

**A metabolic engineering approach for iron and zinc
biofortification of bread wheat (*Triticum aestivum* L.):
impacts on plant growth, grain nutrition and food
processing**

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Submitted in total fulfilment of the requirements for the degree of

Doctor of Philosophy

2020

School of BioSciences

The University of Melbourne

Australia

Abstract

Human iron (Fe) and zinc (Zn) deficiencies are among the most prevalent nutritional disorders in the world and manifest as a range of health issues including fatigue, impaired cognitive development and increased mortality. Micronutrient supplements and fortificants are frequently used to increase human Fe and Zn intakes yet these strategies require continuous investment and frequently miss rural populations; improving the density and/or bioavailability of Fe and Zn in staple crops (a process known as biofortification) represents a powerful alternative.

Bread wheat (*Triticum aestivum* L.) is cultivated on more land than any other crop and is processed into a range of food products to supply ~20% of the daily calories consumed by humans. The wheat grain is mostly comprised of starch and protein, with micronutrients such as Fe and Zn concentrated in the outer aleurone layer of the grain. In the aleurone layer, Fe and Zn are complexed to compounds that inhibit their absorption (bioavailability) in the human gut and the aleurone layer is removed during grain milling to produce white flour. White flour (representing the inner wheat endosperm) contains low concentrations of dietary Fe and Zn, and high consumption of either wholemeal flour or white wheat flour coincides with high prevalences of human Fe and Zn deficiencies. Generating Fe and Zn biofortified wheat through conventional breeding in modern wheat cultivars is hindered by inherently low grain Fe and Zn concentrations and a lack of genetic variation for these traits.

Nicotianamine (NA) is a low-molecular weight metal chelator present in all higher plants with high affinity for Fe^{2+} , Zn^{2+} , and other divalent metal cations. In graminaceous plant species such as wheat, NA serves as the biosynthetic precursor to 2'-deoxymugineic acid (DMA), a root-secreted mugineic acid family phytosiderophore that chelates ferric iron (Fe^{3+}) in the rhizosphere for subsequent uptake by the plant. Both NA and/or DMA are the major chelators of Fe within white wheat flour, and NA is known to enhance Fe bioavailability in cereal grain. For these reasons, increasing the biosynthesis of NA/DMA through upregulation of nicotianamine synthase (*NAS*) genes has emerged as a popular strategy for Fe and Zn biofortification of cereal crops.

In this study we employed constitutive expression (CE) of the rice (*Oryza sativa* L.) nicotianamine synthase 2 (*OsNAS2*) gene in bread wheat to upregulate the biosynthesis of NA and DMA, and evaluate plant growth, grain nutrition and food processing properties of CE-*OsNAS2* wheat. Our lead CE-*OsNAS2* wheat transgenic event (CE-1) demonstrated higher concentrations of Fe, Zn, NA and DMA in wholemeal flour, white flour and white bread, altered distribution of Fe in the grain, and higher white flour Fe bioavailability relative to null segregant (NS) control. Protein composition, dough rheology and breadmaking properties were similar between CE-1 and NS white flour, and a

chicken (*Gallus gallus*) feeding study over a period of six weeks demonstrated that chickens consuming CE-1 white flour had improved Fe status, intestinal morphology and microbial populations relative to chickens that consumed NS white flour. Multi-location confined field trial (CFT) evaluation over three field seasons demonstrated no differences between CE-1 and NS agronomic performance apart from plant height. Throughout all CFTs, grain yield was negatively correlated with grain Fe, Zn, and protein concentrations yet not correlated with grain NA and DMA concentrations. White flour Fe bioavailability was positively correlated with white flour NA concentrations, and we determined NA to be the strongest enhancer of *in vitro* Fe bioavailability identified to date. Together these results suggest the proportion of Fe that is chelated to enhancers of bioavailability (such as NA and DMA) should be prioritized in future crop biofortification efforts and highlight new strategies for developing Fe and Zn biofortified wheat as a more nutritious staple food.

Declaration

I hereby declare that the work presented in this thesis is to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree thesis of any other university.

I also declare that the current thesis is fewer than 100,000 words in length, exclusive of tables, figures, bibliographies and appendices.

Jesse Taylor Beasley

May 2020

Preface

Plant transformation and initial selection of *OsNAS2* wheat events was conducted prior to my enrolment.

The synchrotron images in Chapter 4 were produced by Tonatiuh Sanchez, Enzo Lombi and Alexander Johnson.

Field work and grain milling analyses (Chapters 4 and 5) are original work and were conducted in collaboration with Adrian Cox, Leigh Smith, Shahajahan Miyan and Ed Barrett-Lennard at the Department of Primary Industries and Regional Development, WA, Australia.

Large scale milling and straight/rapid ferment breadmaking analyses (Chapters 5, 6 and 7) are original work and were conducted in collaboration with Marcus Newberry and Crispin Howitt at the Commonwealth Scientific and Industrial Research Organisation, Canberra, ACT, Australia.

In vitro and *in vivo* micronutrient bioavailability analyses (Chapters 3 and 6) are original work and were conducted in the laboratories of Ray Glahn and Elad Tako, Robert W. Holley Center for Agriculture and Health, USDA-ARS, Ithaca, NY, United States.

Quantification of nicotianamine and 2'-deoxymugineic acid is original work and was conducted in collaboration with Thusitha Rupasinghe and Himasha Mendis at the Metabolomics Australia Laboratory, The University of Melbourne, VIC, Australia.

Wheat prolamin analyses are original work and were conducted in collaboration with Chris Florides at the Western Australian State Agricultural Biotechnology Centre, Murdoch University, WA, Australia.

Dough rheology and breadmaking analyses are original work and were conducted in collaboration with Kate Howell at the Faculty of Veterinary and Agricultural Science, The University of Melbourne, VIC, Australia.

Publication status of all chapters

Chapter 3 – Investigation of Nicotianamine and 2' Deoxymugineic Acid as Enhancers of Iron Bioavailability in Caco-2 Cells

Published by *Nutrients* on 30.06.19 (Appendix 3)

JTB, JJH, ET, RPG, and AATJ contributed to conceptualization; JTB and JJH performed the analysis and collated the data; JTB drafted the work; JTB, JJH, ET, RPG, and AATJ revised the work.

Chapter 4 – Metabolic engineering of bread wheat improves grain iron concentration and bioavailability

Published by *Plant Biotechnology Journal* on 08.01.19 (Appendix 4)

JPB and AATJ contributed to the initial selection of material; JTSP, EL and AATJ contributed to the synchrotron analyses; JTB and DLC contributed to the metabolomic analyses; JTB and JPB contributed to the glasshouse analyses; JTB, JPB and LTMM contributed to the field analyses; JTB produced the white flour and JTB, ET and RPG contributed to Fe bioavailability analyses; JTB drafted the work; JPB, JTSP and AATJ revised the work.

Chapter 5 – Agronomic and nutritional evaluation of iron and zinc biofortified bread wheat across multiple field environments

Submitted for publication to *Nature Plants* on 20.12.19

JTB, JPB, LTMM and AATJ contributed to the field analyses; JTB and DLC contributed to the metabolomic and elemental analyses; JTB produced the white flour and JTB, KSH and RA contributed to the breadmaking analyses; JTB; ET and RPG contributed to Fe bioavailability analyses; JT contributed to statistical analyses; JTB drafted the work; JPB, LTMM and AATJ revised the work.

Chapter 6 – Nicotianamine-chelated iron positively affects iron status, intestinal morphology and microbial populations in vivo (*Gallus gallus*)

Accepted for publication by *Scientific Reports* on 21.12.19

JTB, AATJ, RPG, OK and ET contributed to the conception and design of the work; JTB, NK, JPB, LO, OK and ET contributed to the acquisition, analysis and interpretation of data; JTB drafted the work; JPB, AATJ and ET revised the work.

Funding Sources

This research project was supported by grants from the following agencies:

HarvestPlus Challenge program to A.A.T.J

United States Department of Agriculture, Agricultural Research Service to R.P.G and E.T

Australian Government Research Training Program Scholarship to J.T.B

Acknowledgements

Firstly, I thank my principal supervisor Alexander Johnson. Since meeting him for the first time almost eight years ago, he has inspired me to challenge myself and strive for my professional goals. He never failed to be a source of optimism and salient advice throughout my graduate studies. I look forward to continuing our collaboration in the future.

Secondly, I wish to thank my co-supervisors throughout my PhD: Julien Bonneau, Laura Moreno, and Rudi Appels. Over the past four years they have guided me and supported my research goals without fault. I owe each of them a debt of gratitude.

I thank all the members of the Plant Nutrition lab both past and present: Jamie Selby-Pham, Marianne Weisser, Martin O'Brien, Mayu Yamamoto, Natasha Nyangoma, Oscar Fung, Ouda Khammy, Ronan Broad, Rucha Patil, Sonia Sharma, and Tonatiuh Sanchez. Over the past four years they have helped me when I needed it and contributed to a great working environment.

I thank Ray Glahn and Elad Tako for their guidance and support and the members of their labs: Mary Bodis, Pei Pei Chang, Jason Wiesinger, Shree Giri, Nikolai Kolba, Jon Hart, and Desiree Dias, for welcoming me during my time in the United States.

Within the University of Melbourne, I thank Thusitha Rupasinghe, Himasha Mendis, and Roshan Cheetamun from Metabolomics Australia, and Sameera Sirisena, Michela Winters, Daphne Panayotides, and Senaka Ranadheera from the Faculty of Veterinary and Agricultural Science. I thank Kate Howell for her enthusiasm and lengthy discussions. I look forward to our future collaboration.

Outside the University of Melbourne, I thank Damien Callahan, Marcus Newberry, Crispin Howitt, and Chris Florides for their expert assistance in this project. I thank Leigh Smith, Ed Barrett-Lennard, Shahajahan Miyan, Jan Nield, and Julian Taylor for assistance relating to the field trial component of this research project and thank Troy Adriansz for teaching me how to mill. While carrying out field work in Western Australia I worked alongside Adrian Cox, who is sadly no longer with us. Adrian was a lovely man and a good friend; I won't forget the times we shared.

To my partner Lily, who has only known me as a PhD student, thank you for your love, support and understanding as I undertake this journey. I look forward to the next chapter.

Lastly, I thank my parents, Joanne and Malcolm, my brothers, James, Ben, Chris and Sam, and their families for believing in me and for reminding me what is important.

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Keywords

Nicotianamine, 2'-Deoxymugineic Acid, Caco-2, X-Ray Fluorescence Microscopy, Iron Deficiency Anemia, Iron Absorption, Ferritin, Bioavailability, Milling, White Flour, Bread, Confined Field Trials, Multi-Location, Nutrition, Hidden Hunger, Microbiome, Animal Feeding Trial.

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List of Abbreviations

ACE	Angiotensin-converting enzyme
AGS	Albumins and globulins
AKP	Alkaline phosphatase
Al	Aluminium
ANOVA	Analysis of variance
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
AsA	Ascorbic acid
AT1R	Angiotensin II receptor type 1
B	Boron
B WF	Biofortified white flour
BU	Brabender units
C	Organic carbon
C WF	Control white flour
Ca	Calcium
CaCl ₂	Calcium chloride
Caco-2	A human colon carcinoma cell line of blood group O phenotype
CaMV	Cauliflower mosaic virus
CD	Cell density
CE	Constitutive expression
CE-1	Lead wheat transgenic event constitutively expressing the <i>Oryza sativa</i> nicotianamine synthase 2 gene
CE-1.1	CE-1 sibling line 1
CE-1.2	CE-1 sibling line 2
CE-1.2	CE-1 sibling line 3
CE-OsNAS2	Constitutive expression <i>Oryza sativa</i> nicotianamine synthase 2 gene
CFT	Confined field trial
CIMMYT	International Maize and Wheat Improvement Center
CO ₂	Carbon dioxide
COX	Cytochrome c oxidase
CRA	Color reagent A
CRB	Color reagent B
CSIRO	The Commonwealth Scientific and Industrial Research Organisation
Cu	Copper
Cv.	Cultivar
DAA	Days after anthesis
DADA2	Divisive amplicon denoising algorithm
DcytB	Duodenal cytochrome B
DGLA	Dihomo- γ -linolenic acid
dH ₂ O	Distilled H ₂ O
DMA	2' -Deoxymugineic acid
DMEM	Dulbecco's modified Eagle's medium

DMSO	Dimethyl sulfoxide
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DraI	Restriction endonuclease
DTPA	Diethylenetriaminepentaacetic acid
DW	Dry weight
EAR	Estimated average requirement
eBLUE	Empirical best linear unbiased estimator
EC	Soil conductivity
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
Epi	Epicatechin
FA	Formic acid
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FCR	Feed conversion ratio
FDR	False discovery rate
Fe	Iron
Fe EDTA	EDTA-chelated Fe
Fe NA	NA-chelated Fe
Fer	Ferritin
FeSO ₄	Ferrous sulfate
FMOC-Cl	9-fluorenylmethoxycarbonyl chloride
FP	Free phosphorus
G x E	Genotype by environment
Gli	Gliadin gene
Glu	Glutenin gene
GM	Genetic modification
H ₂ O ₂	Hydrogen peroxide
Hb	Hemoglobin
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HClO ₄	Perchloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HindIII	Restriction endonuclease
HMA	Heavy metal ATPase
HME	Hemoglobin maintenance efficiency
HMW-GS	High-molecular weight glutenin subunit
HNO ₃	Nitric acid
HPLC	High-performance liquid chromatography
Hyg	Hygromycin phosphotransferase
ICP-MS	Inductively coupled plasma - mass spectrometry

ICP-OES	Inductively coupled plasma - optical emission spectrometry
IDA	Iron-deficiency anemia
IRT-1	Iron-regulated transporter 1
K	Potassium
KEGG	Kyoto Encyclopedia of Genes and Genomes
LA	Linoleic acid
LC-MS	Liquid chromatography-mass spectrometry
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LMM	Linear mixed model
LMW-GS	Low-molecular weight glutenin subunit
M/Z	Mass-to-charge ratio
MALDI-TOF-MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MCA	Mean cell area
MEM	Eagle's minimal essential medium
MENAP	Middle East, North Africa, Afghanistan and Pakistan
Mg	Magnesium
Mn	Manganese
MTP	Metal tolerance protein
Myr	Myricetin
N	Nitrogen
NA	Nicotianamine
Na	Sodium
NaB	Sodium borate
NaHCO ₃	Sodium bicarbonate
NAM-B1	A NAC Transcription factor
NaOH	Sodium hydroxide
NAS	Nicotianamine synthase
NH ₄	Ammonium
NO ₃	Nitrate
NOS	Nopaline synthase
NS	Null segregant
OsNAS2	Oryza sativa Nicotianamine synthase 2
P	Phosphorus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Phylogenetic diversity
PERMANOVA	Permutational multivariate analysis of variance
PICRUST	Phylogenetic investigation of communities by reconstruction of unobserved states
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]

PPM	Parts per million
QIIME	Quantitative insights into microbial ecology
qRT-PCR	Quantitative reverse transcription PCR
REML	Residual maximum likelihood
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Reverse phase
SAM	S-adenosyl methionine
SCFA	Short-chain fatty acid
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SE-HPLC	Size-exclusion high performance liquid chromatography
SEM	Standard error of the mean
SO ₄	Sulfate
TaVIT2	Wheat vacuolar iron transporter 2
TCA	Trichloroacetic acid
T-DNA	Transfer-DNA
TGW	Thousand grain weight
T _n	Transgenic generation, n = generation number
TP	Total phosphorus
TrACEES	University of Melbourne Trace Analysis for Chemical, Earth and Environmental Sciences laboratory
UBI-1	Ubiquitin 1
USDA	United States Department of Agriculture
UV	Ultraviolet
VF	Void fraction
VIT	Vacuolar iron transporter
WA	Water absorption
WHO	World Health Organization
WT	Wild-type
XFM	X-ray fluorescence microscopy
XRF	Elemental X-ray fluorescence
YSL	Yellow stripe-like
ZIP	ZRT-IRT-like protein
Zn	Zinc
°C	Degrees Celsius
18 MΩ	Deionized H ₂ O
35S	Cauliflower mosaic virus promoter

1. Chapter 1 – The importance of wheat for humans and improving wheat grain as a dietary source of iron and zinc

1.1. Wheat as an important staple crop for humans

Wheat (*Triticum spp.*) was first cultivated ~10,000 years ago in southeastern Turkey as diploid einkorn (*Triticum monococcum* L., $2n = AA$) and tetraploid emmer (*Triticum turgidum* ssp. *dicoccoides*, $4n = AABB$) species. Later domestication in the Near East (modern day Middle East) occurred alongside hybridization of emmer with an unrelated diploid wild grass (*Aegilops tauschii*, $2n = DD$) to form hexaploid bread wheat (*Triticum aestivum* L., $6n = AABBDD$). The two major modern wheat species are bread wheat and durum wheat (*Triticum turgidum* ssp. *durum* L., $4n = AABB$) and over 770 million tonnes of wheat was produced in 2017 (Figure 1.1, FAOSTAT, <http://www.fao.org/faostat>), making wheat the second most produced crop behind maize (*Zea mays* L.).

Wheat is unique among cereal crops given its adaptability to different climate conditions, the particularly long-term storage capability of whole wheat grain and the properties of wheat flour that allow processing into a range of food products (Shewry 2009; Shewry and Hey 2015). Bread wheat accounts for 95% of wheat production and produces grain that is processed into a variety of foods including bread, noodles, and biscuits whereas durum wheat accounts for ~5% of wheat production and is primarily processed into pasta (Shewry 2009). Together bread and durum wheat are currently cultivated on more land than any other crop, with over 210 million hectares of wheat harvested in 2018. Einkorn, emmer and spelt (*Triticum spelta* L.), a bread wheat subspecies, are also produced in small amounts throughout southern Europe and India.

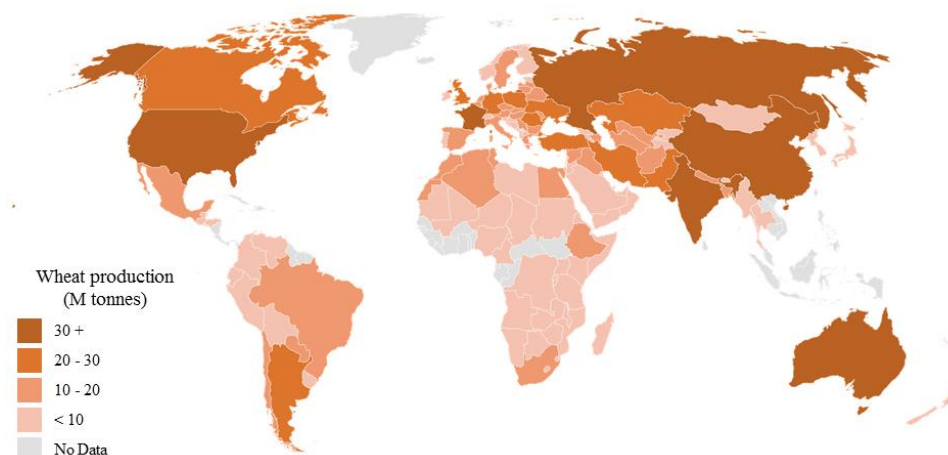


Figure 1.1 Wheat production throughout the world. The map highlights durum and bread wheat production in million (M) tonnes within each country of the world in 2017. Data were obtained from FAOSTAT (<http://www.fao.org/faostat>).

1.1.1. Wheat is a major source of dietary carbohydrates and protein and a poor source of dietary iron and zinc

The grain of wheat is mostly comprised of carbohydrates (80-85%) and protein (10 – 15%) and supplies approximately a fifth of total human dietary calorie and protein intakes (FAOSTAT, <http://www.fao.org/faostat>). The major carbohydrate in wheat grain is the polysaccharide starch (~70% of total grain weight) while other carbohydrate compounds including resistant starch, dietary fiber and mono-, di- and oligosaccharides contribute to the health benefits associated with wheat consumption (Shewry and Hey 2015). Most of the protein (>80%) within wheat grain is comprised of storage proteins (commonly referred to as prolamins) that together form the ‘gluten’ protein fraction of wheat dough (Shewry et al. 2002). Wheat grain contains all essential amino acids at adequate amounts for an adult human diet except for lysine, due to the low amount of lysine found in prolamins (Shewry and Hey 2015).

The wheat grain also contains essential micronutrients such as iron (Fe) and zinc (Zn) at relatively high concentrations compared to other cereals (between 25 – 85 mg/kg), although the Fe and Zn in wheat grain is largely unavailable within the human diet and populations that are heavily reliant on wheat as a staple food have high prevalences of human micronutrient deficiencies (Bouis et al. 2011; Cakmak, Pfeiffer, and McClafferty 2010; Monasterio and Graham 2000; Tang et al. 2008; Velu et al. 2014). Currently over two billion people, mostly women and children, are estimated to suffer from deficiencies of Fe and Zn (Beal et al. 2017; Black et al. 2008; Lopez et al. 2016; Wessells and Brown 2012). Severe Fe deficiency causes anemia, a condition defined by reduced blood hemoglobin (<120 g/L) that impairs work productivity and performance, reduces early mental development and lowers quality of life (WHO 2015). Zinc deficiency increases the risk of premature births, respiratory infection and diarrhea and reduces childhood weight gain and mental development, although it is difficult to estimate the prevalence of Zn deficiency due to the lack of valid biomarkers (Brown, Hambidge, and Ranum 2010; Wessells and Brown 2012). Within less developed countries of wheat primary production, such as countries in the MENAP (Middle East, North Africa, Afghanistan and Pakistan) region, wheat is the major staple food and consumption can exceed 300 grams per day and supply >40% of daily calories (Figure 1.2). Of the 1 billion people within these major wheat-consuming countries, approximately 400 million people are estimated to suffer from anemia (Figure 1.2) and improving Fe and Zn concentrations in wheat represents an effective strategy to boost micronutrient intakes in these populations.

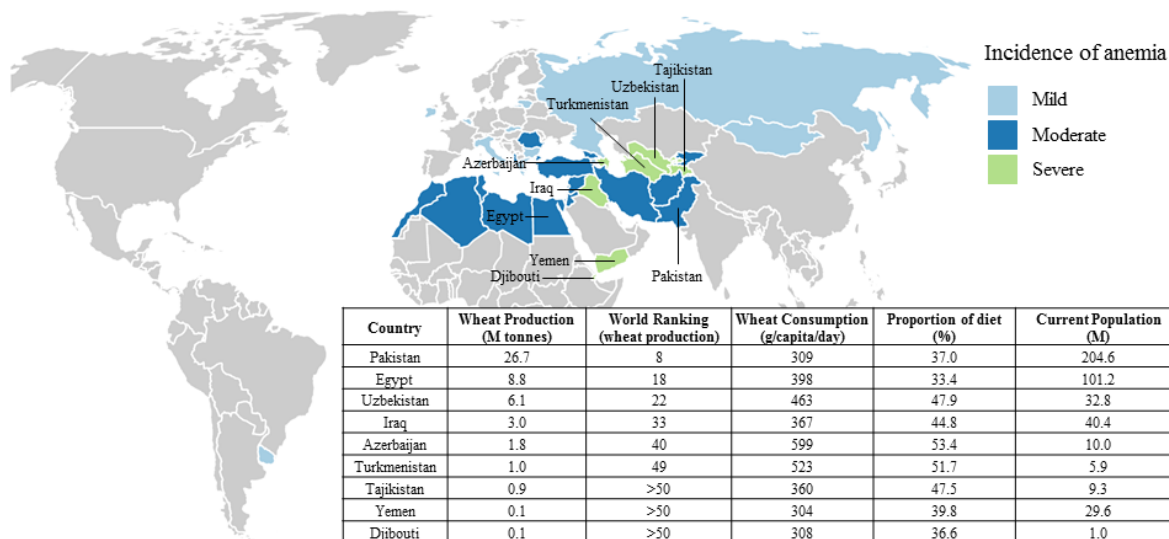


Figure 1.2. The incidence of anemia in major wheat consuming countries where per capita wheat consumption exceeds 300 g a day. Countries highlighted in light blue, dark blue and green have anemia prevalence of less than 20% (mild), between 20-40% (moderate) and over 40% (severe) in non-pregnant women of reproductive age, respectively. The table displays data for wheat production, wheat consumption and total populations for countries with severe incidences of anemia, as well as two of the biggest wheat producing countries in the MENAP region – Pakistan and Egypt. Wheat production is shown in terms of amount and world ranking in columns 2-3, respectively. Column 4 and 5 show daily wheat consumption and the proportion of energy that this represents within each country, respectively. Data were obtained from FAOSTAT (<http://www.fao.org/faostat>).

1.2. Wheat iron and zinc homeostasis

1.2.1. The importance of iron and zinc in plants

Iron and Zn are the most abundant transition metals within all organisms (Andresen, Peiter, and Küpper 2018). In plants, Fe is involved in fundamental cellular processes such as photosynthesis, respiration and chlorophyll biosynthesis given its unique redox properties and ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states (Briat, Curie, and Gaymard 2007; Connorton, Balk, and Rodríguez-Celma 2017). The most common forms of Fe cofactors are haem and Fe-S clusters. Haem contains one Fe atom in a tetrapyrrole ring allowing it to bind proteins such as c-type cytochromes in photosynthesis whereas Fe-S clusters contain 2 or 4 Fe atoms that are bridged by acid-labile sulfides (H_2S) and are able to bind many photosynthetic, respiratory, and biosynthetic proteins via cysteine residues (Connorton, Balk, et al. 2017). Zinc exists in a 2+ oxidation state throughout all biological processes and serves as a catalytic and structural co-factor for many enzymes and regulatory proteins such as those involved in transcription (Olsen and Palmgren 2014; Sinclair and Krämer 2012; White and Broadley 2011). When grown under Fe and Zn deficient conditions, wheat plants exhibit leaf

chlorosis, stunting and reduced grain yield (Cakmak et al. 1997, 1998; Mortvedt 1991). As well as negatively affecting plant growth, inadequate plant uptake of Fe and Zn exacerbates human Fe and Zn deficiencies as plants provide a major gateway for these micronutrients to enter the food system (Cakmak, McLaughlin, and White 2017).

1.2.2. Plant mechanisms to uptake soil iron and zinc

Iron is the fourth most abundant element in the earth's crust and found at concentrations well above that needed for plant growth (Hiradate, Ma, and Matsumoto 2007). In the soil, the majority of Fe is found as insoluble Fe^{3+} oxyhydroxide polymers that have limited availability for plant uptake, particularly under aerobic or calcisol (high pH) conditions (Guerinot and Yi 1994; Kobayashi, Nozoye, and Nishizawa 2018). For each unit increase in soil pH, Fe solubility decreases 1,000-fold and high pH soils represent approximately 30% of arable land. All plants, except for grasses, secrete protons from roots to acidify the rhizosphere and uptake soluble Fe^{2+} ions through the iron-regulated transporter 1 (IRT1) as part of a mechanism commonly referred to as 'Strategy I' Fe uptake (Andresen et al. 2018; Eide et al. 1996; Vert et al. 2002). Gramineous species have developed a separate mechanism, commonly referred to as 'Strategy II' Fe uptake, and acquire Fe by secreting mugineic acid phytosiderophores (MAs) into the rhizosphere which chelate Fe^{3+} ions and improve Fe solubility (Morrissey and Guerinot 2009). Following chelation of Fe^{3+} , the MAs- Fe^{3+} complexes are reabsorbed through yellow stripe-like (YSL) transporters at the root surface (Curie et al. 2001, 2009). Gramineous cereals such as wheat and barley (*Hordeum vulgare* L.) secrete large amounts of MAs relative to rice and maize and are more tolerate to high pH soils (Römheld and Marschner 1990). Rice is unique among cereals as it possesses both Strategy I and Strategy II Fe uptake and has the capability to uptake either Fe^{2+} ions through IRT1 transporters or MAs-chelated Fe^{3+} ions (Kobayashi et al. 2018). More recent research has suggested that Fe uptake regulation is highly conserved between rice and *Arabidopsis* (the model dicotyledonous plant), and calls into question the mutual exclusivity of Strategy I and Strategy II Fe uptake between grasses and non-grasses (Grillet and Schmidt 2019). Zinc is a limiting micronutrient in almost a third of global arable soils and the most common micronutrient deficiency in crops (Broadley et al. 2007; Noulas, Tziouvalekas, and Karyotis 2018). Soil Zn availability is limited under conditions of high soil pH and the vast majority (>90%) of soil Zn is insoluble and unavailable for plant uptake (Broadley et al. 2007). Plants uptake Zn primarily as soluble Zn^{2+} ions through ZRT-IRT-like protein (ZIP) transporters although it is thought that cereals may also uptake Zn^{2+} -MA complexes through YSL transporters (Andresen et al. 2018; Broadley et al. 2007; Olsen and Palmgren 2014; White and Broadley 2009).

1.2.3. The role of chelators in iron and zinc nutrition

The oxidation of Fe^{2+} to Fe^{3+} catalyzed by the Fenton reaction can cause cellular damage through the generation of hydroxyl radicals, and Fe within plant tissues is mostly bound to chelating compounds such as the multimeric protein ferritin (Figure 1.3) to prevent this reaction from occurring (Guerinot and Yi 1994). The long-distance transport of Fe and Zn from the roots and/or senescing vegetative (source) tissues to the developing grain (sink) also relies on the action of metal chelators in plants (Andresen et al. 2018). Low-molecular weight compounds like citrate, malate and nicotianamine (NA; Figure 1.3) are major chelators of Fe and Zn within the xylem and phloem of all higher plants (Connorton, Balk, et al. 2017). Nicotianamine is a non-protein amino acid found in all higher plants and was originally described as the ‘normalising factor’ in the NA auxotroph tomato (*Lycopersicon esculentum* L.) mutant *chloronerva* (Budčinský et al. 1980; Noma and Noguchi 1976; Noma, Noguchi, and Tamaki 1971). *Chloronerva* plants exhibit poor growth and symptoms of Fe deficiency, despite containing high Fe concentrations in vegetative tissues due to a mutation in the nicotianamine synthase (NAS) gene (Ling et al. 1999). Nicotianamine is synthesized from methionine via the trimerisation of S-adenosyl methionine (SAM) in a reaction catalyzed by NAS enzymes, and the application of NA reversed the symptoms of poor growth in *chloronerva* plants (Higuchi et al. 1994). Subsequent studies confirmed that NA plays an important role in both long- and short-distance (intercellular) transport of Fe, Zn, and other metal cations in all higher plants (Stephan and Scholz 1993; von Wirén et al. 1999). In cereals, NA functions as the biosynthetic precursor to 2'-deoxymugineic acid (DMA), an additional Fe chelator in cereal tissues and the major MA species that is secreted into the surrounding rhizosphere to chelate Fe^{3+} (Kobayashi et al. 2018; Takahashi et al. 2003). The biosynthetic pathway of NA and DMA in plants is provided in Figure 1.4. Zinc accumulates primarily as unbound Zn^{2+} ions in leaf and root cell vacuoles, although Zn is also chelated by citrate, malate and other organic acids in the apoplast and by NA in the cytosol (White and Broadley 2011). Zinc is predominately transported as free Zn^{2+} ions from root to leaf tissues via the xylem and remobilized as NA-chelated Zn from leaf tissues to the developing grain via the phloem (Olsen et al. 2016; Olsen and Palmgren 2014; White and Broadley 2009). Due to xylem discontinuity in wheat, all remobilized Fe and Zn enters the wheat grain via the phloem and into the crease, a grain region comprised of the vascular bundle and nucellar projection (Borrill et al. 2014; Maillard et al. 2015; Pottier et al. 2014, 2019; Waters et al. 2009).

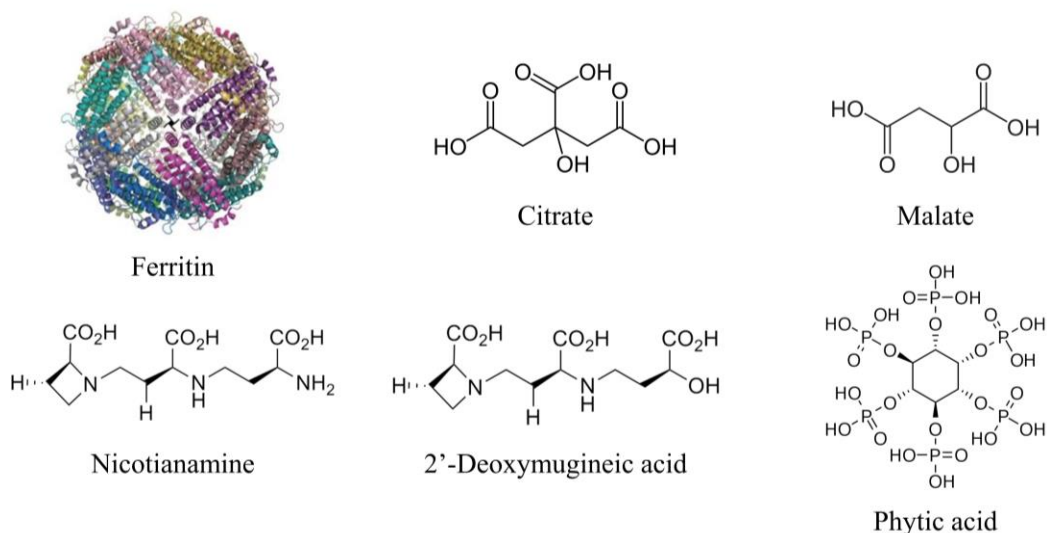


Figure 1.3. Metal chelators in plants. The molecular structures of six major metal chelators within plant tissues is provided. A ribbon diagram of the whole soybean ferritin (SFER4) oligomer is shown down a 4-fold symmetry axis. Figure adapted from (Masuda et al. 2010).

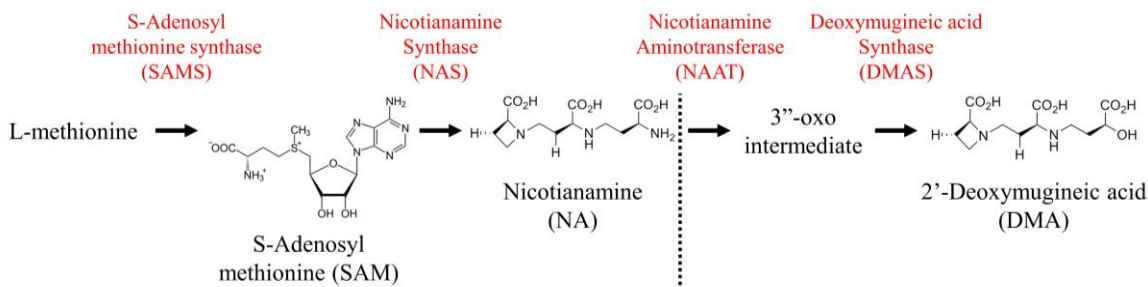


Figure 1.4. The biosynthetic pathway of nicotianamine and 2'-deoxymugineic acid in plants. The molecular structures of S-adenosyl methionine, nicotianamine and 2'-deoxymugineic acid are provided, and the enzymes that catalyze each biosynthetic step are shown in red. The pathway to the right of the dotted line is specific to graminaceous plants. Figure adapted from (Takahashi et al. 2003).

1.3. Wheat grain nutrition and processing

1.3.1. Grain composition and localization of iron and zinc

The wheat grain is heterogenous and comprised of embryo and endosperm tissues surrounded by an outer layer containing the testa and pericarp (Figure 1.5). High-resolution imaging techniques such as X-ray fluorescence microscopy (XFM) allow the spatial analysis of elements *in planta* and clearly demonstrate the separation of these tissues within the wheat grain (De Brier et al. 2016). Iron, Zn and other nutrients such as dietary fiber and lipids are particularly concentrated (200 – 400 mg/kg of Fe and Zn) in the outer aleurone layer of the endosperm and in the embryo, which forms a new plant and is commonly referred to as 'wheat germ' (Balk et al. 2019; Brandolini and Hidalgo 2012; Shewry and

Hey 2015). By contrast, the inner endosperm contains very low concentrations of micronutrients (10 – 20 mg/kg of Fe and Zn) and is mostly comprised of starch and protein to act as an energy reserve for the developing embryo (Balk et al. 2019; Moore et al. 2012; Shewry and Hey 2015; Singh et al. 2013). The vascular bundle and nucellar projection are localized to the crease region of the wheat grain and contain very high concentrations of Fe (>1000 mg/kg) that is most likely chelated to NA (De Brier et al. 2016). From the crease Fe is redistributed to the aleurone layer of the wheat endosperm, whereas Zn remains restricted to crease tissues and the embryo (De Brier et al. 2016; Singh et al. 2013; Stomph, Choi, and Stangoulis 2011). Within cells of the aleurone layer, Fe and Zn are primarily stored in protein storage vacuoles where they colocalize with phytate (inositol hexaphosphate) in small intracellular phytin globoids (Balk et al. 2019; De Brier et al. 2016; Moore et al. 2012). Phytate is a high affinity chelator of Fe, Zn, magnesium (Mg) and calcium (Ca) in grain tissues (Figure 1.3) and is an important storage form of phosphorus (P) for the germinating grain (Schlemmer et al. 2009). Wheat grain is milled into wholemeal flour using either a stone/hammer mill, or milled into white flour using a roller mill, which allows precise separation of the endosperm (white flour) from aleurone, embryo, testa and pericarp tissues which together form wheat bran (Balk et al. 2019; Shewry and Hey 2015). Wholemeal flour contains higher concentrations of protein, fiber and micronutrients relative to white flour, for example a selection of 43 wheat cultivars demonstrated white flour Fe and Zn concentrations between 6.7 – 8.4 mg/kg and wholemeal flour Fe and Zn concentrations between 28.2 – 28.6 mg/kg (Tang et al. 2008). Wholemeal flour has reduced end-use capability and shelf life (as low as 3-months) due to the inclusion of outer grain layers and the associated high enzymatic activity and lipid concentration (Doblado-Maldonado et al. 2012). By contrast, white wheat flour is mostly comprised of starch (85%) and protein (13%), has improved shelf-life (9-15 months) and sensory properties, and is historically associated with prosperity in the western world (Doblado-Maldonado et al. 2012; Shewry 2009; Shewry and Hey 2015). The small amount of Fe in white flour is mostly derived from inner endosperm tissues and chelated to NA and/or DMA, and the small amount of Zn is mostly derived from non-endosperm crease tissues that are difficult to remove during roller milling and chelated to phytate and/or NA (Campbell 2007; Eagling, Neal, et al. 2014). Despite the recognized health benefits of consuming wholemeal flour compared to white flour on reduced risk of cardio-vascular disease, type 2 diabetes, and forms of cancer, the vast majority (>90%) of wheat produced in western countries such as the United Kingdom and the United States is milled into white flour (Balk et al. 2019; Shewry and Hey 2015; USDA-FAS, <https://www.fas.usda.gov/commodities/wheat>).

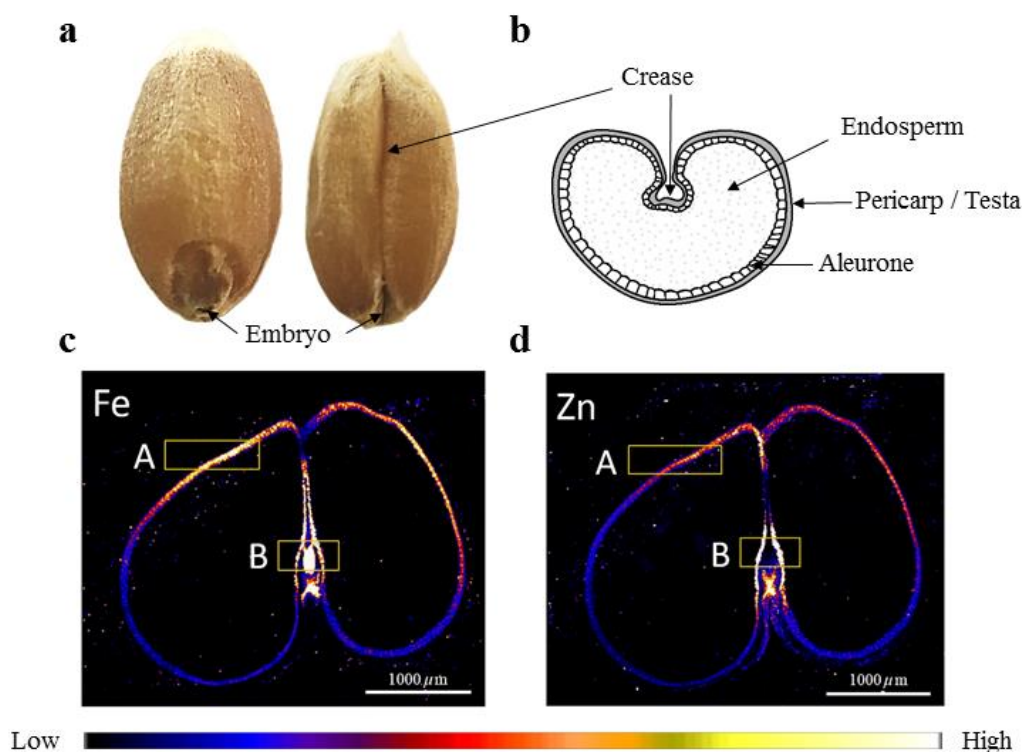


Figure 1.5. Wheat grain composition and localization of Fe and Zn. The location of major tissue types in **a)** a whole wheat grain and **b)** a transverse section of wheat grain. Elemental maps of **c)** Fe and **d)** Zn within a transverse section of a wheat grain, respectively. Yellow boxes highlight cells with high elemental concentrations within the (A) aleurone and (B) crease region, respectively. Color bar represents high (white) and low (black) elemental concentration. Figure was adapted from (De Brier et al. 2016) and Chapter 4.

1.3.2. Downstream processing characteristics of wheat flour

The interactions between prolamins and starch granules in the gluten fraction are responsible for the unique properties of wheat dough that allow processing into a range of food products (Shewry 2009). Mixing wheat flour with water produces a continuous network of prolamins and starch granules, and the strength (measured as both elasticity and extensibility) and viscosity of this network are crucial for food processing (Shewry et al. 1986, 1995). For example, the elasticity of the network allows the trapping of carbon dioxide produced by yeast during dough fermentation and is a critical component of breadmaking. For the last century, wheat prolamins have been classified into two groups based on their solubility: gliadins that are readily soluble in alcohol/water mixtures and glutenins that are only soluble in dilute acid/alkali (Shewry and Tatham 2016). Gliadin proteins are generally present as monomers and are the major determinants of dough viscosity and extensibility whereas glutenin proteins form large polymers with inter-molecular disulphide bonds and are the major determinants

of dough elasticity (Wan, Shewry, and Hawkesford 2013). There are three major types of gliadin proteins: α -, γ -, and ω -type and two glutenin subunits: high-molecular weight (HMW-GS) and low-molecular weight (LMW-GS) that are separated by mass and it is estimated that at least 50 individual prolamin proteins are present in the wheat grain (Shewry and Tatham 2016). A more recent classification of prolamins is based on similarities between amino acid sequences and the proportion of sulphur (S) containing residues (Shewry et al. 1986). The S-rich prolamins account for 70-80% of the total gluten fraction and include α -type gliadins, γ -type gliadins and LMW-GS (A- and B-type). The S-poor prolamins account for 10-20% of gluten fraction and include the ω -type gliadins and the D-type LMW-GS. The HMW-GS have a separate classification and despite representing ~12% of total grain protein these subunits account for the greatest variation in breadmaking performance between wheat flours, due to repetitive amino acid sequences and the number and distribution of disulphide bonds (Shewry et al. 2002; Shewry and Tatham 1997; Z. Wang et al. 2017). In addition to gliadins and glutenins, the wheat grain may contain additional S-rich proteins such as those encoded by the wheat bread making (*wbm*) gene that contribute to downstream processing and bread-making quality (Furtado et al. 2015; Guzmán et al. 2016). Although related proteins exist in the grain of other cereals they do not possess the same functional properties as wheat prolamins. It is therefore critical to determine whether altering wheat grain nutrition has unwanted effects on dough rheology and breadmaking properties.

Determining the functional properties of wheat flour such as elasticity, viscosity and breadmaking performance can be achieved through rheological test methods (Dobraszczyk and Morgenstern 2003). Universal rheological test methods such as the Farinograph, Mixograph, Extensograph, and Alveograph were developed by the cereal industry to provide single parameter values that directly relate to these functional properties. For example, Farinograph torque values are measured in Brabender Units (BU) and strong wheat flours produce Farinograph curves that are stable above 500 BU and are associated with good breadmaking performance (Migliori and Correra 2013). Although these tests allow accurate comparison between dough samples, their relationship to breadmaking performance is only valid within a limited range of commercial flours and conditions that are widely used across the cereals industry (Dobraszczyk and Roberts 1994). When testing flours outside of this range (i.e. non-commercial genotypes) these rheological tests can produce conflicting results and invalidate their relationship to breadmaking performance. The usefulness of more fundamental rheological tests such as dynamic oscillation and creep and stress relaxation methods is subject to debate given that these methods cannot be compared to practical dough processing (Dobraszczyk and Salmanowicz 2008). The biaxial extensional method is considered the most appropriate rheological

test to date, as it stretches dough samples in two perpendicular directions and closely resembles conditions experienced by wheat dough during proof and oven rise (Dobraszczyk and Morgenstern 2003). Differences in dough extensional hardening determined by the biaxial method is closely related to breadmaking performance, and the use of rheological tests such as the biaxial method allows screening for wheat dough quality without the need for more time-consuming test baking assays (Dobraszczyk and Roberts 1994). Fermentation and aeration of wheat dough is also a crucial component of the breadmaking process, although many dough rheological tests are performed without yeast and have so far failed to incorporate the effect of fermentation in their prediction.

1.4. Improving wheat as a dietary source of iron and zinc

The main strategies to combat human micronutrient deficiencies are supplementation, fortification and biofortification. Supplements deliver a large dose of highly absorbable (bioavailable) micronutrient and are effective in cases of severe deficiency (Hurrell and Egli 2010; Prentice et al. 2017; WHO 2015). By contrast, fortification involves the addition of micronutrient-containing fortificants to staple foods (such as wheat flour) during manufacture and is an effective population-based strategy for improving micronutrient intakes (Hurrell et al. 2010). Biofortification is the process of enhancing micronutrient density and/or bioavailability in staple crops through agronomic practices, conventional breeding, and modern biotechnology. There are numerous, comprehensive reviews that discuss the merits of micronutrient supplementation (Allen 2002; Gregory et al. 2017; Pasricha et al. 2010; Prentice et al. 2017); the remainder of this review will focus on Fe and Zn fortification and biofortification efforts in wheat.

1.4.1. Wheat flour iron and zinc fortification

Fortifying cereal flour with Fe and Zn is currently mandatory in 86 countries and 34 countries, respectively (Flour Fortification Initiative; <https://fortificationdata.org/>) however, the tendency of highly bioavailable Fe fortificants such as ferrous sulfate (FeSO_4) to oxidize and cause undesired organoleptic and sensory properties pose significant challenges for Fe fortification (Abbaspour, Hurrell, and Kelishadi 2014; Blanco-Rojo and Vaquero 2019; Boccio and Iyengar 2003; Rosado 2003). A survey in 2010 found that almost 90% of countries with mandatory wheat flour Fe fortification were utilizing cost-effective compounds that have low bioavailability (i.e. electrolytic Fe) or fortifying at sub-optimal concentrations (Hurrell and Egli 2010). Current recommendations therefore suggest Fe bound (chelated) to ethylenediaminetetraacetic acid (EDTA) as a fortificant to maximize bioavailability and minimize sensory alterations (Hurrell and Egli 2010; Rebellato et al. 2018) and recent meta analyses suggest that Fe fortification can reduce symptoms of Fe-deficiency

anemia (Sadighi, Nedjat, and Rostami 2019), despite several studies disputing the effectiveness of fortification programs (Aaron et al. 2012; Blanco-Rojo and Vaquero 2019; Pachón et al. 2015; Teshome et al. 2017). Micro- and/or nanoencapsulation could further improve the bioavailability of Fe fortificants whilst preventing alterations to sensory properties (Bryszewska et al. 2019; Mahler et al. 2012; Rebellato et al. 2018), although the use of highly bioavailable Fe requires careful management given that excess Fe consumption can negatively affect gastrointestinal health and the gut microbiome (Allen 2002; Hibberd et al. 2017; Kortman et al. 2014; Ma et al. 2016; Prentice et al. 2017; Sazawal et al. 2006; Tolkien et al. 2015; Yilmaz and Li 2018; Zimmermann et al. 2010). Certain studies describe the efficacy of Zn fortification and cost-effectiveness of Zn oxide as a fortificant (Brown et al. 2010; Hackl et al. 2017, 2019; Hess and Brown 2009), although whether Zn fortification reliably improves Zn status is also unclear (Shah et al. 2016). The costs of appropriately chelated and/or encapsulated Fe fortificants that are often premixed with other micronutrient fortificants (\$2 USD per ton to fortify white wheat flour with EDTA-chelated Fe alone), and the requirement of centralized cereal processing and industrial milling infrastructure limits flour fortifications in less developed countries (Aaron et al. 2012; Allen et al. 2006; Balk et al. 2019). By contrast, biofortification efforts represent a more economically sustainable strategy for increasing micronutrient intakes in rural populations (Bouis et al. 2011).

1.4.2. Wheat iron and zinc biofortification

Iron and Zn biofortification target concentrations for wheat grain have been established by the biofortification organization HarvestPlus (<https://www.harvestplus.org>). Assuming a baseline micronutrient concentration of 30 mg/kg⁻¹ dry weight (DW) for Fe and 25 mg/kg⁻¹ DW for Zn in wheat grain, it is estimated that achieving 59 mg/kg⁻¹ DW grain Fe and 38 mg/kg⁻¹ DW grain Zn is required to meet ~30 – 40% of the estimated average requirement (EAR) for Fe and Zn in wheat consuming populations (Bouis et al. 2011). Achieving these target concentrations requires improved wheat micronutrient uptake, remobilization and/or loading into the grain, and researchers and plant breeders working in wheat biofortification have so far utilized three major strategies to target these processes: agronomic practices, conventional breeding, and modern biotechnology that are described below (Figure 1.6).

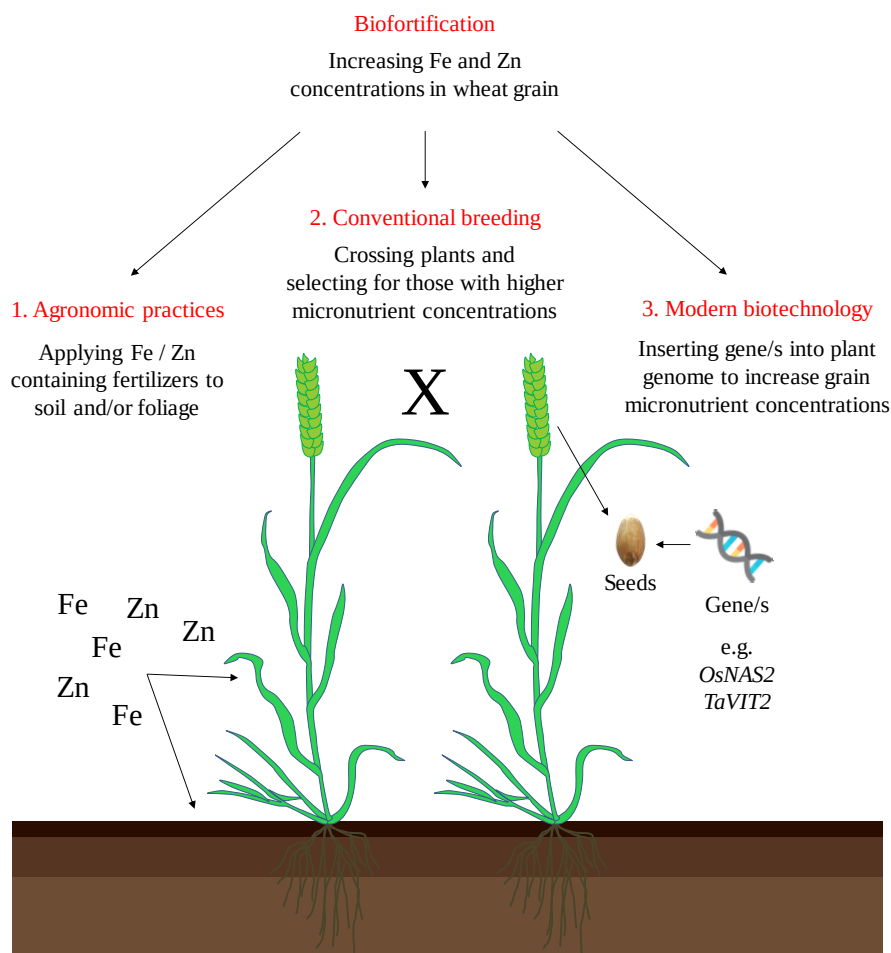


Figure 1.6. The three main strategies to biofortify wheat grain with Fe and Zn. 1. Agronomic practices aim to increase plant uptake and remobilization of Fe and Zn to the developing grain through the application of micronutrient-containing fertilizers to the soil and/or foliage. 2. Conventional breeding involves crossing plants and selecting for increased grain Fe and Zn. 3. Modern biotechnology strategies aim to manipulate the expression of genes involved in nutrient transport and/or bioavailability, for example the rice nicotianamine synthase 2 (*OsNAS2*) gene and the wheat vacuolar Fe transporter 2 (*TaVIT2*) gene.

1.4.2.1. Developing iron and zinc biofortified wheat through agronomic practices

Agronomic biofortification aims to overcome the barriers to plant Fe and Zn uptake by providing additional micronutrients in the form of chemical fertilizers to the soil and/or directly to the plant. Soil and/or foliar application of Fe fertilizers result in a minor increase in grain Fe (up to 1.3-fold) although this depends on the availability of nitrogen (N) and occurs only under certain soil conditions (Aciksoz et al. 2011; Erenoglu et al. 2011; Li et al. 2015; Zhang et al. 2010; Zou et al. 2019). Soil applications of Zn are largely ineffective at improving grain Zn concentration, however foliar

applications of Zn-containing fertilizers increase grain yield in wheat (Cakmak 2009) and are an effective method for improving grain Zn concentration (up to 1.6-fold) and bioavailability (Cakmak and Kutman 2017; Cakmak et al. 2010; Knez et al. 2018; Li et al. 2015; Lowe et al. 2018; Reed et al. 2018; Signorell et al. 2019; Zou et al. 2012, 2019). Despite improving plant growth in micronutrient deficient soils, the costs associated with purchasing and applying chemical fertilizers place an economic burden on rural farmers in developing countries (Cakmak 2008; Velu et al. 2014; Welch and Graham 2004). Agronomic biofortification is therefore considered a short-term solution to improve crop micronutrient concentration alongside conventional and modern genetic-based strategies.

1.4.2.2. Developing iron and zinc biofortified wheat through conventional breeding

Many studies have demonstrated variability in wheat grain Fe and Zn concentrations, particularly in wild species such as einkorn, emmer and *Aegilops tauschii* (Borrill et al. 2014; Cakmak et al. 2004; Monasterio and Graham 2000; Ortiz-Monasterio et al. 2007; Velu et al. 2019; White and Broadley 2005). Analysis of grain samples from the Broadbalk Wheat Experiment determined that Fe and Zn concentrations have decreased significantly since the introduction of semi-dwarf, high yielding cultivars (Fan et al. 2008). Despite significant genotype by environment (G x E) interactions on grain nutrition and the negative correlation between grain nutrition and yield, researchers and breeders for the last two decades have exploited the variability in grain Fe and Zn concentrations to generate synthetic hexaploids and pre-breeding lines as a part of global wheat biofortification breeding programs (Cakmak et al. 2010; Listman et al. 2019; Tabbita, Pearce, and Barneix 2017; Velu et al. 2014; Welch and Graham 2004; White and Broadley 2009). Breeding efforts to increase wheat grain Fe concentration have been hindered by the complexity of the hexaploid bread wheat genome and a lack of genetic variation for this trait among wild species (Appels et al. 2018; Borrill et al. 2014; Listman et al. 2019; Velu et al. 2012). So far the largest increase in wholegrain Fe (up to 1.2-fold) comes from breeding for the *NAM-B1*, or grain protein content (*Gpc-B1*) gene from wild emmer (Distelfeld et al. 2007; Uauy et al. 2006). The *Gpc-B1* gene encodes a transcription factor that regulates flag leaf senescence and the remobilization of Fe, Zn and N from leaf tissue to the developing grain during grain filling, and *Gpc-B1* is now an included trait within global wheat breeding programs (Borrill et al. 2014; Distelfeld, Avni, and Fischer 2014; Tabbita et al. 2017; Velu et al. 2017). Further research is underway to identify additional transcription factors regulating leaf senescence and the remobilization of Fe and Zn from source to sink tissues to improve upon *Gpc-B1*-based biofortification efforts (Borrill et al. 2019). More recently, the International Maize and Wheat

Improvement Center (CIMMYT), in concert with HarvestPlus, has developed disease resistant and high-yielding biofortified wheat cultivars with improved grain Zn concentration (up to 1.3-fold) to mainstream biofortification within wheat breeding programs (Velu et al. 2018, 2019). Field evaluation of these biofortified wheat cultivars in nine different locations demonstrated significant G x E interactions on grain Fe and Zn concentrations, and that grain nutrition was more affected by environment than genotype (Velu et al. 2012). Together these results highlight the importance of conducting multi-location field trials in the development of Fe and Zn biofortified wheat (Velu et al. 2012).

Given most grain Fe and Zn localizes to aleurone and embryo regions that are removed during roller milling, genetic improvements to grain Fe and Zn through conventional breeding are often lost when wheat grain is processed into white wheat flour (De Brier et al. 2016). To maximize the impact of Zn biofortified wholemeal flour, CIMMYT biofortified wheat cultivars have been released to farmers in Pakistan, India and Bangladesh, and are being tested in several other target countries such as Afghanistan and Nepal that traditionally consume wholemeal flour products (Listman et al. 2019; Velu et al. 2015). The relatively minor ~10 mg/kg increase in wholemeal flour Zn has been found to reduce the number of days with pneumonia and vomiting in Indian children but does not improve Zn status (Sazawal et al. 2018). Whole grain cereal-based diets often contain high concentrations of compounds that inhibit Fe and Zn bioavailability such as phytate, polyphenols and dietary fiber (Eagling, Wawer, et al. 2014; Gillooly et al. 1984; Mattila, Pihlava, and Hellström 2005; Reddy and Love 1999; Schlemmer et al. 2009). Phytate forms complexes with Fe and Zn in wholemeal flour that have low solubility in the human intestine, reducing Fe and Zn absorption. It is therefore unclear whether improvements to wholemeal flour Fe and Zn concentrations through conventional breeding will improve Fe and Zn status in wheat consuming populations.

Breeding for increased Fe and Zn concentrations in wheat grain endosperm would improve bioavailability by decreasing the colocalization with phytate and by minimizing the losses of Fe and Zn associated with roller milling and white flour production (Balk et al. 2019; Borrill et al. 2014; Olsen and Palmgren 2014; Singh et al. 2013). So far the Fe and Zn target concentrations set by HarvestPlus have only been established for wholemeal flour rather than white wheat flour (Bouis et al. 2011). Rice biofortification efforts focus on achieving Fe and Zn target concentrations in the polished (white) rice grain, and similar target concentrations in white wheat flour that consider the losses of Fe and Zn during grain milling may be established in the future (Balk et al. 2019; Bouis et al. 2011). Where possible, researchers have instead utilized mandatory fortification requirements as

a guide for target Fe and Zn concentrations within white wheat flour, for example white flour in the United Kingdom must be fortified to contain at least 16.5 mg/kg⁻¹ Fe (Connorton, Jones, et al. 2017). Genetic approaches to increase Fe and Zn concentrations in wheat white flour and achieve these target concentrations appear to require the use of modern biotechnology to overcome the physiological barriers to the loading of Fe and Zn in wheat endosperm.

1.4.2.3. Developing iron and zinc biofortified wheat through modern biotechnology

The low transformation efficiency and recalcitrant nature of wheat to transformation has delayed genetic engineering research in wheat relative to other crops, and to date wheat transformation has had most success in non-commercial cultivars such as Bobwhite and Fielder (Borrill et al. 2014; Harwood 2012; Li et al. 2012). The genomic complexity of wheat further limits the understanding of genes involved in Fe and Zn homeostasis, and much of the early plant biotechnology work in wheat biofortification has relied on knowledge derived from diploid crop species such as rice and barley (Bonneau et al. 2019). Modern biotechnology strategies to successfully biofortify cereal endosperm with Fe and Zn have so far focused on enhancing endosperm sink strength and/or increasing the biosynthesis of plant metal chelators such as NA and DMA.

The loading of Fe and Zn into seeds is tightly regulated and governed by transporters with high metal specificity (Balk et al. 2019; Olsen et al. 2016). The transporters responsible for loading NA-bound Fe into rice grain and distributing Fe to the embryo are encoded by *OsYSL2* and *OsYSL9* genes, respectively (Koike et al. 2004; Senoura et al. 2017). Although some Fe distributed to the aleurone region is bound to large phytoferritin complexes, the majority is loaded into vacuoles by vacuolar iron transporter (*OsVIT*) genes *OsVIT1* and *OsVIT2* (Kim et al. 2006; Zhang et al. 2012; Zielińska-Dawidziak 2015). Manipulating *YSL* and *VIT* gene expression in rice has improved endosperm Fe concentration between 2- to 4.4-fold, particularly when driven by endosperm-specific promoters (Bashir et al. 2013; Bonneau et al. 2019; Ishimaru et al. 2010; Masuda et al. 2012). In wheat, endosperm specific expression of the wheat *TaVIT2* gene (using the wheat high-molecular-weight glutenin-D1 promoter) increased endosperm vacuolar Fe storage and doubled white flour Fe concentration without increasing total grain Fe (Balk et al. 2019; Connorton, Jones, et al. 2017). Whether increasing vacuolar Fe in the endosperm (which may be bound to phytate) is bioavailable and/or whether redirecting aleurone Fe negatively influences embryonic viability in endosperm-specific *TaVIT2* wheat has yet to be determined (Bastow et al. 2018). Endosperm-specific expression of a wheat ferritin (*Fer*) gene (*TaFer1A*) resulted in 1.4-fold higher Fe concentration in white flour, although this was later found to be due to accumulation of large phytate-Fe complexes in the wheat

crease (Borg et al. 2012; Neal et al. 2013). It is unclear whether endosperm-specific expression of *Fer* alone is effective at increasing endosperm Fe concentration as most successful attempts have occurred in combination with constitutive expression of chelator biosynthetic genes (e.g. *NAS*) or root-specific expression of the Fe transporter *IRT1* (Boonyaves et al. 2017; Singh et al. 2017; Trijatmiko et al. 2016; Wu, Gruissem, and Bhullar 2018). The transport of Zn into developing seeds is governed by heavy metal ATPases (HMAs) and metal tolerance proteins (MTPs) (Menguer et al. 2018; Olsen et al. 2016). Recently endosperm-specific expression of the *HvMTP* gene in barley resulted in 1.3-fold higher Zn concentration in the endosperm, although whether applying a similar strategy would improve wheat endosperm Zn concentration has yet to be determined (Menguer et al. 2018).

Given that NA functions in the short- and long-distance transport of both Fe and Zn and serves as the biosynthetic precursor to DMA, increasing NA biosynthesis by *NAS* gene overexpression has emerged as a popular strategy for Fe and Zn biofortification in cereals (Bonneau et al. 2019; Johnson et al. 2011; Lee et al. 2012; Masuda et al. 2009; Trijatmiko et al. 2016). Overexpression of the *OsNAS2* gene in rice under regulatory control of the cauliflower mosaic virus (CaMV) 35S promoter (35S) improved NA concentration in unpolished grain (up to 9-fold) and resulted in higher concentrations of Fe (up to 4-fold) and Zn (up to 2-fold) in polished grain, suggesting that increased NA and/or DMA production improves the loading of Fe and Zn into rice endosperm (Johnson et al. 2011; Kyriacou et al. 2014). Combining 35S::*OsNAS2* overexpression with endosperm specific expression of soybean *Fer* achieved up to 7.5-fold higher Fe concentration in polished grain of an elite rice cultivar (Trijatmiko et al. 2016). Constitutive expression of *OsNAS2* in wheat driven by the maize *ubiquitin* promoter resulted in higher Fe (up to 2-fold) and Zn (up to 4-fold) concentrations in white flour, and combining constitutive expression of *OsNAS2* with endosperm-specific expression of the common bean (*Phaseolus vulgaris* L.) *Fer* gene did not result in further increases to grain Fe concentration (Singh et al. 2017). These results indicate that constitutive *OsNAS2* expression is an effective Fe and Zn biofortification strategy in wheat, although several questions remain in the development of CE-*OsNAS2* wheat.

1.5. Questions remaining in the development of iron and zinc biofortified wheat through constitutive expression of the rice nicotianamine synthase 2 gene

As NA and DMA participate in long-distance micronutrient transport it is likely that plants with constitutive expression (CE) of the *OsNAS2* gene have upregulated remobilization of Fe and Zn from vegetative tissues to the developing grain, although determining the mechanism by which constitutive *OsNAS2* expression increases Fe and Zn loading into wheat grain warrants further investigation

(Andresen et al. 2018; Clemens et al. 2013; Olsen and Palmgren 2014; Stephan and Scholz 1993). The study by (Singh et al. 2017) passed wholemeal flour through a nylon sieve (250 µm) to separate outer grain layers from the endosperm to produce white flour, and it is therefore unclear whether similar fold increases in CE-OsNAS2 white flour Fe and Zn concentrations will be achieved following industry standard practices of grain conditioning and roller milling. Given that NA and/or DMA are the major chelators of Fe in white wheat flour, it is likely that increased Fe in CE-OsNAS2 white flour is the result of additional Fe being loaded into CE-OsNAS2 grain endosperm tissues (Eagling, Neal, et al. 2014). Determining the localization of Fe and Zn in CE-OsNAS2 grain tissues using microscopy (e.g. XFM analysis) would clarify whether the additional Fe and Zn in CE-OsNAS2 white flour is derived from endosperm and/or outer grain tissues. Determining the colocalization of micronutrients with P in CE-OsNAS2 grain will uncover whether Fe and Zn are likely to be chelated to phytate and/or NA and could have implications for Fe and Zn bioavailability.

Previous studies have flagged NA as a potential enhancer of Fe bioavailability, although the extent of this promotion and whether DMA is an enhancer of Fe bioavailability is unknown (Eagling, Wawer, et al. 2014; Lee et al. 2012; Trijatmiko et al. 2016; Zheng et al. 2010). The coupled *in vitro* digestion/Caco-2 cell line assay is the recommended *in vitro* method for measuring Fe bioavailability in food samples and employing the Caco-2 cell method to CE-OsNAS2 white flour would accurately determine Fe bioavailability (Etcheverry, Grusak, and Fleige 2012). As there is no robust *in vitro* method for measuring Zn bioavailability, an *in vivo* animal feeding trial would be required to determine the extent of Zn bioavailability in CE-OsNAS2 white flour.

As grain Fe and Zn concentrations are heavily influenced by environmental and abiotic factors (soil pH, soil composition, rainfall, etc.) and the availability of soil micronutrients, it is necessary to determine whether Fe and Zn biofortification target levels are achieved across a variety of environments (Ortiz-Monasterio et al. 2007; Velu et al. 2012, 2014; Welch and Graham 2004). The CE-OsNAS2 wheat study was conducted under glasshouse conditions with little description of plant morphology and agronomical characteristics (Singh et al. 2017). Multi-site field trial evaluation of CE-OsNAS2 wheat is therefore required to ensure high grain nutrition is unaffected by G x E interactions and stable under realistic farming conditions (Trijatmiko et al. 2016).

Finally, increasing the concentration of grain NA and DMA (both nitrogenous non-protein amino acids) could alter grain N concentration, the composition and concentration of grain prolamins, as well as more downstream processing properties of wheat flour such as dough rheology and breadmaking (Luo et al. 2000). Phytate-chelated Fe is thought to negatively affect white flour gluten

development (Park, Fuerst, and Baik 2016) and Zn fertilization is known to alter wheat grain prolamin composition and polymerization (Peck, McDonald, and Graham 2008), and determining whether increased Fe and Zn concentrations in CE-*OsNAS2* white flour effect food processing properties is also integral to the development of Fe and Zn biofortified wheat.

1.6. Research aim and objectives

The aim of this thesis was to characterise NA and DMA as enhancers of Fe bioavailability and evaluate plant growth, grain nutrition, and food processing properties of Fe and Zn biofortified bread wheat developed through constitutive *OsNAS2* expression and increased biosynthesis of NA and DMA. This aim was accomplished through five major research objectives:

1. Investigation of NA and DMA as enhancers of Fe bioavailability in Caco-2 cells.
2. Development and characterisation of Fe and Zn biofortified bread wheat via CE-*OsNAS2* expression.
3. Evaluation of agronomic performance and grain nutrition in conventional and Fe and Zn biofortified CE-*OsNAS2* bread wheat grown at multiple field environments.
4. Investigation of micronutrient status and gastrointestinal health in chickens (*Gallus gallus*) following short-term consumption of NA-chelated Fe and long-term consumption of conventional and Fe and Zn biofortified CE-*OsNAS2* bread wheat-based diets.
5. Evaluation of food processing in conventional and Fe and Zn biofortified CE-*OsNAS2* bread wheat.

2. Chapter 2 – General Methods

Each of the research Chapters in this PhD thesis has an inherent methods section, although as Chapters 3-6 were written in publication format the respective methods sections contain limited information. The following Chapter therefore aims to describe general methodologies used throughout this PhD project and provide additional detail to the method sections of Chapters 3-6.

2.1. Plant material

2.1.1. Generation of plant material

The full-length coding sequence of the rice nicotianamine synthase 2 (*OsNAS2*) gene (LOC_Os03g19420) was polymerase chain reaction (PCR) amplified from rice (*Oryza sativa* L.) cv. Nipponbare genomic DNA as described in (Johnson et al. 2011). The *OsNAS2* gene was placed under transcriptional control of the maize (*Zea mays* L.) ubiquitin 1 (UBI-1) promoter and the nopaline synthase (NOS) terminator through recombination of *OsNAS2* into the transfer-DNA (T-DNA) region of a modified pMDC32 vector as described in (Curtis and Grossniklaus 2003). The UBI-1 promoter is highly active in monocots and results in constitutive expression (CE) of the downstream protein coding sequence. The T-DNA region of the pMDC32 vector also contains a hygromycin phosphotransferase (*hyg*) plant-selectable marker under transcriptional control of a dual cauliflower mosaic virus (2 x 35S) promoter that allows for high-throughput screening of successful T-DNA integration. Particle bombardment of the pMDC32 vector containing the *OsNAS2* gene into immature wheat (*Triticum aestivum* L.) embryos of cultivar (cv.) Bobwhite (1.0-1.5 mm in length) was performed at The University of Adelaide (Adelaide, SA, Australia) using established protocols as described in (Kovalchuk et al. 2009). Wheat plants that had successful integration of the UBI-1::*OsNAS2* construct grew on hygromycin-containing media and were transferred to fertilized soil (a mixture of coconut peat and sand) and grown to maturity under glasshouse conditions (12 hr photoperiod, 23°C day/12°C night, 50% humidity) at the University of Adelaide (Adelaide, SA, Australia). Six independent CE-*OsNAS2* wheat transformation events were successfully regenerated from tissue culture to produce the first generation (T_1) grain and were termed CE-1, CE-5, CE-7, CE-8, CE-13 and CE-15.

2.1.2. Initial selection of CE-*OsNAS2* events

Genomic DNA (10 µg) was isolated from leaf tissue of each of the six events and digested with *Dra*I and *Hind*III restriction enzymes. Restriction fragments were separated by gel electrophoresis (0.8% agarose) alongside a positive barley control and blotted to a nylon membrane. To determine T-DNA insert copy number, two independent hybridizations of a ³²P-labelled probe to both the NOS terminator and 2 x 35S promoter were performed using established protocols as described in (Pallotta

et al. 2014). The T-DNA insert copy number ranged from 1-7 among the six events. The T₂ plants were grown in 2.5 kg of soil mixture (equal parts clay-loam soil and coconut peat) containing Osmocote® fertilizer in plastic pots (14 × 19 cm) and plants were grown to maturity under glasshouse conditions (12 hr photoperiod, 24°C day/18°C night, 50–90% humidity) in the phenotyping platform of The Plant Accelerator (Adelaide, SA, Australia). The recovered phenotypic data including projected shoot area (cm²), plant height (cm), grain number and thousand grain weight (TGW) were used alongside elemental iron (Fe) and zinc (Zn) concentration data of the T₂ grain and determined a single homozygous double-insert event (CE-1) as the most promising event to advance into future generations. Three sibling lines of CE-1 (termed CE-1.1, CE-1.2 and CE-1.3) and the corresponding null segregant control (NS) were therefore selected for additional analyses from the T₅ to T₇ generations and were the main plant material used in this PhD study.

2.2. Plant growth conditions

2.2.1. Glasshouse growth conditions

Unless otherwise specified, all glasshouse-grown plants mentioned in this PhD thesis were grown in Hortico® potting mix and/or sterilized soil with Osmocote® fertilizer (4.5 g / L) in plastic pots (2.8 L). Plants were grown to maturity under glasshouse conditions (12 hr photoperiod, 18°C day/13°C night, 40–80% humidity) between May and December of each year at the University of Melbourne (Parkville, VIC, Australia).

2.2.2. Field growth conditions

Confined field trials (CFTs) were conducted from May to December in 2015, 2016 and 2017 at Glenthorne (35.0543° S, 138.5524° E) in South Australia and at the New Genes for New Environment facilities located in Merredin (31.4837°S, 118.2771°E) and Katanning (33.6894°S, 117.5551°E) in Western Australia. Grain were sown in 1 m² plots at Glenthorne across all three seasons and in 1.8 m² plots in 2015, 8.5 m² plots in 2016, and 18 m² plots in 2017 at Katanning and Merredin. At least three replicate plots per genotype were grown in 2015, five replicate NS and CE-1 sibling line plots and four replicate Mace plots were grown in 2016, and five replicate plots per genotype were grown in 2017. All plots were arranged in a randomized block design at each site. Within plots, rows were spaced 30 cm apart and grain were sown at a density of 60 kg ha⁻¹ in 2015 and 2016, and 75 kg ha⁻¹ in 2017 at all three sites.

Fertilizers were applied as urea and/or AGRAS (CSBP limited, Kwinana, WA, Australia) at a rate of 40 – 100 kg ha⁻¹ as needed for each field site. At maturity, plant height and tiller number were determined from three representative measurements per plot and biomass, TGW, and harvest index

were determined from 0.15 m² subsamples per plot. Grain yield was calculated from the amount of grain harvested per m² and extrapolated to kg/ha. Soil analysis was performed at sowing at CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia). Soil phosphorus (P) and potassium (K) concentrations were determined using the Colwell P and K extraction methods (Mason et al. 2013; Zhang et al. 2017). A diethylenetriaminepentaacetic acid (DTPA) extraction method was used to calculate soil copper (Cu), Fe, manganese (Mn), and Zn concentrations (Lindsay and Norvell 1978).

2.2.3. Plant material harvesting, cleaning and processing

Leaves and flag leaves (2 – 3 g) were harvested within two weeks post-anthesis from each plot in 2015, 2016 and 2017. To remove contamination of dust and soil, leaf samples were submerged (10 sec) in a non-phosphate detergent solution (0.1% v/v, Tween®20, Sigma Aldrich, St. Louis, MO, USA) and rinsed (10 sec) with distilled H₂O (dH₂O). At maturity, plots were manually harvested at all sites in 2015 and at Glenthorne in 2016 and 2017 and threshed using a Kimseed CW09 Multi-Thresher (Kimseed Australia Pty Ltd, Wangara, WA, Australia). At Katanning and Merredin in 2016 and 2017, plots were harvested using a plot harvester (Kingaroy Engineering Works, Kingaroy, QLD, Australia) and the resulting grain was passed through a Kimseed CW09 Multi-Thresher to further separate the grain from remaining plant material (Figure 2.1). To produce wholemeal flour, grain samples (3 – 5 g) were placed into stainless steel mesh infuser tongs (www.t2tea.com), submerged in a 0.1% Tween®20 solution and rinsed with dH₂O. All plant tissues were dried (60°C, 48 hr) before weighing and grinding to a powder using an IKA tube mill (www.ika.com). Where applicable, micronutrient content of plant tissues was calculated using the post-oven drying tissue weight.

