Iron loading in rice endosperm controlled by cell-type specific over-expression of *OsNAS*

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Abstract

Over 50 years after the attempt to combat worldwide famine with the Green Revolution and the expansion of agricultural practices, we face new and equally challenging battles of combating human micronutrient malnutrition. The prevalence of iron (Fe) deficiency anaemia is tightly associated with the proportion of people reliant on cheap nutrient poor rice diets, particularly women and children in Asia and Africa (Kraemer and Zimmermann, 2007). There is an amazing depth of knowledge available that demonstrates the uptake and transport of Fe in rice plants but little research has successfully resulted in biologically significant Fe enriched rice. This thesis attempts to address this problem with the development and investigation of cell type specific over expression of *OsNAS* genes in rice. The ultimate aim is to develop rice with enriched Fe in the endosperm - the most commonly consumed portion of the grain.

Iron uptake and translocation play a crucial role in the biochemistry of cell development in both animals and plants and as such, Fe must be tightly regulated. Plants acquire Fe via root transport of ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions via transport mechanisms known as Strategy I (in dicots) and Strategy II (in monocots). An important factor in Fe accumulation in both dicots and monocots is the synthesis of nicotianamine (NA) molecules upon the activity of NA synthases. In Strategy I plants, NA is a well characterised Fe chelator, acting as a vehicle for Fe long distance transport. Strategy II plants, like rice, catalyse NA in the production of phytosiderophores, Fe^{3+} chelating molecules used to draw Fe in from the rhizosphere. There exist three NA synthase genes in rice; *OsNAS1*, *OsNAS2* and

OsNAS3. It is known that each gene is spatially controlled across the plant at differing periods of Fe homeostasis (Cheng et al., 2007, Inoue et al., 2003). What is not known is why this happens and whether these genes present any redundancy value or if they have highly specified roles in Fe acquisition in rice. Chapter Two of this thesis investigates the effect of Fe accumulation in rice (*Oryza sativa ssp. Japonica cv* Nipponbare) endosperm when manipulated using transgenic lines over-expressing the three rice NAS genes *OsNAS1*, *OsNAS2* and *OsNAS3*. The hypothesis states that a single *OsNAS* gene may lead to a more efficient Fe accumulator in the grain and that the cell type specific expression of this gene may influence this effect. In order to achieve cell type specific expression of these genes, GAL4 was used to control the enhancer trap system in root stele, root cortex and lodicules of developing grain, along with ovary of developing grain and leaf shoot tissues. Subsequent ICP-OES analysis of T1 grains indicated that Fe was enhanced up to two-fold in brown rice of root cortex-specific *OsNAS2* over-expression and developing grain lodicules-specific *OsNAS3* over-expression lines compared to the corresponding wild type. Furthermore, polished grains from transgenic plants retained up to 30% more Fe in the endosperm post-milling. In some cases 2-3 times more Fe was found in the grain of the transgenic than wildtype. We subsequently suggest there is not a redundancy between the three *OsNAS* genes, but that over-expression of *OsNAS2* may in fact trigger endogenous expression of *OsNAS1* and *OsNAS3*. This refers to the 'heterocot' nature of rice, where both strategies of Fe uptake (strategy I of dicots and strategy II of monocots) are utilised, enabling the NA to be produced for PS sequestration, Fe influx and Fe chelation and transport.

The exciting results from Chapter Two were further investigated in Chapter Three with the use of synchrotron radiation to visualise this increase in Fe accumulation in the grain. Traditionally, relatively little Fe is present in the endosperm, a fact worsened by the loss of 75-80% of Fe with the removal of the Fe rich bran and maternal tissue during milling (Briat et al., 1995). Levels of 14 μg g^{-1} Fe in the rice endosperm have been reported as the 'goal' for biofortified rice- over three times the typical concentration in consumed rice around the world. Grain developed in Chapter Two resulted in Fe concentrations of over 13 μg g^{-1} in the endosperm. It was therefore imperative to visualise the Fe distribution across the whole transgenic grain to ensure we had developed Fe enhanced rice that would provide increased nutritional benefit once milled. Interestingly, SXRF maps showed Fe was not limited to the aleurone and embryo but instead extended further into the sub aleurone and endosperm. Furthermore this increase in Fe was not co localised with P, a key indicator of the anti-nutrient phytate. This was further established with SXANES and nano-SIMS technology, highlighting the utility of multi-spectroscopic analysis of micronutrients in grain as well as suggesting a more bioavailable Fe enhanced rice grain.

