAS3	Nicotianamine synthase3	NM_001067242.1	GCACCAGAAGATGGAGGACA	TGGTGAGGTAGCAAGCGATG
	OsNAAT1	Nicotianamine aminotransferase1	AB182275.1	GATGGCGACTTGGTTGGGT	GCTCCCTGAATGAAAGTTGCT
	OsNAAT2	Nicotianamine aminotransferase2	BR000703.1	CCACACAAGCCAGAGGGAT	TTCAAAGGCATCCACAAGAG
	OsDMAS1	Deoxymugenic acid synthase1	AB269906	TCCAAGGGCAAGACCGTAG	ATCCTCTGCCTCTCCTCCTC
Phytosiderophore extrusion pump	*OsENA1*	NA extrusion pump1	AK102457.1	TAAGCCAGAAGCCAAATGC	TTCAGTTCCTTGCCAATTTG
Transcription factors	*OsIRO2*	Iron regulated *bLH* transcription factor	BR000688.1	GAGCAATGTGGCGGGTTCA	AATGTCCTGTTCCCAAATCCG
	OsIDEF1	Iron deficiency responsive element rcognition transcription factor1	AK107456	GTCTTCAGGCTGGGGATGT	GGGATTTGTCTGCTGATG
	OsIDEF2	Iron deficiency responsive element rcognition transcription factor2	AK099540.1	GAGGGGCTTGTATTGGAGG	CTTCAGCCTCAGACATTGGTG
Reference gene	*OsACTIN1*		AB047313.1	TCATGTCCCTCACAATTTCC	GACTCTGGTGATGGTGTCAGC

4.3.7 Statistical analysis

Differences between transgenic and wild type plants were tested by comparison of means using the *t* test ($p<0.05$).

4.4 Results

4.4.1 Recovery and identification of differential OsNAS1 and HvNAATb co-expressing transgenic lines

Mature rice seed-derived embryos were co-transformed with *OsNAS1*, *HvNAATb* and *hpt* by particle bombardment. A total of 20 hygromycin-resistant transgenic plants were regenerated and mRNA blot analysis distinguished among the lines which expressed both transgenes simultaneously but at different levels (Figure 4.1). All 20 lines accumulated *OsNAS1* transcripts at high levels, whereas *HvNAATb* transcripts accumulated at high levels in some lines and at low levels in others.

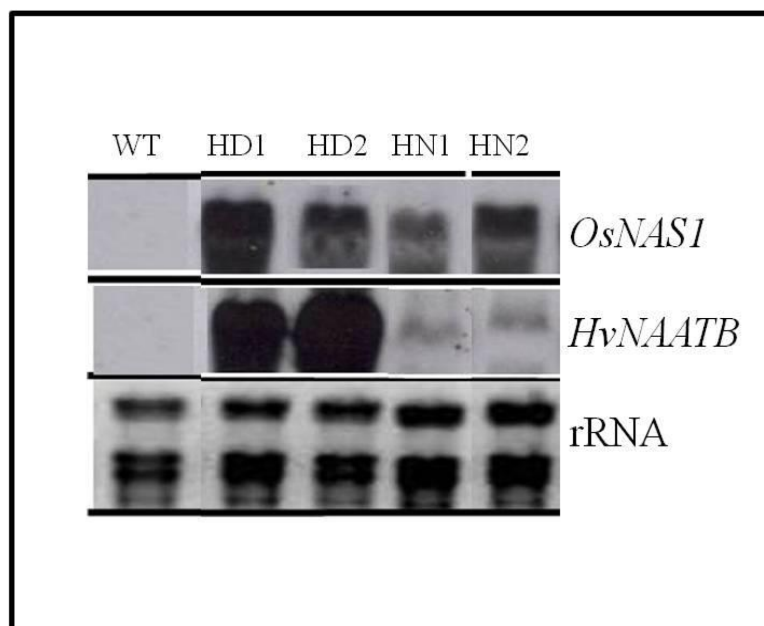

Figure 4.1 mRNA blot analysis showing transgene expression in leaf tissue from wild-type (WT) and transgenic lines. rRNA: ribosomal RNA; *OsNAS1*: rice nicotianamine synthase; *HvNAATb*: barley nicotianamine aminotransferase.

4.4.2 NA and DMA levels

The influence of *OsNAS1* and *HvNAATb* expression on the levels of NA and DMA was determined by measuring both metabolites in roots, leaves and seeds. The relative expression levels of the two transgenes in each line determined the NA/DMA ratio. Lines with high levels of *Osnas1* mRNA and low levels of *Hvnaatb* mRNA accumulated significantly higher levels of NA and DMA compared to wild-type plants, and the amount of NA was greater than that of DMA (NA/DMA ratio in roots, leaves and seeds = 11, 14 and 6, respectively). This metabolic phenotype was therefore described as “high NA” or HN (Figure 4.2). In contrast, the lines accumulating both *Osnas1* and *Hvnaatb* mRNA at high levels produced less NA than wild-type plants but more DMA. The amount of DMA in these lines was therefore higher than that of NA (NA/DMA ratio in roots, leaves and seeds = 0.09, 0.01 and 0.09, respectively). This metabolic phenotype was therefore described as “high DMA” or HD (Figure 4.2).

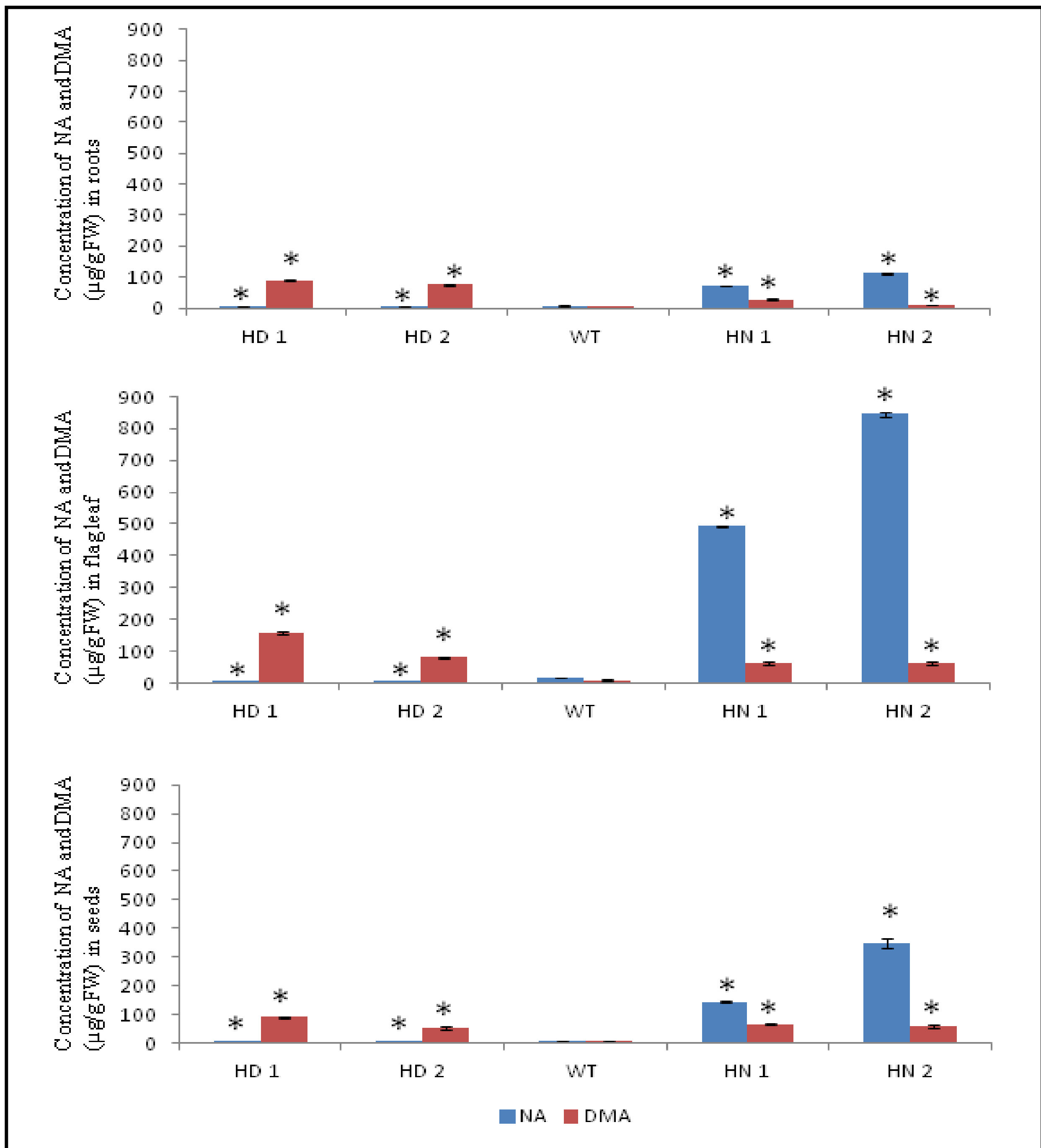

Figure 4.2 NA and DMA concentrations (µg/g FW) in the roots , flag leaf and seeds of transgenic lines co-expressing *OsNAS1*and *HvNAATb* compared to a wild-type (WT) control. Lines HD1 and HD2 have a low NA/DMA ratio, whereas lines HN1 and HN2 have a high NA/DMA ratio. Data are means ± SE, n = 3. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3).

4.4.3 Mineral accumulation

4.4.3.1 Cd levels in roots, middle leaf, flag leaf and seeds

Cadmium uptake, translocation and seed loading in the HD and HN lines were investigated by measuring the Cd concentration in the roots, middle leaf, flag leaf and seeds (polished seeds, unpolished seeds, and husk). In the roots, Cd levels were 5.6-fold (HD) and 2.3-fold (HN) higher than wild-type (Figure 4.3A), suggesting that both phytosiderophores promoted Cd uptake. In the middle leaf, Cd levels were 2.3-fold (HD) and 1.9 fold (HN) higher then wild-type (Figure 4.3B), and importantly the flag leaf of both lines accumulated up to 2.3-fold more Cd than wild-type plants (Figure 4.3C). These data indicate that root-to-shoot Cd translocation is boosted in both transgenic lines but there is less Cd remobilization from the middle leaf to the flag leaf, irrespective of the phytosiderophore ratio.

Cadmium levels in the seed husk were 2-fold higher than wild-type in the HD lines but remarkably 6-fold lower than wild-type in the HN lines (Figure 4.3D). Although unpolished HD seeds contained the same amount of Cd as wild-type seeds, the polished seeds contained 1.7-fold less Cd than wild-type polished seeds. In contrast, the unpolished and polished seeds of the HN lines contained less Cd than wild-type seeds. Specifically, the unpolished HN seeds contained 2.9-fold less Cd than wild-type seeds (Figure 4.3E) and the polished HN seeds contained 2.8-fold less Cd than wild-type seeds (Figure 4.3F). These data are consistent with the conclusion that neither phytosiderophores is involved in Cd endosperm loading.

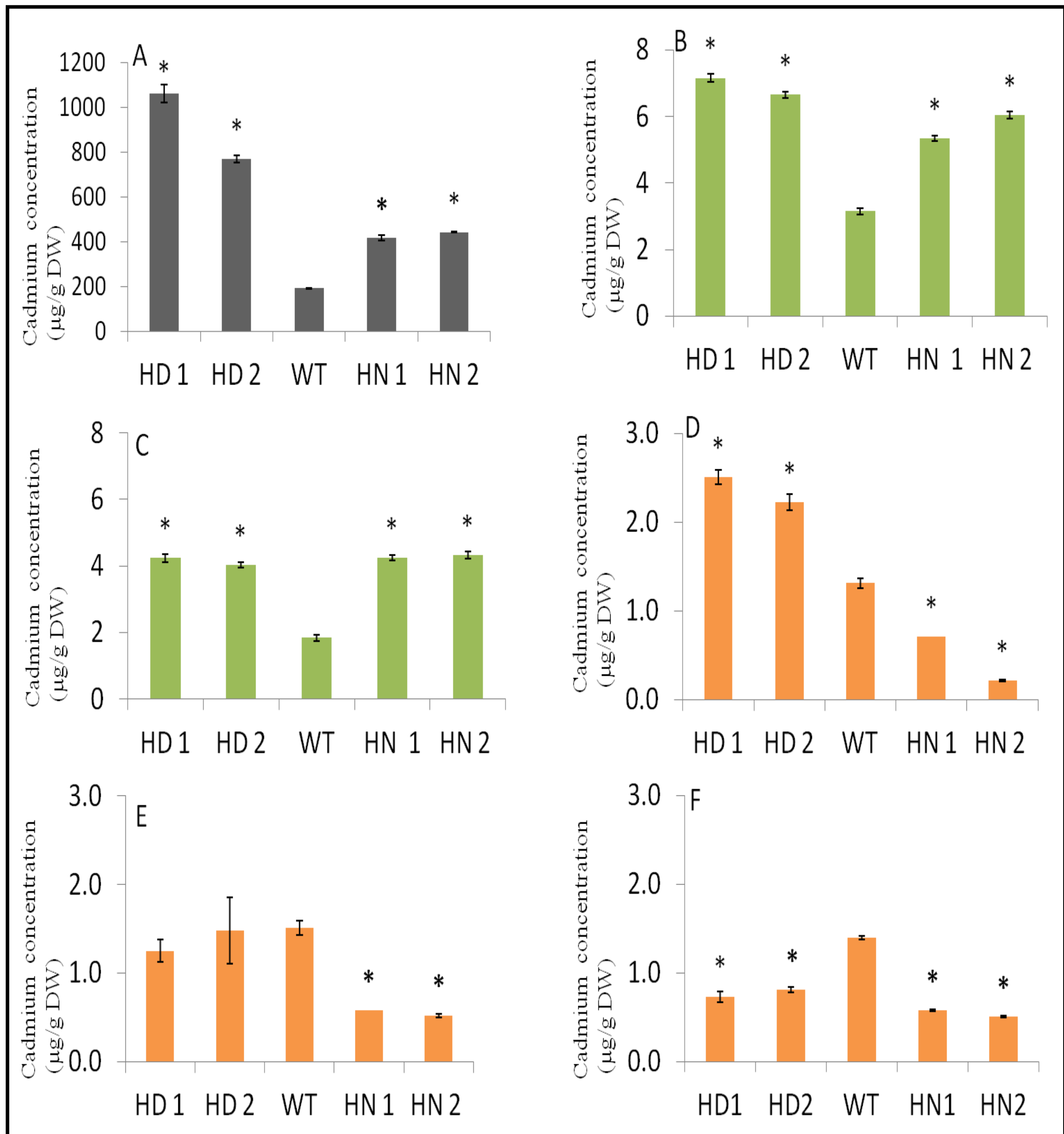

Figure 4.3 Cd concentrations (µg/g DW) in the roots (A), middle leaf (B), flag leaf (C), husk (D), unpolished seeds (E), and polished seeds (F) of wild-type (WT), HD and HN lines grown in the presence of 10 µM $CdCl_2$. Data are means ± SE, n = 6. Asterisks indicate a statistically significant difference between wild-type and transgenic plants as determined by Student's t test ($p < 0.05$; n = 6). Both HD and HN lines accumulated more Cd in the roots, middle leaf and flag leaf compared to wild-type plants but the Cd level in polished seeds is lower than in wild-type seeds.

4.4.3.2 Fe and Zn levels in roots, middle leaf, flag leaf and seeds

Fe and Zn uptake, translocation and seed loading in the HD and HN lines were also investigated when Cd was present in the nutrient solution, focusing on the same tissues listed in the previous section. In the roots, Zn levels were 7.5-fold (HD) and 3.6-fold (HN) higher than wild-type (Table 4.2A). These data suggest that phytosiderophores mobilize much more Zn than Cd, perhaps compensating for the fact that these metals compete for the same sites and that Cd can displace Zn from certain enzymes. In contrast, the roots of the HD plants accumulated similar levels of Fe to wild-type roots, whereas the HN roots accumulated 1.5-fold less Fe than wild-type roots (Table 4.2A). These data indicate that the higher levels of NA in the HN lines allow Cd to be taken up at the expense of Fe. In the middle leaf, Zn levels were 3-fold higher in both the HD and HN lines than wild-type plants, but Fe levels were 3.2-fold (HD) and 2.2-fold (HN) lower than in wild-type plants (Table 4.2A). Similarly in the flag leaf, Zn levels were 3.2-fold (HD) and 2.4-fold (HN) higher than wild-type, whereas Fe levels were 2.6-fold (HD) and 1.8-fold (HN) lower than wild-type (Table 4.2A), confirming a lower rate of Zn mobilization but a higher rate of Fe mobilization from the middle leaf and flag leaf in both the HD and HN lines.

In the seed husk, the HD lines accumulated more Zn (2.3-fold) and Fe (2-fold) than wild-type seeds, whereas the HN accumulated less Zn (2-7-fold) and similar levels of Fe compared to wild-type seeds (Table 4.2B). Fe and Zn mobilization to the husk therefore appears to be enhanced in the HD lines, whereas Zn is mobilized from the husk to the seed in the HN lines. In the unpolished seeds, the HD lines accumulated similar levels of Fe and Zn compared to wild-type seeds whereas the HN lines accumulated 2.3-fold more Fe and 1.9-fold more Zn than wild-type seeds (Table 4.2B). In the polished seeds (endosperm), Fe levels were 1.6-fold (HD) and 1.7-fold (HN) higher in the transgenic lines compared to

wild-type seeds. In contrast, Zn levels in the HD endosperm were similar to wild-type and Zn levels in the HN lines were 1.8-fold higher than wild-type (Table 4.2B). These data suggest that the high levels of DMA in the HD endosperm promote Fe accumulation, whereas the high levels of NA in the HN endosperm promote the accumulation of both Fe and Zn. NA and DMA in the HD and HN lines appear to promote endosperm loading with Fe and Zn in preference to Cd.

Table 4.2A Fe and Zn concentrations in roots, middle leaf and flag leaf of wild-type (WT), high-DMA (HD) and high-NA (HN) phenotypes grown in the presence of 10 µM $CdCl_2$

Tissue	Sample	Fe (µg/g DW)	Zn (µg/g DW)	Fe fold over WT	Zn fold over WT
Root	HD 1	918 ± 35.65	377.18 ± 21 *	NC	7.5↑
	HD 2	1161 ± 14.6	211 ± 4.13 *		
	WT	983 ± 18.2	49.97 ± 0.9		
	HN 1	647 ± 24.85 *	176.54 ± 1.6 *	1.5↓	3.6↑
	HN 2	775 ± 4.14 *	182.68 ± 2.71 *		
Middle leaf	HD 1	84.7 ± 4.46 *	32.28 ± 1.42 *	3.2↓	3↑
	HD 2	78.69 ± 2.7 *	41.55 ± 0.66 *		
	WT	251.92 ± 4.42	13.75 ± 0.61		
	HN 1	121.88 ± 5.37 *	41.95 ± 2.36 *	2.2↓	3↑
	HN 2	112.72 ± 2.7 *	34.66 ± 0.83 *		
Flag leaf	HD 1	88.56 ± 2.04 *	45.7 ± 1.95 *	2.6↓	3.2↑
	HD 2	105.39 ± 5.46 *	54.04 ± 3.32 *		
	WT	227.45 ± 6.95	16.78 ± 0.51		
	HN 1	147.18 ± 3.37 *	44.42 ± 3.2 *	1.8↓	2.4↑
	HN 2	122.9 ± 7.9 *	37.16 ± 2.11 *		

Symbols: ↓ = lower than WT; ↑ = higher than WT; Data are means ± SE, n = 6. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6).

Table 4.2B Fe and Zn concentrations in husk, unpolished and polished seeds of wild-type (WT), high-DMA (HD) and high-NA (HN) phenotypes grown in the presence of 10 µM $CdCl_2$

Tissue	Sample	Fe (µg/g DW)	Zn (µg/g DW)	Fe fold over WT	Zn fold over WT
Polished seeds	HD 1	5.1 ± 0.39*	33.36 ± 0.55	1.6↑	NC
	HD 2	5.19 ± 0.22*	24.6 ± 5.44		
	WT	3.24 ± 0.23	32.28 ± 0.67		
	HN 1	5.46 ± 0.59*	50.15 ± 0.63*	1.7↑	1.8↑
	HN 2	5.83 ± 0.41*	58 ± 0.71*		
Unpolished seeds	HD 1	22.09 ± 1.03	40.98 ± 2.47*	NC	NC
	HD 2	23.02 ± 1.48	37.77 ± 1.03*		
	WT	19.18 ± 1.26	33.39 ± 0.37		
	HN 1	43.52 ± 4.21*	55.95 ± 12.03*	2.3↑	1.9↑
	HN 2	44.30 ± 0.63*	63.06 ± 0.51*		
Husk	HD 1	36.30 ± 1.04*	44.49 ± 0.75*	2↑	2.3↑
	HD 2	41.94 ± 5.55*	33.53 ± 1.16*		
	WT	21.63 ± 1.63	19.44 ± 1.99		
	HN 1	24.16 ± 1.47	7.14 ± 0.61*	NC	2.7↓
	HN 2	21.67 ± 0.66	9.87 ± 0.81*		

Symbols: ↓ = lower than WT; ↑ = higher than WT; Data are means ± SE, n = 6. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6).