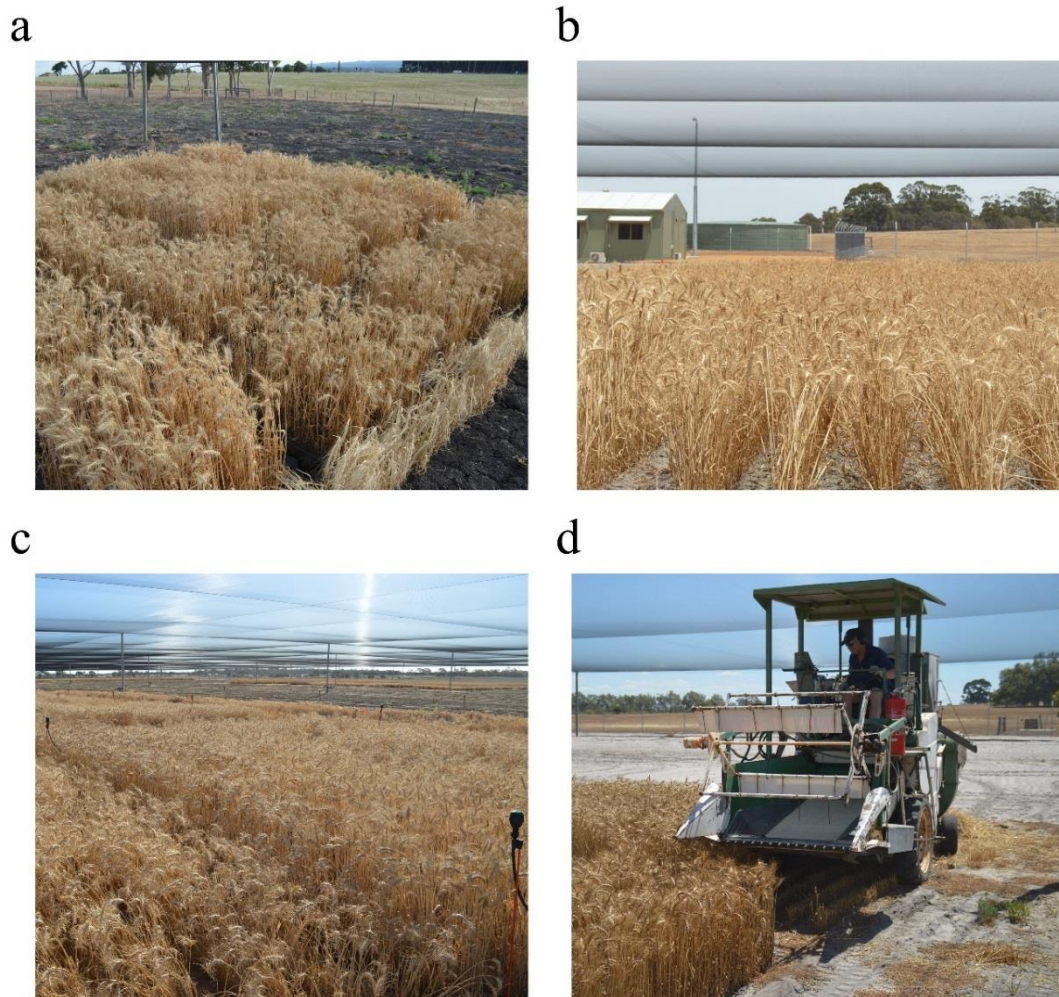


Figure 2.1. Confined field trials at **a)** Glenthorne, **b)** Katanning and **c)** Merredin at harvest in 2017. **d)** A plot harvester at Katanning in 2017.

2.3. Grain conditioning and white flour production

Grain samples (20 g) of each genotype from each CFT were dried (120°C, 48 hr) and reweighed to determine moisture content (%). Grain samples (50 – 200 g) were conditioned to 13% moisture content for 24 hr prior to milling. Each sample was milled using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) at constant temperature and run through a 280 µm sieve to isolate the white flour fraction from the bran fraction. Flour extraction rate across all samples was between 68 – 77% in 2015, 61 – 74% in 2016, and 71 – 78% in 2017 (Appendix 2.1 – 2.3).

For the feeding trial study as well as rapid and straight bread fermentation, a NS grain sample (32.3 kg) and a blended CE-1.2 and CE-1.3 grain sample (32.3 kg) from Merredin in 2017 were conditioned to 15% moisture content. Both samples were milled into eight grain fractions (three break flours, three reduction flours, pollard, and bran) using an MLU-202 laboratory mill (Bühler Group, Uzwil, Switzerland) at The Commonwealth Scientific and Industrial Research Organisation (CSIRO, ACT, Australia). All break and reduction flour fractions were combined to form a white flour fraction and the flour extraction rate was 72% for the NS sample and 73% for the CE-1 sample (Appendix 2.4).

2.4. Nutrient quantification analyses

2.4.1. Inductively coupled plasma-optical emission spectrometry (ICP-OES)

Micronutrient concentrations ($\mu\text{g/g}$) within plant tissues was measured using inductively coupled plasma optical emission spectrometry (ICP-OES). At the Robert W. Holley Centre for Agriculture and Health (USDA-ARS, Ithaca, NY, USA) plant samples (500 mg) were placed into a Pyrex glass tube and digested in 3 mL of a 60:40 nitric acid (HNO_3) : perchloric acid (HClO_4) mixture overnight to destroy all organic matter. The mixture was heated (120°C , 2 hr) in a heating block and 250 μL of Yttrium (40 $\mu\text{g/g}$, Sigma Aldrich, St. Louis, MO, USA) was added as an internal standard to compensate for any drift over the course of the analysis. The mixture was further heated to 145°C (2 hr) and then to 190°C (10 min) before cooling. Cooled samples were diluted to 20 mL with deionized H_2O (18 M Ω), vortexed and transferred to auto sample tubes for ICP-OES analysis on a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA). At the CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia) and the University of Melbourne Trace Analysis for Chemical, Earth and Environmental Sciences (TrACEES) laboratory (Parkville, VIC, Australia), ICP-OES analysis was conducted following a hydrogen peroxide (H_2O_2) / HNO_3 digestion of dry plant samples as described in (CSBP Lab methods, April 2019, www.csbp.com.au). All sample batches included a standard reference material sample (Wheat Flour, 1567b, National Institute of Standards and Technology).

2.4.2. Nitrogen and total protein analyses

Nitrogen concentration (%) analysis of wholemeal, white flour and bran samples (150 mg) was performed using the Dumas combustion method at the CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia) and the University of Melbourne Trace Analysis for Chemical, Earth and Environmental Sciences (TrACEES) laboratory (Parkville, VIC, Australia) and measured using a TruMac N analyzer (LECO Corporation, St. Joseph, MI, United States). Total protein concentrations

of white flour and bran samples were determined as: white flour total protein (%) = N concentration x 5.70, and bran total protein (%) = N concentration x 6.31 as described in (WHO, 2002).

2.4.3. Inductively coupled plasma-mass spectrometry (ICP-MS)

To measure micronutrient concentrations in low quantities of plant tissues (i.e. < 100 mg), inductively coupled plasma-mass spectrometry (ICP-MS) analysis was conducted at the Environmental Analysis Laboratory (Lismore, NSW, Australia) and at Deakin University (Burwood, VIC, Australia). Working in a laboratory fume hood, plant samples (20 mg) were placed in 10 mL tubes and digested in 300 μ L of a 25:75 HNO₃ : hydrochloric acid (HCl) aqua regia mixture (Fluka TraceSELECT™, Honeywell Research Chemicals, Charlotte, NC, United States) before heating (80°C, 90 min). Samples were diluted to 10 mL with deionized H₂O (18 M Ω) and centrifuged (5,000 rpm, 5 min) prior to ICP-MS analysis on a NexION 300D ICP Mass Spectrometer (PerkinElmer, Waltham, MA, United States).

2.5. Metabolite quantification analyses

2.5.1. Extraction of nicotianamine and 2'-deoxymugineic acid

Grain samples (three individual grain) were placed in a 2 mL tube (Eppendorf® Safe-Lock, Hamburg, Germany) with one 7 mm stainless steel bead (Qiagen, Hilden, Germany) and pulverized (30 rps, 5 min) in a tissue lyser (Qiagen, Hilden, Germany) to produce wholemeal flour. Wholemeal flour, white flour and plant tissue samples (25 mg) were combined with 500 μ L methanol (100%, analytical grade, Sigma Aldrich, St. Louis, MO, USA) in a 2 mL tube and incubated (30°C, 15 min, 1400 rpm) in a Thermomixer (Eppendorf, Hamburg, Germany) before centrifugation (10 min, 15,000 rpm). The resulting supernatant (~500 μ L) was placed into a clean 2 mL tube. The pellet was combined with 500 μ L deionized H₂O (18 M Ω), briefly vortexed (30 sec), centrifuged (10 min, 15,000 rpm) and the resulting supernatant (~500 μ L) collected and combined with the methanol supernatant to form the final extract (~1000 μ L). The extract was centrifuged (10 min, 15,000 rpm) before a subsample (20 μ L) was taken from each sample and combined into a single 2 mL tube – this tube was labelled as the pooled biological quality control (PBQC). All extracts and the PBQC were stored at -80°C until derivatization.

2.5.2. Derivatization of nicotianamine and 2'-deoxymugineic acid

Derivatization of nicotianamine (NA) and 2'-deoxymugineic acid (DMA) was achieved using a 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) method as described in (Selby-Pham et al. 2017). Prior to derivatization, a sodium borate (NaB) buffer (1M, pH = 8.0) was produced in a 500 ml Schott Duran® bottle by dissolving 30.92 g of boric acid (granular analytical grade, Chem-Supply Pty Ltd,

Gillman, SA, Australia) with ~400 mL of dH₂O and raising the pH to 8.0 by adding sodium hydroxide (NaOH), first at concentration 10M and then at concentration 1M. The NaB buffer was then topped up with dH₂O to 500 mL total volume. An ethylenediaminetetraacetic acid (EDTA) buffer (50 mM, pH = 8.0) was produced in a 500 mL Schott Duran® bottle by dissolving 9.31 g of EDTA 2H₂O (Chem-Supply Pty Ltd, Gillman, SA, Australia) and raising the pH to 8.0 by adding NaOH (first 10M and then 1M). The EDTA buffer was then topped up with dH₂O to 500 mL total volume and the NaB and EDTA buffers were stored at 4°C. On the day of derivatization, the required amount of 50mM FMOC-Cl solution (e.g. 3 mL) was made by dissolving 38.1 mg FMOC-Cl into 3 mL of acetonitrile (100%, analytical grade, Sigma Aldrich, St. Louis, MO, USA) into a 10 mL yellow cap tube (Sarstedt), and a formic acid (FA) solution (5%, pH = 4) was made by combining 50 µL FA with 1 mL deionized H₂O (18 MΩ) in a 2 mL tube.

Extract samples were defrosted, briefly vortexed (30 sec) and centrifuged (10 min, 15,000 rpm) before adding 5 µL of the supernatant to a clean 1.5 mL tube (Eppendorf, Hamburg, Germany), followed by the addition of 10 µL NaB buffer, 10 µL EDTA buffer and 40 µL FMOC-Cl solution. Samples were then incubated in a Thermomixer (60°C, 15 min, 700 rpm), centrifuged (1 min, 15,000 rpm) before adding 8.9 µL of FA solution and quickly vortexing to quench the reaction. An aliquot (70 µL) of the sample solution was subsequently added to a 2 mL screw top brown glass vial with a clear insert (Agilent Technologies Inc., Santa Clara, CA, United States).

2.5.3. Quantification of nicotianamine and 2'-deoxymugineic acid using liquid chromatography-mass spectrometry (LC-MS)

Quantification of NA and DMA was performed via reverse phase (RP) liquid chromatography-mass spectrometry (LC-MS) on a 1290 Infinity II and 6490 Triple Quadrupole LC-MS system (Agilent Technologies Inc., Santa Clara, CA, United States). A Zorbax Eclipse XDB-C18 Rapid Resolution HS 2.1 x 100 mm, 1.8 µm particle size column (Agilent Technologies Inc., Santa Clara, CA, United States) was used during chromatography with aqueous (0.1 % v/v FA in dH₂O) and organic (0.1 % v/v FA in acetonitrile) mobile phases. Derivatized NA and DMA were observed at retention times (RT) 2.75 min, and 2.88 min respectively. Instrument parameters were: high pressure RF 210V, low pressure RF 150V, sheath gas temperature 400 °C, sheath gas flow 12 L/min, gas temperature 290 °C, gas flow 16 L/min, nebulizer 20 psi, capillary 4500V, nozzle voltage 2000V, cell accelerator voltage 2V, and delta EMV 250V. A derivatized PBQC sample was included every 6-7 samples to identify any drift over the course of the analysis and a 50% methanol (100%, analytical grade, Sigma Aldrich, St. Louis, MO, USA), 50% deionized H₂O (18 MΩ) sample was included as a blank and analyzed after each PBQC sample. For quantification, a stock aqueous solution containing both NA

and DMA (Toronto Research Chemicals, Toronto, ON, Canada) was prepared at 750 μM . Two calibration sets of NA and DMA were generated in the range of 0.005 μM to 75 μM , derivatized and analyzed at the beginning and end of the samples set. All data was processed using MassHunter Quantitative Analysis software (vB.07.00, Agilent Technologies Inc., Santa Clara, CA, United States). Derivatized NA and DMA were identified by retention time and MRM match, and integrated peak areas were used for quantification relative to the NA and DMA standard calibration curves.

2.6. Phytic acid quantification analyses

2.6.1. Extraction of phytic acid

Wholemeal flour, white flour and white bread samples (500 mg) were weighed in a 50 mL tube and combined with 10 mL of a freshly made hydrochloric acid (HCl) solution (0.66 M). Tubes were shaken vigorously to dislodge the pellet, placed in a tube rack and attached to low speed lab shaker (Orbit™, Labnet International Inc., Edison, NJ, United States) overnight (for a minimum of 16 hr). The extract solution was then mixed with a wide-bore pipette tip and 1 mL distributed into a 1.5 mL tube (Eppendorf, Hamburg, Germany), centrifuged (10 min, 13,000 rpm) and 500 μL of the resulting supernatant placed into a clean 1.5 mL tube. Extract solutions were neutralized by the addition of 500 μL NaOH solution (0.75 M, Sigma Aldrich, St. Louis, MO, USA), centrifuged (1 min, 13,000 rpm) and stored at -20°C until quantification.

2.6.2. Quantification of phytic acid

Dietary phytic acid (phytate) concentrations were calculated using a colorimetric assay of total phosphorus released from flour and bread samples by phytase and alkaline phosphatase enzymes using the Phytic Acid Assay Kit according to manufacturer's instructions (Megazyme International, Bray, Ireland). On the day of quantification, a trichloroacetic acid (TCA) solution (50% w/w) was made by dissolving 100 g TCA (Sigma Aldrich, St. Louis, MO, USA) in 200 mL deionized H_2O (18 $\text{M}\Omega$), a color reagent A (CRA) solution was made by dissolving 5 g of L-ascorbic acid (Sigma Aldrich, St. Louis, MO, USA) into 47.3 mL deionized H_2O (18 $\text{M}\Omega$) and 2.7 mL sulfuric acid (99.999%, Sigma Aldrich, St. Louis, MO, USA), and a color reagent B (CRB) solution was made by dissolving 12.5 g of ammonium molybdate (Sigma Aldrich, St. Louis, MO, USA) into 200 mL deionized H_2O (18 $\text{M}\Omega$). The final color solution was made by combining 50 mL CRA with 10 mL CRB. The phosphorus standard solutions (5 mL) were produced by combining the standard solution of the kit (Bottle 5) with deionized H_2O (18 $\text{M}\Omega$) at the following volumes: 0 / 5 mL, 0.05 / 4.95 mL, 0.25 / 4.75 mL, 0.50 / 4.50 mL, and 0.75 / 4.25 mL, respectively. The phosphorus standard solutions (1 mL) were pipetted into new 2 mL tubes to form two standard series.

Thawed sample extracts (50 mL) were combined with 200 μ L buffer solution of the kit (Bottle 1) and either 620 μ L dH₂O for free phosphorus (FP) analysis or 600 μ L dH₂O and 20 μ L phytase enzymes (Bottle 2) for total phosphorus (TP) analysis. All samples were incubated (40°C, 10 min) and combined with 200 μ L buffer solution of the kit (Bottle 3) and FP samples were combined with 20 μ L dH₂O as TP samples were combined with 20 μ L alkaline phosphatase enzymes (Bottle 4). All samples were again incubated (40°C, 15 min) and combined with 300 μ L TCA solution to quench the reaction before centrifugation (10 min, 13,000 rpm). The resulting supernatant (1 mL) was carefully pipetted into new 2 mL tubes, and 500 μ L of the color solution was added to each TP and FP supernatant solution as well as the two phosphorus standard series. Absorbance of all samples was measured at 655 nm. Phytic acid (g / 100g) concentration was calculated based on the values of the two standard series using the Phytic Acid Assay Kit spreadsheet (Megazyme International, Bray, Ireland). An oat flour control (Bottle 6) was used as a positive control between sample batches.

2.7. *In vitro* iron bioavailability analyses

Prior to cell culturing, Dulbecco's modified Eagle's medium (DMEM) was produced in an acid-washed 2000 mL (10% HCl) beaker with an acid-washed stir bar. Two sachets of DMEM high glucose powder (Gibco™, Thermo Fisher Scientific, Waltham, MA, United States) were dissolved in 1600 mL deionized H₂O (18 M Ω), after which 7.4 g sodium bicarbonate (NaHCO₃, Sigma Aldrich, St. Louis, MO, USA) and 11.916 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma Aldrich, St. Louis, MO, USA) was added. The pH of the DMEM solution was raised to 7.2 by addition of NaOH (5M), poured into an acid-washed 2000 mL volumetric flask and transferred to a biosecurity cabinet (Nuair, Plymouth, MN, USA). Using an acid-washed funnel, 200 mL of fetal bovine serum (Optima, Atlanta Biologicals, Flowery Branch, GA, USA) was added to the DMEM solution and deionized H₂O (18 M Ω) added to a total of 2000 mL. The DMEM solution was then sterilized using a 500 mL sterile disposable filter unit (Nalgene™ Rapid-Flow™, Thermo Fisher Scientific, Waltham, MA, United States) and stored at 4°C in sterile 225 cm² cell culture flasks (Corning Inc., Corning, NY, USA). Eagle's minimal essential medium (MEM) was produced by dissolving two sachets of MEM powder (Gibco™, Thermo Fisher Scientific, Waltham, MA, United States) into 1400 mL deionized H₂O (18 M Ω) in an acid-washed 2000 mL (10% HCl) beaker with an acid-washed stir bar, and the following components were added: 7.0 g of D-glucose (Sigma Aldrich, St. Louis, MO, USA), 7.337 g of piperazine-N,N'-bis(2-ethanesulfonic acid) (Sigma Aldrich, St. Louis, MO, USA), 4.4 g of NaHCO₃ (Sigma Aldrich, St. Louis, MO, USA), 8 mg of hydrocortisone (Sigma Aldrich, St. Louis, MO, USA), 20 ml of an antimicrobial-antimycotic solution (Gibco™, Thermo Fisher Scientific, Waltham, MA, United States), a 4 ml solution of acetic acid (Sigma

Aldrich, St. Louis, MO, USA) containing 10 mg of dissolved insulin, and 20 μ L of a sodium selenite solution (for final concentration of 5 ng/mL). The pH of the MEM solution was lowered to 7.0 by addition of HCl (5M), poured into an acid-washed 2000 mL volumetric flask and transferred to a biosecurity cabinet (Nuair, Plymouth, MN, USA). Finally, 1 mL of a triiodo-L-thyronine solution and 2 mL of an epidermal growth factor (EGF) solution were added to the MEM solution and an acid-washed funnel was used to add deionized H₂O (18 M Ω) to a total of 2000 mL. The MEM solution was then sterilized using a 500 mL sterile disposable filter unit (Nalgene™ Rapid-Flow™, Thermo Fisher Scientific, Waltham, MA, United States) and stored at 4°C in sterile 50 mL tubes.

Frozen stocks (passage 27) of Caco-2 cells (a human colon carcinoma cell line of blood group O phenotype) in a 1 mL 10% dimethyl sulfoxide (DMSO) / 90% DMEM solution were thawed and added to 9 mL of DMEM in a sterile 15 mL tube before centrifugation (5 min, 800 rpm). The DMEM solution was poured off and the cell pellet resuspended in 13 mL DMEM before seeding in a 75 cm² cell culture flask (Corning Inc., Corning, NY, USA). Cell cultures were incubated (37°C, 4% CO₂) in a sterile CO₂ incubator (Nuair, Plymouth, MN, USA) and fresh DMEM was applied every second day. Cells were seeded from the 75 cm² sterile cell culture flask by aspirating the DMEM solution, adding 1.3 mL of sterile trypsin-EDTA solution (10x concentration, Sigma Aldrich, St. Louis, MO, USA) and incubating in the CO₂ incubator for 7 min before thoroughly mixing with 13 mL of DMEM to neutralize the trypsin solution. 5 μ L of the cell-DMEM solution was mixed with 5 μ L of trypan blue and added to a hemocytometer slide to calculate cell number per mL. Cells were subsequently seeded in 75 cm² or 225 cm² sterile cell culture flasks at a density of 30,000 cells / cm² and a total volume of 13 mL or 39 mL cell-DMEM solution, respectively. Reseeding of cells occurred weekly and cell-free DMEM solution was added to a sterile untreated 6-well plate (9.5 cm² per well, Corning Inc., Corning, NY, USA) and stored alongside cell culture flasks as a negative control for DMEM contamination.

To perform Caco-2 cell iron bioavailability analyses, sterile untreated 6-well plates (9.5 cm² per well, Corning Inc., Corning, NY, USA) were coated (1.5 mL per well) with a solution containing 147.7 mL glacial acetic acid (0.02M) and 2.23 mL rat tail collagen (Corning Inc., Corning, NY, USA) for 1 hr before aspirating. Hank's balanced salt solution (HBSS) was produced by dissolving one sachet of HBSS powder (Gibco™, Thermo Fisher Scientific, Waltham, MA, United States) with 350 mg of NaHCO₃ and 5.957 g HEPES in 800 mL deionized H₂O (18 M Ω), raising the pH to 7.2 with NaOH (1M) and deionized H₂O (18 M Ω) added to a total of 1000 mL. 2 mL of HBSS was added to each well and aspirated off prior to adding 2.0 mL of a cell-DMEM solution to each well at a cell density

of 50,000 cells / cm². Cells were incubated (37°C, 4% CO₂) in a CO₂ incubator for 13 days prior to the experiment day. Fresh DMEM was reapplied to the cell cultures and a cell-free treated 6-well plate every second day until day 11 after which fresh MEM was applied. All cells were cultured between passage 28 to 34.

3. Chapter 3 – Investigation of Nicotianamine and 2'-Deoxymugineic Acid as Enhancers of Iron Bioavailability in Caco-2 Cells

3.1. Abstract

Nicotianamine (NA) is a low-molecular weight metal chelator in plants with high affinity for ferrous iron (Fe^{2+}) and other divalent metal cations. In graminaceous plant species, NA serves as the biosynthetic precursor to 2' deoxymugineic acid (DMA), a root-secreted mugineic acid family phytosiderophore that chelates ferric iron (Fe^{3+}) in the rhizosphere for subsequent uptake by the plant. Previous studies have flagged NA and/or DMA as enhancers of Fe bioavailability in cereal grain although the extent of this promotion has not been quantified. In this study, we utilized the Caco-2 cell system to compare NA and DMA to two known enhancers of Fe bioavailability—epicatechin (Epi) and ascorbic acid (AsA)—and found that both NA and DMA are stronger enhancers of Fe bioavailability than Epi, and NA is a stronger enhancer of Fe bioavailability than AsA. Furthermore, NA reversed Fe uptake inhibition by Myricetin (Myr) more than Epi, highlighting NA as an important target for biofortification strategies aimed at improving Fe bioavailability in staple plant foods.

3.2. Introduction

Iron (Fe) possesses unique redox properties that are critical to fundamental biological processes such as cellular respiration and photosynthesis [1]. Although abundant in soil, Fe is largely unavailable for plant uptake under aerobic or calcisol (high pH) conditions (representing ~30% of arable land), due to the formation of insoluble ferric (Fe^{3+}) ion precipitates [2]. As well as negatively impacting on plant growth, inadequate plant Fe uptake translates to human Fe deficiency, as plants provide a major gateway for Fe into human food systems [3]. Plants have evolved sophisticated mechanisms to absorb Fe from the rhizosphere through reduction and/or chelation of Fe^{3+} [2]. Non-graminaceous plants such as common bean (*Phaseolus vulgaris* L.) reduce soil Fe^{3+} ions to the more soluble ferrous (Fe^{2+}) form for uptake into plant roots [4]. By contrast, graminaceous plants such as bread wheat (*Triticum aestivum* L.) secrete mugineic acid phytosiderophores, the most common of which is 2'-deoxymugineic acid (DMA), into soil to chelate Fe^{3+} for plant uptake [5]. Some plant species such as rice (*Oryza sativa* L.) utilize aspects of both strategies to maximize Fe uptake under a variety of soil and pH conditions [2].

Within the plant cell, Fe is complexed to chelating agents or is sequestered into plant vacuoles to avoid cellular damage caused by Fe^{2+} oxidation and reactive oxygen species (ROS) formation [3]. Low-molecular weight compounds like citrate, malate and nicotianamine (NA) are major chelators of phloem/xylem Fe within all higher plants while DMA is an additional chelator in graminaceous

plants. Citrate, NA and DMA all function in the transport of Fe from source tissues (i.e., root, leaf) to sink tissues (i.e., leaf, seed) for Fe storage and/or utilization [4]. Within the leaf, most Fe is bound in a phytoferritin complex within the chloroplast [6]. Leaf Fe is liberated from the phytoferritin complex during senescence and chelated by citrate, NA and/or DMA for transport to the developing seed [4]. Once in the seed of non-graminaceous plants, the proportion of Fe stored in embryonic, seed coat, and provascular tissues is heavily influenced by species, genotype and environment [7,8]. The Fe within embryonic tissue is primarily bound to phytoferritin and represents between 18% to 42% of total seed iron in soybeans (*Glycine max* L.) and peas (*Pisum sativum* L.), respectively [9]. The Fe within the seed coat of common bean ranges between 4% and 26% of total seed iron and is bound primarily to polyphenolic compounds, such as flavonoids and tannins [8,10,11]. The majority of seed Fe therefore accumulates in cotyledonary tissues and is likely bound to inositol hexakisphosphate (also known as phytate) within cell vacuoles, or to small metal chelators like NA in the cytoplasm [7,12]. Certain leguminous plants like soybean and chickpea (*Cicer arietinum* L.) accumulate seed NA to very high concentrations (up to a 1:2 molar ratio with Fe), suggesting that a large proportion of seed Fe is cytoplasmic in these species [13,14]. Graminaceous plant seeds (i.e., grain) store the majority of Fe (~80% of total grain Fe) as phytate complexes in vacuolar regions of the outer aleurone layer [3,15,16]. The remaining Fe within the sub-aleurone and endosperm regions (~20% of total grain Fe) is bound to phytate in intracellular phytin-globoids or chelated to NA and/or DMA (1:0.1 molar ratio with Fe) within the cytoplasm [17–20].

The absorption of dietary Fe in humans (bioavailability) depends on several factors apart from Fe concentration alone. The Fe within plant-based foods is mostly comprised of low-molecular weight (i.e., phytate, NA) and high-molecular weight (i.e., ferritin) compounds and is collectively referred to as non-heme Fe [6]. Non-heme Fe bioavailability is generally low (5–12%) and influenced by the concentration of inhibitors (phytate, polyphenols, calcium, etc.) and enhancers, like ascorbic acid (AsA), in the diet [21,22]. Phytate is the major inhibitor of Fe bioavailability in whole-grain foods, although certain polyphenolic compounds such as myricetin (Myr) and quercetin exhibit a greater inhibitory effect in bean-based diets [10,21,22]. Both phytate and Myr form high affinity complexes with Fe^{3+} that are poorly absorbed across the human intestinal surface [23–25]. Other polyphenolic flavanoids present in wheat embryonic and bean seed coat tissues are widely presumed to inhibit Fe bioavailability through pro-oxidation of Fe^{2+} and/or chelation of Fe^{3+} [21,26,27]. Enhancers of Fe bioavailability such as AsA (the strongest enhancer identified to date) are typically antioxidants that reduce Fe^{3+} and prevent polyphenols binding to newly formed Fe^{2+} ions that are highly bioavailable [22]. Some polyphenols such as epicatechin (Epi) are also thought to reduce Fe^{3+} to Fe^{2+} and can

therefore act as potent Fe bioavailability enhancers [21]. Another mechanism of promoting Fe bioavailability is thought to be through direct chelation of Fe^{2+} for uptake in the human small intestine such as that proposed for glycosaminoglycans and proteoglycans [22,28,29]. Nicotianamine has been suggested to enhance Fe bioavailability in Fe biofortified polished rice grains and Fe biofortified white wheat flour, although the extent of this promotion is unclear [17,18,30–32]. Whether DMA, also enhances or inhibits Fe bioavailability is unknown and increased knowledge regarding NA and DMA promotion of Fe bioavailability is needed. Identification of enhancers and inhibitors of Fe bioavailability has traditionally relied on manipulation of dietary components in lengthy human or animal feeding trials [33]. By contrast, the Caco-2 cell bioassay allows rapid investigation of diverse dietary components and accurate estimation of Fe uptake by human intestinal epithelial cells [21,34–36].

Due in large part to high consumption of cereal-based diets that are low in bioavailable Fe, human Fe deficiency is the most common nutritional disorder worldwide and is particularly widespread in less-developed countries [37]. Severe Fe deficiency causes iron-deficiency anemia (IDA), a condition that impairs cognitive development and increases maternal and child mortality, affecting over 40% of pregnant women and preschool-age children worldwide [38–40]. Biofortification efforts aimed at increasing micronutrient intake from staple food consumption represent a key component of alleviating global human IDA, yet there is heavy bias towards increased micronutrient concentration with less regard for bioavailability [41,42]. Recent biofortification studies in rice and wheat have increased NA and/or DMA biosynthesis to enhance both Fe concentration and bioavailability [17,20,28,29,43,44]. Increasing NA/DMA biosynthesis in wheat results in higher Fe accumulation in grain endosperm and increased Fe bioavailability in white flour that is highly correlated with NA and DMA concentration [17]. Understanding the extent to which NA and/or DMA enhance Fe bioavailability is therefore critical to determining the effectiveness of these Fe biofortification programs. Here we utilize modifications of the Caco-2 cell bioassay to characterize NA and DMA as enhancers of in vitro Fe bioavailability through comparison to known enhancers (Epi and AsA) and in a competitive assay with the inhibitor Myr.

3.3. Materials and Methods

3.3.1. Chemicals

Epicatechin, Myr, dimethyl sulfoxide (DMSO), glucose, hydrocortisone, insulin, selenium, triiodothyronine, and epidermal growth factor were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nicotianamine and DMA were purchased from Toronto Research Chemicals Inc. (Toronto,

Canada). Sodium bicarbonate and piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES) were purchased from Fisher Scientific (Waltham, MA, USA). Iron standard (1000 µg/mL in 2% HCl) was from High-Purity Standards (Charleston, SC, USA). Modified Eagle's medium (MEM), Dulbecco's modified Eagle's medium (DMEM), and 1% antibiotic-antimycotic solution were purchased from Gibco (Grand Island, NY, USA).

3.3.2. Preparation of Metabolite and Fe solutions

Epicatechin and Myr were dissolved in DMSO (100%) to a concentration of 1.6 mM and NA and DMA were dissolved in DMSO (50%) and 18 MΩ H₂O (50%) to a concentration of 0.8 mM. All solutions were diluted with pH 2 saline solution (140 mM NaCl, 5 mM KCl, adjusted to pH 2 with HCl) to achieve 400 µM stock solutions and subsequently diluted with pH 2 saline solution to appropriate concentrations for use in Caco-2 assays. To minimize toxicity to Caco-2 cells, the maximum DMSO concentration in 30 µM Epi/Myr treatments was 1.9% (2.5% in 40 µM polyphenol treatments). Fe stock solutions were prepared from 1000 mg/mL Fe standard in pH 2 saline solution. A 50 µL aliquot of Fe²⁺ stock solution of appropriate concentration was added to 150 µL of prepared metabolite solutions to achieve the desired Fe/metabolite concentration.

3.3.3. Caco-2 Assays

The Caco-2 cell assays were performed as previously described [21]. Briefly, cells were cultured in 24-well plates (Corning Costar 24 Well Clear TC-Treated Multiple Well Plates) coated with collagen and maintained in supplemented DMEM [3.7 g/L sodium bicarbonate, 25 mM HEPES (pH 7.2), 10% fetal bovine serum] for 12 days postseeding. Twenty-four hours prior to experiments, DMEM was replaced with iron-free supplemented MEM as previously described [36]. The Fe/metabolite solution (200 µL) was incubated (~22 °C, 15 minutes) and combined with 1 mL of MEM before an aliquot (500 µL) was directly applied to Caco-2 cell monolayers. After overnight incubation (37 °C), cells were washed twice with a buffered saline solution (130 mM NaCl, 5 mM KCl, 5 mM PIPES (pH 6.7)) and lysed by the addition of 0.5 mL 18 MΩ H₂O. In an aliquot of the lysed Caco-2 cell solution, ferritin content was determined using an immunoradiometric assay (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein content was determined using a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA) as previously described [36]. As Caco-2 cells synthesize ferritin in response to intracellular Fe, we used the proportion of ferritin/total cell protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake and refer to this as 'Fe uptake' throughout the manuscript.

3.3.4. Graphical Representation and Statistical Analysis

Each figure includes three control treatments as indicated by the white, grey and black bars on the right side of each panel. The first control treatment, “cell baseline”, represents ferritin formation in Caco-2 cells in the absence of any metabolite or Fe. The second control treatment, either “+ 4 μM Fe” or “+ 40 μM Fe”, represents ferritin formation in the presence of 4 μM Fe (typical Fe concentration for Caco-2 cell assays) or 40 μM Fe alone, respectively. A dotted line between both y-axes is provided to easily compare treatments to this control. The third control treatment, either “+ 80 μM AsA” or “+ 800 μM AsA”, represents ferritin formation in the presence of 4 μM Fe with 80 μM AsA or 40 μM Fe with 800 μM AsA (i.e., an Fe:AsA ratio of 1:20), respectively. In Figures 3.1–3.3, triangular data points represent a fourth control treatment of ferritin formation in Caco-2 cells in the presence of NA, DMA, Epi or AsA solutions without Fe, and displayed values equivalent to the Cell Baseline. Data represent ng ferritin/mg protein in Caco-2 assays and were generated using SigmaPlot software (v.13.0, Systat Software, San Jose, CA). Statistical differences between means were analyzed by unpaired Student’s t-test, and differences among means were assessed using one-way analysis of variance (ANOVA) with Tukey or Hsu’s MCB post-hoc tests, using Minitab software (v 18.0, Minitab, State College, PA).

3.4. Results

All low concentrations (≤ 2 μM) of NA, DMA and Epi enhanced Fe uptake into Caco-2 cells, with NA and DMA promoting ferritin formation more than Epi (Figure 3.1a). Higher concentrations (between 2–20 μM) of NA and Epi enhanced Fe uptake, and higher concentrations of DMA inhibited Fe uptake (Figure 3.1b). Between concentrations of 2 and 8 μM , NA enhanced Fe uptake more significantly than Epi with peak ferritin formation at 8 μM (i.e., a 1:2 molar ratio with Fe). At Fe molar ratios of 1:3, 1:4 and 1:5, NA and Epi promoted ferritin formation at the same level (Figure 3.1b and Table S3.1).

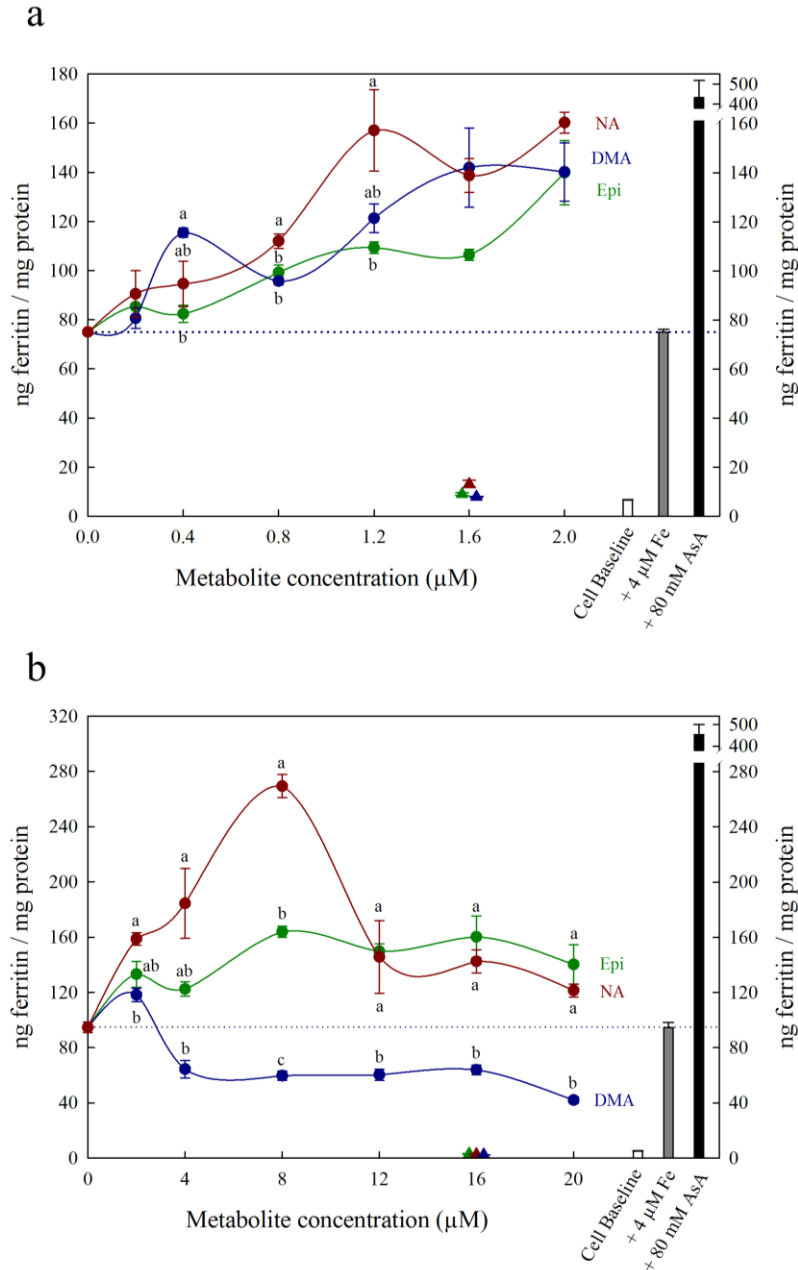


Figure 3.1. Ferritin formation in Caco-2 cells in response to nicotianamine (NA, red), 2' deoxymugineic acid (DMA, blue) and epicatechin (Epi, green) at concentrations varying between (a) 0 and 2.0 μM and (b) 0 and 20 μM in solution with Fe (4 μM). Triangles indicate ferritin formation at metabolite solutions (1.6 μM or 16 μM) without Fe. Dotted line indicates ferritin response to 4 μM Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Different letters indicate significantly different ferritin formation between metabolites of the same concentration as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$).

At a higher concentration of Fe (40 μ M), NA enhanced Fe uptake significantly more than Epi at all concentrations apart from 2 μ M, and DMA enhanced Fe uptake significantly more than Epi at 16 and 20 μ M (Figure 3.2). Together, these results demonstrate that NA > DMA > Epi in the promotion of Fe uptake into Caco-2 cells.

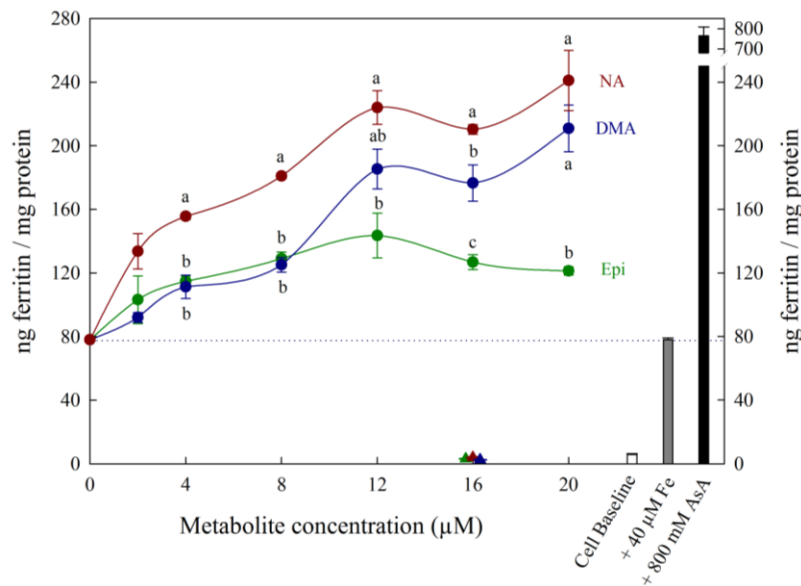


Figure 3.2. Ferritin formation in Caco-2 cells in response to varying concentrations (0–20 μ M) of nicotianamine (NA, red), 2' deoxymugineic acid (DMA, blue) and epicatechin (Epi, green) at concentrations varying between 0–20 μ M in solution with Fe (40 μ M). Triangles indicate ferritin formation in metabolite solutions (16 μ M) without Fe. Dotted line indicates ferritin response to 40 μ M Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Different letters indicate significantly different ferritin formation between metabolites of the same concentration as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$).

As the stronger enhancer, NA was compared in subsequent assays alongside Epi and AsA. All low concentrations (<2 μ M) of NA, Epi and AsA enhanced Fe uptake into Caco-2 cells at similar levels, with NA showing significantly higher ferritin formation at 2 μ M (Figure 3.3a). Above 2 μ M, NA enhanced Fe uptake significantly more than both Epi and AsA with peak ferritin formation at 8 μ M, demonstrating that NA > AsA > Epi in the promotion of Fe uptake into Caco-2 cells (Figure 3.3b).

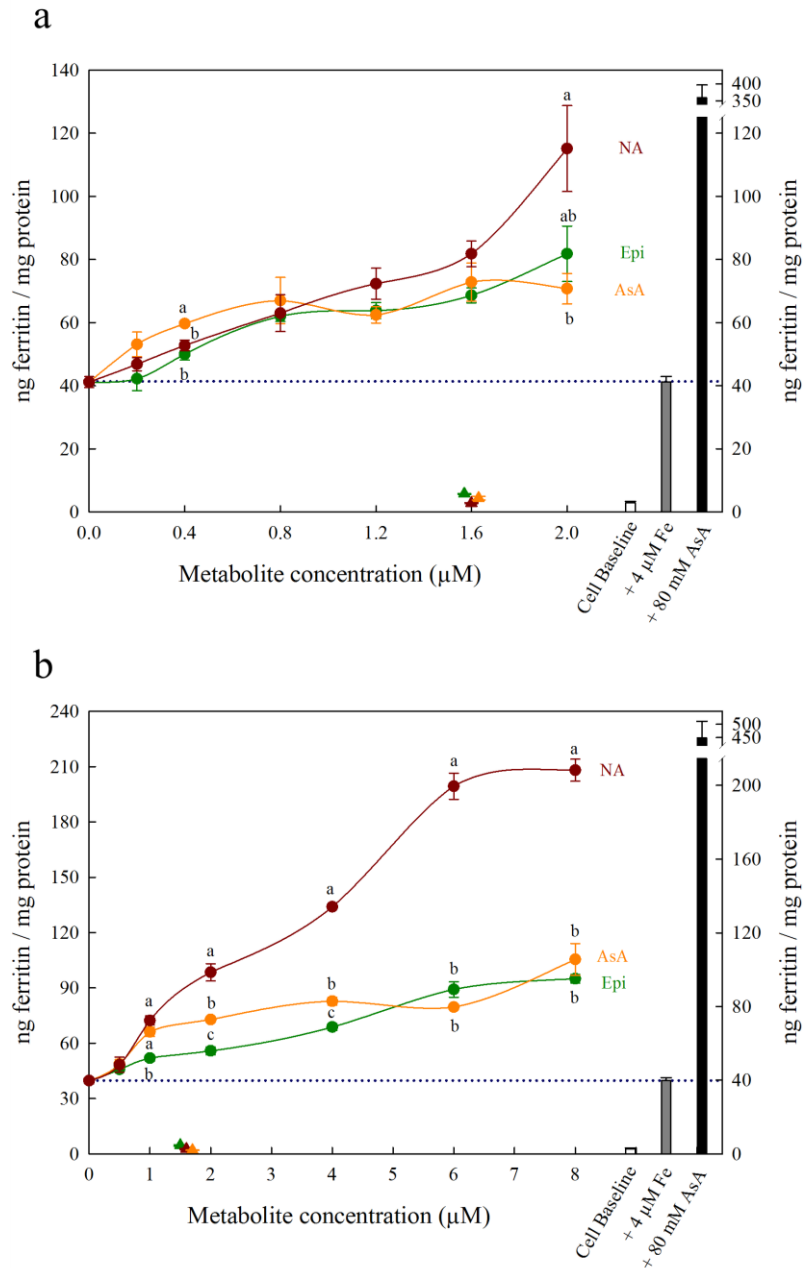


Figure 3.3. Ferritin formation in Caco-2 cells in response to nicotianamine (NA, red), ascorbic acid (AsA, orange) and epicatechin (Epi, green) at concentrations varying between (a) 0–2.0 μM and (b) 0–8 μM in solution with Fe (4 μM). Triangles indicate ferritin formation in metabolite solutions (1.6 μM) without Fe. Dotted line indicates ferritin response to 4 μM Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Different letters indicate significantly different ferritin formation between metabolites of the same concentration as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$).

Across all experiments with Fe:metabolite molar ratios $\leq 1:2$, the fold increase in ferritin formation over the Fe control was significantly higher in Caco-2 cells exposed to NA compared to AsA, Epi or DMA (Figure 3.4).

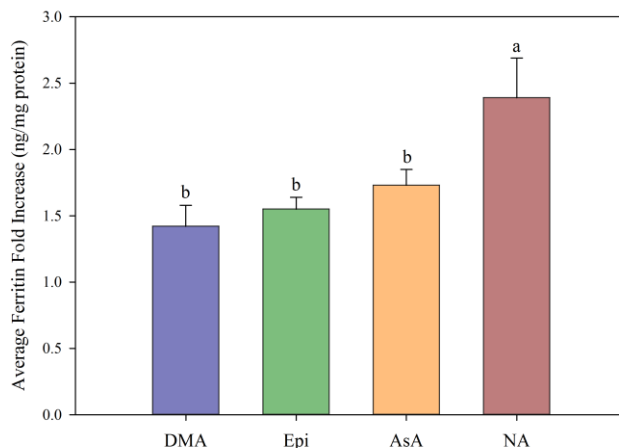


Figure 3.4. Average fold increase in Caco-2 cell ferritin formation in response to 2' deoxymugineic acid (DMA, blue), epicatechin (Epi, green), ascorbic acid (AsA, orange), and nicotianamine (NA, red) at Fe:metabolite molar ratios $\leq 1:2$ compared to ferritin formation in the presence of Fe alone. Error bars represent standard error of the mean of at least eight replicates. Different letters indicate significant differences between mean fold increase in ferritin formation between metabolites as analyzed by one-way ANOVA with Hsu's MCB post-hoc test ($p < 0.05$).

To further characterize NA as a strong enhancer of Fe bioavailability, ferritin formation was measured in response to the Fe uptake inhibitor Myr in combination with NA (NA:Myr) or Epi (Epi:Myr). At 4 μ M, total metabolite concentration, all NA:Myr solutions enhanced Fe uptake significantly more than Epi:Myr, and a NA:Myr solution of ratio 30:70 increased ferritin formation more than the Fe control (Figure 3.5a). At 30 μ M total metabolite concentration, NA:Myr solutions of ratio 70:30, 80:20 and 90:10 enhanced Fe uptake significantly more than Epi:Myr, and a NA:Myr solution of ratio 90:10 increased ferritin formation more than the Fe control (Figure 3.5b). Together these results demonstrate that NA is stronger than Epi in counteracting the inhibitory effect of Myr and enhancing Fe uptake into Caco-2 cells.

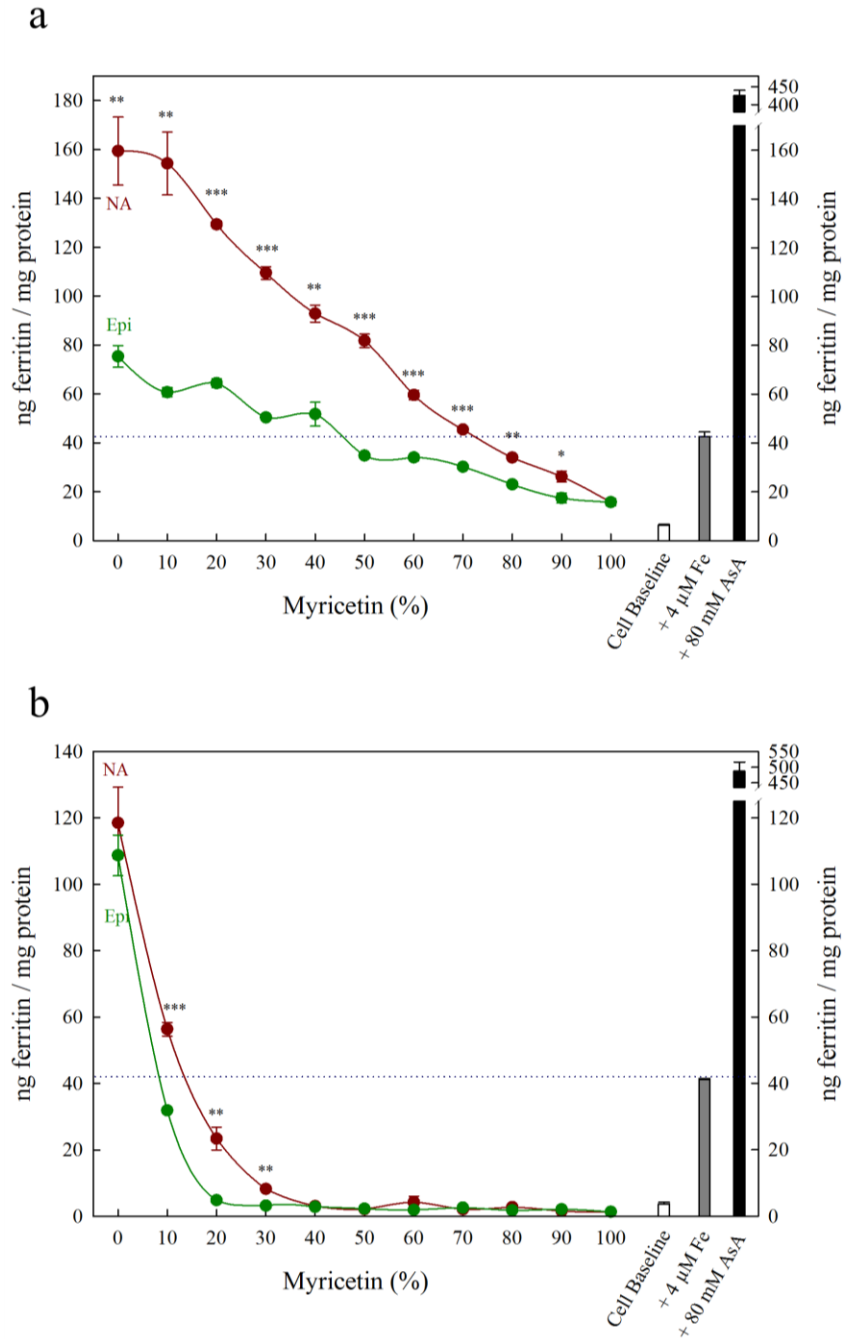


Figure 3.5. Ferritin formation in Caco-2 cells in response to various molar ratios of nicotianamine:myricetin (NA, red) and epicatechin:myricetin (Epi, green). Total metabolite concentration at each data point was (a) 4 μ M and (b) 30 μ M. Dotted line indicates ferritin response to 4 μ M Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Asterisks denote the significance between NA and Epi at each molar ratio for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

3.5. Discussion

At low ($\leq 1:2$) Fe:metabolite molar ratios, NA and DMA enhanced Caco-2 cell ferritin formation more than Epi. Interestingly, the level of promotion was more pronounced at 40 μM Fe concentration compared to 4 μM Fe, despite maintaining the same Fe:metabolite molar ratios (Figures 3.1a and 3.2). As ferritin formation was similar in the 'Fe control' at both 4 μM and 40 μM , it is likely that maximum Fe solubility is exceeded somewhere between 4 μM and 40 μM Fe in the absence of any Fe bioavailability enhancer (Figures 3.1 and 3.2). Although we provide strong evidence to support NA and DMA as enhancers of Fe bioavailability, the exact mechanism of how NA and DMA facilitate Fe uptake into Caco-2 cells remains unknown and is likely dependent on the proportion of readily bioavailable ferrous Fe^{2+} ions in solution [21]. Both NA and DMA form high affinity 1:1 complexes with Fe^{3+} ions, however only NA is thought to be capable of binding Fe^{2+} [45,46]. Here we demonstrate that DMA promotes ferritin formation at Fe:DMA molar ratios less than 1:1, suggesting that DMA is capable of binding Fe^{2+} and/or reducing Fe^{3+} ions to some degree and requires further investigation. The decreased ferritin formation after exposure to Fe:DMA solutions with molar ratio $> 1:1$ (Figure 3.1b) could be due to oxidation of Fe^{2+} ions in solution and excess formation of Fe^{3+} –DMA complexes that have low bioavailability for Caco-2 cell Fe uptake [45]. Some polyphenol compounds capable of complexing Fe^{3+} (e.g., delphinidin and delphinidin 3-glucoside) also demonstrate this biphasic pattern of promoting Fe uptake at molar ratios $< 1:1$ and inhibiting Fe uptake at molar ratios $> 1:1$ [21].

Nicotianamine promoted Caco-2 cell Fe uptake at all molar ratios observed, likely due to the formation of stable Fe^{2+} –NA complexes and facilitation of Fe^{2+} uptake. As Fe is provided in a reduced Fe^{2+} state when preparing the assay, Fe^{2+} –NA complexes rapidly form after NA addition and maintain the reduced Fe^{2+} state during exposure to Caco-2 cells. Certain polyphenols are also suggested to promote Caco-2 cell Fe uptake via a similar mechanism of binding Fe^{2+} and slowing the oxidation of Fe^{2+} to Fe^{3+} [21]. The reduced ferritin formation at Fe:NA molar ratios over 1:2 may be due to excess binding of Fe^{2+} by NA, preventing the release of Fe^{2+} for Caco-2 cell uptake, and suggests that an Fe:NA molar ratio less than 1:2 is optimal for promoting Fe bioavailability (Figure 3.1b, Table S3.1). Alternatively, NA could enhance Fe bioavailability via direct uptake of Fe^{2+} -NA in a similar mechanism to that proposed for glycosaminoglycans and proteoglycans and exploring this mechanism will be the subject of future research [28,29].

Ascorbic acid promotes Fe uptake into Caco-2 cells through de novo reduction of Fe^{3+} ions and maintenance of the Fe^{2+} ions in solution [9,21,22]. Here we demonstrate that at Fe:metabolite molar

ratios less than 1:2, NA enhances Caco-2 cell ferritin formation significantly more than AsA and is the strongest enhancer of in vitro Fe bioavailability identified to date (Figures 3.3b and 3.4). Together, these results suggest that the ability to bind Fe^{2+} ions is critical to enhancing Fe uptake into human intestinal cells. At an Fe:AsA molar ratio of 1:20, complete de novo reduction of Fe^{3+} ions in solution (without Fe^{2+} binding) leads to an ~8-fold increase in Caco-2 cell ferritin levels compared to Fe alone (Table S3.1). An Fe:NA molar ratio of 1:20 was not tested as this ratio does not occur naturally in plant foods, although it is unlikely to show high ferritin formation given the effect of ratios greater than 1:2 (Table S3.1). Instead, molar ratios less than 1:2 capture the highest ratio of Fe:NA measured to date in conventional plants foods (Fe:NA ratio of 1:1.6 in biofortified soybean) and provide realistic targets for plant breeders to improve Fe bioavailability [13].

To rule out the possibility that NA, DMA, Epi or AsA were promoting Caco-2 cell ferritin formation independent of Fe, Caco-2 cells were exposed to these metabolites alone in concentrations equal to 1.6 μM or 16 μM (Figures 3.1–3.3). There was no sign that the metabolite presence alone significantly increased Caco-2 ferritin formation relative to the cell baseline or disrupted the Caco-2 cell monolayer at harvest. Thus, the increased ferritin formation observed in Caco-2 cells exposed to Fe:metabolite solutions is due to the Fe uptake promoting properties of these metabolites.

Although Myr is a potent antioxidant with Fe-reducing properties (normally characteristic of Fe uptake enhancers), it strongly inhibits Caco-2 cell Fe uptake as it forms highly stable Fe–Myr complexes with low Fe bioavailability [47,48]. Nicotianamine increased ferritin formation compared to Epi at all 4 μM solutions and at 30 μM solutions containing over 70% NA or Epi, demonstrating that NA is stronger than Epi at preventing Fe uptake inhibition by Myr (Figure 3.5). At 30 μM , ferritin formation was equivalent to cell baseline for solutions containing less than 70% NA or 90% Epi, demonstrating that Myr inhibits Fe uptake more effectively than NA/Epi promotes Fe uptake (Figure 3.5b). At 4 μM (the equivalent of a 1:1 molar ratio with Fe), 30% NA combined with 70% Myr promoted ferritin formation above the Fe control, suggesting that NA can outcompete Myr and form Fe^{2+} –NA complexes with high bioavailability (Figure 3.5a). An additional assay at 10 μM demonstrated an intermediate response, with low ferritin formation at 20% Myr due to improper Caco-2 cell growth (Figure S3.1).

Whether NA would enhance Fe bioavailability in the presence of several enhancers and inhibitors (i.e., a bean-based food sample) is unclear. To date, increased NA has not overcome the inhibitory effect of polyphenols/phytate to enhance in vitro Fe bioavailability within biofortified whole wheat grain [17]. To further address this question, future studies should explore NA biofortification within

plant foods that contain high endogenous NA levels and low Fe:polyphenol molar ratios (e.g., 1:2 in carioca beans) [21]. Nonetheless, plant species with inherently low polyphenol levels (i.e., wheat) serve as ideal candidates for enhanced Fe bioavailability through the overproduction of NA [26,49]. The additional role of NA in the biosynthesis of DMA (itself an enhancer of Fe bioavailability) further reinforces increased NA biosynthesis as an effective cereal biofortification strategy to improve global human Fe nutrition.

3.6. Conclusions

We utilized a modified Caco-2 cell bioassay to characterize two low-molecular-weight plant metal chelators – NA and DMA – as strong enhancers of Fe bioavailability and demonstrate that NA is also capable of reversing Fe uptake inhibition by Myr. In doing so we highlight NA and DMA as important targets for biofortification strategies aimed at improving Fe bioavailability in cereals. Although we suspect that NA and DMA promote Fe bioavailability in Caco-2 cells by maintaining Fe^{2+} ions in solution, uncovering the exact mechanism by which these metal chelators promote Fe absorption will be the subject of future research studies.

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3.8. Supplementary Information

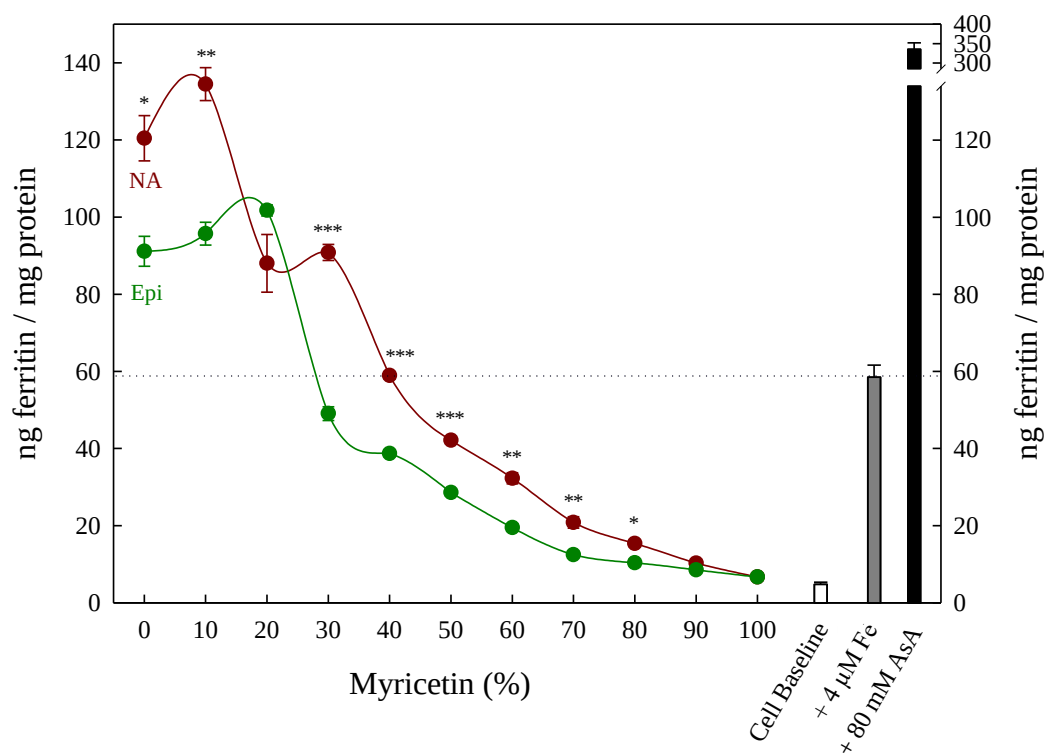


Figure S3.1 Ferritin formation in Caco-2 cells in response to various molar ratios of nicotianamine : myricetin (NA, red) and epicatechin : myricetin (Epi, green). Total metabolite concentration at each data point was 10 μ M. Dotted line indicates ferritin response to 4 μ M Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Asterisks denote the significance between NA and Epi at each molar ratio for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

Table S3.1 Fold increases in Caco-2 cell ferritin formation in response to different molar ratios of 2'-deoxymugineic acid (DMA, blue), epicatechin (Epi, green), ascorbic acid (AsA, orange), and nicotianamine (NA, red) compared to ferritin formation in the presence of Fe alone. For each metabolite the color intensity indicates peak fold increase across the range of molar ratios to Fe.

Molar Ratio to Fe	DMA	Epi	AsA	NA
0.05	1.13	1.16	1.29	1.35
0.1	1.48	1.26	1.45	1.51
0.125	-	1.15	1.23	1.22
0.2	1.44	1.5	1.63	1.78
0.25	-	1.31	1.67	1.82
0.3	2	1.62	1.52	2.24
0.4	2.08	1.57	1.77	2.18
0.5	1.94	1.64	1.72	2.43
1	0.68	1.51	2.09	2.66
1.5	-	2.25	2.01	5.03
2	0.63	2.06	2.66	4.05
3	0.64	1.58	-	1.54
4	0.68	1.69	-	1.51
5	0.44	1.48	-	1.28
20	-	-	8.08	-

4. Chapter 4 – Metabolic engineering of bread wheat improves grain iron concentration and bioavailability

4.1. Abstract

Bread wheat (*Triticum aestivum* L.) is cultivated on more land than any other crop and produces a fifth of the calories consumed by humans. Wheat endosperm is rich in starch yet contains low concentrations of dietary iron (Fe) and zinc (Zn). Biofortification is a micronutrient intervention aimed at increasing the density and bioavailability of essential vitamins and minerals in staple crops; Fe biofortification of wheat has proved challenging. In this study we employed constitutive expression (CE) of the rice (*Oryza sativa* L.) nicotianamine synthase 2 (*OsNAS2*) gene in bread wheat to upregulate biosynthesis of two low molecular weight metal chelators – nicotianamine (NA) and 2'-deoxymugineic acid (DMA) – that play key roles in metal transport and nutrition. The CE-*OsNAS2* plants accumulated higher concentrations of grain Fe, Zn, NA and DMA and synchrotron X-ray fluorescence microscopy (XFM) revealed enhanced localization of Fe and Zn in endosperm and crease tissues, respectively. Iron bioavailability was increased in white flour milled from field-grown CE-*OsNAS2* grain and positively correlated with NA and DMA concentrations.

4.2. Introduction

Micronutrient mineral deficiencies affect over two billion people worldwide with women and children most acutely at risk (Beal et al., 2017; Lopez et al., 2016). Iron (Fe) deficiency is the leading cause of anemia, a condition that impairs cognitive development and work productivity and increases maternal and child mortality (Kassebaum et al., 2014; Lopez et al., 2016). Zinc (Zn) deficiency causes a variety of disorders including stunted growth in children. Human Fe and Zn deficiencies are most prevalent in SEA (South-East Asia) and MENA (Middle East and North Africa), in part due to high consumption of micronutrient-poor staple crops such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) (Beal et al., 2017). Rising atmospheric CO₂ concentrations will likely decrease Fe and Zn concentrations in C₃ grains and could further exacerbate micronutrient deficiencies in SEA and MENA (Myers et al., 2014; Smith et al., 2017).

The wheat grain is comprised primarily of starch (60-75%) and protein (12-14%) with only low concentrations of Fe, Zn and other micronutrients (Shewry, 2009; Velu et al., 2014). Iron and Zn are remobilized from senescing vegetative tissues and/or translocated from roots during grain filling (Maillard et al., 2015; Pottier et al., 2014; Waters et al., 2009) and transported into developing grain via the vascular bundle in the crease region. In mature grain, the highest concentrations of Fe and Zn are found in the aleurone cells in conjunction with compounds such as phytic acid, polyphenols and

fiber that inhibit bioavailability, while only low Fe and Zn concentrations are localized to endosperm tissues (Brouns et al., 2012; De Brier et al., 2016; Moore et al., 2012; Schlemmer et al., 2009; Singh et al., 2013; Stomph et al., 2011). Grain milling removes inhibitors of micronutrient bioavailability but also a significant proportion of the grain Fe and Zn (Hourston et al., 2017; Zhang et al., 2010).

Wheat biofortification aims to complement micronutrient supplementation and fortification programs by improving grain micronutrient density and/or bioavailability (Bouis et al., 2011; Prentice et al., 2017). Generation of Fe biofortified wheat through conventional breeding is hindered in modern wheat cultivars by inherently low grain Fe concentrations and a lack of genomic resources for this trait (Borrill et al., 2014; Velu et al., 2014), indicating that genetic engineering strategies may be required to generate novel genetic variation (Connorton et al., 2017; Singh et al., 2017).

Nicotianamine (NA) is a non-protein amino acid formed by trimerization of S-adenosylmethionine (SAM) in a process mediated by NA synthase (NAS) enzymes (Clemens et al., 2014). Nicotianamine plays key roles in the chelation and transport of metals such as Fe, Zn, manganese (Mn) and copper (Cu) in all higher plants (Takahashi et al., 2003; von Wirén et al., 1999). In graminaceous plant species, NA serves an additional purpose as the precursor to biosynthesis of 2'-deoxymugenic acid (DMA), a root-secreted phytosiderophore that chelates ferric Fe (Fe^{3+}) in the rhizosphere and is subsequently re-absorbed. Nicotianamine and/or DMA are also the main chelators of Fe in white wheat flour (Eagling et al., 2014a), and increased NA is known to increase Fe bioavailability in polished rice (Lee et al., 2012; Trijatmiko et al., 2016; Zheng et al., 2010). For these reasons, increased NA biosynthesis has emerged as a popular strategy for Fe biofortification of cereals (Johnson et al., 2011; Lee et al., 2012; Singh et al., 2017; Trijatmiko et al., 2016).

In this study of glasshouse- and field-grown plants, we demonstrate that constitutive expression of the rice *OsNAS2* gene (CE-*OsNAS2*) in bread wheat causes not only significant Fe and Zn enrichment of whole grain and flour fractions but also increased concentrations of NA and DMA that are positively correlated with Fe bioavailability in CE-*OsNAS2* white flour. Interestingly, our results indicate that Fe bioavailability is higher in NA and DMA-enriched white flour regardless of Fe concentration and that white flour NA and DMA concentrations are more critical than absolute Fe concentration in determining Fe bioavailability. These results provide unique insights into cereal Fe biofortification and highlight the importance of metabolic engineering strategies that enhance not only micronutrient density but also promoters of micronutrient bioavailability.

4.3. Results

Generation of independent transformation events with constitutive OsNAS2 expression and selection of biofortified material for advanced nutritional analysis

Bread wheat cultivar (cv.) Bobwhite transformants with constitutive expression (CE) of the rice nicotianamine synthase 2 (*OsNAS2*) gene were generated through biolistic transformation of a cassette containing the *OsNAS2* gene under regulatory control of the maize ubiquitin 1 promoter (UBI-1) (Figure 4.1a). Six independent CE-*OsNAS2* transformation events termed CE-1, CE-5, CE-7, CE-8, CE-13 and CE-15 were regenerated from tissue culture and Southern blot analysis showed that insert copy number ranged from 1-7 among the six events (Figure 4.1b). Automated imaging facilities at The Plant Accelerator (Adelaide, Australia) were used to phenotype T₁ progeny of the six events; two events (CE-1 and CE-5) showed no phenotypic differences from a null segregant (NS) line derived from CE-1 nor wild-type (WT) wheat with respect to shoot area, plant height, total grain number and thousand grain weight (Figure 4.1c-g). Elemental analysis showed that four of the CE-*OsNAS2* events (CE-1, CE-8, CE-13, CE-15) produced T₂ grain with significantly increased Fe and Zn concentrations relative to NS and WT and one CE-*OsNAS2* event (CE-5) produced T₂ grain with significantly increased Zn concentration relative to NS and WT (Figure 4.1h,i). Wild-type plants did not differ from NS plants for any trait measured and had mean values of 1465 cm² shoot area, 65 cm plant height; 375 total grain number, 38 g thousand grain weight, 40 µg g⁻¹ DW grain Fe and 78 µg g⁻¹ DW grain Zn.

Based on low insert copy number, no difference in plant phenotype, and increased grain Fe and Zn concentrations from the T₀ to T₂ generations, homozygous progeny of the double-insert event CE-1 and corresponding NS line were selected for a range of additional analyses from the T₃ to T₆ generation as depicted in Figure S4.1. Glasshouse-grown CE-1 seedlings displayed high *OsNAS2* expression in roots and shoots (Figure 4.1j,k). Expression of a range of endogenous *TaNAS*, *TaNAAT* and *TaDMAS* genes involved in NA and DMA biosynthesis was not significantly different between CE-1 and NS seedlings, however, a trend towards slightly reduced expression was detected in CE-1 (Figure S4.2). The CE-1 and NS seedlings did not differ with respect to shoot Fe, Zn and DMA concentration while shoot NA concentration was 1.3-fold higher in CE-1 seedlings (Figure S4.3). Nicotianamine concentration was 15-fold higher in CE-1 mature grain relative to NS (Figure 4.1l).

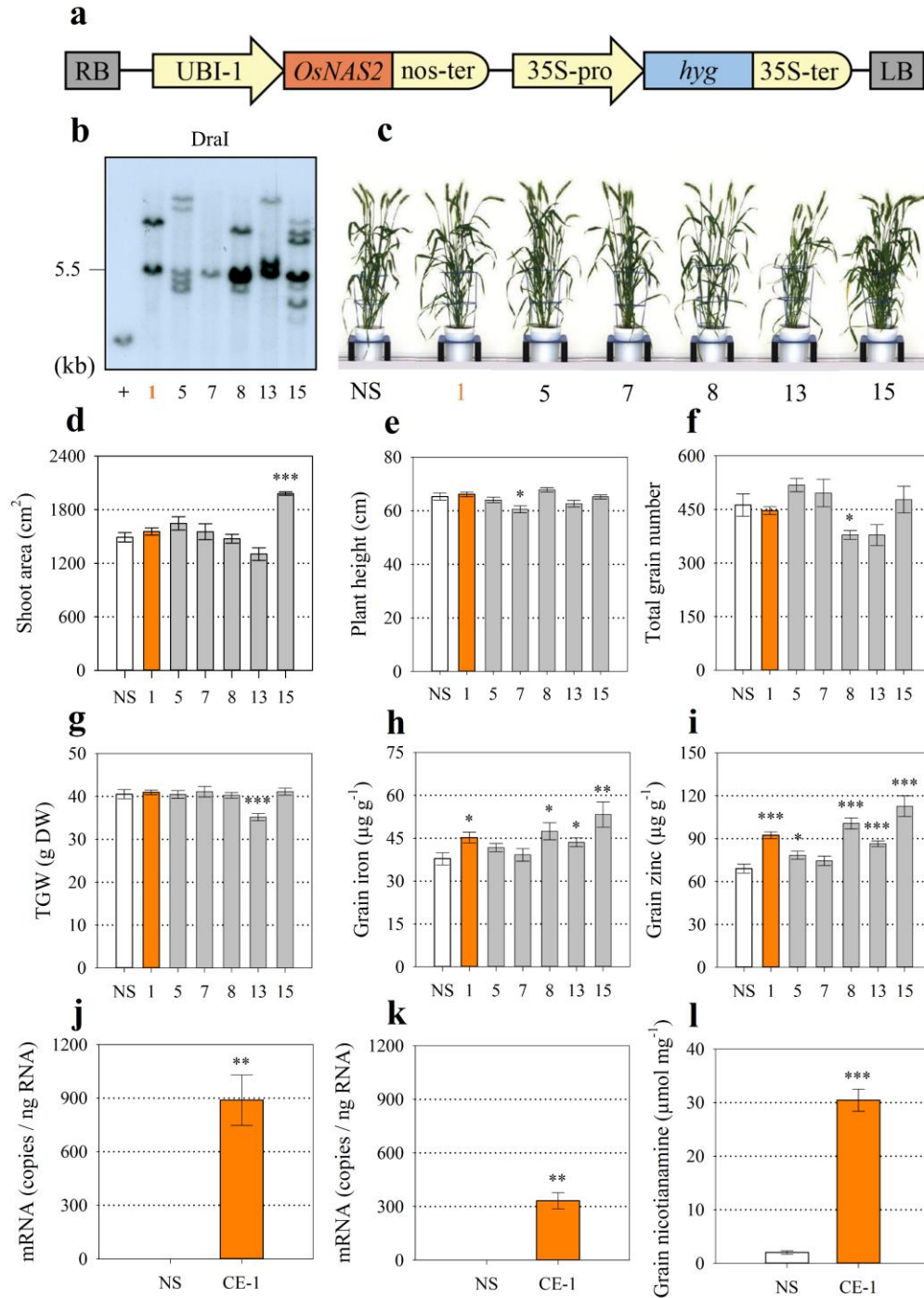


Figure 4.1. Generation and characterization of independent bread wheat transformation events constitutively expressing the rice nicotianamine synthase 2 (*OsNAS2*) gene. (a) Schematic representation of the T-DNA construct. RB and LB: right and left borders, respectively; UBI-1: maize ubiquitin 1 promoter; *OsNAS2*: rice nicotianamine synthase 2 gene (LOC_Os03g19420); nos-ter: nopaline synthase terminator; 35S-pro: dual promoter of 35S cauliflower mosaic virus gene; *hyg*:

hygromycin phosphotransferase gene; 35S-ter: terminator of 35S cauliflower mosaic virus gene. **(b)** Southern blot analysis of T₀ wheat events to determine T-DNA insertion number. DraI: restriction endonuclease; + indicates positive control. **(c)** Representative plants of null segregant (NS) and the 6 transformation events (CE-OsNAS2) 100 days after sowing. **(d-g)** Projected shoot area (cm²), plant height (cm), total grain number and thousand grain weight (TGW) of NS (white), leading CE-OsNAS2 event (CE-1, orange) and other CE-OsNAS2 events (grey) at the T₁ generation. Bars represent mean $\pm$ SEM of at least 7 biological replicates. **(h-i)** Iron and zinc concentration ($\mu\text{g g}^{-1}$ DW) in T₂ whole grain of NS, CE-1 and other CE-OsNAS2 events. Bars represent mean $\pm$ SEM of at least 7 biological replicates. **(j-k)** Relative quantification of OsNAS2 transcript levels in NS and CE-1 shoots and roots. Bars represent mean $\pm$ SEM of three bulked biological replicates, each with three technical replicates of quantitative RT-PCR. **(l)** Nicotianamine concentration ($\mu\text{mol mg}^{-1}$) in whole grain of NS and CE-1 plants at the T₂ generation. Bars represent mean $\pm$ SEM of three biological replicates. Asterisks denote the significance between NS and CE-OsNAS2 events for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test. Wild-type plants did not differ from NS plants for any trait measured and therefore only NS data is presented.

Constitutive OsNAS2 expression alters Fe and Zn accumulation in grain, bracts, rachis and flag leaf at multiple points during grain filling

The main stem flag leaf, rachis, bracts and grain of glasshouse-grown CE-1 and NS plants were harvested at five timepoints post anthesis to determine Fe and Zn accumulation patterns in vegetative and grain tissues during grain filling. The CE-1 grain had significantly higher Fe content at 5 – 8 days after anthesis (DAA) and 19 – 21 DAA relative to NS grain ($P = 0.006$ and $P = 0.046$; respectively) and showed non-significant trends towards higher grain Fe and Zn content at maturity (Figure 4.2a,b). Bracts of CE-1 plants had significantly higher Fe and Zn contents at 12 – 15 DAA relative to NS ($P = 0.013$ and $P \leq 0.001$; respectively) but did not differ from NS bracts at maturity (Figure 4.2c,d). Rachis of CE-1 plants had significantly higher Fe content at 5 – 8 DAA ($P = 0.012$), and significantly higher Zn contents at 12 – 15 DAA and 19-21 DAA ($P = 0.014$ and $P = 0.020$, respectively), but did not differ from NS rachis at maturity (Figure 4.2e,f). Flag leaf of CE-1 and NS plants contained similar Fe content throughout grain filling. Conversely, CE-1 flag leaf contained significantly lower Zn content at 19 - 21 DAA and 26-29 DAA relative to NS ($P = 0.009$ and $P = 0.038$, respectively) but did not differ from NS flag leaf at maturity (Figure 4.2g,h). Iron and Zn concentration data followed similar trends to the content data although more significant differences were detected (Table S4.1). Vegetative and grain tissue biomass was largely unchanged between CE-1 and NS plants throughout anthesis and was not significantly different for any tissue at maturity (Table S4.2).