Research into biofortified foods designed to combat human micronutrient deficiencies would be unrealistic if geographic considerations of rice production were ignored. Over 50% of the world's population consume rice, and the majority of this is grown in Asia. Due to anthropogenic contamination of crop soils with heavy metals such as cadmium (Cd), it is of no surprise that the greatest vehicle for Cd consumption and toxicity in humans is in fact rice. Chapter Four explores the potential for heavy metal accumulation in the transgenic grain, as NA is known to

bind several metals such as Zn, Mn and Ni. It was hypothesised that the root specific over-expression of *OsNAS2* would maintain enhanced levels of Fe in the grain when grown in Cd contaminated soils without increased the grain Cd concentrations. Interestingly, the converse of this was recorded, with Cd levels exceeding those allowed for human consumption in Australia and parts of Asia (Arao and Ae, 2003, Hseu et al., 2010) and Fe concentrations decreased from those found in previous generations. This demonstrates a relationship between NA and Cd - a relationship that has otherwise not been described in literature.

As we have shown in this thesis and other publications (Johnson et al., 2011), there is an increase in endosperm Fe in transgenic rice over expressing *OsNAS2*. The final chapter of this thesis challenges the source-sink relationship of Fe accumulation and loading in the grain. We ask whether an endosperm specific promoter controlling the over-expression of a vacuolar iron transporter (*OsVIT1*) in the background of the *OsNAS2* lines would result in further enhanced Fe loading than previously seen in the single transformant 35S *OsNAS2* lines (Johnson et al., 2011). It is expected that as there is already in increase Fe accumulation in *OsNAS2* grain, endosperm specific *OsVIT1* will further drive Fe into the grain, beyond the aleurone and into the centre of the endosperm. Preliminary results suggest we were able to produce a rice line with relatively higher grain Fe concentrations and this gives rise to a multitude of future research possibilities in the future.

Appendix One of this thesis contains the full manuscript of Johnson et al. (2011) as work from this thesis contributed to second authorship. The 35S *OsNAS* lines referred to in this thesis are further investigated in the manuscript of Appendix One. This work was published in PlosONE.

Declaration of Authenticity

I, Bianca Kyriacou, certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Bianca Kyriacou

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There is always concern that presenting one's gratitude to a number of people will leave some with a feeling of contrite or un-heartfelt emotion. It is with great sincerity that I say this is not my intention.

Often as students we were told to enjoy the PhD journey and reflect on its ups and downs- 'they bring out the best in you as a scientist'. To be honest I feel the past few years have been too blurred and rushed. As I looked out from inside the cyclone, I wanted to click my heels three times and wake up from this thrilling and frightening dream. Now that I am awake and reviewing with the eyes of hind sight, I can confidently say I loved the whole rainbow this PhD presented me with.