4.4.4 Expression of endogenous metal homeostasis genes in roots, flag leaf and immature seeds

The mechanistic basis of Cd, Fe and Zn behavior in the roots, flag leaf and seeds of rice plants exposed to Cd was investigated by measuring the expression of endogenous genes involved in metal homeostasis (Table 4.3). These genes included those involved in metal uptake (*OsIRT1, OsYSL15, OsNRAMP5, OsZIP1* and *OsZIP4*), vacuolar sequestration (*OsHMA3* and *OsVIT1*), long distance transport (*OsNRAMP1, OsHMA2, OsLCT1, OsYSL2, OsYSL16, OsYSL18* and *OsFRDL1*), iron storage (*OsFERRITIN1*), phytosiderophore biosynthesis (*OsSAMS2, OsNAS2, OsNAS3, OsNAAT1, OsNAAT2* and *OsDMAS1*), nicotianamine extrusion (*OsENA1*), and those encoding transcription factors (*OsIRO2, OsIDEF1* and *OsIDEF2*).

As anticipated, the expression of several of these genes was modulated in the roots of HD and HN lines compared to wild-type plants (Table 4.3). The plasma membrane-bound Fe/Zn/Cd transporter *OsIRT1,* the Fe-DMA transporter *OsYSL15* and the Fe/Cd transporter *OsNRAMP5* were upregulated in HD and HN lines, suggesting that higher Cd/Zn levels in the roots reflected the upregulation of these transporters. Although the upregulation of these transporters did not cause Fe levels to increase in the roots of the HD lines, it may have favored Cd/Zn uptake and thus reduced Fe levels in the HN lines. Likewise, the Cd/Zn vacuolar transporter *OsHMA3* was upregulated in both transgenic lines, indicating that most of the Cd/Zn was sequestered into vacuoles. The long distance Cd/Fe transporter *OsNRAMP1* and the long distance Cd/Zn transporter *OsHMA2* behaved in a similar manner, suggesting that root-to-shoot translocation of all three metals was increased in the transgenic plants. Interestingly, the Fe-deficiency-inducible basic helix-loop-helix (bHLH) transcription factor, *OsIRO2*, which controls the expression of genes for phytosiderophore synthesis and Fe uptake, was upregulated in both transgenic lines, suggesting that the

enhanced Cd/Zn uptake, Cd/Zn vacuolar sequestration and Cd/Fe/Zn long distance transport may be regulated by OsIRO2. In contrast, the plasma membrane-bound Zn transporters *OsZIP1* and *OsZIP4*, the Fe/Zn vacuolar transporter *OsVIT1,* the long distance Cd/Mn transporter *OsLCT1*, the Fe-NA transporter *OsYSL2*, and the Fe-DMA transporter *OsYSL18* were downregulated in the transgenic plants. Concerning the phytosiderophore biosynthesis pathway, *OsNAS2* and the NA extrusion pump *OsENA1* were upregulated in both transgenic lines, suggesting that endogenous NA synthesis and NA extrusion were induced in response to changes in Cd/Fe/Zn levels. However, the upregulation of *OsNAS2* may have suppressed *OsNAS3*. In contrast, *OsNAAT1* and *OsNAAT2* were down regulated in both transgenic lines, suggesting that the induction of NA synthesis and secretion was not mirrored by the induction of DMA synthesis.

Several endogenous metal homeostasis genes were also modulated in the flag leaves of the HD and HN lines (Table 4.3). In the HD lines, the Fe/Zn/Cd transporter *OsIRT1* and the Fe-DMA transporter *OsYSL15* were upregulated, whereas in the HN lines *OsIRT1*, *OsYSL15* and the Fe-Cd transporter *OsNRAMP5* were upregulated, suggesting that DMA and NA promote the transfer of Fe/Zn/Cd from the leaf epidermis towards the cortex. Vacuolar transporters play a key role in metal sequestration, and accordingly the Fe/Zn vacuolar transporter *OsVIT1* was downregulated in both transgenic lines, whereas the Cd/Zn vacuolar transporter *OsHMA3* was downregulated in the HD lines but upregulated in the HN lines. The behavior of *OsVIT1* and *OsHMA3* in the HD lines suggested that Cd/Fe/Zn in the cytoplasm is translocated to the seeds most likely for sequestration in the husk, whereas the behavior of the same genes in the flag leaf of the HN lines suggests that Zn/Cd is sequestered into vacuoles thus suppressing seed loading with Cd. The Cd phloem transporter *OsLCT1* was downregulated in both lines whereas the long distance Zn/Cd

transporter *OsHMA2* and the long distance Fe/Cd transporter *OsNRAMP1* were upregulated in both lines. Although *OsLCT1* was downregulated in the HD lines suggesting the suppression of Cd phloem loading, the presence of Cd/Fe/Zn in the cytoplasm caused by the downregulation of *OsHMA3* and *OsVIT1*, followed by the upregulation of *OsHMA2* and *OsNRAMP1*, indicates the transfer of Fe/Zn/Cd towards the husk and Fe/Zn towards endosperm as reflected in the corresponding metal levels in those tissues. In contrast, phloem loading of Cd in the HN lines by *OsLCT1* was inhibited, reducing the mobilization of Cd from flag leaf, possibly reflecting the higher level of Fe and Zn in the flag leaf compared to Cd. The higher levels of *OsNRAMP1* and *OsHMA2* may have encouraged Fe and Zn mobilization from the flag leaf towards the seeds, resulting in higher Fe and Zn concentrations. The lower level of Fe in the flag leaf in both the HD and HN was supported by the downregulation of the iron storage protein *OsFERRITIN1*. Although the behavior of the plasma membrane transporters (*OsIRT1*, *OsYSL15* and *OsNRAMP5*), vacuolar transporters (*OsHMA3* and *OsVIT1*) and long distance transporters (*OsLCT1*, *OsHMA2* and *OsNRAMP1*) in the HD and HN lines did not inhibit long distance Fe-NA transporters (*OsYSL2* and *OsYSL16*), or long distance Fe-DMA transporters (*OsYSL18*), the Fe-citrate long distance transporter *OsFRDL1* was suppressed. Similarly *OsNAS2* and *OsNAS3* were downregulated in the flag leaf of both transgenic lines but *ONAAT1* was upregulated, suggesting that endogenous NA biosynthesis is repressed but the conversion of NA to DMA is enhanced. Whereas many of the genes listed in Table 4.3 were modulated in the roots and flag leaf of the transgenic plants, only a few were modulated in the seeds. *OsNRAMP1* and *OsHMA2* were upregulated in HD seeds whereas only *OsNRAMP1* was upregulated in HN seeds, suggesting that the accumulation of Fe and Zn in the seeds may be regulated by these transporters.

Table 4.3 Fold increase or decrease in the relative expression level of endogenous genes involved in metal homeostasis in roots, flag leaf and immature seeds in the presence of 10 µM CdCl2

Role of Genes	Gene	Roots		Flag leaf		Seeds	
		HD Cd	HN Cd	HD Cd	HN Cd	HD Cd	HN Cd
Metal transporters	*OsYSL15*	6.6 ↑↑	10.1↑↑	2.4↑	3.5↑	NA	NA
	OsIRT1	22 ↑↑	15.75 ↑↑	2.2↑	2.5↑	NC	NC
	OsNRAMP5	17↑↑↑	21↑↑↑	NC	6↑	NA	NA
	OsZIP1	7↓↓	4.1 ↓	6.2↓↓	NC	NA	NA
	OsZIP4	11↓↓	12↓↓	NC	21↑↑↑	NC	NC
Vacuolar sequestration	*OsHMA3*	4 ↑	3.7 ↑	3.1 ↓	2.8↑	NA	NA
	OsVIT1	30↓↓↓	8↓↓	9 ↓↓	6 ↓	2.9↑	NC
Long distance transport	*OsNRAMP1*	38↑	18 ↑	2.4↑	3.5↑	3.9↑	3.3↑
	OsHMA2	24↑↑	11↑↑	4.2↑	2↑	3.7↑	NC
	OsLCT1	4.9↓	6↓↓	8.1↓	13.4↓↓	NA	NA
	OsYSL2	5.9↓↓	7.6 ↓↓	NC	NC	NA	NA
	OsFRDL1	NC	NC	8.7↓↓	7.1↓↓	NC	NC
	OsYSL16	NC	NC	NC	NC	NC	NC
	OsYSL18	34.6↓↓↓	24.2↓↓↓	NC	NC	NC	NC
Iron storage	*OsFERRITIN1*	NC	NC	6.2↓↓	3.9↓	NC	NC
Phytosiderophore synthesis pathway	*OsSAMS2*	NC	NC	NC	NC	NC	NC
	OsNAS2	7.3↑↑	11↑↑	3.4↓	17↓↓	NA	NA
	OsNAS3	32↓↓↓	26↓↓↓	3.5↓	6.7↓↓	NC	NC
	OsNAAT1	4.5↓	5.1↓	35↑↑↑	33↑↑↑	NC	NC
	OsNAAT2	5.3↓	4.6↓	2.8↓	NC	NA	NA
	OsDMAS1	NC	2.5↑	2.4↓	NC	NC	NC
Phytosiderophore extrusion pump	*OsENA1*	28↑↑↑	24↑↑↑	NC	NC	5.1↓↓	2.4↓
Transcription factors	*OsIDEF1*	5.3 ↓↓	6.6 ↓	NC	NC	NA	NA
	OsIDEF2	NC	NC	NC	NC	NA	NA
	OsIRO2	43↑↑↑	21↑↑↑	28↑↑	2.4↑	NA	NA

Note: HD = high DMA, HN = high NA, NC = No change, NA = not analyzed, Symbols: ↑ Upregulation (2–5 fold), ↑↑ Upregulation (5–10 fold), ↑↑↑ Upregulation (>10 fold), ↓ Downregulation (2–5 fold), ↓↓ Downregulation (5–10 fold), ↓↓↓ Downregulation (>10 fold). Each value is the average of three independent experiments. Transcript levels are represented by the ratio between mRNA levels of genes listed in the table and those of Os*ACTIN1*. Upward and downward arrows indicate a statistically significant upregulation and downregulation of endogenous metal homeostasis genes in transgenic lines compared to wild type as determined by Student's t test (p <0.05; n=3).

4.5 Discussion

The phytosiderophores DMA and NA promote the uptake, translocation, and seed loading of Fe and Zn by modulating the expression of metal transporters. Genetic engineering can therefore be used to increase DMA and NA levels in plants and thus improve the Fe and Zn content of seeds. However, metal transporters are promiscuous, i.e. many of them can also transport the strictly toxic heavy metal Cd along with Fe and Zn, but the ability of transgenic plants with enhanced levels of phytosiderophores to mobilize Cd is unclear. Similarly, in plants accumulating higher levels of phytosiderophores, Fe and Zn homeostasis in the presence of Cd has not been investigated.

To investigate the impact of higher levels of DMA and NA on Fe, Zn and Cd homeostasis and the corresponding molecular mechanisms, I generated transgenic plants co-expressing nicotianamine synthase (OsNAS1) and nicotianamine amino transferase (HvNAATb). All the transgenic lines expressed *OsNAS1* at high levels but differences in the level of *HvNAATb* mRNA (Figure 4.1) yielded two different phenotypes with contrasting NA/DMA ratios. The HN lines, expressing *HvNAATb* at lower levels than *OsNAS1*, produced more NA and DMA than wild-type plants, and the NA/DMA ratios in the roots, leaves and seeds were 11, 14 and 6, respectively (Figure 4.2). Similarly, the expression of *AtNAS1* (Wirth et al 2009), *HvNAS1* (Masuda et al 2008; 2009; 2012; 2013), and *OsNAS1-3* (Johnson et al 2011) was predicted to convert S-adenosylmethionine (SAM) to NA to achieve higher levels of both NA and DMA. Although in these previous studies NA levels in seeds increased by up to 20-fold (Cheng et al 2007; Lee et al 2009; Masuda et al 2008; Masuda et al 2009; Johnson et al 2011; Lee et al 2011; Masuda et al 2012) and DMA levels in seeds increased by up to 5-fold (Lee et al 2009; Masuda et al 2009; Lee et al 2011; Masuda et al 2012), our HN lines achieved a 43-fold increase in NA and a 12-fold

increase in DMA compared to wild-type seeds, indicating that the co-expression of *OsNAS1* and relatively low levels of *HvNAATb* was clearly advantageous over single gene expression by enhancing the conversion of SAM-NA and NA to DMA. In contrast to our HN lines, the HD lines expressed *OsNAS1* and *HvNAATb* at similar levels and this resulted in the conversion of more NA to DMA resulting in low NA/DMA ratios of 0.09, 0.01 and 0.09 in roots, leaves and seeds, respectively (Figure 4.2). Although previous studies achieved higher levels of DMA alone by expressing *HvNAATa* and *HvNAATb*, there was only a moderate increase over wild-type levels perhaps due to the limited NA pool available for conversion (Takahashi et al 2001; Takahasi et al 2003). In our lines, the co-expression of *OsNAS1* and *HvNAATB* at high levels increased the NA pool by converting SAM to NA, providing a more abundant substrate for *HvNAATb* to synthesize DMA. Phytosiderophore biosynthesis enzymes from barley are more active than those from other cereals such as rice, wheat and sorghum (Ma et al 1999) so the expression of *HvNAATb* in our HD transgenic lines was sufficient to convert the majority of the NA pool into DMA resulting in the observed low NA/DMA ratios.

Next I investigated the impact of the HD and HN phenotypes on Cd uptake, translocation and seed loading. The HD and HN lines respectively had 5.6-fold and 2.3-fold higher levels of Cd in the roots compared to wild-type plants (Figure 4.3A). Although DMA can mobilize Cd in the soil, the weak Cd-DMA are not taken up by the plants (Shenkar et al 2000, Kudo et al 2007; Meda et al 2007). This suggests that the higher level of Cd in the HD lines results from the ability of DMA to mobilize Cd in solution, followed by uptake via metal transporters. Furthermore, *OsNAAT1* mutations which block the synthesis of DMA accumulate more NA and take more Cd into the roots than wild-type plants, similar to our HN phenotype (Cheng et al 2007). The enhanced uptake of Cd by the HD and HN

lines is accompanied by higher Cd root-to-shoot translocation, resulting in higher levels of Cd in the middle leaf and flag leaf compared to wild-type plants (Figure 4.3B; Figure 4.3C). Although the role for DMA is unclear, NA is known to facilitate Cd root-to-shoot translocation (Cheng et al 2007). Our results therefore confirm previous reports and also provide evidence that DMA also promotes Cd root-to-shoot translocation. Interestingly, whereas the accumulation of Cd in unpolished seeds was similar in HD lines and wild-type plants, the concentration of Cd in the husk was 1.9-fold higher than wild-type seeds and the concentration of Cd in the endosperm was 1.7-fold lower than wild-type seeds (Figure 4.3D; Figure 4.3E; Figure 4.3F). Surprisingly, the HN lines accumulated less Cd in all seed tissues (husk, unpolished seeds and polished seeds) compared to wild-type plants (Figure 4.3D; Figure 4.3E; Figure 4.3F). Although previous studies of transgenic plants with higher levels of NA (Cheng et al 2007) or both NA and DMA (Masuda et al 2012) did not look at Cd levels in the husk and polished seeds separately, the unpolished seeds contained less Cd than wild-type seeds. Our results suggest that although the HD and HN lines are characterized by enhanced Cd uptake and root-to-shoot translocation, the Cd is sequestered in the roots, leaves and husk (HD lines) or roots and leaves (HN lines) to prevent accumulation in the endosperm. Cd is toxic to humans (Meda et al 2007; Ueno et al 2010; Uraguchi et al 2011; Uraguchi et al 2012; Clemens et al 2013; Slamet-Loedin 2015), and the relative depletion in the endosperm of HD lines (1.7-fold) and HN lines (2.8-fold) may therefore help to remove Cd from the soil and divert it to the non-edible portions of the rice plants.

All metals are toxic once they reach a certain threshold concentration so plants have evolved regulatory mechanisms to maintain homeostasis (Lee and An 2009; Lee et al 2009; Lee et al 2011; Wang et al 2013). The enhanced Cd uptake and root-to-shoot

translocation in the HD and HN did not result in Cd seed loading, possibly due to the competition by other metals for shared chelators and transporters. Fe and Zn homeostasis in the HD and HN lines was therefore investigated along with Cd homeostasis. Zn levels were higher than wild-type controls in the roots of both HD and HN plants, but Fe levels were unchanged in the HD lines and were significantly lower in the HN lines (Table 4.2A). This suggests that the presence of Cd in the HD and HN lines does not affect Zn uptake but interferes with Fe uptake. Cd is a strictly toxic heavy metal which induces the synthesis of reactive oxygen species and lipid peroxidation (Aravind et al 2005). Zn is the closest analogue of Cd, and many plants take up more Zn to inhibit the negative effect of Cd (Tuner 1971; Aravind and Prasad 2004; 2005). Both the HD and HN lines took up more Zn to mitigate the potential oxidative stress caused by Cd. Although both NA and DMA are known to promote the uptake of Fe, the lower levels of Fe in the HN lines suggest that Fe uptake is compromised by Cd/Zn uptake and particularly by Zn. A similar trend for Zn was observed in the middle leaf and flag leaf, with both the HD and HN lines containing more Zn than wild-type plants, whereas both lines contained lower levels of Fe than wild-type controls (Table 4.2A). Because DMA and NA help to remobilize the metals from leaves towards the seeds, the higher level of Zn in the HD and HN lines suggest that Zn is held in the vegetative tissues to mitigate the effects of Cd, whereas Fe is transferred from the leaves to the seeds. Our results are consistent with impact of Cd on the mobilization of Fe and Zn in barley (Astolfi et al 2014), wheat (Nan et al 2002), maize (Nan et al 2002) and lettuce (Mckenna et al 1993).

In the HD seeds, the concentration of Fe and Zn in the husk increased 2-fold and 2.3-fold respectively, whereas in the HN lines there was no change in Fe levels and a decline in Zn levels. This suggests that the HD lines tend to direct metals to accumulate in the husk,

including Cd. In the HN lines however, the concentration of Zn decreased 2.7-fold in the husk, which appears to inhibit the accumulation of Cd and Zn in husk. Although the HD lines contained similar amounts of Fe and Zn in the unpolished seeds as wild-type controls, the HD polished seeds contained 1.6-fold more Fe than wild-type seeds and similar levels of Zn to wild-type seeds. Therefore, it appears that DMA promotes the preferential loading of Fe into the endosperm, which competes with Cd and displaces it. In contrast the level of Fe increased 1.7-fold and Zn 1.8-fold in the unpolished and polished HN seeds, displacing Cd from the endosperm. The HN lines accumulated more Fe and Zn than the HD lines thus displacing more Cd. Similarly, boosting the levels of NA (Cheng et al 2007), or both NA and DMA (Masuda et al 2012), or the expression of metal transporters and ferritin (Aung et al 2013), resulted in the significant accumulation of Fe and Zn with a concomitant reduction of up to 20% Cd.

Most metal transporters are promiscuous, so competition among different metals for uptake, transport and seed accumulation in the HD and HN plants reflects the behavior of the transporters and the ability of DMA and NA to regulate this process. Therefore, the expression of number of these transporters was investigated along with genes representing the phytosiderophore biosynthesis pathway and transcription factors (Table 4.3). In roots, the transporters *OsYSL15, OsIRT1* and *OsNRAMP5* were upregulated in both the HD and HN lines. Although *OsYSL15* is a Fe(III)-DMA specific transporter (Inoue et al 2009; Lee et al 2009), *OsIRT1* can transport Cd in addition to Fe and Zn (Lee and An 2009) and *OsNRAMP5* can transport Cd in addition to Fe (Sasaki et al 2012). The upregulation of *OsIRT1* and *OsNRAMP5* in the HD and HN lines therefore promoted the uptake of Cd and Zn whereas the upregulation of *OsYSL15* probably boosted the transport of Fe. Because Cd is detrimental to plant health, plants have evolved vacuolar sequestration as a strategy to

minimize the impact of Cd toxicity (Ueno et al 2010; Sasaki et al 2012). The upregulation of the Cd/Zn vacuolar transporter *OsHMA3* suggests that most of the Cd in the HD and HN lines was sequestered into vacuoles along with significant amounts of Zn, explaining why the roots accumulated the highest levels of both metals in our lines and in other transgenic plants expressing *OsHMA3* (Ueno et al 2010; Sasaki et al 2012).

The root-to-shoot translocation of metal through the xylem is one of the key factors that determine the rate of translocation of metals, and thus the availability of metals for remobilization from leaves to seeds. Cd/Fe/Zn xylem loading is considered to be a rate limiting step, which determines the amount of Cd/Fe/Zn in the seeds, and *OsNRAMP1* (an Fe/Cd transporter) and *OsHMA2* (a Zn/Cd transporter) are important in regulating this process (Takahashi et al 2011; Takahashi et al 2012). Therefore, the higher expression of *OsNRAMP1* and *OsHMA2* in the HD and HN lines suggests higher xylem loading followed by root-to-shoot translocation of Cd/Fe/Zn. The relative abundance of the three metals in wild-type leaves was Fe>Zn>Cd, suggesting discrimination against Cd for xylem-mediated root-to-shoot translocation (Takahashi et al 2011; Takahashi et al 2012; Clemens et al 2013). More importantly, transcription factors play an important role in the regulation of metal uptake, vacuolar sequestration, and root-to-shoot translocation (Astolfi et al 2014). Interestingly, transcription factor *OsIRO2* was upregulated in the HD and HN lines, and may have induced the expression of *OsYSL15*, *OsIRT1* and *OsNRAMP5* (for Cd/Fe/Zn uptake), *OsHMA3* (for Cd/Zn vacuolar sequestration), *OsNRAMP1* and *OsHMA2* (for Cd/Fe/Zn root-to-shoot translocation).