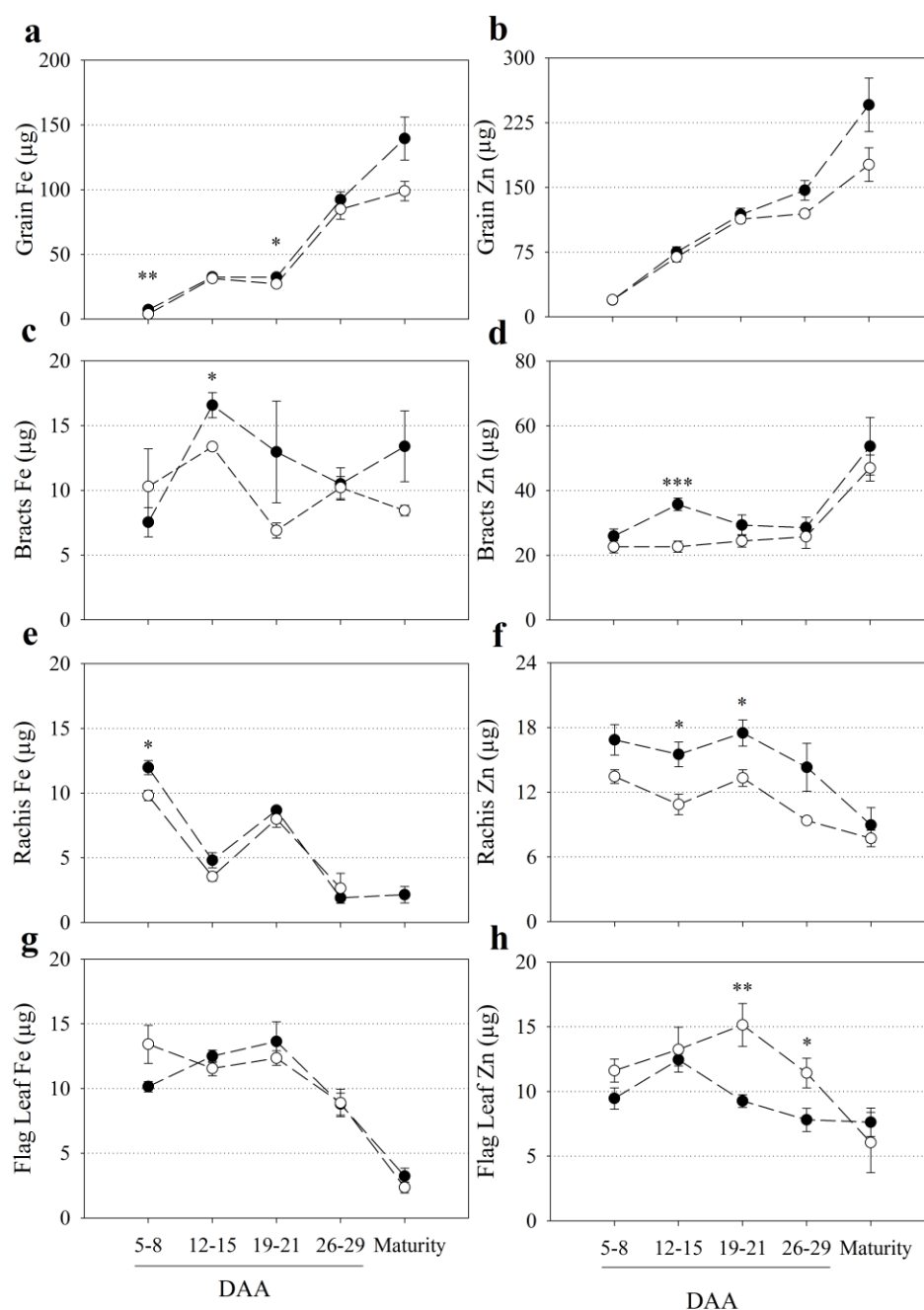


Figure 4.2. Fe and Zn content in vegetative and grain tissues during grain filling of CE-OsNAS2 and NS wheat lines. Fe and Zn content (µg) in NS (open circles) and CE-1 (closed circles) plant tissues between 5-8, 12-15, 19-21 and 26-29 days after anthesis (DAA) as well as at maturity. **(a-b)** grain; **(c-d)** bracts; **(e-f)** rachis; and **(g-h)** flag leaf tissues were sampled for Fe and Zn content, respectively. Each symbol represents mean $\pm$ SEM of at least 3 biological replicates. Asterisks denote the significance between NS and CE-1 for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

Constitutive OsNAS2 expression increases Fe accumulation in grain endosperm tissue and Fe and Zn accumulation within the crease region

Elemental maps of Fe and Zn in transverse cross-sections of two representative CE-1 and NS grain showed that CE-1 grain had higher Fe signal intensities in all grain tissue types relative to NS with the largest difference detected in endosperm tissues (Figure 4.3c,d). The Zn signal in both CE-1 and NS grain was localized to the aleurone and crease regions and was not detectable in the endosperm. Line scans across the mid-section demonstrated that CE-1 grain had higher Fe signal intensities relative to NS in all regions of the grain, particularly in the endosperm, while CE-1 grain had slightly higher Zn signal intensities in the aleurone cells relative to NS (Figure 4.3e,f). The line scans also revealed different Fe and Zn distribution patterns within the crease region of NS and CE-1 grain, with Zn signals appearing as two distinct peaks on either side of the crease while Fe signals clustered into one central peak. Tri-color elemental maps of Fe, Zn and Cu distribution within the crease region demonstrated that these distinct peaks result from extensive Zn localization in modified aleurone cells bordering the crease in contrast to prominent Fe localization in the centrally located nucellar projection (Figure S4.4). While CE-1 and NS grain showed similar Zn signal intensities in the modified aleurone cells of the crease, the CE-1 grain had slightly higher Zn signal intensity in the nucellar projection relative to NS (Figure 4.3f). The CE-1 and NS grain had similar distribution and signal intensities for Cu and Mn (Figure S4.5).

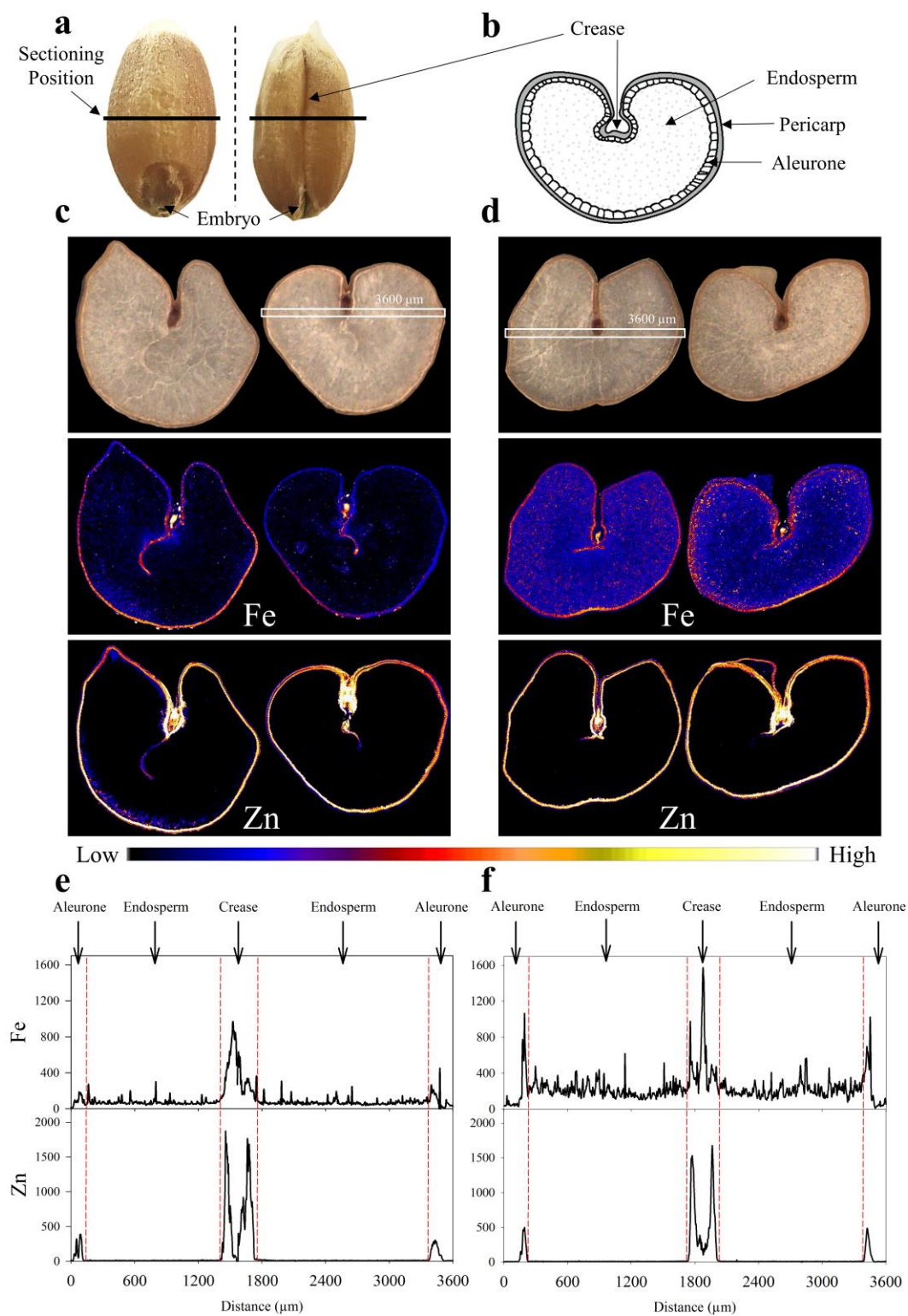


Figure 4.3. Distribution of Fe and Zn in CE-OsNAS2 and NS wheat grain. (a) Grain position where transverse cross-sections were made. **(b)** Location of major tissue types in transverse section

of wheat grain. (c) Bright field images of two representative NS grain sections and corresponding XFM elemental maps of Fe and Zn. (d) Bright field images of two representative CE-1 grain sections and corresponding XFM elemental maps of Fe and Zn. Color bar represents high (white) and low (black) elemental concentration. White boxes in the bright field images represent areas in two grain sections (one each for NS and CE-1) used to generate line scans. (e) Line scans showing the distribution and signal intensity of Fe and Zn across NS grain. (f) Line scans showing the distribution and signal intensity of Fe and Zn across CE-1 grain. Units for the y-axis are elemental counts per pixel.

Tri-color elemental maps of Fe, Zn and P near the grain edge of one representative CE-1 and NS grain demonstrated that the Fe signal in CE-1 grain was enhanced within pericarp, aleurone and endosperm regions relative to NS grain (Figure 4.4c,d). The Zn signal in CE-1 grain was enhanced primarily within the pericarp and aleurone cells, relative to the NS, and co-localized with Fe in that region. The P signal in both NS and CE-1 grain did not differ in intensity nor distribution and was localized to the aleurone cells. The trends observed in CE-1 and NS tri-color maps were further confirmed by line scans (Figure 4.4e,f). In both CE-1 and NS grain, P was localized exclusively to the aleurone cells with equal signal intensity, indicating that the significant enrichment of Fe in CE-1 endosperm was not associated with phytic acid (a P containing compound). The distribution and intensity of S (a proxy for protein) appeared slightly reduced in the CE-1 aleurone, relative to NS, but similar to NS in the endosperm (Figure 4.4e,f).

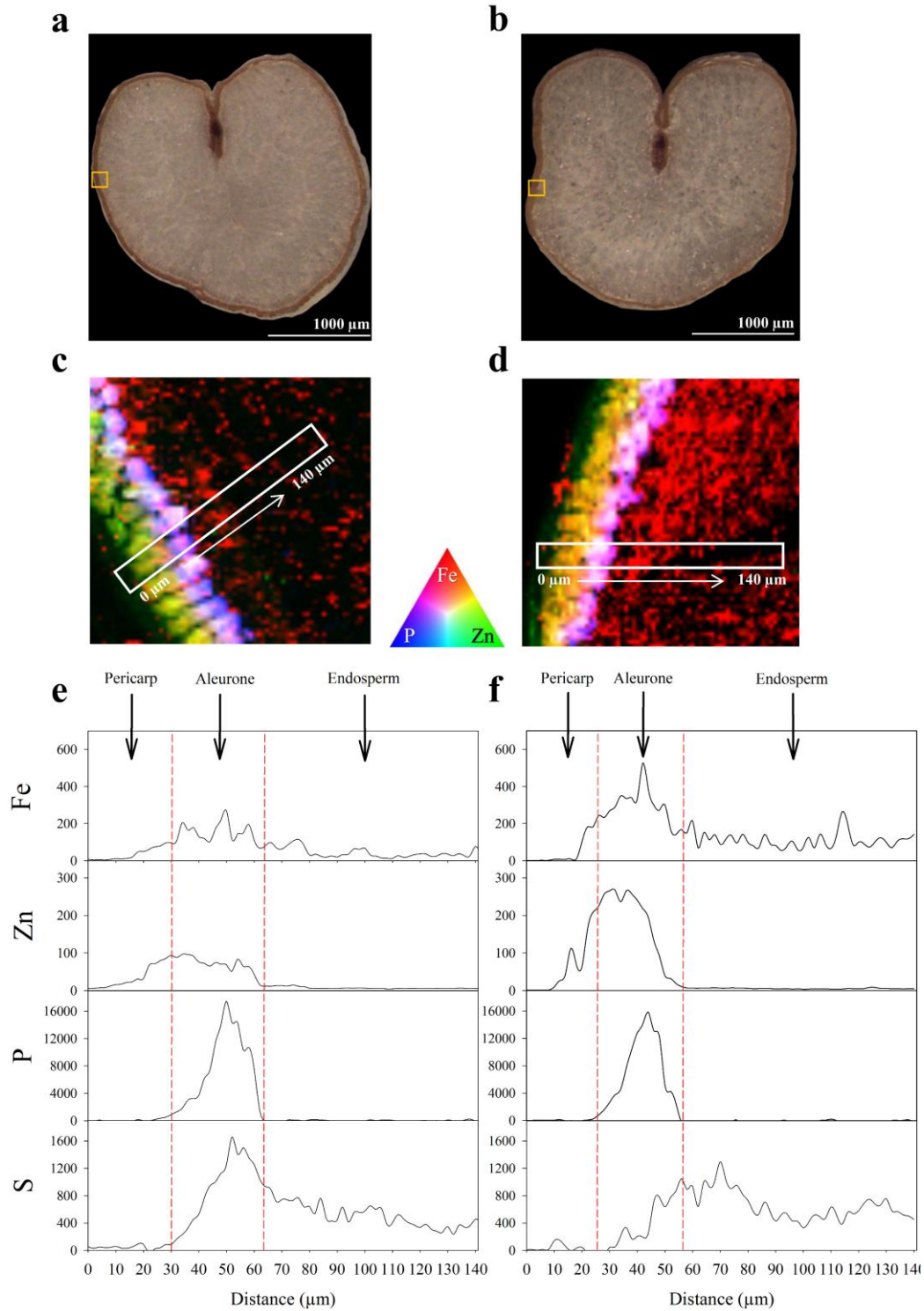


Figure 4.4. Distribution of Fe, Zn and P in CE-OsNAS2 and NS wheat grain. (a-b) Bright field images of NS and CE-1 grain sections, respectively. Yellow boxes represent areas used to generate tri-color elemental maps. (c) Tri-color XFM elemental map of Fe (red), Zn (green) and P (blue) in

NS grain. White box represents the area used to generate line scans. **(d)** Tri-color XFM elemental map of Fe (red), Zn (green) and P (blue) in CE-1 grain. White box represents the area used to generate line scans. **(e)** Line scans showing the distribution and signal intensity of Fe, Zn, P and S in NS grain. **(f)** Line scans showing the distribution and signal intensity of Fe, Zn, P and S in CE-1 grain. Units for the y-axis are elemental counts per pixel.

Constitutive OsNAS2 expression does not alter the phenotype of field-grown plants and increases Fe, Zn, NA and DMA concentrations in grain and white flour

Replicated plots of three CE-1 sibling lines designated CE-1.1, CE-1.2 and CE-1.3 and the NS were evaluated in confined field trials at Katanning and Merredin, Western Australia in 2015. With the exception of CE-1.1 at Merredin, phenotype of the CE-1 sibling lines and NS did not differ with respect to plant height, spike number, biomass, thousand grain weight (TGW) and grain yield (Table S4.3). Whole grain Fe concentrations were significantly higher for CE-1.1 at both Katanning ($P = 0.042$) and Merredin ($P = 0.002$) relative to NS (Figure 4.5a). Whole grain Zn and P concentrations did not differ between the three CE-1 sibling lines and NS at both Merredin and Katanning. All lines (CE-1 and NS) had higher whole grain Zn and P concentrations at Katanning relative to Merredin, possibly due to lower TGW at Katanning (Figure 4.5b,c, Table S4.3). Analysis of NA and DMA showed that whole grain NA concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P = 0.005$ and $P \leq 0.001$, respectively), and for all three CE-1 sibling lines at Merredin ($P = 0.004$, $P = 0.008$ and $P = 0.016$, respectively), relative to NS. Whole grain DMA concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P = 0.014$ and $P = 0.045$, respectively), and CE-1.2 and CE-1.3 at Merredin ($P = 0.022$ and $P \leq 0.001$, respectively), relative to NS (Figure 4.5d,e).

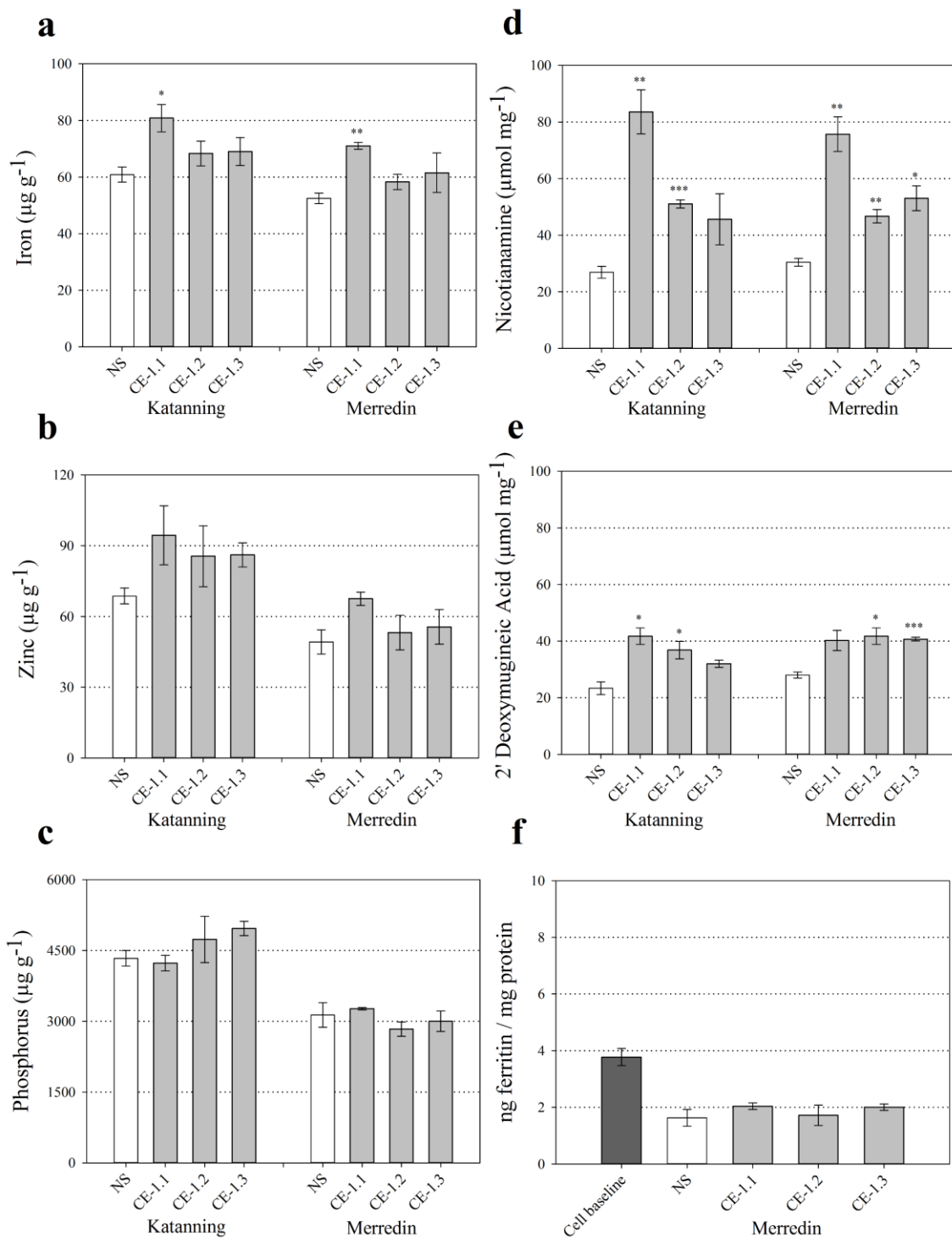


Figure 4.5. Whole grain nutrition of field grown CE-1 and NS wheat lines. Nutrient and metabolite concentrations in whole grain samples of NS (white) and three CE-1 sibling lines (CE-1.1, 1.2 and 1.3, grey) at the T₆ generation. (a-c) Whole grain Fe, Zn and P concentrations ($\mu\text{g g}^{-1}$) of NS and CE-1 plants grown at Katanning and Merredin field sites. (d-e) Whole grain NA and DMA

concentrations ($\mu\text{mol mg}^{-1}$) of NS and CE-1 plants grown at Katanning and Merredin field sites. (f) Whole grain Fe bioavailability of NS and CE-1 plants grown at Merredin field site. Bars represent mean $\pm$ SEM of 3 biological replicates. Asterisks denote the significance between NS and each CE-1 wheat line for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

White flour Fe concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P \leq 0.001$ and $P = 0.012$, respectively) relative to NS (Figure 4.6a) but did not differ between any line at Merredin. White flour Zn concentrations were significantly higher for all three CE-1 sibling lines at Katanning ($P = 0.002$, $P = 0.010$ and $P = 0.036$, respectively) relative to NS (Figure 4.6b) but did not differ between any line at Merredin. White flour P concentration did not differ between any line at both Merredin and Katanning (Figure 4.6a-c). White flour NA concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P = 0.004$ and $P \leq 0.001$, respectively) and for all three CE-1 sibling lines at Merredin ($P \leq 0.001$, $P = 0.002$ and $P = 0.002$, respectively), relative to NS (Figure 4.6d). White flour DMA concentrations were significantly higher for all three CE-1 sibling lines at both Katanning ($P = 0.010$, $P = 0.020$ and $P = 0.035$, respectively) and Merredin ($P = 0.006$, $P \leq 0.001$ and $P = 0.002$, respectively) relative to NS (Figure 4.6e).

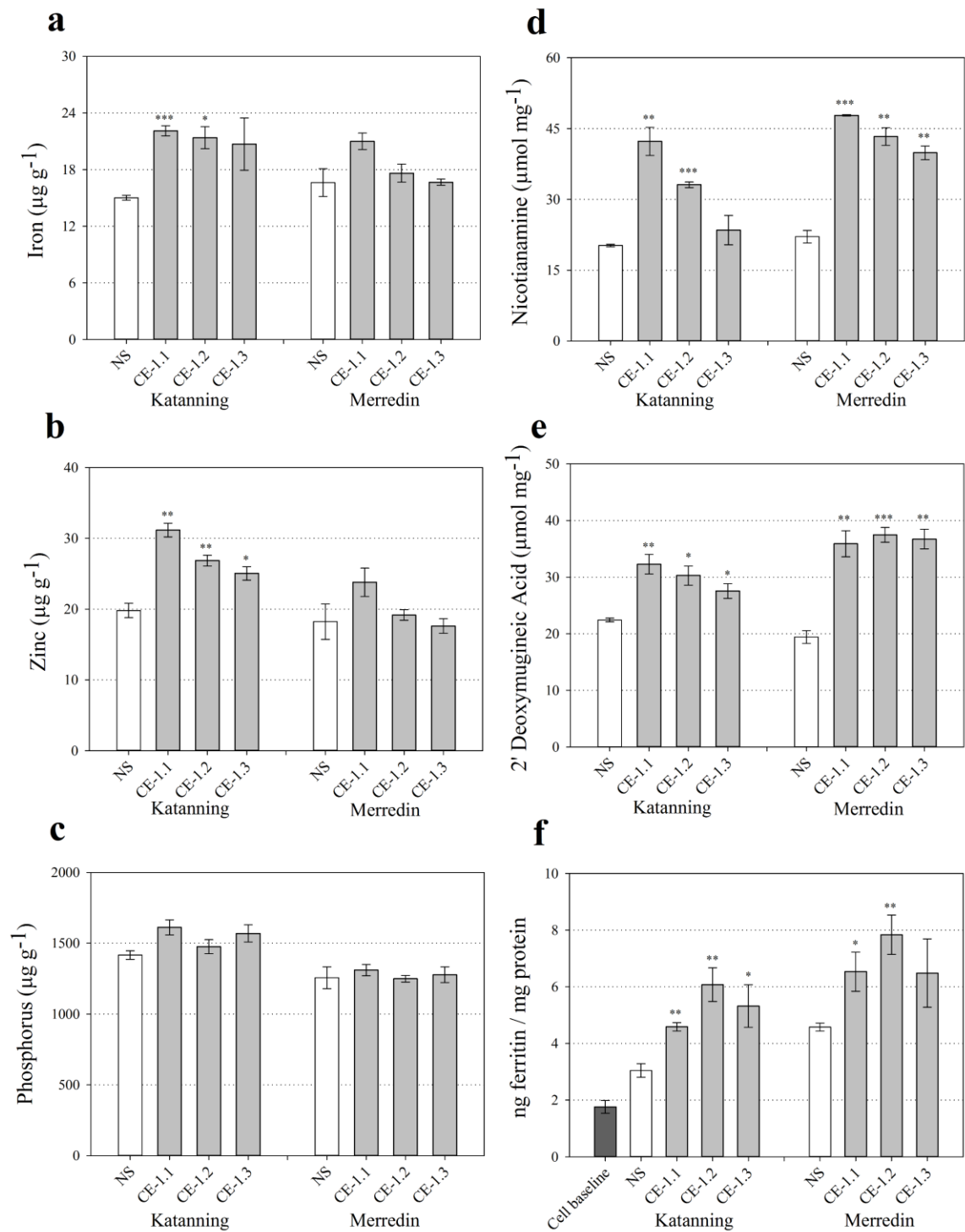


Figure 4.6. White flour nutrition of field grown CE-1 and NS wheat lines. Nutrient and metabolite concentrations in white flour samples of NS (white) and three CE-1 sibling lines (CE-1.1, 1.2 and 1.3, grey) at the T₆ generation. **(a-c)** White flour Fe, Zn and P concentrations ($\mu\text{g g}^{-1}$) of NS and CE-1 plants grown at Katanning and Merredin field sites. **(d-e)** White flour NA and DMA concentrations

($\mu\text{mol mg}^{-1}$) of NS and CE-1 plants grown at Katanning and Merredin field sites. (f) White flour Fe bioavailability of NS and CE-1 plants grown at Merredin and Katanning field sites. Bars represent mean $\pm$ SEM of 3 biological replicates. Asterisks denote the significance between NS and each CE-1 wheat line for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

Iron bioavailability is increased in white flour containing increased concentrations of NA and DMA

Iron bioavailability in CE-1.1, CE-1.2, CE-1.3 and NS whole grain and white flour produced from field-grown grain was determined through ferritin assays of *in vitro* Caco-2 cells incubated with flour digests. The levels of bioavailable Fe in CE-1.1, CE-1.2, CE-1.3 and NS whole grain flour were negligible and did not differ significantly; a result likely due to phytic acid and other inhibitory compounds in the outer layers of wheat grain (Figure 4.5f). By contrast, levels of bioavailable Fe were significantly increased in CE-1.1, CE-1.2, CE 1.3 white flour digests from Katanning ($P = 0.005$, $P = 0.009$ and $P = 0.045$, respectively), and CE-1.1 and CE-1.2 white flour digests from Merredin ($P = 0.050$ and $P = 0.010$, respectively) relative to NS (Figure 4.6f). White flour Fe bioavailability was significantly correlated with NA concentration ($r = 0.711$, $P = 0.048$) and DMA concentration ($r = 0.812$, $P = 0.014$). White flour Fe bioavailability was not significantly correlated with concentrations of Fe ($r = 0.242$, $P = 0.563$), Zn ($r = -0.132$, $P = 0.755$) and P ($r = -0.433$, $P = 0.284$) (Table 4.1).

Table 4.1. Pearson's correlation analysis of NA, DMA, Fe, Zn and P concentrations in NS and CE-1 white flour and ferritin production in Caco-2 cells. Bold and non-bold numbers represent correlation coefficients and P values, respectively. Asterisks denote significant correlation between factors for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***)

	DMA ($\mu\text{mol/mg}$)	Fe ($\mu\text{g/g}$)	Zn ($\mu\text{g/g}$)	P ($\mu\text{g/g}$)	Ferritin (ng/mg protein)
NA ($\mu\text{mol/mg}$)	0.912** 0.002	0.440 0.276	0.239 0.569	-0.180 0.670	0.711* 0.048
DMA ($\mu\text{mol/mg}$)		0.355 0.389	0.132 0.755	-0.145 0.732	0.812* 0.014
Fe ($\mu\text{g/g}$)			0.893** 0.003	0.627 0.096	0.242 0.563
Zn ($\mu\text{g/g}$)				0.854** 0.007	-0.132 0.755
P ($\mu\text{g/g}$)					-0.433 0.284

4.4. Discussion

S-adenosylmethionine (SAM) is a ubiquitous cosubstrate involved in the production of polyamines, ethylene, biotin, NA and DMA in plants (Mao et al., 2015; Roeder et al., 2009). Because increased

NAS gene activity diverts more endogenous SAM towards NA and DMA production, it is important to carefully phenotype Fe and Zn biofortified plants with constitutive NAS expression to identify any pleiotropic effects that may negatively impact on plant phenotype. In our study we analyzed multiple CE-*OsNAS2* transformation events in several glasshouse experiments, and the lead CE-1 event in multi-location field trials in Western Australia, and found that important agronomical traits such as shoot area, plant height, total grain number and thousand grain weight did not differ from NS in most cases (Figure 4.1, Tables S4.2 and S4.3). These results are similar to those reported for glasshouse and field-grown 35S::*OsNAS2* rice (Johnson et al., 2011; Trijatmiko et al., 2016), as well as glasshouse grown UBI-1::*OsNAS2* wheat (Singh et al., 2017), and demonstrate that constitutive expression of the *OsNAS2* gene to biofortify bread wheat with Fe and Zn does not negatively impact on plant phenotype and yield. Determining whether the changes observed in Fe and Zn accumulation patterns during grain filling (Figure 4.2) relate to increased remobilization of Fe and Zn from vegetative to grain tissues in CE-*OsNAS2* plants will require further analysis using radioactive or stable Fe and Zn isotopes (Pottier et al., 2014).

The most striking result from our synchrotron X-ray fluorescence microscopy (XFM) analyses was the increased Fe signal in endosperm tissue of CE-1 grain relative to NS (Figures 4.3d and 4.4d). Line scans showed that the increased Fe signal extended throughout CE-1 endosperm and did not co-localize with P (Figures 4.3f and 4.4f), indicating that Fe is not associated with phytic acid in this tissue nor the inhibitory effects that phytic acid has on mineral absorption in humans (Hurrell et al., 2003). By contrast, both CE-1 and NS grain showed high Zn signal co-localized with P in aleurone cells and essentially no Zn signal in the endosperm (Figures 4.3f and 4.4f). The observed differences in grain Fe and Zn localization demonstrate that wheat white flour contains Zn that is derived almost entirely from outside of the endosperm. We postulate that the wheat crease, which is difficult to remove through milling (Campbell et al., 2007), represents the major source of Zn in white flour. Line scans across the crease region showed similar Zn signal intensities in CE-1 and NS modified aleurone cells yet higher Zn signal intensity in the CE-1 nucellar projection (Figure 4.3e,f; Figure S4.4), a tissue which has been shown to contain NA-bound Fe (De Brier et al., 2016), and this difference likely accounts for the observed Zn concentration increase in CE-1 white flour (Figure 4.6b). Interestingly, synchrotron XFM studies of rice grain reveal a radically different Zn distribution pattern that extends throughout the endosperm and shows no obvious barrier to endosperm loading (Johnson et al., 2011; Kyriacou et al., 2014). Determining whether differences in transport mechanisms or sink strength account for the Zn distribution differences between wheat and rice merits further investigation.

White flour milled from CE-1 grain contained significantly higher concentrations of NA and DMA at both the Merredin and Katanning field sites, relative to NS, while white flour Fe and Zn concentrations only differed from NS at Katanning (Figure 4.6a-e). The NS and CE-1 wheat plants were larger and higher yielding at the Merredin field site (Table S4.3), which may have had a dilution effect on grain micronutrient concentrations and thereby minimized differences between the genotypes. This result demonstrates the importance of multi-location field trials for accurate assessment of wheat Fe and Zn biofortification traits, yet also uniquely enabled us to examine the effects of NA and DMA as promoters of Fe bioavailability (Eagling et al., 2014b; Glahn et al., 1998; Lee et al., 2012; Tako et al., 2016) under differing Fe concentrations. We used the *in vitro* Caco-2 cell line model to measure Fe bioavailability in NS and CE-1 white flour produced at both field sites and found that white flour NA and DMA concentrations were significantly and positively correlated with Fe bioavailability at both sites while white flour Fe concentration was not significantly correlated with Fe bioavailability (Figure 4.6, Table 4.1). This result was most evident at the Merredin field trial, where white flour Fe concentrations of two CE-1 sibling lines (CE-1.2 and CE-1.3) were virtually identical to that of NS, however, CE-1.2 white flour contained 1.7-fold more bioavailable Fe than NS (Figures 4.6a and 4.6f). In addition to Fe, white flour P concentrations were unchanged between NS and CE-1 sibling lines, indicating that altered white flour phytate/Fe ratios are not likely to be responsible for the increase in Fe bioavailability (Figure 4.6c). These results reinforce the finding that NA and/or DMA bind Fe in a readily bioavailable form within wheat white flour (Eagling et al., 2014a) and suggests that NA and/or DMA mediate increased uptake of Fe into human cells independent of endosperm Fe concentration. As such, NA and DMA can be viewed as important phytonutrients that increase Fe bioavailability in plant foods (Martin et al., 2017; Nozoye et al., 2018), and metabolic engineering strategies focused on increasing their concentrations could help offset the effects of decreased mineral concentrations forecast for C₃ grains by mid-century.

4.5. Methods

4.5.1. Vector construction and generation of wheat transformation events

The full-length coding sequence of *OsNAS2* (LOC_Os03g19420) was PCR amplified from rice (*Oryza sativa* L.) cv. Nipponbare genomic DNA (Johnson et al., 2011). Recombination into a modified pMDC32 vector (Curtis et al., 2003) with the hygromycin phosphotransferase plant-selectable marker gene placed *OsNAS2* under transcriptional control of the maize (*Zea mays* L.) ubiquitin 1 promoter. Particle bombardment of the construct into immature wheat (*Triticum aestivum* L.) cv. Bobwhite embryos (1.0-1.5 mm in length) was performed at The University of Adelaide (Adelaide, Australia) using established protocols (Kovalchuk et al., 2009). Plants were grown in

glasshouse conditions (12 hr photoperiod, 23°C day/12°C night, 50% humidity) in soil (coconut peat and sand mixture) with complete fertilizers.

4.5.2. Insert copy number analysis

Genomic DNA (10 µg) was isolated from CE-OsNAS2 leaf tissue and digested with *DraI* and *HindIII* restriction enzymes. Restriction fragments were separated by gel electrophoresis (0.8% agarose) alongside a positive barley control and blotted to a nylon membrane. Two independent hybridizations of a ³²P-labelled probe to both the nopaline synthase terminator and dual 35S promoter were performed using established protocols (Pallotta et al., 2014).

4.5.3. Automated phenotyping

Grain were sown in white plastic pots (14 × 19 cm) containing 2.5 kg of soil mixture (equal parts clay-loam soil and coconut peat) and Osmocote® fertilizer. Plants were maintained under glasshouse conditions (12 hr photoperiod, 24°C day/18°C night, 50–90% humidity) in the phenotyping platform of The Plant Accelerator (Adelaide, Australia). Projected shoot area and plant height was measured 100 days after sowing using the conveyer automated imaging system (Berger et al., 2012). Grain number and TGW was manually determined at harvest.

4.5.4. Inductively coupled plasma optical emission spectrometry (ICP-OES)

Plant tissues were submerged in 1% Tween 20 solution, rinsed with dH₂O and oven dried for 48 hr at 60°C before grinding to a powder using an IKA tube mill (www.ika.com). Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis was conducted at Waite Analytical Services (Adelaide, SA, Australia), the Robert W. Holley Centre for Agriculture and Health (USDA-ARS, Ithaca, NY, USA) and the CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia).

4.5.5. Quantitative reverse transcription PCR (qRT-PCR)

Shoot and root tissues (without the crown) of 4-week-old plants were separated, cleaned with dH₂O and snap frozen. Three plants of each genotype (representing one biological replicate) were combined and total RNA was extracted from pulverized frozen plant tissue (100 – 150 mg) using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) and a commercial kit (Direct-zol™, ZymoResearch). Genomic DNA was removed from RNA (2 µg) using a DNase I treatment (Promega) and reverse transcription was performed using a commercial kit (Bioline).

Consensus primers were designed to amplify homeologous groups of *TaNAS*, *TaNAAT* and *TaDMAS* gene families using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0>) software. Each biological replicate was analyzed in triplicate and transcripts were quantified against four replicates of ten-fold serial

dilutions (10^2 – 10^8) for each purified PCR template (DNA Clean & Concentrator™-5, ZymoResearch). Expression levels of *OsNAS2*, *TaNAS*, *TaNAAT* and *TaDMAS* were measured in root and shoot tissues using qRT-PCR analysis (CFX384- BioRad). The geometric mean expression of three housekeeping genes: *TaCyclophilin*, *TaGAPDH* and *TaELF* and *TaGAPDH*, *TaActin* and *TaELF*, was used to normalize *OsNAS2*, *TaNAS*, *TaNAAT* and *TaDMAS* gene expression within shoot and root tissues, respectively (Schreiber et al., 2009; Vandesompele et al., 2002). All primers had annealing temperatures between 61-65°C and primer sequences and efficiencies are provided (Table S4.4).

4.5.6. Quantification of NA and DMA

Quantification of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized NA in whole grain was performed via liquid chromatography-mass spectrometry (LC-MS) using established protocols (Callahan et al., 2007; Johnson et al., 2011). Quantification of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) derivatized NA and DMA in whole grain (Figure 4.5d,e) and white flour (Figure 4.6d,e) was performed via reverse phase (RP) LC-MS on an 1290 Infinity II and 6490 Triple Quadrupole LC/MS system (Agilent Technologies Inc.) using established protocols (Selby-Pham et al., 2017). Briefly, sequential methanol (100%) and deionized H₂O (18M Ω) extractions of pulverized wheat grain or white flour (25 mg) were combined and added (5 μ L) to sodium borate buffer (pH = 8, 1 M, 10 μ L), EDTA buffer (pH = 8, 50 mM, 10 μ L) and fresh FMOC-Cl solutions (50 mM, 40 μ L). After incubation (60 °C, 700 rpm, 15 mins), the derivatization reaction was quenched via the addition of formic acid (FA; pH = 4, 5%, 8.9 μ L). A Zorbax Eclipse XDB-C18 Rapid Resolution HS 2.1x100 mm, 1.8 μ m particle size column (Agilent Technologies Inc.) was used during chromatography with aqueous (0.1 % v/v FA in dH₂O) and organic (0.1 % v/v FA in acetonitrile) mobile phases. For quantification, a stock aqueous solution of NA and DMA (Toronto Research Chemicals) was prepared at 750 μ M and a calibration set was generated in the range of 0.005 μ M to 75 μ M.

4.5.7. Analysis of Fe and Zn accumulation post anthesis

Plants were grown in glasshouse conditions (12 hr photoperiod, 18°C day/13°C night, 40–80% humidity) in Hortico® potting mix with Osmocote® fertilizer at The University of Melbourne (Victoria, Australia). The main stem flag leaf, rachis, bracts and grain were harvested at 5-8 DAA (days after anthesis), 12-15 DAA, 19-21 DAA, 26-29 DAA and maturity. Samples were washed, oven dried for 48 hr at 60°C and ground to a powder before analysis by inductively coupled plasma mass spectrometry (ICP-MS) at the Environmental Analysis Laboratory (Lismore, NSW, Australia).

4.5.8. Synchrotron X-ray fluorescence microscopy (XFM)

Elemental X-ray fluorescence (XRF) maps of Fe, Zn, Cu and Mn in transverse cross-sections of two representative CE-1 and NS grain (4 sets of maps total) were collected at the XFM beamline at the Australian Synchrotron (Melbourne, Australia) as previously described (Van Malderen et al., 2017). Briefly, the beam energy was set at 15.6keV and the beam focused to approximately $2 \times \mu\text{m}^2$ using Kirkpatrick-Baez mirrors. Samples were analyzed continuously in the horizontal direction with a sampling interval of $4\mu\text{m}$ and a step size of $4\mu\text{m}$ in the vertical direction (pixel transit time was set at 5.2 ms). The XRF signal from the $80 \mu\text{m}$ transverse grain sections was collected using a 384-element Maia detector system. Tri-color elemental maps showing the distribution of Fe, Zn and P near the grain edge of one representative CE-1 and NS grain (2 sets of maps total; different grain from those used with the Maia detector) were collected using a separate Vortex-EM detector. The tri-color maps were used as guides to select rectangular areas of approximately $14 \times 140 \mu\text{m}$ near the grain edge for the generation of Fe, Zn, P and S line scans. Elemental maps were generated using GeoPIXE (<http://nmp.csiro.au/GeoPIXE.html>) software. The NS grain contained $38 \mu\text{g g}^{-1}$ DW Fe, $71 \mu\text{g g}^{-1}$ DW Zn, $4700 \mu\text{g g}^{-1}$ DW P and $1630 \mu\text{g g}^{-1}$ DW S while CE-1 grain contained $69 \mu\text{g g}^{-1}$ DW Fe, $122 \mu\text{g g}^{-1}$ DW Zn, $5300 \mu\text{g g}^{-1}$ DW P and $2100 \mu\text{g g}^{-1}$ DW S (Table S4.5).

4.5.9. Confined field trials

Confined field trials were conducted in Western Australia from June to December 2015 at the New Genes for New Environment facilities located in Merredin (31.4837° S, 118.2771° E) and Katanning (33.6894° S, 117.5551° E). Grain were sown in 2 m^2 plots with three replicate plots per genotype and arranged in a randomized block design at each site. Rows were spaced at 30 cm and grain were sown at a rate of 60 kg ha^{-1} . At maturity, average plant height was determined from three representative measurements per plot and spike number, total biomass and TGW was determined from 0.15 m^2 subsamples per plot (Table S4.3). Grain yield was calculated from the amount of grain harvested per 2 m^2 plot and extrapolated to kg/ha^{-1} . Soil properties of both field sites are provided in Table S4.6.

4.5.10. Production of white flour

Whole grain samples harvested at Merredin and Katanning were conditioned to 13% moisture content for 24 hr prior to milling. Each sample was milled using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) at constant temperature and run through a $280 \mu\text{m}$ sieve to isolate the white flour fraction. Average flour extraction for all lines from Merredin and Katanning was $71.5 \pm 0.2 \%$.

4.5.11. Caco-2 cell culture assessment of Fe bioavailability

Whole grain and white flour samples were digested for Caco-2 cell Fe-bioavailability analysis as previously described (Glahn et al., 1998; Trijatmiko et al., 2016). The Caco-2 cells were maintained in supplemented Dulbecco's modified Eagle medium (DMEM) for 11 days post-seeding and replaced with supplemented minimum essential media (MEM) solution 48 hr prior to the experiment. On the experiment day, gastric-digested samples (1.5 mL) were added to cylindrical Transwell inserts (Corning) fitted with a semipermeable (15000 Da MWCO) basal membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The inserts were placed within wells containing Caco-2 cell monolayers and incubated for 2 hr (37 °C), after which the inserts were removed and additional MEM (1 mL) added to the cells before incubation for 22 hr (37 °C). After incubation, growth medium was removed by aspiration and the Caco-2 cells were washed twice with a solution (pH = 7.0) containing NaCl (140 mmol/L), KCl (5 mmol/L) and PIPES (10 mmol/L) and harvested with the addition of dH₂O (1.5 mL) and brief sonication (Lab-Line Instruments, Melrose Park, IL). In an aliquot of the Caco-2 cell solution, ferritin content was determined using an immunoradiometric assay (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein content was determined using a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA). As Caco-2 cells synthesize ferritin in response to intracellular Fe, we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake.

4.5.12. Statistical analysis

All graphs and statistical analyzes were generated using Minitab (version 17.0) and SigmaPlot (version 13.0) software. Data are presented as mean $\pm$ SEM with biological replicate numbers noted in table and figure legends. Student's t-test were used to determine significant differences between means. Data are available upon request to the corresponding author of this paper.

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4.7. Supplementary Information

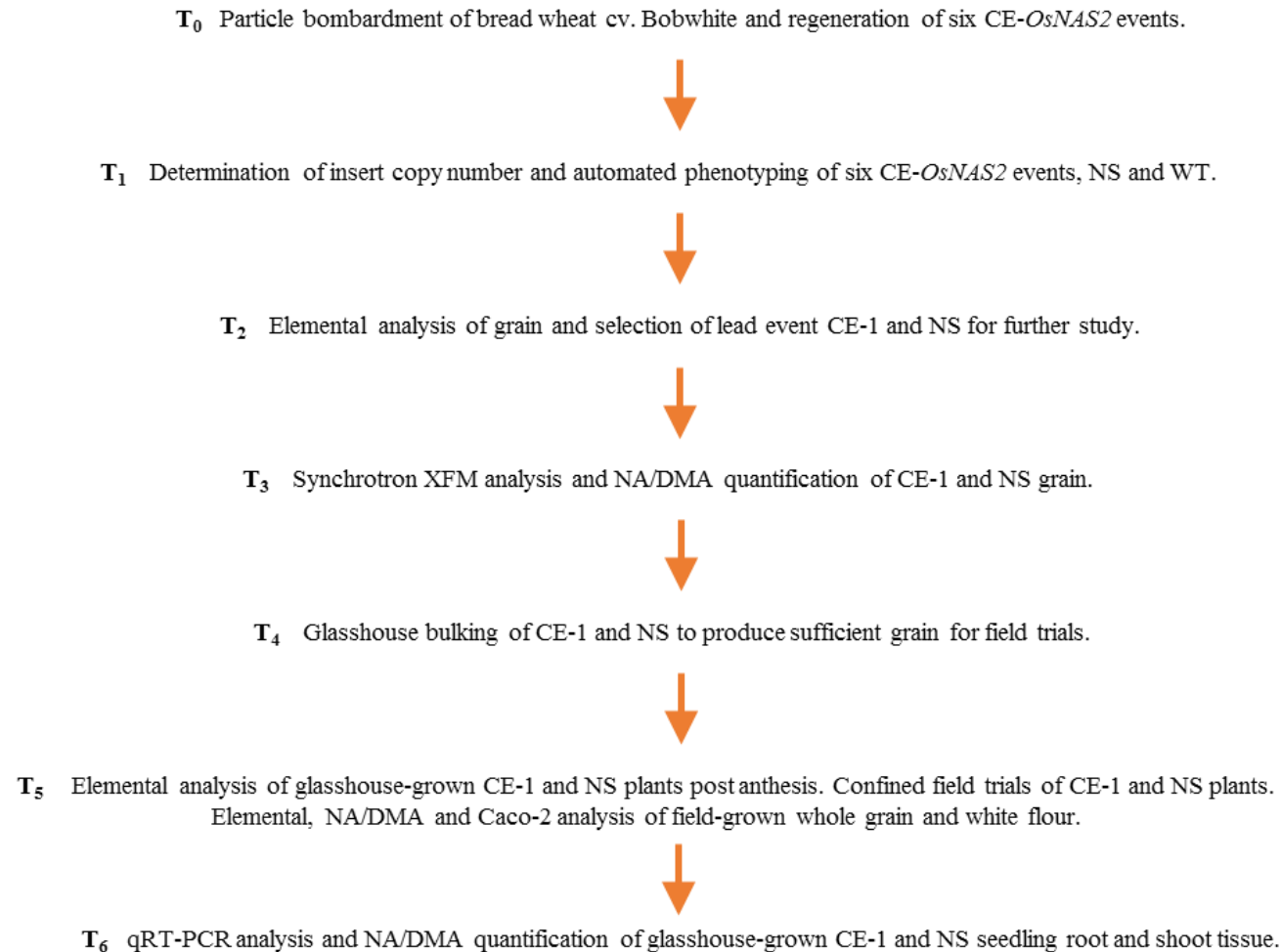


Figure S4.1. Flowchart detailing the analyses performed for each generation of the Ubi::*OsNAS2* lead event (CE-1) from transformation (T₀) to T₆ generation. The NS line was derived from CE-1 at the T₁ generation and included in each subsequent analysis.

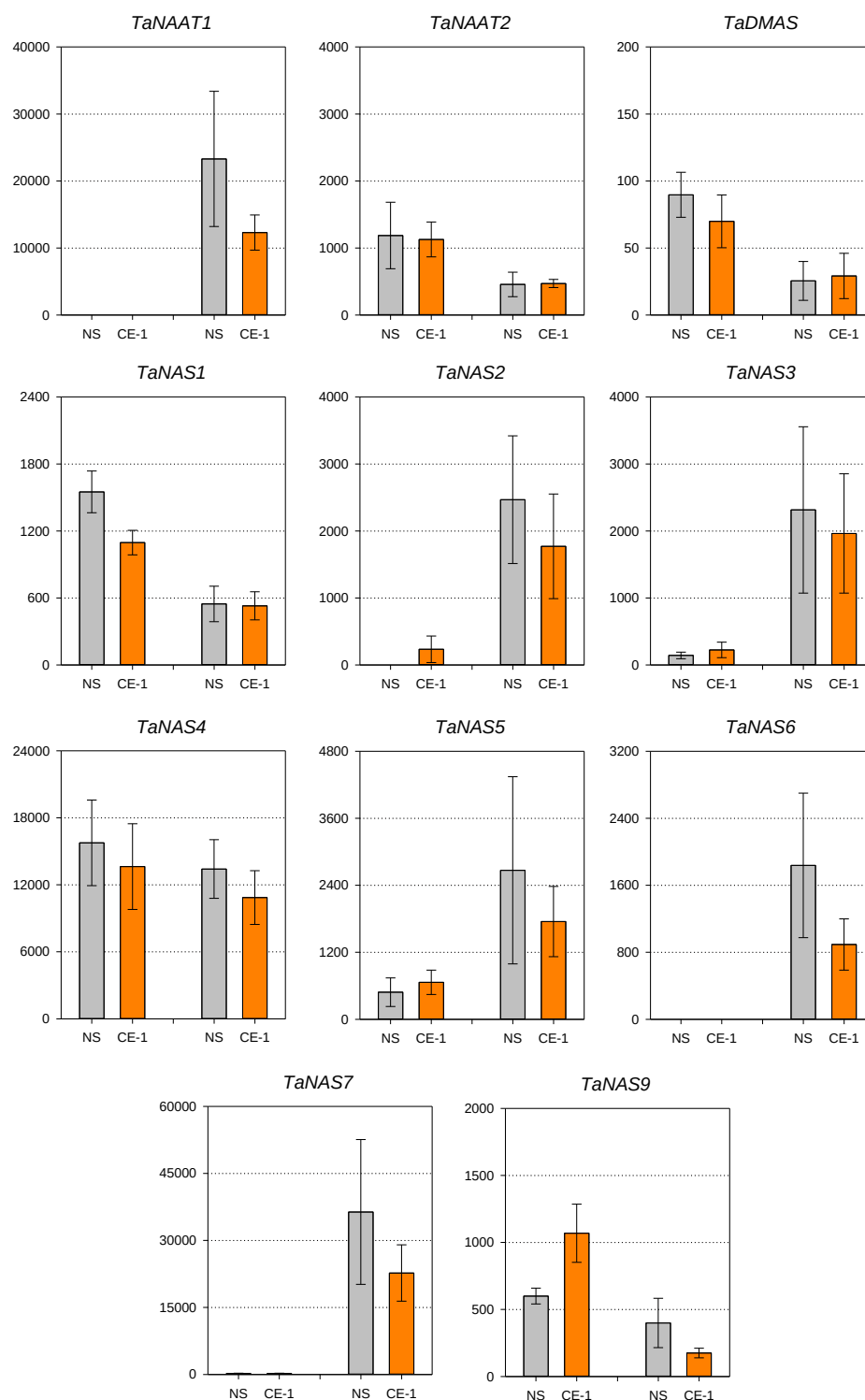


Figure S4.2. Relative quantification of DMA biosynthetic gene transcript levels in NS and CE-1 shoots (left) and roots (right). Units on the y-axis are mRNA copies per μg of total RNA. Bars represent mean $\pm$ SEM of three bulked biological replicates, each with three technical replicates of quantitative RT-PCR.

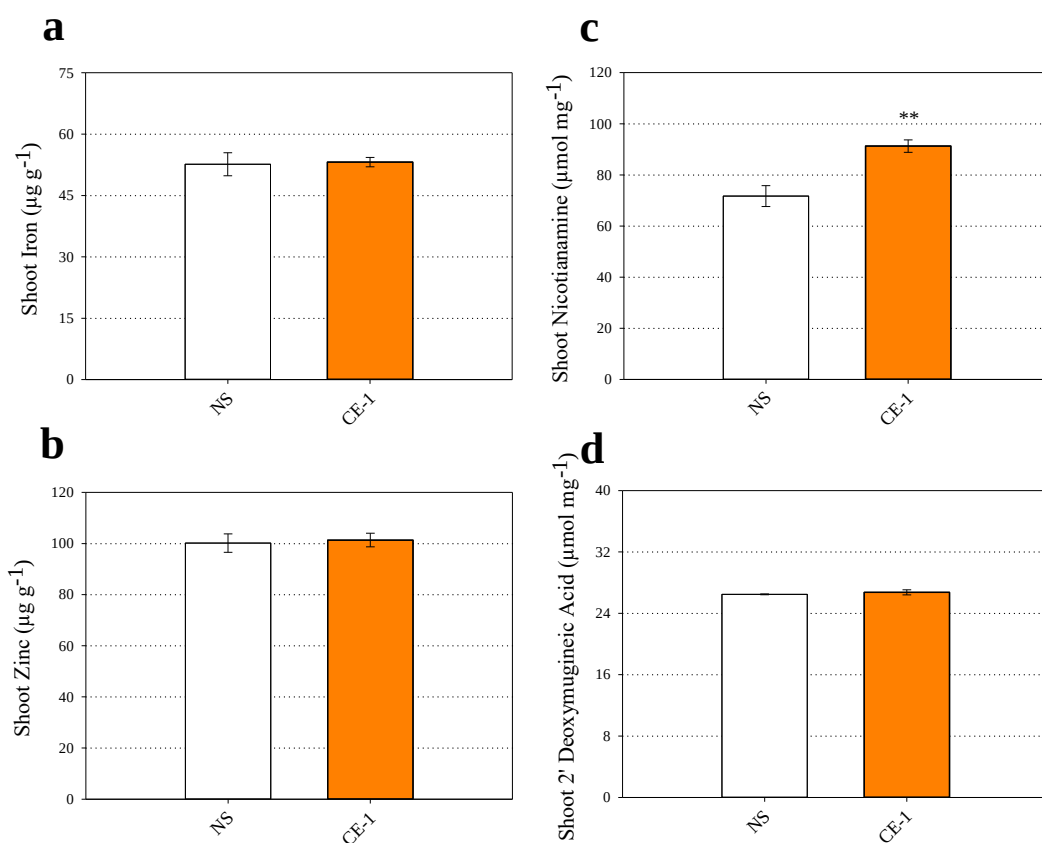


Figure S4.3. (a-b) Iron and zinc concentrations ($\mu\text{g g}^{-1}$ DW) in the shoots of NS (white) and CE-1 (orange) plants, respectively. (c-d) Nicotianamine and 2-deoxymugineic acid concentrations ($\mu\text{mol mg}^{-1}$) in the shoots of NS and CE-1 plants, respectively. Bars represent mean $\pm$ SEM of three biological replicates. Asterisks denote the significance between NS and CE-OsNAS2 events for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

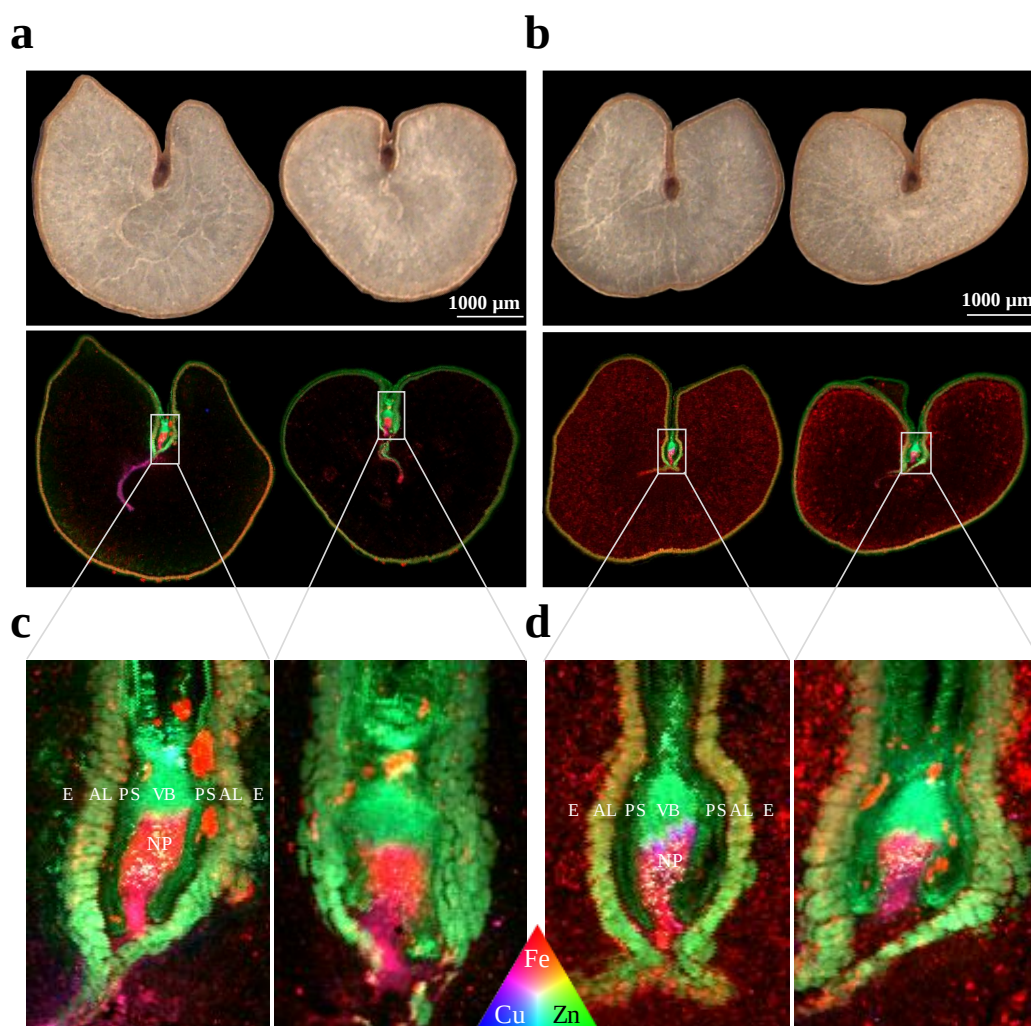


Figure S4.4. (a-b) Bright field images of NS and CE-1 grain sections and corresponding tri-colour XFM elemental maps of Fe (red), Zn (green) and Cu (blue). (c-d) Enlarged view of the crease region in NS and CE-1 grain, respectively. The endosperm (E), modified aleurone cells (AL), pigment strand (PS), vascular bundle (VB) and nucellar projection (NP) are labelled.

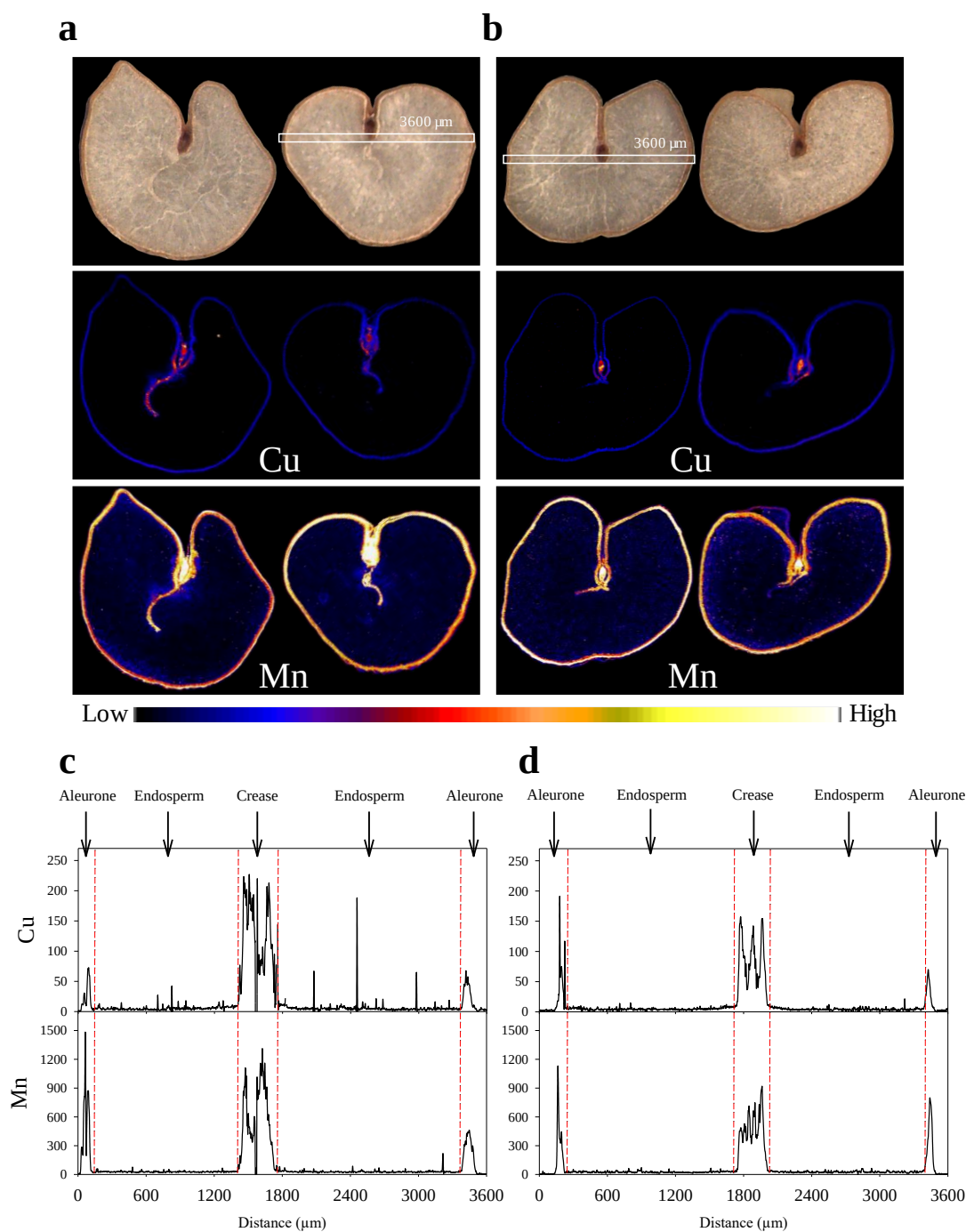


Figure S4.5. (a-b) Bright field images of NS and CE-1 grain sections and corresponding Cu and Mn elemental maps. Colour bar represents high (white) and low (black) elemental concentrations. White boxes in bright field images represent areas used to obtain line scans. (c) Line scans showing the distribution and signal intensity of Cu and Mn across NS grain. (d) Line scans showing the distribution and signal intensity of Cu and Mn across CE-1 grain. Units for the y-axis are elemental counts per micron.

Table S4.1. Iron and zinc concentrations ($\mu\text{g g}^{-1}$ DW) in NS and CE-1 plant tissues at 5-8, 12-15, 19-21 and 26-29 days after anthesis (DAA) as well as maturity. Values represent mean $\pm$ SEM of at least three biological replicates. Asterisks denote the significance between NS and CE-1 for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

	Timepoint	Fe ($\mu\text{g g}^{-1}$)		Zn ($\mu\text{g g}^{-1}$)	
		CE-1	NS	CE-1	NS
<i>Grain</i>	5-8 DAA	40 $\pm$ 7.1*	21 $\pm$ 5.5	99 $\pm$ 5.3*	83 $\pm$ 2.4
	12-15 DAA	52 $\pm$ 4.6*	41 $\pm$ 2.2	121 $\pm$ 11.1*	91 $\pm$ 4.5
	19-21 DAA	30 $\pm$ 1.9**	20 $\pm$ 1.3	110 $\pm$ 4.5***	82 $\pm$ 3.1
	26-29 DAA	63 $\pm$ 4.9	51 $\pm$ 5.6	98 $\pm$ 3.5**	72 $\pm$ 5.2
	Maturity	71 $\pm$ 5.3**	51 $\pm$ 2.5	124 $\pm$ 7.6**	90 $\pm$ 4.6
<i>Bracts</i>	5-8 DAA	17 $\pm$ 2.8	19 $\pm$ 3.4	57 $\pm$ 4.2*	47 $\pm$ 0.6
	12-15 DAA	29 $\pm$ 1.8	25 $\pm$ 1.4	62 $\pm$ 3.0***	42 $\pm$ 2.5
	19-21 DAA	23 $\pm$ 7.5	11 $\pm$ 0.2	51 $\pm$ 3.0*	41 $\pm$ 2.2
	26-29 DAA	20 $\pm$ 1.5	17 $\pm$ 2.0	55 $\pm$ 4.3*	42 $\pm$ 1.7
	Maturity	21 $\pm$ 4.4**	14 $\pm$ 1.2	84 $\pm$ 11.4	76 $\pm$ 7.7
<i>Rachis</i>	5-8 DAA	118 $\pm$ 7.9*	93 $\pm$ 6.9	165 $\pm$ 11.8*	127 $\pm$ 5.5
	12-15 DAA	42 $\pm$ 4.4*	31 $\pm$ 2.9	139 $\pm$ 15.6*	93 $\pm$ 5.8
	19-21 DAA	68 $\pm$ 1.8	61 $\pm$ 5.4	137 $\pm$ 10.0**	101 $\pm$ 6.9
	26-29 DAA	17 $\pm$ 1.3	22 $\pm$ 9.5	122 $\pm$ 17.6*	81 $\pm$ 4.1
	Maturity	20 $\pm$ 4.7	-	73 $\pm$ 9.2	64 $\pm$ 4.1
<i>Flag leaf</i>	5-8 DAA	66 $\pm$ 4.4	79 $\pm$ 13.8*	61 $\pm$ 4.6	66 $\pm$ 2.8
	12-15 DAA	66 $\pm$ 1.4	63 $\pm$ 1.3	67 $\pm$ 4.7	72 $\pm$ 8.3
	19-21 DAA	82 $\pm$ 11.7	62 $\pm$ 3.9	55 $\pm$ 3.1	75 $\pm$ 4.8**
	26-29 DAA	53 $\pm$ 2.3	50 $\pm$ 3.2	48 $\pm$ 5.3	64 $\pm$ 1.8**
	Maturity	28 $\pm$ 4.3	20 $\pm$ 1.1	62 $\pm$ 6.2	49 $\pm$ 13.6

Table S4.2. Biomass (mg DW) of NS and CE-1 plant tissues at 5-8, 12-15, 19-21 and 26-29 days after anthesis (DAA) as well as maturity. Values represent mean $\pm$ SEM of at least three biological replicates. Asterisks denote the significance between NS and CE-1 for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

	Timepoint	Biomass (mg DW)	
		CE-1	NS
<i>Grain</i>	5-8 DAA	195 $\pm$ 19.4	235 $\pm$ 32.5
	12-15 DAA	628 $\pm$ 40.9	764 $\pm$ 56.2*
	19-21 DAA	1083 $\pm$ 68.8	1390 $\pm$ 100.5*
	26-29 DAA	1491 $\pm$ 97.1	1687 $\pm$ 73.7
	Maturity	1956 $\pm$ 177.1	1949 $\pm$ 155.3
<i>Bracts</i>	5-8 DAA	451 $\pm$ 12.6	486 $\pm$ 44.0
	12-15 DAA	582 $\pm$ 25.4	537 $\pm$ 32.3
	19-21 DAA	574 $\pm$ 26.7	599 $\pm$ 20.6
	26-29 DAA	523 $\pm$ 57.8	602 $\pm$ 63.0
	Maturity	627 $\pm$ 51.3	625 $\pm$ 36.4
<i>Rachis</i>	5-8 DAA	102 $\pm$ 3.0	107 $\pm$ 6.0
	12-15 DAA	114 $\pm$ 7.6	117 $\pm$ 3.4
	19-21 DAA	128 $\pm$ 2.7	132 $\pm$ 2.8
	26-29 DAA	117 $\pm$ 3.4	116 $\pm$ 3.5
	Maturity	120 $\pm$ 9.6	120 $\pm$ 9.8
<i>Flag leaf</i>	5-8 DAA	155 $\pm$ 8.4	175 $\pm$ 9.6
	12-15 DAA	187 $\pm$ 8.4	182 $\pm$ 5.9
	19-21 DAA	169 $\pm$ 8.9	200 $\pm$ 12.0*
	26-29 DAA	164 $\pm$ 9.8	175 $\pm$ 14.6
	Maturity	122 $\pm$ 11.6	112 $\pm$ 15.3

Table S4.3. Agronomic performance of NS and CE-1 sibling lines grown at the Katanning and Merredin field sites. Values represent mean $\pm$ SEM of three biological replicates. Asterisks denote the significance between NS and CE-1 sibling lines for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

Field Site	Genotype	Plant height (cm)	Spike number (m ²)	Biomass (kg ha ⁻¹)	TGW (g)	Grain yield (kg ha ⁻¹)
Katanning	NS	59.7 $\pm$ 2.1	320 $\pm$ 9	5176 $\pm$ 488	26.1 $\pm$ 1.8	1358 $\pm$ 234
	CE-1.1	51.7 $\pm$ 3.6	225 $\pm$ 31	3720 $\pm$ 697	29.8 $\pm$ 1.9	860 $\pm$ 175
	CE-1.2	56.8 $\pm$ 2.9	236 $\pm$ 22	3882 $\pm$ 678	26.7 $\pm$ 2.3	846 $\pm$ 111
	CE-1.3	58.8 $\pm$ 0.8	314 $\pm$ 9	4667 $\pm$ 342	25.6 $\pm$ 1.0	1328 $\pm$ 195
Merredin	NS	69.6 $\pm$ 0.5	419 $\pm$ 14	10433 $\pm$ 870	32.7 $\pm$ 1.0	2633 $\pm$ 345
	CE-1.1	65.3 $\pm$ 0.3*	354 $\pm$ 22	8776 $\pm$ 519	31.5 $\pm$ 0.5	1831 $\pm$ 343
	CE-1.2	66.7 $\pm$ 1.4	317 $\pm$ 51	9176 $\pm$ 1236	33.0 $\pm$ 0.7	2120 $\pm$ 375
	CE-1.3	70.3 $\pm$ 2.0	424 $\pm$ 5	11147 $\pm$ 461	33.4 $\pm$ 2.2	2953 $\pm$ 698

Table S4.4. Wheat genes and primers used for quantitative reverse transcription PCR (qRT-PCR) analysis of CE-1 and NS seedling shoot and root tissue. The table provides gene name, forward and reverse primer sequences, qRT-PCR primer efficiency and PCR product length.

Gene name	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')	Primer efficiency (%)	Product length (bp)
<i>TaGAPDH</i>	TTCAACATCATTCCAAGCAGCA	CGTAACCCAAAATGCCCTTG	98.1	220
<i>TaELF</i>	CAGATTGGCAACGGCTACG	CGGACAGCAAAACGACCAAG	96.3	227
<i>TaWIN</i>	GGACAGCTTAGGCGAGGAAT	GCTGGGGCTTCCTTAATCTC	97.7	126
<i>TaVIP2</i>	AAGGGTGGATGGTGATAGCC	TTGATGTTGCCATGTGCCC	98.6	138
<i>UbiOsNAS2</i>	GTTCCAGAAGGCGGAAGAGT	AACGATCGGGGAAATTCG	95	166
<i>TaNAS1</i>	GAATGACGTCCGAGGAGAAG	CGATATCGTCCAGCTCCACT	98.3	135
<i>TaNAS2</i>	CGGCTTCCTGTACCCCATC	CTCCATCTTGGTGGAGAAGC	97.7	216
<i>TaNAS3</i>	TCCAGAAGATCACCGGACTC	CGAGCATGTCGGAGTAGTGC	96	225
<i>TaNAS4</i>	GTCTTCCTGGCCGCACTT	G TTCACCACGTCGTCGTCT	99.6	213
<i>TaNAS5</i>	GCGGGTTCCTATACCCGAT	TGCATGTCCTTCGACTTGTG	99.7	130
<i>TaNAS6</i>	CTCTTCACCGACCTGGTCAC	TG TAGTTGCTGTAGTAGGGGAAGAT	99.2	208
<i>TaNAS7</i>	GAGGCGGGTTCGAGGTGCTC	CACCATCTCGCCGAACCT	92.6	179
<i>TaNAS9</i>	GAGGAGGCCCTGGTGAAGA	GGATGCAGGACGTCACCA	99.1	118
<i>TaNAAT1</i>	CACATTGCCCCTGTCTTGTC	CTGGGTCCGTTGAGACGTTA	97.3	160
<i>TaNAAT2</i>	GGACCCAGCAACCTTCATT	GATCCTTCTGGCTTGTGAGG	96.7	165
<i>TaDMAS</i>	ATGGAGGAGTGCCACAGG	AGTAGGCGCACAGCTGGAT	96.1	193

Table S4.5. Elemental concentrations ($\mu\text{g g}^{-1}$ DW) of T₃ grain harvested from 10 NS and 9 CE-1 plants including the two batches used for synchrotron XFM analysis (in bold). Asterisks denote the significance between mean values of NS and CE-1 for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t-test.

Genotype	Plant	Grain number	Al	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	P	S	Zn
NS	1	213	0.10	370	7.0	44	4700	1390	161	1.5	7.4	< 0.4	4500	1520	78
	2	196	0.13	430	8.3	52	4900	1580	176	1.5	8.2	< 0.4	5100	1760	91
	3	318	0.19	480	7.8	43	4600	1510	143	1.6	7.6	< 0.4	4900	1580	69
	4	262	< 0.1	450	6.3	36	4600	1440	168	1.6	8.0	< 0.4	4600	1540	64
	5	243	0.14	400	5.6	34	4600	1310	142	1.5	8.4	< 0.4	4400	1340	60
	6	275	0.15	390	6.1	35	4700	1300	141	1.4	7.1	< 0.4	4300	1410	62
	7	192	0.38	450	7.3	44	4400	1520	179	1.5	9.7	< 0.4	4700	1650	79
	8	211	0.16	450	6.0	33	4600	1390	136	1.6	9.3	< 0.4	4400	1450	57
	9	185	0.12	410	6.0	38	4400	1420	156	1.5	8.5	< 0.4	4700	1630	71
	10	240	< 0.1	460	8.1	39	4400	1540	137	1.6	8.6	< 0.4	4900	1620	69
	Mean	234	0.2	429	7	40	4590	1440	154	2	8	na	4650	1550	70
	SEM	13	0.03	11	0.3	2	50	30	5	0.02	0.25	na	82	40	3
CE-1	1	246	0.33	420	9.2	55	4600	1620	152	1.2	11	< 0.4	4900	1750	96
	2	354	< 0.1	480	9.0	57	4700	1530	144	0.69	10	0.65	4900	1730	84
	3	253	< 0.1	510	11	69	4700	1840	112	0.92	9.2	0.40	5300	2100	122
	4	242	0.14	420	7.2	38	4400	1550	71	1.4	11	< 0.4	4600	1550	76
	5	254	< 0.1	460	8.4	44	4400	1670	58	1.4	9.1	< 0.4	5000	1770	90
	6	211	< 0.1	470	9.9	60	4400	1770	76	1.2	8.9	< 0.4	5300	1970	110
	7	294	0.14	530	11	64	4600	1750	107	0.83	12	< 0.4	5100	1990	111
	8	111	0.11	420	16	98	5900	2200	131	1.3	22	0.52	6000	2500	192
	9	276	< 0.1	470	9.5	64	5400	1690	125	0.87	13	< 0.4	5400	1960	114
	Mean	249	0.18	464	10***	61***	4789	1736***	108**	1.09***	12**	0.53	5167**	1924***	110***
	SEM	22	0.05	13	0.78	6	173	67	11	0.09	1	0.07	133	91	11

*elements not listed because below the detection limit: As (<1), Cd (<0.1), Co (<0.4), Cr (<0.2), Pb (<1), Se (<3) and Ti (<0.02).

Table S4.6. Soil properties of Katanning (K) and Merredin (M) field sites. Values represent mean $\pm$ SEM of three replicates and are provided in $\mu\text{g g}^{-1}$ DW unless otherwise specified. pH was determined using CaCl_2 . C = organic carbon.