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Bianca Kyriacou

Abbreviations List

[x]	Concentration of element
°C	Degrees Celcius
$^{56}Fe^{16}O^{-}$	Radiolabelled iron oxide
AA	Ascorbic acid
AAS	Atomic absorption spectroscopy
AQC	6-aminoquinolyl-n-hydroxysuccinimidyl carbamate
ACPFG	Australian centre for plant functional genomics
AQIS	Australian quarantine inspection services
At	*Arabidopsis thaliana*
B	Boron
BO_3	Borate
Ca	Calcium
Caco-2	Immortalised human colonic epithelial cells
CCC1	Cation ca^{2+}-sensitive cross-complementer
Cd	Cadmium
CdCS	Cadmium contaminated soils
cDNA	Complementary deoxyribonucleic acid
CER	Controlled environment growth rooms
CGIAR	Consultative group on international agricultural research
Cs	Caesium
Cu	Copper
DAB	Diaminobenzidine
DF	Developing flower
DF:L	Lodicules of developing flowers
DF:O ; LC	Ovary of developing flower; and leaf collar
DF:VB ; LC	Vascular bundle of developing flower; and leaf collar
DMA	Deoxymugenic acid/s
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive x-ray microanalysis
eV	Electron volts
FAO	Food and agriculture organisation of the United Nations
FDRL	Ferric reductase defective-like
Fe	Iron
Fe^{2+}	Ferrous ions
Fe^{3+}	Ferric ions
$FeCl_2$	Ferrous chloride
$FeNO_3$	Ferric nitrate

FRO	Ferric reductases/oxidases
gDNA	Genomic DNA
Ge	Germanium
GFP	Green fluorescent protein
HCl	Hydrochloric acid
ICP-OES	Inductively coupled plasma optical emission spectroscopy
IRRI	International rice research institute
IRT	Iron-regulated transporters
K	Potassium
keV	Kilo electron volts
KOH	Potassium hydroxide
L, ml	Litre, millilitre
LC	Liquid chromatography
µ	Micro
M, mM	Molar, millimolar
MA	Mugenic acid/s
µg g^{-1}	Micrograms per gram
Mn	Manganese
MS	Mass spectroscopy
NA	Nicotianamine
Na	Sodium dodecyl sulfate
NAAT	Nicotianamine aminotransferase
NAS	Nicotianamine synthase
NCdS	Non-(cadmium) contaminated soils
Ni	Nickel
nmol	Nanomole/s
NPTII	Neomycin phosphotransferase selective marker
NRAMP	Natural resistance associated macrophage protein/s
Os	*Oryza sativa L.*
OsIRO2	*Oryza sativa iron regulated transporter 2*
OsNAS	*Oryza sativa* nicotianamine synthase gene
OsNAS	*Oryza sativa* nicotianamine synthase protein
OsNAS1	*Oryza sativa* nicotianamine synthase gene 1
OsNAS2	*Oryza sativa* nicotianamine synthase gene 2
OsNAS3	*Oryza sativa* nicotianamine synthase gene 3
P	Phosphorous
pA	Pico Amperes (pico Amps)
PCR	Polymerase chain reaction
pmol	Picomole/s
PPB	Perl's Prussian blue stain
PS	Phytosiderophore/s

PSV	Protein storage vacuole
qPCR	Real time PCR
R:C	Root cortex
R:S	Root stele
RO	reverse osmosis
RNA	Ribonucleic acid
RNAi	Interference ribonucleic acid
S	Sulfur
SAM	S-adenosylmethionine
SARDI	South australian research and development institute
Sc	*Saccharomyces cerevisiae*
SDS	Sodium dodecyl sulfate
SE	Secondary electron
SEM	Scanning electron microscopy
SR	Synchrotron radiation
SXANES	Synchrotron radiation x-ray absorption near edge spectroscopy
SXRF	Synchrotron radiation x-ray fluorescence microscopy
T0, T1, T2	Transgenic material from original transformation, T1 is progeny of T0…
TAE	Tris-acetate EDTA
TEM	Transmission electron microscopy
UAS	Upstream activation sequence
UC Davis	University of California, Davis campus
UK	United Kingdom
UNICEF	United Nations children's fund
US	United States of America
USDA	United States department of agriculture
v/v	Volume per volume
VIT1	Vacuolar iron transporter 1
w/v	Weight per volume
w/w	Weight per weight
WAS	Waite analytical service
XFM	X-ray fluorescence microprobe
YS1	Yellow stripe 1
YSL	Yellow stripe-like
Zn	Zinc