The remobilization of metals from the flag leaf during grain filling is necessary for seed loading. Transporters and phytosiderophores play important roles in this process. The behavior of transporters in the flag leaf of the HD and HN lines was therefore considered

in relation to Cd/Fe/Zn mobilization (Table 4.3). In the HD lines, *OsIRT1* and *OsYSL15* were upregulated, whereas in the HN lines, *OsIRT1*, *OsYSL15*, *OsNRAMP5* and *OsZIP4* were upregulated. This suggests that both lines promoted the translocation of Cd/Fe/Zn from the epidermis/endodermis region to the cortex/stele region, where vacuolar sequestration, xylem loading and phloem loading takes place (Clemens et al 2013; Yoneyama et al 2015). *OsHMA3*, *OsVIT1* and *OsLCT1* were downregulated whereas *OsNRAMP1* and *OsHMA2* were upregulated, suggesting that Cd/Fe/Zn vacuolar sequestration was strongly suppressed by the inhibition of *OsHMA3* and *OsVIT1* allowing more Cd/Fe/Zn to be loaded in the vascular tissues. Similarly, the mutation of *OsVIT1* increased the availability of Cd/Fe/Zn for xylem and phloem loading, boosting the concentrations of these metals in the seeds (Zhang et al 2012). *OsLCT1* is necessary for Cd remobilization from the flag leaf (Clemens et al 2013; Uraguchi et al 2011; Uraguchi and Fujiwara et al 2012) and this gene was downregulated in the HD lines suggesting that Cd phloem loading was suppressed. Although *OsHMA2* and *OsNRAMP1* can load Cd into the xylem, the relative abundance of Cd in the flag leaf was 27-fold lower than Fe and 14-fold lower than Zn, so more Fe and Zn are transported compared to Cd. Furthermore, *OsYSL2*, *OsYSL6* and *OsYSL18* are expressed at the same level in HD and wild-type lines, suggesting that Fe and Zn phloem loading occurs normally in HD lines. The HD lines therefore demonstrated a higher level of endosperm Fe loading, as well as Cd/Fe/Zn loading in the husk. Only *OsHMA3* was upregulated in the HN lines, whereas *OsVIT1, OsLCT1, OsNRAMP1, OsHMA2, OsYSL2, OsYSL6* and *OsYSL18* were expressed at similar levels in the HN and HD lines, indicating that the HN lines had a greater capacity for Cd/Zn vacuolar sequestration in the flag leaf but less Cd xylem and phloem loading, resulting in much lower Cd concentrations in all parts of the seed. Few of the tested endogenous genes were modulated in the immature seeds of the transgenic lines (Table

4.3). In rice plants expressing *AtNAS1*, the number of metal homeostasis and phytosiderophore biosynthesis genes that were modulated by transgene expression was lower than the number in roots and leaves, which is consistent with the outcome observed in our HD and HN lines (Wang et al 2013).

4.6 Conclusions and future prospects

The co-expression of *OsNAS1* and *HvNAATb* resulted in two metabolic phenotypes: low NA/high DMA (HD) and high NA/high DMA (HN). Both phenotypes demonstrated higher root uptake and root-to-shoot translocation of Cd compared to wild-type plants, but seed loading with Cd was not increased. In contrast, these phenotypes showed higher Zn uptake, Fe/Zn root-to-shoot translocation, Fe remobilization from the flag leaf to seeds, and endosperm accumulation of Fe/Zn (HN lines) or Fe (HD lines). More detailed analysis of these phenotypes revealed both the HD and HN lines preferentially transfer Fe and Zn from roots to shoots and remobilize the two metals from flag leaf to seeds, while discriminating against Cd. This resulted in the preferential delivery of Fe/Zn (HN lines) or Fe (HD lines) to the endosperm, which suppresses Cd loading and retention resulting in a lower level of Cd in the seeds. This strategy therefore offers a simple and effective approach to engineer crops with increased Fe and Zn levels but lower Cd levels in the endosperm, while removing the strictly toxic heavy metal Cd from soil and diverting it to the straw which is not usually used for human nutrition.

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Chapter 5

5 Heterologous expression of the Fe (III) Phytosiderophore transporter HvYS1 leads to increased Fe levels in rice endosperm with the simultaneous exclusion of heavy metals

5.1 Abstract

A low iron (Fe) level in the endosperm is a major factor contributing towards iron deficiency anemia in many parts of the developing world where rice is the major staple. Rice engineered with metal transporters for Fe fortification reported thus far use promiscuous transporters which also promote the simultaneous uptake of Zn, an important micronutrient, but in addition toxic metals such as Cu, Mn and Cd. Therefore, in order to achieve the selective transport of Fe and preferential increase of Fe levels in the endosperm simultaneously excluding Cd I transformed rice with *HvYS1*. Transgenic rice lines expressing the input transgene had increased Fe uptake, root to shoot translocation, seed accumulation and endosperm loading. In contrast, although Zn, Mn, and Cu levels did not change in roots and leaves, Cu level in the endosperm was significantly reduced in the transgenic lines, confirming the selectivity of this Fe transporter. Simultaneously, transgenic lines had reduced uptake, root to shoot translocation and accumulation of Cd in seeds.

5.2 Aims

The aims of this study were to generate transgenic rice lines expressing the barley Fe (III)-DMA transporter YS1 and evaluate these lines for:

a) Fe uptake, translocation and seed accumulation

b) Uptake, translocation and accumulation of Zn, Cu, Mn and Cd

c) Impact on endogenous Fe homeostasis genes

5.3 Materials and methods

5.3.1 Rice transformation

Mature rice seed derived embryos (*Oryza sativa* L. cv EYI 105) were co-transformed with *HvYS1* and the selectable marker *hpt* by particle bombardment as described in matrials and methods in chapter 2.

5.3.2 mRNA blot analysis

Expression analysis for *HvYS1* was conducted using 20µg of total leaf RNA as described in materials and methods in chapter 2.

5.3.3 Cd uptake studies

Seeds from three representative transgenic rice lines (Lines 1, 2, and 3) were germinated on ½ MS medium supplemented with 50 mg l^{-1} hygromycin, and WT seeds were germinated on ½ MS medium without hygromycin. After 7 days, fifteen uniform seedlings from WT and transgenic lines were transferred to nutrient solution (Kobayashi et al 2005) containing 10µM $CdCl_2$. Sampling of roots, leaves and seeds was performed as described in materials and methods in chapter 3.

5.3.4 Measurement of mineral levels

Concentration of metals was measured in roots, leaves and seeds as described in materials and methods in chapter 3.

5.3.5 qRT-PCR for endogenous metal homeostasis genes

Total RNA was extracted from roots, leaves and immature seeds as described in materials and methods in chapter 4. qRT-PCR for endogenous genes listed in table 5.1 was also performed as described in materials and methods in chapter 4.

5.3.6 Statistical analysis

Differences between transgenic and wild type plants were tested by comparison of means using the *t* test ($p<0.05$).

Table 5.1 Genes and primers used for quantitative real-time RT-PCR analysis

Gene function	Full name of gene	Abbreviated gene name	Gene Bank ID	Forward primer	Reverse primer
Metal transporter	Yellow stripe like 15 Fe (III)-DMA transporter 15	*OsYSL15*	AB190923.1	CTGGTGCTGT TTGCTTGGA	ATGAGCCCAG ACGCAACAG C
	Fe regulated transporter 1	*OsIRT1*	BAB85123.1	GCATCATGCA ATTCGCTGC	CCTGAACAAC CACGCTACAA
	natural resistance-associated macrophage proteins 5	*OsNRAMP5*	AB690551.1	GCTGCCGTTT GCTCTCATCC	CCGATGATGA GCAGACCCA GGA
Vacuolar sequestration	Vacuolar iron transporter 1	*OsVIT1*	NM_0010595 45.1	CCACAGCACA GAACGCCAT	GCTGAGGAAT GGACGGTTT
Long distance transport	natural resistance-associated macrophage proteins1	*OsNRAMP1*	DQ431468.1	CGGTCGTCTC ATCGTCATTG C	ACCTATCTTG TTGCTGCTGC TGC
	Yellow stripe like 2 transporter	*OsYSL2*	AB164646	GGTGGAGAG AGTTGTGGGT T	TCATTCCCGC ACCAACATA
	Yellow stripe like 16 transporter	*OsYSL16*	AB673449.1	GGCTCTACTG GACTGCTTTC G	GCTGCGACAA AGAAGACCG
	Ferric redictase defective citrate extrusion transporter 1	*OsFRDL1*	NM_0010559 21.1	GCATTCCTTT TGTCGCTGG	GGAGATAGC AGCCACACCA
	Yellow stripe like 18 transporter	*OsYSL18*	NM00105122 7.1	CGGAGTTCGG TCGGATGATG	TTGATGAGGT GAGCCGTCGC
Iron storage	*Ferritin1*	*OsFERRITIN 1*	AF519570.1	GCTTGAATGG AGGAGACTGT G	CTCCATTGCT ACTGCGTGCT
Reference gene	*Actin1*	*OsACTIN1*	AB047313.1	TCA TGT CCC TCA CAA TTT CC	GAC TCT GGT GAT GGT GTC AGC

5.4 *Results*

5.4.1 Recovery of transgenic plants expressing *HvYS1*

I transformed 7 day old mature seed-derived zygotic rice embryos with a plasmid encoding barley yellow stripe 1 transporter (*HvYS1*) driven by the constitutive maize ubiquitin-1 (*ubi1*) promoter, under hygromycin selection. *HvYS1* expression in 15 independent transgenic lines was confirmed by mRNA blot analysis (Figure 5.1). These lines and corresponding wild type plants were grown to maturity and T_1 seed was collected. Five transgenic lines which expressed *HvYS1* at high levels were advanced to the next generation for further in depth analysis.

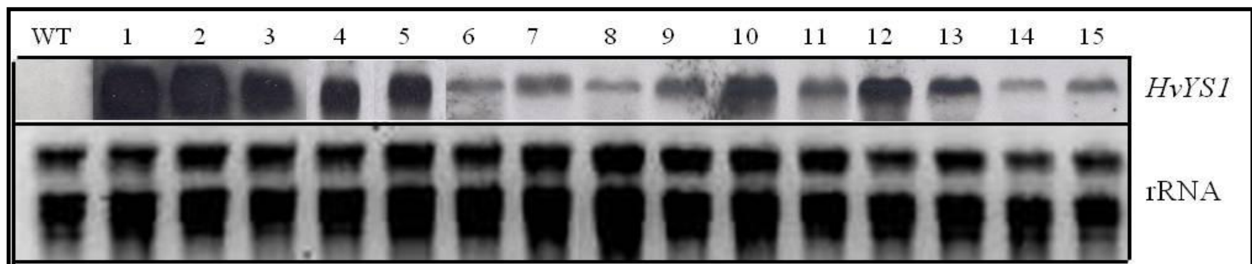

Figure 5.1 mRNA blot analysis showing transgene expression in leaves of wild type (WT) and transgenic lines expressing *HvYS1*. rRNA: ribosomal RNA; *HvYS1*: barley yellow stripe 1 like Fe (III)-DMA transporter

5.4.2 Mineral concentrations

5.4.2.1 Fe concentration

To understand the influence of *HvYS1* expression on Fe uptake, root to shoot translocation, and seed loading, I quantified Fe levels in roots, leaves and seeds. Iron content in roots of the transgenic lines increased up to ca: 64% over wild type (Figure 5.2). The roots of transgenic plants contained 566±38 µg/g DW of Fe compared to 345±10 µg/g DW in the wild type. The data are consistent with improved Fe uptake due to the expression of

HvYS1. In turn, this resulted in enhanced root to shoot translocation of Fe, reflected as an increase of up to ca: 208% (231±10 µg/g DW of Fe) in leaf Fe over wild type (ca: 104±4.99 µg/g DW of Fe) (Figure 5.2). This increase in Fe uptake and translocation due to *HvYS1* expression resulted in higher Fe seed loading and endosperm retention in the transgenic lines. In the unpolished seeds transgenic lines had up to ca: 60% (24± 0.46 µg/g DW of Fe) increases in Fe concentration over wild type (15.42±0.42 µg/g DW of Fe) (Figure 5.2). In the polished seeds transgenic lines accumulated up to ca: 216% (8.66±0.32 µg/g DW of Fe) higher levels of Fe than wild type (4±0.11 µg/g DW of Fe) (Figure 5.2). These results suggest that *HvYS1* expression in the transgenic lines improved Fe endosperm loading.

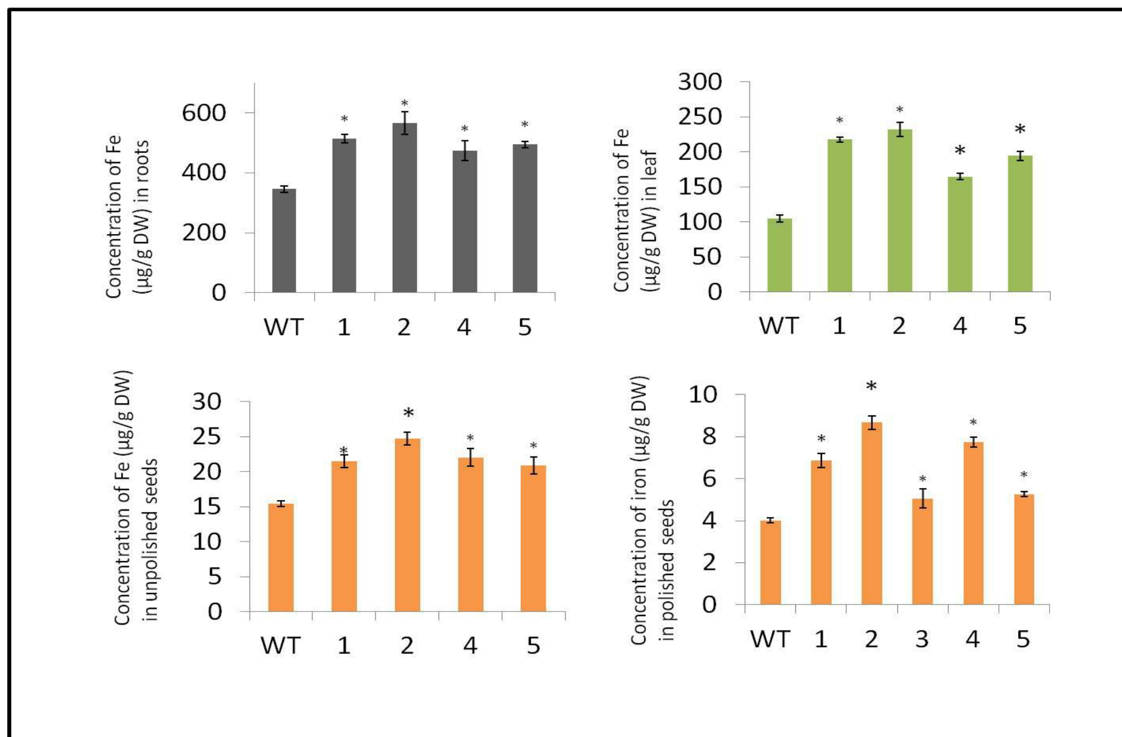

Figure 5.2 Concentrations of Fe (µg g^{-1} DW) in roots, leaf, unpolished and polished seeds of wild type (WT) and transgenic lines expressing *HvYS1* (lines 1, 2, 3, 4, 5). Data are means ± SE, n = 6. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6). DW: dry weight.

5.4.2.2 Zn, Mn, and Cu concentrations

Co-transport ability of many Fe transporters makes it possible that Zn, Cu, and Mn can be co-transported along with Fe. In order to understand whether *HvYS1* might also exhibit this promiscuous behavior in rice, I measured the levels of these three metals in roots, leaves and seeds. In roots of the transgenic lines concentration of the three metals did not differ in comparison with the wild type (Figure 5.3A), suggesting that *HvYS1* preferentially excludes the uptake of Zn, Cu, and Mn. Similarly to roots, levels of the three metals in the leaves of *HvYS1* lines did not differ from wild type (Figure 5.3B), suggesting that *HvYS1* did not alter the root to shoot translocation of Zn, Cu, or Mn.

In order to understand if there were seed-specific changes in the levels of the three metals, I measured their levels in seeds (unpolished as well as polished) of wild type and *HvYS1* lines. Although transgenic lines and wild type did not exhibit any differences in terms of Zn or Mn accumulation, levels of Cu in the transgenic lines were lower compared to the wild type. In unpolished seeds, transgenic lines had ca: 371% (3±0.1 μg/g DW) lower levels of Cu over wild type (11.48±0.13 μg/g DW) (Figure 5.3C). In polished seeds, transgenic lines had ca: 361% (2.4±0.13 μg/g DW) lower Cu over wild type (9±0.09 μg/g DW) (Figure 5.3D), suggesting that in both cases, Fe accumulation impacted the levels of seed Cu but not that of Zn or Mn.

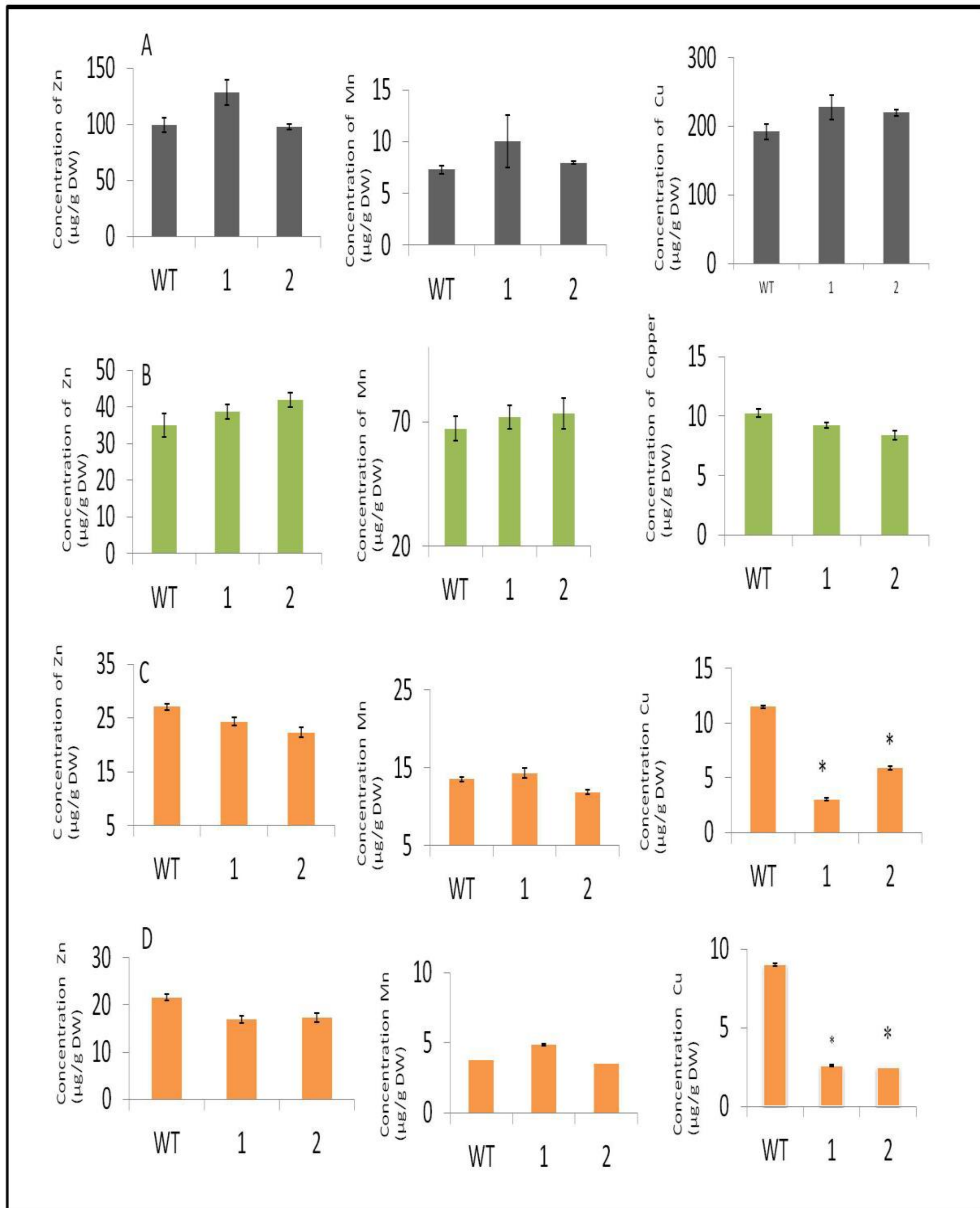

Figure 5.3 Levels of Zn, Mn, and Cu (µg g^{-1} DW) in roots (A), leaves (B), unpolished seeds (C) and polished seeds (D) of wild type (WT) and transgenic lines expressing *HvYS1* (lines 1 and 2). Data are means ± SE, n = 6. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6). DW: dry weight.