Site	Type	NH_4^+	NO_3^-	P	K	S	Cu	Fe	Mn	Zn	Al	Ca	Mg	Na	B	C (%)	pH
K	Sand	6.3 ± 0.5	9.7 ± 1.9	37.7 ± 1.5	21.0 ± 2.8	4.4 ± 0.4	0.7 ± 0.1	76.0 ± 1.2	6.8 ± 0.4	2.0 ± 0.1	15.1 ± 1.3	62.6 ± 4.8	28.8 ± 1.5	9.2 ± 0.0	0.4 ± 0.0	1.5 ± 0.1	4.6 ± 0.1
M	Sand	4.7 ± 1.0	1.7 ± 0.3	65.0 ± 2.5	113.7 ± 11.8	3.9 ± 0.1	0.7 ± 0.0	123.4 ± 3.5	3.4 ± 0.1	1.8 ± 0.1	17.3 ± 1.9	87.3 ± 8.2	38.4 ± 3.0	8.4 ± 1.3	0.8 ± 0.0	1.1 ± 0.1	4.6 ± 0.0

5. Chapter 5 – Agronomic and nutritional evaluation of iron and zinc biofortified bread wheat across multiple field environments

5.1. Abstract

Conventional breeding efforts for iron (Fe) and zinc (Zn) biofortification of bread wheat (*Triticum aestivum* L.) have been hindered by a lack of genetic variation for these traits and negative correlation between grain Fe and Zn concentrations and yield. We have employed constitutive expression (CE) of the rice nicotianamine synthase 2 (*OsNAS2*) gene to upregulate biosynthesis of two metal chelators – nicotianamine (NA) and 2'-deoxymugineic acid (DMA) – in bread wheat, resulting in increased Fe and Zn concentrations in wholemeal and white flour. Here we describe multi-location confined field trial (CFT) evaluation of a lead transgenic CE-*OsNAS2* wheat event (CE-1) over three years and demonstrate higher concentrations of Fe, Zn, NA and DMA in CE-1 wholemeal flour, white flour and white bread, and higher Fe bioavailability in CE-1 white flour and white bread relative to a null segregant (NS) control. For all genotypes, grain yield was negatively correlated with wholemeal and white flour Fe, Zn, and total protein concentrations and not correlated with wholemeal and white flour NA and DMA concentrations. White flour Fe bioavailability was only positively correlated with white flour NA concentrations, suggesting that the chelated form of Fe should be a critical consideration in crop biofortification efforts.

5.2. Introduction

The two major types of wheat, bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* L.), are produced on more land than any other crop and processed into a variety of foods such as bread, noodles and pasta to supply ~20% of daily calorie intake to humans^{1,2}. Wheat production exceeded 770 million tonnes in 2017 and superseded rice (*Oryza sativa* L.) as the second most produced crop behind maize (*Zea mays* L.) in that year (FAOSTAT, <http://www.fao.org/faostat>). Within less developed countries of wheat primary production, such as countries in the MENAP (Middle East, North Africa, Afghanistan and Pakistan) region, wheat consumption can supply >40% of daily calorie intake and coincides with high prevalences of human iron (Fe) and zinc (Zn) deficiency^{3,4}. Crop biofortification represents a sustainable strategy to increase human micronutrient intake⁵, although Fe and Zn biofortification of wheat has largely been hindered by a lack of genomic resources, significant genotype (G) x environment (E) effects and a negative correlation with grain yield^{6–12}.

International breeding efforts over the last two decades have developed elite disease resistant and high yielding biofortified wheat with increased grain Zn (up to 1.3-fold) by exploiting grain micronutrient variation in *Triticum dicoccoides* wild varieties and developing synthetic hexaploids^{7,13,14}. Conventional breeding efforts to biofortify wheat with Fe have had limited success, with the largest increase in grain Fe (up to 1.2-fold) achieved by selecting for the

functional allele of *NAM-B1* (commonly referred to as *Gpc-B1*) which encodes a transcription factor that regulates flag leaf senescence and the remobilization of Fe and Zn from leaf tissue to the grain^{15–20}. Cereal biofortification through conventional breeding is also limited by the presence of dietary compounds such as phytate (inositol hexaphosphate), polyphenols and fibre that inhibit Fe and Zn absorption (bioavailability) in the human gut^{21–24}. Most Fe and Zn (70–80%) in wheat grain colocalizes with phytate-containing globoids in outer grain tissues such as the aleurone layer of the endosperm, and increased Fe and Zn concentrations in wholemeal flour per se may therefore not improve dietary micronutrient absorption among wheat consuming populations^{25–29}. The limited Fe and Zn (20–30%) in wheat inner endosperm does not colocalize with phytate and is therefore considered more bioavailable, and modern plant biotechnology allows researchers to overcome the physiological barriers to endosperm loading and increase the concentrations of Fe and Zn in white flour³⁰. Using genetic modification (GM) to improve crop nutritional quality and/or processing traits was recently coined second-generation GM with great potential to benefit human health in less developed countries³¹.

The loading of Fe and Zn into cereal grain is regulated by transporters and chelators with high metal specificity^{27,32}. In wheat all Fe and Zn enters the grain via the phloem and into the crease, a grain region comprised of the vascular bundle and nucellar projection, prior to redistribution of Fe to the aleurone and Zn to embryo and crease tissues^{26,28,33–36}. In the aleurone Fe is loaded and stored in cell vacuoles by vacuolar iron transporter (VIT) genes^{37–39} and expression of the wheat *TaVIT2* gene under the control of an endosperm-specific high-molecular-weight glutenin promoter (HMW) redirects Fe from the aleurone into the endosperm, doubling white flour Fe concentration without increasing total grain Fe^{27,40}. Phloem Fe is mostly bound to nicotianamine (NA), a non-protein amino acid that chelates Fe, Zn and other transition metals in higher plants and serves as a biosynthetic precursor to 2'-deoxymugenic acid (DMA), an endogenous Fe chelator in cereals that is secreted as a phytosiderophore to chelate Fe in the rhizosphere^{41–45}. The majority of Fe in white wheat flour (representing the inner endosperm) is chelated to NA and/or DMA⁴⁶ and constitutive expression (CE) of the rice nicotianamine synthase 2 (*OsNAS2*) gene increases both Fe (up to 2-fold) and Zn (up to 4-fold) concentrations in white flour^{30,47}. Multi-location confined field trial (CFT) evaluation of transgenic biofortified wheat plants such as HMW-*TaVIT2* and CE-*OsNAS2* is necessary to determine whether increased grain nutrition is maintained under realistic farming conditions while maintaining agronomic performance⁴⁸.

We have developed biofortified CE-*OsNAS2* wheat that accumulates additional grain Fe and Zn in endosperm and aleurone tissues, respectively and demonstrates no apparent yield penalty under glasshouse and field conditions³⁰. Here we comprehensively investigate agronomy and wholemeal flour, white flour and white bread nutrition in CE-*OsNAS2* wheat in multi-location CFTs over three consecutive seasons.

5.3. Results

Soil properties and environmental conditions varied between confined field environments

Three transgenic sibling lines of bread wheat cultivar (cv.) Bobwhite constitutively expressing the rice nicotianamine synthase 2 (*OsNAS2*) gene (Figure 5.1a) and derived from a double-insert transgenic event (hereafter referred to as CE-1.1, CE-1.2, CE-1.3) were grown alongside a null segregant control derived from the same event (hereafter referred to as NS). Mace, an elite Australian wheat cv. was included as a check. Replicated plots of all genotypes were evaluated in CFTs at three representative wheat growing environments in Australia: Glenthorne in South Australia, and Katanning and Merredin in Western Australia in 2015, 2016 and 2017 (Figures S5.1, S5.2). Soil pH, soil conductivity (EC) and the concentrations of soil macronutrients including ammonium (NH₄), nitrate (NO₃), phosphorus (P) potassium (K), sulfate (SO₄), and organic carbon (C) and soil micronutrients including aluminium (Al), calcium (Ca), magnesium (Mg), sodium (Na), copper (Cu), iron (Fe) manganese (Mn), zinc (Zn) and boron (B) at Glenthorne, Katanning and Merredin across the three field seasons are provided in Table S5.1. Total rainfall across the three field seasons varied between 493 to 949 mm at Glenthorne, 291 to 504 mm at Katanning, and 284 to 517 mm at Merredin (Figure S5.3). Merredin and Katanning have more temperate climates relative to Glenthorne with warmer temperatures in the summer months and cooler temperatures in the winter months. Daily temperatures (minimum/maximum) at Glenthorne varied from 12.6/21.8 °C in 2015, 12.7/21.4 °C in 2016, and 13.1/22.0 °C in 2017, at Katanning from 9.3/23.5 °C in 2015, 9.3/21.9 °C in 2016, and 9.5/22.9 °C in 2017, and at Merredin from 12.5/26.0 °C in 2015, 10.8/23.8 °C in 2016, and 12.0/25.9 °C in 2017 (Figure S5.3). Significant G x E components between CFTs were detected for all agronomic performance, wholemeal flour nutrition, white flour nutrition or leaf nutrition traits (Table S5.2).

Constitutive OsNAS2 expression had variable effects on agronomic performance and improved the nutritional composition of wholemeal flour, white flour and bran across multiple field environments

Glenthorne

Over three years of CFTs at Glenthorne (Figure 5.1b), soil pH varied from 7.1 – 7.6 and the concentrations of soil Fe and Zn varied from 4.5 – 32 mg/Kg (Figure 5.1c). Plant height (cm) was lower ($p < 0.05$) in all CE-1 sibling lines over three field seasons, and thousand grain weight was lower in CE-1.1 ($p \leq 0.001$) and CE-1.3 ($p < 0.05$) in 2017 relative to NS (Figure 5.1d,e). Grain yield (kg ha⁻¹) was lower ($p < 0.05$) in all CE-1 sibling lines relative to NS, apart from CE-1.3 in 2016 and CE-1.2 in 2017 (Figure 5.1f). Grain number (m²) was lower ($p \leq 0.01$) in all CE-1 sibling lines in 2015, and grain number (m²), tiller number (m²) and harvest index (%) were lower ($p < 0.05$) in CE-1.1 in 2016 relative to NS (Table S5.3). Wholemeal and white flour Fe

concentrations were higher ($p \leq 0.01$) in CE-1.1 over three field seasons, and wholemeal Fe concentrations were higher ($p \leq 0.001$) in CE-1.2 and CE-1.3 in 2016 relative to NS (Figure 5.1g). Wholemeal and white flour Zn concentrations ($p < 0.05$), and nicotianamine (NA) and 2'-deoxymugineic acid (DMA) concentrations ($p \leq 0.01$) were higher in all CE-1 sibling lines over three field seasons relative to NS, apart from white flour DMA concentrations in CE-1.1 and CE-1.3 in 2015 (Figure 5.1h-j). Bran Fe concentrations were higher ($p \leq 0.01$) in all CE-1 sibling lines in 2016 and CE-1.1 in 2017, and bran Zn concentrations were higher ($p \leq 0.01$) in all CE-1 sibling lines over three field seasons relative to NS (Table S5.4). Sibling line CE-1.1 had higher ($p \leq 0.01$) wholemeal flour P concentrations in 2016, white flour P and total protein concentrations in 2015 and 2016, and wholemeal flour total protein concentrations across three field seasons relative to NS (Table S5.5). In 2016, wholemeal Cu, Mg, Mn and S concentrations, white flour S concentrations, and bran Cu and S concentrations were higher ($p < 0.05$) in all CE-1 sibling lines relative to NS (Tables S5.6-S5.8).

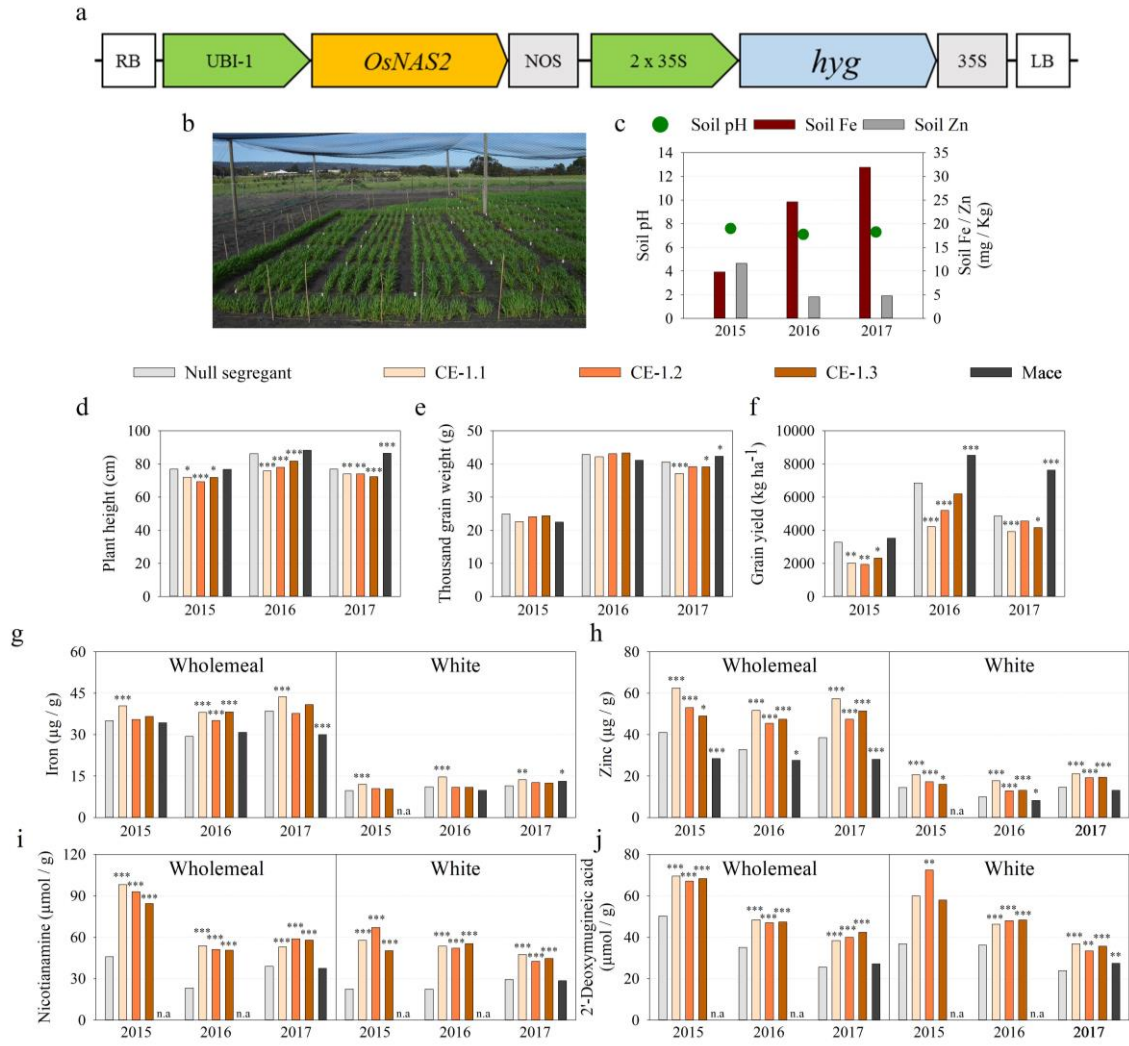


Figure 5.1. Agronomic performance of bread wheat constitutively expressing the rice nicotianamine synthase 2 (*OsNAS2*) gene (CE-1.1, CE-1.2, and CE-1.3) alongside the null segregant and cv. Mace over three field seasons at Glenthorne. **a)** Schematic representation of the T-DNA construct. RB and LB: right and left borders, respectively; UBI-1: maize ubiquitin 1 promoter; *OsNAS2*: rice nicotianamine synthase 2 gene (LOC_Os03 g19420); NOS: nopaline synthase terminator; 2 x 35S: dual promoter of 35S cauliflower mosaic virus gene; *hyg*: hygromycin phosphotransferase gene; 35S: terminator of 35S cauliflower mosaic virus gene. **b)** an image of replicated plots at Glenthorne during the 2017 field season. **c)** Soil pH (green circles), as well as soil Fe (red bars) and Zn (dark grey bars) concentrations (mg / Kg) are provided for the three field seasons. **d-j)** Bars indicate best linear unbiased estimators of the null segregant (light grey), CE-1.1 (light brown), CE-1.2 (orange), CE-1.3 (dark brown), and Mace (black) for **d)** plant height (cm), **e)** thousand grain weight (g), **f)** grain yield (kg ha⁻¹) and the concentrations of **g)** Fe (µg / g), **h)** Zn (µg / g), **i)** NA (µmol / g), and **j)** DMA (µmol / g) in wholemeal flour (left panel) and white flour (right panel). A minimum of three replicates per genotype were included for each field season and asterisks represent significant differences to the NS for p < 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) as determined by Wald test. n.a = not applicable.

Katanning

Over three years of CFTs at Katanning (Figure 5.2a), soil pH varied between 4.4 – 5.3, soil Fe concentrations varied between 41.5 – 85.6 mg/Kg, and soil Zn concentrations varied between 1.4 – 13.6 mg/Kg (Figure 5.2b). Plant height (cm) and grain yield (kg ha^{-1}) were lower ($p < 0.05$) in CE-1.1 and CE-1.2 in 2015, and plant height (cm) was lower ($p \leq 0.01$) in all CE-1 sibling lines in 2016 relative to NS (Figure 5.2c-e). Tiller number (m^2), harvest index (%), biomass (kg ha^{-1}) and grain number (m^2) were lower ($p < 0.05$) in CE-1.1 and CE-1.2 in 2015 relative to NS (Table S5.3). Wholemeal and white flour Fe, Zn, NA and DMA concentrations were higher ($p < 0.05$) in all CE-1 sibling lines over three field seasons relative to NS, apart from wholemeal flour Fe and Zn in CE-1.2 in 2015, white flour Fe in CE-1.2 in 2017, and wholemeal flour Zn in CE-1.3 in 2015 and CE-1.2 in 2016 (Figure 5.2f-i). Bran Fe concentrations were higher in CE-1.1 ($p \leq 0.001$) in 2015, CE-1.1 and CE-1.3 ($p \leq 0.01$) in 2016 and all CE-1 sibling lines ($p < 0.05$) in 2017 relative to NS (Table S5.4). Bran Zn concentrations were higher ($p < 0.05$) in all CE-1 sibling lines over three field seasons relative to NS, apart from CE-1.2 and CE-1.3 in 2015. Sibling line CE-1.1 had higher ($p < 0.05$) wholemeal flour P concentrations in 2016 and 2017 and white flour P concentrations in 2015, and sibling line CE-1.3 had higher ($p < 0.05$) white flour P concentrations in 2015 and 2017 relative to NS (Table S5.5). All CE-1 sibling lines had higher ($p \leq 0.01$) white flour total protein and S concentrations in 2015, white flour Ca concentrations in 2016, and white flour Mg concentrations over three field seasons relative to NS (Tables S5.5, S5.7).

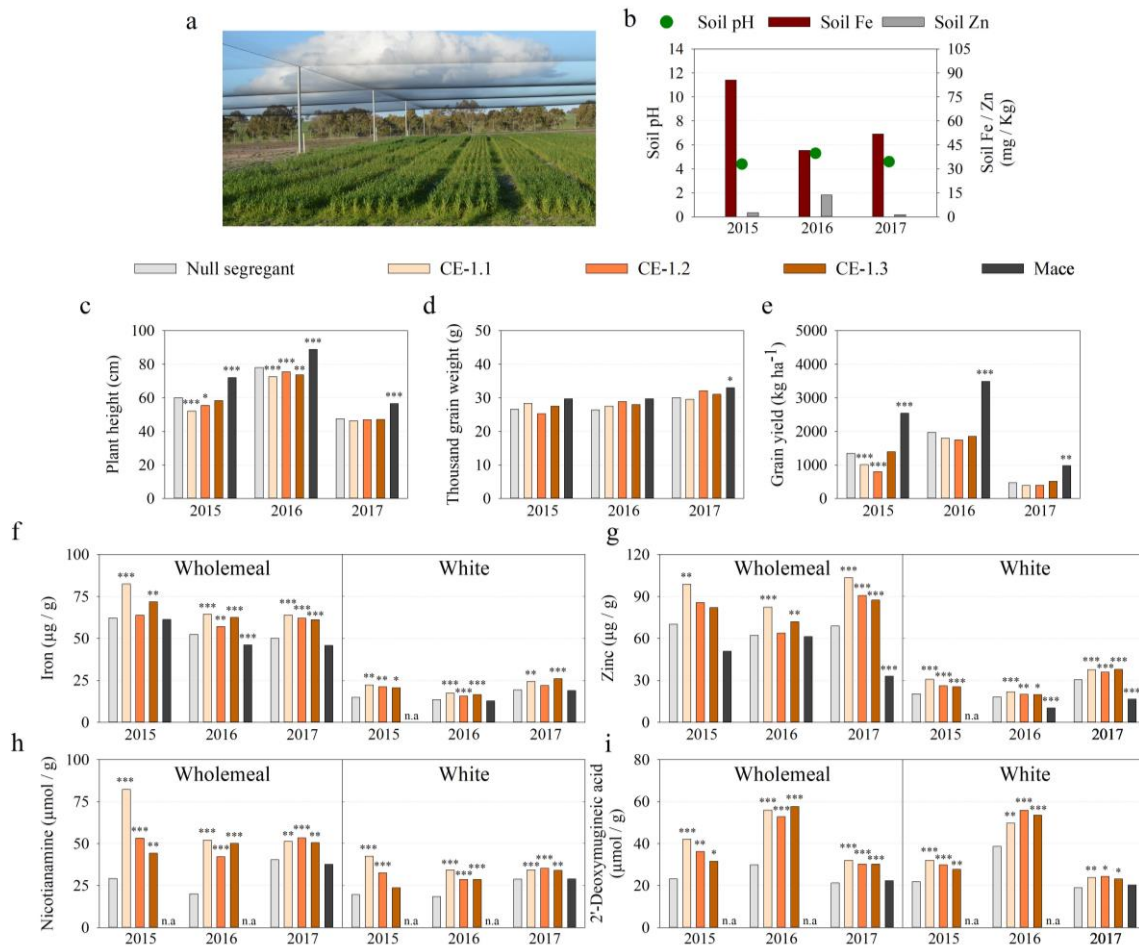


Figure 5.2. Agronomic performance, wholemeal and white flour nutritional composition of null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace over three field seasons at Katanning. **a)** An image of 18m² replicated plots for the 2017 field season is provided. **b)** Soil pH (green circles), as well as soil Fe (red bars) and Zn (dark grey bars) concentrations (mg / Kg) are provided for the three field seasons. **c-i)** Bars indicate best linear unbiased estimators of the null segregant (light grey), CE-1.1 (light brown), CE-1.2 (orange), CE-1.3 (dark brown), and Mace (black) for **c)** plant height (cm), **d)** thousand grain weight (g), **e)** grain yield (kg ha⁻¹) and the concentrations of **f)** Fe (µg / g), **g)** Zn (µg / g), **h)** NA (µmol / g), and **i)** DMA (µmol / g) in wholemeal flour (left panel) and white flour (right panel). A minimum of three replicates per genotype were included for each field season and asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable.

Merredin

Over three years of CFTs at Merredin (Figure 5.3a), soil pH varied between 4.6 – 4.8, soil Fe concentrations varied between 51.0 – 124.0 mg/Kg, and soil Zn concentrations varied between 1.4 – 2.3 mg/Kg (Figure 5.3b). Plant height (cm) and thousand grain weight were lower ($p \leq 0.01$) in CE-1.1 in 2016, plant height (cm) was lower ($p < 0.05$) in all CE-1 sibling lines in 2017, and grain yield (kg ha^{-1}) was lower ($p \leq 0.01$) in CE-1.1 in 2017 relative to NS (Figure 5.3c-e). Tiller number (m^2) was lower ($p < 0.05$) in CE-1.2 in 2015, biomass (kg ha^{-1}) was lower ($p \leq 0.01$) in CE-1.1 in 2016 and harvest index (%) was lower ($p < 0.05$) in CE-1.1 in 2017 relative to NS (Table S5.3). Wholemeal flour Fe concentrations were higher ($p \leq 0.001$) in CE-1.1 in 2015, and wholemeal flour Fe and Zn concentrations were higher ($p < 0.05$) in CE-1.1 and CE-1.2 in 2016 and all CE-1 sibling lines in 2017 relative to NS (Figure 5.3f-g). White flour Fe concentrations were higher ($p \leq 0.01$) in CE-1.1 in 2015 and 2016, and white flour Zn concentrations were higher ($p \leq 0.001$) in all CE-1 sibling lines in 2016 and CE-1.1 in 2017 relative to NS. Wholemeal and white flour NA and DMA concentrations were higher ($p < 0.05$) in all CE-1 sibling lines over three field seasons relative to NS, apart from white flour DMA concentrations in CE-1.3 in 2016 (Figure 5.3h-i). Bran Fe concentrations were higher in CE-1.1 ($p \leq 0.001$) in 2015 and CE-1.3 ($p < 0.05$) in 2016 relative to NS (Table S5.4). Bran Zn concentrations were higher ($p < 0.05$) in CE-1.1 over three field seasons and CE-1.3 in 2017 relative to NS. Sibling line CE-1.1 had higher ($p < 0.05$) wholemeal flour P and Mn concentrations in 2016 and 2017, total protein concentrations in 2015 and 2016, and Mg and S concentrations over three field seasons relative to NS (Tables S5.5, S5.6). All CE-1 sibling lines had higher ($p < 0.05$) white flour Cu concentrations in 2016, white flour Mg concentrations in 2015 and 2017, white flour S concentrations in 2015 and 2016 relative to NS (Tables S5.5, S5.7). All CE-1 sibling lines had lower ($p < 0.05$) bran Mg concentrations in 2016 relative to NS (Table S5.8). Across all CFTs Bobwhite had overall lower ($p < 0.05$) agronomic performance and higher wholemeal flour, white flour and bran nutrition relative to the check, apart from wholemeal and white flour K concentrations (Figures 5.1-5.3, Tables S5.4- S5.8).

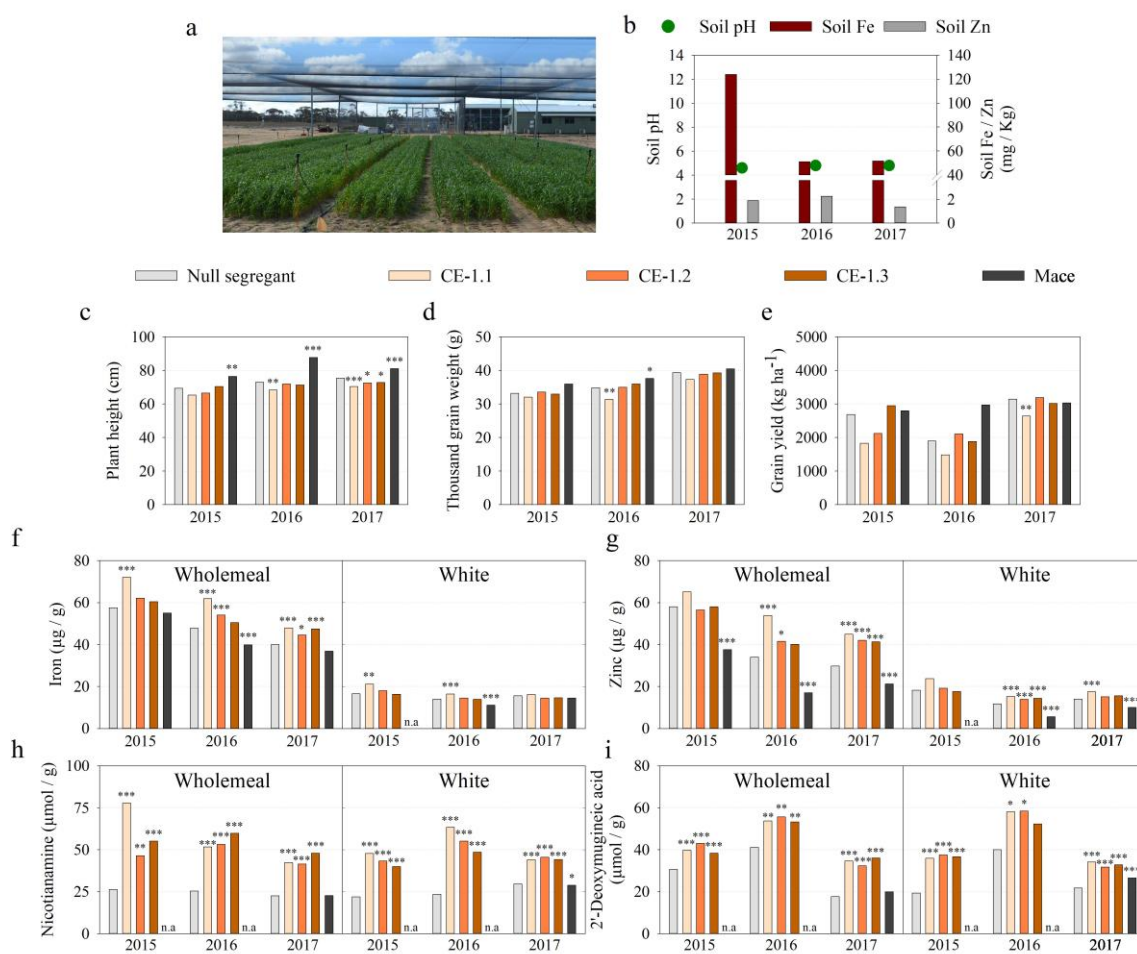


Figure 5.3. Agronomic performance, wholemeal and white flour nutritional composition of null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace over three field seasons at Merredin. **a**) An image of 18m² replicated plots for the 2017 field season is provided. **b**) Soil pH (green circles), as well as soil Fe (red bars) and Zn (dark grey bars) concentrations (mg / Kg) are provided for the three field seasons. **c-i**) Bars indicate best linear unbiased estimators of the null segregant (light grey), CE-1.1 (light brown), CE-1.2 (orange), CE-1.3 (dark brown), and Mace (black) for **c**) plant height (cm), **d**) thousand grain weight (g), **e**) grain yield (kg ha⁻¹) and the concentrations of **f**) Fe (µg / g), **g**) Zn (µg / g), **h**) NA (µmol / g), and **i**) DMA (µmol / g) in wholemeal flour (left panel) and white flour (right panel). A minimum of three replicates per genotype were included for each field season and asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable.

Constitutive OsNAS2 expression had variable effects on leaf nutrition across multiple environments

Flag leaf Fe concentrations were lower ($p < 0.05$) in CE-1.1 at Katanning in 2016 and Merredin in 2015, in CE-1.2 at Katanning in 2015 and Merredin over three field seasons, and in CE-1.3 in Katanning and Merredin in 2016 (Table S5.9). All CE-1 sibling lines had higher ($p < 0.05$) flag leaf P concentrations at Glenthorne in 2016 and Merredin in 2015, higher ($p \leq 0.01$) flag leaf K

concentrations at Glenthorne in 2016 and Merredin in 2017, and higher ($p < 0.05$) flag leaf and leaf S concentrations at Merredin in 2015 relative to NS (Tables S5.9- S5.11). Across all CFTs Bobwhite had overall lower concentrations of flag leaf Fe, P and Mn relative to the check. Leaf Fe and S were negatively correlated with leaf P ($r < -0.80$, $p < 0.01$) and leaf Fe was negatively correlated with flag leaf Zn ($r < -0.80$, $p < 0.05$) (Figure S5.4). Leaf Cu and K were positively correlated ($r > 0.90$, $p \leq 0.001$) and negatively correlated with leaf Ca ($r < -0.90$, $p \leq 0.001$), Mg, Mn, and P ($r < -0.70$, $p < 0.05$). Flag leaf removal at anthesis did not affect the concentration of grain Fe in CE-1.2, CE-1.3 and NS at Katanning, yet in the glasshouse, flag leaf removal reduced grain Fe concentrations in CE-1.2 and CE-1.3 and not NS (Figure S5.5). Flag leaf removal at anthesis resulted in a higher ($p \leq 0.01$) concentration of grain Zn in CE-1.2 and CE-1.3 relative to NS at Katanning.

White flour Fe bioavailability was positively correlated with grain NA and DMA concentrations and grain yield, and negatively correlated with grain Fe, Zn, P and protein concentrations

Iron bioavailability determined through the coupled *in vitro* digestion/Caco-2 cell model was higher ($p < 0.05$) in white flour of all CE-1 sibling lines at Glenthorne and Katanning over three field seasons relative to NS, apart from CE-1.1 and CE-1.2 at Glenthorne in 2016, CE-1.3 at Katanning in 2016, and CE-1.1 and CE-1.2 at Glenthorne and Katanning in 2017 (Figure S5.6). Across all CFTs white flour Fe bioavailability was positively correlated with white flour NA concentration ($r = 0.77$, $p < 0.05$) (Figure 5.4). Wholemeal and white flour Fe, Zn and total protein concentrations were positively correlated ($r \geq 0.93$, $p \leq 0.001$), and negatively correlated with tiller number ($r < -0.75$, $p < 0.05$), plant height ($r \leq -0.95$, $p \leq 0.001$), plant biomass ($r \leq -0.86$, $p \leq 0.001$), grain number ($r \leq -0.93$, $p \leq 0.001$), and grain yield ($r \leq -0.92$, $p \leq 0.001$). All wholemeal and white flour nutrition traits clustered separately from agronomic performance traits except for wholemeal and white flour NA, DMA and Cu concentrations, white flour K concentrations and white flour Fe bioavailability (Figure S5.7).

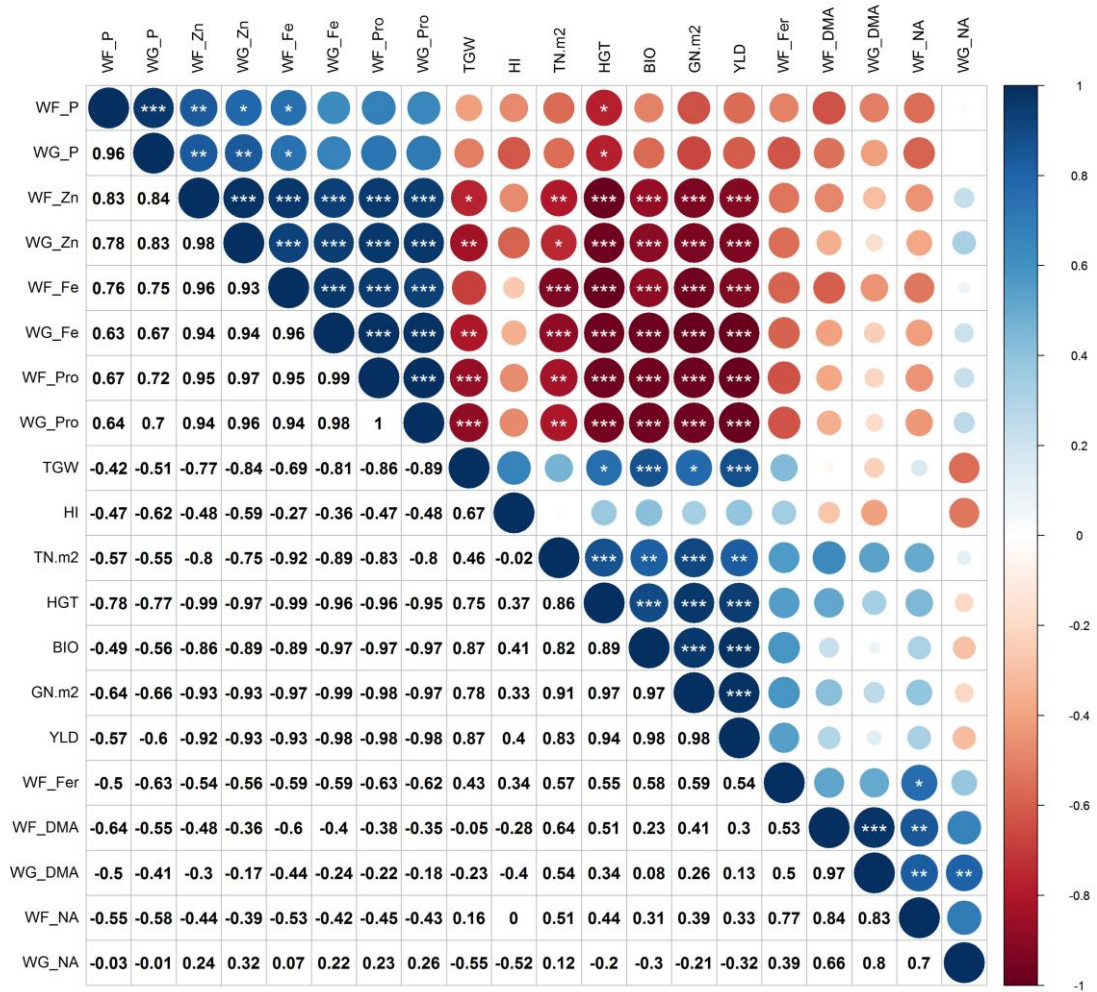


Figure 5.4. Multi-trait correlation of empirical best linear unbiased estimators (eBLUEs) obtained from the multi-environment models of agronomic performance, grain nutrition and white flour nutrition of NS, CE-1 and Mace. Agronomic performance variables include grain yield (YLD), harvest index (HI), thousand grain weight (TGW), total biomass (BIO), tiller number per m² (TN.m2), grain number per m² (GN.m2), plant height (HGT). Wholemeal (WG) and white flour (WF) nutrition variables include Fe, Zn, P, NA, and DMA concentrations, total protein (Pro) and Caco-2 cell ferritin production (Fer). Each circle represents a positive (blue) or negative (red) pairwise Pearson correlation coefficient between variables, with the diameter of the circle proportional to the absolute value. Asterisks denote significant correlations for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Constitutive OsNAS2 expression improved grain fraction and white bread nutritional composition across multiple field environments, and white bread Fe bioavailability at Katanning and Merredin

Iron, Zn, NA and DMA concentrations were higher ($p < 0.05$) in all CE-1 grain fractions relative to NS at Merredin in 2017, except for Fe and Zn in break 1 and 2 and Fe in reduction 1 (Table 5.1). Phytate concentrations were higher ($p < 0.05$) in break 3, reduction 2 and bran of CE-1 grain

relative to NS, and in CE-1.3 white flour relative to the NS at Katanning in 2017 (Table 5.1, Figure S5.8). White bread Fe, Zn, NA and DMA concentrations were higher ($p \leq 0.01$) in all CE-1 sibling lines relative to NS at all field locations in 2016, apart from Fe concentrations in all CE-1 sibling lines at Glenthorne (Figure 5.5a-d). Caco-2 cell ferritin formation was higher ($p < 0.05$) following exposure to digests of white bread from CE-1.1 and CE-1.2 at Katanning and from CE-1.2 at Merredin, relative to digests of NS white bread (Figure 5.5e). Caco-2 cell ferritin formation was lower ($p \leq 0.01$) following exposure to digests of white bread from CE-1.2 and CE-1.3 at Glenthorne relative to digests of NS white bread.

Table 5.1. The concentrations of Fe ($\mu\text{g} / \text{g}$), Zn ($\mu\text{g} / \text{g}$), NA ($\mu\text{mol} / \text{g}$), DMA ($\mu\text{mol} / \text{g}$), and phytate (mg / g) in null segregant (NS) and CE-1 grain fractions at Merredin in 2017. Values represent mean $\pm$ SEM of three biological replicates with three technical replicates for Fe and Zn, four biological replicates for NA and DMA, and two biological replicates with three technical replicates for phytate. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

Grain Region	Fraction	Iron ($\mu\text{g} / \text{g}$)	Zinc ($\mu\text{g} / \text{g}$)	Nicotianamine ($\mu\text{mol} / \text{g}$)	2'-Deoxymugineic Acid ($\mu\text{mol} / \text{g}$)	Phytate (mg / g)
Endosperm	NS Break 1	17.9 $\pm$ 0.4	11.4 $\pm$ 0.1	33.9 $\pm$ 0.1	25.8 $\pm$ 0.3	1.3 $\pm$ <0.1
	CE-1 Break 1	19.5 $\pm$ 0.7	13.2 $\pm$ 0.6	50.6 $\pm$ 1.1 ***	35.5 $\pm$ 0.8 ***	1.4 $\pm$ <0.1
	NS Break 2	19.3 $\pm$ <0.1	11.6 $\pm$ 0.1	34 $\pm$ 0.2	25.2 $\pm$ 0.7	1.2 $\pm$ 0.1
	CE-1 Break 2	22 $\pm$ 0.9	12.4 $\pm$ 0.2	51.5 $\pm$ 0.7 ***	34.5 $\pm$ 0.6 ***	1.2 $\pm$ <0.1
	NS Break 3	37.8 $\pm$ 1.4	16.8 $\pm$ 0.4	33.7 $\pm$ 0.1	25 $\pm$ 0.2	2.8 $\pm$ <0.1
	CE-1 Break 3	45.8 $\pm$ 0.8 *	22.6 $\pm$ 0.2 ***	51 $\pm$ 0.4 ***	36.8 $\pm$ 1.3 ***	3.3 $\pm$ <0.1 *
	NS Reduction 1	13.8 $\pm$ 0.3	12.1 $\pm$ 0.1	33.9 $\pm$ 0.3	25.6 $\pm$ 0.3	1.5 $\pm$ <0.1
	CE-1 Reduction 1	16.1 $\pm$ 1.1	14.3 $\pm$ 0.1 ***	53.2 $\pm$ 0.2 ***	36.6 $\pm$ 0.7 ***	1.7 $\pm$ 0.1
	NS Reduction 2	18.6 $\pm$ 0.1	16.6 $\pm$ 0.1	35.9 $\pm$ 0.3	27.5 $\pm$ 0.6	1.9 $\pm$ <0.1
	CE-1 Reduction 2	24.9 $\pm$ 0.1 **	24 $\pm$ 0.2 ***	52.7 $\pm$ 0.7 ***	35.9 $\pm$ 0.4 ***	2.5 $\pm$ <0.1 ***
	NS Reduction 3	30 $\pm$ 0.6	25.3 $\pm$ <0.1	35.8 $\pm$ 0.5	25.4 $\pm$ 0.7	3.4 $\pm$ 0.2
	CE-1 Reduction 3	35.5 $\pm$ 0.4 ***	33.3 $\pm$ 0.2 ***	53.4 $\pm$ 0.3 ***	36.3 $\pm$ 1.3 ***	3.2 $\pm$ 0.1
Aleurone	NS Pollard	107.3 $\pm$ 1.1	84.6 $\pm$ 0.9	45.3 $\pm$ 0.4	25.9 $\pm$ 0.2	9.4 $\pm$ 0.3
	CE-1 Pollard	123.2 $\pm$ 0.9 ***	102.1 $\pm$ 0.7 ***	69.7 $\pm$ 0.4 ***	34.7 $\pm$ 0.4 ***	7.6 $\pm$ 0.2
Pericarp / Testa	NS Bran	135.2 $\pm$ 0.4	95.7 $\pm$ 0.4	50.2 $\pm$ 0.7	27.4 $\pm$ 0.4	10.4 $\pm$ 0.2
	CE-1 Bran	148.2 $\pm$ 1.5 ***	115.5 $\pm$ 0.5 ***	71.5 $\pm$ 0.7 ***	34.8 $\pm$ 0.5 ***	13.8 $\pm$ 0.5 *

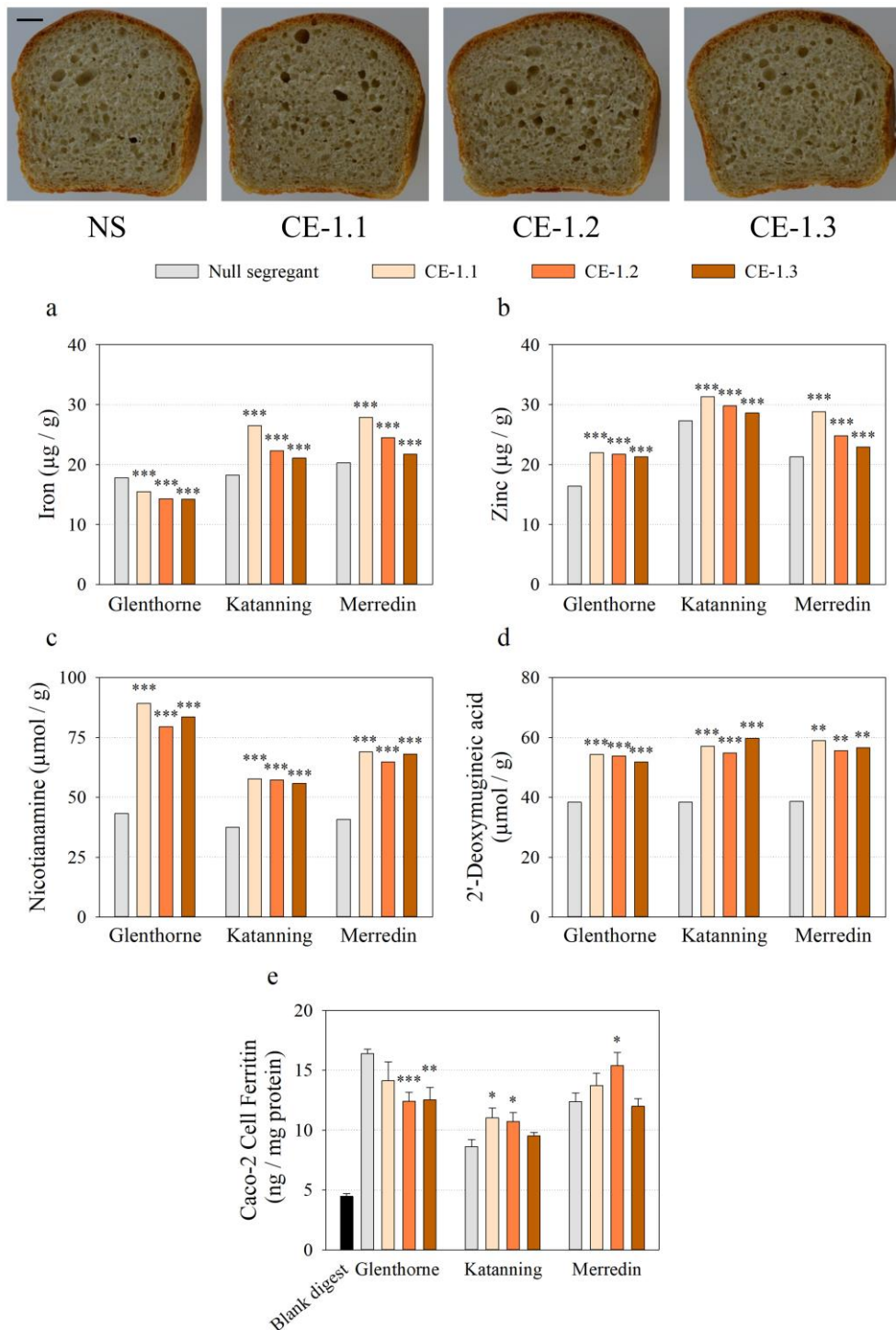


Figure 5.5. White bread nutritional composition of NS and CE-1 sibling lines at Glenthorne, Katanning and Merredin. Bars indicate best linear unbiased estimators for the concentrations of **a)** Fe ($\mu\text{g/g}$), **b)** Zn ($\mu\text{g/g}$), **c)** NA ($\mu\text{mol/g}$), and **d)** DMA ($\mu\text{mol/g}$) at each field site in 2016. Three replicates per genotype were included in the analysis and asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. **e)** Caco-2 cell ferritin formation following exposure to white bread digests at each field site in 2017. Bars represent the mean $\pm$ SEM of six replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test. Photos show representative cross sections of NS and CE-1 loaves from Katanning, 2017 prior to analysis. Scale bar = 1cm.

5.4. Discussion

A key component of biofortified crop development involves multi-location CFTs to determine if target micronutrient concentrations are affected by genotype x environment (G x E) interactions and/or have impacts on yield^{5,49}. Previous field evaluation of Zn biofortified wheat cultivars at nine different locations highlighted significant G x E interactions on grain Fe and Zn concentrations, and confirmed that grain nutrition was more affected by environment than genotype¹⁵. More recent multi-location CFT evaluation of two leading Fe and Zn biofortified rice transgenic events overexpressing the *OsNAS2* gene in combination with endosperm-specific expression of the soybean (*Glycine max* L.) ferritin (*Sfer-H1*) gene uncovered that one event (termed NASFer-274) exhibited no yield penalty relative to the null control⁴⁸. We previously reported that CE-1 wheat plants exhibited no apparent agronomic difference to NS plants when grown under glasshouse and field conditions, however, the field component of that research was limited to two sites and one season³⁰. In this study we comprehensively evaluated three CE-1 sibling lines in multi-location CFTs over three field seasons and detected a small but consistently significant reduction in plant height for all three CE-1 sibling lines relative to NS (Figures 5.1-5.3). Increased *NAS* gene activity may reduce plant height through altered biosynthesis of the phytohormone ethylene, given that S-adenosyl methionine (SAM) is the biosynthetic precursor to both ethylene and NA, and ethylene plays fundamental roles in leaf development, senescence and fruit ripening⁵⁰. Modifying ethylene biosynthesis and the downstream targets of ethylene improves crop yield and abiotic stress tolerance in corn (*Zea mays*, L.) and rice^{51,52} and determining whether altered ethylene biosynthesis is responsible for the reduced plant height in CE-1 sibling lines warrants further investigation. Variability in agronomic performance, wholemeal flour nutrition and white flour nutrition (Figures 5.1-5.3 and Tables S5.3-S5.7) between CE-1 sibling lines is likely due to somaclonal variation⁵³ and backcrossing of CE-1 lines to wild-type wheat will be required to remove this variation prior to dissemination. Similar findings were documented during field trial evaluation of barley (*Hordeum vulgare* L.), where agronomic performance varied between sibling lines of the same transgenic event at the second (T₂) and fourth (T₄) generations⁵⁴. Together these results suggest that CFT evaluation of transgenic plants should occur over multiple generations and utilize multiple sibling lines per transgenic event. Importantly although we detected significant G x E components in our study (Table S5.2), between field sites all CE-1 sibling lines exhibited similar agronomic performance and grain nutrition relative to the NS suggesting that CE-*OsNAS2* exhibits the same effect regardless of field condition.

Plant Fe is mostly stored as phytoferritin in leaf tissues and during grain filling metal chelators such as NA and/or DMA facilitate long-distance remobilization of Fe from leaf tissues to the developing grain^{41,55}. To determine the proportion of Fe and Zn that is remobilized from CE-1

and NS leaves to the grain during grain filling, we removed flag leaves and leaves from CE-1 and NS plants at anthesis when grown in the glasshouse (soil pH ~ 6.0) and at Katanning (soil pH ~4.8). Flag leaf removal reduced grain Fe and Zn concentrations in CE-1 plants more than in NS plants when grown in the glasshouse but not at Katanning, suggesting that soil pH influences the remobilization of micronutrients from CE-1 leaves to the developing grain (Figure S5.5). We hypothesize that under conditions of higher soil pH, CE-1 plants remobilize a greater proportion of leaf Fe and Zn to the developing grain, as higher soil pH limits the availability of soil Fe and Zn for plant uptake^{56,57}. By contrast, the lower soil pH at Katanning increases the availability of soil Fe and Zn (Figure 5.1c, Table S5.1) and may therefore allow more direct remobilization from below ground sources to the grain during CE-1 grain filling. Mature CE-1 flag leaves contained lower ($p < 0.05$) Fe than NS leaves (Table S5.9), suggesting that CE-1 plants remobilize additional Fe from leaf tissue to the grain during grain filling relative to NS plants. It is likely that this remobilization is mediated by increased NA concentrations in CE- leaves given that CE-1 shoots contain higher NA concentrations relative to NS shoots³⁰. As NA chelates other micronutrients such as Cu and Mn⁴³, increased NA-mediated remobilization from CE-1 leaves could explain increased concentrations of Cu and Mn in wholemeal and white flour of CE-1 relative to NS (Tables S5.6-S5.8). Grain Cu concentrations were negatively correlated with all other grain nutrients (apart from white flour K) and positively correlated with agronomic performance traits, although whether or not breeding for higher grain NA-chelated Cu concentration may indirectly improve grain yield requires further investigation (Figure S5.7). The positive correlation of white flour K with agronomic performance traits is likely the result of lower white flour K concentrations in Bobwhite relative to the check (Table S5.7). Both NA and DMA are synthesized from S-adenosyl-methionine (a S-containing cosubstrate)⁴⁴, and upregulated biosynthesis of NA and/or DMA in CE-1 grain could explain the higher wholemeal and white flour S concentrations measured in all CE-1 sibling lines relative to the NS (Tables S5.6, S5.7). Throughout our study we measured higher wholemeal and white flour Mg concentrations in all CE-1 sibling lines relative to NS, a result that was demonstrated in glasshouse grown wheat constitutively overexpressing *OsNAS2*⁴⁷, and suggests that NA may be capable of chelating and remobilizing Mg^{2+} ions. Future radioisotope and metabolomic studies are required to elucidate these hypothetical nutrient dynamics in CE-1 plants and to confirm the contribution of increased leaf NA and/or DMA concentrations to wheat grain nutrition³⁵.

At Glenthorne, Katanning and Merredin over three field seasons we consistently measured significantly higher concentrations of Fe, Zn, NA and DMA in CE-1 wholemeal flour, and significantly higher concentrations of Zn, NA and DMA in CE-1 white flour relative to NS (Figures 5.1-5.3). White flour Fe concentrations were higher ($p < 0.05$) in all CE-1 sibling lines over three field seasons at Katanning (apart from CE-1.2 in 2017) yet were only higher ($p \leq 0.01$)

in sibling line CE-1.1 at Glenthorne and Merredin. To further determine whether both Fe and Zn concentrations were higher throughout all CE-1 grain regions relative to NS, we performed industrial-scale milling (>30 kg per genotype) and obtained eight grain fractions: three break, three reduction, pollard, and bran, that represent the innermost and outermost grain layers (Table 5.1). We detected significantly higher Fe and Zn concentrations in CE-1 break, reduction, pollard (aleurone), and bran fractions relative to NS, providing strong evidence to suggest that Fe and Zn concentrations are higher throughout CE-1 grain (Table 5.1, Table S5.4). Synchrotron X-ray fluorescence microscopy (XFM) analysis of glasshouse-grown CE-1 and NS grain demonstrated that CE-1 grain contained enhanced Fe and Zn in aleurone tissues and only an enhanced Fe signal in endosperm tissues³⁰. Together these results indicate that break/reduction flour Fe and Zn concentrations measured in our study are most likely derived from endosperm and crease tissues, respectively that are retained during roller milling⁵⁸. We detected significantly ($p \leq 0.001$) higher NA and DMA concentrations in all CE-1 grain fractions relative to NS, suggesting that constitutive *OsNAS2* expression increases NA and DMA throughout the wheat grain (Table 5.1). Increased NA and DMA concentrations in break and reduction fractions would likely increase Fe bioavailability given that these fractions contain low concentrations of phytate ($< 3.5 \mu\text{g/g}$) and that these chelators are strong enhancers of Fe bioavailability⁵⁹. White flour Fe bioavailability was higher in all CE-1 sibling lines relative to the NS throughout our study and positively correlated with white flour NA and DMA concentrations (Figure 5.4, Figures S5.6, S5.7). We have previously reported on improved white flour Fe bioavailability independent of Fe concentration³⁰ and similar results were observed in this study for sibling line CE-1.3 (Figure 5.1g, Figure S5.6). These results provide additional evidence that NA and DMA are phytonutrients that strongly enhance Fe bioavailability in white flour and mediate the uptake of Fe into human cells^{60,61}. In our study we demonstrate that wholemeal flour and white flour Fe and Zn concentrations per se were negatively correlated ($r \leq -0.92$, $p \leq 0.001$) with grain yield and uncover for the first time that white flour NA and DMA concentrations had a modest positive correlation ($r \geq 0.42$) with grain yield (Figure 5.4, Figure S5.7). Together these results suggest that a shift in focus towards breeding for increased metal chelator concentration (such as NA and/or DMA) could improve both grain Fe bioavailability and agronomic performance within wheat biofortification programs.

Iron bioavailability was higher in CE-1.2 white bread relative to NS white bread at Katanning and Merredin in 2017 despite a minor ($< 5 \text{ ppm}$) difference in Fe concentration (Figure 5.5e) and no difference in phytate/Fe ratios (Figure S5.8), suggesting that NA and DMA are thermostable and persist the breadmaking process to enhance white bread Fe bioavailability (Figure 5.5c,d). It is therefore worth investigating whether enhanced NA and DMA concentrations would also improve wholemeal bread Fe bioavailability, given that wholemeal breads contain higher

concentrations of Fe bioavailability inhibitors such as phytate, polyphenols and dietary fibre^{21,24}. Furthermore, due to a lack of *in vitro* screening tools we could not determine whether consistently higher concentrations of Zn in CE-1 wholemeal and white flour translates into higher Zn bioavailability⁶². Future analysis of CE-1 wheat may therefore involve more comprehensive *in vivo* studies that have the capacity to simultaneously evaluate Fe and Zn bioavailability. White bread Fe concentrations and bioavailability were higher in NS relative to CE-1 only at Glenthorne, suggesting that properties of the soil (particularly soil pH) influence white bread Fe nutrition (Figure 5.5a, 5.5e). The high soil pH at Glenthorne likely results in greater biosynthesis of NA and DMA in CE-1 plants relative to NS plants and higher molar ratios of NA/DMA to Fe⁴⁵. We hypothesize that a greater proportion of DMA-chelated Fe in CE-1 white bread relative to NS white bread at Glenthorne results in lower Fe bioavailability given that DMA preferentially binds Fe³⁺ ions and Fe bioavailability is inhibited at high DMA: Fe molar ratios⁵⁹. Higher white flour S and total protein concentrations in CE-1 white flour relative to NS white flour (Tables S5.5 and S5.7) likely affected dough rheology given that S and protein play important roles in the gluten complex^{63,64}. Such alterations may have resulted in a lower proportion of Fe in CE-1 crumb relative to CE-1 crust (which was not sampled in this study) and future research will determine if these alterations could therefore account for in the differences in white bread Fe concentrations in CE-1 and NS observed at Glenthorne. Determining the localization and speciation of Fe bound to NA and/or DMA within both the crumb and crust of CE-1 and NS white bread also warrants further investigation⁶⁵.

Throughout our study sibling line CE-1.3 demonstrated wholemeal flour, white flour and white bread Fe, Zn, NA and DMA concentrations and white flour Fe bioavailability that were significantly higher than the NS. The slight reduction in plant height observed in this line could be advantageous by helping to prevent lodging. As CE-1.3 maintained an equivalent grain yield to the NS, particularly at Katanning and Merredin, it therefore represents the strongest candidate to advance for comprehensive food processing and *in vivo* micronutrient bioavailability studies. Together our study highlights the potential of CE-OsNAS2 wheat as a biofortified second-generation GM crop for improving human micronutrient intakes in less developed countries.

5.5. Methods

5.5.1. Generation of iron and zinc biofortified wheat

Vector construction, plant transformation and the initial selection of our lead transgenic event CE-1 is previously described³⁰. In brief, the full-length coding sequence of OsNAS2 (LOC_Os03g19420) was PCR amplified from rice (*Oryza sativa* L.) cultivar (cv.) Nipponbare and recombined into a modified pMDC32 vector under transcriptional control of the maize (*Zea mays* L.) ubiquitin 1 promoter with a hygromycin phosphotransferase plant-selectable marker (Figure 5.1a). Bombardment of the construct into immature wheat (*Triticum aestivum* L.) cv.

Bobwhite embryos was performed at the University of Adelaide (Adelaide, Australia) to generate six independent CE-OsNAS2 transgenic events, and one double-insert event termed CE-1 was selected for additional analyses based on no difference in plant phenotype and increased grain Fe and Zn concentrations. Three sibling lines of CE-1 (termed CE-1.1, CE-1.2 and CE-1.3) and the corresponding null segregant control (NS) were selected for confined field trial (CFT) evaluation and were grown at the T₅, T₆ and T₇ generations consecutively alongside the check cv. Mace, an elite Australian bread wheat cultivar.

5.5.2. Confined field trials (CFTs)

Confined field trials were conducted from May to December in 2015, 2016 and 2017 at Glenthorne (35.0543° S, 138.5524° E) in South Australia and at the New Genes for New Environment facilities located in Merredin (31.4837°S, 118.2771°E) and Katanning (33.6894°S, 117.5551°E) in Western Australia. Grain were sown in 1 m² plots at Glenthorne across all three seasons and in 1.8 m² plots in 2015, 8.5 m² plots in 2016, and 18 m² plots in 2017 at Katanning and Merredin (Figures S5.1, S5.2). At least three replicate plots per genotype were grown in 2015, five replicate NS and CE-1 sibling line plots and four replicate Mace plots were grown in 2016, and five replicate plots per genotype were grown in 2017. All plots were arranged in a randomized block design at each site. Rows were spaced 30 cm apart and grain were sown at a density of 60 kg ha⁻¹ in 2015 and 2016, and 75 kg ha⁻¹ in 2017 at all three sites. Fertilizers were applied as urea and/or AGRAS (CSBP limited, Kwinana, WA, Australia) at a rate of 40 – 100 kg ha⁻¹ as needed for each field site. Leaves and flag leaves were harvested post-anthesis from each plot, cleaned with 0.1% Tween®20 (Sigma Aldrich, St. Louis, MO, USA), rinsed with dH₂O and oven dried for 48 h at 60°C before homogenization. At maturity, plant height and tiller number were determined from three representative measurements per plot and biomass, TGW, and harvest index were determined from subsamples of each plot as previously described³⁰. Grain yield was calculated from the amount of grain harvested per m² and extrapolated to kg/ha. Soil analysis was performed at sowing at CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia). Soil phosphorus (P) and potassium (K) concentrations were determined using the Colwell P and K extraction methods^{66,67}. A diethylenetriaminepentaacetic acid (DTPA) extraction method was used to calculate soil copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) concentrations⁶⁸. Soil properties of each site for each season are shown in Table S5.1 and field environmental conditions are shown in Figure S5.3.

5.5.3. Analysis of iron and zinc remobilization post anthesis

Plants were grown under glasshouse conditions at The University of Melbourne (Victoria, Australia) as previously described³⁰. At flowering, plants grown in the glasshouse and in the field at Katanning received one of three treatments: 1) all tillers apart from the main tiller removed (detillered), 2) detillered and main stem flag leaf removed (detillered - flag leaf), 3) detillered and

all leaves removed (detillered - all leaves). Grain was harvested at maturity, cleaned, oven dried for 48 h at 60°C and ground into a powder. A subsample (20 mg) was digested with 300 µL aqua regia (1 HNO₃: 3 HCl) heated (80°C, 90 min), diluted to 10 mL with deionized H₂O (18 MΩ) and centrifuged (5,000 rpm, 5 min) prior to analysis by inductively coupled plasma mass spectrometry (ICP-MS) at Deakin University (Burwood, Australia).

5.5.4. White flour and white bread production

Whole grain samples were conditioned to 13% moisture content for 24 h prior to milling using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) as previously described³⁰ and using a Buhler MLU-202 laboratory mill at The Commonwealth Scientific and Industrial Research Organisation (CSIRO, ACT, Australia) for grain fraction analysis of NS and a combined sample of CE-1.2 and CE-1.3 at Merredin, 2017. Flour extraction across all samples was between 70-75%. White bread was produced using a baker's yeast protocol as previously described⁶⁹ with minor modifications. White flour (500 g) was mixed with H₂O (325 g), 10 g of dry baker's yeast (Lowan® whole foods, NSW, Australia) and 10 g of cooking salt (Black & Gold, NSW, Australia) in a BM2500 Compact Bakehouse (Sunbeam, NSW, Australia) for 25 min, prior to fermentation for at least 1 hr. Dough was divided into 150 g pieces, shaped and placed into loaf tins (14.6 x 7.6 cm) for proofing (30 min, 30°C) and baking (20 min, 200°C) in a commercial oven (Convotherm 4 EasyDial 10.10, Moffat, VIC, Australia). Loaves were cooled (1 hr) before the crumb was separated from the crust as described in⁷⁰, lyophilized and ground to a powder for analysis.

5.5.5. Inductively coupled plasma optical emission spectrometry (ICP-OES)

Plant tissues were cleaned and ground using an IKA tube mill (www.ika.com) prior to inductively coupled plasma optical emission spectrometry (ICP-OES) analysis at the CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia) as previously described³⁰. White flour and white bread micronutrient concentrations were determined by nitric / perchloric acid digestion as previously described⁷¹ followed by ICP-OES using a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA). Nitrogen analysis of white flour was performed using the Dumas combustion method at CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia) and total protein concentration of white flour and bran were determined as: white flour total protein (%) = N concentration x 5.70, and bran total protein (%) = N concentration x 6.31⁷².

5.5.6. Quantification of nicotianamine and 2'-deoxymugineic acid

Quantification of 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) derivatized NA and DMA was performed via RP LC-MS on a 1290 Infinity II and 6490 Triple Quadrupole LC/MS system (Agilent Technologies Inc., Santa Clara, CA) as previously described^{30,73}. Briefly, sequential methanol (100%) and deionized H₂O (18MΩ) extractions of pulverized wheat material (25 mg) were combined and derivatized with fresh Fmoc-Cl solutions (50 mM, 40 µL). After incubation

(60°C, 700 rpm, 15 min), the derivatization reaction was quenched by adding formic acid (FA; pH = 4, 5%, 8.9 µL) and run through a Zorbax Eclipse XDB-C18 Rapid Resolution HS 2.1 x 100 mm, 1.8 µm particle size column (Agilent Technologies Inc.) using aqueous (0.1% v/v FA in dH₂O) and organic (0.1% v/v FA in acetonitrile) mobile phases and NA and DMA quantified using a stock calibration set (Toronto Research Chemicals, Toronto, ON, Canada).

5.5.7. In vitro Fe bioavailability bioassay

White flour samples (500 mg) were subjected to the Caco-2 cell bioassay as previously described^{30,48,74}. Caco-2 cells were maintained in supplemented Dulbecco's modified Eagle medium (DMEM) for 11 days post-seeding and replaced with supplemented minimum essential media (MEM) solution containing less than 80 µg Fe/L at 48 hr prior to the experiment. Gastric-digested samples (1.5 mL) were added to cylindrical Transwell inserts (Corning Life Sciences, Corning, NY) fitted with a semipermeable (15,000 Da MWCO) basal membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA) and placed within wells containing Caco-2 cell monolayers. After incubation for 2 hr (37 °C) the inserts were removed and additional MEM (1 mL) was added to the cells before incubation for 22 hr (37 °C). Cells were washed twice with a solution (pH = 7.0) containing NaCl (140 mmol/L), KCl (5 mmol/L) and PIPES (10 mmol/L), harvested and analysed for ferritin (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein contents (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA). Caco-2 cell ferritin synthesis occurs in response to increases in intracellular Fe and we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake.

5.5.8. Statistical Analysis

Agronomic, grain nutrition, white flour nutrition, and leaf nutrition traits for each environment were analysed using a multi-environment linear mixed model (LMM) that partitioned and accounted for genetic and non-genetic sources of variation. The LMM had the form:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\tau} + \mathbf{Z}_r\mathbf{u}_r + \mathbf{Z}_C\mathbf{u}_C + \mathbf{Z}_R\mathbf{u}_R + \boldsymbol{\epsilon}$$

and a vector of observed responses across the nine environments had the form: $\mathbf{y} = (\mathbf{y}_1^T \dots \mathbf{y}_9^T)^T$.

The vectors of fixed effects ($\boldsymbol{\tau}$) were conformably partitioned to be $\boldsymbol{\tau} = [\boldsymbol{\tau}_g^T \boldsymbol{\tau}_e^T \boldsymbol{\tau}_{eg}^T]^T$ where $\boldsymbol{\tau}_g$, and $\boldsymbol{\tau}_e$ were vectors of genotype and environment main effects and $\boldsymbol{\tau}_{eg}$ was a vector of genotype by environment interaction fixed effects, with associated explanatory matrix $\mathbf{X} = [\mathbf{X}_g \mathbf{X}_e \mathbf{X}_{eg}]$.

For $i = (r, C, R)$ each of the random effects were also partitioned $\mathbf{u}_i = [\mathbf{u}_{i1}^T \dots \mathbf{u}_{i9}^T]^T$ where $\mathbf{u}_i \sim N(\mathbf{0}, \bigoplus_{j=1}^9 \sigma_{ij}^2 \mathbf{I})$ and $\bigoplus$ is the direct sum operator⁷⁵ used to indicate block diagonality of the associated variance matrix. Identical to $\mathbf{y}$, the multi-environment LMM error term was also conformably partitioned $\boldsymbol{\epsilon} = [\boldsymbol{\epsilon}_1^T \dots \boldsymbol{\epsilon}_9^T]^T$ and assumed to be distributed $\boldsymbol{\epsilon} \sim N(\mathbf{0}, \bigoplus_{j=1}^9 \sigma_j^2 \mathbf{I})$. From this full model the genotype (G) x environment (E) interaction was tested and if the interaction

was not statistically significant it was removed. The multi-environment model was then refitted with fixed effects τ_g and τ_e , and eBLUEs for each genotype extracted and compared against NS using a single degree of freedom Wald test. Single and multi-environment linear mixed modelling was computationally conducted using the functionality of the R package ASReml-R⁷⁵. ASReml-R uses the Residual Maximum Likelihood (REML) algorithm⁷⁶ for estimation of model parameters and provides a suite of functions for diagnostic assessment of models.

To visualize relationships between traits eBLUEs were extracted from each of the full multi-environment models, compared pairwise using a Pearson correlation and hierarchically clustered. Significant correlations were determined by forming a set of 5,000 permutations of each trait eBLUE, storing the maximum Pearson correlation and calculating p-values based on the number of maximum correlations exceeding the observed pairwise correlation⁷⁷. Hierarchical clustering and graphical visualization of correlation matrices were conducted using the R package corplot⁷⁸. Other graphs were generated using SigmaPlot v13 (Systat Software Inc., San Jose) software and data are presented as mean $\pm$ SEM with biological replicate numbers noted in table and figure legends. Student's *t*-test was used to determine significant differences between means and was conducted using Minitab 17 Statistical software (Minitab, State College, PA).

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5.7. Supplementary Information

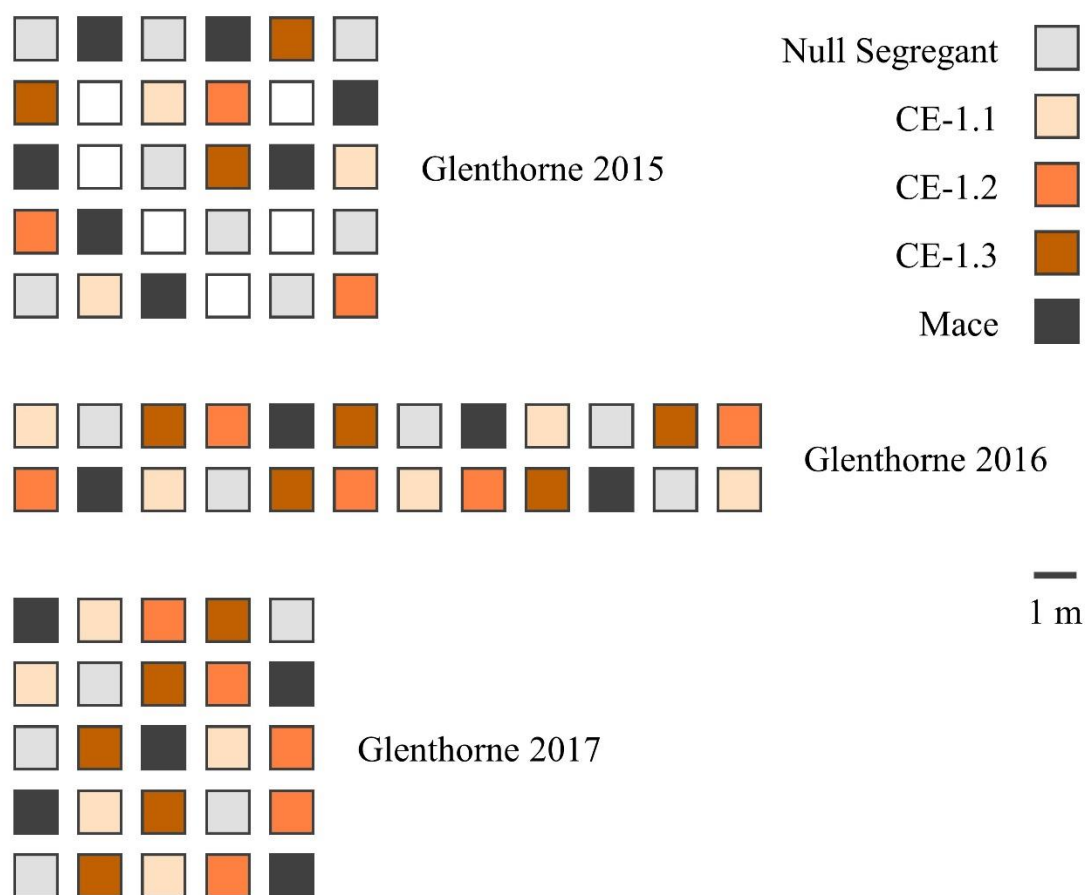


Figure S5.1. Replicated plot design of confined field trials conducted at Glenthorne in 2015, 2016 and 2017. The blank plots during the 2015 field season indicate the position of genotypes that were not included in this study. Scale bar = 1 meter.

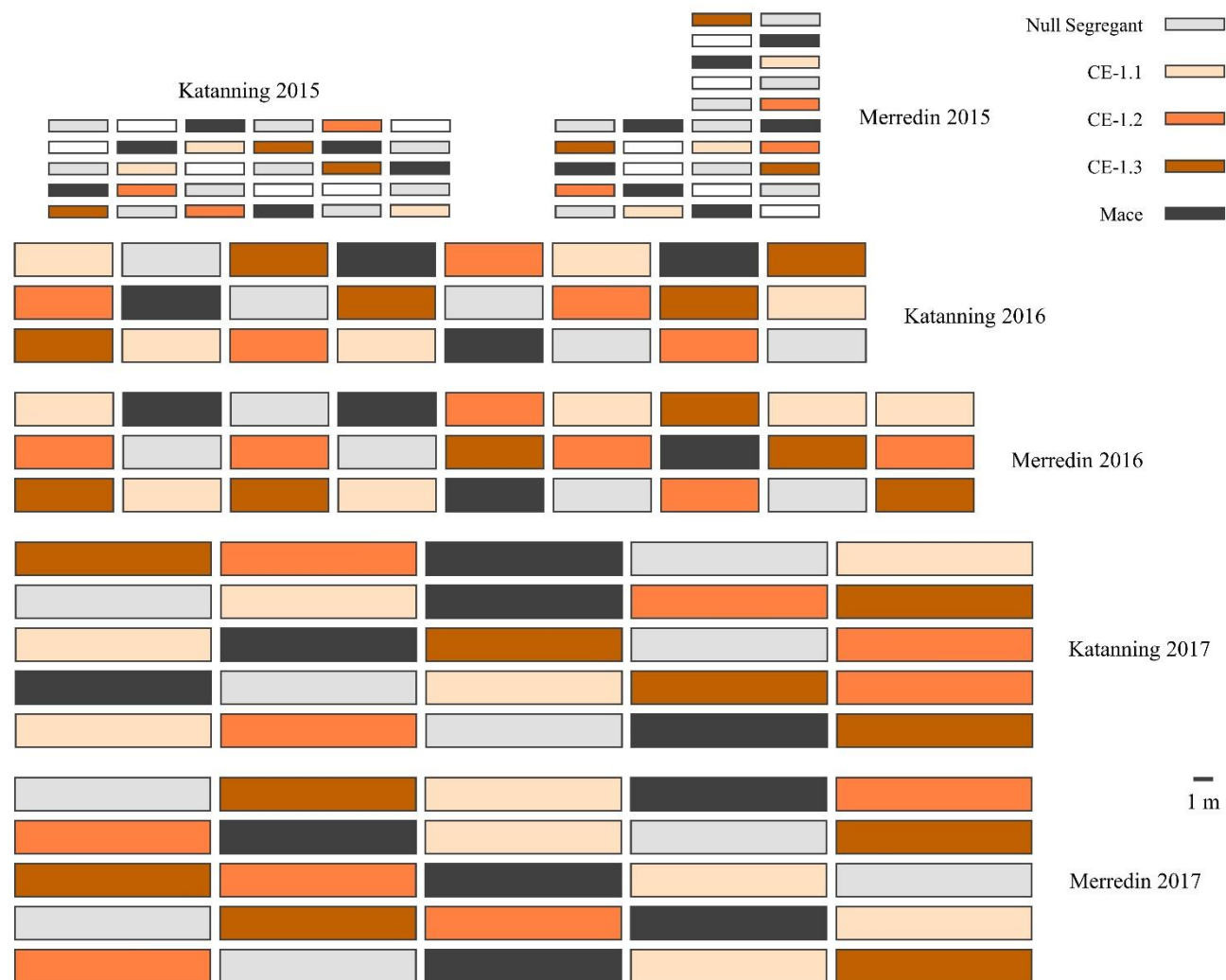


Figure S5.2. Replicated plot design of confined field trials conducted at Katanning and Merredin in 2015, 2016 and 2017. The blank plots during the 2015 field season indicate the position of genotypes that were not included in this study. Scale bar = 1 meter.

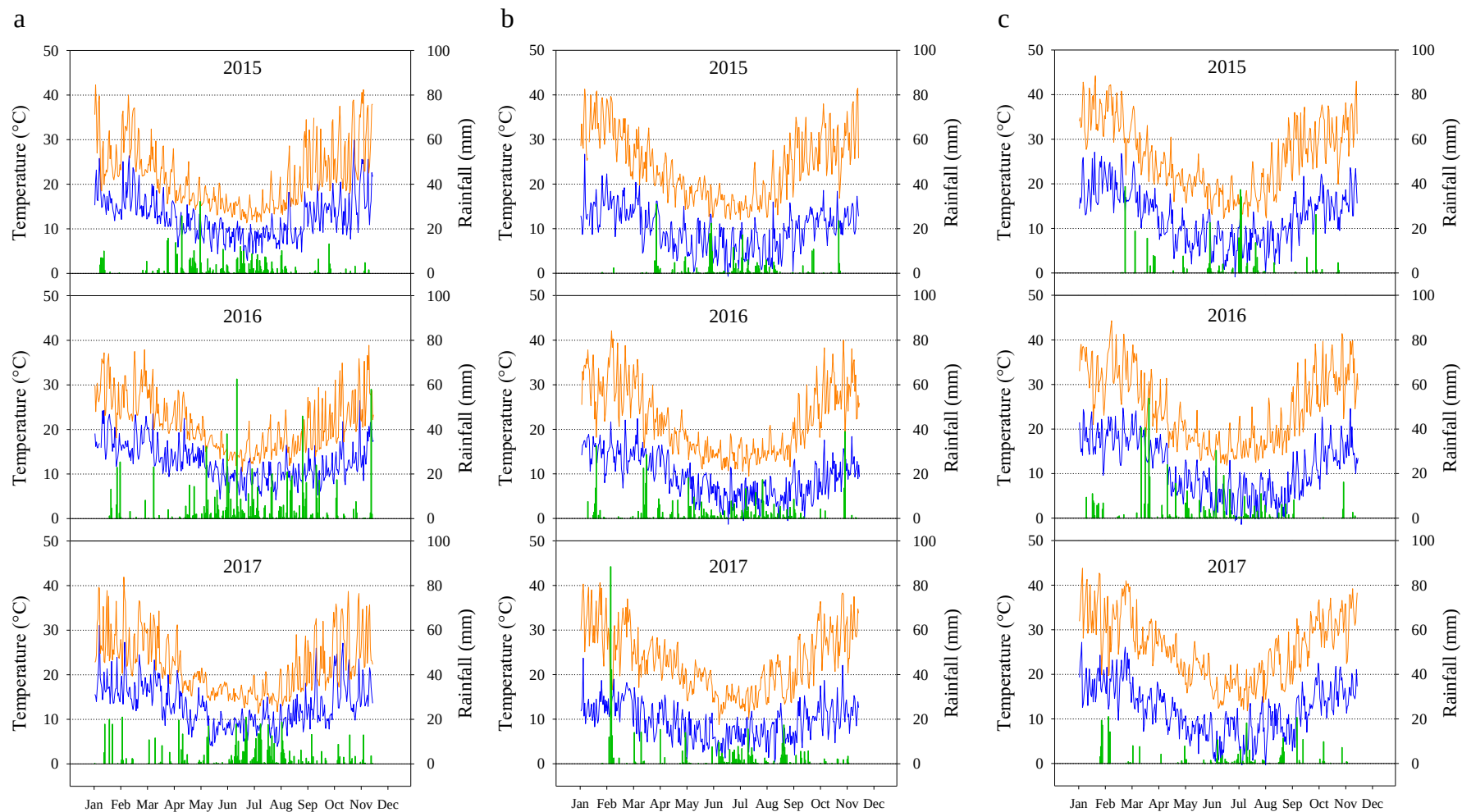


Figure S5.3. Temperature and rainfall throughout 2015, 2016, and 2017 in **a)** Glenthorne, **b)** Katanning and **c)** Merredin. Daily maximum (orange) and minimum (blue) temperatures ($^{\circ}\text{C}$) are shown alongside daily rainfall (green, mm).

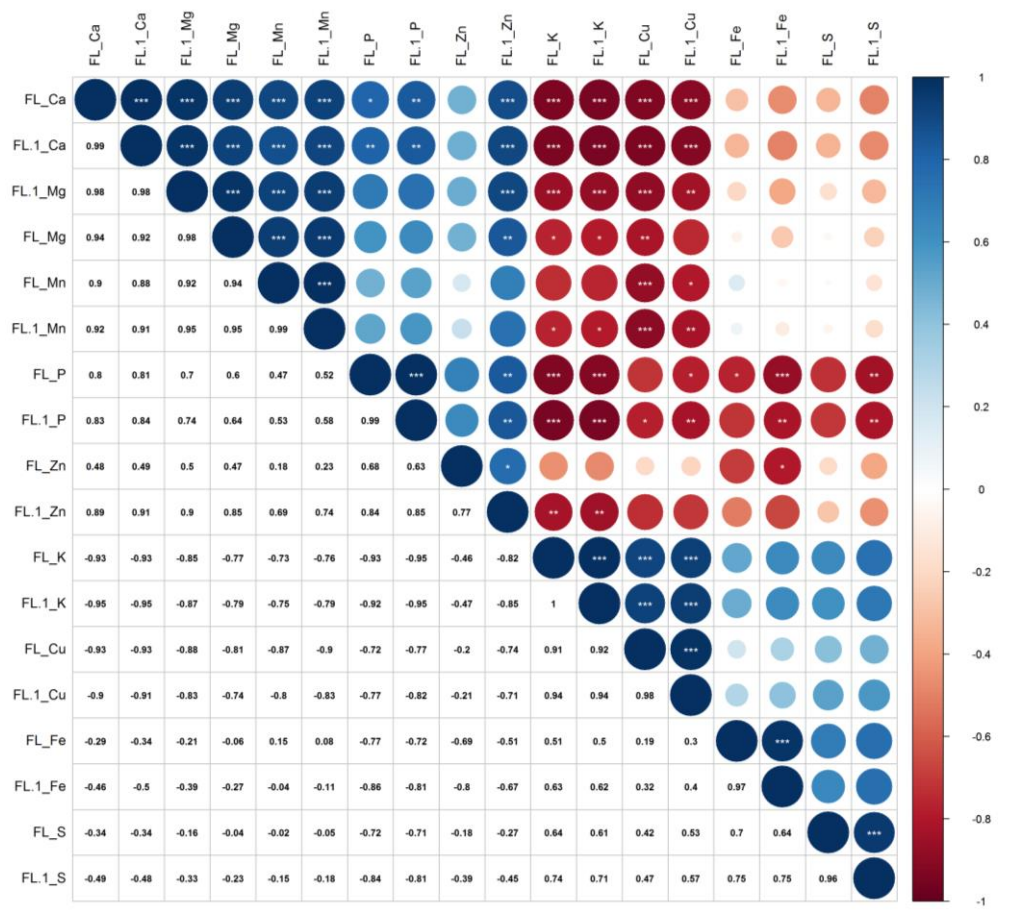


Figure S5.4. Multi-trait correlation of empirical best linear unbiased estimators (eBLUEs) obtained from the multi-environment models of leaf nutrition of NS, CE-1 and Mace. Flag leaf (FL) and non-flag leaf (FL.1) nutrition variables include B, Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn concentrations. Each circle represents a positive (blue) or negative (red) pairwise Pearson correlation coefficient between variables, with the diameter of the circle proportional to the absolute value. Asterisks denote significant correlations for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

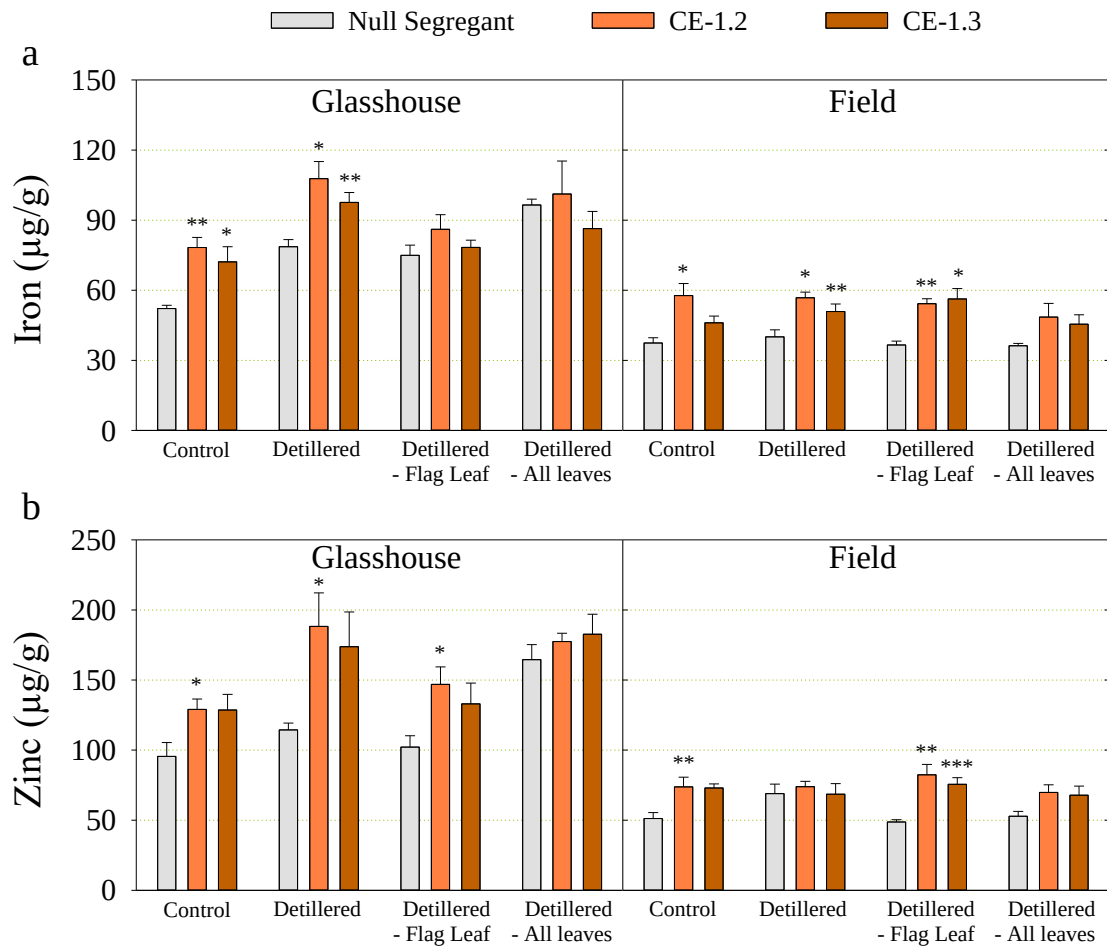


Figure S5.5. Grain **a)** Fe, and **b)** Zn concentrations in null segregant, CE-1.2 and CE-1.3 plants grown in glasshouse (University of Melbourne) and field (Katanning) conditions in 2016. At anthesis, plants had all tillers apart from the main tiller removed (detillered), tillers and flag leaf removed (detillered - flag leaf), or tillers and all leaves removed (detillered - all leaves). Control plants received no treatment. Bars represent the mean $\pm$ SEM of five biological replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), as determined by Student's t-test.

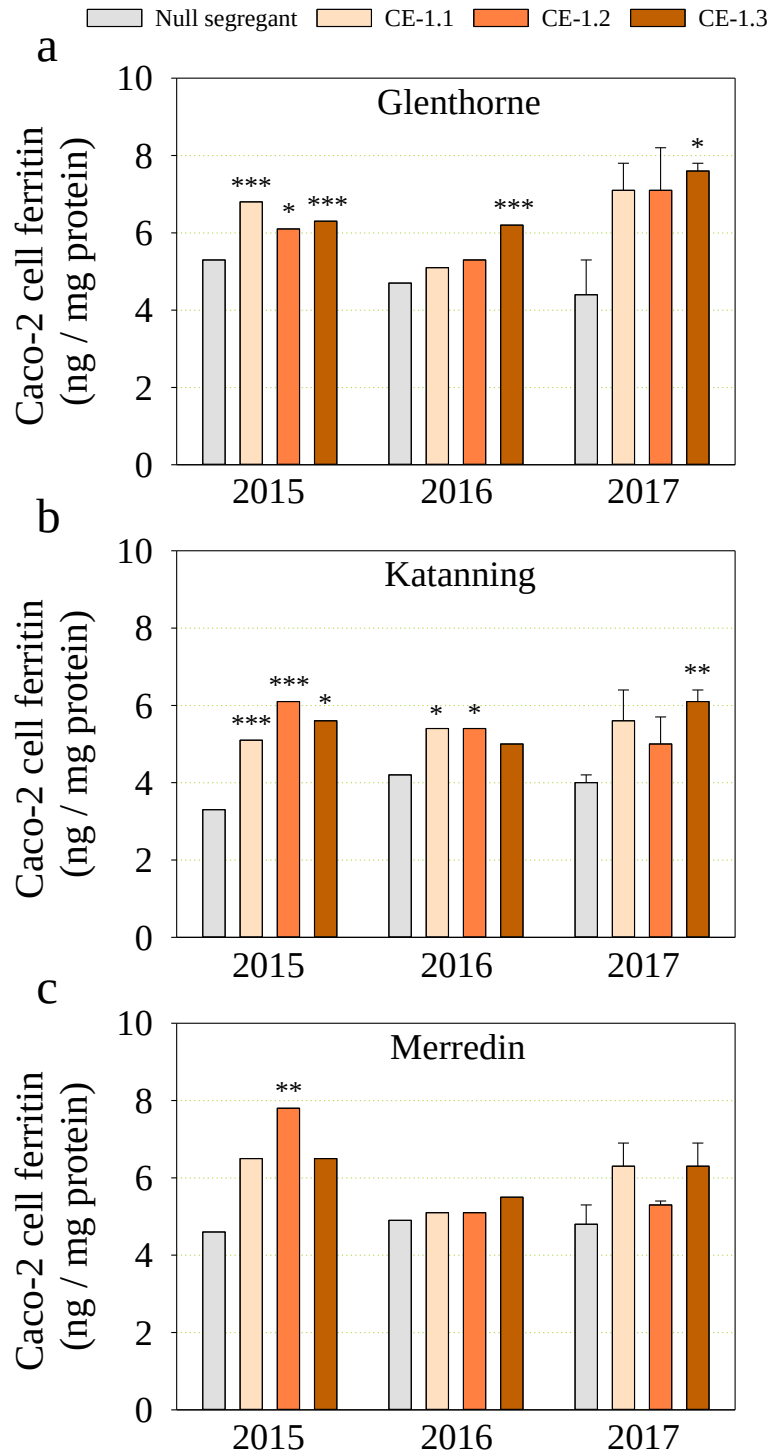


Figure S5.6. White flour Fe bioavailability of null segregant, CE-1.1, CE-1.2 and CE-1.3 at **a)** Glenthorne, **b)** Katanning and **c)** Merredin over three field seasons. Bars in 2015 and 2016 indicate best linear unbiased estimators for three replicates per genotype. Bars in 2017 represent the mean $\pm$ SEM of three replicates derived from a bulk of five biological replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test for 2015 and 2016, and by Student's t-test for 2017.

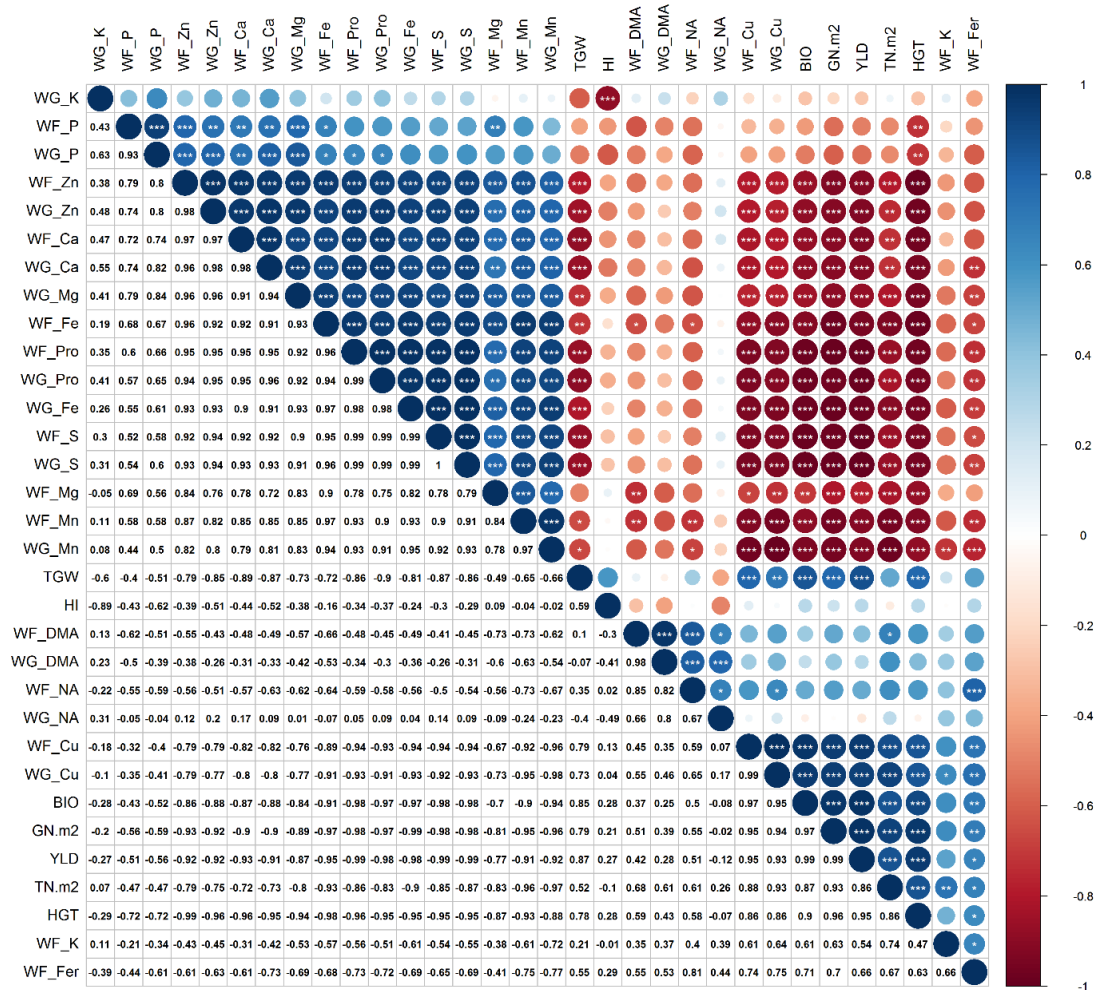


Figure S5.7. Multi-trait correlation of empirical best linear unbiased estimators (eBLUEs) obtained from the multi-environment models of agronomic performance, wholemeal flour, and white flour nutrition of NS, CE-1 and Mace. Agronomic performance variables include grain yield (YLD), harvest index (HI), thousand grain weight (TGW), total biomass (BIO), tiller number per m² (TN.m2), grain number per m² (GN.m2), plant height (HGT). Wholemeal flour (WG) and white flour (WF) nutrition variables include B, Ca, Cu, Fe, K, Mg, Mn, P, S, Zn, NA, and DMA concentrations, total protein (Pro) and Caco-2 cell ferritin production (Fer). Each circle represents a positive (blue) or negative (red) pairwise Pearson correlation coefficient between variables, with the diameter of the circle proportional to the absolute value. Asterisks denote significant correlations for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)

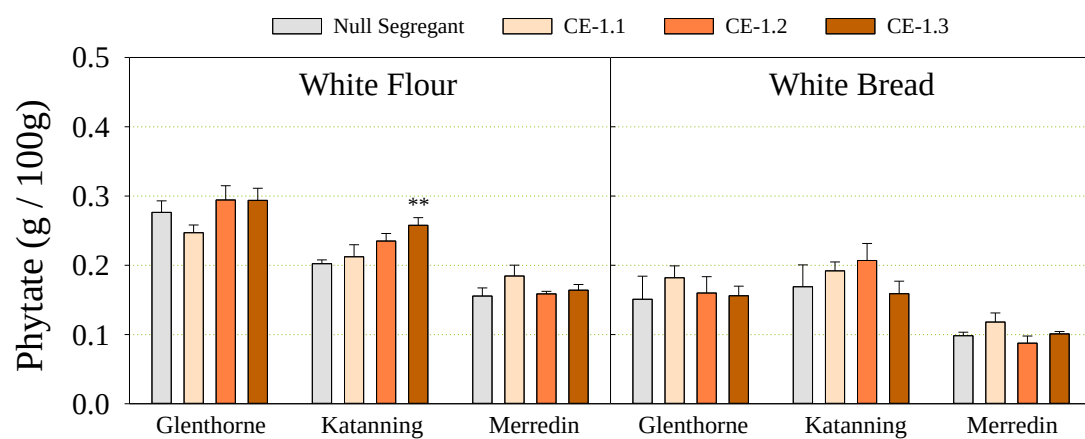


Figure S5.8. Phytate concentration in white flour (left) and white bread (right) of null segregant, CE-1.1, CE-1.2 and CE-1.3 at Glenthorne, Katanning and Merredin in 2017. Bars represent the mean $\pm$ SEM of at least five replicates. Asterisks denote significant differences for $p \leq 0.01$ (**) as determined by Student's t-test.

Table S5.1. Soil properties of Glenthorne, Katanning and Merredin field sites over three years. Values represent mean of three replicates and are provided in $\mu\text{g g}^{-1}$ DW unless otherwise specified. S = sand, Sy Loam = sandy loam, ^ = determined using Colwell P and K extraction method, # = determined using a DTPA extraction method.

Soil property	Glenthorne			Katanning			Merredin		
	2015	2016	2017	2015	2016	2017	2015	2016	2017
Texture	Sy loam/ Loam	Sy loam/ Loam	Sy loam/ Loam	S	S / Sy Loam	S / Sy Loam	S	S / Sy Loam	S / Sy Loam
NH ₄	2.00	5.00	4.00	4.00	11.0	2.30	2.00	4.00	2.00
NO ₃	12.0	10.0	19.6	33.0	22.0	25.5	2.00	7.00	18.6
P [^]	27.0	31.0	25.3	40.0	44.0	41.3	46.0	35.0	48.0
K [^]	480	538	476	35.0	48.0	68.5	116	75.0	61.0
SO ₄	19.6	13.4	8.10	7.30	8.50	6.20	5.10	4.00	3.40
Al	0.50	0.60	1.00	2.30	0.70	1.60	2.30	2.60	1.60
Ca	343	371	357	33.3	46.9	3.00	8.90	0.60	130
Mg	39.8	41.3	37.1	2.80	3.80	1.70	3.10	1.80	1.70
Na	5.50	7.20	5.50	0.80	1.40	0.90	0.10	0.10	1.00
Cu [#]	0.85	1.50	0.79	1.18	13.2	0.82	0.81	3.69	0.82
Fe [#]	9.79	24.6	31.9	85.6	41.5	51.8	124	51.0	51.7
Mn [#]	2.19	2.76	2.70	7.51	7.91	2.84	4.04	3.93	2.84
Zn [#]	11.6	4.54	4.73	2.59	13.6	1.38	1.90	2.27	1.38
B	1.95	2.12	2.13	0.46	0.95	0.32	0.65	0.59	0.61
Organic C	1.78	1.86	1.76	1.48	1.21	1.28	1.05	0.39	0.74
EC (dS / m)	0.17	0.23	0.21	0.09	0.08	0.06	0.02	0.03	0.05
pH (CaCl ₂)	7.6	7.1	7.3	4.4	5.3	4.6	4.6	4.8	4.8

Table S5.2. Interaction of genotype and environment on plant traits at Glenthorne Katanning and Merredin field sites in 2015, 2016 and 2017. Asterisks denote significant interactions for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. NA: nicotianamine, DMA: 2'-deoxymugineic acid.

Plant Trait	Interaction	Wald test	p-value
Plant height (cm)	$y = G + E$ $y = G + E + G \times E$	151.01	***
Tiller number (m ²)	$y = G + E$ $y = G + E + G \times E$	151.15	***
Biomass (kg ha ⁻¹)	$y = G + E$ $y = G + E + G \times E$	168.23	***
Grain yield (kg ha ⁻¹)	$y = G + E$ $y = G + E + G \times E$	274.35	***
Harvest index (%)	$y = G + E$ $y = G + E + G \times E$	74.56	***
Thousand grain weight (g)	$y = G + E$ $y = G + E + G \times E$	53.85	**
Wholemeal flour iron (µg / g)	$y = G + E$ $y = G + E + G \times E$	152.27	***
Wholemeal flour zinc (µg / g)	$y = G + E$ $y = G + E + G \times E$	144.05	***
Wholemeal flour NA (µmol / g)	$y = G + E$ $y = G + E + G \times E$	334.96	***
Wholemeal flour DMA (µmol / g)	$y = G + E$ $y = G + E + G \times E$	65.55	***
White flour iron (µg / g)	$y = G + E$ $y = G + E + G \times E$	113.93	***
White flour zinc (µg / g)	$y = G + E$ $y = G + E + G \times E$	371.31	***
White flour NA (µmol / g)	$y = G + E$ $y = G + E + G \times E$	25.68	*
White flour DMA (µmol / g)	$y = G + E$ $y = G + E + G \times E$	144.68	***
Leaf iron (µg / g)	$y = G + E$ $y = G + E + G \times E$	192.99	***
Leaf zinc (µg / g)	$y = G + E$ $y = G + E + G \times E$	82.13	***
Flag leaf iron (µg / g)	$y = G + E$ $y = G + E + G \times E$	89.03	***
Flag leaf zinc (µg / g)	$y = G + E$ $y = G + E + G \times E$	74.66	***

Table S5.3. Tiller number (m²), biomass (kg ha⁻¹), harvest index (%), and grain number (m²) in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for p < 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***) as determined by Wald test.

Field Site	Genotype	2015	2016	2017	2015	2016	2017
		Tiller number (m ²)			Biomass (kg ha ⁻¹)		
Glenthorne	NS	602.5	522.1	507.6	12197.4	17527.0	14737
	CE-1.1	604.4	409.8 ***	522.0	10986.0	16089.8	14604.5
	CE-1.2	642.2	447.7 **	509.4	12220.0	15022.7	15621.1
	CE-1.3	635.6	487.3	511.2	10317.1	19299.2	14067.6
	Mace	597.1	507.9	648.0 ***	12297.1	20029.0	23908.8 ***
Katanning	NS	436.7	506.4	323.6	5283.2	6451.7	4559.2
	CE-1.1	317.4 **	407.9 ***	332.4	3721.9 *	5684.8	4211.6
	CE-1.2	326.5 *	487.1	321.8	3663.2 **	4807.2 **	4753.7
	CE-1.3	402.3	426.5 **	313.8	4589.7	5331.6	4807.4
	Mace	378.9	502.9	364.4	7509.6 ***	8237.2 **	7057.4 **
Merredin	NS	423.9	362.5	420.1	10426.9	2482.8	14070.0
	CE-1.1	369.8	347.7	398.0	8570.1	1537.2 **	12011.6
	CE-1.2	328.1 *	409.0	405.5	9236.3	2418.2	12956.8
	CE-1.3	451.2	367.2	452.8	11352.8	2185.6	14919.7
	Mace	379.0	336.3	313.0 **	10774	3152.2 *	11577.2 *
		Harvest index (%)			Grain number (m ²)		
Glenthorne	NS	27.2	26.7	36.3	13090.1	13757.2	12102.5
	CE-1.1	22.0	21.4 *	32.7 *	8950.2 **	8625.2 ***	10715.4
	CE-1.2	24.9	25.7	35.1	8004.5 ***	10466.4	11877.6
	CE-1.3	24.3	24.3	33.3	9637.9 **	12275.4 ***	10918.2
	Mace	29.5	27.7	37.3	15554.8 *	18032.8 ***	18171.1
Katanning	NS	31.5	20.1	26.0	5001.5	6880.2	1552.0
	CE-1.1	25.0 *	22.1	24.6	3552.0 ***	5911.1 *	1322.0
	CE-1.2	23.7 **	22.3	26.4	3615.8 **	5666.0 **	1183.6
	CE-1.3	32.6	23.2	29.0	4456.5	6231.2	1603.7
	Mace	41.0 ***	27.6 ***	37.8 ***	8637.0 ***	10987.6 ***	2908.7 **
Merredin	NS	43.4	33.0	45.2	8202.1	5261.4	7983.8
	CE-1.1	41.4	35.9	42.8 *	5838.8	4856.0	7168.0
	CE-1.2	45.5	35.0	45.0	6490.5	5732.0	8202.8
	CE-1.3	44.0	36.2	45.6	8894.5	5059.9	7617.2
	Mace	47.8 ***	37.2 *	45.0	7764.5	7487.2 **	7663.9

Table S5.4. Bran iron ($\mu\text{g} / \text{g}$), zinc ($\mu\text{g} / \text{g}$), phosphorus ($\mu\text{g} / \text{g}$), and total protein (%) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable

Field Site	Genotype	2015	2016	2017	2015	2016	2017
		Bran Iron ($\mu\text{g} / \text{g}$)			Bran Zinc ($\mu\text{g} / \text{g}$)		
Glenthorne	NS	91.3	64.7	85.4	107.8	63.7	90.3
	CE-1.1	95.5	84.3 **	107.5 ***	148.8 ***	97.3 ***	141.5 ***
	CE-1.2	87.8	83.8 **	85.8	133.6 ***	94.5 ***	106.7 **
	CE-1.3	87.8	84.1 **	86.2	129.7 **	99.0 ***	115.9 ***
	Mace	n.a	69.2	78.5	n.a	57.8	72.9 ***
Katanning	NS	129.4	99.6	135.4	137.1	115.8	181.7
	CE-1.1	195.3 ***	122.9 ***	155.0 ***	230.4 ***	140.3 **	269.3 ***
	CE-1.2	145.9	110.3	148.5 *	179.5	133.5 *	245.0 ***
	CE-1.3	143.5	115.5 **	152.2 ***	167.4	135.1 *	253.1 ***
	Mace	n.a	106.1	114.8 ***	n.a	73.2 ***	80.5 ***
Merredin	NS	121.0	119.7	111.8	116.0	77.8	87.3
	CE-1.1	167.6 ***	126.4	120.9	175.9 *	103.4 ***	112.7 ***
	CE-1.2	146.8	112.6	113.8	129.6	79.2	97.8
	CE-1.3	134.1	104.9 *	121.2	123.3	77.1	105.7 **
	Mace	n.a	99.0 **	101.2	n.a	37.5 ***	59.6 ***
		Bran Phosphorus ($\mu\text{g} / \text{g}$)			Bran Total Protein (%)		
Glenthorne	NS	8766.7	8331.7	8984.1	15.9	11.3	13.9
	CE-1.1	8300.0	8614.6	10280.1 *	16.9	12.9 ***	15.1 *
	CE-1.2	8200.0	9403.0 *	8263.8	15.7	11.8	13.7
	CE-1.3	7533.3	9191.3	8096.1	16.4	12.3 *	14.1
	Mace	n.a	8574.2	7975.9	n.a	11.7	13.5
Katanning	NS	10131.0	8140.0	10280.0	17.8	17.9	19.2
	CE-1.1	12026.5 *	8180.0	10580.0	19.2 **	18.1	19.7
	CE-1.2	9764.3	8720.0	9640.0	18.9 *	19.0 **	19.6
	CE-1.3	9959.8	8620.0	9760.0	19.1 *	18.5	19.2
	Mace	n.a	8525.0	7820.0 ***	n.a	16.9 *	17.4 ***
Merredin	NS	7433.3	6908.0	7000.0	16.4	15.0	15.7
	CE-1.1	7500.0	6722.8	6960.0	18.6 ***	16.9 ***	16.1
	CE-1.2	6766.7	6149.1	6640.0	17.4	16.1 ***	16.0
	CE-1.3	7100.0	5608.0 **	6820.0	17.1	15.4	16.1 *
	Mace	n.a	6290.0	6880.0	n.a	13.9 ***	15.9

Table S5.5. Wholemeal and white flour phosphorus ($\mu\text{g} / \text{g}$) and total protein (%) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable

Field Site	Genotype	2015	2016	2017	2015	2016	2017
		Wholemeal flour Phosphorus ($\mu\text{g} / \text{g}$)			White flour Phosphorus ($\mu\text{g} / \text{g}$)		
Glenthorne	NS	3091.9	3909.5	4380.0	1140.7	1306.9	1668.2
	CE-1.1	3280.3	4458.6 ***	4460.0	1383.7 ***	1699.3 ***	1738.1
	CE-1.2	3194.2	4080.0	4300.0	1269.4 **	1387.2	1855.4 **
	CE-1.3	2949.1	4061.9	4460.0	1188.8	1319.1	1804.6 *
	Mace	3070.0	3712.4	3600.0 ***	n.a	1349.8	1693.6
Katanning	NS	4538.3	3987.6	4397.8	1410.1	1442.6	1797.4
	CE-1.1	4413.9	4477.9 *	4780.7 *	1630.2 ***	1487.6	1844.0
	CE-1.2	4770.6	4272.2	4480.6	1461.9	1489.6	1841.6
	CE-1.3	4786.1	4171.5	4444.0	1550.7 ***	1525.9	1915.3 *
	Mace	4061.6	3663.5	3656.9 ***	n.a	1397.4	1708.3
Merredin	NS	3133.3	2898.6	2740.0	1256.2	1123.0	1266.6
	CE-1.1	3266.7	3362.1 **	3200.0 **	1309.8	1348.7 ***	2809.3
	CE-1.2	2833.3	2984.3	3260.0 **	1248.8	1267.1 ***	1100.5
	CE-1.3	3000.0	2753.4	2960.0	1278.1	1159.6	1116.9
	Mace	2866.7	2577.0	3040.0	n.a	980.2 ***	1254.6
		Wholemeal flour Total Protein (%)			White flour Total Protein (%)		
Glenthorne	NS	13.0	8.8	10.0	11.5	8.7	10.2
	CE-1.1	14.2 **	10.9 ***	11.2 ***	12.7 ***	10.0 ***	10.9
	CE-1.2	13.2	9.7 *	9.7	11.7	9.2	10.3
	CE-1.3	13.1	9.5	10.3	11.1 *	9.3	10.1
	Mace	13.1	9.6	9.4	n.a	8.2	9.1 **
Katanning	NS	18.3	16.7	16.9	13.9	15.9	16.8
	CE-1.1	18.7	17.0	17.7	16.1 ***	16.0	17.4
	CE-1.2	19.0	16.1	17.2	15.1 **	15.9	16.7
	CE-1.3	19.2	16.2	16.6	15.4 ***	16.2	17.0
	Mace	16.2	13.9 ***	14.1 ***	n.a	13.4 ***	13.8 ***
Merredin	NS	14.0	12.9	11.8	12.3	12.7	11.8
	CE-1.1	16.1 ***	13.9 ***	12.3	14.3 ***	14.2 ***	12.3 **
	CE-1.2	14.6	14.0 ***	11.9	12.5	13.1	11.8
	CE-1.3	14.2	13.3 *	12.3	12.5	12.8	12.2 *
	Mace	12.8 *	11.3 ***	11.0 *	n.a	10.9 ***	10.7 ***

Table S5.6. Wholemeal flour calcium ($\mu\text{g/g}$), copper ($\mu\text{g/g}$), potassium ($\mu\text{g/g}$), magnesium ($\mu\text{g/g}$), manganese ($\mu\text{g/g}$), and sulphur ($\mu\text{g/g}$) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test.

Field Site	Genotype	2015	2016	2017	2015	2016	2017	2015	2016	2017
		Wholemeal flour Calcium ($\mu\text{g/g}$)			Wholemeal flour Copper ($\mu\text{g/mg}$)			Wholemeal flour Potassium ($\mu\text{g/g}$)		
Glenthorne	NS	724.5	519.0	600.0	3.5	3.4	4.4	4313.0	4164.5	4139.9
	CE-1.1	712.6	541.0	620.0	4.5 ***	4.4 ***	5.0 **	4119.0	4236.2	4139.9
	CE-1.2	744.5	521.0	640.0	3.7	4.2 ***	4.6	4592.5	4283.7	4100.0
	CE-1.3	716.5	559.0	640.0	3.3	4.2 ***	4.8	4380.9	4244.1	4240.0
	Mace	533.9 ***	400.0 ***	420.0 ***	3.3	3.5	4.1	4949.4 **	4564.1 ***	4079.9
Katanning	NS	1066.7	896.8	1057.8	1.9	2.4	1.8	5125.9	4629.8	3799.9
	CE-1.1	833.3	829.8	1122.6	2.1	2.4	2.1	4663.5	4511.2	4199.9 ***
	CE-1.2	1033.3	894.9	1000.8	2.3	2.2	1.8	5362.5	4352.2	3960.0
	CE-1.3	1133.3	882.6	1039.9	2.3	2.4	1.9	4903.1	4183.4 **	4040.0 *
	Mace	800	644.8 ***	738.9 ***	2.1	1.8 ***	1.7	5090.0	4603.9	3959.9
Merredin	NS	574	506.7	541.5	2.0	1.8	2.5	3599.9	3623.0	3520.0
	CE-1.1	549.4	588.2 *	562.3	2.7	2.5 **	2.6	3566.6	4038.1 ***	3679.9
	CE-1.2	587.9	505.1	577.2	2.2	2.0	2.8	3666.6	3894.5 *	3759.9
	CE-1.3	583.9	526.7	557.7	2.3	2.3 *	2.8	3566.6	3747.6	3540.0
	Mace	522.5	391.5 **	401.3 ***	2.3	1.9	2.8	3999.9 *	3770.8	3799.9 *
		Wholemeal flour Magnesium ($\mu\text{g/g}$)			Wholemeal flour Manganese ($\mu\text{g/mg}$)			Wholemeal flour Sulphur ($\mu\text{g/g}$)		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Glenthorne	NS	1233.9	1234.2	1500.9	20.7	29.4	31.5	1623.1	1212.1	1439.9
	CE-1.1	1348.9	1420.3 ***	1520.1	21.0	36.3 ***	33.3	1787.8 ***	1435.3 ***	1539.9 **
	CE-1.2	1193.7	1324.7 **	1439.2	21.5	32.8 **	30.5	1652.0	1312.0 **	1400.0
	CE-1.3	1160.0	1319.6 *	1459.2	21.7	32.3 *	31.3	1624.3	1343.1 ***	1420.0
	Mace	1005.5 **	1176.5	1220.7 ***	22.0	29.8	28.1	1449.8 ***	1221.5	1339.9 **
Katanning	NS	1666.6	1482.0	1435.8	68.4	64.4	70.0	2101.4	1892.5	2034.8
	CE-1.1	1695.6	1579.1 *	1529.5 *	76.2	71.3 *	72.6	2407.4 **	2025.5 ***	2168.5
	CE-1.2	1642.5	1461.6	1475.9	72.1	67.3	69.4	2177.9	1933.7	1987.4
	CE-1.3	1771.0 *	1460.4	1482.4	85.2 **	62.4	71.3	2240.8	1971.1 *	2006.2
	Mace	1447.7 ***	1271.1 ***	1216.5 ***	76.8	69.5	60.9 **	2038.7	1771.3 **	1742.8 ***
Merredin	NS	1425.6	1226.0	1180.0	68.4	63.6	52.5	1800.0	1791.8	1544.9
	CE-1.1	1640.0 **	1403.7 ***	1300.0 **	57.8	76.5 *	63.7 *	2333.3 ***	2093.8 ***	1693.7 **
	CE-1.2	1432.1	1326.5 **	1300.0 **	61.0	63.3	70.1 ***	1866.5	1796.7	1646.1 *
	CE-1.3	1431.4	1218.9	1240.0	61.5	64.9	65.1 *	1899.9	1759.1	1647.8 *
	Mace	1333.3	1081.2 ***	1180.0	65.8	62.0	64.1 *	1800.0	1498.0 ***	1447.3

Table S5.7. White flour calcium ($\mu\text{g} / \text{g}$), copper ($\mu\text{g} / \text{g}$), potassium ($\mu\text{g} / \text{g}$), magnesium ($\mu\text{g} / \text{g}$), manganese ($\mu\text{g} / \text{g}$), and sulphur ($\mu\text{g} / \text{g}$) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable

Field Site	Genotype	2015	2016	2017	2015	2016	2017	2015	2016	2017
		White flour Calcium ($\mu\text{g} / \text{g}$)			White flour Copper ($\mu\text{g} / \text{mg}$)			White flour Potassium ($\mu\text{g} / \text{g}$)		
Glenthorne	NS	375.9	255.6	290.6	1.2	1.3	1.8	1589.6	1136.1	1379.6
	CE-1.1	411.3 *	266.7	288.1	1.3 ***	1.9 ***	2.1 **	1683.5	1222.7	1356
	CE-1.2	420.5 **	258.6	315.5 **	1.2	1.4	2	1902.3 ***	1192	1473.8
	CE-1.3	389.7	260	322.6 ***	1.1	1.4	1.9	1684.4	1149.5	1437.5
	Mace	n.a	223.8 *	230.7 ***	n.a	1.6 ***	1.9	n.a	1825 ***	1797.4 ***
Katanning	NS	396	395.7	494.9	0.5	0.7	0.6	1306.8	1176.9	1317.7
	CE-1.1	362.4	310 ***	498.3	0.8 **	0.9 *	0.7	1374.6	1086.5 **	1391.3
	CE-1.2	433.4	360 ***	498.4	0.7	0.7	0.5	1445.4	1151.4	1339.4
	CE-1.3	512.8 ***	352 ***	498.8	0.5	0.7	0.6	1670.4 ***	1184.9	1261.6
	Mace	n.a	298.3 ***	422 ***	n.a	0.5 ***	0.7	n.a	1395.3 ***	1546.4 ***
Merredin	NS	317.6	261.3	318.2	0.9	0.6	0.9	1357.5	1192.6	1328.1
	CE-1.1	334.3	269.3	319.6	1	0.8 ***	1.1	1380.9	1083.1 **	1292.4
	CE-1.2	384.6 ***	285.6 ***	265.7 ***	0.8	0.7 **	0.9	1576.4 ***	1201.7	1204.3 **
	CE-1.3	406.4 ***	269.8	270.6 ***	0.8	0.9 ***	0.9	1473.8 ***	1172.9	1169.1 ***
	Mace	n.a	199.2 ***	223.8 ***	n.a	0.6	0.9	n.a	1401.3 ***	1378.3
		White flour Magnesium ($\mu\text{g} / \text{g}$)			White flour Manganese ($\mu\text{g} / \text{mg}$)			White flour Sulphur ($\mu\text{g} / \text{g}$)		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Glenthorne	NS	338.8	312.3	443.7	4.5	4.6	7	1260.3	970.6	1087
	CE-1.1	410 ***	434 ***	460.1 ***	4.5	6.3	7	1436.8	1217.1 ***	1243.6 ***
	CE-1.2	357 *	333.9 *	499.3 *	4.4	4.8	7.9	1264.8	1077.1 ***	1119.4
	CE-1.3	345.2	325.4	462.9	4.5	4.4	7.5	1279.1	1077 ***	1119.8
	Mace	n.a	350.1	494	n.a	5.2	9.6 ***	n.a	1134.7 ***	987.9 ***
Katanning	NS	407.4	414.6	473.8	17.4	12.5	22.6	1474.7	1553.4	1560.6
	CE-1.1	454.3 **	409.4 **	468.5 **	17.5	13.8	21.8	1753.8 ***	1624.1 ***	1630.9 *
	CE-1.2	443.6 ***	404.1 ***	481 ***	16.4	13.2	21.8	1543.9 ***	1547.8	1588.9
	CE-1.3	489 ***	404.8 ***	506.5 ***	16.2 *	11.9	23.9	1594.1 ***	1569.1	1603.8
	Mace	n.a	383.8	469.9	n.a	19.7 ***	23.9	n.a	1392.5 ***	1351.9 ***
Merredin	NS	494.6	414.6	427.6	11.4	12.3	15.2	1450.8	1346.7	1219.9
	CE-1.1	568.8 **	438.8	414.3 **	10.8	12	15.2	1783.1 ***	1560.3 ***	1427.3 **
	CE-1.2	541.2 *	436.7	360.5 *	12.4	13.1	14.3	1601.7 ***	1461.7 ***	1271.1
	CE-1.3	548.2 *	408.6	357.6 *	11.5	12.8	14.4	1569.8 ***	1408.3 ***	1267.8
	Mace	n.a	361.6 ***	390.7	n.a	13.1	18.7 *	n.a	1126.1 ***	1121.4

Table S5.8. Bran calcium ($\mu\text{g} / \text{g}$), copper ($\mu\text{g} / \text{g}$), potassium ($\mu\text{g} / \text{g}$), magnesium ($\mu\text{g} / \text{g}$), manganese ($\mu\text{g} / \text{g}$), and sulphur ($\mu\text{g} / \text{g}$) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable

Field Site	Genotype	2015	2016	2017	2015	2016	2017	2015	2016	2017
		Bran Calcium ($\mu\text{g} / \text{g}$)			Bran Copper ($\mu\text{g} / \text{mg}$)			Bran Potassium ($\mu\text{g} / \text{g}$)		
Glenthorne	NS	1800.0	996.2	1238.4	8.5	5.6	9.4	12960.8	8841.9	9222.8
	CE-1.1	1733.3	1024.3	1296.0	7.2 **	6.9 ***	11.0 ***	11013.6 *	8208.0	10098.9
	CE-1.2	1700.0	1024.0	1158.3	7.5 *	7.3 ***	9.2	12082.1	9410.1	8856.8
	CE-1.3	1633.3	1056.0	1137.7	7.0 **	7.0 ***	9.7	12086.4	9296.9	8496.1
	Mace	n.a	824.4 **	829.7 ***	n.a	6.8 ***	9.2	n.a	9478.8	9905.4
Katanning	NS	2331.7	1947.9	2660.0	3.0	3.6	3.1	9951.8	9529.8	9577.5
	CE-1.1	2059.6	1463.1 ***	2540.0	4.0 ***	3.9	3.4	10827.3	8165.6 **	9826.5
	CE-1.2	2050.5	1827.9	2360.0 **	3.3	3.8	3.0	10151.8	9289.8	9560.2
	CE-1.3	2273.7	1709.0	2380.0 **	2.8	4.2	3.2	9827.3	9044.6	9602.4
	Mace	n.a	1315.1 ***	1640.0 ***	n.a	4.2	2.8	n.a	10662.7 *	9953.3
Merredin	NS	1133.3	1130.9	1221.8	4.4	3.5	5.6	8433.3	9889.6	9048.3
	CE-1.1	1166.7	1074.5	1135.2	5.4	4.6 *	5.8	8600.0	8809.8	8841.3
	CE-1.2	1166.7	898.7 **	1102.4	4.2	3.6	5.7	8500.0	8360.6 *	8587.7
	CE-1.3	1233.3	906.6 **	1161.0	5.0	4.0	6.2 *	9566.7	7889.6 ***	8933.4
	Mace	n.a	736.6 ***	839.5 ***	n.a	3.6	6.4 **	n.a	8838.0	9389.2
	Genotype	Bran Magnesium ($\mu\text{g} / \text{g}$)			Bran Manganese ($\mu\text{g} / \text{mg}$)			Bran Sulphur ($\mu\text{g} / \text{g}$)		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Glenthorne	NS	3900.0	2899.3	3438.8	69.1	69.9	77.6	1900.0	1339.9	1640.0
	CE-1.1	3500.0	2979.1	3796.5	56.0 ***	78.3	84.9	2033.3 *	1601.9 ***	1840.0 ***
	CE-1.2	3433.3	3244.6	3215.8	59.8 **	82.1 *	74.0	1866.7	1521.0 ***	1580.0
	CE-1.3	3133.3 **	3150.8	2997.7 *	62.2 *	79.0	72.0	1800.0	1478.1 **	1660.0
	Mace	n.a	2857.7	2991.2 *	n.a	80.8 *	82.9	n.a	1498.7 **	1680.0
Katanning	NS	4333.3	3106.4	3740.0	193.0	149.9	168.6	2195.2	1980.3	2204.5
	CE-1.1	5033.3 *	2919.6	3700.0	222.2	152.7	167.8	2464.9 ***	2080.2 *	2141.0
	CE-1.2	3800.0	3026.4	3640.0	161.2	151.9	166.0	2128.5	2060.3	2125.7
	CE-1.3	3966.7	3014	3700.0	180.4	140.4	169.4	2198.2	2059.5	2088.3 *
	Mace	n.a	3042	3120.0 ***	n.a	166.4	151.4 ***	n.a	2124.6 ***	1980.5 ***
Merredin	NS	3529.3	3336.9	3360.0	165.3	175.3	165.9	2066.7	1958.9	1936.7
	CE-1.1	4018.6	2876.3 *	3300.0	154.8	169.3	160.9	2533.3 ***	2156.5 ***	1936.8
	CE-1.2	3560.7	2626.7 ***	3200.0	160.0	140.0 *	166.5	2166.7	1992.2	1871.7
	CE-1.3	3719.2	2436.9 ***	3300.0	157.1	142.2 *	170.7	2266.7	1918.1	1960.0
	Mace	n.a	2653.8 ***	3160.0	n.a	153.9	173.7	n.a	1892.9	1874.7

Table S5.9. Flag leaf and leaf iron ($\mu\text{g} / \text{g}$), zinc ($\mu\text{g} / \text{g}$), and phosphorus ($\mu\text{g} / \text{g}$) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable

Field Site	Genotype	2015	2016	2017	2015	2016	2017	2015	2016	2017
		Flag leaf Iron ($\mu\text{g} / \text{g}$)			Flag leaf Zinc ($\mu\text{g} / \text{mg}$)			Flag leaf Phosphorus ($\mu\text{g} / \text{g}$)		
Glenthorne	NS	75.2	67.9	69.1	20.4	13.9	15.2	2538.4	2100.0	2399.1
	CE-1.1	67.9	72.2	67.7	41.5	15.1	17.4	2936.3 ***	2700.0 ***	2771.8 ***
	CE-1.2	67.4	80.7	71.9	19.3	14.0	17.7	2601.4	2500.0 *	2791.9 ***
	CE-1.3	64.9 *	73.6	70.7	20.9	14.9	16.0	2226.5 ***	2500.0 *	2532.6
	Mace	76.1	63.5	74.8	27.2	17.1 **	20.7	2754.3 *	2450.0	3124.6 ***
Katanning	NS	89.9	56.4	68.1	23.8	20.7	19.8	n.a	2676.2	3617.2
	CE-1.1	81.3	46.9 ***	64.1	28.7	19.2	21.6	n.a	2805.9	3820.9
	CE-1.2	71.5 *	55.7	71.4	25.4	21.0	20.3	n.a	2957.8 *	3860.3
	CE-1.3	77.2	50.5 *	68.7	26.3	18.1 *	21.9	n.a	2663.0	3934.7
	Mace	114.1 **	66.3 **	88.7 ***	21.1	19.8	19.4	n.a	2971.4 *	3546.9
Merredin	NS	124.9	104.9	103.3	21.7	14.7	15.2	1831.5	2118.3	2002.2
	CE-1.1	97.8 **	109.2	93.6	22.2	14.8	14.3	2591.6 ***	2353.5	2228.2
	CE-1.2	103.5 *	93.8 *	90.3 **	31.2	14.8	13.8	2575.9 ***	2172.5	2220.1
	CE-1.3	109.0	94.2 *	98.0	19.2	13.9	15.2	2475.1 ***	1999.7	2172.3
	Mace	116.2	105.6	109.2	17.9	14.2	18.5 *	1989.2	2195.0	2697.2 ***
	Genotype	Leaf Iron ($\mu\text{g} / \text{g}$)			Leaf Zinc ($\mu\text{g} / \text{mg}$)			Leaf Phosphorus ($\mu\text{g} / \text{g}$)		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Glenthorne	NS	61.6	96.7	86.8	19.9	13.1	14.4	2237.1	1938.6	2480.0
	CE-1.1	59.7	66.8 *	79.6	21.2	11.8	15.4	2431.3	2150.6	2680.0
	CE-1.2	57.2	84.0	83.8	18.5	11.3	14.7	2280.3	1967.7	2620.0
	CE-1.3	58.6	79.2	77.9	18.5	11.6	15.1	2102.0	2186.1	2660.0
	Mace	64.7	83.9	64.7 ***	19.4	14.4	16.3 **	2372.2	2546.3 ***	3420.0 ***
Katanning	NS	n.a	54.2	59.1	n.a	20.2	22.4	n.a	2572.1	3932.9
	CE-1.1	n.a	52.0	63.5	n.a	18.7	24.7	n.a	2995.6 *	4729.8 *
	CE-1.2	n.a	52.1	55.3	n.a	21.2	20.9	n.a	2892.1	4111.7
	CE-1.3	n.a	50.7	61.2	n.a	17.2 *	23.2	n.a	2712.4	4254.3
	Mace	n.a	55.1	77.9 ***	n.a	15.9 **	15.9 *	n.a	3284.9 ***	3831.3
Merredin	NS	125.7	108.7	95.7	24.3	15.6	14.6	2012.6	2065.9	2100.0
	CE-1.1	89.4 **	111.8	93.0	24.1	15.3	19.3 *	2263.4	2340.6	2520.0 **
	CE-1.2	105.0	100.0	96.5	21.9	15.3	13.9	2354.4	1871.1	1820.0
	CE-1.3	96.5 *	99.5	94.4	24.4	13.2	16.1	2438.6 *	1779.5	1960.0
	Mace	103.8	106.9	94.5	20.7	11.6	13.4	1922.8	2053.6	2580.0 **

Table S5.10. Flag leaf calcium ($\mu\text{g / g}$), copper ($\mu\text{g / g}$), potassium ($\mu\text{g / g}$), magnesium ($\mu\text{g / g}$), manganese ($\mu\text{g / g}$), and sulphur ($\mu\text{g / g}$) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable

Field Site	Genotype	2015	2016	2017	2015	2016	2017	2015	2016	2017
		Flag leaf Calcium ($\mu\text{g / g}$)			Flag leaf Copper ($\mu\text{g / mg}$)			Flag leaf Potassium ($\mu\text{g / g}$)		
Glenthorne	NS	6676.6	6175.8	5699.1	6.0	3.5	3.3	24933.3	15620.0	21934.1
	CE-1.1	6244.6	5956.9	5126.2	7.6 **	4.1	3.7	28333.3	19740.0 ***	24086.6 *
	CE-1.2	6363.0	5598.2 *	5609.2	5.9	4.0	3.2	27033.3	19520.0 ***	23744.7 *
	CE-1.3	6944.7	6392.5	5647.1	5.0 *	4.8 ***	3.7	27100.0	19560.0 ***	22652.5
	Mace	5295.2	5195.8 ***	5458.4	6.4	4.1	5.2 ***	22900.0	16275.0	21202.2
Katanning	NS	n.a	9368.0	10200.0	2.4	2.5	2.6	14208.6	9689.9	5960.0
	CE-1.1	n.a	8220.9	10460.0	2.6	2.0	3.2	14128.3	11754.0	5400.0
	CE-1.2	n.a	10568.0	9380.0	3.2	2.1	1.7	10629.5 *	11629.9	7200.0
	CE-1.3	n.a	9391.1	11040.0	2.4	1.7 **	2.3	13153.9	9336.1	6680.0
	Mace	n.a	10015.0	11760.0 *	2.6	2.1	2.8	11753.3	8812.6	6240.0
Merredin	NS	9315.9	6209.6	10160.0	3.8	2.4	3.3	25166.7	19879.5	18387.7
	CE-1.1	6694.1 **	7075.3	8700.0	4.1	2.9	3.4	29100.0	22179.6	21591.2 **
	CE-1.2	8908.3	5904.0	7820.0 *	3.6	2.3	3.9	33000.0 **	22240.9	23221.7 ***
	CE-1.3	8106.9	6075.3	8220.0	5.4 ***	2.9	4.3	32133.3 *	23219.5	23033.6 ***
	Mace	8381.7	6094.8	8480.0	4.3	3.7 **	4.8 **	19300.0 *	15400.6 *	16565.8
		Flag leaf Magnesium ($\mu\text{g / g}$)			Flag leaf Manganese ($\mu\text{g / mg}$)			Flag leaf Sulphur ($\mu\text{g / g}$)		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Glenthorne	NS	2257.8	1557.5	1582.2	32.1	24.3	16.6	4983.9	2363.4	2360.0
	CE-1.1	2469.6	1627.3	1594.6	29.2	26.0	17.2	5007.9	2474.9	2580.0
	CE-1.2	2186.6	1441.1	1549.9	33.2	22.6	18.1	4533.3	2400.4	2720.0 **
	CE-1.3	2150.3	1559.6	1332.4 ***	32.3	26.2	15.8	4992.1	2655.2 **	2380.0
	Mace	1927.2	1293.1 **	1560.9	39.2	32.2 ***	20.2 *	4016.5	2157.7 *	2620.0 *
Katanning	NS	3877.4	2400.0	2140.0	237.8	126.4	123.9	3676.7	3340.3	2770.9
	CE-1.1	4173.4	1940.0 **	2180.0	323.6	126.0	153.6	4362.8 *	2992.6	2756.9
	CE-1.2	3163.9 *	2400.0	2060.0	282.3	173.8	111.3	3635.3	3480.3	3079.9
	CE-1.3	4300.2	2080.0 *	2240.0	404.6 ***	111.8	143.6	4229.5	3487.0	3077.6
	Mace	4827.5 **	2800.0 *	2860.0 ***	444.5 ***	176.4 **	213.9 ***	4348.9 *	3174.6	2654.8
Merredin	NS	2822.5	1703.6	2760.0	204.5	165.5	169.8	4266.7	4110.3	3933.1
	CE-1.1	3141.8	1910.0	2400.0	144.1	207.2	179.5	5166.7 *	4823.3	4145.6
	CE-1.2	3301.4	1640.2	1960.0 **	242.8	132.7	165.8	5266.7 *	3795.7	3719.0
	CE-1.3	2923.1	1638.6	2140.0 *	207.6	138.9	163.3	5333.3 *	4859.6	4157.5
	Mace	3111.1	1834.5	2680.0	280.6	206.2	291.5 **	4533.3	3813.9	3604.9

Table S5.11. Leaf calcium ($\mu\text{g / g}$), copper ($\mu\text{g / g}$), potassium ($\mu\text{g / g}$), magnesium ($\mu\text{g / g}$), manganese ($\mu\text{g / g}$), and sulphur ($\mu\text{g / g}$) concentrations in null segregant, CE-1.1, CE-1.2, CE-1.3 and Mace at Glenthorne, Katanning and Merredin over three field seasons. Values indicate best linear unbiased estimators for a minimum of three replicates per genotype. Asterisks represent significant differences to the NS for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Wald test. n.a = not applicable

Field Site	Genotype	2015	2016	2017	2015	2016	2017	2015	2016	2017
		Leaf Calcium ($\mu\text{g / g}$)			Leaf Copper ($\mu\text{g / mg}$)			Leaf Potassium ($\mu\text{g / g}$)		
Glenthorne	NS	6419.6	5960.0	6560.0	5.5	3.3	2.9	37260.8	22985.4	29167.2
	CE-1.1	6788.6	6360.0	6780.0	5.8	4.4 **	3.8 *	40545.0	22919.5	27391.1 *
	CE-1.2	5799.2	6080.0	6720.0	5.4	3.9	3.4	31616.7 *	23042.0	27569.5
	CE-1.3	6911.4	6840.0 *	6960.0 *	4.9	3.8	3.1	36126.1	23393.3	27963.8
	Mace	4334.9	4650.0 **	4640.0 ***	6.0	4.5 **	4.4 ***	29415.5 *	25149.6 ***	29328.3
Katanning	NS	n.a	12860.0	12660.0	n.a	2.8	2.3	n.a	9118.1	4000.0
	CE-1.1	n.a	12760.0	11280.0	n.a	2.5 *	2.4	n.a	13699.8	7060.0 **
	CE-1.2	n.a	13940.0	12120.0	n.a	2.5 *	2.5	n.a	11858.6	5100.0
	CE-1.3	n.a	13220.0	13500.0	n.a	1.9 ***	3.0	n.a	9984.2	5880.0
	Mace	n.a	8000.0 ***	9020.0 ***	n.a	2.4 *	2.7	n.a	10774.1	7440.0 ***
Merredin	NS	7627.5	7718.7	9170.8	4.5	2.7	3.7	32790.8	27628.2	25637.3
	CE-1.1	7127.9	8669.3	10376.3 *	5.1	3.5 *	4.3	36759.5 *	26449.6	26158.0
	CE-1.2	8255.4	8637.1	9264.0	4.9	2.5	3.7	30703.7	27747.5	26551.1
	CE-1.3	9458.0 ***	7194.7	10078.2	4.8	2.6	3.9	38740.4 **	27247.8	27375.4
	Mace	7342.0	5575.2 *	7670.6 **	5.3	3.6 *	4.0	26492.1 **	23533.7 *	22118.2 **
		Leaf Magnesium ($\mu\text{g / g}$)			Leaf Manganese ($\mu\text{g / mg}$)			Leaf Sulphur ($\mu\text{g / g}$)		
		2015	2016	2017	2015	2016	2017	2015	2016	2017
Glenthorne	NS	1973.1	1301.5	1344.4	22.4	21.7	15.3	4433.3	2477.8	2550.5
	CE-1.1	2274.1	1460.2	1388.9	28.4	25.8 **	16.0	5066.7	2439.9	2828.4 *
	CE-1.2	1817.3	1320.1	1308.4	27.0	22.0	15.6	3800.0	2403.0	2924.9
	CE-1.3	2025.9	1457.4	1264.6	30.7 *	24.6	14.6	4333.3	2811.2 **	2757.9
	Mace	1685.0	1176.1	1193.7 *	29.4	25.9 **	15.7	3400.0	2160.2 **	2298.3
Katanning	NS	n.a	3580.0	2760.0	n.a	171.5	166.7	n.a	3680.8	2718.8
	CE-1.1	n.a	3040.0 *	2520.0	n.a	175.0	185.1	n.a	3739.9	2572.3
	CE-1.2	n.a	3480.0	2480.0	n.a	211.3 *	170.1	n.a	3720.8	2487.7
	CE-1.3	n.a	3180.0	2760.0	n.a	149.8	218.4 *	n.a	3719.3	2449.1
	Mace	n.a	2525.0 ***	2400.0	n.a	148.2	179.3	n.a	2599.0 ***	2252.2
Merredin	NS	2733.3	1991.1	2300.0	232.6	214.6	171.9	4044.6	4745.9	3881.3
	CE-1.1	2933.3	2372.5	2520.0	188.4	265.6	188.3	5073.6 *	5449.4	4898.8 ***
	CE-1.2	3000.0	2256.4	2340.0	230.9	201.0	211.0	4961.9 *	4811.0	4149.2
	CE-1.3	3100.0	1711.1	2560.0	255.3	180.3	198.1	5659.7 ***	5857.7 *	4371.7
	Mace	2666.7	1711.1	2360.0	286.2	203.0	220.1	3980.0	3620.1 *	3099.0 **

6. Chapter 6 – Nicotianamine-chelated iron positively affects iron status, intestinal morphology and microbial populations *in vivo* (*Gallus gallus*)

6.1. Abstract

Wheat flour iron (Fe) fortification is mandatory in 75 countries worldwide yet many Fe fortificants, such as Fe-ethylenediaminetetraacetate (EDTA), result in unwanted sensory properties and/or gastrointestinal dysfunction and dysbiosis. Nicotianamine (NA) is a natural chelator of Fe, zinc (Zn) and other metals in higher plants and NA-chelated Fe is highly bioavailable *in vitro*. In graminaceous plants NA serves as the biosynthetic precursor to 2'-deoxymugineic acid (DMA), a related Fe chelator and enhancer of Fe bioavailability, and increased NA/DMA biosynthesis has proved an effective Fe biofortification strategy in several cereal crops. Here we utilized the chicken (*Gallus gallus*) model to investigate impacts of NA-chelated Fe on Fe status and gastrointestinal health when delivered to chickens through intraamniotic administration (short-term exposure) or over a period of six weeks as part of a biofortified wheat diet containing increased NA, Fe, Zn and DMA (long-term exposure). Striking similarities in host Fe status, intestinal functionality and gut microbiome were observed between the short-term and long-term treatments, suggesting that the effects were largely if not entirely due to consumption of NA-chelated Fe. These results provide strong support for wheat with increased NA-chelated Fe as an effective biofortification strategy and uncover novel impacts of NA-chelated Fe on gastrointestinal health and functionality.

6.2. Introduction

Iron (Fe) supplementation and fortification are the two most widely used strategies to combat human Fe deficiencies that affect over 2 billion people worldwide¹⁻³. Iron supplementation involves large dose delivery of highly absorbable (bioavailable) Fe to humans and is effective in treating severe cases of Fe deficiency anemia^{2,4,5}. Iron fortification involves low dose delivery of bioavailable Fe fortificants to food products during manufacture (or point-of-use) and is an effective population-based strategy to boost Fe intakes. Iron fortification of wheat flour is now mandatory in 75 countries worldwide (Flour Fortification Initiative; <https://fortificationdata.org/>), however, the tendency of Fe fortificants such as ferrous sulfate (FeSO₄) to oxidize and cause undesired organoleptic and sensory properties pose significant challenges^{6,7}. Almost 90% of countries utilize fortificants with poor bioavailability or fortify at sub-optimal concentrations, although recent evidence suggests that Fe fortification can effectively reduce symptoms of Fe-deficiency anemia when correctly implemented⁸⁻¹⁰. Iron chelated by ethylenediaminetetraacetate (EDTA) is a commonly recommended fortificant for cereal flour to minimize sensory alterations while providing Fe in a bioavailable form^{3,6,8,11,12}. Fortificants that utilize micro- and/or nanoencapsulation can further improve bioavailability¹²⁻¹⁴.

although the cost of using appropriately chelated and/or encapsulated Fe fortificants (\$2 USD per ton to fortify wheat flour with EDTA-chelated Fe alone), and the requirement for centralized cereal processing and industrial milling limits flour fortification programs in less developed countries^{8,11,15,16}. Furthermore, and perhaps more importantly, both supplementation and fortification frequently deliver excess dietary Fe to the human intestinal lumen which can cause severe gastrointestinal disruption, dysbiosis and the proliferation of non-beneficial gut bacteria^{3,17–22}.

Nicotianamine (NA) is a non-protein amino acid that functions as an endogenous chelator of Fe, zinc (Zn) and other transition metals in higher plants. In graminaceous cereals NA serves as the biosynthetic precursor to 2'-deoxymugenic acid (DMA), a related Fe chelator in plant tissues that also functions as a root-secreted phytosiderophore to chelate ferric Fe in the rhizosphere²³. Both NA and/or DMA are major Fe chelators in white wheat (*Triticum aestivum* L.) flour and enhancers of *in vitro* Fe bioavailability^{24–26} and increased NA/DMA biosynthesis has been employed to biofortify wheat^{25,27} and rice (*Oryza sativa* L.)^{28–30} with Fe and Zn. While both NA and DMA chelate ferric (Fe³⁺) ions, only NA is capable of chelating highly-bioavailable Fe²⁺ ions^{31,32}. Iron biofortified rice with increased NA biosynthesis has also reversed anemia symptoms in mice, suggesting that NA-chelated Fe is bioavailable *in vivo*^{33,34}. Taken together these results reveal NA-chelated Fe as a natural and highly bioavailable Fe fortificant that improves host Fe status.

The chicken (*Gallus gallus*) model is physiologically relevant for estimating dietary micronutrient absorption in humans due to similarities in intestinal morphology and enteric microbiota, and has been used in numerous studies to evaluate Fe and Zn bioavailability in staple foods^{35–44}. Here we utilized the chicken model to investigate the impact of NA-chelated and EDTA-chelated Fe on Fe status and gastrointestinal health when delivered alongside extracts of control and biofortified white wheat flour containing increased NA, Fe, Zn and DMA through intraamniotic administration four days prior to hatch (short-term exposure). Short-term exposure to NA-chelated Fe and extracts of biofortified wheat flour had similar effects on gastrointestinal health; we therefore conducted a separate feeding trial study of control and biofortified wheat-based diets over a period of six weeks (long-term exposure). Together this study highlights the versatility of the chicken model and demonstrates novel positive effects of NA-chelated Fe on host Fe status and gastrointestinal health when administered as an Fe fortificant or as part of a biofortified diet.

6.3. Results

6.3.1. Experiment 1 – Intraamniotic administration of EDTA-chelated and NA-chelated Fe fortificants

Intraamniotic administration of NA-chelated Fe improves Fe status and alters expression of Fe homeostasis/hypertension genes

Blood serum Fe concentration was significantly elevated in chickens that received intraamniotic administration of EDTA-chelated Fe ('Fe EDTA') and NA-chelated Fe ('Fe NA') relative to unchelated Fe ('Fe') and non-injected ('NI') treatment groups (Figure 6.1a). Blood serum Zn, liver Fe and liver Zn concentrations were not significantly different between treatment groups (Figure 6.1b-d).

Duodenal cytochrome B (DcytB), divalent metal transporter 1 (DMT1) and Zn transporter (ZIP4) expression was significantly upregulated in intestinal tissue of chickens that received 'Fe' relative to all treatment groups, except for *DcytB* and *DMT1* expression in control white flour extract ('C WF') and biofortified white flour extract ('B WF') treatment groups (Figure 6.1e). Both *alkaline phosphatase (AKP)* and *Zn transporter 1 (ZnT1)* expression were significantly upregulated in chickens that received intraamniotic administration of H₂O ('H₂O') relative to 'Fe', 'C WF' and 'B WF' treatment groups (Figure 6.1e). No differences in heart gene expression were observed between treatment groups (Figure 6.1f).

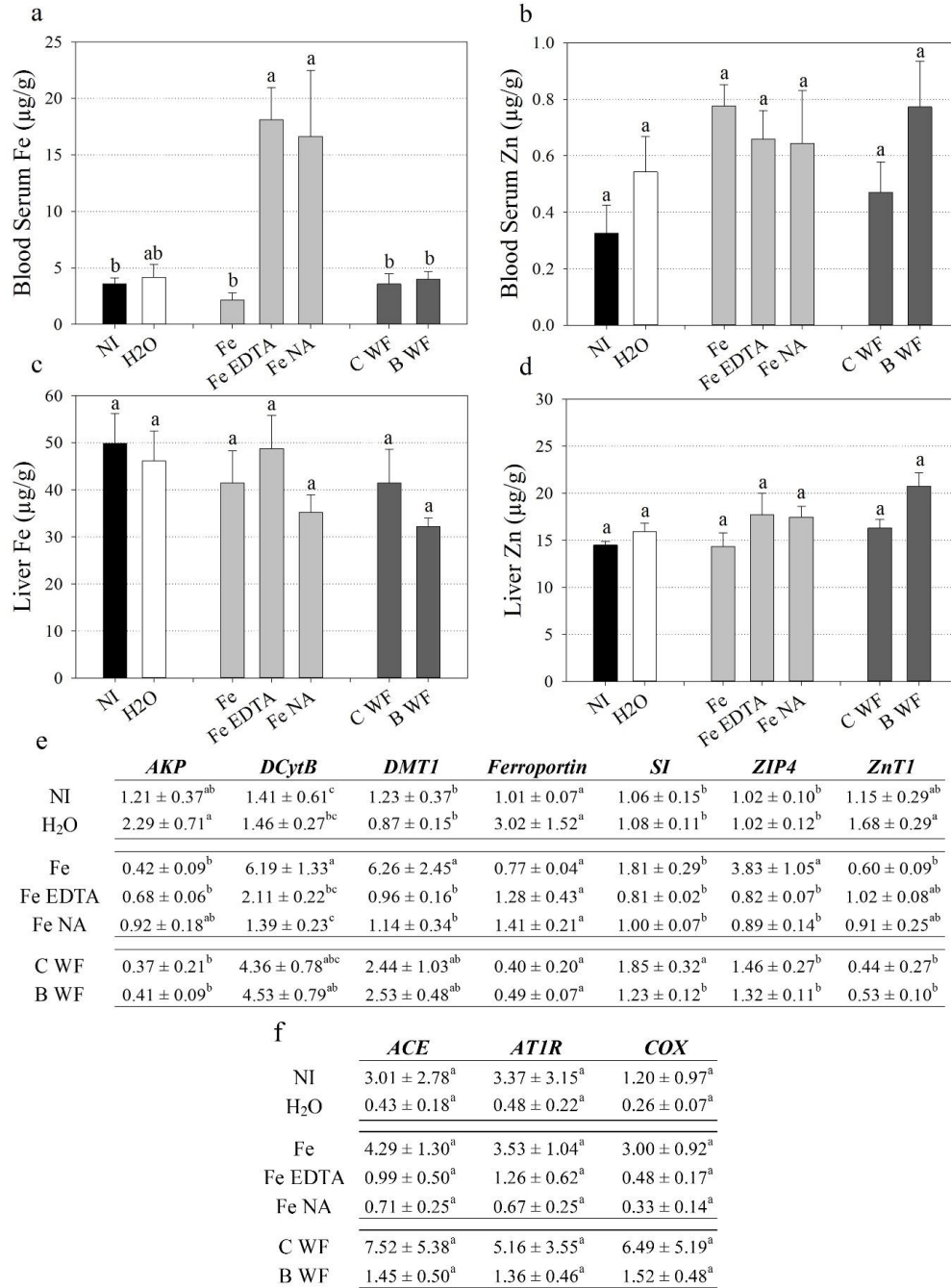


Figure 6.1. Biomarkers of Fe and Zn status following intraamniotic administration. Fe and Zn concentration (µg/g) in chicken (a-b) blood serum, respectively; and (c-d) liver, respectively. Bars represent mean ± SEM of at least three biological replicates. (e-f) Transcript quantification of genes in chicken duodenal and heart tissue, respectively. Values (expression ratio relative to 18S) represent mean ± SEM of at least three biological replicates, each with two technical replicates of quantitative RT-PCR. Different letters indicate significantly different values between treatment groups as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$). NI: non-injected, C WF: control white flour extract, B WF: biofortified white flour extract.

Intraamniotic administration of NA-chelated Fe positively affects intestinal morphology and microbial population density

Goblet cell number increased significantly in 'Fe NA' intestinal villi relative to all treatment groups and in 'B WF' relative to all groups except for 'Fe NA' (Figure 6.2a). Goblet cell number decreased significantly in 'Fe EDTA' intestinal villi relative to all treatment groups. Intestinal villi length increased significantly in 'Fe EDTA' relative to all treatment groups except for 'H₂O' and in 'H₂O', 'Fe NA' and 'B WF' treatment groups relative to 'NI', 'Fe' and 'B WF' treatment groups (Figure 6.2b). Intestinal villi width increased significantly in 'Fe EDTA' relative to all treatment groups, and in 'H₂O' relative to 'Fe NA' (Figure 6.2c). Intestinal villi width decreased significantly in 'C WF' relative to all treatment groups.

The abundance of *Bifidobacterium* significantly increased in 'Fe NA' cecum relative to all treatment groups apart from 'Fe' and 'C WF', and significantly decreased in 'H₂O' relative to all treatment groups apart from 'Fe EDTA' (Figure 6.2d). The abundance of both *Escherichia* significantly increased in 'Fe EDTA' cecum relative to 'Fe NA' and *Clostridium* significantly increased in 'H₂O' and 'Fe EDTA' relative to 'C WF' (Figure 6.2d).

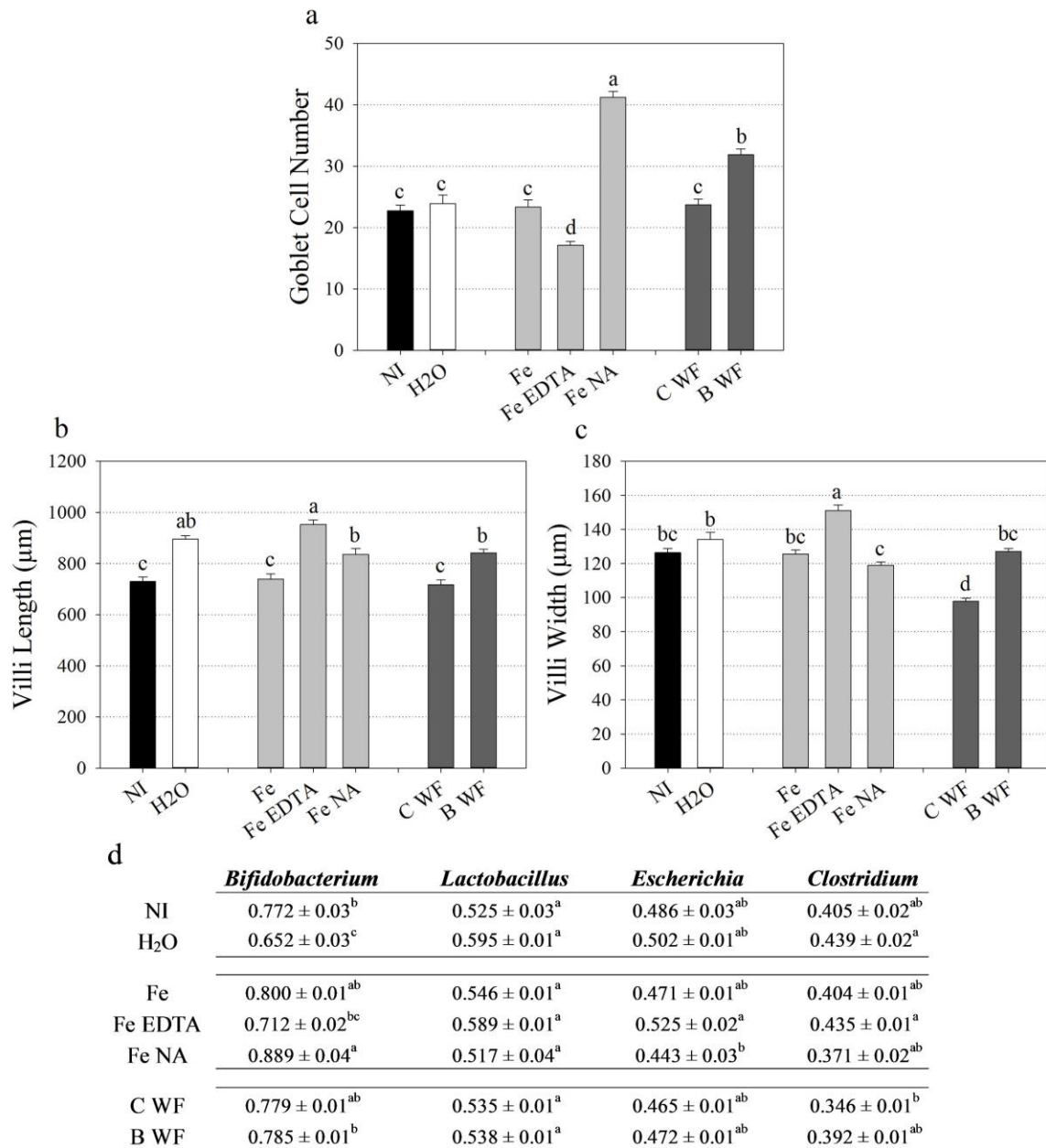


Figure 6.2. Intestinal functionality and cecal microbial composition following intraamniotic administration. (a) Chicken intestinal goblet cell number. (b-c) Chicken intestinal villi length and width (μm), respectively. Bars represent mean ± SEM of five biological replicates. (d) Bacterial proportions relative to a universal bacterial population present in ceca. Values (arbitrary units, AU) represent mean ± SEM of five biological replicates. Different letters indicate significantly different values between treatment groups as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$). NI: non-injected, C WF: control white flour extract, B WF: biofortified white flour extract.