5.4.3 Expression of endogenous Fe homeostasis genes

Fe homeostasis in crops, including rice, is complex and involves expression of a number of genes specific for metal uptake, root to shoot translocation, remobilization from flag leaf, and accumulation in seeds (Table 5.2). In order to investigate the potential impact of increased Fe levels in roots, leaves and seeds of the transgenic lines on the expression of endogenous Fe homeostasis genes I carried out expression analysis for a number of Fe homeostasis genes in the transgenic and wild type lines (Table 5.2).These genes included transporters for Fe UPTAKE: Fe regulated transporter 1 (*OsIRT1*), Yellow stripe like Fe (III)-DMA transporter 15 (*OsYSL15*), natural resistance-associated macrophage proteins 5 (*OsNRAMP5*); Fe VACUOLAR SEQUESTRATION: vacuolar iron transporter 1 (*OsVIT1*); transporter for Fe LONG DISTANCE TRANSPORT: Yellow stripe like 2, 16, 18 (*OsYSL2*, *OsYSL16*, *OsYSL18*), natural resistance associated macrophage protein 1 (*OsNRAMP1*); and Fe STORAGE PROTEIN: FERRITIN1 (*OsFERRITIN1).*

In roots of transgenic plants the expression of a number of Fe homeostasis genes was altered, relatively to the wild type (Table 5.2). Expression of *OsIRT1*, *OsYSL15* was down regulated, whereas *OsNRAMP5* expression was upregulated, suggesting that uptake of Fe (III)-DMA as a result of *HvYS1* expression impacted negatively on *OsIRT1* and *OsYSL15* expression, which in turn triggered the expression of *OsNRAMP5*. Expression of *OsVIT1* declined in the transgenic lines whereas levels of *OsNRAMP1* mRNA increased, suggesting that higher levels of Fe in roots of the transgenic plants suppressed vacuolar sequestration caused by *OsVIT1*, allowing higher levels of Fe for root to shoot translocation mediated by *OsNRAMP1*. The increased expression of *OsNRAMP1* downregulated the expression of long distance Fe (III)-DMA transporter; *OsYSL18*, and Fe (III)-citrate transporter; *OsFRDL1*. In addition, *OsFERRITIN1* expression in the roots of

the transgenic plants was reduced. Since, FERRITIN is an iron storage protein and its expression increases with increases in Fe levels in a given tissue. Down regulation of FERRITIN in *HvYS1* lines suggests that higher level of Fe in roots of the transgenic lines did not impact negatively on cells in roots, most likely because *HvYS1* transports Fe (III)-DMA complex rather than elemental Fe. In leaves of the transgenic plants the expression of a number of Fe homeostasis genes was also altered relatively to the wild type (Table 5.2). *OsVIT1* expression did not change, whereas expression of *OsFERRITIN1* was upregulated in leaves of the transgenic plants. It appears that higher Fe levels in leaves did not promote vacuolar sequestration through *OsVIT1* but instead a large proportion of Fe was present in the cytoplasm. The increase in Fe concentration in the cytosol might have triggered Fe *OsFERRITIN* expression. In leaves, expression of *OsNRAMP5*, *OsYSL16*, *OsYSL18* was upregulated suggesting that these genes might play a synergistic role in the translocation of Fe from leaves towards seeds. Though *OsNRAMP5*, *OsYSL16*, *OsYSL18* was upregulated, modulation of *OsFERRITIN* expression suggests that Fe translocation from leaves to seeds through *OsNRAMP5*, *OsYSL16*, *OsYSL18* is limited.

In contrast to the changes in the expression patterns of a number of Fe homeostasis genes in roots and leaves of the transgenic plants, only a relatively small number of these endogenous genes altered their expression patterns in seeds of the transgenic lines (Table 5.2). Expression of *OsYSL15* and *OsIRT1* was downregulated, indicating that expression of *HvYS1* influenced negatively the expression of these endogenous metal transporters. Similarly, expression of *OsVIT1* was downregulated in the transgenic lines, suggesting that the amount of Fe in the embryo and the aleurone was reduced. This compensated by allowing Fe to enter into the endosperm. *OsYSL18* expression was also down regulated suggesting that delivery of Fe (III)-DMA in to the seeds by *HvYS1* expression,

downregulated the expression of *OsYSL18* which is also an important Fe (III)-DMA transporter in seeds.

Table 5.2 Fold change in relative expression levels of endogenous genes in transgenic plants

Genes		Roots	Flag leaf	Seeds
Metal transporters	*OsYSL15*	↓ 2.2	*NC*	*7.2*↓↓
	OsIRT1	↓ 3	*NC*	*2.8*↓
	OsNRAMP5	↑3.2	*2.6*↑	*NC*
Vacuolar sequestration	*OsVIT1*	↓↓7.7	*NC*	*9*↓↓
Long distance transport	*OsYSL2*	NC	*NC*	*NC*
	OsYSL16	↓3.7	*3*↑	*NC*
	OsFRDL1	↓↓5.1	*NC*	*2.7*
	OsYSL18	NC	*2.2*↑	*7.5*↓↓
	OsNRAMP1	↑4.4	*NC*	*NC*
Iron storage	*OsFERRITIN1*	↓5.6	*3.1*↑	*NC*

Arrows show corresponding up- or down-regulation of the expression levels of the genes. Note: ↓- down regulation 2-5 fold, ↓↓-down regulation 5-10 fold, ↑- upregulation 2-5 fold, NC = no change. Each value is the average of three independent experiments. Transcript levels are represented by the ratio between mRNA levels of endogenous metal homeostasis genes and those of Os*ACTIN1*. Upward and downward arrow indicate a statistically significant upregulation and downregulation respectively, between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3).

5.4.4 Cd concentration

Many Fe transporters are able to co-transport the toxic heavy metal Cd in roots, leaves and seeds (Lee et al 2009; Sasaki et al 2012; Takahasi et al 2011). In order to investigate whether *HvYS1* exhibited such promiscuity in transporting Cd along with Fe in roots, leaves and seeds, I grew a number of *HvYS1* expressing lines along with wild type in a nutrient solution supplemented with external Cd, and I measured the levels of Fe and Cd in roots, leaves and seeds. Transgenic lines had significantly lower levels of Cd in roots, leaves and seeds (Figure 5.4A). In contrast, Fe concentration in roots, leaves and seeds of the transgenic lines was significantly higher (Figure 5.4B), suggesting that Cd uptake, root to shoot translocation and seed accumulation were suppressed due to the enhanced uptake of Fe and its subsequent root to shoot translocation, and seed loading.

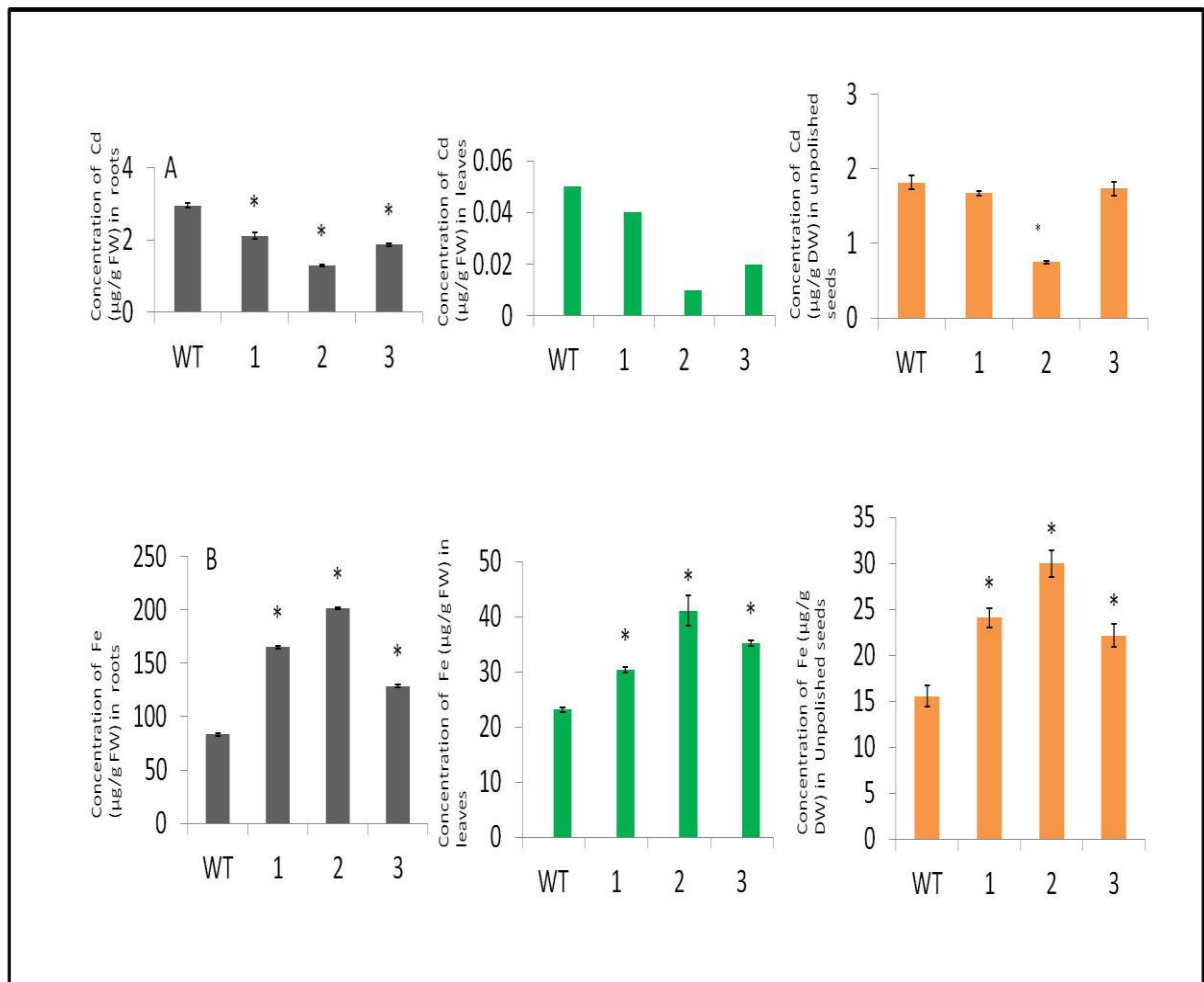

Figure 5.4 Levels of (A) Cd (µg g^{-1} DW) and (B) Fe (µg g^{-1} DW) in roots, leaves, and unpolished seeds of wild type (WT) and T_3 generation transgenic lines expressing *HvYS1* (lines 1, 2, and 3). Data are means±SE. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6). DW: dry weight.

5.5 Discussion

Rice takes up Fe as Fe (II) and Fe (III)-DMA complexes (Lee et al 2009). As a result of O_2 release from the roots, Fe (II) is oxidized to form Fe (III) precipitate over the root surface. This has a negative impact on Fe uptake (Chen et al 2006). Rice plants excrete deoxy-mugenic acid (DMA) from the root surface to chelate Fe (III), and the resulting Fe (III)-DMA complex is taken up through Fe(III)-DMA complex transporters (Inoue et al 2009); along with Fe solubilisation and uptake, DMA is also important in Fe internal translocation and seed deposition (Ayoma et al 2009). However, in comparison with barley, wheat, corn, and sorghum, the ability of rice to excrete DMA into the rhizosphere and take up Fe (III)-DMA complex is weaker (Inoue et al 2009; Gomez-Galera et al 2012). As a result among staple cereal crops, rice exhibits the lowest Fe uptake and translocation capacity (Gomez-Galera et al 2012). Overexpression of endogenous transporters or heterologous expression of Fe transporters, provide strategies to address this deficiency. Efforts to engineer rice with metal transporters for improved Fe uptake, translocation, and seed accumulation, have thus far used non-selective transporters, such as *OsIRT1*, *MxIRT1*, *OsYSL2, OsNRAMP1*, *OsZIP4* with a broad range of metal transport specificity including the highly toxic metals Cd and Ni (Lee and An et al 2009; Ishimaru et al 2006; Ishimaru et al 2010; Takahashi et al 2011; Tan et al 2015). In view of the importance of Fe in human health and the serious detrimental health effects caused by heavy metals to both animals and plants, efforts to engineer rice with metal transporters must consider transporters with strict Fe transport specificity.

Barley Yellow Stripe1 like transporter (*HvYS1*) and rice Yellow Stripe 15 (*OsYSL15*) like transporter have been reported to be strictly specific for Fe (III)-DMA (Murata et al 2006, 2008; Inoue et al 2009; Lee et al 2009). In comparison with other cereals, barley exhibits

the strongest ability for Fe uptake and translocation (Ma et al 1999). This ability of barley has been attributed to Fe (III)-DMA uptake mechanisms in which YS1 plays a major role.

I generated transgenic rice lines constitutively expressing *HvYS1* to investigate Fe uptake, translocation, and seed accumulation. *HvYS1* expression in transgenic lines resulted in improved Fe uptake in roots, enhanced root to shoot translocation, seed accumulation and importantly endosperm loading (Figure 5.2). The transgenic lines accumulated similar levels of Zn, Mn and Cu in roots and leaves as the wild type. However, in seeds of the transgenic lines, Cu levels were significantly lower (Figure 5.3). This suggests that *HvYS1* effectively transports Fe into rice roots, followed by translocation to shoots and delivery to the seeds. Similarly, when *OsIRT1, MxIRT1, OsYSL15* were over expressed in rice, resulting transgenic plants had increased Fe uptake, translocation and seed loading (Lee and An 2009; Lee et al 2009; Tan et al 2015).Unlike transgenic lines expressing *HvYS1* as reported in our study, others have reported that *OsIRT1, MxIRT1,OsYSL15* expression resulted in increased Zn, Cu, Mn, Cd, and Ni uptake, translocation and seed accumulation (Lee and An 2009; Lee et al 2009; Tan et al 2015). Therefore, expression of *HvYS1* followed by a selective increase in Fe levels appears to be a unique attribute of this particular transporter. The lower amounts of Cu in seeds of the transgenic lines expressing *HvYS1* (compared with the wild type) might be attributed to the enhanced Fe deposition in the endosperm of the transgenic lines which replaces Cu in the endosperm. The recommended daily intake of Cu is 0.2 ppm/day/person and the safe upper limit of Cu intake is 1 ppm/day/person. Even though Cu is an essential element it is toxic above this limit (Uauy et al 1998; Gaetke and Chow 2003). Accumulation of Cu in polished seeds of wild type plants in our experiments was ca: 9µg/g DW. This translates to 1.8 ppm Cu/day/person in 200 g rice consumed per day. In contrast, the *HvYS1* transgenic lines

accumulated 2.4 µg/g DW Cu in the seed endosperm (3.6 fold lower compared to wild type), thus, making it safe for human consumption by avoiding Cu toxicity.

HvYS1 expression resulted in a 2 fold increase in Fe in seeds over WT. A similar enhancement in Fe levels was also reported following expression of *Malus xiaojinensis IRT1* transporter in rice (Tan et al 2015). Iron homeostasis is complex, involving many genes and the whole process is tightly regulated (Sperotto et al 2012; Wang et al 2013).It appears that the Fe homeostasis machinery in plants limits the uptake of Fe and its translocation and accumulation in seeds. Consequently, the levels of Fe in rice seeds have been limited to a maximum of 2 fold increase over WT. Therefore, I investigate the impact of *HvYS1* expression on Fe uptake, translocation and seed loading with emphasis on: a) metal uptake transporters such as Fe regulated Fe-Zn-Mn transporter (*OsIRT1*), Fe regulated Fe (III)-DMA transporter (*OsYSL15*), Fe-Mn transporter (*OsNRAMP5*); b) vacuolar Fe-Zn transporter (*OsVIT1*); c) long distance transporters Fe-Mn transporter (*OsYSL2*), Fe transporter (*OsYSL1* and *OsNRAMP1*), Fe-citrate transporter (*OsFRDL1*), Fe (III)-DMA transporter (*OsYSL18*) (Table 5.2).

In roots, expression of *OsIRT1* and *OsYSL15* was slightly down regulated. Since *OsIRT1* and *OsYSL15* are Fe regulated transporters and expression of these transporters is induced by Fe deficiency and repressed under Fe sufficiency (Lee and An 2009; Inoue et al 2009) it appears that higher Fe levels in the *HvYS1* transgenic lines created a situation of Fe sufficiency resulting in downregulation of these two transporters. Since, *OsIRT1* can co-transport Fe-Zn-Mn, down regulation of *OsIRT1* under Fe sufficiency conditions might have triggered expression of the Fe-Mn transporter *OsNRAMP5* to take up Mn. Fe mobilisation from roots through xylem contributes towards Fe seed loading (Yoneyama et al 2015). The transporter *OsNRAMP1* is involved in xylem loading of Fe (Takhashi et al

2011) and *OsNRAMP5* has the ability for Fe uptake and xylem loading (Yang et al 2014). Up-regulation of these two transporters in the *HvYS1*- expressing lines suggests an increase in Fe xylem loading and seed delivery.

Remobilisation of Fe from the flag leaf through phloem contributes towards seed loading of the metal. Transporters *OsYSL16* (Kakei et al 2012) and *OsYSL18* (Ayoma et al 2009) are important in the seed delivery of Fe through the phloem. In the *HvYS1*-expressingtransgenic lines, expression of *OsYSL16* and *OsYSL18* was upregulated in comparison to wild type, suggesting that transgenic lines had enhanced phloem loading of Fe over WT, resulting higher level of Fe in seeds. Ferritin is an iron storage protein and its expression is regulated by the amount of Fe present in the cell (Jain and Connolly 2013). Interestingly, up-regulation of *OsFERRITIN1* in the flag leaf suggests that Fe in the flag leaf was not freely available for remobilisation through phloem. Therefore, it appears that Fe storage in Ferritin acts as a buffer mechanism to control the remobilisation of the metal through phloem.

Similarly to the uptake of metals by roots, and their remobilisation from the flag leaf, transporters also contribute to loading of Fe in seeds. Surprisingly, compared to wild type plants, in the seeds of the *HvYS1* transgenic lines *OsYSL15* (, *OsIRT1* and *OsYSL18* expression was downregulated (by ca: over 7-fold over wild type). It was suggested that lower levels of expression of *OsYSL15*, *OsIRT1*, *OsYSL18* leads to lower seed Fe levels (Lee et al 2009; Lee and An 2009; Ayoma et al 2009). Hence, limited seed loading of Fe in transgenic lines can be attributed to negative Fe homeostasis mechanism on endogenous transporters to limit Fe accumulation to certain levels (i.e. 2 fold Fe increase over wild type in the transgenic lines expressing *HvYS1*). Therefore, Fe homeostasis mechanism operating at roots, flag leaf and seeds controls Fe levels in seeds.

One of the advantages of engineering crop plants with Fe specific transporters is that expression of transporters followed by specific uptake of Fe might limit the uptake of the toxic heavy metal Cd. Therefore, I investigated the uptake, translocation and seed loading of Cd in *HvYS1*-expressing lines versus wild type. Under an external supply of Cd, the transgenic lines exhibited lower Cd uptake, translocation and seed accumulation. In contrast, Fe uptake, translocation and seed loading was higher in the transgenic lines (Figure 5.4A; Figure 5.4B). It has been reported that plants take up more Fe in the presence of Cd and that Fe acquisition in the presence of Cd might act as a defence mechanism to mitigate Cd induced stress (Meda et al 2007; Astolfi et al 2014). Similarly, *NtPIC1* a chloroplast Fe transporter, when over expressed in tobacco, resulted in higher Fe leaf levels and lower Cd levels and improved tolerance to Cd (Gong et al 2015). Rice engineered for enhanced accumulation of Fe in seeds through over expression of *HvNAS1* accumulated lower amounts of Cd in seeds compared to wild type (Masuda et al 2012). In contrast, rice plants subjected to an iron deficiency treatment in the presence of Cd, had higher Cd uptake, translocation and seed accumulation (Nakanishi et al 2006). Thus, it appears that specific uptake, root to shoot translocation and seed loading of Fe by *HvYS1* in transgenic lines inhibits Cd uptake, root to shoot translocation and seed loading. Considering the ill health effects of Cd and positive health effects of Fe on animals and humans (Clemens et al 2013; Slamet-Loedin et al 2015) the higher levels of Fe in the endosperm of the *HvYS1*-expressing lines along with the lower levels of Cd can help to solve Fe deficiency related illnesses simultaneously reducing the risk of Cd toxicity.

5.6 Conclusions and future prospects

In conclusion, I have shown that ectopic expression of *HvYS1* in rice leads to increased Fe uptake, translocation and seed loading without any increase in the uptake of Zn, Mn, and

Cu uptake and translocation. Even though the Fe specific transport nature of *HvYS1* does not impact negatively on Zn or Mn levels in the seeds, it appears to influence Cu levels in seeds. I have established that even though Fe levels in rice seeds can be increased to a certain extent (up to 2-fold over wild type) through the expression of *HvYS1*, the endogenous Fe homeostasis machinery limits the levels of Fe in seeds. Furthermore preferential uptake, translocation and seed loading of Fe in the *HvYS1*-expressing transgenic lines resulted in the competitive inhibition of Cd uptake, translocation and seed loading. Thus, *HvYS1* is a Fe specific transporter, and is thus safe to use in biofortification programmes to alleviate malnutrition.

5.7 References

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General Conclusions

General conclusions

1. Combinatorial nuclear genetic transformation of rice with four transgenes involved in metal homeostasis resulted in the recovery of a population of transgenic plants expressing the input genes in different combinations. I used this population to identify key synergistic and antagonistic mechanisms controlling Fe and Zn accumulation in rice endosperm.

2. Co-expression of nicotianamine synthase (*OsNAS1*) and nicotianamine aminotransferase (*HvNAATb*) exhibited synergistic effects on the synthesis and accumulation of nicotianamine (NA) and deoxy mugenic acid (DMA) in the endosperm. Such synergistic effects resulted in the recovery of transgenic rice plants accumulating higher levels of Fe and Zn in the endosperm compared to what has been reported thus far. When the metal transporters *OsIRT1* and *HvYS1* were over-expressed in *OsNAS1+HvNAATB* expressing-lines, the transporters exhibited an antagonistic effect on the synthesis of NA and DMA, and reduced the levels of Fe and Zn in the endosperm.

3. I established experimentally that additional Fe in high Fe-accumulating lines was bioavailable. These findings suggest that transgenic lines not only contained higher Fe levels in seeds but also the Fe in transgenic lines is more bioavailable.

4. Transgenic lines accumulating high levels of phytosiderophores impose a ceiling on Fe and Zn accumulation in rice endosperm. This occurs because Fe and Zn uptake in the roots is regulated by the modulation of the expression of endogenous metal transporters in response to increased Fe levels, through sequestration of Fe and Zn in the roots, culm, middle leaf and flag leaf, and by controlling phloem Fe and Zn remobilization from the flag leaf to the seeds.. In particular, vacuolar transporters are modulated substantially in the flag leaf to promote vacuolar sequestration of Fe and Zn.

5. The Fe and Zn accumulation ceiling in the endosperm of transgenic lines accumulating high levels of NA and DMA creates a competition for metal accumulation in the endosperm. Due to this competition Cd is displaced from the endosperm of transgenic lines and it appears to accumulate in the bran.

6. NA and DMA promote the uptake and root-to-shoot translocation of Cd through modulation of the expression of endogenous metal transporters. However, most of the Cd is sequestered in roots and leaves through modulation of vacuolar transporters. Thus NA and DMA do not mediate Cd accumulation in the endosperm.

7. In the presence of external Cd, NA and DMA mediate the uptake of Zn, root-to-shoot translocation of Fe and Zn by modulating endogenous metal homeostasis genes. More importantly, NA and DMA mediate phloem remobilisation of Fe and Zn from leaves and promote the accumulation of Fe and Zn in the endosperm.

8. Transgenic lines expressing *HvYS1* had modest increases in Fe levels in roots, leaves and seeds. In *HvYS1*-expressing lines the levels of Zn, Cu, and Mn did not change in roots and leaves. Even though Zn and Mn levels did not change in seeds, Cu levels were lower in seeds of these lines. These results suggest that *HvYS1* is a selective Fe transporter and the resulting selective Fe delivery to seeds, suppresses Cu deposition in seeds. In addition, the selective transport of Fe by *HvYS1* inhibited Cd uptake, translocation, and seed loading.

9. In *HvYS1*-expressing lines the endogenous metal homeostasis machinery allows only a modest increase of Fe levels in seeds by down-regulating endogenous genes for metal uptake, root-to-shoot translocation and seed accumulation.

10. My findings provide a strong basis to improve the levels of nutritionally important Fe and Zn in rice endosperm while simultaneously minimising the levels of toxic Cd.

Outputs

1. Manuscript Submitted

- **Banakar R,** Alvarez Fernandez A, Díaz-Benito P, Abadia J, Capell T, Christou P (2016) Enhanced accumulation of phytosiderophores reveals a threshold for iron accumulation in rice endosperm but allows strictly toxic cadmium to be replaced with iron and zinc. **Submitted to *Nature Communications* (Annex 1)**

2. Manuscripts in preparation

- **Banakar R,** Curia R, Go´mez-Galera S, Albacete A, Alvarez Fernandez A, Serrano J, Portero-Otin M, Abadia J, Watanabe M, Hoefgen R, Capell T, Christou P (2016) Combinatorial genetic transformation of rice with iron and zinc homeostasis genes identifies synergistic mechanism for the development of iron and zinc biofortified rice.

- **Banakar R,** Albacete A, Alvarez Fernandez A, Abadia J, Capell T, Christou P (2016) The relative ratio of the phytosiderophors nicotianamine and 2'-deoxy mugenic acid influences cadmium uptake and translocation in rice in an organ specific manner, excludes cadmium deposition in the endosperm and facilitates preferential accumulation of iron and zinc in the endosperm.

- **Banakar R,** Alvarez Fernandez A, Abadia J, Capell T, Christou P (2016) Expression of Iron (III) phytosiderophore transporter in rice leads to modest increases in Fe uptake, translocation, and seed loading, but limits heavy metal acquisition, translocation and seed loading through selective Fe transport.

- **Banakar R,** Alvarez Fernandez A, Abadia J, Capell T, Christou P (2016) The expression of two genes involved in phytosiderophore biosynthesis reveals the complex regulation of metal homeostasis and mobilization in rice plants.

- **Banakar R,** Watanabe M, Albacete A Alvarez Fernandez A, Abadia J, Hoefgen R, Capell T, Christou P (2015) Rice engineered with nicotianamine synthase and nicotianamine aminotransferase unravels nicotianmine and deoxy mugenic acid role in metal homeostasis in reproductive organs.

- **Banakar R,** Oromi-Bosch A, Zanga D, Alvarez Fernandez A, Abadia J, Capell T, Christou P (2016) Unravelling the nickel homeostasis in rice through metabolic engineering for enhanced nicotianamine and deoxymugenic acid.

- **Banakar R,** Alvarez Fernandez A, Abadia J, Capell T, Christou P (2016) Engineering rice with rice nicotianamine synathase and metal transporters reveals bottlenecks in iron and zinc biofortification of rice.

- **Banakar R,** Albacete A, Alvarez Fernandez A, Abadia J, Capell T, Christou P (2016) Phytosiderophores influence hormone homesotasis for preferential mobilization of metals from vegetative tissues and accumulation in reproductive tissues to suppress toxic cadmium accumulation in reproductive tissues.

3. Publications related to nutritional enhancement of crop plants

- Zorrilla-López U, Masip G, Arjó G, Bai C, **Banakar R,** Bassie L, Berman J, Farré G, Miralpeix B, Pérez-Massot E, Sabalza M, Sanahuja G, Vamvaka E, Twyman RM, Christou P, Zhu C, Capell T (2013) Engineering metabolic pathways in plants by multigene transformation. *Int J Dev Biol*. 57:565-76

- Masip G, Sabalza M , Pérez-Massot E, **Banakar R,** Cebrian, D, Twyman RM, Capell T, Christou P (2013) Paradoxes in EU agricultural policies and the role of Genetically Engineered crops. *Trends Plant Sci* 18:312-24

- Berman J, Zhu C, Pérez-Massot E, Arjó G, Zorrilla-López U, Masip G, **Banakar R,** Sanahuja G, Farré G, Miralpeix B, Bai C, Vamvaka E, Sabalza M, Twyman RM, Bassié L, Capell T, Christou P (2013) Can the world afford to ignore biotechnology solutions that address food insecurity? *Plant Mol Biol* 83:5-19

- Pe´rez-Massot E, **Banakar R*,** Go´mez-Galera S, Zorrilla-Lo´pez U, Sanahuja G,Arjo´ G, Miralpeix B, Vamvaka E, Farre´ G, Rivera SM, Dashevskaya S,Berman J, Sabalza M, Yuan D, Bai C, Bassie L, Twyman RM, Capell T, Christou P, Zhu C (2012) The contribution of transgenic plants to better health through improved nutrition: opportunities and constraints. *Genes Nutr* 8:29-41

- Zhu C, Sanahuja G, YuanY, Farre´ G, Arjo´G, Berman J, Zorrilla-Lo´pez U, **Banakar R,** Bai C, Pe´rez-Massot E, Bassie L, Capell T, Christou P (2012) Biofortification of plants with altered antioxidant content and composition: genetic engineering strategies, *Plant Biotechnol J* 11:129-41

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4. Other Publications

- Sanahuja G, **Banakar R*,** Twyman R, Capell T, Christou P (2011) *Bacillus thuringiensis* – a century of research, development and commercial applications. *Plant Biotechnol J* 9: 283-300

Note: * Joint first author

5. Oral presentation in conference

- **Banakar R,** Go´mez-Galera S, Capell T, Christou P. Multi gene engineering of rice for iron biofortification. Plant Biology congress, Freiburg, Germany, July 30th-3rd August 2012.

6. Poster presentations in conference

- **Banakar R,** Capell T, Christou P. Combinatorial genetic transformation of rice for iron and zinc biofortification. Second international hidden hunger congress. Stuttgart, Germany, 3-5 March 2015

- Díaz-Benito P, **Banakar R,** Fernández B, Abadía J, Morales F, Pereiro R, Christou P, Álvarez-Fernández A. Localization of metals and metal ligands in rice seeds. XIV Congreso Hispano-Luso de Fisiología Vegetal. Toledo, Spain, 14-17 June 2015

- **Banakar R,** Capell T, Christou P. Combinatorial genetic transformation of rice for iron biofortification. 4th International Rice Congress, Bangkok, Thailand, 7 October – 1 November 2014.

- Fernández B, Carrasco-Gil S, Diaz-Benito P, **Banakar R,** Álvarez-Fernández A, Abadía J' Christou P, García JI, Pereiro R. Imaging studies of trace elements in biological samples by LA-ICP-MS: Analysis of leaf and seed sections. 17th International Symposium on Iron Nutrition and Interactions in Plants (ISINIP). Gatersleben, Germany, 06-10 July 2014

- **Banakar R,** Capell T, Christou P. Co-expression of rice nicotianamine synthase and barley nicotianamine amino transferase increases the level of iron and zinc in rice endosperm. Joint *EPSO-FESPB* Conference, Dublin, Ireland, 22-26 June 2014

- **Banakar R,** Capell T, Christou P. Heterologous expression of barley YS1 transporter in rice improves iron uptake and seed iron levels. Seventh EPSO conference, Plants for a Greening Economy, Porto Heli, Greece, 1-4th Septemeber 2013.

Annex 1

Feedback limitations affecting iron and zinc levels in the seeds of rice plants producing excess phytosiderophores reveal independent competitive mechanisms controlling metal uptake, translocation and seed loading that can displace cadmium from the endosperm

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Abstract

Plants producing high levels of phytosiderophores can promote mineral accumulation in the endosperm by facilitating the uptake, translocation and seed loading of Fe and Zn. We generated transgenic rice lines coexpressing rice nicotianamine synthase (OsNAS1) and barley nicotianamine amino transferase (HvNAATb), which produced 165-fold more nicotianamine and 29-fold more 2'-deoxymugenic acid than wild-type plants. These plants also accumulated four times the normal levels of Fe and Zn in the polished seeds. The uptake and internal mobilization of both metals was influenced by increasing the external availability of Fe using three different feeding regimes, but seed loading was subject to strict feedback limitations. The amount of Fe and Zn in the seeds was not influenced by the external Fe supply, suggesting there is an intrinsic upper limit for the accumulation of each metal. However, Fe and Zn levels in the roots were affected by Fe feeding, suggesting competition for the same transporters, and the distribution profiles of Fe and Zn differed in the culm and middle leaf suggesting the preferential displacement of Zn by Fe. The transgenic plants accumulated more Fe and Zn in the flag leaf than wild-type controls, suggesting a bottleneck between the flag lead and seed that may involve sequestration of excess metals into the vacuoles. The external supply of Fe modulated the expression of metal transporters in the roots and aboveground vegetative tissues that mirror the distribution of each metal. Plants exposed to 10 μM Cd partition this toxic heavy metal between the endosperm and bran. We found that the transgenic plants divert more Cd to the bran and less to the endosperm than wild-type plants. Our data therefore show that although homeostasis imposes a ceiling of 22 μg Fe and 84 μg Zn per gram dry weight of endosperm, strategies to enhance seed loading with Fe and Zn can also displace Cd and thus further increase the nutritional value of polished rice.

Introduction

Iron (Fe) and zinc (Zn) are essential metal elements in plants (1, 2). For example, Fe mediates electron transport during photosynthesis and respiration (3), and both Fe and Zn act as cofactors in a large number of enzymes and regulatory proteins (2). However, both metals are toxic at high concentrations and plants have therefore evolved homeostasis mechanisms to regulate their accumulation in vegetative and reproductive tissues (2, 4).

Rice (*Oryza sativa*) has two Fe acquisition strategies, namely the direct uptake of Fe^{2+} (Strategy I) and the uptake of Fe^{3+} complexes following the secretion of phytosiderophores that chelate metals (Strategy II). In strategy I, Fe^{2+} is taken up into the root epidermis by the membrane-bound Fe-regulated transporters OsIRT1 and OsIRT2, which also transport Zn^{2+} (5, 6). Once inside the plant, both Fe^{2+} and Zn^{2+} form complexes with nicotianamine (NA) and the complexes are translocated and remobilized internally for seed loading (7, 8, 9, 10). The Fe-regulated ZIP family transporter proteins OsZIP1–4 and the heavy metal ATPase family transporter OsHMA2 can also transport either Fe^{2+} or Zn^{2+} (11, 12). In strategy II, Fe^{3+} in the soil is chelated by the phytosiderophore 2'-deoxymugenic acid (DMA), which is secreted into the rhizosphere by the membrane-bound transporter OsTOM1 (13, 14). Fe^{3+}-DMA complexes are taken up into the roots of rice plants by OsYSL15 and internally transported by OsYSL18 for seed loading (14, 15). DMA can also bind Zn^{2+}, and Zn-DMA complexes are favored over free Zn^{2+} for internal transport and seed loading (16).

The uptake and redistribution of Fe and Zn in rice is under strict homeostatic control, which maintains the endosperm concentration of Fe in the range 2–5 μg g^{-1} dry weight (DW), and that of Zn in the range 15–20 μg g^{-1} DW (17, 18). Both NA and DMA are involved in this process (19), and therefore increasing the availability of NA and DMA can

increase the accumulation of Fe and Zn in rice seeds (19, 20, 21). NA and DMA are synthesized from the precursor S-adenosylmethionine (SAM) in three sequential steps involving the enzymes nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT) and DMA synthase (DMAS) (13). The disruption of NAAT blocks the pathway and causes NA to accumulate in preference to DMA, whereas the overexpression of NAAT increases flux through the pathway and causes DMA to accumulate in preference to NA, but both strategies promote the uptake, mobilization and seed loading of Fe (8, 19, 21).

Although modulating the NA/DMA pathway can increase the accumulation of Fe and Zn in seeds, the increase in concentration tends not to exceed a certain threshold, which is approximately four-fold the level found in wild-type seeds. This suggests that compensatory homeostatic mechanisms come into effect when the threshold is reached. Because the metal chelation and transport pathways in rice are promiscuous, we hypothesized that the modulation of specific elements of these pathways in the context of an upper limit for seed loading could lead to competition among metal ions and the preferential loading of seeds with micronutrients such as Fe and Zn. This in turn would allow us to control the levels of strictly toxic heavy metals such as Cd, which is often found in rice growing areas.

If the proportions of different metals can be controlled in this manner, it would be beneficial because rice is a staple crop for more than 50% of the world's population (22, 23, 24). Insufficient levels of Fe and Zn in the rice endosperm is a major cause of deficiency diseases in populations subsisting on rice-based diets (25, 26, 27): more than 2 billion people suffer from Fe deficiency anemia (IDA) and/or Zn deficiency (ZnD) (28, 29). Furthermore, rice grown on contaminated soils can also be a source of Cd toxicity in animals and humans (29, 30, 31, 32, 33, 34). Therefore, biofortification strategies to

enhance Fe and Zn levels in rice endosperm should also attempt to minimize Cd levels in the grain (29, 35).

Here we show that transgenic rice plants co-expressing NAS and NAAT accumulate up to 22.5 μg g^{-1} dry weight (DW) of Fe in polished seeds under both normal and excess Fe feeding regimes, suggesting that strict Fe homeostasis occurs in the roots, middle leaf, flag leaf and seeds. The higher levels of Fe and Zn also displace Cd from the endosperm, resulting in the sequestration of this toxic metal in the bran. The modulation of metal loading pathways in rice can therefore be used to generate beneficial micronutrient profiles for human nutrition.

Results

Transgenic rice plants co-expressing NAS and NAAT produce higher levels of phytosiderophores and accumulate more Fe and Zn in the seeds than wild-type plants

Mature seed-derived zygotic rice embryos were bombarded with constructs encoding rice NAS (*OsNAS1*), barley NAAT (*HvNAATb*) and the selectable marker HPT, each controlled by a constitutive promoter. Subsequent mRNA blot analysis revealed the co-expression of *OsNAS1* and *HvNAATb* in 19 independent lines (Figure 1). T_1 seeds were harvested from the first 10 of these lines to flower, and these lines were selfed to produce the next generation. The unpolished seeds (T_2 generation) were screened by inductively coupled plasma mass spectrometry (ICP-MS) to determine the concentrations of Fe and Zn (Table S1). The Fe concentration range in the unpolished seeds was 22–57 µg g^{-1} DW, 1.4–3.7 times higher than that in wild-type seeds, and the Zn concentration range was 22–78 µg g^{-1} DW, 1.2–4.2 times higher than that in wild-type seeds.

T_2 seeds from the three best-performing lines (EYI-9, EYI-89 and EYI-98) were polished to determine the levels of Fe and Zn in the endosperm. The endosperm from these lines contained 8, 16 and 10 µg Fe g^{-1} DW, respectively, which was up to four-fold more than found in wild-type seeds (4 µg Fe g^{-1} DW). Similarly, the endosperm from the three transgenic lines contained 36, 42 and 65 µg Zn g^{-1} DW, respectively, which was also up to four-fold more than found in wild-type seeds (16 µg Zn g^{-1} DW).

T_2 seeds from the same lines (EYI-9, EYI-89 and EYI-98) were also extracted and analyzed by HPLC-ESI-TOF-MS to determine the concentrations of NA and DMA (Figure 2). In all three lines, both phytosiderophores accumulated to higher levels than in wild-type seeds. The concentration range of NA was 14–347 µg g^{-1} fresh weight (FW) compared to

2.1 µg g^{-1} FW in wild-type seeds, representing a 7–165-fold increase. The concentration range of DMA was 58–161 µg g^{-1} FW compared to 5.5 µg g^{-1} FW in wild-type seeds, representing an 11–29-fold increase. The NA/DMA ratios differed among the three lines, with EYI-9 and EYI-89 producing more NA than DMA and EYI-98 producing more DMA than NA. These data indicate that the increased concentration of Fe and Zn in the seeds of transgenic lines is caused by the greater abundance of NA and/or DMA.

The uptake, translocation, remobilization of Fe but not Fe seed loading is influenced by external Fe supply

To determine whether seed loading with metals could be increased by boosting the external availability of metal ions and to investigate the mechanism of Fe homeostasis, we grew T_2 plants representing the three transgenic lines discussed above (plus wild-type controls) and applied three different Fe feeding regimes: normal (100 µM Fe), double (200 µM Fe) and triple (300 µM Fe). We then measured the Fe concentrations in the roots, culm, middle leaf, flag leaf and seeds.

Under normal feeding conditions there was no significant difference in Fe concentrations between the roots of transgenic and wild-type plants (Figure 3). When the feed was doubled, the roots of the transgenic lines accumulated significantly more Fe than the wild-type roots, but when the feed was tripled the roots of the transgenic lines accumulated significantly less Fe than the wild-type roots, suggesting that excess Fe triggers a compensatory mechanism that restricts Fe uptake into root cells (Figure 3). The culms of the transgenic lines accumulated more Fe than the wild-type controls under all three treatments, indicating that Fe root-to-shoot translocation was enhanced in the transgenic plants (Figure 3). The middle leaf of the transgenic lines accumulated more Fe than wild-type controls under all three treatment regimes, with plants receiving 200 µM Fe

containing the highest concentrations of Fe (Figure 3). However, although the flag leaves of the transgenic lines accumulated more Fe than the corresponding wild-type plants, there were no significant differences among the three treatments (Figure 3).

Under the normal feeding regime, the polished T_3 transgenic seeds accumulated 2.6–4.3 times more Fe in the endosperm compared to wild-type seeds, whereas the corresponding increments under the double and triple Fe regimes were 2.2–4.5-fold and 1.9–4.6-fold, respectively (Figure 4). Thus, there was no statistically significant difference in Fe seed loading among the three treatments, strongly supporting the presence of a strict homeostatic mechanism that limits the accumulation of Fe in the endosperm to a maximum of 22.5μg g^{-1} DW regardless of any excess of external Fe.

The uptake, translocation, and remobilization of Zn are influenced by the external supply of Fe but this does not affect Zn seed loading

The promiscuity of ligands and transporters that mobilize Fe and Zn makes it likely that the homeostatic mechanisms controlling the distribution of Fe may also affect Zn. Therefore, we analyzed the same tissues described above under the same three Fe feeding regimes, but this time measured the concentrations of Zn. We found that the accumulation of Zn in different plant tissues differed according to the Fe supply, but there was a clear difference in the distribution of the two metals that indicated competition during their mobilization from the roots to the seeds (Figure 3).