6.3.2. Experiment 2 – Feeding trial of control and biofortified white wheat flour

Biofortified white wheat flour increases total body hemoglobin and hemoglobin maintenance efficiency with lower feed intake and feed conversion ratio

The concentrations of Fe, Zn, NA and DMA were significantly higher in white flour derived from field-grown bread wheat expressing the rice nicotianamine synthase (*OsNAS2*) gene compared to control white flour (Figure S6.1, Table S6.1) and significantly increased 1.1- to 1.2-fold (Fe and Zn) and 1.7- to 1.8-fold (NA and DMA) in diet containing 80% biofortified white flour ('Biofortified') relative to diet containing 80% control white flour ('Control') (Table 6.1). Caco-2 cell ferritin significantly increased after exposure to biofortified white flour relative to control white flour (Figure S6.2). At week 2, hemoglobin (Hb), total body Hb and hemoglobin maintenance efficiency (HME) decreased significantly in 'Biofortified' relative to 'Control' chickens (Table 6.2). From week 4 onwards, a trend of lower cumulative feed intake (g) and cumulative feed conversion ratio (FCR) was present in 'Biofortified' relative to 'Control' chickens. No differences in body weight between 'Biofortified' and 'Control' chickens were observed throughout the study (Table 6.2).

Table 6.1. Composition of the experimental diets. Component values represent mean $\pm$ SEM of at least four technical replicates. Asterisks denote significant differences for $p \leq 0.001$ (***) as determined by Student's t-test.

Diet Ingredient	Control	Biofortified
	g/Kg (by formulation)	
Control white wheat flour	800	-
Biofortified white wheat flour	-	800
skim milk, dry	99.75	99.75
DL-methionine	2.5	2.5
corn oil	27	27
choline chloride	0.75	0.75
vitamin / mineral premix (no Fe/Zn)	70	70
Selected Components	Control	Biofortified
Dietary Fe ($\mu\text{g/g}$)	25.9 ± 0.12	$28.9 \pm 0.13^{***}$
Dietary Zn ($\mu\text{g/g}$)	16.6 ± 0.06	$19.2 \pm 0.03^{***}$
Dietary NA ($\mu\text{mol/g}$)	18.1 ± 0.32	$33.0 \pm 0.21^{***}$
Dietary DMA ($\mu\text{mol/g}$)	19.5 ± 0.16	$34.1 \pm 0.74^{***}$
Dietary Phytate (mg/g)	0.5 ± 0.09	0.5 ± 0.08
Total Fiber ($\mu\text{g/g}$)	19.9 ± 0.18	23.8 ± 1.12
Total Protein (%)	13.47 ± 0.08	13.67 ± 0.08
Total Carbon (%)	41.90 ± 0.13	41.30 ± 0.13
Phytate : Fe molar ratio	1.63	1.46

Table 6.2. Body weight, biomarkers of Fe status and feed consumption throughout the study. Values represent mean $\pm$ SEM of at least nine biological replicates. Asterisks denote significant differences between diet treatments for $p \leq 0.001$ (***) as determined by Student's t-test. Hb: hemoglobin, HME: hemoglobin maintenance efficiency, FCR: feed conversion ratio.

Variable	Diet	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Body Weight (kg)	Control	0.125 $\pm$ 0.007	0.158 $\pm$ 0.004	0.195 $\pm$ 0.007	0.236 $\pm$ 0.010	0.286 $\pm$ 0.013	0.355 $\pm$ 0.016	0.365 $\pm$ 0.029
	Biofortified	0.122 $\pm$ 0.006	0.160 $\pm$ 0.004	0.191 $\pm$ 0.007	0.222 $\pm$ 0.010	0.260 $\pm$ 0.014	0.318 $\pm$ 0.018	0.353 $\pm$ 0.029
Hb (g/L)	Control	72.7 $\pm$ 2.3	96.5 $\pm$ 1.6	112.2 $\pm$ 1.2***	103.5 $\pm$ 3.5	99.6 $\pm$ 3.7	82.4 $\pm$ 3.7	94.9 $\pm$ 3.6
	Biofortified	72.7 $\pm$ 2.3	93.2 $\pm$ 1.6	92.5 $\pm$ 1.5	97.8 $\pm$ 3.7	104.7 $\pm$ 4.0	91.4 $\pm$ 3.4	101.2 $\pm$ 3.5
Total Body Hb (mg)	Control	2.59 $\pm$ 0.14	4.26 $\pm$ 0.124	6.15 $\pm$ 0.17***	7.09 $\pm$ 0.35	7.87 $\pm$ 0.33	8.48 $\pm$ 0.67	9.83 $\pm$ 1.01
	Biofortified	2.52 $\pm$ 0.13	4.20 $\pm$ 0.124	4.72 $\pm$ 0.17	6.21 $\pm$ 0.39	7.57 $\pm$ 0.36	7.74 $\pm$ 0.70	10.06 $\pm$ 0.98
HME (%)	Control		12.16 $\pm$ 0.879	13.80 $\pm$ 0.67***	10.21 $\pm$ 0.77	8.32 $\pm$ 0.50	4.36 $\pm$ 0.76	3.14 $\pm$ 1.02
	Biofortified		11.17 $\pm$ 0.879	7.14 $\pm$ 0.71	8.56 $\pm$ 0.85	8.41 $\pm$ 0.55	4.65 $\pm$ 0.76	5.84 $\pm$ 1.00
FCR	Control		5.85 $\pm$ 0.738	4.82 $\pm$ 0.55	6.10 $\pm$ 0.52	7.98 $\pm$ 0.51	4.09 $\pm$ 0.24	22.30 $\pm$ 3.19
	Biofortified		4.86 $\pm$ 0.736	5.98 $\pm$ 0.55	6.14 $\pm$ 0.57	6.89 $\pm$ 0.53	3.63 $\pm$ 0.25	19.81 $\pm$ 2.99
Feed Intake (g)	Control		180.6 $\pm$ 20.0	157.8 $\pm$ 13.3	251.0 $\pm$ 27.4	299.9 $\pm$ 41.9	284.5 $\pm$ 29.8	243.7 $\pm$ 25.2
	Biofortified		171.0 $\pm$ 20.0	152.6 $\pm$ 13.3	201.3 $\pm$ 27.4	244.8 $\pm$ 41.9	190.8 $\pm$ 29.8	185.6 $\pm$ 21.8
Cumulative Feed Intake (g)	Control			338.5 $\pm$ 31.6	589.5 $\pm$ 50.7	889.4 $\pm$ 91.2	1174.0 $\pm$ 119.0	1333.0 $\pm$ 153.0
	Biofortified			323.6 $\pm$ 31.6	524.9 $\pm$ 50.7	769.7 $\pm$ 91.2	960.0 $\pm$ 119.0	1096.0 $\pm$ 153.0
Cumulative FCR	Control			4.56 $\pm$ 0.38	5.00 $\pm$ 0.39	5.45 $\pm$ 0.63	4.92 $\pm$ 0.52	6.12 $\pm$ 0.85
	Biofortified			5.15 $\pm$ 0.38	5.25 $\pm$ 0.39	4.73 $\pm$ 0.63	4.16 $\pm$ 0.52	4.66 $\pm$ 0.73

Biofortified white wheat flour improves iron status and glycogen storage

No differences in blood serum Fe and Zn concentrations were observed between ‘Biofortified’ and ‘Control’ chickens throughout the study (Figure 6.3a,b). At week 2, blood linoleic acid:dihomo- γ -linolenic acid ratio (LA:DGLA) was significantly decreased in ‘Biofortified’ relative to ‘Control’ chickens (Figure 6.3c). At the conclusion of the study, liver Fe concentration and glycogen storage in both liver and pectoral tissue was significantly elevated in ‘Biofortified’ relative to ‘Control’ chickens (Figure 6.3d,e). No differences in nail or feather Fe and Zn concentrations were observed throughout the study (Figure S6.3). Expression of *cytochrome c oxidase (COX)* was significantly upregulated in ‘Biofortified’ heart tissue relative to ‘Control’ (Figure 6.3f).

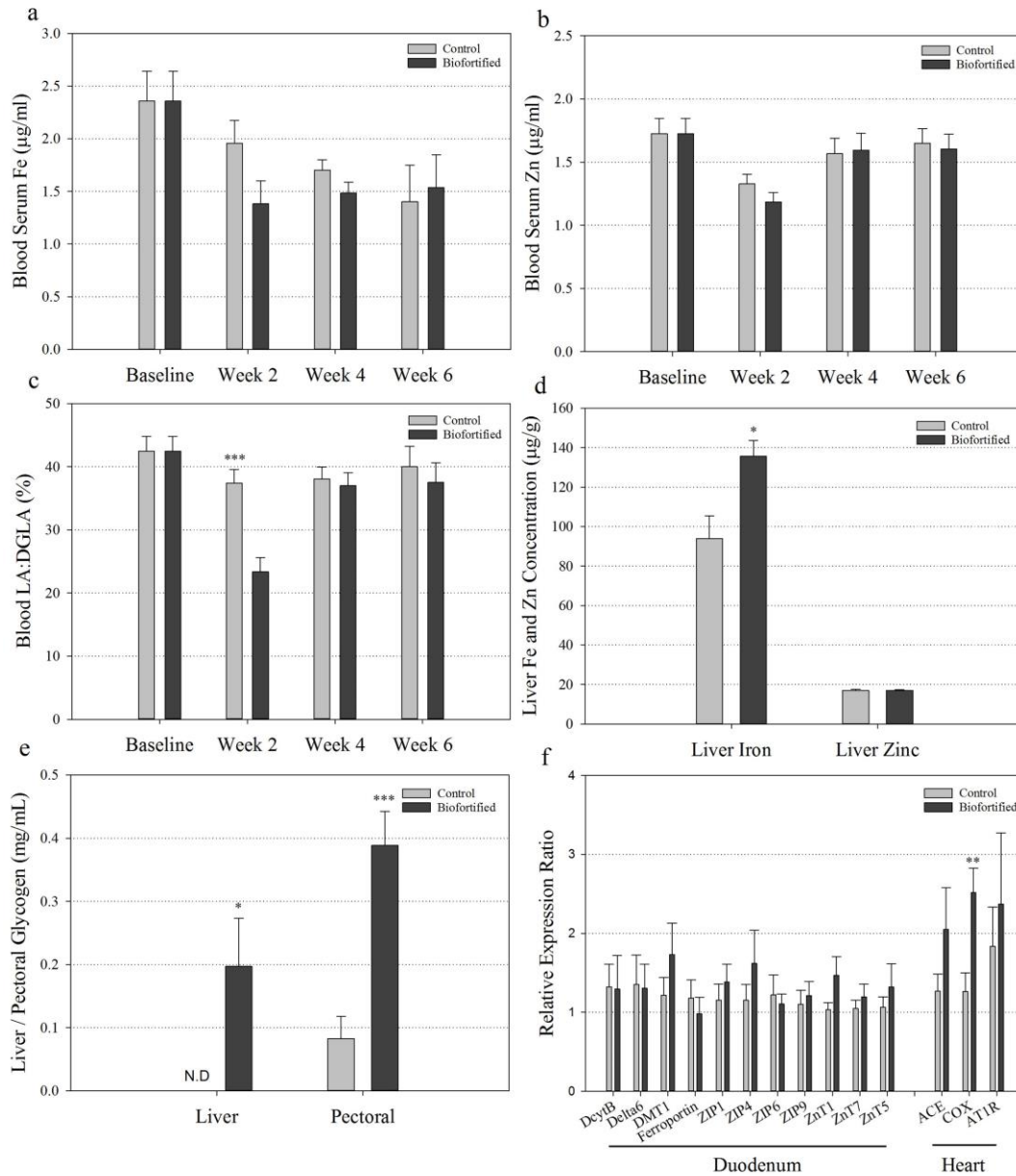


Figure 6.3. Biomarkers of Fe and Zn status and glycogen storage following consumption of experimental diets. (a-b) Fe and Zn concentration ($\mu\text{g/g}$) in chicken blood serum, respectively. (c) Ratio of LA:DGLA in chicken blood cells. Measurements were taken at the beginning (Baseline) and in the 2nd, 4th and 6th week of the study. (d) Fe and Zn concentration ($\mu\text{g/g}$) in chicken liver. (e) Glycogen (mg/mL) concentration in chicken liver and pectoral tissue. Bars represent mean $\pm$ SEM of nine biological replicates. (f) Transcript quantification relative to 18S in chicken duodenal and heart tissue. Bars represent mean $\pm$ SEM of at least eight biological replicates, each with two technical replicates of quantitative RT-PCR. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.001$ (***) as determined by Student's t-test.

Biofortified white wheat flour increases goblet cell number and positively alters gut health and the microbiome

The number of intestinal goblet cells significantly increased, and the diameter of intestinal goblet cells significantly decreased in 'Biofortified' relative to 'Control' chickens (Figure 6.4a). 'Biofortified' chickens had significantly more goblet cells that were a mixture of both acidic and neutral relative to 'Control' chickens (Figure S6.4). No differences in intestinal villi length and width were detected (Figure 6.4b). Short-chain fatty acid (SCFA) production significantly increased for acetic acid, propionic acid and valeric acid and decreased for butanoic acid in 'Biofortified' relative to 'Control' chickens (Figure 6.4c). For major bacteria phyla the proportion of Actinobacteria increased 1.9-fold while the proportion of Firmicutes and Proteobacteria decreased 1.2- and 2.0-fold, respectively in 'Biofortified' ceca relative to 'Control' (Figure 6.4d). For major bacterial genera the proportion of *Bifidobacterium* and *Lactobacillus* increased 1.9- and 1.5-fold, respectively while the proportion of *Streptococcus* (1.7-fold), *Coprococcus* (1.4-fold), *Ruminococcus* (1.2-fold) *Faecalibacterium* (2-fold), and *Escherichia* (2-fold) decreased in 'Biofortified' relative to 'Control' (Figure 6.4d). The proportion of family Lachnospiraceae decreased 1.7-fold and was significantly ($p = 0.045$) lower in 'Biofortified' relative to 'Control' (Figure 6.4d). Only one genus, *Enterococcus*, was significantly ($p = 0.010$) more abundant in 'Biofortified' (3.5 %) relative to 'Control' (> 1.0 %). The abundance of all families and genera detected decreased 1.5-fold in 'Biofortified' cecum relative to 'Control'.

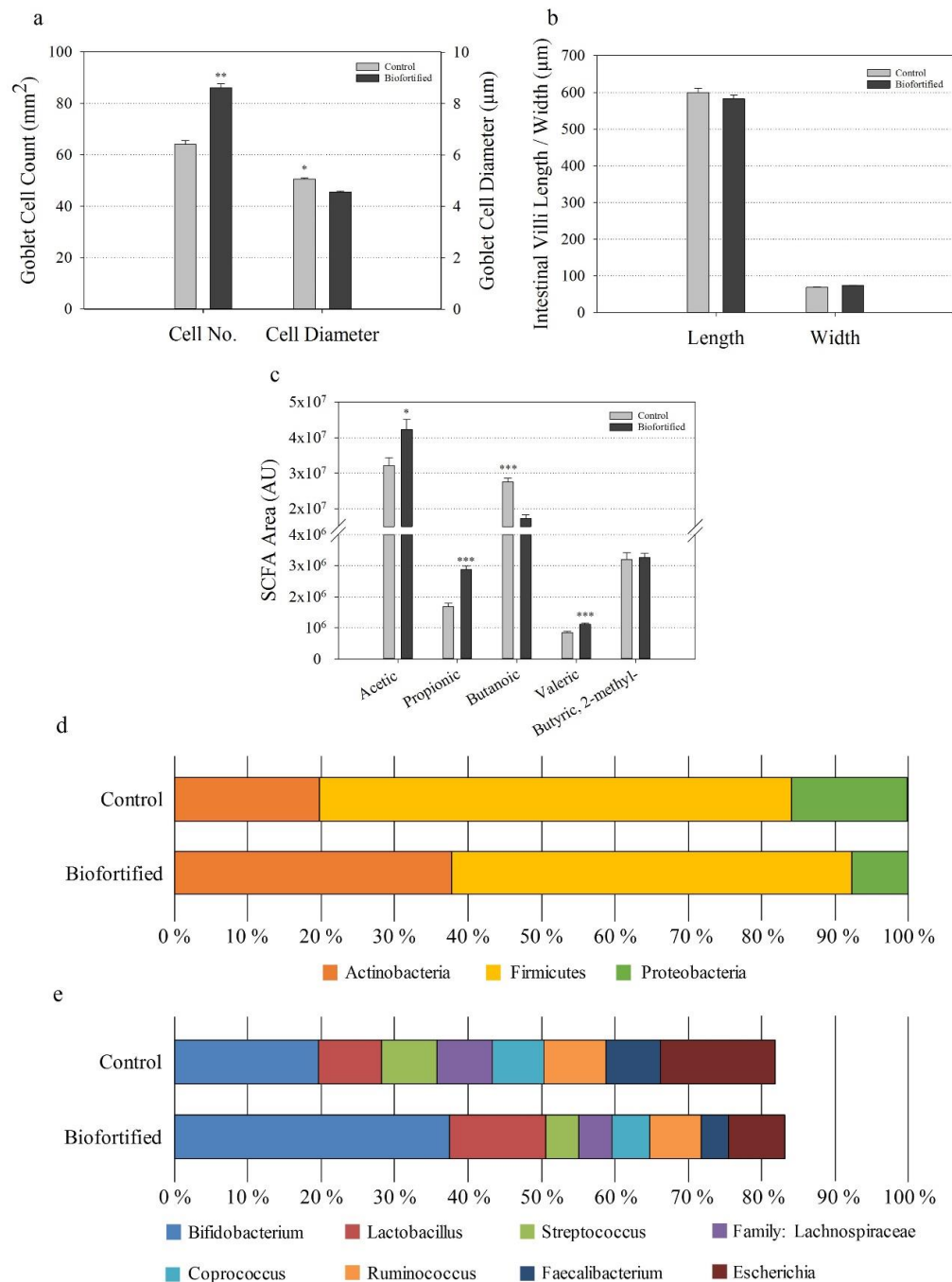


Figure 6.4. Intestinal functionality, short-chain fatty acid production and cecal microbial composition following consumption of experimental diets. (a) Chicken intestinal goblet cell number and diameter (μm). (b) Chicken intestinal villi length and width (μm). (c) Cecal short-chain fatty acid (SCFA) composition. Bars represent mean ± SEM of nine biological replicates. Relative abundance of microbial populations at the levels of (d) phyla; and (e) families and genera. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.001$ (***) as determined by Student's t-test. AU: arbitrary units.

Biofortified white wheat flour significantly alters diversity and metagenomic potential of the intestinal microbiota

Microbial population diversity (α -diversity) represented as Faith's phylogenetic diversity (PD) significantly decreased in 'Biofortified' cecum relative to 'Control' (Figure 6.5a). Significant ($q = 0.042$) separate clustering (β -diversity) of weighted 'Biofortified' and 'Control' microbial populations was observed (Figure 6.5b) with family Enterococcaceae (including an unspecified genus) significantly more abundant and genus *Dorea* significantly less abundant in 'Biofortified' relative to 'Control' (Figure S6.5). Microbial glycolysis/gluconeogenesis significantly increased and microbial tropane piperidine and pyridine alkaloid biosynthesis significantly decreased in 'Biofortified' microbial populations relative to 'Control' (Figure 6.5c).

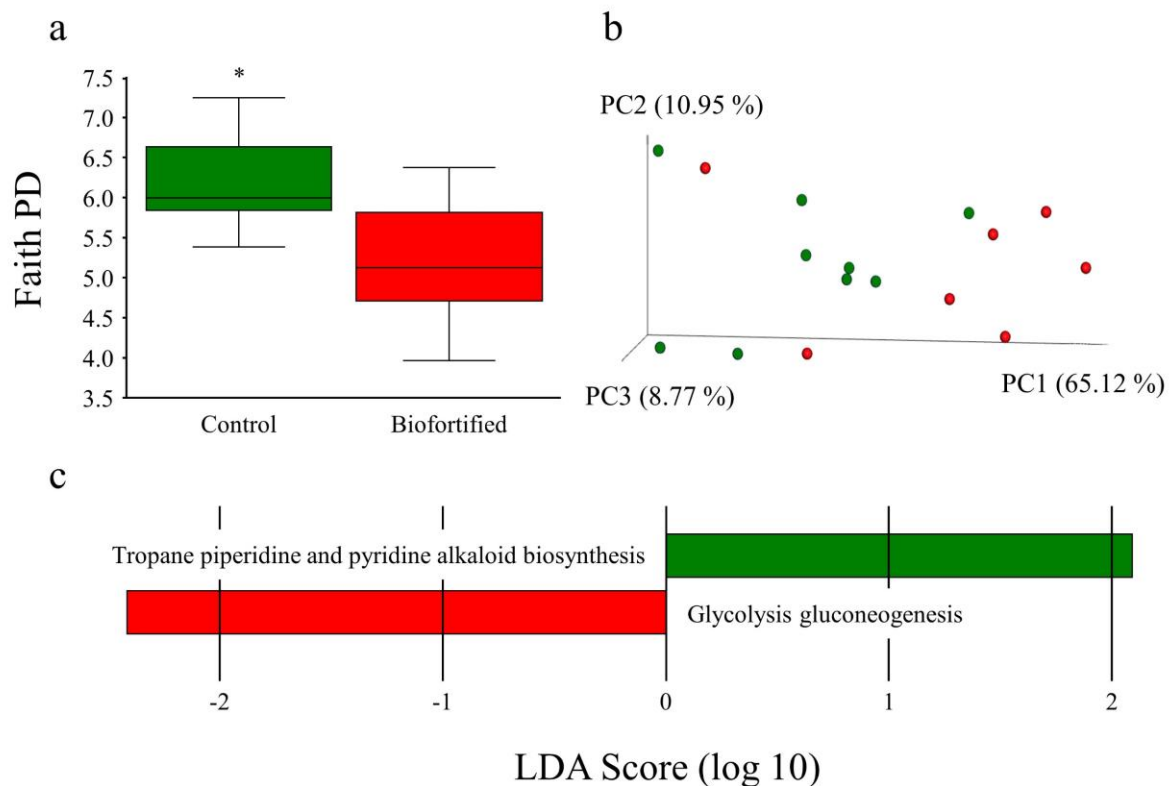


Figure 6.5. Microbial diversity and metabolic capacity following consumption of experimental diets. (a) Microbial α -diversity of chicken ceca using Faith's phylogenetic diversity (PD). (b) Microbial β -diversity of chicken ceca using unweighted UniFrac distances separated by three principal components (PC). Each dot represents either a 'Control' (green) or 'Biofortified' (red) chicken. (c-d) computed linear discriminant analysis (LDA) scores of differences in microbial relative abundance and metabolic capacity, respectively. Positive LDA scores (green) are enriched in 'Control' and negative LDA scores (red) are enriched in 'Biofortified'. Asterisks denote significant differences for $p < 0.05$ (*) as determined by Kruskal-Wallis test.

6.4. Discussion

Both NA and DMA form high affinity 1:1 complexes with Fe^{3+} (formation constants of 10^{20} and 10^{18} , respectively) and NA complexes Fe^{2+} with a formation constant of 10^{13} ^{31,32}. By contrast, EDTA forms a pentagonal bipyramidal complex surrounding a single Fe^{3+} atom with a formation constant of 10^{25} , and likely provides Fe^{3+} ions to the small intestine that require reduction by *DCytB* before absorption^{31,45,46}. Intraamniotic administration of ‘Fe EDTA’ and ‘Fe NA’ significantly increased blood serum Fe and significantly downregulated the expression of intestinal *DMT1* (a major Fe transporter) relative to administration of unchelated ‘Fe’ (Figure 6.1a, 6.1e) suggesting that both EDTA-chelated and NA-chelated Fe are readily absorbed into the small intestine before export into the blood stream^{3,47}. Decreased expression of *DCytB* (which catalyzes the reduction of Fe^{3+} to Fe^{2+}) in ‘Fe NA’ relative to unchelated ‘Fe’ is evidence that NA delivers relatively more Fe^{2+} ions, and that administration of an unchelated Fe solution delivers relatively more oxidized Fe^{3+} ions to the intestine⁴⁸. The expression of *Ferroportin* (the only known intestinal Fe exporter) was similar between treatment groups, and determining whether these NA-chelated Fe^{2+} ions would be preferentially absorbed into intestinal enterocytes or transferred paracellularly into the blood stream requires further investigation⁴⁷. Given that low expression of duodenal *DMT1/DCytB* relative to *Ferroportin* is linked with a positive gut microbiome⁴⁹ and that ‘Fe NA’ administration resulted in proliferation of probiotic *Bifidobacterium* in the ceca relative to *Escherichia* and *Clostridium* (Figure 6.2d), we hypothesize that NA-chelated Fe is readily absorbed by the host and not available to Fe-responsive pathogenic bacteria^{20,21,50–52}. Increased cecal *Escherichia* abundance in ‘Fe EDTA’ instead suggests that EDTA-chelated Fe persists in the intestinal lumen and contributes to the proliferation of non-beneficial bacteria (Figure 6.2d). Within the intestine, goblet cells are responsible for the synthesis and secretion of mucus, a polysaccharide/protein rich layer that physically protects epithelial cells, provides microbial habitat and facilitates nutrient exchange^{53–55}. We hypothesize that highly bioavailable NA-chelated Fe is readily absorbed by the intestinal epithelia, leading to significantly increased goblet cell number (Figure 6.2a) and a mucosal habitat that supports probiotic *Bifidobacterium*⁵⁶. By contrast, reduced intestinal goblet cells (and mucus production) coupled with increased villi surface area in ‘Fe EDTA’ may amplify the risk of bacterial infection due an increased proportion of potentially pathogenic *Escherichia* and *Clostridium* relative to probiotic *Bifidobacterium*⁵⁵. Together these results suggest NA-chelated Fe is highly bioavailable to the host and improves intestinal functionality without causing dysbiosis and proliferation of pathogenic bacteria as commonly seen in traditional Fe supplements and fortificants^{20,22}.

Biofortification is a cost-effective strategy to combat human micronutrient deficiencies by improving the density and/or bioavailability of micronutrients in staple crops through agronomic practices, conventional breeding, or modern biotechnology^{57,58}. Biofortification efforts in pearl millet (*Pennisetum glaucum* L.) and common bean (*Phaseolus vulgaris* L.) have increased seed Fe concentration (up to 3.9-fold and 1.7-fold, respectively), and consuming these crops improves both Fe status and cognitive performance^{59–62}. Traditional biomarkers of Fe status including blood serum Fe concentration and Fe homeostasis gene expression⁴⁷ were unchanged between chickens that received ‘Biofortified’ and ‘Control’ diets (Figures 6.3a, 6.3f). Caco-2 cell ferritin formation, a commonly used biomarker for measuring Fe bioavailability *in vitro*³⁷, increased in biofortified white flour relative to control white flour but did not differ between digests of ‘Biofortified’ and ‘Control’ diets (Figure S6.2). Instead we observed a trend of increasing blood Hb and HME from week 4 onwards (Table 6.2), and significantly increased liver Fe concentration and heart *COX* gene expression⁶³ at week 6 (Figures 6.3d, 6.3f), indicating that ‘Biofortified’ chickens had improved Fe status relative to ‘Control’ chickens and demonstrating the importance of a holistic approach in evaluating host Fe status³⁷. Interestingly we observed significantly decreased blood Hb, total body Hb and HME in ‘Biofortified’ chickens relative to ‘Control’ chickens at week 2 (Table 6.2). The ‘Control’ chicken blood Hb and HME values at week 2 were the highest values obtained throughout the study and may be evidence of a carryover effect from consuming a nutrient-rich commercial diet prior to commencement of the study. We hypothesize that ‘Control’ chickens utilized greater amounts of Fe (and likely other nutrients) from the commercial diet as part of an adaptive response to the poor nutrient concentrations within the ‘Control’ diet (Table 6.1), and that ‘Biofortified’ chickens did not utilize the additional Fe within this commercial diet to the same extent. Together these results highlight the importance of conducting long-term feeding studies to accurately evaluate biofortified diets and more comprehensive investigation of this hypothetical nutrient utilization mechanism is warranted. Short-term exposure to extracts of biofortified white flour was insufficient to alter liver Fe storage in ‘B WF’ (Figure 6.1c) and further highlights the importance of long-term exposure when evaluating biofortified diets. Given the large difference in feed consumption (Table 6.2) and relatively small (3 ppm) difference in dietary Fe concentration, ‘Biofortified’ chickens had lower Fe intake than ‘Control’ chickens over the course of the study (31.6 mg compared to 34.5 mg Fe). The increased liver Fe concentration to ‘Biofortified’ chickens relative to ‘Control’ chickens must therefore be the result of improved Fe bioavailability in the biofortified diet, likely due to increased concentration of NA- and/or DMA-chelated Fe given that both NA and DMA enhance Fe bioavailability *in vitro*^{26,33,34}. Separating the effect of NA and DMA on dietary Fe bioavailability requires a follow-up study evaluating diets fortified with NA-chelated or DMA-chelated Fe and together these results reinforce

the importance of the chelated form of Fe rather than target levels as a consideration for future Fe fortification and biofortification programs.

Here we show for the first time that the benefits of consuming a biofortified diet include altered intestinal functionality, enteric microbiota and feed energy conversion. Biofortified wheat consumption increased the abundance of *Bifidobacterium* and *Lactobacillus* in 'Biofortified' ceca relative to Clostridiales (comprising *Coprococcus Ruminococcus*, *Faecalibacterium* and family Lachnospiraceae) and *Escherchia* (Figure 6.4e) which is strikingly similar to the results obtaining following intraamniotic administration of NA-chelated Fe (Figure 6.2d) and provides further evidence that NA- and/or DMA-chelated Fe is highly bioavailable and does not persist in the intestinal lumen where it can contribute to the proliferation of pathogenic bacteria^{51,52}. The major phyla observed in this study: Firmicutes, Actinobacteria and Proteobacteria are shared between humans and chickens^{36,64}. Typically Firmicutes are the most abundant (70 - 80%) and Actinobacteria least abundant (~5%) phyla in human and poultry, suggesting the atypical microbial composition of both 'Control' and 'Biofortified' (~20% and 38% Actinobacteria, respectively) is due to nutritional insufficiencies in both diets⁶⁵⁻⁶⁷. *Bifidobacterium* and *Lactobacillus* are major probiotic genera within Actinobacteria and Firmicutes respectively, and both genera symbiotically harvest additional nutrients and energy from the diet for the host^{68,69}. These probiotic populations likely inhabit the additional intestinal mucin secreted by increased goblet cells in 'Biofortified' chickens (Figure 6.4a, Figure S6.4) that are both acidic and neutral and provide mucin with an appropriate chemical composition to support these populations⁷⁰. We hypothesize that additional *Bifidobacterium* and *Lactobacilli* in the mucosal layer upregulate glycolysis/gluconeogenesis enzymes and increase the production of acetic, propionic and valeric SCFAs (Figures 6.4c, 6.5c), leading to improved host Fe absorption and carbohydrate metabolism in 'Biofortified' chickens relative to 'Control'^{66,69,71}. Improved metabolic capacity in 'Biofortified' chickens manifested as reduced cumulative FCR (consuming ~20% less for the same weight gain) and increased glycogen storage in both liver and pectoral tissues relative to 'Control' (Table 6.2, Figure 6.3e). Improved food energy conversion due to increased *Bifidobacterium/Lactobacillus* relative to *Escherchia* was observed following prebiotic supplementation in broiler chickens⁷², suggesting these effects may be due to NA and/or DMA acting as prebiotics in the biofortified diet (Table 6.1). Administering extracts of biofortified white flour (containing NA and DMA) increased intestinal goblet cell number and villi surface in 'B WF' relative to 'C WF' (Figure 6.1d,e, Figure 6.2), suggesting that even short-term exposure to biofortified wheat positively affects intestinal morphology.

Traditional biomarkers of Zn status such as *ZIP4* and *ZnT1* gene expression and Zn concentration in blood serum, nails, and feathers^{39,73} were unchanged in ‘Biofortified’ chickens relative to ‘Control’, suggesting that Zn status was also unchanged (Figure 6.3b, 6.3f, Figure S6.3). Given the small differences in dietary Zn concentration (< 3 ppm), ‘Biofortified’ chickens had lower Zn consumption than ‘Control’ chickens over the course of the study (21.0 mg compared to 22.1 mg Zn, respectively). Together these results suggest that ‘Biofortified’ chickens had improved Zn bioavailability likely due to consumption of increased dietary NA and/or DMA, although whether NA and/or DMA increase Zn bioavailability requires further investigation. We observed significantly decreased LA:DGLA at week 2 and a trend of decreased LA:DGLA from week 4 onwards in ‘Biofortified’ relative to ‘Control’ (Figure 6.3c). As the LA:DGLA is a sensitive novel biomarker for evaluating Zn status⁷⁴, these results suggest that longer-term (6 months) exposure to ‘Biofortified’ diet may demonstrate clearer improvements to Zn status and is warranted. Zinc deficiency in chickens is known to negatively alter the gut microbiome, and improved microbial composition in ‘Biofortified’ chickens may be an additional symptom of improved Zn status⁷⁵. Given NA and DMA enhance Fe bioavailability we have previously argued these natural metal chelators function as phytonutrients in cereal foods^{25,26,76}. It is well established that NA exhibits anti-hypertensive effects *in vivo*^{34,77} although we did not detect differences in heart *angiotensin-converting enzyme (ACE)* and *angiotensin II receptor type 1 (AT1R)* gene expression throughout our study (Figure 6.1f, Figure 6.3f). We suspect similar heart *ACE* and *AT1R* expression between treatment groups in both the intraamniotic administration and feeding trial experiments is due to the relatively short exposure time to Fe solutions (4 days) or experimental diets (6 weeks) and it is worth investigating whether longer-term (6 months) exposure to increased dietary NA reduces hypertension. Nevertheless, the improved Fe status, gastrointestinal health and microbial composition in chickens following short and long-term exposure to NA-chelated Fe reinforces the idea of NA as an important phytonutrient in plant foods. Furthermore, utilization of NA-chelated Fe in food fortification and crop biofortification programs shows great potential to improve human health.

6.5. Methods

6.5.1. Plant material and white flour production

Vector construction, plant transformation and the initial selection of biofortified wheat material is described in²⁵. In brief, the full-length coding sequence of *OsNAS2* (LOC_Os03g19420) was PCR amplified from rice (*Oryza sativa* L.) cv. Nipponbare and recombined into a modified pMDC32 vector under transcriptional control of the maize (*Zea mays* L.) ubiquitin 1 (UBI-1) promoter with a hygromycin phosphotransferase plant-selectable marker (Figure S1). Bombardment of the construct

into immature wheat (*Triticum aestivum* L.) cv. Bobwhite embryos was performed at the University of Adelaide (Adelaide, Australia). One double-insert event and corresponding null segregant (termed ‘Biofortified’ and ‘Control’, respectively) were grown were grown for two seasons for use in intra-amniotic administration (2016 field season) and feeding trial (2017 field season) in New Genes for New Environment facilities located in Merredin, Western Australia (Figure S6.1, Table S6.1). Whole grain samples from Merredin were conditioned to 15% moisture content and milled (70-75% extraction) using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) for intraamniotic administration or a Buhler MLU-202 laboratory mill at The Commonwealth Scientific and Industrial Research Organisation (CSIRO, ACT, Australia) for the feeding trial. All break and reduction fractions of ‘Biofortified’ or ‘Control’ grain were combined to form either ‘Biofortified’ white flour or ‘Control’ white flour (Table 6.1).

6.5.2. Preparation of extracts, solutions and diets

Wheat extracts were generated as described in⁷⁸. In brief, ‘Biofortified’ white flour or ‘Control’ white flour was mixed in dH₂O (50 g/L), filtered (600 µm) and centrifuged, and the resulting supernatant was dialyzed (MWCO 12–14 kDa, Medicell International Ltd., London, UK) exhaustively against dH₂O (48 hrs.). The dialysate was lyophilized, and the resulting powder dissolved in 18MΩ H₂O (0.05 g/mL) forming the white wheat flour extracts for intra-amniotic administration. Iron solutions were prepared by combining an Fe standard (1000 µg/mL, 2% HCl, High-Purity Standards, Charleston, SC, USA) with either 18MΩ H₂O (‘Fe’), or 1.6 mM NA (Toronto Research Chemicals Inc., Toronto, Canada) dissolved in 18MΩ H₂O (‘Fe NA’). The (‘Fe EDTA’) solution was achieved by combining ferric nitrate (Fe(NO₃)₃ 9H₂O, Sigma, St. Louis, MO, USA) with hydroxyethyl ethylenediamine triacetic acid (H₃HEDTA, Sigma, St. Louis, MO, USA) dissolved in sodium hydroxide (NaOH, Sigma, St. Louis, MO, USA) to represent an anionic chelate of dissolved NaFeEDTA⁴⁵ with final Fe concentration of 77 µM. Osmolarity and final Fe concentration of extracts / Fe solutions for intraamniotic administration is provided (Table S6.2).

6.5.3. Dietary analysis (phytate, protein, carbon, fiber, NA, DMA)

Dietary phytate was calculated relative to total phosphorus released from diet and flour samples by phytase and alkaline phosphatase enzymes according to manufacturer’s instructions (K-PHYT 11/15. Megazyme International. Bray, Ireland). Total dietary carbon (%) and nitrogen (%) was measured via Dumas combustion using a TruMac® CN (LECO Corporation, St. Joseph, MI, USA) with total protein (%) for wheat diet samples equal to 5.7 x total nitrogen (%). Total dietary fiber was measured via enzymatic digestion using heat-resistant amylase, protease and amyloglucosidase according to manufacturer’s instructions (Total Dietary Fiber Assay Kit, Sigma, St. Louis, MO, USA).

Quantification of NA and DMA in diet and flour samples was performed as described in²⁵. Briefly, sequential MeOH (100%) and 18MΩ H₂O sample were derivatized by 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) and quantified via RP LC-MS on a 1290 Infinity II and 6490 Triple Quadrupole LC/MS system (Agilent Technologies Inc., Santa Clara, CA, USA).

6.5.4. Caco-2 Fe bioavailability bioassay

Diet and flour samples were subjected to the Caco-2 cell bioassay as previously described²⁵. Briefly, gastric-digested samples (1.5 mL) were added to cylindrical Transwell inserts (Corning Life Sciences, Corning, NY) fitted with a semipermeable (15 000 Da MWCO) basal membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA) and placed within wells containing Caco-2 cell monolayers. Following overnight incubation, cells were washed, harvested and analyzed for ferritin (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein contents (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA). As Caco-2 cells synthesize ferritin in response to intracellular Fe, we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake.

6.5.5. Micronutrient analysis

Micronutrient concentration in white flour, diets and extracts, Fe solutions, blood serum, and all animal tissues was determined by nitric / perchloric acid digestion as previously described³⁸ followed by inductively coupled plasma-optical emission spectrometry (ICP-OES) using a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA).

6.5.6. Animals and study design

Cornish-cross fertile broiler eggs (n = 70) were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA) and incubated at the Cornell University Animal Science poultry farm until hatching. All animal protocols were approved by Cornell University Institutional Animal Care and Use committee (protocol number: 2007-0129). All methods were performed in accordance with the relevant guidelines and regulations. For intraamniotic administration, eggs (n = 40) containing viable embryos were weighed and randomly assigned to seven groups (n ≥ 5) based on weight distribution. At day 17 of incubation, extracts / Fe solutions (1 mL) were injected into the amniotic fluid via a 21-gauge needle for the seven treatment groups as follows: (1) non-injected (NI); (2) 18MΩ H₂O (H₂O); (3) Fe solution (Fe); (4) Fe-EDTA solution (Fe-EDTA); (5) Fe-NA solution (Fe-NA); (6) 'Control' white flour extract (C WF); (7) 'Biofortified' white flour extract (B WF) and eggs were subsequently incubated for four days until hatch as described in^{79,80}. Chicks were euthanized by CO₂ exposure after hatching and all tissues were collected. The remaining hatchlings (n = 30) were allocated based on body weight into two treatment groups: (1) 80% 'Control' white flour diet

(‘Control’) and (2) 80% ‘Biofortified’ white flour diet (‘Biofortified’) as described in³⁸. All chickens received a commercial diet (Nutrena® Chick Starter Grower 18% Crumble, Cargill Inc, Wayzata, MN, USA) for one week prior to consumption of ‘Control’ and ‘Biofortified’ diets for six weeks. ‘Control and Biofortified’ diet formulations met the Nutrient Requirements for Poultry (NRC Poultry reference) excluding Fe and Zn. Chickens (n = 3) were housed in cages (1m²) and provided *ad libitum* access to food and H₂O. Feed intakes were measured daily, and body weight and blood samples were obtained weekly. Feed conversion ratio (FCR) represents weekly feed intake (g) proportional to the weekly increase in body weight (g). Chickens were euthanized by CO₂ exposure seven weeks post-hatch and tissues collected.

6.5.7. Blood measurements

Wing-vein blood samples (100 µL) were collected using micro-hematocrit heparinized capillary tubes (Fisher, Pittsburgh, PA, USA). Blood plasma Hb concentrations were determined spectrophotometrically using the Triton® / NaOH method according to manufacturer’s instructions (Hemoglobin Assay Kit, Sigma, St. Louis, MO, USA). The Hb maintenance efficiency (HME) was calculated as previously described³⁸. Blood serum Linoleic Acid:Dihomo-γ-Linolenic Acid ratio (LA:DGLA) was determined as previously described³⁹.

6.5.8. Gene expression analysis (Tissue harvesting, RNA isolation, cDNA synthesis, primer design)

Total RNA extraction from duodenal and heart tissue (30 mg) using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA), cDNA synthesis and real time-polymerase chain reaction (RT-PCR) analysis were performed as previously described^{38,81} with minor adjustments. In brief, the cycle product (Cp) of each gene was quantified using a seven-point standard curve in duplicate. Gene expression was obtained relative to 18S (Cp), primer pair efficiency, and control treatments: ‘NI’ for intraamniotic administration and ‘Control’ for feeding trial⁸². *Alkaline phosphatase (AKP)* and *sucrase isomaltase (SI)* acted as intestinal reference genes following intraamniotic administration (Figure 6.1e). All primers used for gene expression analysis are provided in Table S6.3.

6.5.9. Ferritin and glycogen analysis

Liver ferritin was determined as previously described⁷⁸. In brief, samples (1 g) were homogenized in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM) and heat treated (75°C, 10 min) before centrifugation. Native polyacrylamide gel electrophoresis (PAGE) gels were stained with Coomassie blue G-250 stain or potassium ferricyanide [K₃Fe(CN)₆] and quantified using the Quantity-One 1-D analysis program (Bio-Rad, Hercules, CA). Liver and pectoral glycogen was

determined colorimetrically as described in⁸³ with minor adjustments. After centrifugation and mixing with petroleum ether, homogenized tissue was mixed with color reagent (300 µL) and total glycogen determined on an ELISA plate reader (450 nm) according to a standard curve.

6.5.10. Intestinal functionality and short-chain fatty acid (SCFA) analysis

Duodenal samples were fixed in fresh 4% (v/v) buffered formaldehyde, dehydrated, and embedded in paraffin as previously described³⁸. Serial sections (5 µm) were deparaffinized in xylene and stained with hematoxylin and eosin before goblet cell number and villi surface area examination under light microscopy using EPIX XCAP software (Standard version, Olympus, Waltham, MA, USA). Cecal samples were homogenized in HCl (2 ml, 3 %, 1M), centrifuged and combined with ethyl acetate (100 µL) and acetic acid-d4 (1 µg / mL) before collecting the organic phase to determine short chain fatty acid (SCFA) composition. Samples were quantified via GC-MS using a TRACETM 1310 gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) and a TraceGOLD™ TG-WaxMS A column (Thermo Fisher Scientific, Waltham, MA, USA).

6.5.11. Microbial population analysis

Lactobacillus, *Bifidobacterium*, *Escherichia*, and *Clostridium* density in intraamniotic administration treatment groups was determined as previously described⁷⁹. In brief, cecal contents were homogenized with phosphate-buffered saline (PBS, 9 ml), centrifuged and the pellet resuspended in ethylenediaminetetraacetic acid (EDTA, 50 mM) and treated (37°C, 45 min) with lysozyme (10 mg / mL, Sigma Aldrich CO., St. Louis, MO, USA). Bacterial genomic DNA was isolated according to manufacturer's instructions (Wizard® Genomic DNA Purification Kit, Promega Corp., Madison, WI, USA) and bacterial genera are presented in relative proportions. All primers used for microbial population analysis are provided in Table S6.4.

6.5.12. 16S rRNA gene sequencing and analysis

Microbial genomic DNA extraction from 'Control' and 'Biofortified' cecal samples, gene sequencing and analysis was conducted as previously described³⁸. In brief, 16S rRNA gene sequences were amplified from the V4 hypervariable region of microbial genomic DNA (Powersoil DNA isolation kit, MoBio Laboratories Ltd., Carlsbad, CA, USA, purified (AMPure, Beckman Coulter, Atlanta, GA, USA) and quantified according to manufacturer's instructions (Quant-iT™ PicoGreen™ dsDNA Assay Kit, Invitrogen, Carlsbad, CA, USA). Samples were sequenced at Bar Ilan University (Safed, Israel) using an Illumina MiSeq Sequencer (Illumina, Inc., Madison, WI, USA). Amplicon reads were analyzed using 'Divisive Amplicon Denoising Algorithm' (DADA2) and 'quantitative insights into microbial ecology' (QIIME) software before taxonomic classification using Greengenes database (<http://greengenes.lbl.gov>)⁸⁴⁻⁸⁶. Faith's phylogenetic diversity (PD) was used to assess α -diversity and

principal component (PC) analysis of weighted UniFrac distances was used to assess β -diversity^{87,88}. Relative abundance was determined using linear discriminant analysis effect size (LEfSe) and metabolic capacity was determined using 'phylogenetic investigation of communities by reconstruction of unobserved states' (PICRUST) software compared to known pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg>)^{89,90}.

6.5.13. Statistical analyses

A mixed linear model was utilized to normalize body weight, Hb, total body Hb, HME, FCR and feed intake relative to baseline total body Hb as presented in Table 6.2 using MiniTab software (v 18.0, MiniTab). Significant differences between intraamniotic administration treatment groups was determined by ANOVA with a Tukey post-hoc test ($p < 0.05$) with additional significant differences between 'Fe', 'Fe EDTA' and 'Fe NA' as well as between 'C WF' and 'B WF' determined by Student's t-test ($p < 0.05$). Significant differences in physiological measurements between 'Control' and 'Biofortified' samples were determined by Student's t-test ($p < 0.05$). Significant differences in Faith's PD and weighted UniFrac distances was determined by Kruskal-Wallis and permutational multivariate analysis of variance (PERMANOVA) tests, respectively and LEfSe significant differences were corrected for false discovery rate (FDR).

6.6. References

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6.7. Supplementary Information

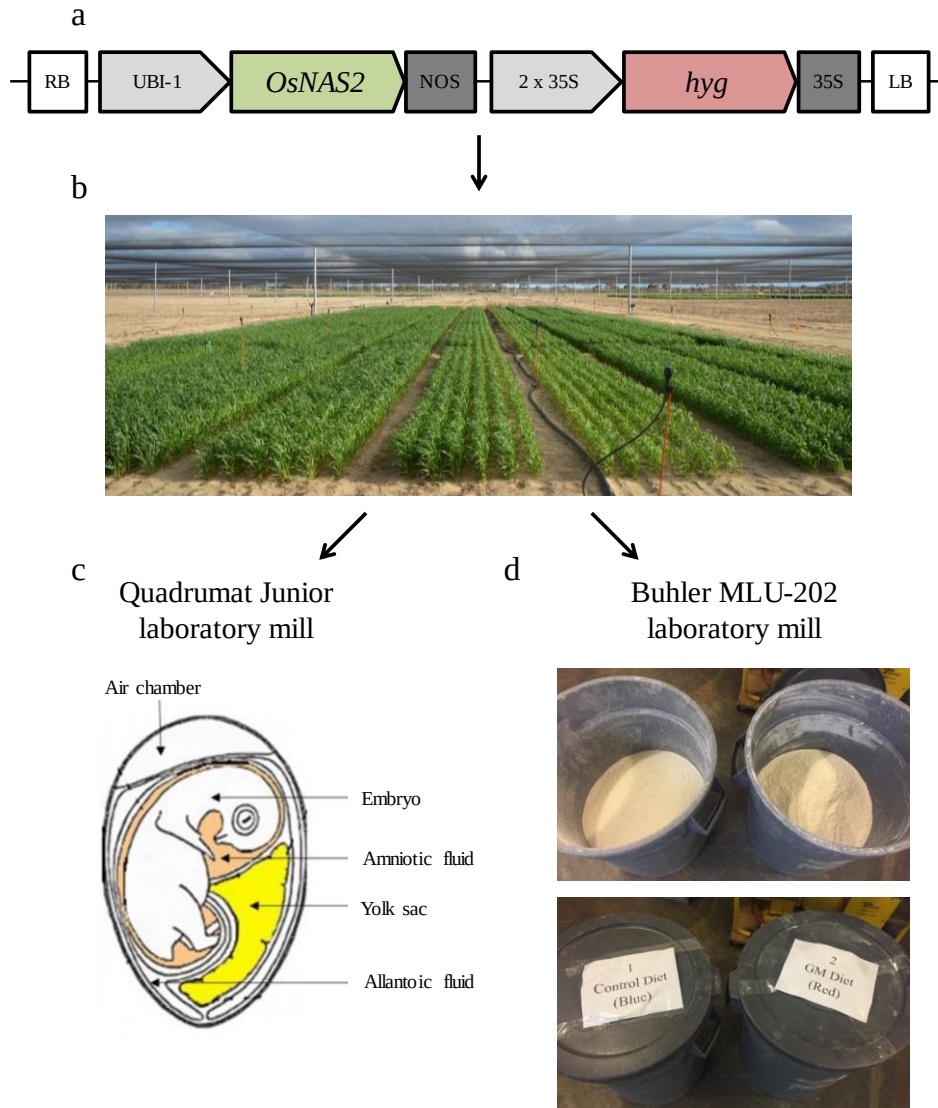


Figure S6.1. Generation of plant material and white flour. (a) Schematic representation of the T-DNA construct used to transform wheat (*Triticum aestivum* L.) as previously described¹. RB and LB: right and left borders, respectively; UBI-1: *Zea Mays ubiquitin 1* gene promoter; *OsNAS2*: *Oryza sativa nicotianamine synthase 2* gene (LOC_Os03 g19420); NOS: nopaline synthase terminator; 2 x 35S: dual promoter of 35S cauliflower mosaic virus gene; *hyg*: *hygromycin phosphotransferase* gene (selectable marker); 35S: 35S cauliflower mosaic virus gene terminator. (b) Confined field trial of ‘Biofortified’ and ‘Control’ in the New Genes for New Environment facilities located in Merredin, Western Australia. (c) ‘Biofortified’ and ‘Control’ wheat grown at Merredin in 2016 was milled using a Quadrumat Junior laboratory mill and extracts of the resulting white flour used in the intraamniotic administration assay. The image was adapted from² with permission from the authors. (d) ‘Control’ and ‘Biofortified’ wheat grown at Merredin in 2017 was milled using a Buhler MLU-202 laboratory mill and the resulting white flour mixed into ‘Control’ and ‘Biofortified’ (GM) diets for a six-week long feeding trial.

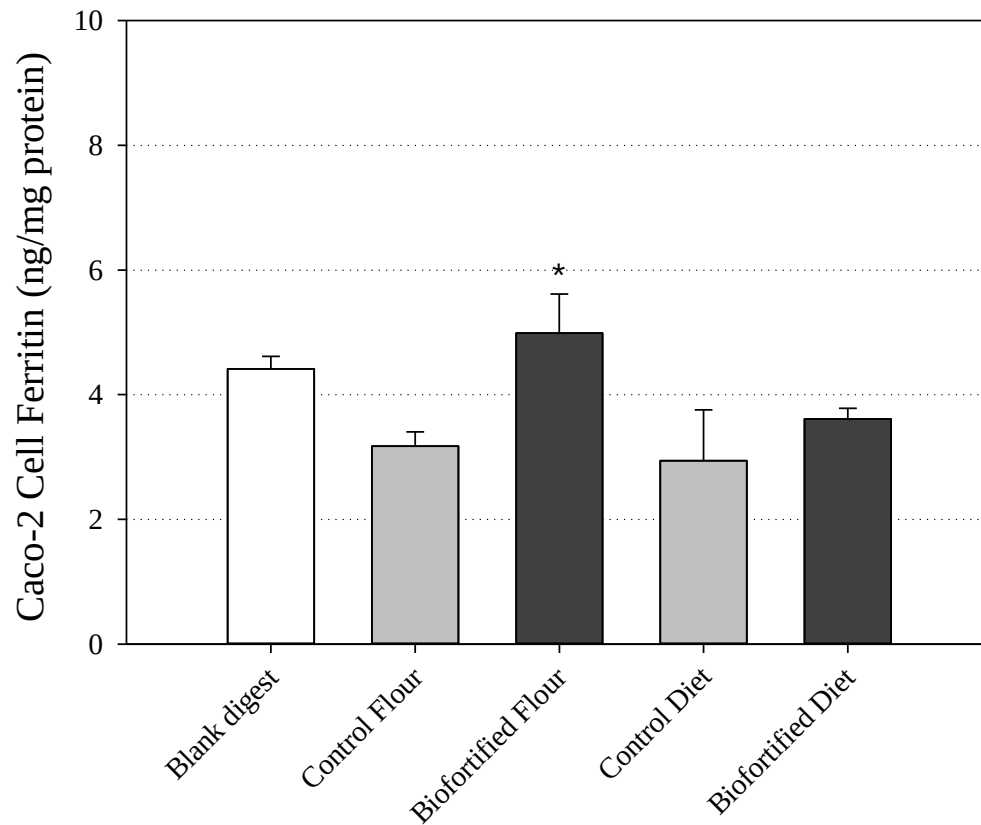


Figure S6.2. Iron bioavailability in Control and Biofortified white flour and experimental diets. Bars represent mean $\pm$ SEM of three biological replicates and asterisks denote the significance between Control and Biofortified flour for $p < 0.05$ (*) as determined by Students' *t*-test.

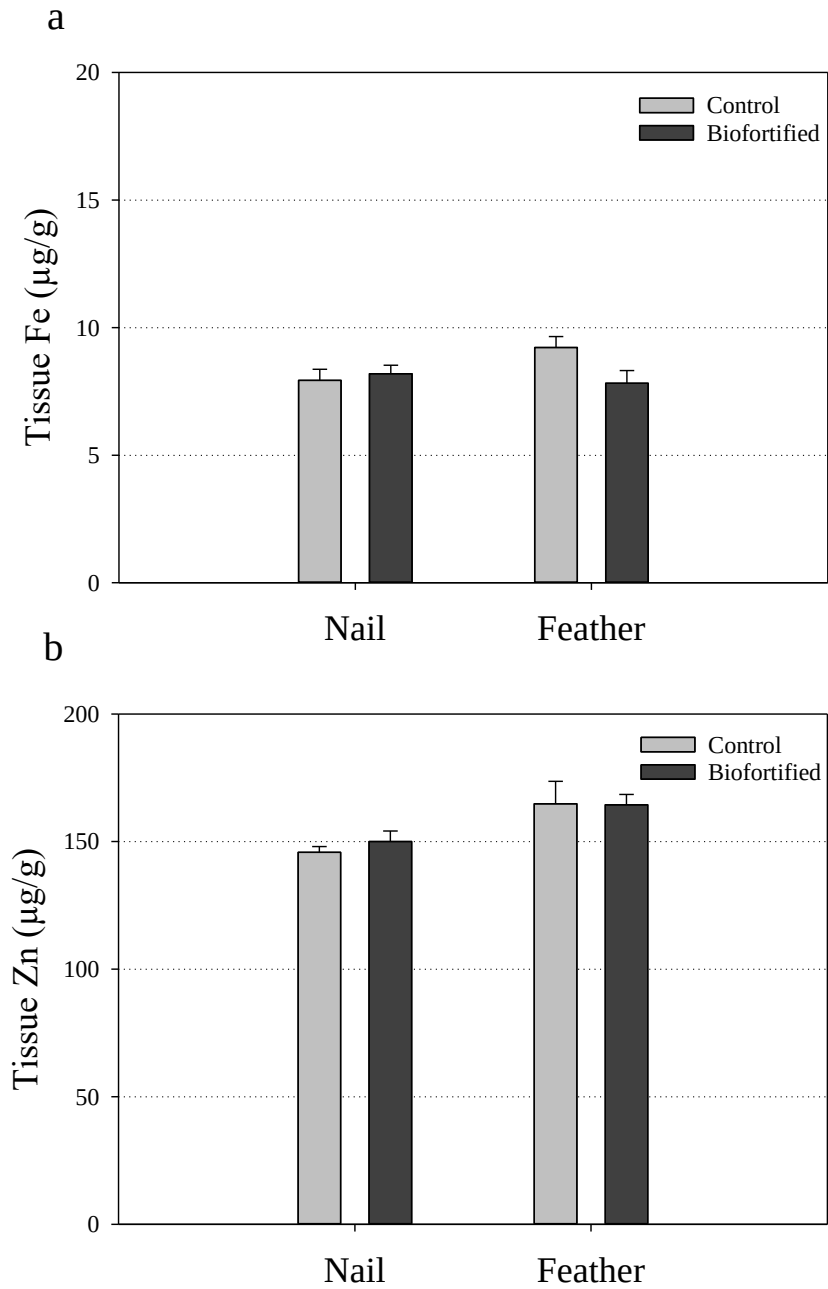


Figure S6.3. Nail and feather Fe and Zn concentration following consumption of experimental diets. The concentration (µg/g) of (a) Fe and (b) Zn in Control and Biofortified chicken nail and feather is provided. Bars represent mean ± SEM of nine biological replicates.

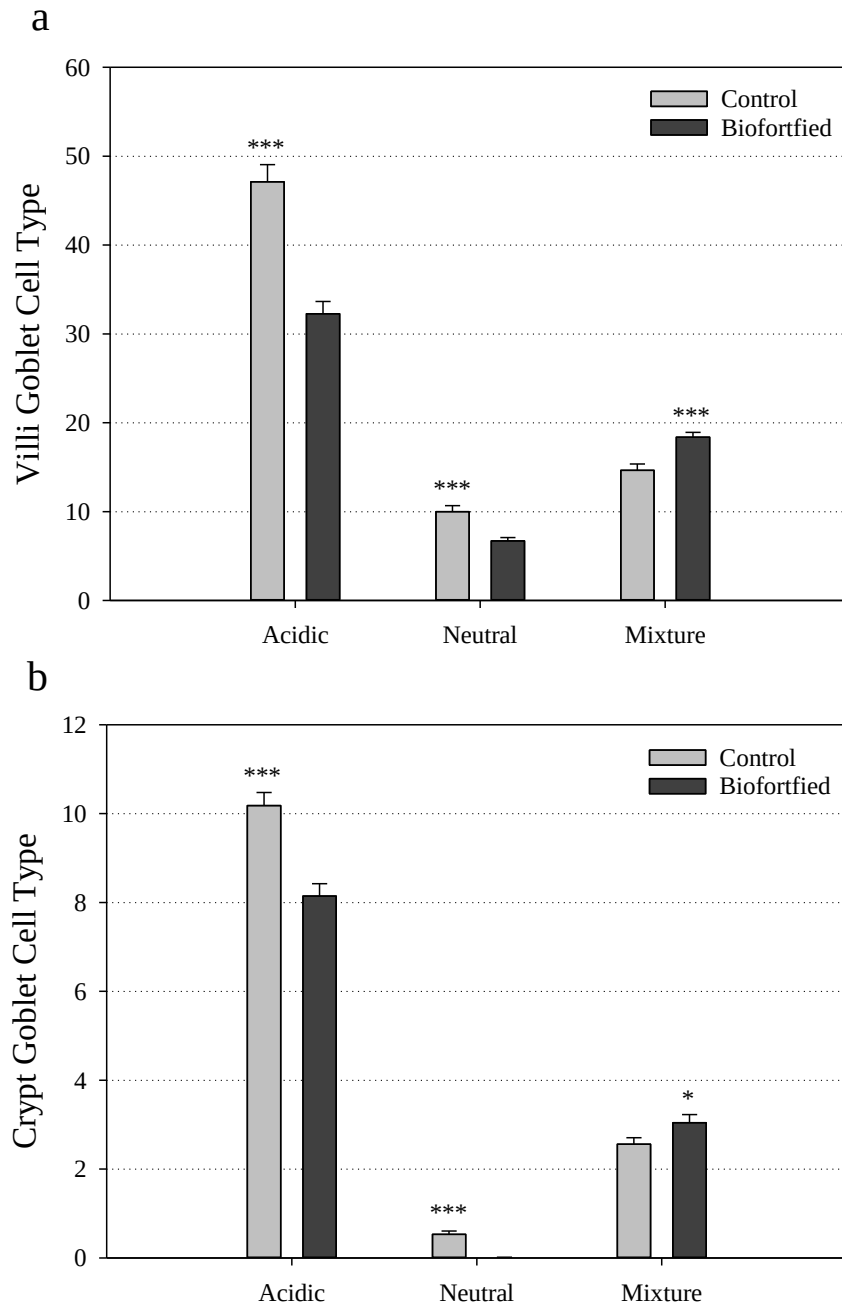


Figure S6.4. Chicken intestinal goblet cell type following consumption of experimental diets. The number of acidic, neutral and both acidic and neutral (mixture) goblet cells between Control and Biofortified chicken intestinal (a) villi and (b) crypts is provided. Bars represent mean $\pm$ SEM of nine biological replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.001$ (***) as determined by Student's t-test.

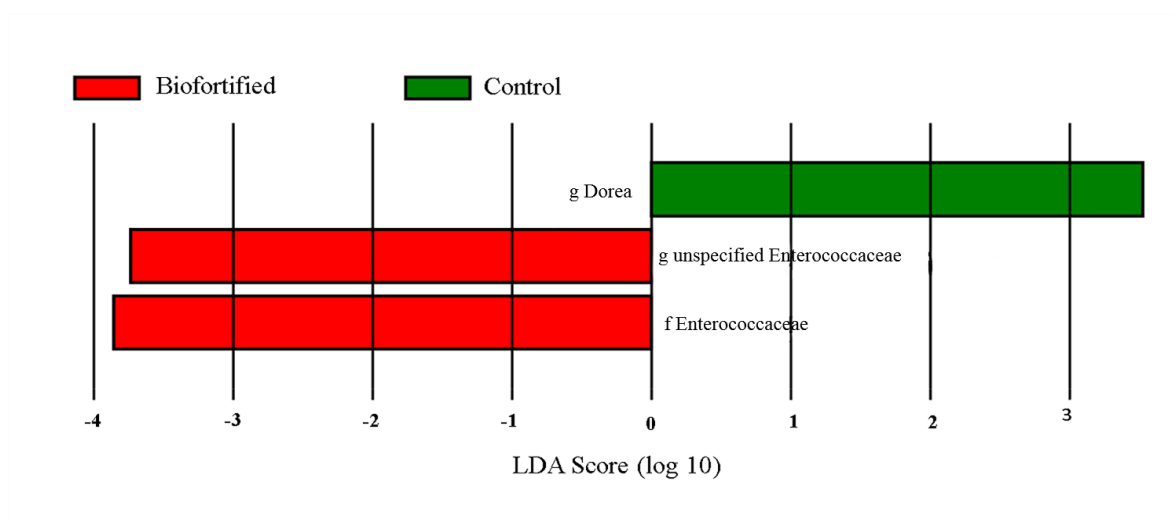


Figure S6.5. Differentially enriched taxa following consumption of experimental diets. Computed linear discriminant analysis (LDA) scores of differences in microbial relative abundance. Positive LDA scores (green) are enriched in ‘Control’ and negative LDA scores (red) are enriched in ‘Biofortified’.

Table S6.1. Nutritional composition of Control and Biofortified white flour. Values represent mean $\pm$ SEM of at least four technical replicates. Asterisks denote significant differences for $p \leq 0.001$ (***) as determined by Student's t-test.

Selected Components	Control	Biofortified
White Flour Fe ($\mu\text{g/g}$)	19.0 ± 0.07	$24.5 \pm 0.14^{***}$
White Flour Zn ($\mu\text{g/g}$)	16.0 ± 0.06	$21.1 \pm 0.03^{***}$
White Flour NA ($\mu\text{mol/g}$)	19.0 ± 0.21	$37.4 \pm 0.88^{***}$
White Flour DMA ($\mu\text{mol/g}$)	17.7 ± 0.09	$23.6 \pm 0.20^{***}$
White Flour Phytate (mg/g)	2.0 ± 0.03	1.9 ± 0.05
Phytate : Fe molar ratio	8.91	6.56

Table S6.2. Osmolarity and Fe concentration of extracts / Fe solutions for intraamniotic administration.

Group	Osmolality	Fe ($\mu\text{g/g}$)
NI	-	-
H ₂ O	87	ND
Fe	168	11.68
Fe EDTA	82	11.19
Fe NA	188	11.68
C WF	129	0.91
B WF	120	0.82

Table S6.3. The sequences of the qPCR primers used for gene expression analysis. The PCR product size and GenInfo identifier for each gene is provided.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	GenInfo identifier
18s rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT	100	7262899
ACE	CATGGCCTTGTCTGTCTCC	GAGGTATCCAAAGGGCAGG	142	424059
AKP	CTCATTCCAGCGTACTCTTCTT	GTGTGTAGATCAAAGGGCTACT	100	424936
AT1R	TCATCTGGCTCCTTGCTGG	AACCTAGCCCAACCCTCAG	138	396065
COX	GCAGG GTTTCCTCCAT	GGTTGCGGTTCGGTAAGT	150	420624
Delta6	GGCGAAAGTCAGCCTATTGA	AGGTGGGAAGATGAGGAAGA	93	261865208
DcytB	CATGTGCATTCTCTTCCAAAGTC	CTCCTTGGTGACCGCATTAT	103	20380692
DMT1	TTGATTCAGAGCCTCCCATTAG	GCGAGGAGTAGGCTTGTATTT	101	206597489
Ferroportin	CTCAGCAATCACTGGCATCA	ACTGGGCAACTCCAGAAATAAG	98	61098365
SI	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
ZIP1	TGCCTCAGTTTCCCTCAC	GGCTCTTAAGGGCACTTCT	144	107055139
ZIP4	TCTCCTTAGCAGACAATTGAG	GTGACAAACAAGTAGGCGAAAC	95	107050877
ZIP6	GCTACTGGGTAATGGTGAAGAA	GCTGTGCCAGAACTGTAGAA	99	66735072
ZIP9	CTAAGCAAGAGCAGCAAAGAAG	CATGAACTGTGGCAACGTAAAG	100	237874618
ZnT1	GGTAACAGAGCTGCCTTAACT	GGTAACAGAGCTGCCTTAACT	105	54109718
ZnT5	TGGTTGGTATCTGTGCCTTTAG	GGCTGTGTCCATGGTAAGATT	99	56555150
ZnT7	GGAAGATGTCAGGATGGTTCA	CGAAGGACAAATTGAGGCAAAG	87	56555152

18S rRNA, 18S ribosomal subunit; ACE, angiotensin-converting enzyme; AKP, alkaline phosphatase; AT1R, angiotensin II receptor type 1; COX, cytochrome c oxidase; Delta6, delta-6-desaturase; DcytB, duodenal cytochrome b; DMT1, divalent metal transporter 1; SI, Sucrose isomaltase; ZIP1, Zinc transporter 1; ZIP4, Zinc transporter 4; ZIP6, Zinc transporter 6; ZIP9, Zinc transporter 9; ZnT 1, Zinc transporter 1; Znt 5, Zinc transporter 5; Znt 7, Zinc transporter 7.

Table S6.4. The sequences of the primers used for microbial analysis. Sequences were obtained from³.

Bacterial Population	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>16s rRNA</i> (Universal)	CGTGCCAGCCGCGGTAATACG	GGGTTGCGCTCGTTGCGGGACTTAACCCAACAT
<i>Bifidobacterium</i>	GGGTGGTAATGCCGGATG	CCACCGTTACACCGGGAA
<i>Lactobacillus</i>	CATCCAGTGCAAACCTAAGAG	GATCCGCTTGCCTTCGCA
<i>Escherichia</i>	GACCTCGGTTTAGTTCACAGA	CACACGCTGACGCTGACCA
<i>Clostridium</i>	AAAGGAAGATTAATACCGCATAA	ATCTTGCGACCGTACTCCCC

18S rRNA, 18S ribosomal subunit.

Supplementary Information References

1. Beasley, J. T. *et al.* Metabolic engineering of bread wheat improves grain iron concentration and bioavailability. *Plant Biotechnol. J.* 1–13 (2019). doi:10.1111/pbi.13074
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7. Chapter 7 – Impacts of bread wheat iron and zinc biofortification on white flour protein composition and concentration, rheology and breadmaking

7.1. Introduction

Successful biofortification efforts involve the completion of certain activities within three phases generally described as discovery, development and dissemination (Bouis, Saltzman, and Birol 2019). The discovery phase involves determining target levels of micronutrients in focus crops and obtaining these levels by exploiting genetic variation within existing banks of germplasm. Genetic transformation may be utilized if there is insufficient variability in food micronutrient concentration. A key component of the discovery and early development phases is demonstrating that improved micronutrient concentration is unaffected by genotype x environment (G x E) interactions and does not compromise yield, and determining these traits requires growing biofortified crops in multi-location trials (see Chapter 5). Activities in later development and dissemination phases include demonstrating that increased micronutrient concentrations in biofortified foods are retained and bioavailable (available for physiological use following consumption) following food processing, and that biofortification does not result in unwanted side effects for the food industry and/or consumers (Meenakshi 2009).

Biofortified crops that have been released to date include Fe and Zn biofortified cowpea (*Vigna unguiculata* L.), sorghum (*Sorghum bicolor* L.), and lentil (*Lens culinaris*), Fe biofortified bean (*Phaseolus vulgaris* L.), and pearl millet (*Pennisetum glaucum* L.), and Zn biofortified maize (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.) (Bouis and Saltzman 2017; Bouis et al. 2019). Conventional breeding efforts to increase Fe concentrations in maize, rice and wheat have been hindered by a lack of genetic variability for this trait, and Fe biofortification programs in wheat and rice have relied on modern biotechnology to achieve target biofortification levels for grain Fe concentration (Connorton, Jones, et al. 2017; Johnson et al. 2011; Singh et al. 2017; Trijatmiko et al. 2016). Most Fe and Zn biofortified crops are commonly consumed as whole foods (i.e. bean, lentil, cowpea, and rice) or as whole flours/breads (sorghum, pearl millet and maize) that require relatively minimal processing such as grinding, polishing and cooking prior to consumption, and biofortification efforts in these crops have primarily focussed on the discovery and early development phases. By contrast, wheat grain is milled into wholemeal flour using either a stone/hammer mill, or more commonly milled into white flour using a roller mill, which allows precise separation of the endosperm (white flour) from outer grain layers (Balk et al. 2019; Shewry and Hey 2015). Wheat is unique among crop species in that interactions between grain storage proteins (commonly referred to as prolamins or gluten proteins) allow wheat flour to be processed into a range of food products

including breads, noodles, pasta, cakes and biscuits. Mixing wheat flour with water produces a continuous network of prolamins and starch granules, and the strength (measured as both elasticity and extensibility) and viscosity of this network are crucial for food processing (Shewry et al. 1986, 1995). For example, the elasticity of the network allows the trapping of carbon dioxide produced by yeast during dough fermentation and is a critical component of breadmaking.

Prolamins (i.e. gliadins and glutenins) represent ~80% of grain protein and act as an energy source for the developing grain and non-storage proteins (commonly referred to as metabolic proteins) such as albumins and globulins (AGS) comprise most of the remaining 20% (Békés and Wrigley 2015; Merlino et al. 2009; Uthayakumaran and Wrigley 2017). In the bread wheat genome, the gliadin proteins are encoded by three *Gli-1* loci (A, B and D) and three *Gli-2* loci (A, B and D) on the short arms of group 1 and group 6 chromosomes, respectively, although other minor loci (*Gli-3* and *Gli-5*) have also been reported (Z. Wang et al. 2017). The LMW-GS are encoded by three *Glu-3* loci (A, B and D) also on the short arms of group 1 chromosomes, and each locus encodes several LMW-GS genes that demonstrate extensive allelic variation (Appelbee et al. 2009). The *Gli-1* and *Glu-3* loci encode proteins of similar sequence and are closely linked, adding to the difficulty of distinguishing gliadin and LMW-GS proteins (Ruiz and Carrillo 1993). The loci encoding HMW-GS are relatively simple and consist of three *Glu-1* loci (A, B and D) on the long arms of group 1 chromosomes with each locus having two types (x and y). Not all *Glu-1* loci are expressed in all wheat genotypes and most contain between three (Bx, Dx, and Dy) to five (Ax, Bx, By, Dx, and Dy) HMW-GS alleles (Shewry 2019). The *Glu-A1* loci encodes either 1Ax1, 1Ax2* or Null allele, the *Glu-B1* loci encodes the 1Bx6 + 1By8, 1Bx7 + 1By8, 1Bx7 + 1By9, 1Bx13 + 1By16, 1Bx14 + 1By15, and 1Bx17 + 1By18 allelic pairs and the *Glu-D1* loci encodes the 1Dx2 + 1Dy12, 1Dx3 + 1Dy12, 1Dx4 + 1Dy12, and 1Dx5 + 1Dy10 allelic pairs (Halford et al. 1992; Liu et al. 2009; Shewry et al. 2002; Zhang et al. 2018). The combination of HMW-GS alleles varies between wheat genotypes and certain alleles, such as the 1Dx5 + 1Dy10 allelic pair, are associated with greater loaf volume and breadmaking performance (Liu et al. 2009). Traditionally the composition of glutenin alleles was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), which allows protein separation based on molecular mass (Jackson, Holt, and Payne 1983). More recent methods such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) provide greater resolution and the precise separation of HMW-GS and LMW-GS between wheat genotypes (Liu et al. 2009, 2010). The ratio of glutenins to gliadins in wheat flour also affects food processing properties and prolamins concentrations can be quantified using size-exclusion high performance liquid chromatography (SE-HPLC) (Bangur et al. 1997; Z. Wang et al. 2017).

To assess dough rheology, the Mixolab® system (Chopin Technologies) is adaptable to both commercial and non-commercial flours and more comprehensive than fundamental test methods (see Chapter 1) (Haros, Ferrer, and Rosell 2006; Koksel et al. 2009; Rosell, Collar, and Haros 2007). The Mixolab® test involves varying the mixing speed and temperature of a dough sample within a single measurement (~45 min duration) and measures a range of gluten and starch properties across five separate stages. The first stage involves initial mixing of flour and water, leading to the formation of wheat dough and determination of maximal dough resistance and dough stability. The second stage involves an increase in temperature and weakening of the dough structure. The third stage begins with starch gelatinization and a sharp increase in dough strength until mechanical shear and temperature stress lead to starch breakdown (fourth stage). The fifth stage involves a decrease in temperature and increase in dough strength through starch retrogradation. A Mixolab® curve highlights all five stages alongside three slope measurements: α , β , and γ that indicate dough weakening, starching speed, and enzymatic degradation, respectively, and together these measurements closely reflect results obtained through Alveograph and Farinograph tests. The Mixolab® assay has been used to evaluate the effect that adding common hydrocolloid ingredients or enzymes used in breadmaking has on wheat dough rheology (Haros et al. 2006; Kahraman et al. 2008; Rosell et al. 2007) and a proteomic analysis of Mixolab® dough samples has expanded the known range of prolamins associated with downstream processing (X. Wang et al. 2017).