The roots of the transgenic lines contained higher levels of Zn than wild-type plants under the normal and double Fe feeding regimes, but significantly lower levels of Zn under the triple Fe regime, which was similar to the profile observed for Fe (Figure 3). This suggests that Zn accumulation in the roots may be restricted by the same feedback mechanism that

limits the uptake of Fe when the external supply of Fe is in great excess, and may therefore depend on the same transporters, but that different feedback thresholds operate at lower Fe levels (i.e. under the double Fe feeding regime, the uptake of Fe increase but Zn decreases). In contrast, the Fe and Zn distribution profile differed in the culms. Higher levels of Zn were present in the culms of transgenic plants compared to wild-type controls under normal feeding conditions, indicating that root-to-shoot translocation of Zn was more active in these circumstances, but there was little difference between the transgenic and wild-type culms at the higher feeding levels, suggesting that root-to-shoot translocation of Zn is suppressed by high levels of Fe (Figure 3). This difference between roots and culms suggests that Fe and Zn compete on an equal footing for metal transporters in the root epidermis, but that Fe displaces Zn when they compete for the same root-to-shoot transporters. The profile in the culm was not replicated exactly in the middle leaf but there was a similar trend: the transgenic lines accumulated more Zn in the middle leaf compared to wild-type plants under the normal feeding regime (and also to a lesser extent under the triple Fe feeding regime) but there was no significant difference in Zn levels when comparing the middle leaves of transgenic and wild-type plants under the double Fe feeding regime (Figure 3). Again, these data suggest a competition between Fe and Zn for transporters, which is most prevalent under the double Fe feeding regime when the greatest amount of Fe accumulates in the middle leaves. The amount of Zn in the flag leaves was generally higher in the transgenic lines than wild-type controls under all three treatments, but the lowest level of Zn accumulated in plants treated with 300 μM Fe (Figure 3). This result is intriguing because the similar levels of Fe in the flag leaves under all three treatments suggested an upper threshold for accumulation, but clearly there is still competition between Fe and Zn that differs according to the external supply even when the

internal levels of Fe are similar, suggesting that Zn accumulation in the flag leaf is regulated by signals emanating from other tissues.

Even so, the accumulation of Zn in the endosperm appeared to be governed (like Fe) by an intrinsic threshold that was independent of the external Fe supply. The endosperm of T_3 transgenic seeds accumulated 1.2–2.2 times more Zn than wild-type seeds under the normal feeding regime, and this barely changed under the double Fe regime (1.5–2 times more Zn) and the triple regime (1.2–2 times more Zn) (Figure 4). These data indicate that the external supply of Fe does not alter the threshold level of Zn in the endosperm, which was approximately 84 μg Zn g^{-1} DW.

The external supply of Fe modulates the expression of metal transporters

One potential explanation for the competition between Fe and Zn under different feeding regimes is that the external supply of Fe regulates the expression of genes encoding metal transporters, resulting in the preferential mobilization of Fe. We therefore investigated the expression of three metal transporters in the roots of T_3 transgenic plants from lines EYI9 and EYI89, as well as wild-type controls: *OsYSL15*, which encodes the Fe^{3+}-DMA transporter YSL15, *OsIRT1*, which encodes the IRT1 transporter that mobilizes Fe and Zn, and the Zn transporter *OsZIP1*. We found that *OsYSL15* was upregulated in the roots of the transgenic lines compared to wild-type plants under the normal and double Fe feeding regimes, but was downregulated under the triple Fe feeding regime (Figure 5). The expression of *OsIRT1* was downregulated in the transgenic lines compared to wild-type plants under all three treatments, with the lowest expression level observed under the normal feeding regime (Figure 5). *OsZIP1* was downregulated in the transgenic lines under normal Fe feeding, there was no significant difference under the double Fe feeding regime, and it was upregulated in the transgenic plants under the triple Fe feeding regime (Figure

5). These data suggest that the genes for all three transporters are modulated by the increase in phytosiderophore synthesis and/or by the external Fe supply, although the phytosiderophores may have an indirect effect by increasing the internal concentration of Fe. *OsYSL15* appears to be regulated by a feedback mechanism that suppresses Fe transport when there is an external excess of Fe, whereas *OsIRT1* and *OsZIP1* are suppressed by the higher internal levels of Fe in the transgenic plants but this effect is partially (*OsIRT1*) or fully (*OsZIP1*) alleviated as the external Fe supply increases.

Because the transgenic plants generally accumulated more Fe and Zn in the flag leaf than wild-type controls, it is likely that excess metals are sequestered into the vacuoles thus reducing the amount available for seed loading. We therefore investigated the expression of several further endogenous genes encoding the Fe/Zn vacuolar transporter OsVIT1, the Fe-NA/Mn-NA phloem transporter OsYSL2, the Fe-regulated ZIP family Zn transporters OsZIP1 and OsZIP4, and the Fe storage protein, OsFERRITIN1. Compared to wild-type controls, *OsVIT1* expression was upregulated in the transgenic lines under all treatments and expression levels mirrored the external Fe supply (Figure 5). The expression profile of *OsYSL2* was similar in the transgenic and wild-type plants, and was expressed at the highest level under the double Fe feeding regime (Figure 5). *OsFERRITIN1* and *OsZIP4* were both upregulated in the transgenic lines compared to wild-type controls under all three treatments. *OsZIP1* was expressed at higher levels in the transgenic plants compared to wild-type controls under the normal and triple Fe treatments, but there was no difference between transgenic and wild-type lines under the double Fe treatment (Figure 5). These data suggest that higher levels of Fe and Zn in the flag leaf of the transgenic lines induce the expression of vacuolar transporters to prevent excess seed loading with Fe and Zn. Accordingly, *OsVIT1* expression is regulated in response to the external supply of Fe and

the abundance of Fe and Zn in the flag leaf, whereas *OsYSL2* appears to limit the phloem transport of Fe. The *OsZIP1/OsZIP4* expression profiles suggest a higher capacity for Zn transport over Fe for seed loading.

Cd is displaced from the endosperm of the transgenic lines by Fe and Zn

Our results indicate the existence of absolute threshold concentrations for Fe and Zn in the rice endosperm, but the fold change for each metal is distinct (approximately four-fold for Fe and two-fold for Zn – in the latter case the fold change is different to that observed in unpolished seeds). Thus, the regulation of metal mobilization should in principle allow the preferential loading of endosperm with essential metal nutrients such as Fe and Zn rather than strictly toxic heavy metals such as Cd. T_3 plants representing the three transgenic lines discussed above (plus wild-type controls) were therefore grown in the presence of 10 μM Cd and the seeds were analyzed to determine the metal content. The unpolished seeds of the transgenic lines accumulated 1.5-fold less Cd than wild-type seeds (Figure 6). The polished wild-type seeds contained ~9% less Cd than the unpolished wild-type seeds, suggesting that >90% of the Cd in rice seeds accumulates in the endosperm. Interestingly, the polished transgenic seeds contained 14–18% less Cd than the unpolished transgenic seeds. Therefore, due to the lower Cd seed loading and endosperm retention, the transgenic endosperm accumulated half as much Cd than the wild-type endosperm, possibly because Cd is displaced by the high levels of Fe and Zn (Figure 6). The concentrations of Fe and Zn in polished transgenic seeds were similar when plants were grown in the presence or absence of Cd, whereas the Zn concentration in polished wild-type seeds decreased by 20% in the presence of Cd and the concentration of Fe did not change (Figures 4 and 6).

The rice heavy-metal ATPase2 OsHMA2 and the low-affinity cation transporter1 OsLCT1 play key roles in Cd mobilization through the phloem and therefore regulate the

subsequent accumulation of Cd in the seed. We therefore investigated the expression of *OsHMA2* and *OsLCT1* mRNA in immature T_3 seeds. *OsHMA2* mRNA was expressed at similar levels in the wild-type and transgenic plants, whereas *OsLCT1* was suppressed in the transgenic lines (Figure S1). These data suggest that seed loading with Cd was inhibited in the transgenic lines. Accordingly, the combination of Cd displacement from the endosperm by Fe and Zn, and the inhibition of seed loading with Cd, resulted in lower levels of Cd in the endosperm of the transgenic lines compared to wild-type plants.

Discussion

The uptake and distribution of metal ions in rice is controlled by transporters that recognize either free metal ions or metal complexes with NA and DMA. One strategy to enhance the accumulation of metals is therefore to increase the abundance of phytosiderophores. However, metal accumulation in plants is controlled by strict homeostatic mechanisms that limit toxicity and several key metal transporters are promiscuous. The overall impact of phytosiderophore synthesis is therefore unclear, particularly when the external supplies of metal ions are varied.

We generated transgenic plants expressing two key enzymes in the phytosiderophore biosynthesis pathway (NAS and NAAT, encoded by *OsNAS1* and *HvNAATb*), which together with endogenous DMAS convert the precursor SAM into NA and DMA. T_2 seeds from the best-performing lines produced up to 165-fold more NA and 29-fold more DMA than wild-type seeds. Previous studies have reported up to 20-fold increases in NA (19, 20, 21, 36, 37) and up to 5-fold increases in DMA (19, 20, 36, 37) based on the expression of individual *NAS* or *NAAT* transgenes, suggesting that our higher levels reflect the coexpression of both enzymes.

The T_2 seeds of the best-performing transgenic lines accumulated up to 57 µg Fe g^{-1} DW (3.7-fold higher than wild-type) and up to 78 µg Zn g^{-1} DW (4.2-fold higher than wild-type) in the unpolished grain, similar to the levels reported for rice plants expressing *HvNAS1* (20) and *OsNAS1–3*(21). We observed a positive correlation between the metal and phytosiderophore concentrations in our transgenic seeds (Figure S2) which agrees with the outcome of previous studies (Figure S3) (19, 21, 36, 37). The accumulation of Fe and Zn in seeds therefore appears to be limited by phytosiderophore levels as previously reported (19, 21). After polishing, the transgenic endosperm was shown to contain up to 16 µg Fe g^{-1} DW and 65 µg Zn g^{-1} DW, in each case a four-fold increase over the wild-type, suggesting that metal loading into the endosperm had been enhanced. Similarly, *AtNAS1* , *HvNAS1* , and *OsNAS1–3* enhanced the loading of Fe and Zn into the endosperm (20, 21, 38), suggesting that the modulation of NA and DMA synthesis is a useful strategy to enhance the mineral content of the endosperm in rice.

Next we investigated whether different Fe feeding regimes might affect metal homeostasis to different extents in the transgenic plants and wild-type controls. Transgenic plants supplied with 200 µM Fe took up more Fe into the roots than wild-type plants, suggesting an increased uptake capacity, but when this was increased to 300 µM Fe the wild-type plants performed better. The high external concentration of Fe caused by the presence of DMA thus increased the concentration of Fe immediately above the root epidermis, resulting in the precipitation of Fe^{3+} generated by the release of O_2 from the roots (39). The remobilization of Fe from the roots to the aboveground vegetative organs (culm and leaves) is necessary for seed loading during grain filling (40). We found that the culms and middle leaves of the transgenic plants generally accumulated more Fe than their wild-type

counterparts under all three treatments, suggesting that the root-to-shoot translocation of Fe was effective under all treatment regimes.

Interestingly, the flag leaves of the transgenic plants generally contained more Fe than those of wild-type plants, but there were no significant differences among the three treatments. The remobilization of Fe from middle and flag leaves is an important determinant of Fe levels in seeds (41, 42). In *OsVIT1/2* knockdown mutants, the impaired vacuolar sequestration of Fe reduced the level of Fe in the flag leaves and higher levels accumulated in the seeds, suggesting that the flag leaf acts as a buffer to prevent seed overloading (43). The higher level of Fe in the middle and flag leaves of our transgenic plants suggests that much of the Fe pool may have been sequestered into the vacuole making it unavailable for remobilization (Figure 7), as observed during foliar applications of Fe in rice (44). Accordingly, we found that the accumulation of Fe in the endosperm of our transgenic plants fell within a similar range regardless of the feeding regime, and never exceeded 4.6-fold more than wild-type levels. These data support the existence of a homeostasis mechanism that prevents Fe overloading during seed development (Figure 7), matching the limited natural genetic diversity of Fe levels in rice (2–5 µg g^{-1} DW in all varieties). The threshold was 6 µg Fe g^{-1} DW in plants expressing *AtNAS1* (38), 14 µg Fe g^{-1} DW in plants expressing *OsNAS2* (21) and 22.5 µg Fe g^{-1} DW in our T_3 plants co-expressing *OsNAS1* and *HvNAATb*.

It is unclear whether approaching the ceiling for Fe accumulation in rice endosperm inhibits the accumulation of other metals that share some of the same transport and loading mechanisms. We found that the amount of Zn taken up by the roots declined as the amount of Fe increased, suggesting there is competition for uptake through common transporters (27, 45). If the transporters demonstrate no preference between the two metals, then the

inhibition of Zn uptake reflects statistical exclusion by the more abundant Fe^{2+} ions, but at higher Fe loads the precipitation of Fe to form plaques on the root surface could also exclude Zn by spatial restriction on the root surface (39). The amount of Zn in the culms and middle leaves of the transgenic plants was inversely related to the amount of Fe, suggesting a knock-on effect from the inhibition of Zn uptake into the roots. In the flag leaf, Zn generally accumulated to higher levels in the transgenic plants than in the wild-type plants under all three treatments, suggesting that Zn levels are unaffected by Fe in this organ, unlike the other vegetative organs. It is also possible that Zn levels in the flag leaf could be regulated as a buffer system to limit Zn levels in the seed (Figure 7). Most of the Zn in the transgenic plants appears to be held in the culm, which acts as a 'stopcock' to regulate the flow of Zn to other tissues (Figure 7). This could be achieved by the sequestration of Zn into vacuoles, e.g., by vacuolar Zn transporters such as MTP1 (46) and OZT1 (47). Although the transgenic lines accumulated up to 84 μg Zn g^{-1} DW, there was no difference in the levels of Zn in the endosperm of our transgenic plants regardless of the external Fe supply, suggesting that the buffering effect of the flag leaf on Fe levels may also act on Zn.

The distinct accumulation profiles of Fe and Zn in tissues supplied with metals by the roots suggests that root- transporters and other downstream components have different preferences for the two metals. We therefore investigated the expression of three metal transporters in roots: YSL15, which is specific for Fe^{3+}-DMA complexes (48, 49), IRT1, which transports Fe and Zn (6), and ZIP1, which is specific for Zn (50). Compared to wild-type plants, the *OsYSL15* gene was induced in the roots of our transgenic lines in response to 100 and 200 μM Fe but suppressed in the presence of 300 μM Fe which mirrors the level of Fe in roots. *OsIRT1* was expressed at lower levels in the transgenic plants than in

wild-type plants at all Fe concentrations, but the gap between transgenic and wild-type plants declined as the Fe concentration increased. The IRT1 protein transports Fe^{2+} whereas we supplied Fe^{3+} in our experiments so the availability of Fe^{2+} remained low. The relative increase in expression at higher concentrations of Fe may reflect the displacement of Zn and the corresponding Zn deficiency. *OsZIP1* was also induced at higher Fe levels, and Zn deficiency may also explain this trend.

The remobilization of Fe and Zn from vegetative tissues to seeds is important during grain filling, and this process is tightly regulated by metal transporters (27). We investigated the expression of the vacuolar Fe transporter VIT1 (43), Fe-NA/Mn-NA transporter YSL2 (7), and the Fe-regulated ZIP family transporters ZIP1 and ZIP4 (11) in flag leaves. *OsVIT1* was upregulated in the transgenic lines under all treatments and this mirrored the levels of Fe and Zn in the flag leaf. *OsYSL2* was expressed at similar levels in transgenic and wild-type plants under all treatments, indicating that Fe remobilization from the flag leaf is limited (Figure 7). *OsZIP4* was upregulated in the transgenic lines under all treatments, reflecting the lower level of Zn compared to Fe in the flag leaf and suggesting a preference for Zn over Fe during endosperm loading in the transgenic lines.

The competition among metal ions during transport and loading could potentially be exploited to exclude strictly toxic heavy metals such as Cd from the endosperm. NA and DMA regulate the accumulation of Fe, Zn, Mn and Cu in rice seeds (19) but they also have a low affinity for Cd, which is detrimental to human health (29, 32, 35 51). We found that transgenic plants grown on medium supplemented with Cd accumulated 1.5-fold and 2-fold lower amounts of this heavy metal in the unpolished and polished seeds, respectively. The endosperm is the preferred site for both Zn and Cd accumulation (20, 21,31, 37, 52, 53) suggesting that strategies to increase Fe and/or Zn accumulation may displace Cd

(Figure 7). Accordingly, Cd levels in seeds can be reduced by using Zn fertilizers (54, 55) and external Fe supplements (56, 57), whereas Fe deficiency increases Cd levels in the seed (58).

The heavy metal ATPase2 (HMA2) and low-affinity cation transporter (LCT1) facilitate seed loading with Cd (12, 33). *OsHMA2* mRNA levels were similar in the wild-type and transgenic lines, whereas *OsLCT1* mRNA was depleted in the transgenic plants, mirroring the lower levels of Cd in the endosperm. The displacement of Cd may therefore reflect a combination of factors including the preferential binding of Fe and Zn in the endosperm (59) and the suppression of *OsLCT1* expression (33).

In conclusion, we have shown that the coexpression of *OsNAS1* and *HvNAATb* in rice increases the abundance of NA and DMA and leads to accumulation of higher levels of Fe and Zn in the endosperm. Increasing the external supply of Fe affected the uptake of Fe and Zn into roots and their mobilization in the aboveground organs, but compensatory mechanisms presumably involving the vacuolar sequestration of metals in the flag leaf have a buffering effect and impose strict limits on the accumulation of metal micronutrients in the endosperm: 22.5 and 84 $\mu g\ g^{-1}$ DW for Fe and Zn, respectively. Furthermore, the preferential retention of Fe and Zn in the endosperm led to the competitive exclusion of Cd, halving the amount of Cd in the endosperm, and this can provide a useful strategy to increase the concentration of metal nutrients in polished rice seeds while ensuring that toxic metals are exported to the bran. Such strategies could help to address simultaneously micronutrient deficiency and heavy metal toxicity in communities that rely predominantly on cereal-based diets.

Materials and methods

Gene cloning

The *OsNAS1* (GenBank: AB021746.2) and *HvNAATb* (GenBank: AB005788.1) cDNAs were cloned from the roots of 2-week-old rice (*Oryza sativa* L cv. EYI 105) and barley (*Hordeum vulgare* L. cv. Ordalie) plants grown *in vitro* on MS medium without Fe (60). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and 1 µg of total RNA was reverse transcribed using the Omniscript RT Kit (Qiagen). The full-size *OsNAS1* cDNA (999 bp) and *HvNAATb* cDNA (1656 bp) were amplified by PCR using primer combinations mentioned in SI Materials and Methods. The products were transferred to the pGEM-T Easy vector (Promega, Madison, Wisconsin, USA) for sequencing and verification. The *OsNAS1* and *HvNAATb* cDNAs were then subcloned using the BamHI and HindIII sites and inserted into the expression vector pAL76 (61) which contains the maize *Ubiquitin-1* promoter and first intron, and an *Agrobacterium tumefaciens nos* transcriptional terminator. A separate vector was used to provide the *hpt* selectable marker (62).

Rice transformation

Mature rice seed-derived embryos (*Oryza sativa* L. cv EYI 105) were cultured and excised as previously described (63, 64). After 7 days, the embryos were bombarded with gold particles carrying the transgenes and the *hpt* selectable marker (65). The rice embryos were incubated on high-osmoticum medium (0.2M mannitol, 0.2M sorbitol) for 4 h prior to bombardment. Bombarded embryos were selected on MS medium supplemented with 30 mg l^{-1} hygromycin, and callus pieces were transferred sequentially to shooting and rooting medium containing hygromycin as above. Regenerated plantlets were transferred to pots

containing Traysubstract soil (Klasmann-Deilmann GmbH, Geeste, Germany) and were grown under flooded conditions in a chamber at 26 ± 2°C, with a 12-h photoperiod (900 μmol m^{-2} s^{-1} photosynthetically active radiation) and 80% relative humidity. Plants were irrigated with a solution of 100 μM Fe provided as Fe (III)-EDDHA in the form of Sequestrene 138 Fe G-100 (Syngenta Agro SA, Madrid, Spain).

mRNA blot analysis

Total leaf RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) and 20-μg aliquots were fractionated on a denaturing 1.2% agarose gel containing formaldehyde before blotting. The membranes were probed with digoxigenin-labeled partial *OsNAS1* or *HvNAATb* cDNAs at 50°C overnight using DIG Easy Hyb (Roche Diagnostics, Mannheim, Germany). After washing and immunological detection with anti-DIG-AP (Roche Diagnostics) according to the manufacturer's instructions, CSPD chemiluminescence (Roche Diagnostics) was detected on Kodak BioMax light film (Sigma-Aldrich, St Louis, Missouri, USA).

Iron uptake studies

T_2 seeds from transgenic rice lines EYI-9, EYI-89 and EYI-98 were germinated on half strength MS medium supplemented with 50 mg l^{-1} hygromycin, and wild-type seeds were germinated on the same medium without hygromycin. After 5 days, seedlings were transferred to floating trays and watered for 3 weeks with nutrient solution (66) containing 100 μM $FeCl_3$. Ten seedlings of equivalent height from each transgenic line (and wild-type controls) were transferred to nutrient solution containing 100 μM $FeCl_3$ (normal feeding), 200 μM $FeCl_3$ (double Fe regime) or 300 μM $FeCl_3$ (triple Fe regime). Fresh nutrient solution was applied every other day and the pH of the solution was adjusted to 5.3 with

0.1 N KOH. Every week, the nutrient solution was completely replaced. Plants were maintained as above until seed maturity. Samples were collected from 10 plants per group and pooled as roots, culm, middle leaf, flag leaf and seeds for analysis of metal concentrations by ICP-MS.

Cadmium uptake studies

T_2 seeds from transgenic and wild-type lines were germinated and transferred to the floating trays containing nutrient solution supplemented with 100 μM $FeCl_3$ as above. Fifteen seedlings of equivalent height from each transgenic line (and wild-type controls) were transferred to nutrient solution containing 10 μM $CdCl_2$ or an equivalent control as described above. The pH of the solution was adjusted to 5.3 with 0.1 N KOH and the plants were maintained as above until seed maturity. Seeds were harvested from all plants and metal concentrations were quantified by ICP-MS.

Measurement of metal concentrations by ICP-MS

Roots and leaves were collected in plastic containers pre-treated with 6.5% HNO_3 to avoid contamination with exogenous minerals. Metals were removed from the surface of each sample by washing three times in double-deionized water followed by 100 μM Na_2EDTA. The EDTA was then removed with two further washes in double-deionized water. To avoid metal contamination during polishing, dehusked wild-type and transgenic seeds were polished using a non-contaminating polisher (Kett, Villa Park, California, USA) and ground using a mortar and pestle pre-treated with 6.5% HNO_3. Roots, leaves and seeds were dried at 70°C for 2 days and 300-mg portions were wet ashed with 4.4 M HNO_3, 6.5 M H_2O_2 and double-deionized water (3:2:2) for 20 min at 230°C using a MarsXpress oven (CEM Corp., Matthews, North Carolina, USA). Metal concentrations were determined in

diluted samples by ICP-MS using an Agilent 7700X (Agilent Technologies, Santa Clara, California, USA).

Quantitation of endogenous gene expression by qRT PCR

Quantitative real-time RT-PCR was carried out to measure the steady state mRNA levels in roots, flag leaf and immature seeds representing the endogenous genes listed in Table S2 (Details of PCR conditions are described in SI Materials and Methods).

Quantitation of NA and DMA by HPLC-ESI-MS

NA (98% purity) was obtained from Hasegawa Co. Ltd. (Kawasaki, Japan), and DMA (98% purity) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Nicotyl-lysine was synthesized as previously described (67). All standards were dissolved in water to a concentration of 1–10 mM and stored in darkness at –80°C. Working standard solutions were prepared by diluting the stock solutions with double-deionized water. Each 5-µL standard solution was diluted with 5 µL 50 mM EDTA, 5 µL nicotyl-lysine and 30 µL of a 1:9 ratio mixture of 10 mM ammonium acetate and acetonitrile (pH 7.3) and the mixture was filtered through polyvinylidene fluoride (Durapore® PVDF) 0.45-µm ultrafree-MC centrifugal filter devices (Merck KGaA, Darmstadt, Germany) before injection into the HPLC-ESI-TOF-MS system (see SI materials and methods).

For the measurement of NA and DMA, seeds were ground to a fine powder in liquid nitrogen and then extracted three times as described by (67), with modifications. Briefly, NA and DMA were extracted from 50 mg seed powder with 300 µL double-deionized water and 18 µL of 1 mM nicotyl-lysine. The supernatant was recovered by centrifugation (15,000 x g, 4ºC, 15 min) and stored at –20°C, and the pellet was resuspended twice in 300 µl double-deionized water, homogenized, centrifuged as above and the supernatant was

recovered. The three supernatant fractions were pooled and the total extract was passed through a 3-kDa centrifugal filter (regenerated cellulose Amicon® Ultra filter units, Merck). The filtered solution obtained by centrifugation at 15,000 x g for 30 min was vacuum concentrated until dry. The residue was dissolved in 10 µl water, and a 5-µl aliquot was diluted with 10 µl 50 mM EDTA, 15 µl double-deionized water and 30 µl of the 1:9 mixture of 10 mM ammonium acetate and acetonitrile (pH 7.3). The mixture was filtered and injected into the HPLC-ESI-TOF-MS system (see SI materials and methods).

Statistical analysis

Differences between transgenic and wild type plants were tested by comparison of means using the *t* test ($p<0.05$).

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Author contributions: R.B and P.C. designed research; R.B., A.A.F., P.D-B., and T.C. performed research; R.B and A.A.F. analyzed data; R.B., A.A.F., J.A., and P.C. wrote the paper.

*Authrs declare no conflict of interest.

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Figure legends

Figure 1 mRNA blot analysis showing transgene expression in leaf tissue of wild type (WT) and 19 independent T_2 generation transgenic lines. rRNA: ribosomal RNA; *OsNAS1*: rice nicotianamine synthase; *HvNAATb*: barley nicotianamine amino transferase.

Figure 2 Concentrations of nicotianamine (NA) and 2' deoxymugenic acid (DMA) (in both cases in µg g^{-1} FW) in unpolished seeds of wild type (WT) and three different T_2 generation transgenic lines co-expressing *OsNAS1* and *HvNAATb* (EYI-9, EYI-89 and EYI-98). FW: fresh weight. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3).

Figure 3 Concentrations of Fe and Zn (in µg g^{-1} DW) in roots, culm, middle leaf and flag leaf in wild type (WT) and three different T_2 generation transgenic lines co-expressing *OsNAS1* and *HvNAATb* (EYI-9, EYI-89 and EYI-98) under 100 (control), 200 and 300 µM Fe treatments. DW: dry weight; R: roots; ML: middle leaf; FL: flag leaf. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3).

Figure 4 Concentrations of Fe and Zn (in µg g^{-1} DW) in polished seeds of wild type (WT) and three different T_3 generation transgenic lines co-expressing *OsNAS1* and *HvNAATb* (EYI-9, EYI-89 and EYI-98) under 100 (control), 200 and 300 µM Fe treatments. DW: dry weight. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6)

Figure 5 quantitative Real-time PCR analysis of *OsYSL15, OsIRT1, OsZIP1* in roots (R) and *OsYSL2, OsVIT1, OsFERRITIN1, OsZIP1, OsZIP4* in flag leaf (FL) under 100 (control), 200 and 300 µM Fe supply at grain filling stage in wild type and T_3 generation transgenic lines co-expressing *OsNAS1* and *HvNAATb* (EYI-9, EYI-89). Each value is the average of three independent experiments. Transcript levels are represented by the ratio between mRNA levels of *OsYSL5, OsIRT1, OsZIP1, OsZIP4, OsVIT1, OsYSL2, OsFERRITIN1* and those of Os*ACTIN1*. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3). Gene-specific primers are listed in Supplementary Table S2.

Figure 6 Concentrations of Cd (unpolished and polished seeds), Fe and Zn (polished seeds) in wild type (WT) and T_3 generation transgenic lines co-expressing *OsNAS1* and *HvNAATb* (EYI-9, EYI-89 and EYI-98) grown with 100 µM $FeCl_3$ and 10 µM $CdCl_2$. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=6). The Cd concentrations in unpolished and polished seeds were significantly different both in WT and transgenic lines.

Note: As transgenic line EYI-9 did not produce enough seeds, quantification of Fe and Zn was not performed.

Figure 7 The mechanistic basis of iron (Fe) and zinc (Zn) homeostasis in rice. We found that transgenic lines accumulating high levels of phytosiderophores impose a ceiling on Fe and Zn accumulation in the endosperm. This phenomenon occurs because Fe and Zn uptake in the roots is regulated by modulating endogenous metal transporter expression in response to Fe levels (1), by sequestering Fe/Zn in the roots (2), culm (3), middle leaf (4) and flag leaf (5), and by controlling phloem Fe/Zn remobilization from the flag leaf to the seeds by the modulation of metal transporter expression in these tissues (6), particularly vacuolar transporters in the flag leaf to promote vacuolar sequestration (5). The accumulation of Fe and Zn in the endosperm can therefore increase by a maximum of four-fold when phytosiderophores are not limiting (7) but this is sufficient to displace the toxic metal Cd into the bran (8).