Starch and protein properties in the wheat grain are strongly influenced by environmental conditions post-anthesis, such as temperature, drought, and the availability of nutrients in the soil (Dupont and Altenbach 2003; Panozzo and Eagles 1998). The concentration of glutenins and gliadins wheat flour, and therefore more downstream properties of dough rheology and breadmaking, are particularly affected by the availability and balance of grain nitrogen (N) and S (Luo et al. 2000). The availability of soil N determines the prolamins content in wheat grain and the ratio of HMW-GS to LMW-GS, with N fertilisation resulting in greater prolamins content and a higher ratio of HMW-GS to LMW-GS (Zhu et al. 1999). The availability of soil S influences the proportion of S-poor to S-rich prolamins, with low S availability reducing the proportion of A- and B-type LMW-GS and the formation of disulphide bonds within glutenin polymers, leading to a reduction in overall gluten quality (Zhao, Salmon, et al. 1999; Zhao, Hawkesford, and McGrath 1999). Combined fertilization of S and N is therefore used by agronomists to produce wheat grain with optimal protein content and composition (Flæte et al. 2005; Järvan et al. 2008; Yu et al. 2018). Fertilizing wheat with Zn alters the composition of glutenins and gliadins, thought to be due to Zn interacting with cysteine residues and altering the properties of disulphide bonds and/or Zn interacting with enzymes involved in polymerisation (Peck et al. 2008). The post-harvest addition of nutrients to wheat flour (a process commonly referred to as

fortification) is also known to influence downstream processing. For example, the addition of Fe-containing fortificants to wheat flour alters sensory characteristics, dough rheology, and breadmaking properties, likely through the oxidation of lipids (Rebellato et al. 2018; Rebellato, Bussi, et al. 2017; Rebellato, Castro Lima, et al. 2017).

Chapters 4 and 5 describe the generation of biofortified bread wheat through constitutive expression (CE) of the rice nicotianamine synthase (*OsNAS2* gene) and upregulation of nicotianamine (NA) and 2'-deoxymugineic acid (DMA). Field evaluation demonstrated that the concentrations of Fe, Zn, S and N were higher in CE-1 whole grain and white flour relative to the null segregant (NS) control (see Chapters 4 and 5). This study therefore aimed to determine if these changes to CE-1 white flour Fe, Zn, S, N concentrations affects wheat grain downstream processing including the composition and concentration of prolamins, dough rheology and breadmaking properties. The specific objectives of this chapter were to:

1. Investigate the effect of altered Fe, Zn, S and N concentrations on CE-1 and NS white flour protein composition and prolamins concentration,
2. Investigate the effect of altered Fe, Zn, S and N concentrations on CE-1 and NS white flour dough rheology,
3. Investigate the effect of altered Fe, Zn, S and N concentrations and fermentation time on CE-1 and NS white flour breadmaking properties.

7.2. Materials and Methods

7.2.1. Plant Material

Vector construction, plant transformation and the initial selection of our lead event CE-1 is previously described in Chapters 4, 5 and 6. In brief, the *OsNAS2* coding sequence (LOC_Os03g19420) from rice (*Oryza sativa* L.) cultivar (cv.) Nipponbare was recombined into a modified pMDC32 vector under transcriptional control of the maize (*Zea mays* L.) ubiquitin 1 promoter with a hygromycin phosphotransferase plant-selectable marker (see Chapter 4). Bombardment of the construct into immature wheat (*Triticum aestivum* L.) cv. Bobwhite embryos was performed at the University of Adelaide (Adelaide, Australia) to generate six independent CE-*OsNAS2* transformation events, and one double-insert fixed event termed CE-1 was selected for additional analyses based on no difference in plant phenotype and higher concentrations of grain Fe and Zn. Three sibling lines of CE-1 (termed CE-1.1, CE-1.2 and CE-1.3) and the corresponding null segregant control (NS) were selected for field trial evaluation at the sixth (T_6) and seventh (T_7) generation in 2016 and 2017, respectively. Plants were grown alongside the check cv. Mace, an elite Australian bread wheat variety that is grown throughout the Australian wheat belt. Field trials of NS, CE-1 sibling lines, and Mace were conducted at the New Genes for New Environment facilities located in Merredin (31.4837°S, 118.2771°E) and Katanning (33.6894°S, 117.5551°E) in Western Australia, and at Glenthorne (35.0543° S, 138.5524° E) in South Australia. Average yearly rainfall across the two field seasons was 804, 483, and 401 mm at Glenthorne, Katanning, and Merredin, respectively. Average daily temperatures (minimum/maximum) across the three field seasons were 11.8/25.3, 9.4/22.4, and 11.4/24.8 degrees Celsius (°C) in Glenthorne, Katanning, and Merredin, respectively. Average soil pH was 7.20, 4.95 and 4.8 at Glenthorne, Katanning, and Merredin, respectively (see Chapter 5).

7.2.2. White flour production

Whole grain samples were conditioned to 13% moisture content and milled using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) as described in Chapter 2 and the resulting white flour was used in prolamin, rheological and breadmaking analysis (Figure S7.1). Rapid and straight bread was produced from white flour composed of all break and reduction fractions following milling of whole grain samples at Merredin in 2017 using a Buhler MLU-202 laboratory mill at The Commonwealth Scientific and Industrial Research Organisation (CSIRO, ACT, Australia) as described in Chapter 2.

7.2.3. Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis

White flour and white bread micronutrient concentration was determined by nitric / perchloric acid digestion as described in Chapter 2, followed by ICP-OES using a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA). Nitrogen analysis of white flour was performed using the Dumas combustion method at CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia) as described in Chapter 2.

7.2.4. Grain protein analysis

7.2.4.1. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

For albumin and globulin (AGS) extraction, white flour (~50 mg) was mixed in dH₂O (300 µL) and centrifuged at 14,000 rpm for 3 min. Gliadin extraction was performed by substituting dH₂O with 70% ethanol (300 µL). Supernatant containing AGS or gliadin proteins (1 µL) was combined with 9 µL of a matrix solution containing sinapinic acid (40 mg / mL) dissolved in 100% acetonitrile/methanol/dH₂O (60:36:4 v/v). The supernatant/matrix solution (2 µL) was deposited onto a MALDI-TOF Voyager DE Pro 100 sample size plate and air dried prior to analysis. Glutenin extraction was performed according to (Wang et al. 2015) with minor modifications. White flour samples (~50 mg) were weighed in a 96 well plate, mixed in 70% ethanol (300 µL) for 30 min and centrifuged (5 min, 14,000 rpm) before discarding the supernatant containing AGS and gliadin fractions. The resulting pellet was mixed with 55% iso-propanol (1000 µL), incubated (65°C, 30 min, 20 rpm), centrifuged (5 min, 14,000 rpm) and the supernatant discarded. This wash step was repeated three times to ensure the removal of any AGS or gliadin proteins. After the final wash, the pellet was combined with 300 µL of glutenin extraction solution (50% iso-propanol, 80 mM Tris-HCl, pH 8.0, and 1% dithiothreitol) and incubated (65°C, 60 min, 20 rpm) before centrifugation (25°C, 45 min, 4450 rpm). Supernatant (240 µL) was transferred to a new 96 well plate, combined with 100% acetone (160 µL) and placed in the freezer (-20°C, 12 hr) before centrifugation (25°C, 45 min, 4450 rpm). The supernatant (300 µL) was combined with 100% acetone (600 µL) for low-molecular weight glutenin subunit (LMW-GS) analysis. The pellet was mixed with glutenin extraction solution (150 µL), incubated (65°C, 60 min, 20 rpm), centrifuged (25°C, 45 min, 4450 rpm), and 120 µL of the supernatant was combined with 100% acetone (80 µL) for high-molecular weight glutenin subunit (HMW-GS) analysis. Both LMW-GS and HMW-GS plates were placed in the freezer (-20°C, 12 hr), removed and centrifuged (4°C, 45 min, 4450 rpm) and the supernatant discarded to air-dry the pellet for 30 min. Samples were combined with 50 µL of resuspension solution (30% acetonitrile, 0.4% trifluoroacetic acid) and gently mixed for 60 min. One µL of the resuspended sample was combined

with 9 μL of the matrix solution, and 2 μL of the solution was deposited onto a MALDI-TOF Voyager DE Pro 100 sample size plate and air dried prior to analysis. Analysis was performed on a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an ultraviolet (UV) nitrogen laser (337 nm) which provides mass spectrum profiles. Laser intensity, mass range, delay time and grid voltage were specific for each prolamin subunit and are outlined in (Juhász et al. 2018; Liu et al. 2009, 2010; Wang et al. 2015). The identification of LMW-GS and HMW-GS alleles in cv. Bobwhite and Mace (Figures 7.1, S7.2, S7.3) was achieved using previously reported data (Gao 2012; Liu et al. 2009, 2010). The composition of LMW-GS in sibling line CE-1.3 and Mace at Merredin in 2017 was not measured due to technical difficulties (Figure S7.3).

7.2.4.2. Size-exclusion high-performance liquid chromatography (SE-HPLC)

The separation and quantification of gliadins and glutenins was performed by size-exclusion high-performance liquid chromatography (SE-HPLC) analysis as described by (Juhász et al. 2018). Briefly, white flour (10 mg) was thoroughly mixed with 1 mL of a 0.05 M phosphate extraction buffer (pH 6.9) containing 0.05% SDS and centrifuged at 20,000 rcf for 15 min using an Eppendorf Centrifuge 5424 (Eppendorf, Hamburg, Germany). The supernatant was aspirated and filtered (0.45 μm) into a HPLC vial prior to analysis on an Agilent 1200 LC system (Agilent Technologies Inc., CA, USA). Samples (10 μL) were injected at a flow rate of 0.350 $\mu\text{L min}^{-1}$ into a Bio SEC-5 4.6 x 300 mm, 300 Å pore size, 5 μm particle size HPLC column (Agilent Technologies Inc., CA, USA). Proteins were separated by a constant gradient of 50% solvent A (0.1% trifluoroacetic acid in dH_2O) and 50% solvent B (0.1% trifluoroacetic acid in acetonitrile) and detected by UV absorbance at 210 nm. All chemicals were HPLC grade (Sigma-Aldrich, MO, USA).

7.2.5. Rheological analysis

Moisture content of white flour samples (10 g) from Glenthorne, 2016 were determined by oven drying (120°C, 24 hr) and was found to be between 14.0 – 15.0% for all genotypes. Dough rheological properties of Glenthorne flour samples was determined using the Mixolab® (Chopin Technologies, Villeneuve La Garenne, France) using the standard ‘Chopin+’ protocol (Figure S7.1). Briefly, white flour (45-50 g) was added based on moisture content (%) to the Mixolab® mixing bowl and water (30°C) was automatically added for a target dough weight of 75 g. Mixing speed of the blades was kept constant at 80 rpm and the target torque for C1 was kept at 1.10 Nm. Temperature of the mixing bowl varied over the course of 45 mins: 30°C for initial 8 min, heating to 90°C over 15 min (rate of 4°C/min), holding at 90°C for 7 min, cooling to 50°C over 10 min (rate of 4°C/min), and holding at 50°C for 5 min. Dough temperature closely reflected the temperature of the mixing bowl (Figure 7.3).

The mixing bowl and blades were thoroughly cleaned between samples. The C1 torque value was used to adjust flour moisture content of subsequent runs and all C1 values were kept between 1.0 – 1.2 Nm.

7.2.6. Breadmaking analysis

7.2.6.1. University of Melbourne method

White bread was produced at the University of Melbourne (Faculty of Veterinary and Agricultural Science) using white flour of all field sites in 2016 and 2017 (Figures S7.1, S7.4, S7.5, S7.6) and a baker's yeast protocol as previously described (Winters et al. 2019). White flour (500 g) was mixed with H₂O (325 g), 10 g of dry baker's yeast (Lowan® whole foods, NSW, Australia) and 10 g of cooking salt (Black & Gold, NSW, Australia) in a BM2500 Compact Bakehouse (Sunbeam, NSW, Australia) for 25 min and fermented at 24°C for 1 hr (1.5 hr total fermentation), followed by fermentation at 4°C for 15 hrs (15 hr total fermentation) or 30 hrs (30 hr total fermentation). Dough was divided into 150 g pieces, shaped and placed into loaf tins (14.6 x 7.6 cm) for proofing (30 min, 30°C) and baking (20 min, 200°C) in a commercial oven (Convotherm 4 EasyDial 10.10, Moffat, VIC, Australia). Loaves were cooled (1 hr) and sliced transversely for crumb analysis as described in (Winters et al. 2019). Briefly, images of loaf cross sections were cropped to 5 cm² squares containing only crumb, converted to grayscale and segmented using a locally adaptive threshold method (sensitivity factor 0.68) with value 0 (black) meaning cell wall and value 1 (white) meaning cells using Matlab® (Mathworks Inc., Matick, MA) software (Appendix 7.1). Cells were defined as any 8-connected region of pixels with value 1 and the following features calculated: void fraction (VF, total cell area / total area x 100), mean cell area (MCA, mm²), cell density (CD, no. of cells/cm²).

7.2.6.2. Rapid and straight fermented dough methods

Rapid and straight dough fermented breads were produced from Merredin white flour in 2017 (Figure S7.1) at CSIRO (ACT, Australia) and based upon the internationally recognized methods for determining wheat baking quality (AACC Approved Methods 10-09.01 and 10-10.03). The rapid dough breadmaking method simulates the large-scale sliced-bread baking method used to produce white bread sold throughout Australian supermarkets. White flour (50 g) produced from Buhler MLU-202 laboratory milling was combined with the following ingredients at percentages of flour weight: dried yeast 1.5%, vegetable fat 2%, salt 2%, baking improver 1.5% and water (at optimal water absorption level less 4%) for rapid fermented dough method, and dried yeast 0.7%, vegetable fat 2%, salt 1%, baking improver 0.5%, sugar 1%, and water (at optimal water absorption level less 4%) for straight fermented dough method. Ingredients were mixed (180 rpm) in a 50 g bowl of a doughLAB mixer (Perten Instruments, Sydney, Australia) to peak consistency, with the bowl

temperature maintained at 22°C. Following mixing, dough for rapid fermentation was divided into 60 g pieces, proofed (15 min, 40°C, 85% humidity), passed through an LRM-1 bread moulder (International Bakery Equipment, Clontarf, Australia) and placed into a baking tin prior to a final proof (40 min, 40°C, 85% humidity). Dough for straight fermentation was divided into 60 g pieces and placed into a sealed plastic container before being proofed for 105 min, 50 min, and 25 min (28°C, 85% humidity), and was passed through an LRM-1 bread moulder between proofs. Following the third proof the straight fermented dough samples were placed into a baking tin prior to a final proof (50 min, 35°C, 85% humidity). Total dough development time for rapid and straight fermentation methods was 55 and 230 min, respectively. Rapid and straight fermented doughs were baked at 220°C for 15 min, frozen and lyophilized before grinding to a powder for analysis.

7.2.7. Phytate analysis

Dietary phytate was calculated relative to total phosphorus released from rapid and straight dough fermented bread samples by phytase and alkaline phosphatase enzymes according to manufacturer's instructions (K-PHYT 11/15. Megazyme International. Bray, Ireland) as described in Chapter 2.

7.2.8. Caco-2 Fe bioavailability analysis

Rapid and straight dough fermented bread samples were subjected to the Caco-2 cell bioassay as described in Chapter 2. Briefly, gastric-digested samples (1.5 mL) were added to cylindrical Transwell inserts (Corning Life Sciences, Corning, NY) fitted with a semipermeable (15 000 Da MWCO) basal membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA) and placed within wells containing Caco-2 cell monolayers. Following overnight incubation, cells were washed, harvested and analyzed for ferritin (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein contents (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA). As Caco-2 cells synthesize ferritin in response to intracellular Fe, we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake.

7.2.9. Statistical analysis

Graphs were generated using SigmaPlot v13 (Systat Software Inc., San Jose) software and data are presented as mean $\pm$ SEM with biological replicate numbers noted in table and figure legends. Student's t-test was used to determine significant differences between means and was conducted using Minitab 17 Statistical software (Minitab, State College, PA).

7.3. Results

7.3.1. Wholemeal and white flour nutritional composition at Glenthorne

Summarizing the results of our multi-year field trial analysis at Glenthorne in South Australia as outlined in Chapter 5, wholemeal flour Fe concentrations were between 1.1- to 1.2-fold higher, and wholemeal flour Zn concentrations were between 1.3- to 1.5-fold higher in all CE-1 sibling lines relative to NS (Table 7.1). Wholemeal flour S and total protein concentrations were 1.1-fold higher in sibling line CE-1.1 relative to NS (Table 7.1). Wholemeal flour NA concentrations were between 1.8- to 1.9-fold higher, and wholemeal flour DMA concentrations were 1.4-fold higher in all CE-1 sibling lines relative to NS. White flour Fe concentrations were 1.2-fold higher in sibling line CE-1.1 relative to NS (7.2). White flour Zn concentrations were between 1.3- to 1.5-fold higher in all CE-1 sibling lines relative to NS. White flour S concentrations were 1.2-fold higher, and white flour total protein concentrations were 1.1-fold higher in sibling line CE-1.1 relative to NS (Table 7.2). White flour NA concentrations were between 2.0- to 2.1-fold higher, and white flour DMA concentrations were between 1.5- to 1.6-fold higher in all CE-1 sibling lines relative to NS.

Table 7.1. Wholemeal flour nutritional composition of NS, CE-1.1, CE-1.2, CE-1.3 and Mace. Values indicate average concentrations over three field seasons at Glenthorne.

Genotype	Iron ($\mu\text{g} / \text{g}$)	Zinc ($\mu\text{g} / \text{g}$)	Sulphur ($\mu\text{g} / \text{g}$)	Total protein (%)	Nicotianamine ($\mu\text{mol} / \text{g}$)	2-Deoxymugineic acid ($\mu\text{mol} / \text{g}$)
NS	33.9	38.0	1412.6	10.6	35.4	37.0
CE-1.1	40.9	56.7	1591.7	12.1	68.3	52.1
CE-1.2	37.1	48.6	1459.9	10.9	66.9	51.1
CE-1.3	38.1	50.1	1446.4	10.9	62.4	52.6
Mace	32.5	29.1	1365.2	10.8	37.5	27.2

Table 7.2. White flour nutritional composition of NS, CE-1.1, CE-1.2, CE-1.3 and Mace. Values indicate average concentrations over three field seasons at Glenthorne.

Genotype	Iron ($\mu\text{g} / \text{g}$)	Zinc ($\mu\text{g} / \text{g}$)	Sulphur ($\mu\text{g} / \text{g}$)	Total protein (%)	Nicotianamine ($\mu\text{mol} / \text{g}$)	2-Deoxymugineic acid ($\mu\text{mol} / \text{g}$)
NS	10.7	12.8	1098.3	10.2	25.1	32.2
CE-1.1	13.3	19.8	1306.6	11.2	52.6	47.5
CE-1.2	11.5	16.6	1164.5	10.5	53.3	51.2
CE-1.3	11.1	16.2	1157.1	10.1	50.1	47.4
Mace	11.5	10.4	1057.6	8.7	29.7	27.4

7.3.2. Grain protein subunit composition

The composition of albumin and globulins (AGS), low-molecular weight glutenin subunits (LMW-GS) and high-molecular weight glutenin subunits (HMW-GS) was the same in NS and all three CE-1 sibling lines at Glenthorpe in 2016 (Figure 7.1). The *Glu A3e*, *Glu B3g* (or *Glu B3j*), and *Glu D3b* LMW-GS alleles and the 1Ax2*, 1Bx7 + 1By9, and 1Dx5 + 1Dy10 alleles were present in cv. Bobwhite. The peak indicating high mass globulins was absent in cv. Mace white flour relative to NS and CE-1 white flour (Figure 7.1e). The *Glu A3c*, *Glu B3h*, and *Glu D3c* LMW-GS alleles and the 1Ax1, 1Bx7 + 1By8, and 1Dx2 + 1Dy12 HMW-GS alleles were present in cv. Mace (Figures 7.1j and 7.1o). The AGS, LMW-GS and HMW-GS profiles that were observed at Glenthorpe were similar to the profiles observed at Katanning and Merredin in 2016 (Figures S7.2-S7.3).

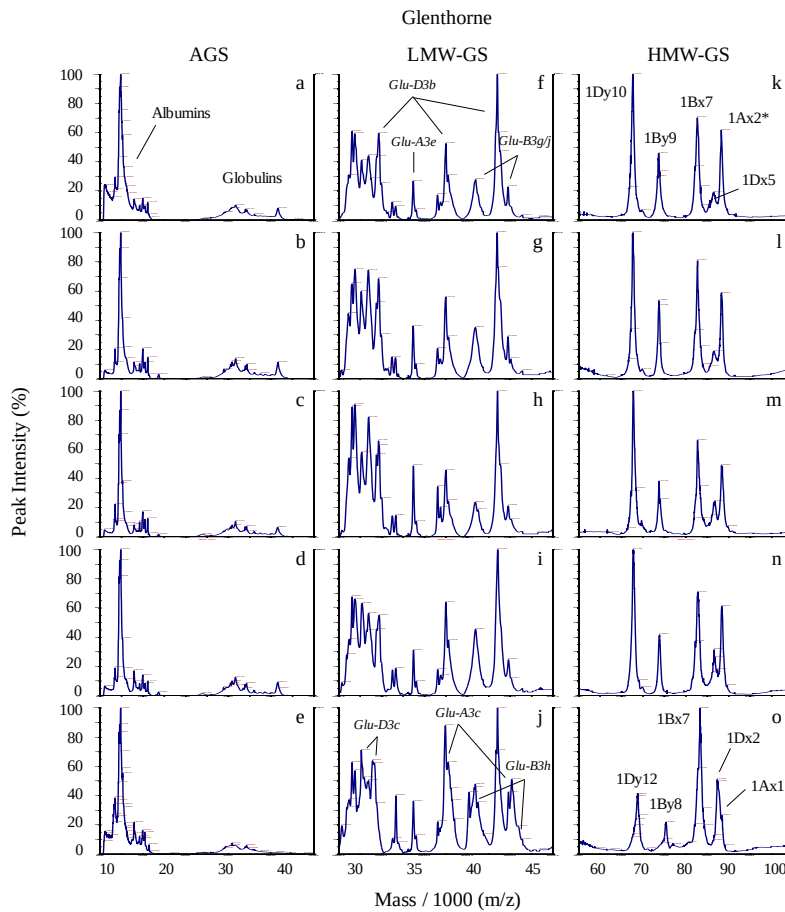


Figure 7.1. Mass spectrum profiles of protein subunits in NS, CE-1 sibling line and Mace white flour at Glenthorpe in 2016. **a-e)** Albumins and globulins (AGS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. **f-j)** Low-molecular-weight glutenin subunits (LWM-GS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. **k-o)** High-molecular-weight glutenin subunits (HMW-GS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. Each peak represents a different subunit. The y-axis represents peak intensity (%) and the x-axis represents mass-to-charge ratio (m/z).

7.3.3. Grain prolamins subunit concentration

Glutenin concentrations (including LMW-GS and HMW-GS) were 1.2-fold and significantly higher in CE-1.1 white flour, and 1.1-fold higher in CE-1.2 white flour relative to NS at Glenthorne in 2016 (Figure 7.2a). Gliadin concentrations were 1.2-fold higher in CE-1.1 white flour relative to NS (Figure 7.2b). The ratio of glutenins to gliadins in white flour was 1:1.8 in NS and all three CE-1 sibling lines and 1:1.3 in Mace.

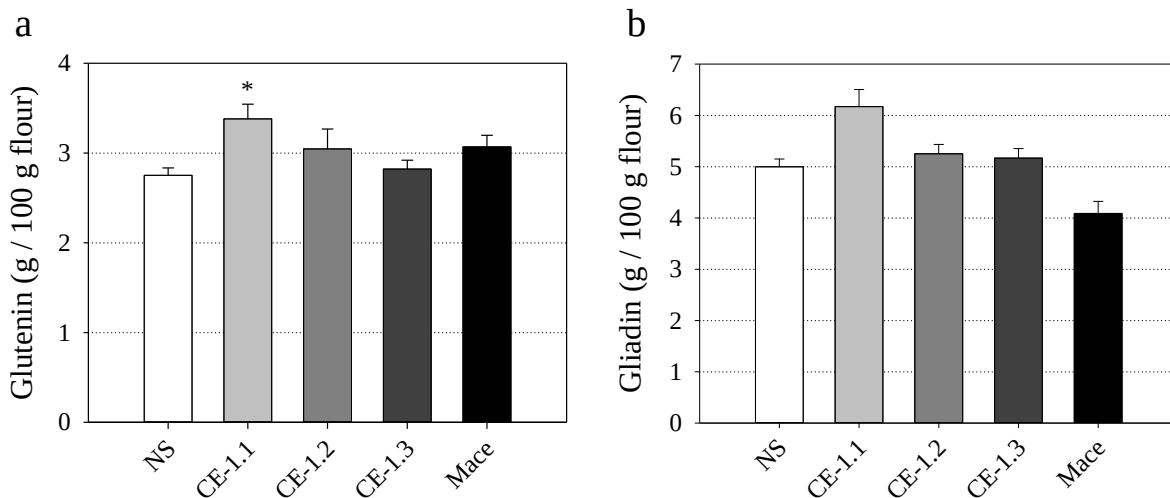


Figure 7.2. White flour **a)** glutenin and **b)** gliadin concentration (g / 100g) in NS, CE-1 sibling lines and Mace at Glenthorne in 2016. Bars represent the mean $\pm$ SEM of three replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

7.3.4. Dough rheology

Mixolab curves of white flour derived from NS and CE-1 sibling lines at Glenthorne revealed that CE-1.1 flour has a greater C1 (water absorption) value, and that CE-1.2 and CE-1.3 flours have greater starching speed (β slope on curve), C4 (starch degradation), and C5 (starch retrogradation) values relative to NS (Figure 7.3). By contrast, cv. Mace flour demonstrated lower C1 and C2 (protein weakening) values and greater C3 (starch gelatinization) and C5 values relative to NS at the same moisture content (Figure 7.3). At optimal moisture content, cv. Mace white flour demonstrated greater dough stability (5 mins at torque = 1.1 Nm) relative to the NS (4 mins at torque = 1.1 Nm) as well as greater C2 and C3 values and total starch degradation after 25 minutes of the Mixolab® assay (Figure 7.3). The collapse of the curve of cv. Mace white flour is thought to be due to starch-protein interactions leading to a breakdown of the gluten complex and loss of mixing resistance.

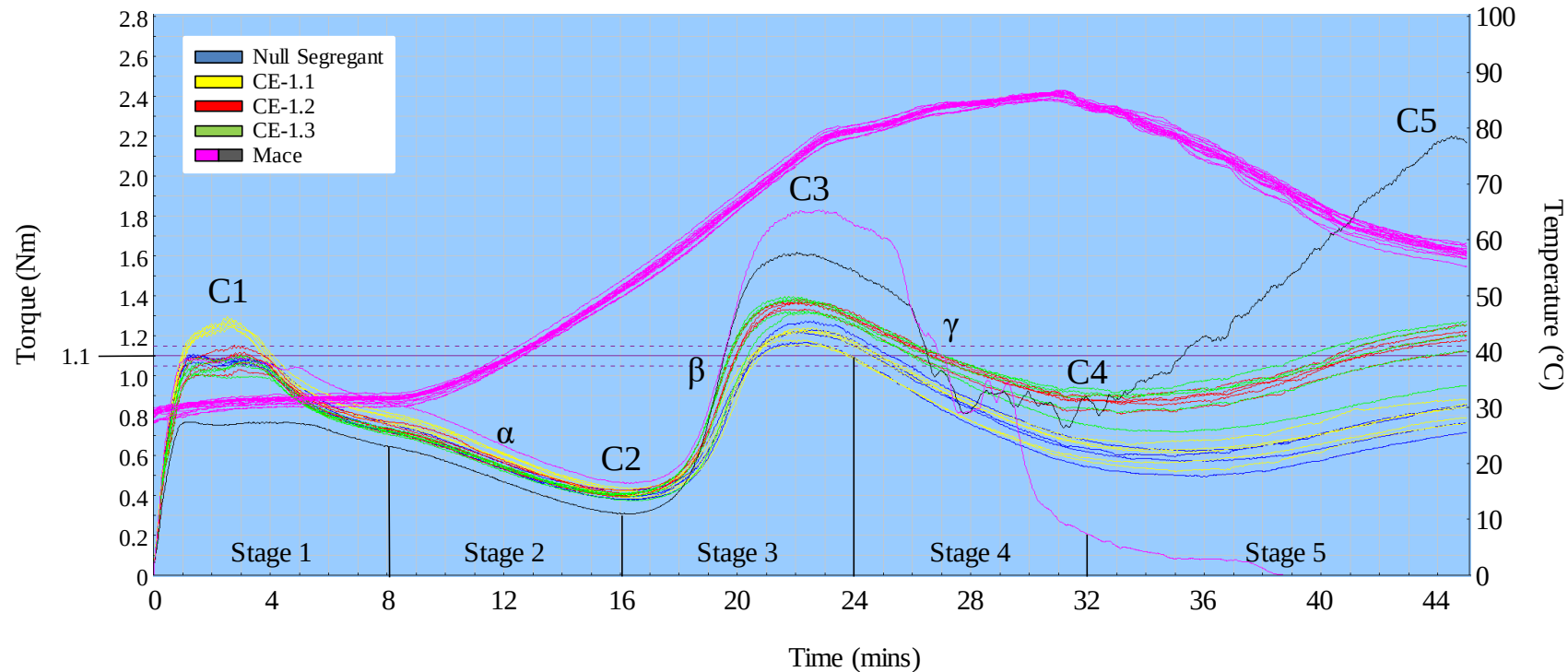


Figure 7.3. Mixolab curves of white flour produced from NS (blue lines), CE-1 sibling lines (yellow, red, and green lines) and Mace (purple and black lines) at Glenthorne in 2016. Representative curves of Mace white flour with equivalent (black) and optimal (purple) moisture content are included. The y axis on the left-hand side indicates the torque required to maintain the dough mixing action (newton/meter, Nm) and the y axis on the right-hand side indicates the temperature change (pink lines) for each curve. The five stages relating to initial mixing (stage 1), protein weakening (stage 2), starch gelatinization (stage 3), starch melting (stage 4) and starch retrogradation (stage 5). The highest and lowest torque values in each stage are labelled C1-C5. α = protein weakening speed, β = starching speed slope, γ = starch degradation speed.

7.3.5. Breadmaking performance

White bread void fraction (VF), mean cell area (MCA) and cell density (CD) are analogous to sensory measurements of bread crumb porosity and were similar between white bread samples of all CE-1 sibling lines and the NS at Glenthorne, Merredin and Katanning in 2017 (Figure 7.4). White bread of Mace demonstrated slightly lower VF and MCA relative to NS white bread, particularly at Glenthorne in 2017 (Figure 7.4, Figure S7.4). White bread MCA was lower, and white bread CD was higher in all genotypes at Katanning relative to Glenthorne and Merredin in 2017. In 2016 we measured significantly higher VF and MCA in white bread of all CE-1 sibling lines at Merredin and significantly lower VF and MCA in white bread of CE-1.3 at Katanning relative to NS (Figure S7.5).

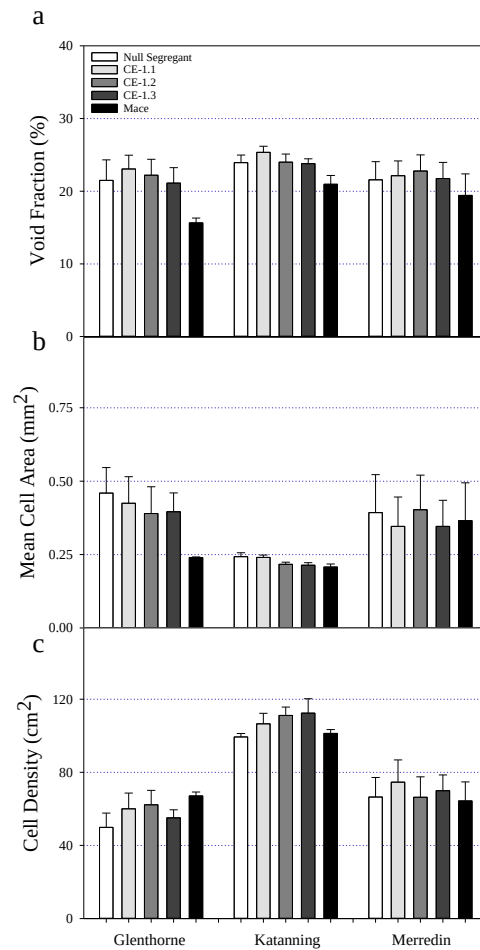


Figure 7.4. White bread properties of field grown NS and CE-1 wheat lines in 2017. **a)** void fraction (%), **b)** mean cell area (mm²), and **c)** cell density (cm²) in white bread of NS (white) and three CE-1 sibling lines (CE-1.1 light grey, CE-1.2 grey, and CE-1.3 dark grey) at Glenthorne, Katanning and Merredin in 2017. Bars represent the mean ± SEM of six replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

7.3.6. White bread nutrition following rapid or straight fermentation

Iron concentration was significantly higher in rapid fermented CE-1 white bread relative to NS (Figure 7.5a). Zinc concentration was significantly higher in rapid and straight fermented CE-1 white bread relative to NS (Figure 7.5b). Phytate concentration was significantly higher in straight fermented CE-1 white bread relative to NS (Figure 7.6). Caco-2 cell ferritin formation was significantly higher following exposure to digests of straight fermented CE-1 white bread relative to digests of NS white bread (Figure 7.7).

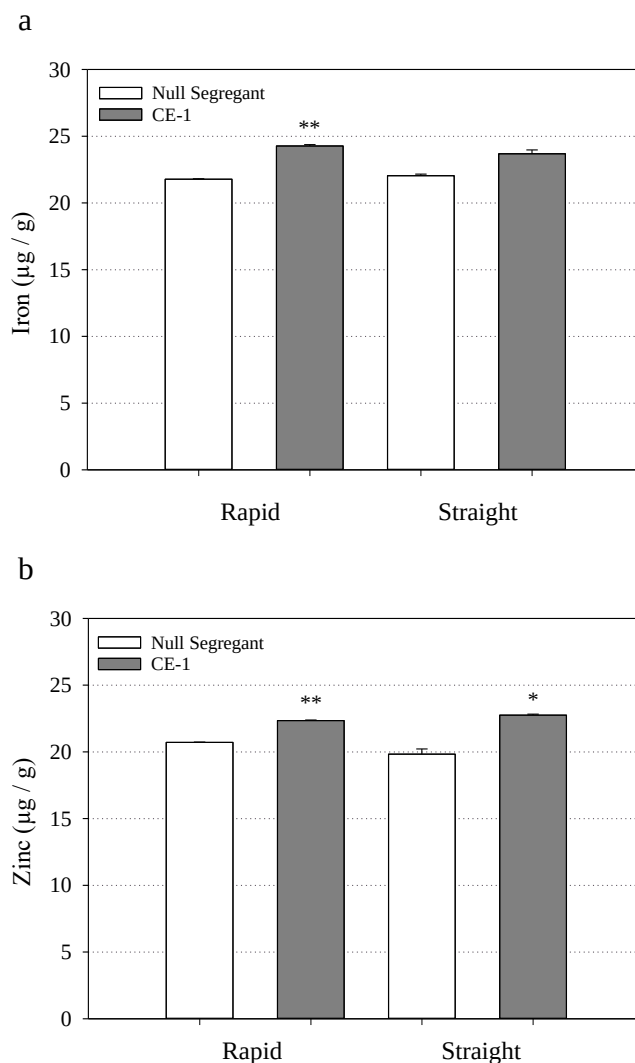


Figure 7.5. Rapid and straight fermented white bread **a)** Fe and **b)** Zn concentration (µg / g) of NS (white) and CE-1 (biofortified, grey) at Merredin in 2017. Bars represent the mean $\pm$ SEM of three replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

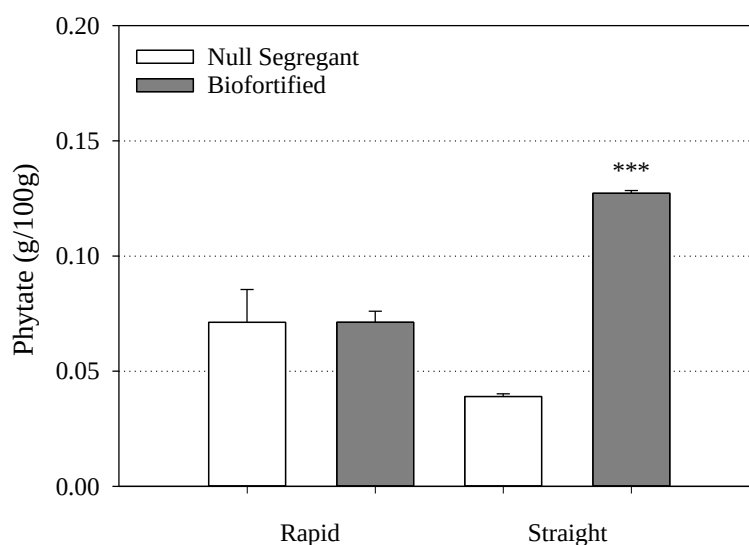


Figure 7.6. Rapid and straight fermented white bread phytate concentration (g / 100g) of NS (white) and CE-1 (biofortified, grey) at Merredin in 2017. Bars represent the mean $\pm$ SEM of two replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

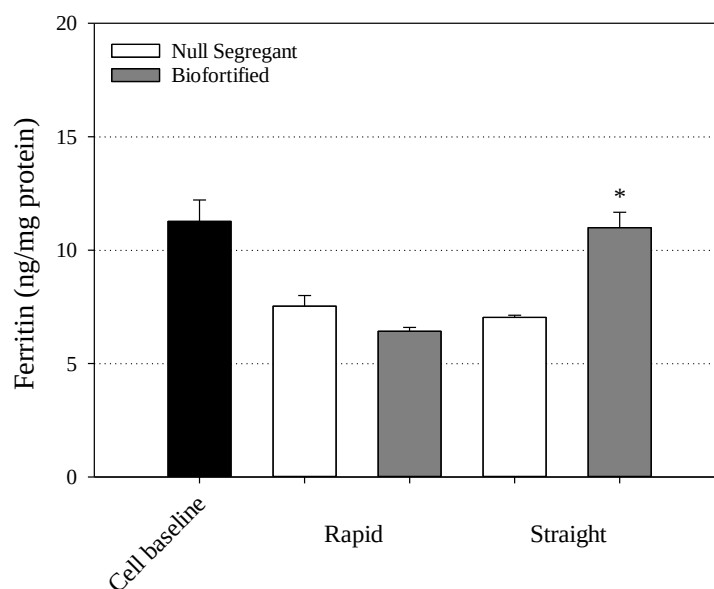


Figure 7.7. Rapid and straight fermented white bread Fe bioavailability (ng ferritin / mg protein) of NS (white) and CE-1 (biofortified, grey) at Merredin in 2017. Bars represent the mean $\pm$ SEM of three replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

7.4. Discussion

Several studies have demonstrated the importance of S and N in prolamin formation and function and that fertilization of wheat with S and/or N can alter the glutenin/gliadin concentrations in the grain and subsequently affect downstream processing (Flæte et al. 2005; Järvan et al. 2008; Luo et al. 2000; Yu et al. 2018; Zhao, Salmon, et al. 1999; Zhao, Hawkesford, et al. 1999; Zhu et al. 1999). Given that both NA and DMA are non-protein amino acids that contain N in their chemical structure and are synthesized from methionine (an S-containing amino acid), it is worthwhile to investigate whether upregulated biosynthesis of NA and DMA in wheat alters grain prolamin characteristics and/or downstream processing (Clemens 2014). In our study we analyzed three CE-OsNAS2 wheat sibling lines containing variable concentrations of grain Fe, Zn, S, N, NA and DMA alongside our NS control and determined the effect of these differences in nutrition on white flour prolamin concentration, dough rheology and breadmaking performance.

7.4.1. Higher concentrations of S and N in CE-1.1 white flour was associated with higher prolamin concentrations without affecting white flour protein composition

At Glenthorne, white flour milled from grain of all CE-1 sibling lines contained higher concentrations of Zn (up to 1.5-fold), NA (up to 2.1-fold) and DMA (up to 1.6-fold), and white flour milled only from sibling line CE-1.1 contained higher Fe (1.2-fold), S (1.2-fold) and N (1.1-fold) relative to NS (Table 7.2). These results indicate that the higher concentrations of N in CE-1.1 white flour cannot be entirely explained by the presence of additional grain NA and/or DMA. Instead it is likely that additional N and S concentrations are remobilized from CE-1.1 vegetative tissues to the grain during grain filling alongside the remobilization of leaf Fe and Zn (see Chapter 5) and confirming these nutrient dynamics between CE-1 tissues will require separate experimentation using radioisotopes (Pottier et al. 2014; Waters et al. 2009). The variability in CE-1 white flour nutrition observed in this study provided us the unique opportunity to examine the effects of S and N separately from the effects of Zn, NA and DMA on wheat grain prolamin characteristics and downstream processing. White flour glutenin composition was unaffected by altered S and N concentrations in CE-1 sibling lines and the NS (Figure 7.1, Figures S7.2-S7.3). By contrast, glutenin concentrations were significantly higher in CE-1.1 white flour relative to NS at Glenthorne (Figure 7.2a), most likely due to the 1.1- to 1.2-fold higher concentrations of S and N in CE-1 white flour as these nutrients play critical roles in glutenin formation and polymerization (Flæte et al. 2005). Glutenin concentration was 1.1-fold higher in CE-1.2 white flour relative to NS, suggesting that even small increases (< 10%) to grain S and N concentrations can affect white flour glutenin concentration. These results are consistent with previous reports indicating that improving N and/or S concentration in wheat grain through

fertilization alters the content and polymerization of glutenins (Luo et al. 2000; Zhao, Salmon, et al. 1999). From our study it is unclear whether the 1.2-fold higher Fe concentration in CE-1 white flour had any effect on glutenin and gliadin concentrations. Previous research has demonstrated that adding phytate (a chelator of Fe, Zn, magnesium (Mg) and calcium (Ca) in cereal grain) to wheat flour negatively affects gluten development due to phytate chelating Fe and/or binding glutenins directly and deciphering the effect of Fe on wheat grain downstream processing warrants further investigation (Park et al. 2016).

7.4.2. Constitutive *OsNAS2* expression in wheat altered water absorption in CE-1.1 white flour and starch characteristics in CE-1.2 and CE-1.3 white flour

We detected no difference in the ratio of glutenins to gliadins in CE-1 sibling lines and the NS and we therefore expected no differences in dough rheology (Figure 7.2). In contrast, previous studies have utilized transgenesis to achieve a 4-fold higher concentration of the 1Dx5 HMW-GS protein in white flour relative to the control (Rakszegi et al. 2005; Shewry 2007). As gliadin concentrations were unchanged in that study, the white flour contained a greater ratio of glutenins to gliadins, an increased HMW-GS to LMW-GS ratio and produced wheat dough with improved stability and reduced extensibility. The most striking result from our study occurred during the rheological Mixolab® analysis, which demonstrated a higher C1 value in CE-1.1 white flour that was not present in other CE-1 sibling lines or the NS (Figure 7.3). The C1 value is a measure of flour water absorption (WA), which is defined as the ratio of water to flour required to achieve a dough of standard consistency (Greer and Stewart 1959). The higher C1 value suggests that CE-1.1 white flour had improved WA relative to the NS, and therefore less CE-1.1 flour would be required to produce dough of a similar consistency to the NS. Improved WA in CE-1.1 white flour was likely due to the higher concentrations of N, S and/or prolamins as previous studies have indicated that 90% of variation in WA is accounted for by differences in protein and starch quality (Greer and Stewart 1959). Dough stability (time at torque = 1.1 Nm) was similar between white flour of all CE-1 sibling lines and the NS, suggesting that the higher prolamins concentrations in CE-1.1 white flour improved WA without affecting dough stability. Starching speed (β slope), starch degradation and starch retrogradation were higher in CE-1.2 and CE-1.3 sibling lines relative to both sibling line CE-1.1 and the NS (Figure 7.3), suggesting these lines have altered starch properties. We hypothesize that the higher concentration of white flour Zn in CE-1.2 and CE-1.3 relative to NS is responsible for these altered starch characteristics, given that Zn is known to alter prolamins polymerization through interaction with enzymes and/or disulphide bonds (Peck et al. 2008). For sibling line CE-1.1 we hypothesize that these altered starch characteristics are masked by the higher concentrations in grain prolamins relative to

NS, CE-1.2 and CE-1.3, given that starch granules are embedded within the gluten network (Shewry et al. 1995). Confirming these hypothesized effects of higher white flour Zn concentration on dough rheology will be the subject of future research.

7.4.3. CE-OsNAS2 expression does not affect breadmaking properties

We initially detected significantly higher VF and MCA in all CE-1 sibling lines relative to NS at Merredin in 2016 (Figures S7.5, S7.6). More comprehensive breadmaking analysis during 2017 revealed no differences in VF, MCA and CD in CE-1 white bread relative to NS at all field sites, suggesting that CE-OsNAS2 expression does not affect breadmaking properties (Figure 7.4, Figure S7.4). Previous studies have shown that white bread VF and MCA were most affected by adding bacteria to baker's yeast to produce sourdough fermented bread, and that increased crumb porosity is associated with improved sensory properties (Rizzello et al. 2012; Settanni et al. 2013; Winters et al. 2019). Measuring the sensory properties of biofortified foods is a necessary component of determining consumer acceptance (Bouis et al. 2019), and given that CE-1 white flour contains higher Fe concentrations (which is known to alter sensory properties) future research would need to include sensory evaluation of CE-1 and NS white bread (Rebellato et al. 2018). These sensory experiments will occur alongside more comprehensive analyses of CE-1 and NS breadmaking performance and will require the use of recognized rheological methods that are associated with breadmaking performance (such as the Alveograph) or by directly measuring bread loaf volume (Dobraszczyk and Morgenstern 2003).

7.4.4. Grain protein content, grain protein composition, dough rheology and breadmaking varies between wheat cultivars and should be considered in future biofortification programs

Bread wheat cv. Bobwhite demonstrated a yield penalty and lower thousand grain weight compared to our agronomic check cv. Mace (a commercially grown Australian hard wheat that is used for breadmaking) when grown over multiple field seasons (see Chapter 5). The ratio of glutenins to gliadins was higher in Mace (1:1.3) relative to Bobwhite (1:1.8), suggesting that Mace white flour would produce wheat dough of greater strength and lower extensibility relative to Bobwhite (Z. Wang et al. 2017). Mixolab® analysis confirmed that Mace dough had greater stability during initial mixing and could resist greater amounts of torque during starch gelatinization (Figure 7.3). Interestingly Mace demonstrated variable starch degradation and retrogradation during Mixolab® Stages 4 and 5 indicating that Mace dough had lower extensibility, which may have been caused by the relatively low gliadin concentrations in Mace white flour (Figure 7.2b). Similar results have been demonstrated in other elite Australian wheat cultivars and suggests that the Mixolab® analysis may be an unsuitable rheological test for Australian hard (high protein) bread wheats (X. Wang et al. 2017). Interestingly,

Mace contains the 1Dx2 + 1Dy12 allelic pair (Figure 7.1m) that has less of a positive effect on breadmaking relative to the 1Dx5 + 1Dy10 allelic pair that is present in Bobwhite (Liu et al. 2009). By contrast, the Bobwhite genome contains a 1BL-1RS translocation from rye that disrupts the LMW-GS Glu-1 loci on the long arm of chromosome 1, resulting in a ‘sticky-dough’ characteristic and lower bread making performance relative to other bread wheats (Furtado et al. 2015; Warburton, Skovmand, and Mujeeb-Kazi 2002). Given that Bobwhite and Mace demonstrated similar breadmaking properties throughout this Chapter, it appears that the 1Dx2 + 1Dy12 allelic pair and 1BL-1RS translocation may both affect breadmaking performance to a similar degree. Future wheat Fe and Zn biofortification programs through constitutive *OsNAS2* expression may therefore benefit from including elite wheat cvs. other than cv. Mace that have genetic profiles conducive to strong breadmaking performance.

7.4.5. Straight fermentation enhances Fe bioavailability in CE-*OsNAS2* bread

To assess the effect of fermentation on CE-*OsNAS2* white bread nutrition we utilized the internationally recognized methods of rapid (short) and straight (long) dough fermentation (Newberry et al. 2018). Measuring *in vitro* Fe bioavailability in rapid and straight dough bread samples was achieved through the coupled *in vitro* digestion/Caco-2 cell assay, which is the recommended method for measuring *in vitro* food Fe bioavailability (Etcheverry et al. 2012). Straight fermented CE-1 white bread contained 1.6-fold more bioavailable Fe relative to NS straight bread, indicating that CE-1 white bread produced to an international standard is likely to improve human Fe status relative to NS white bread (Figure 7.7). These results support a previous study in which we demonstrated higher Fe bioavailability in CE-1 white bread samples relative to NS white bread samples that were generated using an in-house breadmaking method (see Chapter 5). The 3-fold difference in phytate (an Fe bioavailability inhibitor) between CE-1 and NS straight breads suggests white bread phytate was not degraded following longer dough fermentation and that changes to phytate/Fe ratios do not account for the increase in Fe bioavailability (Figure 7.6). Previous studies have demonstrated the effectiveness of bacterial phytase enzymes at degrading phytate in sourdough bread, although total removal of phytate from sourdough bread did not improve Caco-2 cell Fe uptake suggesting that other compounds (i.e. dietary fiber and polyphenols) inhibit Fe bioavailability in bread (Rodriguez-Ramiro et al. 2017). The minor difference (< 2 ppm) in Fe concentration between CE-1 and NS straight breads (Figure 7.5a) is unlikely to account for the increase in Fe bioavailability, and we instead hypothesize that NA and/or DMA mediate the uptake of Fe into Caco-2 cells given these chelators are potent enhancers of *in vitro* Fe bioavailability (see Chapters 3, 4, 5 and 6).

7.5. Conclusions

The results of this Chapter found that higher concentrations of grain Fe and Zn in CE-*OsNAS2* wheat occur alongside higher concentrations of grain S and N, and that the accumulation of all these nutrients in CE-1 grain cannot be attributed to higher concentrations of grain NA and DMA alone. Given wheat is processed into a range of food products prior to consumption, and that S and N play structural and functional roles in wheat gluten, it is worth investigating whether altered S and N concentrations in CE-1 grain result in changes to grain downstream processing. Several preliminary analyses in this Chapter were performed only on white flour derived from Glenthorne in 2016, and more comprehensive multi-location and multi-generation studies are required to confirm these effects on white flour prolamin concentrations and rheology.

Wheat Fe and Zn biofortification through CE-*OsNAS2* generates plants that satisfy the discovery and early development phases and has the potential to enhance human micronutrient intake. We also observed higher Fe bioavailability in CE-1 white bread relative to NS white bread when produced through an internationally recognized straight dough fermentation method, and we therefore expect that CE-*OsNAS2* wheat food products would improve Fe status in humans. It is worth investigating whether similar results would be obtained from CE-1 wholemeal bread (i.e. chapatti), as the most severe incidences of human micronutrient deficiencies occur in countries that traditionally consume wholemeal flour products (see Chapters 1, 4 and 5). Prior to dissemination of CE-*OsNAS2* wheat, further analyses of breadmaking performance using internationally recognized methods as well as sensory evaluation of CE-1 bread are required to satisfy the late development phase of biofortification and to ensure consumer acceptance.

7.6. Supplementary Information

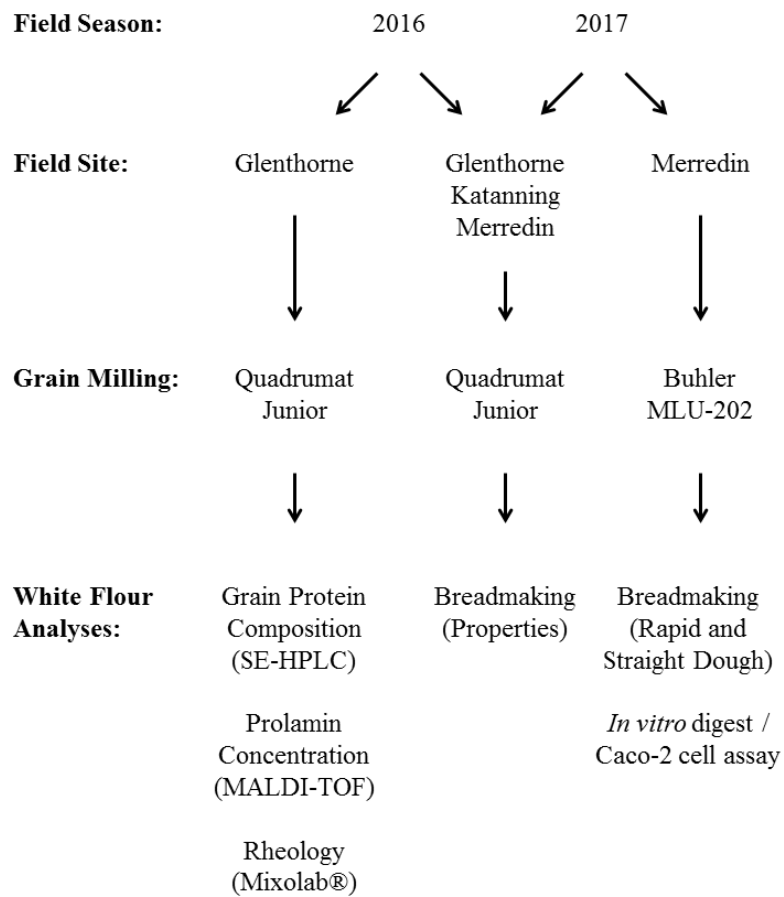


Figure S7.1. Schematic representation of which white flour analyses were performed at each field site in 2016 and 2017.

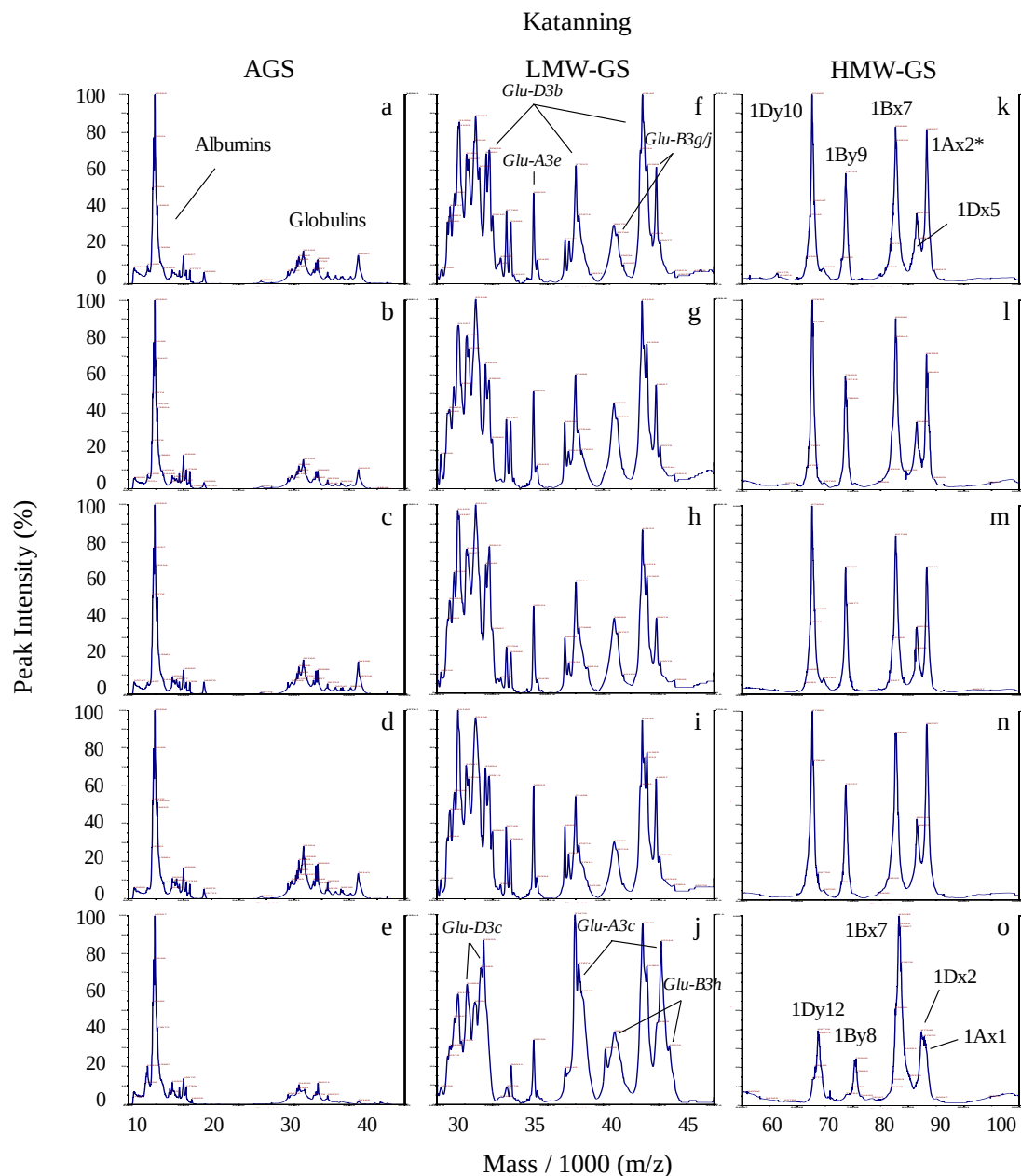


Figure S7.2. Mass spectrum profiles of protein subunits in NS, CE-1 sibling line and Mace white flour at Katanning in 2016. **a-e**) Albumins and globulins (AGS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. **f-j**) Low-molecular-weight glutenin subunits (LWM-GS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. **k-o**) High-molecular-weight glutenin subunits (HMW-GS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. Each peak represents a different subunit. The y-axis represents peak intensity (%) and the x-axis represents mass-to-charge ratio (m/z).

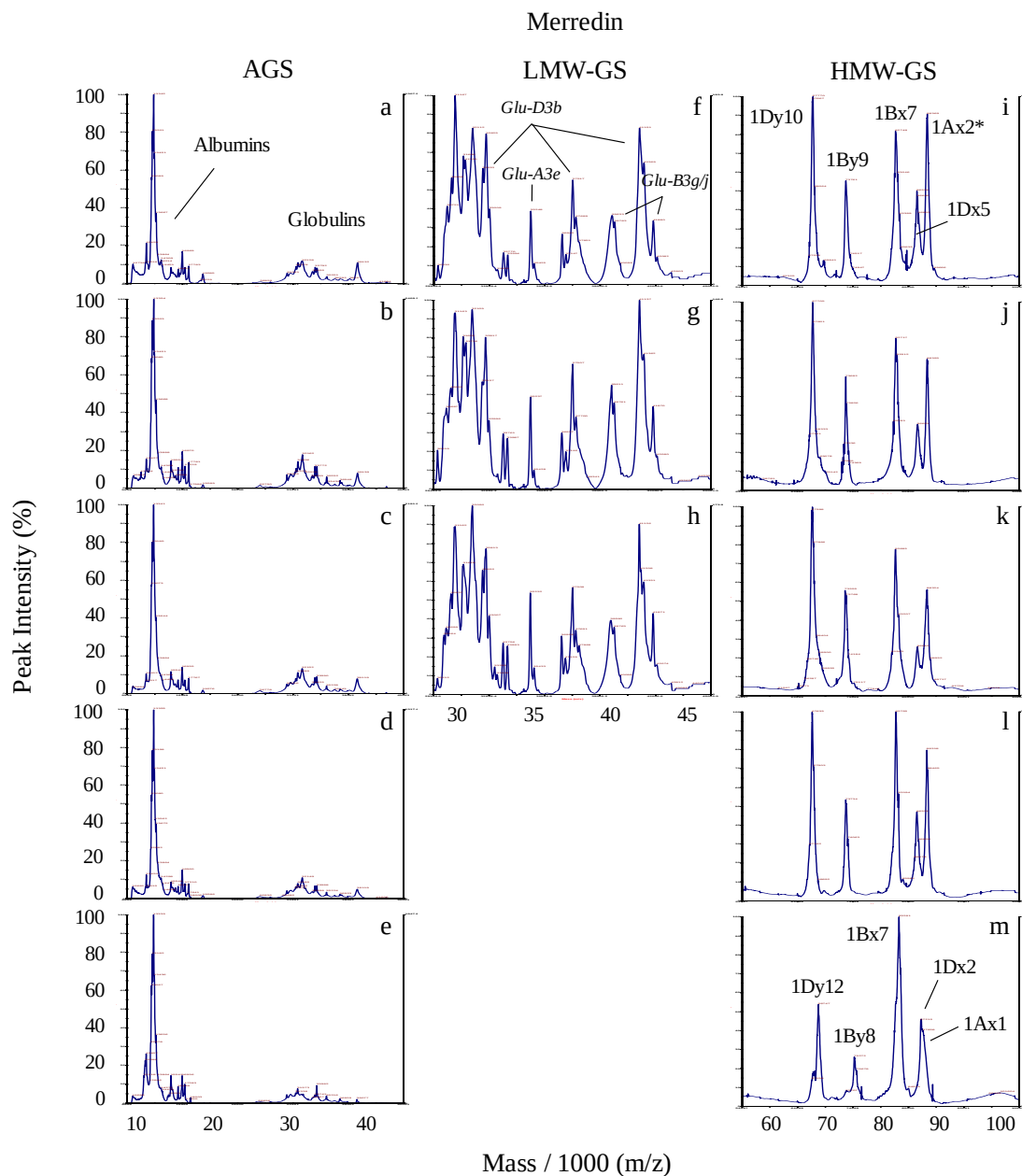


Figure S7.3. Mass spectrum profiles of protein subunits in NS, CE-1 sibling line and Mace white flour at Merredin in 2016. **a-e)** Albumins and globulins (AGS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. **f-h)** Low-molecular-weight glutenin subunits (LWM-GS) in NS, CE-1.1, and CE-1.2, respectively. **i-m)** High-molecular-weight glutenin subunits (HMW-GS) in NS, CE-1.1, CE-1.2, CE-1.3, and Mace, respectively. Each peak represents a different subunit. The y-axis represents peak intensity (%) and the x-axis represents mass-to-charge ratio (m/z).

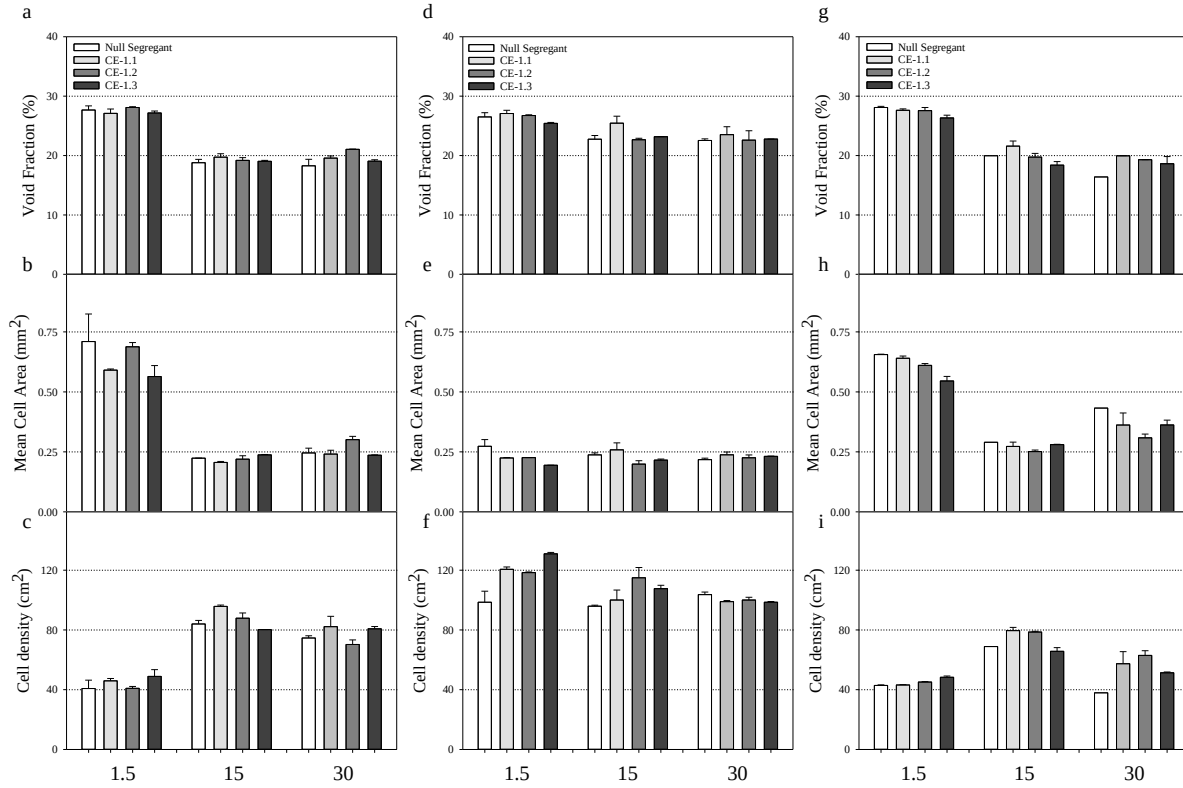


Figure S7.4. White bread properties following different fermentation times of field grown NS and CE-1 wheat lines in 2017. Void fraction (%), mean cell area (mm^2), and cell density (cm^2) in white bread of NS (white) and three CE-1 sibling lines (CE-1.1 light grey, CE-1.2 grey, and CE-1.3 dark grey) fermented for 1.5, 15 or 30 hours from **a-c)** Glenthorne, **d-f)** Katanning and **g-i)** Merredin in 2017. Bars represent the mean $\pm$ SEM of two replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.

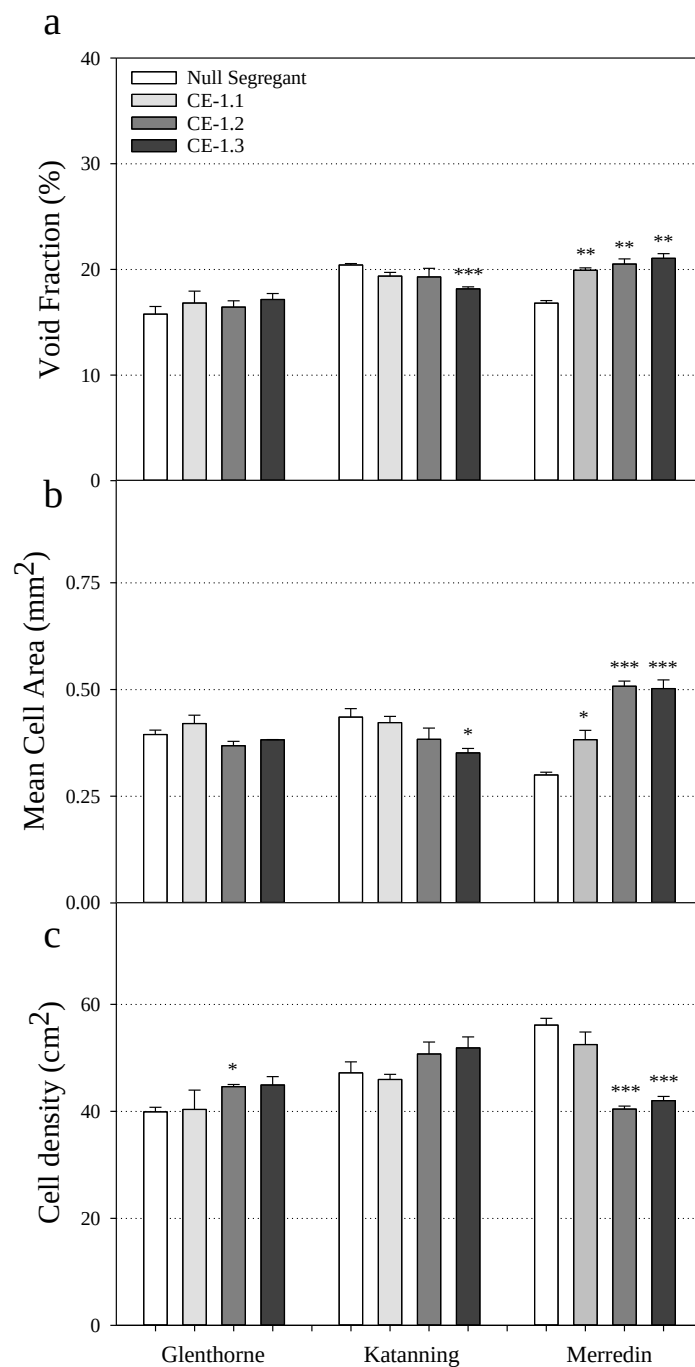


Figure S7.5. White bread properties of field grown NS and CE-1 wheat lines in 2016. **a)** void fraction (%), **b)** mean cell area (mm²), and **c)** cell density (cm²) in white bread of NS (white) and three CE-1 sibling lines (CE-1.1 light grey, CE-1.2 grey, and CE-1.3 dark grey) at Glenthorne, Katanning and Merredin in 2016. Bars represent the mean $\pm$ SEM of three replicates. Asterisks denote significant differences for $p < 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) as determined by Student's t-test.



Figure S7.6. White bread samples and cross sections of field grown NS and CE-1 wheat lines at Merredin in 2016. Scale bar = 1 cm.

8. Chapter 8 – Overall conclusions and future directions

Over two billion people worldwide suffer from iron (Fe) and zinc (Zn) deficiencies, increasing maternal and child mortality and causing a range of health problems including anemia, suppressed immune function and impaired cognitive development (Kassebaum et al. 2014; Lopez et al. 2016). The prevalence of human Fe and Zn deficiencies in countries of the MENAP (Middle East, North Africa, Afghanistan and Pakistan) region are in part due to high consumption of wheat (*Triticum aestivum* L.) and the low concentration and bioavailability of micronutrients within wheat-based diets (Beal et al. 2017). Biofortification efforts to improve micronutrient density and/or bioavailability in staple foods such as wheat were therefore considered the fifth most cost-effective investment that humanity can make towards solving the world's biggest problems in the 2008 Copenhagen Consensus (Meenakshi 2009). The Fe biofortification target levels for wheat wholemeal flour were first established by HarvestPlus (<https://www.harvestplus.org>) and assume both a baseline micronutrient concentration and percentage bioavailability (i.e. 5%) (Bouis et al. 2011). Table 8.1 provides these assumed Fe concentration and bioavailability levels within wholemeal and white flour alongside three hypothetical scenarios of improving Fe concentration and/or Fe bioavailability within a majority wheat-based diet. Improving Fe bioavailability can have a greater impact than improving Fe concentration on the percentage of daily Fe delivered, and small improvements to white flour Fe concentrations can be more effective than improving wholemeal flour Fe concentrations (Table 8.1). To date, most Fe biofortification programs in all crops have focused on improving Fe concentration alone.

Table 8.1. Wheat Fe biofortification levels. Column one shows baseline and target scenarios for Fe biofortification of wheat wholemeal and white flour. Columns two and three show the Fe concentration ($\mu\text{g/g}$) and assumed Fe bioavailability (%), respectively. Columns four and five show daily Fe intake (μg) and the percentage of daily Fe delivered assuming wheat consumption of 300 g/day and a daily Fe requirement of 1,460 $\mu\text{g/day}$, respectively. Current scenarios (light grey) show baseline Fe concentration and assumed Fe bioavailability in wholemeal and white flour. Target scenarios (dark grey) depict the effect of higher Fe concentration and/or bioavailability on daily Fe intake and requirement. Adapted from (Bouis et al. 2011).

	Scenario	Iron concentration ($\mu\text{g} / \text{g}$)	Iron bioavailability (%)	Iron intake ($\mu\text{g} / \text{day}$)	Iron delivered (% / day)
Wholemeal flour	Baseline	30	5	405	28
	Target 1	30	7	810	39
	Target 2	52	5	702	48
	Target 3	52	7	1404	67
White flour	Baseline	15	10	405	28
	Target 1	15	20	810	55
	Target 2	20	10	594	37
	Target 3	20	20	1188	74

This PhD project aimed to characterize Fe and Zn biofortified bread wheat developed through constitutive expression of the rice nicotianamine synthase 2 (*OsNAS2*) gene (CE-1) and increased biosynthesis of two plant metal chelators – nicotianamine (NA) and 2'-deoxymugineic acid (DMA) – with a particular focus on wholemeal and white flour micronutrient concentration and/or bioavailability. The *in vitro* study performed in Chapter 3 aimed to first characterize NA and DMA as enhancers of Fe bioavailability, by comparing NA and DMA to two known enhancers of Fe bioavailability: epicatechin (Epi) and ascorbic acid (AsA). The results revealed that across their molar range in plant foods, DMA was stronger than Epi, and NA was stronger than DMA, Epi and AsA at enhancing Fe bioavailability. Together these results suggest that increased concentrations of NA and/or DMA in wheat would not only facilitate increased transport of Fe and Zn to the developing grain, but that within the grain these increased concentrations of NA and/or DMA would improve Fe bioavailability.

Synchrotron analyses conducted in Chapter 4 revealed greater accumulation of Fe in CE-1 grain aleurone and endosperm tissues relative to null segregant (NS), indicating that upregulated NA/DMA biosynthesis results in greater transportation of Fe to CE-1 grain and additional loading of Fe into CE-1 endosperm. As NA/DMA are the major chelators of Fe in white wheat flour (Eagling, Neal, et

al. 2014), it is likely that NA and/or DMA facilitate the additional loading of Fe into CE-1 endosperm and future research programs should build on these findings and use newly developed techniques such as electrospray ionization-mass spectrometry (ESI-MS) to determine the proportion of Fe bound to NA/DMA in CE-1 white flour (Tsednee et al. 2016). Determining the speciation of Fe in CE-1 white flour will further elucidate the role/s of NA/DMA in the chelating and loading of micronutrients into wheat endosperm. Despite increased Fe, NA and DMA concentrations in CE-1 wholemeal flour, no difference in *in vitro* Fe bioavailability was measured between CE-1 and NS wholemeal flour (Chapter 4), suggesting that milling grain into white flour is necessary to reveal the benefits of wheat Fe biofortification when using *in vitro* assays. Determining the nutritional benefits of biofortified wholemeal flour may instead require an *in vivo* feeding study, as long term (≥ 6 weeks) exposure to higher concentrations of dietary Fe, Zn, NA and DMA in a CE-1 wholemeal flour-based diet is likely to reduce the effect of bioavailability inhibitors (Knez et al. 2018). Multi-location, multi-year field evaluation of CE-1 revealed significantly higher Fe, Zn, NA and DMA concentrations in CE-1 wholemeal and white flour and significantly higher Fe bioavailability in CE-1 white flour relative to NS, and that within all genotypes white flour Fe bioavailability was positively correlated with white flour NA concentrations (Chapter 5). The same study revealed that agronomic performance was negatively correlated with grain Fe and Zn concentrations and not with grain NA and DMA concentrations, suggesting that breeding for increased white flour NA and/or DMA concentrations either conventionally or through modern biotechnology could improve white flour Fe bioavailability without compromising grain yield.

The industrial-scale milling (>30 kg per genotype) of CE-1 grain performed in Chapter 5 produced eight grain fractions: three break flours, three reduction flours, pollard, and bran, that contained significantly higher Fe, Zn, NA and DMA concentrations relative to NS grain fractions. To determine the *in vivo* bioavailability of Fe and Zn in CE-1 and NS grain, a study in Chapter 6 combined all break and reduction flours to form either CE-1 or NS wheat-based diets and a 6-week long feeding study was conducted using the domestic chicken (*Gallus gallus*) animal model. The study revealed that host Fe status and gastrointestinal health was improved in chickens that received the CE-1 diet relative to NS diet and, given the small difference in Fe and Zn concentrations between the diets, these results were most likely due to differences in consumption of dietary NA and/or DMA. These results were supported by a simultaneous study that examined the effect of short-term exposure (intraamniotic administration) of NA-chelated Fe and found strikingly similar effects on Fe status, intestinal functionality and microbial composition between chickens that had received short- or long-term treatments. We therefore suggest that NA should be classified as an important phytonutrient in plant foods (Martin and Li 2017), and that future Fe biofortification programs in all crops should consider

the proportion of Fe that is chelated by compounds that enhance Fe bioavailability such as NA and/or DMA.

Given that wheat is processed into a range of food products prior to consumption, a study was performed in Chapter 7 to examine the effect of constitutive *OsNAS2* expression on the processing properties of wheat grain including white flour protein composition and concentration, dough rheology and breadmaking. The results from this study revealed CE-1 white flour contained higher prolamin concentrations (up to 1.2-fold) and altered rheology (particularly white flour water absorption), although these results were largely preliminary and confined to one field trial (Glenthorne) and one field season. More comprehensive rheological and breadmaking analyses of CE-1 and NS wheat over multiple-locations are therefore required to confirm these effects on CE-1 grain processing. Coupled to these additional analyses, sensory evaluation of CE-1 wheat food products will eventually be required to confirm consumer acceptance (Bouis et al. 2019). Within Chapter 7 we determined that CE-1 white bread has higher Fe bioavailability relative to NS, suggesting that CE-1 wheat food products would improve Fe status in humans and more comprehensive white bread Fe bioavailability analyses (possibly through an animal feeding trial or human efficacy study) are now warranted.

To complement and extend on the findings from Chapters 3 – 6, this discussion Chapter presents results from a final experiment in which a selection panel of fourteen elite wheat cultivars (cvs.) from the Australian Grains Genebank (Horsham, VIC, Australia) were grown under glasshouse conditions until maturity at the University of Melbourne. The concentrations of NA, DMA, Fe and Zn in grain samples from each cultivar were measured using liquid chromatography–mass spectrometry (LC-MS) and inductively coupled plasma-optical emission spectroscopy (ICP-OES) techniques (Chapter 2). Across the selection panel, grain NA concentrations ($\mu\text{mol} / \text{g}$) varied 1.3-fold (Figure 8.1a) and were not correlated with grain DMA concentrations ($\mu\text{mol} / \text{g}$) (Figure 8.1b). These results provide some preliminary insight into natural variation for wheat grain NA and DMA concentrations and suggest that it may be possible to breed for modest increases in grain NA and/or DMA concentrations through conventional wheat breeding programs. By contrast, a 2.7-fold difference in grain NA concentrations ($\mu\text{mol} / \text{g}$) was detected between a CE-1 sibling line (CE-1.1) and corresponding NS under the same glasshouse conditions (Figure 8.2), suggesting that plant biotechnology programs (i.e. constitutive expression of *NAS* genes) are more effective than conventional breeding programs at increasing wheat grain NA concentrations.