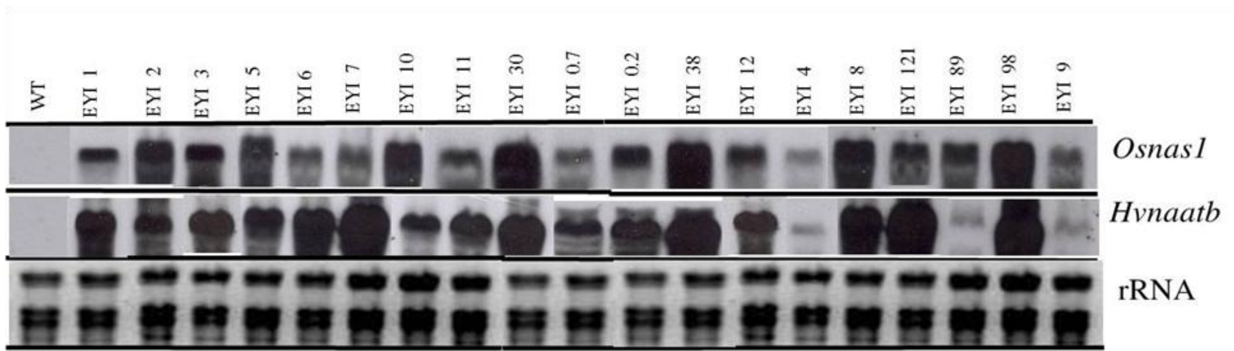

Figure 1

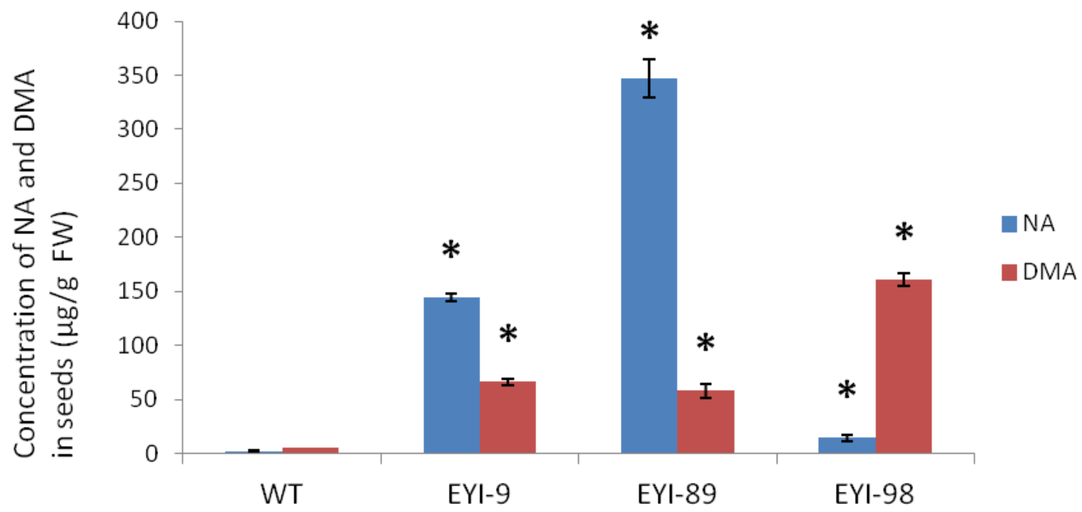

Figure 2

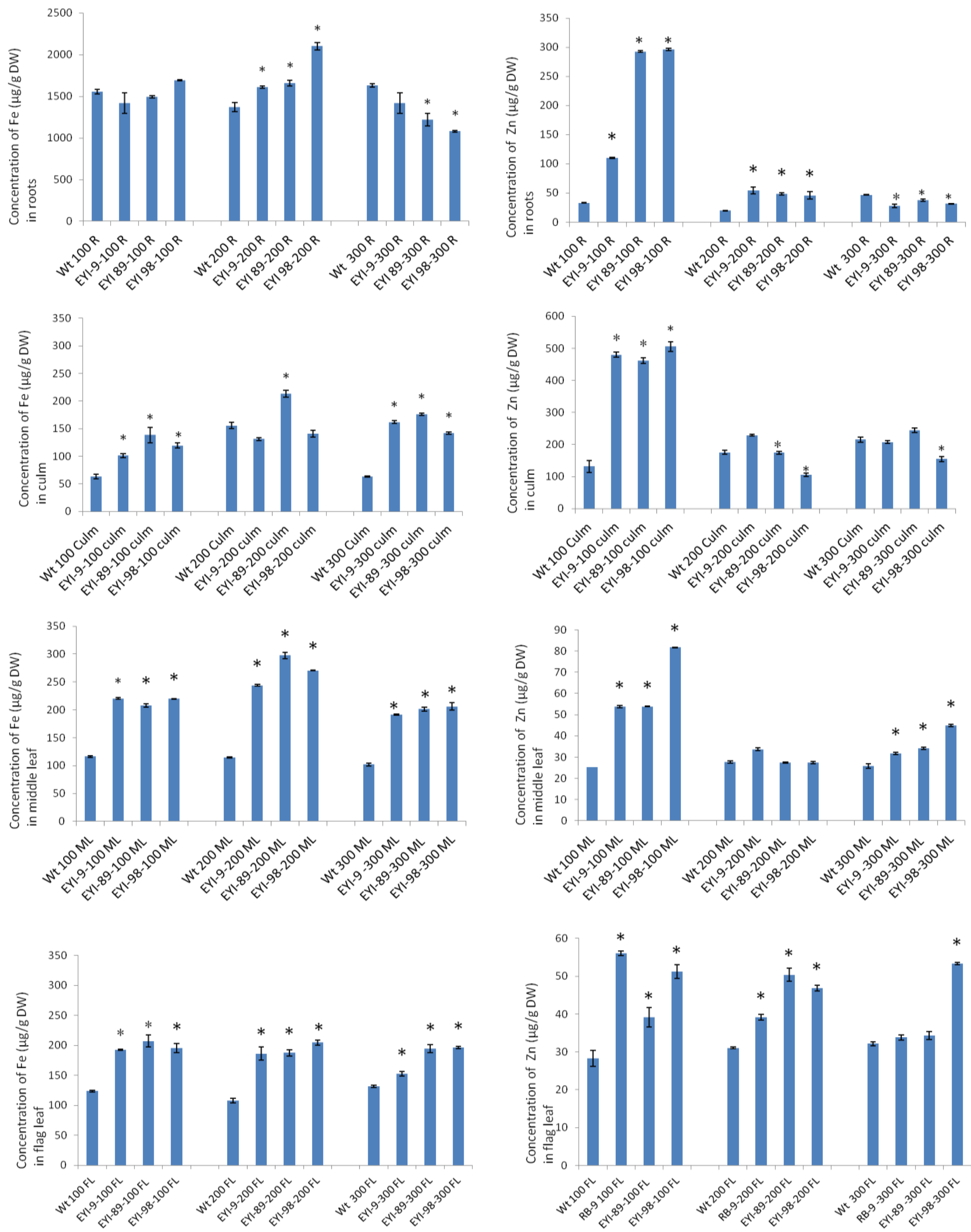

Figure 3

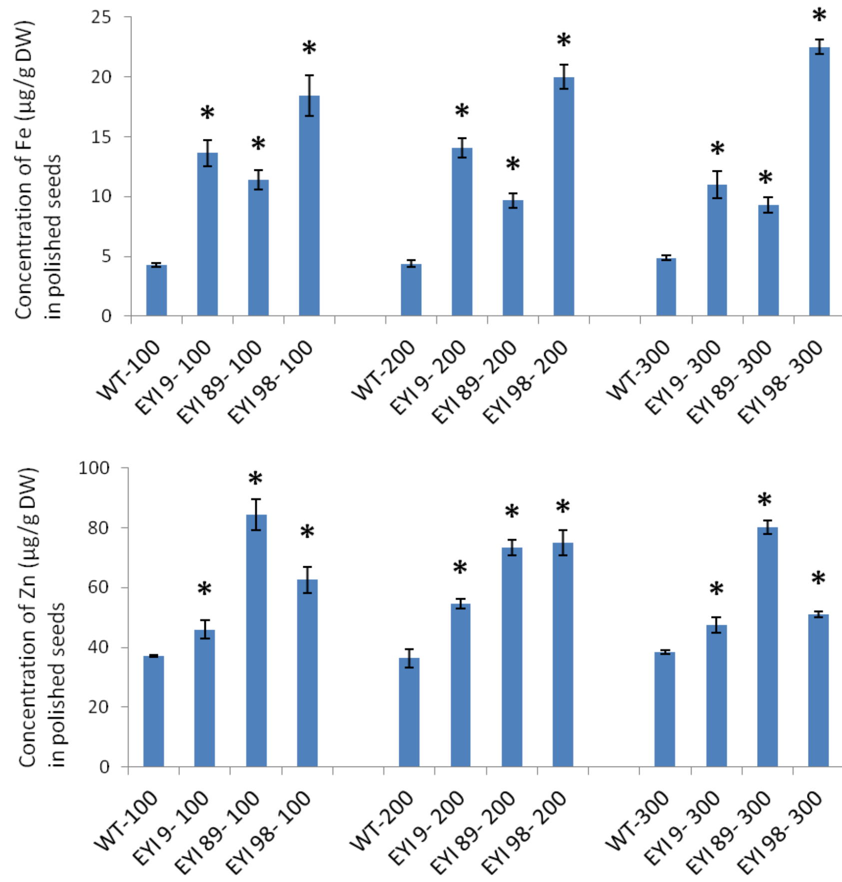

Figure 4

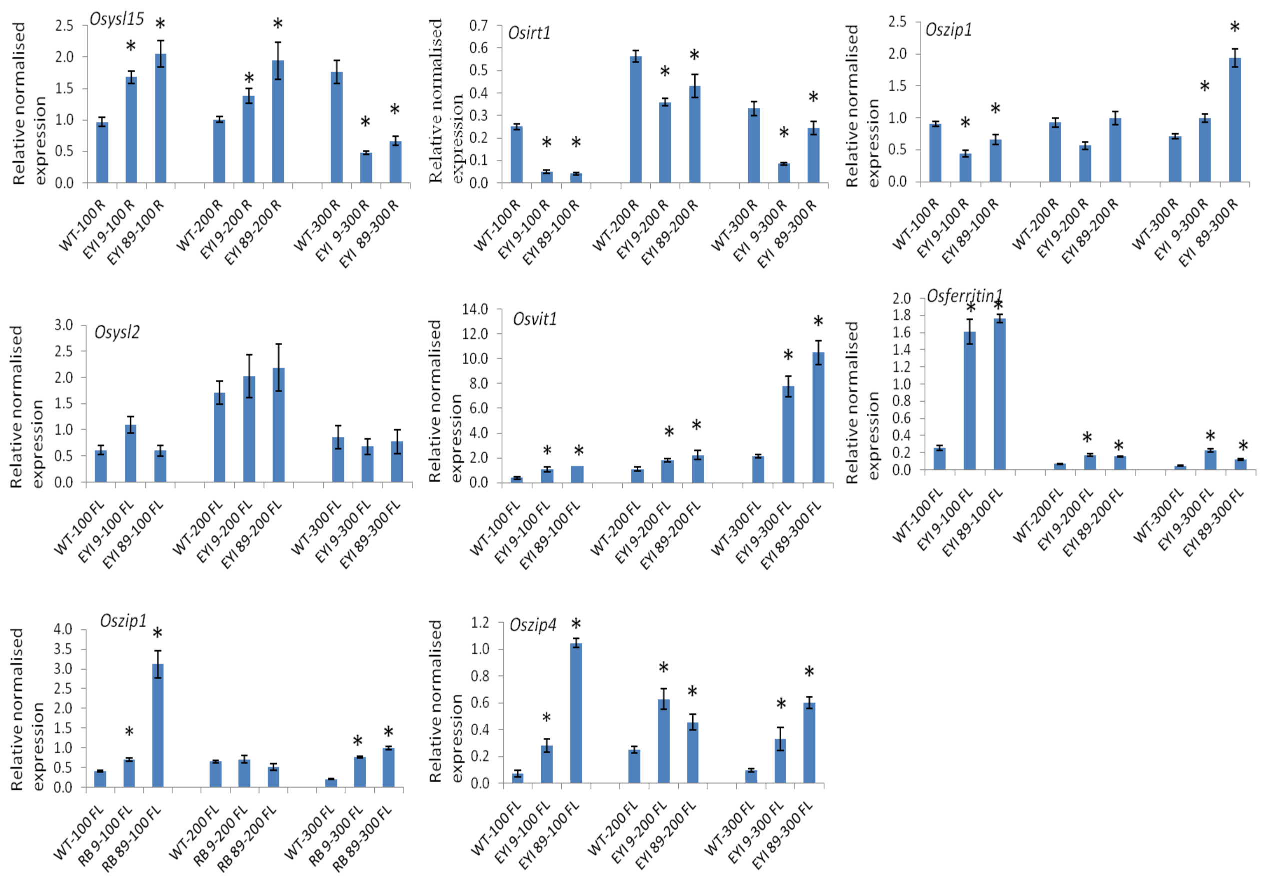

Figure 5

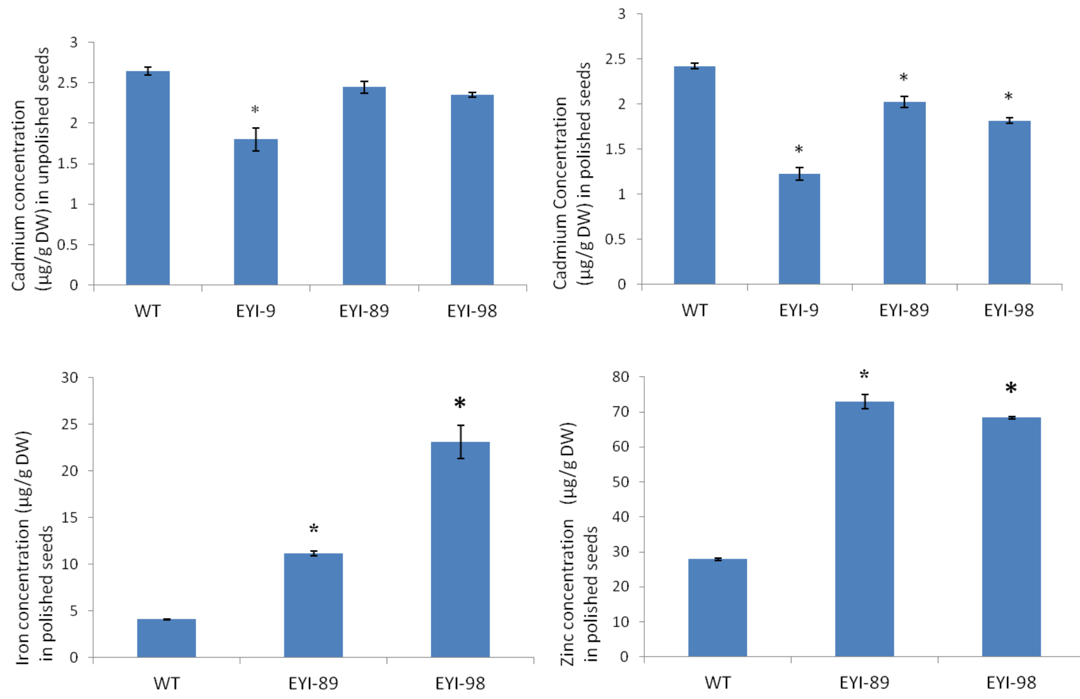

Figure 6

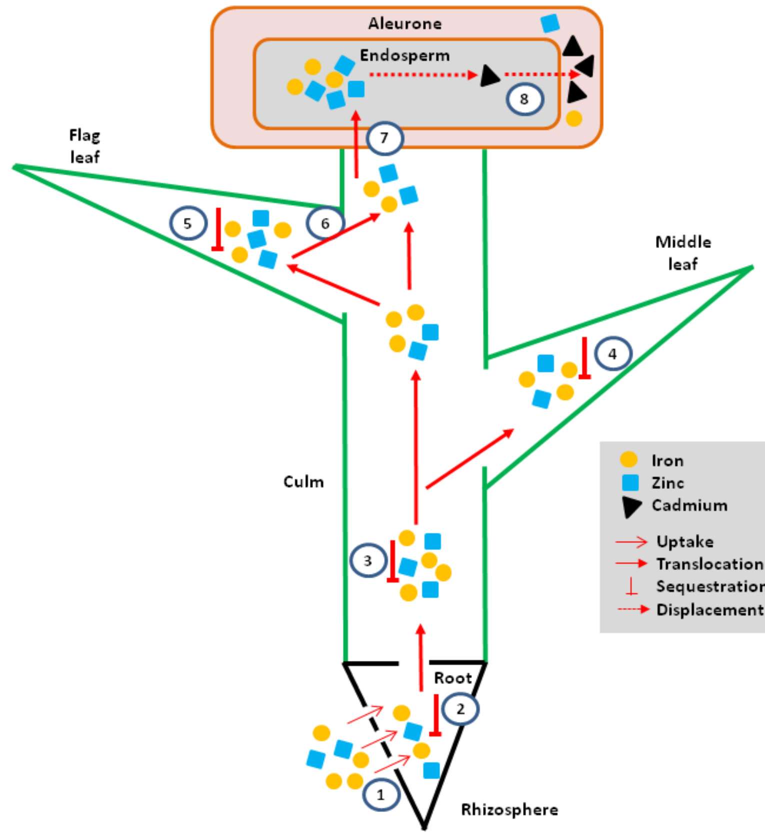

Figure 7

Supporting information

SI Materials and Methods

Primer combinations used for gene cloning

The full-size *OsNAS1* cDNA (999 bp) and *HvNAATb* cDNA (1656 bp) were amplified by PCR using primer combinations combinations mentioned in SI materials and methods. *OsNAS1*-BamHI-FOR (5'-AGG ATC CAT GGA GGC TCA GAA CCA AGA GGT CG-3') and *OsNAS1*-HindIII-REV (5'-AAA GCT TCA TAA TAT AGT GCG CCT TTC GAT CGT CCG GCT GT -3'), or *HvNAATb*-BamHI-FOR (5'-AGG ATC CAT GGC CAC CGT ACG GCC AGA GAG CGA CG-3') and *HvNAATb*-HindIII-REV (5'- AAA GCT TCT AGC AAT CAT CGC TCG CTC GAA TTT CTC -3').

qRT-PCR conditions

Genes mentioned in Table S2 were amplified using a BioRad CFX96^{TM} system and 25-µL mixtures containing 10 ng of cDNA, 1x iQSYBR Green supermix (BioRad, Hercules, California, USA) and 0.2 µM forward and reverse primers (Table S2). Relative expression levels were calculated on the basis of serial dilutions of cDNA (125–0.2 ng) and used to generate standard curves each target gene. Each experiment was carried out in triplicate using 96-well optical reaction plates. Cycling conditions consisted of a single incubation step at 95°C for 5 min followed by 39 cycles of 95°C for 10 s, 59.4°C for 35 s and 72°C for 15 s. Specificity was confirmed by product melt curve analysis over the temperature range 50–90°C with fluorescence acquired after every 0.5°C increase. The fluorescence threshold value and gene expression data were calculated using BioRad CFX96^{TM} software. Values represent the mean of three RT-PCR replicates ± SE. Amplification efficiencies were compared by plotting the DCt values of different primer combinations in

serial dilutions against the log of the starting template concentrations using the CFX96™ software.

Quantification of NA and DMA

NA and DMA in seed extracts were quantified as described by (1), with modifications. The samples were fractionated using an Alliance 2795 HPLC system (Waters, Milford, Massachusetts, USA) with a micro LC column (SeQuant ZIC®-HILIC, 15 cm x 1 mm internal diameter, 5 µm, 200 Å, Merck KGaA), with a mobile phase consisting of solvent A (9:1 10 mM ammonium acetate:acetonitrile, pH 7.3) and solvent B (8:2 30 mM ammonium acetate:acetonitrile, pH 7.3) at a flow rate of 0.15 ml min^{-1}. The gradient program started at 100% solvent A for 3 min, and then decreased linearly to 30% solvent A over the next 7 min, then remained for 7 min at 30% solvent A, and then returned to the initial conditions over the next 8 min. The column was then allowed to stabilize for 10 min at the initial conditions before proceeding to the next injection. The total HPLC run time was 35 min, the injection volume was 10 µl and the auto sampler and column temperatures were 6ºC and 30ºC, respectively. The HPLC was coupled to the MicrOTOF time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. The operating conditions were optimized by the direct injection of 100 µM solutions of NA and DMA standards at a flow rate of 180 µl h^{-1}. Mass spectra were acquired in negative ion mode (see settings in Table S3) over the 150–700 *m/z* range. The mass axis was calibrated externally and internally using Li–formate adducts (10 mM LiOH, 0.2% (v/v) formic acid and 50% (v/v) 2-propanol). Bruker Daltonik software packages micrOTOF Control v2.2, HyStar v3.2 and Data Analysis v4.0 were used to control the MS, HPLC interface and for data processing, respectively. The concentrations

of NA and DMA were quantified by external calibration, with nicotyl-lysine as the internal standard.

Reference

Xuan Y, et al. (2006) Separation and identification of phytosiderophores and their metal complexes in plants by zwitterionic hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry. *J Chromatgr A* 1136:73-81.

Table S1 Concentration of Fe and Zn in unpolished seeds of wild type and transgenic rice seeds (T_2 generation). DW-dry weight; WT- wild type; ten independent transgenic lines co-expressing *OsNAS1* and *HvNAATb* lines are shown. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test ($p < 0.05$). Number of replications (n=6)

Line	Fe in unpolished seeds (μg/g DW)	Zn in unpolished seeds (μg/g DW)
EYI-9	26.35 *	47.49*
EYI-89	56.68*	77.80*
EYI-98	34.47*	58.7*
EYI-0.7	33*	43*
EYI-0.2	30*	42*
EYI-38	25*	37*
EYI-4	26.29*	28.02*
EYI-8	22.96*	21.57*
EYI-12	23.42*	23.24*
EYI-121	21.92*	22.77*
WT	15.33	18.51

Table S2 Details of genes and primers used for quantitative real time RT-PCR

Gene	Gene Bank ID	Amplicon (bp)	Forward primer	Reverese primer
OsYSL15	AB190923.1	78	CTGGTGCTGTTTGCTTGGA	ATGAGCCCAGACGCAACAGC
OsIRT1	BAB85123.1	139	GCATCATGCAATTCGCTGC	CCTGAACAACCACGCTACAA
OsZIP1	AY324148.1	90	CGATGGTCCTCTTCTTCTGC	GTCGGGCTGCTCTCGTTGTA
OsZIP4	AB126089.1	107	TCCACCAGTTCTTTGAAGGC	CGGAGCAGTGAGGGAGAAGA
OsVIT1	NM_001059545.1	107	CCACAGCACAGAACGCCAT	GCTGAGGAATGGACGGTTT
OsYSL2	AB164646	169	GGTGGAGAGAGTTGTGGGTT	TCATTCCCGCACCAACATA
OsFERRITIN1	AF519570.1	130	GCTTGAATGGAGGAGACTGTG	CTCCATTGCTACTGCGTGCT
OsLCT1	AB905364.1	100	GCTCAAGTGGATGGCGGACA	GCCACCATCTTGTCCTTGCTT
OsHMA2	AB697186.1	126	CGAGGAATACACCCGCATCAGC	CAAGCAGAGCCACGAAAAGAGC
OsACTIN1	AB047313.1	120	TCA TGT CCC TCA CAA TTT CC	GAC TCT GGT GAT GGT GTC AGC

Table S3 Operating conditions of the time-of-flight (TOF) mass spectrometer (MS) used for NA and DMA determinations

Source	Electrospray
Polarity	Negative
Endplate voltage	-0.5 kV
Spray tip voltage	3.0 kV
Orifice voltage	107 V
Nebulizer gas	N_2
Nebulizer gas pressure	2.0 bar
Drying gas	N_2
Drying gas (N_2) flow rate	8.5 L min^{-1}
Drying gas temperature	180 ºC

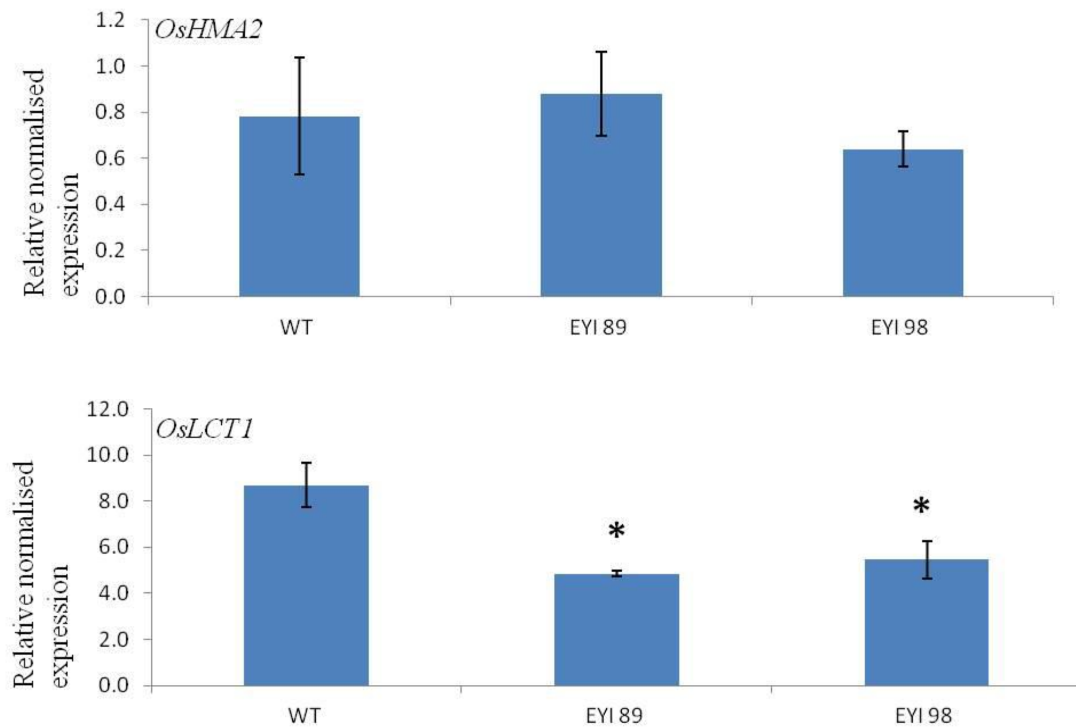

Figure S1 qReal-time PCR analysis of *OsHMA2 and OsLCT1* under Cd supply at grain filling stage in wild type (WT) and T_3 transgenic lines co-expressing *OsNAS1*and *HvNAATb* (EYI-89, EYI-98). Each value is the average of three independent experiments. Transcript levels are represented by the ratio between mRNA levels of *OsHMA2, OsLCT1* and those of *OsACTIN1*. Asterisks indicate a statistically significant difference between wild type and transgenic plants as determined by Student's t test (p <0.05; n=3). Gene-specific primers are listed in Supplementary Table S2.

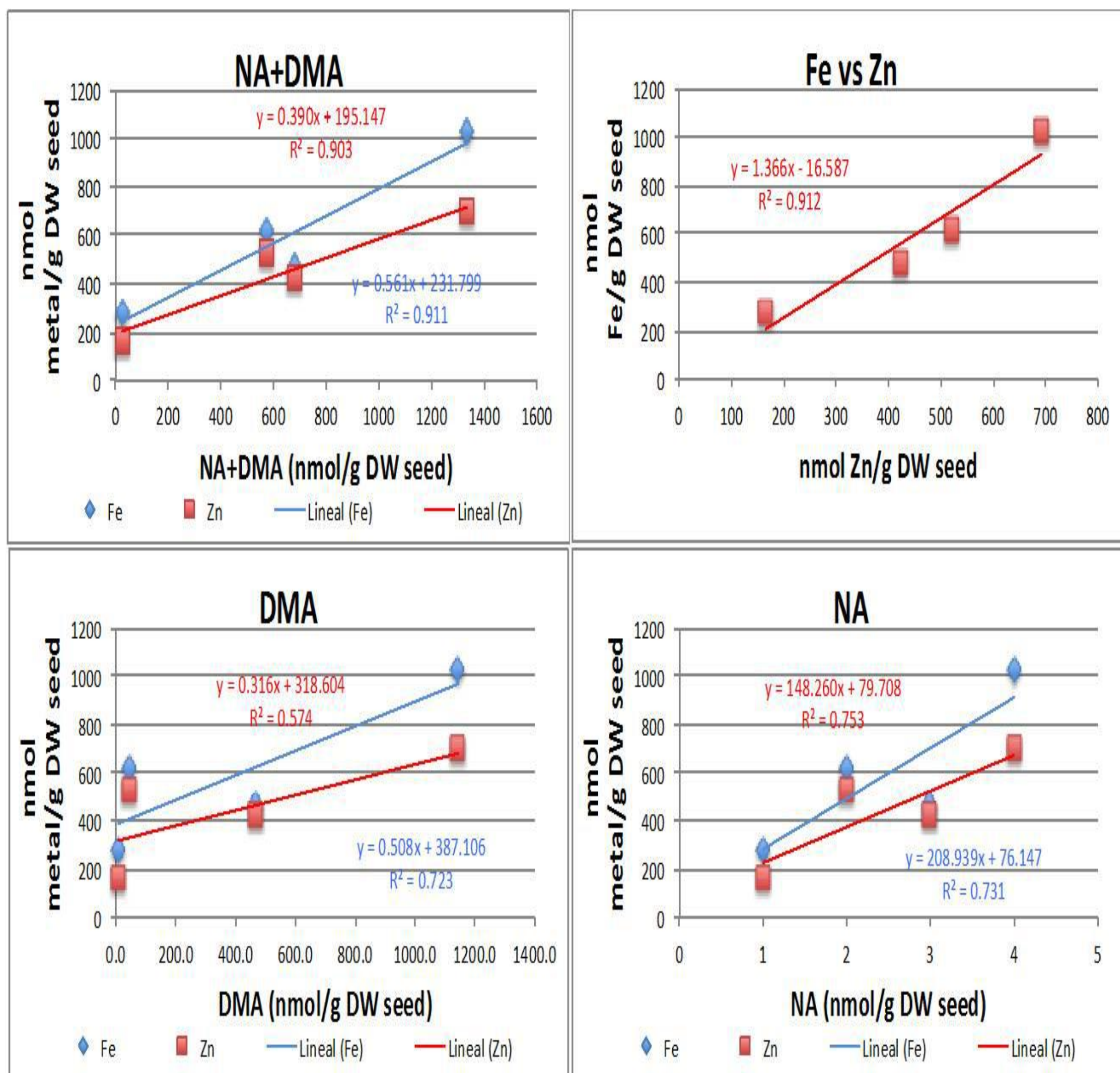

Figure S2 Scatter graph and linear correlations between unpolished grain NA and/or DMA concentration and Fe (blue diamons) and Zn (red square) concentrations for the genotypes in Figure 2.

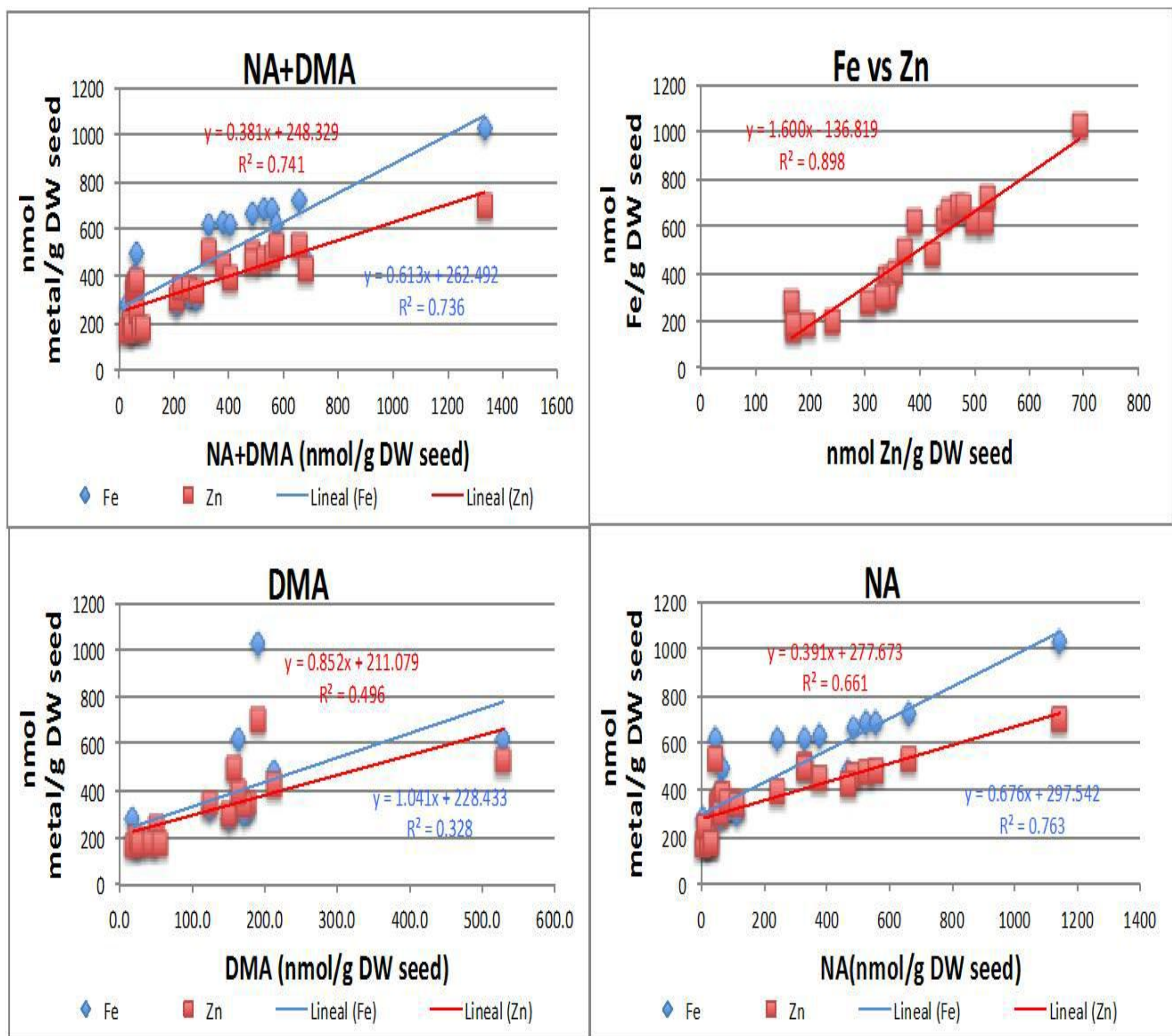

Figure S3 Scatter graph and linear correlations between unpolished grain NA and/or DMA concentration and Fe (blue diamonds) and Zn (red square) concentrations for the genotypes in Figure 2 and for those reported by (1, 2, 3, 4).

References

Johnson AAT, et al. (2011) Constitutive overexpression of the OsNAS gene family reveals single-gene strategies for effective iron- and zinc-biofortification of rice endosperm. *PLoS ONE* 6:e24476-e24487.

Masuda H, et al. (2012) Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Sci Rep* 2:54-549.

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