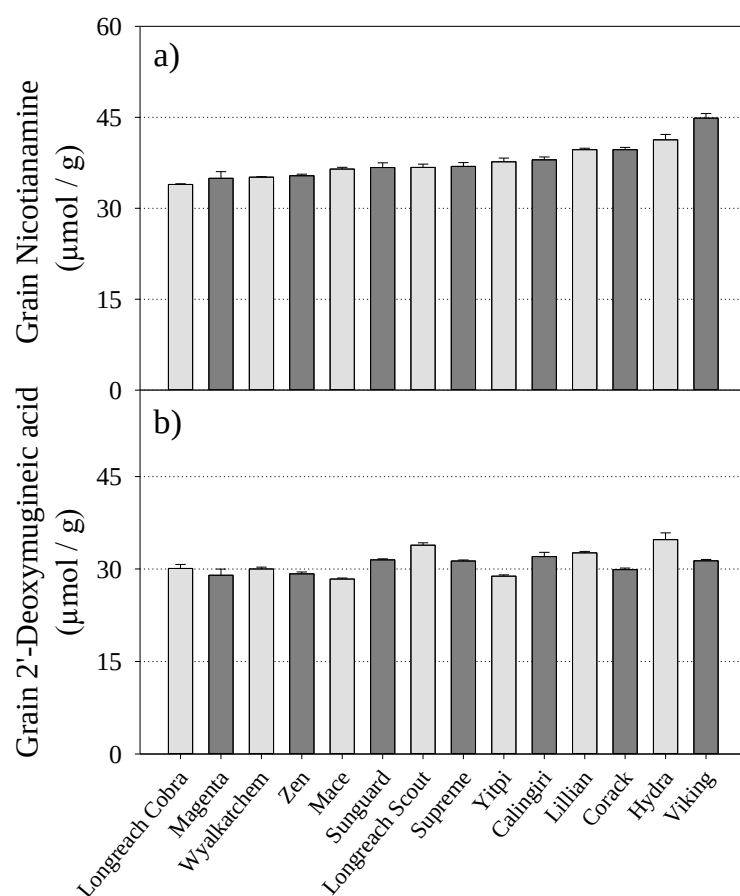


Figure 8.1. Grain **a)** NA and **b)** DMA concentration ($\mu\text{mol} / \text{g}$) in glasshouse-grown elite wheat cvs. at Melbourne University in 2017. Bars represent the mean $\pm$ SEM of five biological replicates. Grain NA and DMA concentrations were determined using liquid chromatography–mass spectrometry (LC-MS) as described in Chapters 2, 4, 5 and 6.

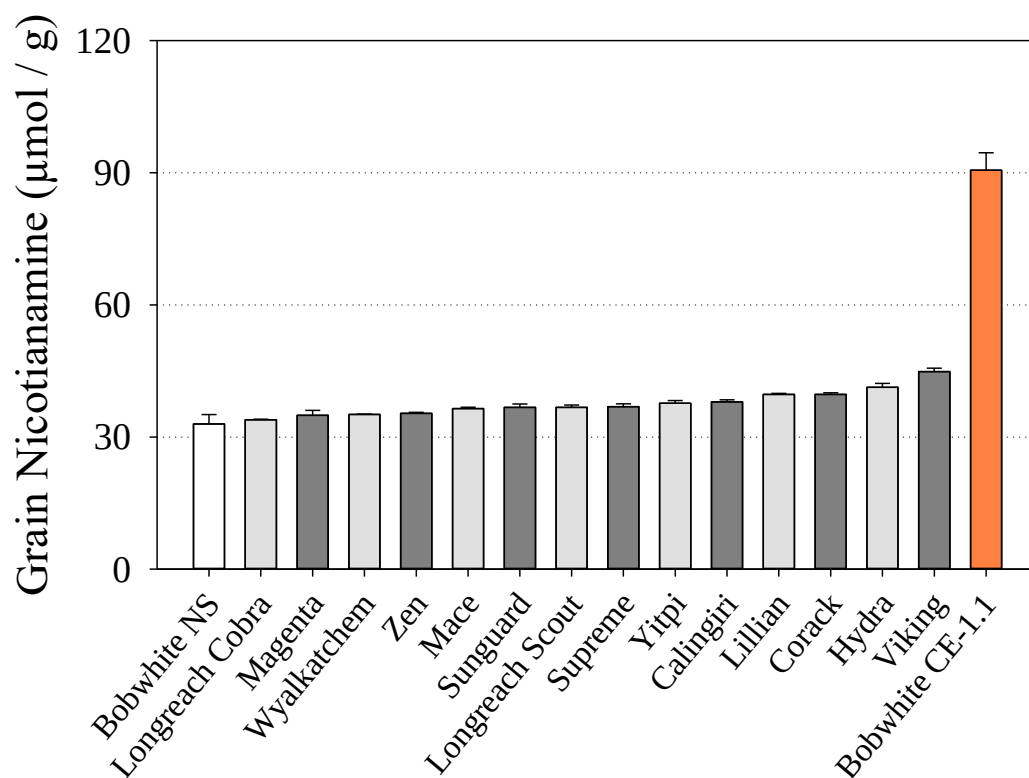


Figure 8.2. Grain NA concentration ($\mu\text{mol} / \text{g}$) in NS (white), CE-1.1 (orange) and glasshouse-grown elite wheat cvs. (grey) at Melbourne University in 2017. Bars represent the mean $\pm$ SEM of five biological replicates.

To determine the relationship between grain nutritional composition and agronomic performance, we correlated grain Fe, Zn, NA and DMA concentrations with grain yield in the elite wheat cv. selection panel. Across all biological replicates, grain weight ($\text{g} / \text{plant}^{-1}$) was negatively correlated ($p < 0.001$) with grain Fe and Zn concentrations ($\mu\text{g} / \text{g}$) (Figure 8.3a) and positively correlated ($p < 0.001$) with grain NA concentrations ($\mu\text{mol} / \text{g}$) (Figure 8.3b). The results from this experiment reinforce the findings from Chapters 3 – 6 which show that increased NA and/or DMA concentrations in wheat grain can improve Fe bioavailability without negatively affecting agronomic performance. As our selection panel included a relatively small number of cvs. (fourteen), future research programs should investigate grain NA/DMA concentrations within larger wheat diversity panels (i.e. hundreds of cvs.) and further explore the potential of improving grain NA/DMA concentrations through conventional breeding.

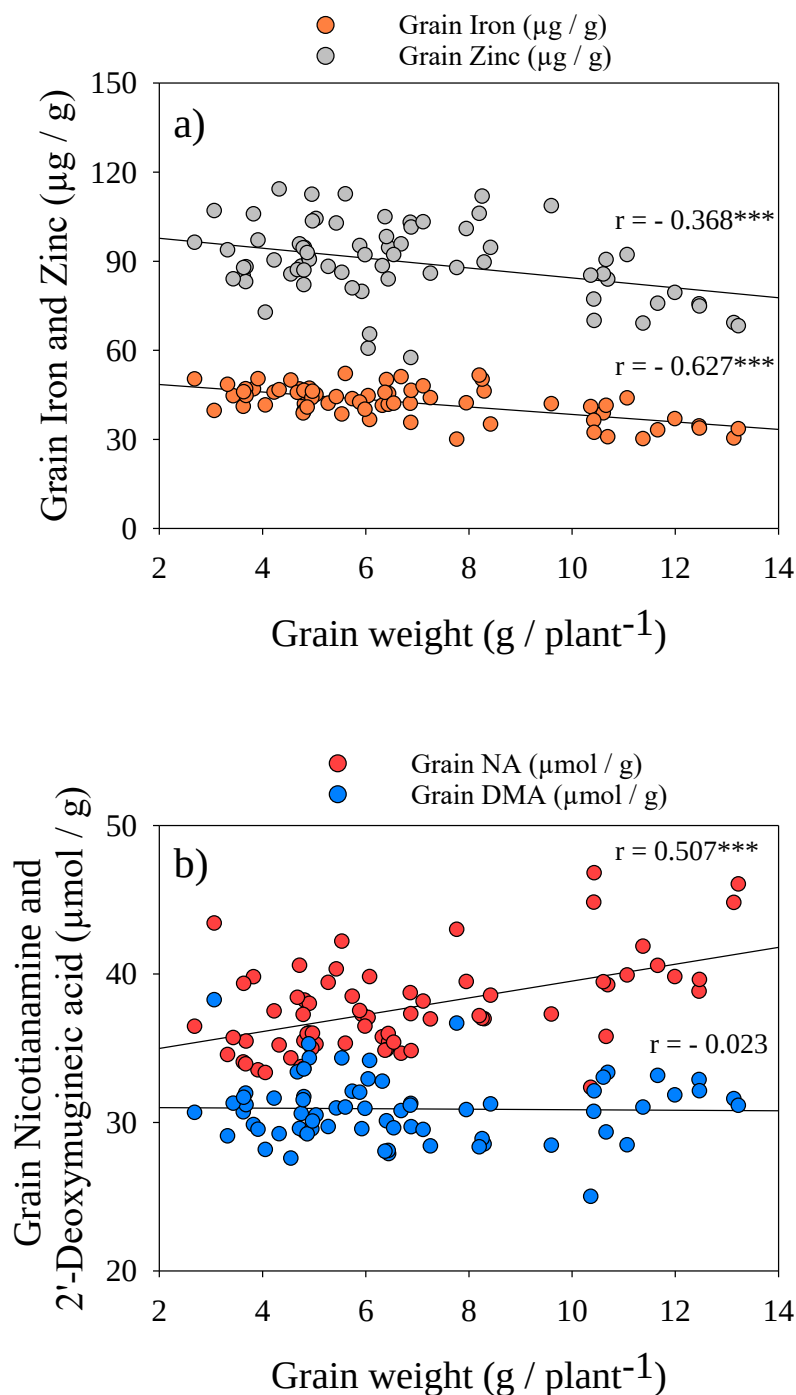


Figure 8.3. Correlation of elite wheat cv. grain weight (g / plant⁻¹) with **a)** grain Fe and Zn concentrations (µg / g) and **b)** grain NA and DMA concentrations (µmol / g). Each point represents grain weight with either the concentration of grain Fe (orange), Zn (grey), NA (red), or DMA (blue) of an individual plant. Pairwise Pearson correlation coefficients (r) are shown between variables with asterisks denoting significant correlations for $p < 0.05$ (***), $p \leq 0.01$ (**), $p \leq 0.001$ (***). Grain Fe and Zn concentrations were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES) as described in Chapters 2, 4, 5, and 6.

Future steps in our laboratory's wheat biofortification program can be guided by lessons learned from more commercially advanced GM biofortified crops, such as an Fe biofortified rice developed at the International Rice Research Institute (Philippines) involving a stacked 35S::OsNAS2 and endosperm-specific soybean *Fer* cassette (Trijatmiko et al. 2016). Several events of this Fe biofortified rice are currently undergoing a range of genetic screens and food safety assessments to allow deregulation and commercialization of the rice in Bangladesh (Ludwig and Slamet-Loedin 2019). Similar genetic screening and safety assessment would need to be performed on CE-1 wheat prior to deployment and eventual adoption in target countries. Major wheat producing countries of the MENAP (Middle East, North Africa, Afghanistan and Pakistan) region would be well suited for commercialization of CE-1 wheat in the future (Chapter 1). Pakistan has a population of 204 million people with an estimated 51% of preschool aged children and 33% of women suffering from anemia (de Benoist et al. 2008). Egypt has a population of 101 million people with an estimated 30% of preschool aged children and 31% of women suffering from anemia. In both Pakistan and Egypt, wheat is the major staple food and consumed as a variety of food products (e.g. chapati, aish baladi, etc.) to provide over a third of daily calories to humans (309 and 398 g wheat consumed / capita / day in Pakistan and Egypt, respectively). Commercializing CE-1 wheat could therefore improve human nutrition within these countries and would first involve backcrossing of our lead CE-1 lines to locally adapted cvs. to increase grain nutrition without negatively affecting yield. As part of the commercialization effort, a long-term human efficacy study would comprehensively determine whether a CE-1 wheat-based diet improves human micronutrient status and should occur in collaboration with local Pakistani and/or Egyptian populations. Such commercialization efforts would be most effective if they occur by mid-century, when atmospheric CO₂ concentrations are estimated to reach 550 ppm and lead to a 5-10% decrease in wheat grain Fe and Zn concentrations (Myers et al. 2014; Smith, Golden, and Myers 2017). Similar decreases in nutrition are forecast for other C₃ crops and are likely to exacerbate micronutrient deficiencies in MENAP and South-East Asia (Beach et al. 2019; Smith and Myers 2018). It is therefore urgent that biofortified crops such as CE-1 wheat are fully embraced as part of the global solution to combat human micronutrient deficiencies over the coming decades.

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Appendices

Appendix 2.1. Milling data from Glenthorne (G), Katanning (K) and Merredin (M) in 2015. Columns provide the genotype, replicate number (rep), grain weight (g), time of day, bran weight (g), grind time (seconds), mill temperature (°C) and flour extraction rate (%). Control samples were cv. Mace grain from Merredin in 2016.

Site	Genotype	Rep	Grain (g)	Time	Bran (g)	Grinding time (sec)	Temp (°C)	Yield (%)
	Control	1	257.43	2:38	58.13	462	24.5	77.4
G	NS	1	51.72	2:55	14.25	78	25.2	72.4
G	NS	2	51.86	3:02	14.5	68	25.5	72
G	NS	3	51.9	3:07	14.48	66	25	72.1
G	CE-1.1	1	52.01	3:14	15.22	66	25.1	70.7
G	CE-1.1	2	51.95	3:23	15.25	64	25.4	70.6
G	CE-1.1	3	51.93	3:28	16.01	70	24.6	69.2
G	CE-1.2	1	51.99	3:34	14.96	70	24.8	71.2
G	CE-1.2	2	51.96	3:39	15.25	73	25.5	70.7
G	CE-1.2	3	51.9	3:43	14.54	72	24.7	72
G	CE-1.3	1	52.02	3:48	15.79	65	24.9	69.6
G	CE-1.3	2	51.94	3:52	14.77	68	24.8	71.6
G	CE-1.3	3	51.98	3:56	14.97	68	24.8	71.2
G	Mace	1	51.91	4:00	13	72	24.6	75
G	Mace	2	51.93	4:04	14.4	74	24.7	72.3
G	Mace	3	51.99	4:08	14.11	77	25.3	72.9
	Control	4	257.5	4:12	60.81	463	25.4	76.4
K	NS	1	50.94	4:28	14.17	81	24.6	72.2
K	NS	2	50.78	4:32	14.4	72	24.5	71.6
K	NS	3	50.89	4:36	14.52	73	24.4	71.5
K	CE-1.1	1	50.91	4:40	14.2	75	24	72.1
K	CE-1.1	2	51	4:44	15.03	68	24.3	70.5
K	CE-1.1	3	49.24	4:48	13.71	73	24.6	72.2
K	CE-1.2	1	50.74	4:52	13.56	82	24.5	73.3
K	CE-1.2	2	50.77	4:56	15.52	73	25.3	69.4
K	CE-1.2	3	50.78	5:00	13.95	78	24.3	72.5
K	CE-1.3	1	50.86	5:04	14.05	77	24.3	72.4
K	CE-1.3	2	51	5:08	15.63	84	24.2	69.4
K	CE-1.3	3	50.64	5:12	14.39	71	24.7	71.6
K	Mace	1	50.92	5:16	12.6	90	24.7	75.3
K	Mace	2	50.85	5:20	11.48	81	24.9	77.4
K	Mace	3	50.87	5:24	12.28	85	24.8	75.9
	Control	4	257.3	5:28	57.1	498	25.1	77.8
M	NS	1	51.79	5:45	16.48	69	24.4	68.2
M	NS	2	51.74	5:49	14.46	73	24.3	72.1
M	NS	3	51.77	5:53	14.7	72	24.1	71.6
M	CE-1.1	1	51.68	5:56	14.77	70	24.3	71.4
M	CE-1.1	2	51.72	6:00	14.63	73	24.3	71.7
M	CE-1.1	3	51.72	6:04	14.49	73	24	72
M	CE-1.2	1	51.71	6:07	15.1	73	24.3	70.8
M	CE-1.2	2	51.75	6:11	14.88	73	24.2	71.2
M	CE-1.2	3	51.66	6:14	14.27	74	24.4	72.4
M	CE-1.3	1	51.8	6:18	14.98	75	24.2	71.1
M	CE-1.3	2	51.82	6:22	13.81	71	24.4	73.4
M	CE-1.3	3	51.82	6:26	14.44	74	24	72.1
M	Mace	1	51.81	6:30	12.35	84	24.5	76.2
M	Mace	2	51.8	6:34	12.25	92	24.1	76.4
M	Mace	3	51.81	6:38	12.57	82	24.5	75.7
	Control	4	257.34	6:42	55.96	500	24.7	78.3

Appendix 2.2. Milling data from Glenthorne (G), Katanning (K) and Merredin (M) in 2016. Control and ‘warm up’ samples were cv. Mace grain from plants grown at Merredin in 2016 that were conditioned and unconditioned, respectively.

Site	Genotype	Rep	Grain (g)	Time	Bran (g)	Grinding time (sec)	Temp (°C)	Yield (%)
	Control	1	209.48	8:40	37.03	260	24.7	82.3
	Control	2	205.89	8:50	36.32	262	25.3	82.4
G	NS	1	153.72	9:05	42.38	160	25.8	72.4
G	NS	2	153.9	9:10	42.89	153	26.1	72.1
G	NS	3	153.58	9:15	43.46	169	25.4	71.7
G	NS	4	153.85	9:24	42.8	150	26	72.2
G	NS	5	153.72	9:29	42.6	149	25.5	72.3
G	CE-1.1	1	153.31	11:48	43.55	133	25.7	71.6
G	CE-1.1	2	154.17	11:56	45.58	147	25.7	70.4
G	CE-1.1	3	153.53	12:10	44.59	153	25.7	71
G	CE-1.1	4	153.28	12:16	46.21	184	26	69.9
G	CE-1.1	5	154.3	12:24	45.47	134	26.5	70.5
	Control	3	208.94	9:31	58.25	290	25	72.1
G	CE-1.2	1	152.9	11:41	59.74	85	25.7	60.9
G	CE-1.2	2	153.74	11:53	57.7	199	26.8	62.5
G	CE-1.2	3	153.68	12:02	48.82	169	25.9	68.2
G	CE-1.2	4	153.72	12:11	48.17	171	25.7	68.7
G	CE-1.2	5	153.64	12:17	48.67	181	25.4	68.3
G	CE-1.3	1	153.85	2:44	55.59	195	26.2	63.9
G	CE-1.3	2	153.68	2:51	55.02	195	25.7	64.2
G	CE-1.3	3	153.94	2:58	55.16	211	25.6	64.2
G	CE-1.3	4	153.84	3:05	53.91	199	26	65
G	CE-1.3	5	153.88	3:11	54.73	197	25.3	64.4
G	Mace	1	153.55	5:39	46.1	210	24.6	70
G	Mace	2	153.84	5:49	48.41	184	23.9	68.5
G	Mace	3	153.49	5:56	45.57	202	24	70.3
G	Mace	4	153.75	6:05	46.07	191	24.8	70
	Control	4	210.16	8:11	52.75	216	25.5	74.9
	Warm up	1	510.97	8:41	142.66	587	26.1	72.1
	Control	5	155.76	9:08	40.66	281	24.8	73.9
	Control	6	155.79	9:16	40	271	25.6	74.3
K	NS	1	153.53	9:29	52.27	243	25.3	66
K	NS	2	153.59	9:39	53.71	227	24.7	65
K	NS	3	153.59	9:46	52.98	243	25.4	65.5
K	NS	4	153.63	9:55	53.63	239	25.2	65.1
K	NS	5	153.63	12:26	51.88	230	24.1	66.2
K	CE-1.1	1	153.61	10:08	53.33	233	25.2	65.3
K	CE-1.1	2	153.63	10:16	52.48	235	25.1	65.8
K	CE-1.1	3	153.65	10:25	51.1	224	25.1	66.7
K	CE-1.1	4	153.53	10:33	51.45	238	25	66.5
K	CE-1.1	5	153.61	10:43	50.99	240	25.3	66.8
K	CE-1.2	1	153.62	10:54	51.31	246	25	66.6
K	CE-1.2	2	153.62	11:03	53.12	243	24.9	65.4
K	CE-1.2	3	153.56	11:11	52.23	244	25.1	66
K	CE-1.2	4	153.61	11:20	52.66	244	24.8	65.7
K	CE-1.2	5	153.58	11:29	52.05	252	25.1	66.1
K	CE-1.3	1	153.56	11:42	53.51	238	24.8	65.2
K	CE-1.3	2	153.61	11:50	54.2	241	25	64.7
K	CE-1.3	3	153.61	11:57	53.68	239	25.2	65.1
K	CE-1.3	4	153.6	12:04	53.02	231	24.9	65.5
K	CE-1.3	5	153.61	12:13	52.83	242	24.8	65.6
K	Mace	1	153.66	12:38	44.05	250	24.1	71.3

K	Mace	2	153.67	12:46	46.05	254	25.3	70
K	Mace	3	153.64	12:54	44.67	254	24.9	70.9
K	Mace	4	153.63	1:04	44.11	271	24.7	71.3
	Control	7	155.7	1:22	39.46	254	24.7	74.7
	Warm up	1	547.26	1:18	122.01	707	26.2	77.7
	Control	8	155.28	1:41	38.97	294	26.9	74.9
	Control	9	155.29	1:52	39.35	281	28.2	74.7
	Control	10	155.26	2:22	41.3	290	26.2	73.4
M	NS	1	155.32	2:38	46.88	232	25.9	69.8
M	NS	2	155.3	2:54	48.52	230	26.5	68.8
M	NS	3	155.32	3:14	47.43	240	25.5	69.5
M	NS	4	155.33	3:27	48.21	238	26	69
M	NS	5	155.35	6:51	44.57	228	24.9	71.3
M	CE-1.1	1	155.32	3:43	52.94	240	25.6	65.9
M	CE-1.1	2	155.42	3:54	46.57	240	26.1	70
M	CE-1.1	3	155.32	4:09	44.46	239	25.6	71.4
M	CE-1.1	4	155.36	4:18	44.87	243	26.2	71.1
M	CE-1.1	5	155.25	4:34	45.65	243	25.6	70.6
M	CE-1.2	1	155.32	4:50	44.84	259	26	71.1
M	CE-1.2	2	155.32	5:15	44.31	246	25.4	71.5
M	CE-1.2	3	155.38	5:23	44.48	248	25.5	71.4
M	CE-1.2	4	155.26	5:32	44.23	259	25.5	71.5
M	CE-1.2	5	155.26	7:04	45.55	230	25.6	70.7
M	CE-1.3	1	155.36	5:48	43.45	247	26	72
M	CE-1.3	2	155.38	6:02	45.07	248	25.7	71
M	CE-1.3	3	155.39	6:12	45.07	241	25.8	71
M	CE-1.3	4	155.3	6:21	45.13	235	25.8	70.9
M	CE-1.3	5	155.27	6:31	44.91	236	26.2	71.1
M	Mace	1	155.3	7:19	40.08	276	25.9	74.2
M	Mace	2	155.35	7:28	40.82	272	25.7	73.7
M	Mace	3	155.19	7:41	41.16	256	25.3	73.5
M	Mace	4	155.21	7:49	41	257	25.5	73.6
	Control	11	155.18	8:07	39	305	26	74.9

Appendix 2.3. Milling data from Glenthorne (G), Katanning (K) and Merredin (M) in 2017. Control and ‘warm up’ samples were cv. Mace grain from plants grown at Merredin in 2016 that were conditioned and unconditioned, respectively.

Site	Genotype	Rep	Grain (g)	Time	Bran (g)	Grinding time (sec)	Temp (°C)	Yield (%)
	Warm up	1	513.73	7:44	104.7	573	23	79.6
	Warm up	2	296.06	7:58	61.9	325	24.2	79.1
	Control	1	155.28	8:08	39.19	277	24.6	74.8
	Control	2	155.37	8:16	39.34	261	25.2	74.7
G	NS	1	205.9	8:27	52.38	277	24.6	74.6
G	NS	2	205.87	8:35	57.22	267	24.4	72.2
G	NS	3	205.91	8:43	54.7	235	24.6	73.4
G	NS	4	205.83	8:51	57.2	261	24.6	72.2
G	NS	5	205.8	8:58	58.5	257	24.3	71.6
G	CE-1.1	1	205.9	9:08	57.38	261	24	72.1
G	CE-1.1	2	205.91	9:16	56.09	256	24.2	72.8
G	CE-1.1	3	205.86	9:23	58.1	261	24.7	71.8
G	CE-1.1	4	205.69	9:30	59.54	243	24.7	71.1
G	CE-1.1	5	205.79	9:37	57.15	251	24.7	72.2
G	CE-1.2	1	205.84	9:49	54.18	270	24.6	73.7
G	CE-1.2	2	205.75	9:57	56.87	276	24.6	72.4
G	CE-1.2	3	205.87	10:04	57.45	275	24.2	72.1
G	CE-1.2	4	205.78	10:11	56.83	271	24.2	72.4
G	CE-1.2	5	205.78	10:18	57.01	270	24.3	72.3
G	CE-1.3	1	205.73	10:29	53.42	274	24.2	74.0
G	CE-1.3	2	205.71	10:37	57.23	254	24.3	72.2
G	CE-1.3	3	205.77	10:43	56.57	271	24.2	72.5
G	CE-1.3	4	205.74	10:51	57.8	260	24.3	71.9
G	CE-1.3	5	205.72	10:58	54.85	275	24.4	73.3
	Control	3	155.28	11:06	38.88	301	24.6	75.0
	Control	4	155.26	11:22	38.72	267	24.5	75.1
K	NS	1	200.7	11:32	52.59	261	23	73.8
K	NS	2	200.77	11:40	50.58	274	23.6	74.8
K	NS	3	200.75	11:47	51.55	259	23.9	74.3
K	NS	4	200.73	11:54	49.2	290	24.2	75.5
K	NS	5	200.75	12:02	49.26	275	24.5	75.5
K	CE-1.1	1	200.71	12:13	50.42	275	23.9	74.9
K	CE-1.1	2	200.73	12:21	49.13	268	23.8	75.5
K	CE-1.1	3	200.71	12:28	50.45	262	24	74.9
K	CE-1.1	4	200.72	12:35	51.23	274	23.9	74.5
K	CE-1.1	5	200.74	12:43	48.4	264	23.9	75.9
K	CE-1.2	1	200.76	12:53	47.35	262	23.3	76.4
K	CE-1.2	2	200.76	1:00	48.18	256	23.7	76.0
K	CE-1.2	3	200.79	1:08	50.07	250	24.4	75.1
K	CE-1.2	4	200.82	1:14	47.87	272	24.5	76.2
K	CE-1.2	5	200.78	1:22	45.54	291	24.3	77.3
K	CE-1.3	1	200.8	1:32	48.32	261	23.7	75.9
K	CE-1.3	2	200.72	1:40	48.21	253	24.1	76.0
K	CE-1.3	3	200.78	1:47	49.38	261	24.1	75.4
K	CE-1.3	4	200.72	1:55	47.94	275	24.3	76.1
K	CE-1.3	5	200.77	2:02	47.88	277	24	76.2
	Control	5	155.2	2:10	32.9	264	24.3	78.8
	Warm up	3	500.37	7:35	92.08	534	22.6	81.6
	Warm up	4	314.81	7:49	57.45	480	24.3	81.8
	Control	6	155.3	8:00	35.18	276	24.3	77.3
	Control	7	155.23	8:08	34.84	263	24.7	77.6
M	NS	1	207	8:18	47.83	279	24.5	76.9

M	NS	2	207.26	8:26	47.67	284	24.6	77.0
M	NS	3	206.93	8:34	47.43	260	25.2	77.1
M	NS	4	207.08	8:41	46.16	273	25.4	77.7
M	NS	5	207.04	8:48	47.89	269	25.4	76.9
M	CE-1.1	1	207.17	8:58	51.36	281	24.8	75.2
M	CE-1.1	2	207.03	9:06	49.84	283	24.8	75.9
M	CE-1.1	3	207.08	9:13	49.66	271	24.8	76.0
M	CE-1.1	4	207.05	9:20	48.46	261	25.4	76.6
M	CE-1.1	5	207	9:27	49.71	264	25.4	76.0
M	CE-1.2	1	207.11	9:38	49.03	279	25.2	76.3
M	CE-1.2	2	206.99	9:45	49.17	284	25	76.2
M	CE-1.2	3	207.08	9:52	47.80	286	24.9	76.9
M	CE-1.2	4	207.01	10:00	48.39	272	24.8	76.6
M	CE-1.2	5	207.04	10:07	48.14	281	24.8	76.7
M	CE-1.3	1	207.06	10:17	48.48	297	24.5	76.6
M	CE-1.3	2	207.11	10:25	48.54	277	24.2	76.6
M	CE-1.3	3	207	10:32	47.54	286	24.2	77.0
M	CE-1.3	4	207.08	10:40	47.57	273	24.7	77.0
M	CE-1.3	5	207.17	10:47	49.46	271	24.5	76.1
	Control	8	155.35	10:55	34.55	268	24.3	77.8
	Control	9	155.34	11:28	36.2	287	24.1	76.7
G	Mace	1	205.95	11:42	47.28	275	24.5	77.0
G	Mace	2	205.9	11:49	46.87	290	24.9	77.2
G	Mace	3	205.91	11:57	45.79	281	25.2	77.8
G	Mace	4	205.91	12:04	44.02	274	24.6	78.6
G	Mace	5	206	12:12	46.56	299	25	77.4
K	Mace	1	200.73	12:21	46.3	269	24.5	76.9
K	Mace	2	200.67	12:29	46.31	284	24.4	76.9
K	Mace	3	200.73	12:37	48.5	281	24.5	75.8
K	Mace	4	200.73	12:44	45.78	285	24.6	77.2
K	Mace	5	200.72	12:52	43.67	295	24.2	78.2
M	Mace	1	207.07	1:02	43.35	342	24.3	79.1
M	Mace	2	207	1:11	43.83	289	24.5	78.8
M	Mace	3	207.16	1:18	44.44	305	24.2	78.5
M	Mace	4	207.06	1:26	43.85	314	24.1	78.8
M	Mace	5	207.18	1:34	46.27	298	24.5	77.7
	Control	10	155.3	1:41	35.61	256	24.7	77.1

Appendix 2.4. Milling data of a NS grain sample and a blended CE-1.2 and CE-1.3 grain sample Merredin in 2017.

Genotype	Null Segregant	CE-1.2 / CE-1.3
Initial grain weight (g)	32313.0	32345.9
Moisture content (%)	9.65	9.59
Final grain weight (g)	33987.9	34094.5
Break 1 (g)	1831.3	1855.0
Break 2 (g)	1740.6	1861.2
Break 3 (g)	779.8	739.7
Reduction 1 (g)	11676.4	12286.5
Reduction 2 (g)	5408.2	5479.5
Reduction 3 (g)	3032.0	2665.9
Pollard (g)	8287.8	8010.1
Bran (g)	521.9	375.2
Total product (g)	33278.0	33273.1
Total flour (g)	24468.3	24887.8
Flour extraction rate (%)	71.99%	73.00%
Recovery Rate (%)	97.91%	97.59%

Appendix 7.1. MATLAB code sample for analysing bread void fraction, mean cell area and cell density.

```
clear all;
close all;
clc;

%Names=importdata('Names.mat');

A = dir("\Users\jbeasley\Desktop\PhD\Research\Breadmaking\2017\MATLAB Images');
Names = {A.name};
Names = Names(4:end);
Names = transpose(Names);

for ii = 1:numel(Names)
    file_name_cell = Names(ii);
    file_name=cell2mat(file_name_cell);
    I=imread(file_name);
    Igray=rgb2gray(I); %convert to grayscale

    BWinv=~imbinarize(Igray,'adaptive', 'Sensitivity',0.68); %segments grayscale image into binary image using
    adaptive otsu thresholding method
    %the ~ inverts binary image so that crumb cells appear as white ie.1s

    se=strel('cube',3);
    BW3=imopen(BWinv,se); %performs image opening operation with a 3x3 cube - erosions followed by dilation
    to remove small pixels and separate cells cleanly
    BW=imfill(BW3,'holes'); %fills any holes in image

    connectcrumb=bwconncomp(BW);
    measurement=regionprops(connectcrumb,'all'); %measuring properties of binary image

    crumbarea=[measurement.Area].'; %crumb area in pixels
    crumbarea2=crumbarea(crumbarea>4); %removing cells less than 4 pixels from cell areas
    crumbarea_cm=crumbarea2/45736; %crumb area in cm2
    crumbarea_mm=crumbarea2/457.36; %crumb area in mm2

    void_fraction=(sum(crumbarea2)/numel(BW))*100; %void fraction as percentage of pixels
    mean_area_mm2=mean(crumbarea_mm); %mean cell area in mm2
    cell_density_cm2=(numel(crumbarea2))/(numel(BW)/45736); %number of cells per unit area in cm2
    range=max(crumbarea_mm)-min(crumbarea_mm);

    small_cell_density_cm2=nnz(crumbarea_mm<=4)/(numel(BW)/45736); %number of cells <1mm2 per unit
    area in cm2
    large_cell_density_cm2=nnz(crumbarea_mm>4)/(numel(BW)/45736);

    ecc=[measurement.Eccentricity].';%eccentricity indicates shape (O=circular, 1=straght line)
    mean_ecc=mean(ecc); %mean eccentricity

    results(ii,:) = [void_fraction    mean_area_mm2    cell_density_cm2    small_cell_density_cm2
    large_cell_density_cm2 range mean_ecc];
end

xlswrite('ResultsRep1.xlsx',results)
```

Article

Investigation of Nicotianamine and 2' Deoxymugineic Acid as Enhancers of Iron Bioavailability in Caco-2 Cells

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Received: 1 June 2019; Accepted: 28 June 2019; Published: 30 June 2019



Abstract: Nicotianamine (NA) is a low-molecular weight metal chelator in plants with high affinity for ferrous iron (Fe^{2+}) and other divalent metal cations. In graminaceous plant species, NA serves as the biosynthetic precursor to 2' deoxymugineic acid (DMA), a root-secreted mugineic acid family phytosiderophore that chelates ferric iron (Fe^{3+}) in the rhizosphere for subsequent uptake by the plant. Previous studies have flagged NA and/or DMA as enhancers of Fe bioavailability in cereal grain although the extent of this promotion has not been quantified. In this study, we utilized the Caco-2 cell system to compare NA and DMA to two known enhancers of Fe bioavailability—epicatechin (Epi) and ascorbic acid (AsA)—and found that both NA and DMA are stronger enhancers of Fe bioavailability than Epi, and NA is a stronger enhancer of Fe bioavailability than AsA. Furthermore, NA reversed Fe uptake inhibition by Myricetin (Myr) more than Epi, highlighting NA as an important target for biofortification strategies aimed at improving Fe bioavailability in staple plant foods.

Keywords: biofortification; iron deficiency anemia; iron absorption; ferritin; ascorbic acid; epicatechin

1. Introduction

Iron (Fe) possesses unique redox properties that are critical to fundamental biological processes such as cellular respiration and photosynthesis [1]. Although abundant in soil, Fe is largely unavailable for plant uptake under aerobic or calcisol (high pH) conditions (representing ~30% of arable land), due to the formation of insoluble ferric (Fe^{3+}) ion precipitates [2]. As well as negatively impacting on plant growth, inadequate plant Fe uptake translates to human Fe deficiency, as plants provide a major gateway for Fe into human food systems [3]. Plants have evolved sophisticated mechanisms to absorb Fe from the rhizosphere through reduction and/or chelation of Fe^{3+} [2]. Non-graminaceous plants such as common bean (*Phaseolus vulgaris* L.) reduce soil Fe^{3+} ions to the more soluble ferrous (Fe^{2+}) form for uptake into plant roots [4]. By contrast, graminaceous plants such as bread wheat (*Triticum aestivum* L.) secrete mugineic acid phytosiderophores, the most common of which is 2' deoxymugineic acid (DMA), into soil to chelate Fe^{3+} for plant uptake [5]. Some plant species such as rice (*Oryza sativa* L.) utilize aspects of both strategies to maximize Fe uptake under a variety of soil and pH conditions [2].

Within the plant cell, Fe is complexed to chelating agents or is sequestered into plant vacuoles to avoid cellular damage caused by Fe^{2+} oxidation and reactive oxygen species (ROS) formation [3]. Low-molecular weight compounds like citrate, malate, nicotianamine (NA) and the oligopeptide transporter family protein (OPT3) are major chelators of phloem/xylem Fe within all higher plants while DMA is an additional chelator in graminaceous plants. Citrate, NA, DMA and OPT3 all function in the transport of Fe from source tissues (i.e., root, leaf) to sink tissues (i.e., leaf, seed) for Fe

storage and/or utilization [4]. Within the leaf, most Fe is bound in a phytoferritin complex within the chloroplast [6]. Leaf Fe is liberated from the phytoferritin complex during senescence and chelated by citrate, NA and/or DMA for transport to the developing seed [4]. Once in the seed of non-graminaceous plants, the proportion of Fe stored in embryonic, seed coat, and provascular tissues is heavily influenced by species, genotype and environment [7,8]. The Fe within embryonic tissue is primarily bound to phytoferritin and represents between 18% to 42% of total seed iron in soybeans (*Glycine max* L.) and peas (*Pisum sativum* L.), respectively [9]. The Fe within the seed coat of common bean ranges between 4% and 26% of total seed iron and is bound primarily to polyphenolic compounds, such as flavonoids and tannins [8,10,11]. The majority of seed Fe therefore accumulates in cotyledonary tissues and is likely bound to inositol hexakisphosphate (also known as phytate) within cell vacuoles, or to small metal chelators like NA in the cytoplasm [7,12]. Certain leguminous plants like soybean and chickpea (*Cicer arietinum* L.) accumulate seed NA to very high concentrations (up to a 1:2 molar ratio with Fe), suggesting that a large proportion of seed Fe is cytoplasmic in these species [13,14]. Graminaceous plant seeds (i.e., grain) store the majority of Fe (~80% of total grain Fe) as phytate complexes in vacuolar regions of the outer aleurone layer [3,15,16]. The remaining Fe within the sub-aleurone and endosperm regions (~20% of total grain Fe) is bound to phytate in intracellular phytin-globoids or chelated to NA and/or DMA (1:0.1 molar ratio with Fe) within the cytoplasm [17–20].

The absorption of dietary Fe in humans (bioavailability) depends on several factors apart from Fe concentration alone. The Fe within plant-based foods is mostly comprised of low-molecular weight (i.e., phytate, NA) and high-molecular weight (i.e., ferritin) compounds and is collectively referred to as non-heme Fe [6]. Non-heme Fe bioavailability is generally low (5–12%) and influenced by the concentration of inhibitors (phytate, polyphenols, calcium, etc.) and enhancers, like ascorbic acid (AsA), in the diet [21,22]. Phytate is the major inhibitor of Fe bioavailability in whole-grain foods, although certain polyphenolic compounds such as myricetin (Myr) and quercetin exhibit a greater inhibitory effect in bean-based diets [10,21,22]. Both phytate and Myr form high affinity complexes with Fe^{3+} that are poorly absorbed across the human intestinal surface [23–25]. Other polyphenolic flavanoids present in wheat embryonic and bean seed coat tissues are widely presumed to inhibit Fe bioavailability through pro-oxidation of Fe^{2+} and/or chelation of Fe^{3+} [21,26,27]. Enhancers of Fe bioavailability such as AsA (the strongest enhancer identified to date) are typically antioxidants that reduce Fe^{3+} and prevent polyphenols binding to newly formed Fe^{2+} ions that are highly bioavailable [22]. Some polyphenols such as epicatechin (Epi) are also thought to reduce Fe^{3+} to Fe^{2+} and can therefore act as potent Fe bioavailability enhancers [21]. Another mechanism of promoting Fe bioavailability is thought to be through direct chelation of Fe^{2+} for uptake in the human small intestine such as that proposed for glycosaminoglycans and proteoglycans [22,28,29]. Nicotianamine has been suggested to enhance Fe bioavailability in Fe biofortified polished rice grains and Fe biofortified white wheat flour, although the extent of this promotion is unclear [17,18,30–32]. Whether DMA, also enhances or inhibits Fe bioavailability is unknown and increased knowledge regarding NA and DMA promotion of Fe bioavailability is needed. Identification of enhancers and inhibitors of Fe bioavailability has traditionally relied on manipulation of dietary components in lengthy human or animal feeding trials [33]. By contrast, the Caco-2 cell bioassay allows rapid investigation of diverse dietary components and accurate estimation of Fe uptake by human intestinal epithelial cells [21,34–36].

Due in large part to high consumption of cereal-based diets that are low in bioavailable Fe, human Fe deficiency is the most common nutritional disorder worldwide and is particularly widespread in less-developed countries [37]. Severe Fe deficiency causes iron-deficiency anemia (IDA), a condition that impairs cognitive development and increases maternal and child mortality, affecting over 40% of pregnant women and preschool-age children worldwide [38–40]. Biofortification efforts aimed at increasing micronutrient intake from staple food consumption represent a key component of alleviating global human IDA, yet there is heavy bias towards increased micronutrient concentration with less regard for bioavailability [41,42]. Recent biofortification studies in rice and wheat have increased NA and/or DMA biosynthesis to enhance both Fe concentration and bioavailability [17,20,28,29,43,44].

Increasing NA/DMA biosynthesis in wheat results in higher Fe accumulation in grain endosperm and increased Fe bioavailability in white flour that is highly correlated with NA and DMA concentration [17]. Understanding the extent to which NA and/or DMA enhance Fe bioavailability is therefore critical to determining the effectiveness of these Fe biofortification programs. Here we utilize modifications of the Caco-2 cell bioassay to characterize NA and DMA as enhancers of *in vitro* Fe bioavailability through comparison to known enhancers (Epi and AsA) and in a competitive assay with the inhibitor Myr.

2. Materials and Methods

2.1. Chemicals

Epicatechin, Myr, dimethyl sulfoxide (DMSO), glucose, hydrocortisone, insulin, selenium, triiodothyronine, and epidermal growth factor were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nicotianamine and DMA were purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Sodium bicarbonate and piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES) were purchased from Fisher Scientific (Waltham, MA, USA). Iron standard (1000 µg/mL in 2% HCl) was from High-Purity Standards (Charleston, SC, USA). Modified Eagle's medium (MEM), Dulbecco's modified Eagle's medium (DMEM), and 1% antibiotic–antimycotic solution were purchased from Gibco (Grand Island, NY, USA).

2.2. Preparation of Metabolite and Fe solutions

Epicatechin and Myr were dissolved in DMSO (100%) to a concentration of 1.6 mM and NA and DMA were dissolved in DMSO (50%) and 18 MΩ H₂O (50%) to a concentration of 0.8 mM. All solutions were diluted with pH 2 saline solution (140 mM NaCl, 5 mM KCl, adjusted to pH 2 with HCl) to achieve 400 µM stock solutions and subsequently diluted with pH 2 saline solution to appropriate concentrations for use in Caco-2 assays. To minimize toxicity to Caco-2 cells, the maximum DMSO concentration in 30 µM Epi/Myr treatments was 1.9% (2.5% in 40 µM polyphenol treatments). Fe stock solutions were prepared from 1000 mg/mL Fe standard in pH 2 saline solution. A 50 µL aliquot of Fe²⁺ stock solution of appropriate concentration was added to 150 µL of prepared metabolite solutions to achieve the desired Fe/metabolite concentration.

2.3. Caco-2 Assays

The Caco-2 cell assays were performed as previously described [21]. Briefly, cells were cultured in 24-well plates (Corning Costar 24 Well Clear TC-Treated Multiple Well Plates) coated with collagen and maintained in supplemented DMEM [3.7 g/L sodium bicarbonate, 25 mM HEPES (pH 7.2), 10% fetal bovine serum] for 12 days postseeding. Twenty-four hours prior to experiments, DMEM was replaced with iron-free supplemented MEM as previously described [36]. The Fe/metabolite solution (200 µL) was incubated (~22 °C, 15 min) and combined with 1 mL of MEM before an aliquot (500 µL) was directly applied to Caco-2 cell monolayers. After overnight incubation (37 °C), cells were washed twice with a buffered saline solution (130 mM NaCl, 5 mM KCl, 5 mM PIPES (pH 6.7)) and lysed by the addition of 0.5 mL 18 MΩ H₂O. In an aliquot of the lysed Caco-2 cell solution, ferritin content was determined using an immunoradiometric assay (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein content was determined using a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA) as previously described [36]. As Caco-2 cells synthesize ferritin in response to intracellular Fe, we used the proportion of ferritin/total cell protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake and refer to this as 'Fe uptake' throughout the manuscript.

2.4. Graphical Representation and Statistical Analysis

Each figure includes three control treatments as indicated by the white, grey and black bars on the right side of each panel. The first control treatment, "cell baseline", represents ferritin formation in

Caco-2 cells in the absence of any metabolite or Fe. The second control treatment, either “+ 4 μM Fe” or “+ 40 μM Fe”, represents ferritin formation in the presence of 4 μM Fe (typical Fe concentration for Caco-2 cell assays) or 40 μM Fe alone, respectively. A dotted line between both y-axes is provided to easily compare treatments to this control. The third control treatment, either “+ 80 μM AsA” or “+ 800 μM AsA”, represents ferritin formation in the presence of 4 μM Fe with 80 μM AsA or 40 μM Fe with 800 μM AsA (i.e., an Fe:AsA ratio of 1:20), respectively. In Figures 1–3, triangular data points represent a fourth control treatment of ferritin formation in Caco-2 cells in the presence of NA, DMA, Epi or AsA solutions without Fe, and displayed values equivalent to the Cell Baseline. Data represent ng ferritin/mg protein in Caco-2 assays and were generated using SigmaPlot software (v.13.0, Systat Software, San Jose, CA, USA). Statistical differences between means were analyzed by unpaired Student’s t-test, and differences among means were assessed using one-way analysis of variance (ANOVA) with Tukey or Hsu’s MCB post-hoc tests, using Minitab software (v 18.0, Minitab, State College, PA, USA).

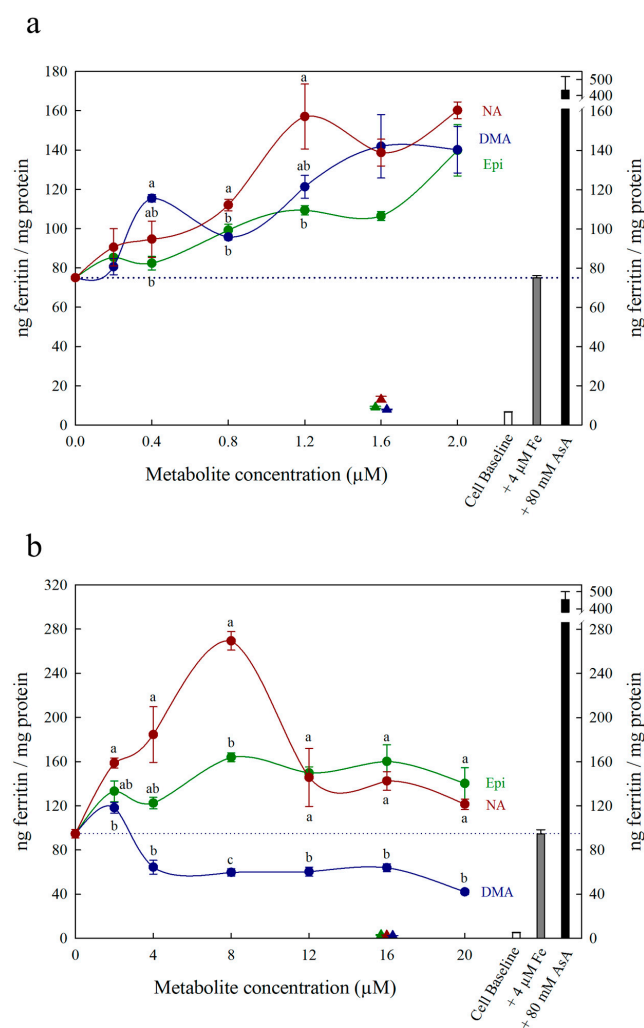


Figure 1. Ferritin formation in Caco-2 cells in response to nicotianamine (NA, red), 2' deoxymugineic acid (DMA, blue) and epicatechin (Epi, green) at concentrations varying between (a) 0 and 2.0 μM and (b) 0 and 20 μM in solution with Fe (4 μM). Triangles indicate ferritin formation at metabolite solutions (1.6 μM or 16 μM) without Fe. Dotted line indicates ferritin response to 4 μM Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Different letters indicate significantly different ferritin formation between metabolites of the same concentration as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$).

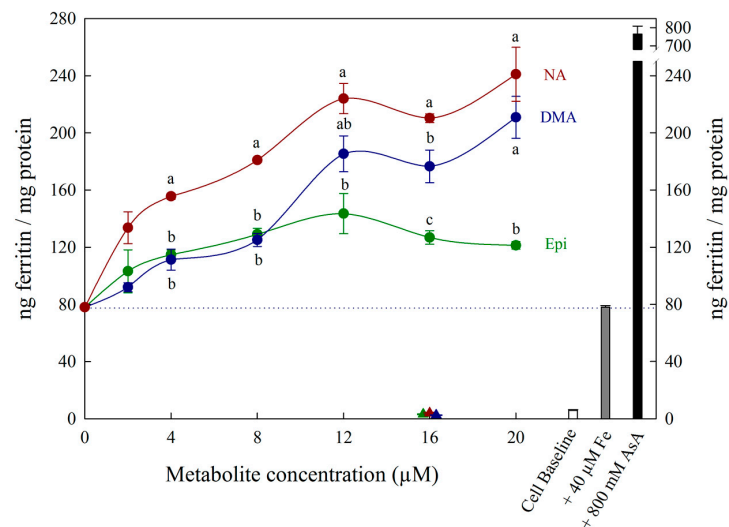


Figure 2. Ferritin formation in Caco-2 cells in response to varying concentrations (0–20 μM) of nicotianamine (NA, red), 2' deoxymugineic acid (DMA, blue) and epicatechin (Epi, green) at concentrations varying between 0–20 μM in solution with Fe (40 μM). Triangles indicate ferritin formation in metabolite solutions (16 μM) without Fe. Dotted line indicates ferritin response to 40 μM Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Different letters indicate significantly different ferritin formation between metabolites of the same concentration as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$).

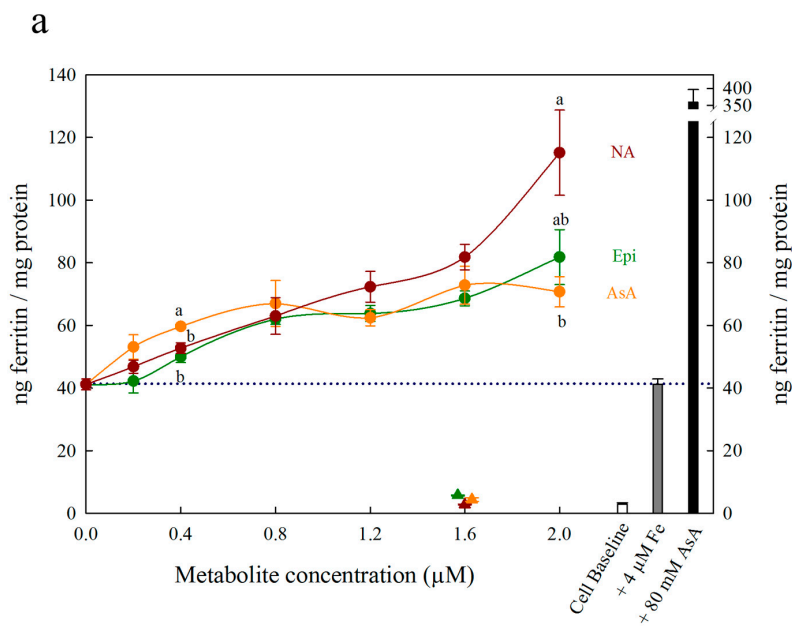


Figure 3. Cont.

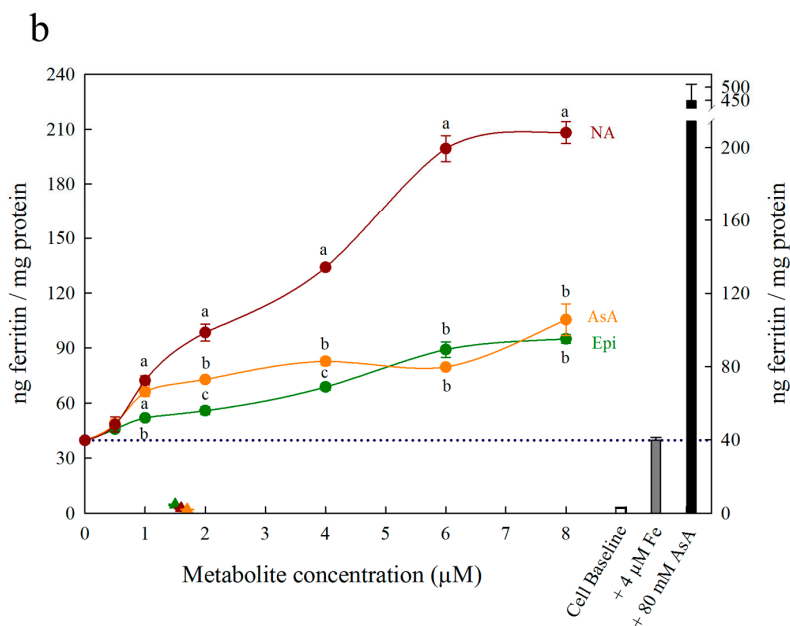


Figure 3. Ferritin formation in Caco-2 cells in response to nicotianamine (NA, red), ascorbic acid (AsA, orange) and epicatechin (Epi, green) at concentrations varying between (a) 0–2.0 μM and (b) 0–8 μM in solution with Fe (4 μM). Triangles indicate ferritin formation in metabolite solutions (1.6 μM) without Fe. Dotted line indicates ferritin response to 4 μM Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Different letters indicate significantly different ferritin formation between metabolites of the same concentration as analyzed by one-way ANOVA with Tukey post-hoc test ($p < 0.05$).

3. Results

All low concentrations ($\leq 2 \mu\text{M}$) of NA, DMA and Epi enhanced Fe uptake into Caco-2 cells, with NA and DMA promoting ferritin formation more than Epi (Figure 1a). Higher concentrations (between 2–20 μM) of NA and Epi enhanced Fe uptake, and higher concentrations of DMA inhibited Fe uptake (Figure 1b). Between concentrations of 2 and 8 μM , NA enhanced Fe uptake more significantly than Epi with peak ferritin formation at 8 μM (i.e., a 1:2 molar ratio with Fe). At Fe molar ratios of 1:3, 1:4 and 1:5, NA and Epi promoted ferritin formation at the same level (Figure 1b and Table S1). At a higher concentration of Fe (40 μM), NA enhanced Fe uptake significantly more than Epi at all concentrations apart from 2 μM , and DMA enhanced Fe uptake significantly more than Epi at 16 and 20 μM (Figure 2). Together, these results demonstrate that NA > DMA > Epi in the promotion of Fe uptake into Caco-2 cells. As the stronger enhancer, NA was compared in subsequent assays alongside Epi and AsA. All low concentrations ($< 2 \mu\text{M}$) of NA, Epi and AsA enhanced Fe uptake into Caco-2 cells at similar levels, with NA showing significantly higher ferritin formation at 2 μM (Figure 3a). Above 2 μM , NA enhanced Fe uptake significantly more than both Epi and AsA with peak ferritin formation at 8 μM , demonstrating that NA > AsA > Epi in the promotion of Fe uptake into Caco-2 cells (Figure 3b). Across all experiments with Fe:metabolite molar ratios $\leq 1:2$, the fold increase in ferritin formation over the Fe control was significantly higher in Caco-2 cells exposed to NA compared to AsA, Epi or DMA (Figure 4). To further characterize NA as a strong enhancer of Fe bioavailability, ferritin formation was measured in response to the Fe uptake inhibitor Myr in combination with NA (NA:Myr) or Epi (Epi:Myr). At 4 μM , total metabolite concentration, all NA:Myr solutions enhanced Fe uptake significantly more than Epi:Myr, and a NA:Myr solution of ratio 30:70 increased ferritin formation more than the Fe control (Figure 5a). At 30 μM total metabolite concentration, NA:Myr solutions of ratio 70:30, 80:20 and 90:10 enhanced Fe uptake significantly more than Epi:Myr, and a NA:Myr solution of ratio 90:10 increased ferritin formation more than the Fe control (Figure 5b).

Together these results demonstrate that NA is stronger than Epi in counteracting the inhibitory effect of Myr and enhancing Fe uptake into Caco-2 cells.

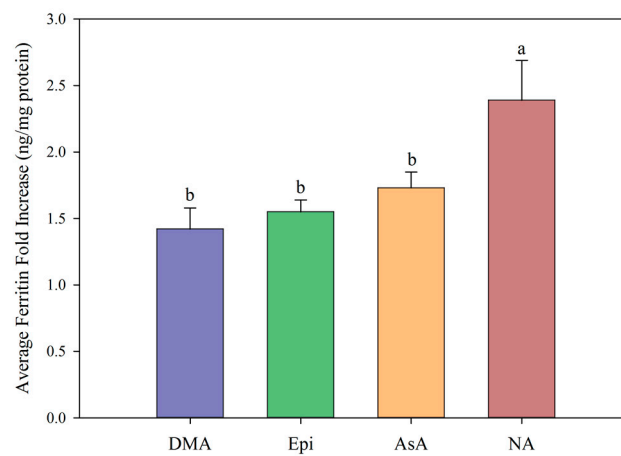


Figure 4. Average fold increase in Caco-2 cell ferritin formation in response to 2' deoxymugineic acid (DMA, blue), epicatechin (Epi, green), ascorbic acid (AsA, orange), and nicotianamine (NA, red) at Fe:metabolite molar ratios $\leq 1:2$ compared to ferritin formation in the presence of Fe alone. Error bars represent standard error of the mean of at least eight replicates. Different letters indicate significant differences between mean fold increase in ferritin formation between metabolites as analyzed by one-way ANOVA with Hsu's MCB post-hoc test ($p < 0.05$).

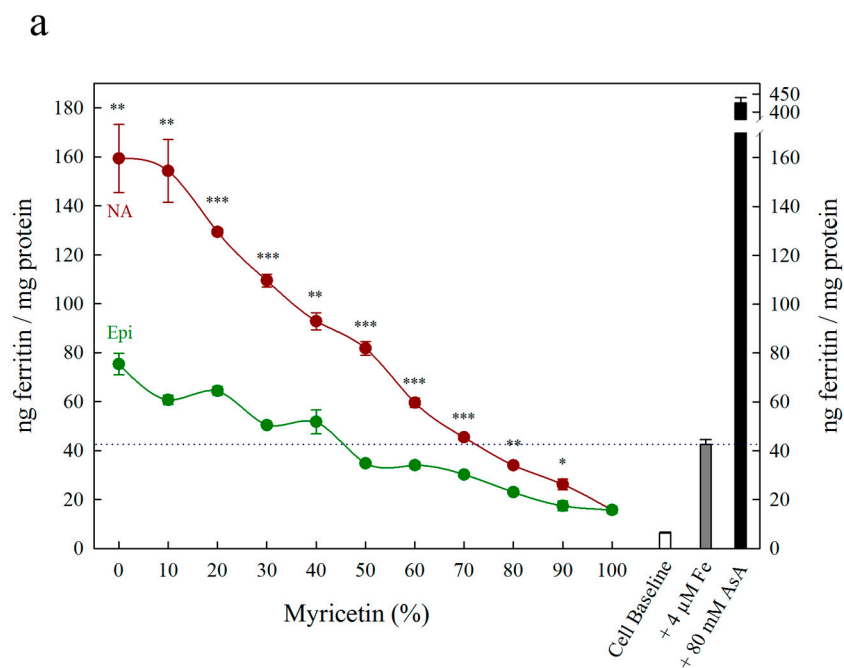


Figure 5. Cont.

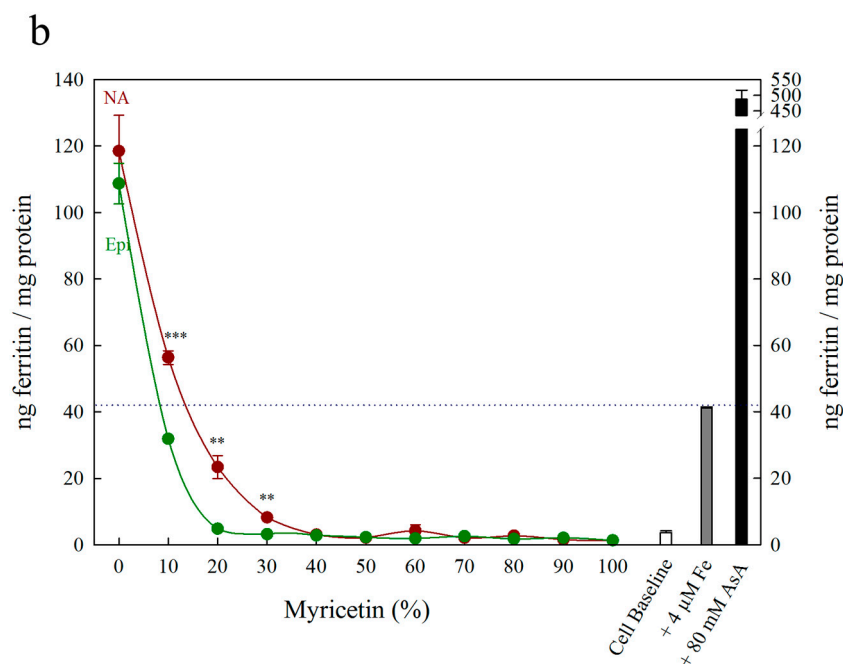


Figure 5. Ferritin formation in Caco-2 cells in response to various molar ratios of nicotianamine:myricetin (NA, red) and epicatechin:myricetin (Epi, green). Total metabolite concentration at each data point was (a) 4 μ M and (b) 30 μ M. Dotted line indicates ferritin response to 4 μ M Fe alone and is extended to both y axes to facilitate comparison with other treatments. Error bars represent standard error of the mean of three replicates. Asterisks denote the significance between NA and Epi at each molar ratio for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) as determined by Student's *t*-test.

4. Discussion

At low ($\leq 1:2$) Fe:metabolite molar ratios, NA and DMA enhanced Caco-2 cell ferritin formation more than Epi. Interestingly, the level of promotion was more pronounced at 40 μ M Fe concentration compared to 4 μ M Fe, despite maintaining the same Fe:metabolite molar ratios (Figures 1a and 2). As ferritin formation was similar in the 'Fe control' at both 4 μ M and 40 μ M, it is likely that maximum Fe solubility is exceeded somewhere between 4 μ M and 40 μ M Fe in the absence of any Fe bioavailability enhancer (Figures 1 and 2). Although we provide strong evidence to support NA and DMA as enhancers of Fe bioavailability, the exact mechanism of how NA and DMA facilitate Fe uptake into Caco-2 cells remains unknown and is likely dependent on the proportion of readily bioavailable ferrous Fe^{2+} ions in solution [21]. Both NA and DMA form high affinity 1:1 complexes with Fe^{3+} ions, however only NA is thought to be capable of binding Fe^{2+} [45,46]. Here we demonstrate that DMA promotes ferritin formation at Fe:DMA molar ratios less than 1:1, suggesting that DMA is capable of binding Fe^{2+} and/or reducing Fe^{3+} ions to some degree and requires further investigation. The decreased ferritin formation after exposure to Fe:DMA solutions with molar ratio $> 1:1$ (Figure 1b) could be due to oxidation of Fe^{2+} ions in solution and excess formation of Fe^{3+} -DMA complexes that have low bioavailability for Caco-2 cell Fe uptake [45]. Some polyphenol compounds capable of complexing Fe^{3+} (e.g., delphinidin and delphinidin 3-glucoside) also demonstrate this biphasic pattern of promoting Fe uptake at molar ratios $< 1:1$ and inhibiting Fe uptake at molar ratios $> 1:1$ [21].

Nicotianamine promoted Caco-2 cell Fe uptake at all molar ratios observed, likely due to the formation of stable Fe^{2+} -NA complexes and facilitation of Fe^{2+} uptake. As Fe is provided in a reduced Fe^{2+} state when preparing the assay, Fe^{2+} -NA complexes rapidly form after NA addition and maintain the reduced Fe^{2+} state during exposure to Caco-2 cells. Certain polyphenols are also suggested to promote Caco-2 cell Fe uptake via a similar mechanism of binding Fe^{2+} and slowing the oxidation of Fe^{2+} to Fe^{3+} [21]. The reduced ferritin formation at Fe:NA molar ratios over 1:2 may be due to excess binding of Fe^{2+} by NA, preventing the release of Fe^{2+} for Caco-2 cell uptake, and suggests that

an Fe:NA molar ratio less than 1:2 is optimal for promoting Fe bioavailability (Figure 1b, Table S1). Alternatively, NA could enhance Fe bioavailability via direct uptake of Fe^{2+} -NA in a similar mechanism to that proposed for glycosaminoglycans and proteoglycans and exploring this mechanism will be the subject of future research [28,29].

Ascorbic acid promotes Fe uptake into Caco-2 cells through *de novo* reduction of Fe^{3+} ions and maintenance of the Fe^{2+} ions in solution [9,21,22]. Here we demonstrate that at Fe:metabolite molar ratios less than 1:2, NA enhances Caco-2 cell ferritin formation significantly more than AsA and is the strongest enhancer of *in vitro* Fe bioavailability identified to date (Figures 3b and 4). Together, these results suggest that the ability to bind Fe^{2+} ions is critical to enhancing Fe uptake into human intestinal cells. At an Fe:AsA molar ratio of 1:20, complete *de novo* reduction of Fe^{3+} ions in solution (without Fe^{2+} binding) leads to an ~8-fold increase in Caco-2 cell ferritin levels compared to Fe alone (Table S1). An Fe:NA molar ratio of 1:20 was not tested as this ratio does not occur naturally in plant foods, although it is unlikely to show high ferritin formation given the effect of ratios greater than 1:2 (Table S1). Instead, molar ratios less than 1:2 capture the highest ratio of Fe:NA measured to date in conventional plants foods (Fe:NA ratio of 1:1.6 in biofortified soybean) and provide realistic targets for plant breeders to improve Fe bioavailability [13].

To rule out the possibility that NA, DMA, Epi or AsA were promoting Caco-2 cell ferritin formation independent of Fe, Caco-2 cells were exposed to these metabolites alone in concentrations equal to 1.6 μM or 16 μM (Figures 1–3). There was no sign that the metabolite presence alone significantly increased Caco-2 ferritin formation relative to the cell baseline or disrupted the Caco-2 cell monolayer at harvest. Thus, the increased ferritin formation observed in Caco-2 cells exposed to Fe:metabolite solutions is due to the Fe uptake promoting properties of these metabolites.

Although Myr is a potent antioxidant with Fe-reducing properties (normally characteristic of Fe uptake enhancers), it strongly inhibits Caco-2 cell Fe uptake as it forms highly stable Fe–Myr complexes with low Fe bioavailability [47,48]. Nicotianamine increased ferritin formation compared to Epi at all 4 μM solutions and at 30 μM solutions containing over 70% NA or Epi, demonstrating that NA is stronger than Epi at preventing Fe uptake inhibition by Myr (Figure 5). At 30 μM , ferritin formation was equivalent to cell baseline for solutions containing less than 70% NA or 90% Epi, demonstrating that Myr inhibits Fe uptake more effectively than NA/Epi promotes Fe uptake (Figure 5b). At 4 μM (the equivalent of a 1:1 molar ratio with Fe), 30% NA combined with 70% Myr promoted ferritin formation above the Fe control, suggesting that NA can outcompete Myr and form Fe^{2+} –NA complexes with high bioavailability (Figure 5a). An additional assay at 10 μM demonstrated an intermediate response, with low ferritin formation at 20% Myr due to improper Caco-2 cell growth (Figure S1).

Whether NA would enhance Fe bioavailability in the presence of several enhancers and inhibitors (i.e., a bean-based food sample) is unclear. To date, increased NA has not overcome the inhibitory effect of polyphenols/phytate to enhance *in vitro* Fe bioavailability within biofortified whole wheat grain [17]. To further address this question, future studies should explore NA biofortification within plant foods that contain high endogenous NA levels and low Fe:polyphenol molar ratios (e.g., 1:2 in carioca beans) [21]. Nonetheless, plant species with inherently low polyphenol levels (i.e., wheat) serve as ideal candidates for enhanced Fe bioavailability through the overproduction of NA [26,49]. The additional role of NA in the biosynthesis of DMA (itself an enhancer of Fe bioavailability) further reinforces increased NA biosynthesis as an effective cereal biofortification strategy to improve global human Fe nutrition.

5. Conclusions

We utilized a modified Caco-2 cell bioassay to characterize two low-molecular-weight plant metal chelators – NA and DMA – as strong enhancers of Fe bioavailability and demonstrate that NA is also capable of reversing Fe uptake inhibition by Myr. In doing so we highlight NA and DMA as important targets for biofortification strategies aimed at improving Fe bioavailability in cereals. Although we suspect that NA and DMA promote Fe bioavailability in Caco-2 cells by maintaining Fe^{2+} ions in

solution, uncovering the exact mechanism by which these metal chelators promote Fe absorption will be the subject of future research studies.

Supplementary Materials: The following are available online <http://www.mdpi.com/2072-6643/11/7/1502/s1>; Figure S1: Ferritin formation in Caco-2 cells in response to various molar ratios of nicotianamine:myricetin (NA, red) and epicatechin:myricetin (Epi, green), Table S1: Fold increases in Caco-2 cell ferritin formation in response to different molar ratios of 2' deoxymugineic acid (DMA, blue), epicatechin (Epi, green), ascorbic acid (AsA, orange), and nicotianamine (NA, red) compared to ferritin formation in the presence of Fe alone.

Author Contributions: Conceptualization, J.T.B., J.J.H., E.T., R.P.G., and A.A.T.J.; Data curation, J.T.B. and J.J.H.; Formal analysis, J.T.B.; Funding acquisition, R.P.G. and A.A.T.J.; Investigation, J.T.B. and J.J.H.; Methodology, J.T.B. and J.J.H.; Project administration, R.P.G.; Resources, R.P.G. and A.A.T.J.; Supervision, A.A.T.J.; Validation, J.T.B.; Visualization, J.T.B. and J.J.H.; Writing—original draft, J.T.B.; Writing—review & editing, J.T.B., J.J.H., E.T., R.P.G., and A.A.T.J. All authors have read and approve the final manuscript.

Funding: Funding was provided by the United States Department of Agriculture, Agricultural Research Service to R.P.G. and the Harvest Plus Challenge program to A.A.T.J.

Acknowledgments: The authors wish to acknowledge Yongpei Chang and Mary Bodis for their excellent technical assistance throughout the study, and Julien Bonneau for advice during manuscript writing. The authors have not received funds for covering the costs to publish in open access.

Conflicts of Interest: The authors declare no conflict of interest.

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Metabolic engineering of bread wheat improves grain iron concentration and bioavailability

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Received 9 July 2018;

revised 13 December 2018;

accepted 17 December 2018.

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Keywords: nicotianamine, 2'-deoxymugineic acid, biofortification, Zinc, Caco-2, X-ray fluorescence microscopy.

Summary

Bread wheat (*Triticum aestivum* L.) is cultivated on more land than any other crop and produces a fifth of the calories consumed by humans. Wheat endosperm is rich in starch yet contains low concentrations of dietary iron (Fe) and zinc (Zn). Biofortification is a micronutrient intervention aimed at increasing the density and bioavailability of essential vitamins and minerals in staple crops; Fe biofortification of wheat has proved challenging. In this study we employed constitutive expression (CE) of the rice (*Oryza sativa* L.) nicotianamine synthase 2 (*OsNAS2*) gene in bread wheat to up-regulate biosynthesis of two low molecular weight metal chelators – nicotianamine (NA) and 2'-deoxymugineic acid (DMA) – that play key roles in metal transport and nutrition. The CE-*OsNAS2* plants accumulated higher concentrations of grain Fe, Zn, NA and DMA and synchrotron X-ray fluorescence microscopy (XFM) revealed enhanced localization of Fe and Zn in endosperm and crease tissues, respectively. Iron bioavailability was increased in white flour milled from field-grown CE-*OsNAS2* grain and positively correlated with NA and DMA concentrations.

Introduction

Micronutrient mineral deficiencies affect over two billion people worldwide with women and children most acutely at risk (Beal *et al.*, 2017; Lopez *et al.*, 2016). Iron (Fe) deficiency is the leading cause of anaemia, a condition that impairs cognitive development and work productivity and increases maternal and child mortality (Kassebaum *et al.*, 2014; Lopez *et al.*, 2016). Zinc (Zn) deficiency causes a variety of disorders including stunted growth in children. Human Fe and Zn deficiencies are most prevalent in SEA (Southeast Asia) and MENA (Middle East and North Africa), in part due to high consumption of micronutrient-poor staple crops such as wheat (*Triticum aestivum* L.), rice (*Oryza sativa* L.) and maize (*Zea mays* L.) (Beal *et al.*, 2017). Rising atmospheric CO₂ concentrations will likely decrease Fe and Zn concentrations in C₃ grains and could further exacerbate micronutrient deficiencies in SEA and MENA (Myers *et al.*, 2014; Smith *et al.*, 2017).

The wheat grain is comprised primarily of starch (60%–75%) and protein (12%–14%) with only low concentrations of Fe, Zn and other micronutrients (Shewry, 2009; Velu *et al.*, 2014). Iron and Zn are remobilized from senescing vegetative tissues and/or translocated from roots during grain filling (Maillard *et al.*, 2015; Pottier *et al.*, 2014; Waters *et al.*, 2009) and transported into developing grain via the vascular bundle in the crease region. In mature grain, the highest concentrations of Fe and Zn are found in the aleurone cells in conjunction with compounds such as phytic acid, polyphenols and fibre that inhibit bioavailability, while

only low Fe and Zn concentrations are localized to endosperm tissues (Brouns *et al.*, 2012; De Brier *et al.*, 2016; Moore *et al.*, 2012; Schlemmer *et al.*, 2009; Singh *et al.*, 2013; Stomph *et al.*, 2011). Grain milling removes inhibitors of micronutrient bioavailability but also a significant proportion of the grain Fe and Zn (Hourston *et al.*, 2017; Zhang *et al.*, 2010).

Wheat biofortification aims to complement micronutrient supplementation and fortification programs by improving grain micronutrient density and/or bioavailability (Bouis *et al.*, 2011; Prentice *et al.*, 2017). Generation of Fe biofortified wheat through conventional breeding is hindered in modern wheat cultivars by inherently low grain Fe concentrations and a lack of genomic resources for this trait (Borrill *et al.*, 2014; Velu *et al.*, 2014), indicating that genetic engineering strategies may be required to generate novel genetic variation (Connorton *et al.*, 2017; Singh *et al.*, 2017).

Nicotianamine (NA) is a non-protein amino acid formed by trimerization of S-adenosylmethionine (SAM) in a process mediated by NA synthase (NAS) enzymes (Clemens *et al.*, 2013). Nicotianamine plays key roles in the chelation and transport of metals such as Fe, Zn, manganese (Mn) and copper (Cu) in all higher plants (Takahashi *et al.*, 2003; von Wiren *et al.*, 1999). In graminaceous plant species, NA serves an additional purpose as the precursor to biosynthesis of 2'-deoxymugineic acid (DMA), a root-secreted phytosiderophore that chelates ferric Fe (Fe³⁺) in the rhizosphere and is subsequently re-absorbed. Nicotianamine and/or DMA are also the main chelators of Fe in white wheat

Please cite this article as: Beasley, J.T., Bonneau, J.P., Sánchez-Palacios, J.T., Moreno-Moyano, L.T., Callahan, D.L., Tako, E., Glahn, R.P., Lombi, E. and Johnson, A.A.T. (2019) Metabolic engineering of bread wheat improves grain iron concentration and bioavailability. *Plant Biotechnol. J.*, <https://doi.org/10.1111/pbi.13074>

flour (Eagling *et al.*, 2014a), and increased NA is known to increase Fe bioavailability in polished rice (Lee *et al.*, 2012; Trijatmiko *et al.*, 2016; Zheng *et al.*, 2010). For these reasons, increased NA biosynthesis has emerged as a popular strategy for Fe biofortification of cereals (Johnson *et al.*, 2011; Lee *et al.*, 2012; Singh *et al.*, 2017; Trijatmiko *et al.*, 2016).

In this study of glasshouse- and field-grown plants, we demonstrate that constitutive expression of the rice *OsNAS2* gene (CE-*OsNAS2*) in bread wheat causes not only significant Fe and Zn enrichment of whole grain and flour fractions but also increased concentrations of NA and DMA that are positively correlated with Fe bioavailability in CE-*OsNAS2* white flour. Interestingly, our results indicate that Fe bioavailability is higher in NA and DMA-enriched white flour regardless of Fe concentration and that white flour NA and DMA concentrations are more critical than absolute Fe concentration in determining Fe bioavailability. These results provide unique insights into cereal Fe biofortification and highlight the importance of metabolic engineering strategies that enhance not only micronutrient density but also promoters of micronutrient bioavailability.

Results

Generation of independent transformation events with constitutive *OsNAS2* expression and selection of biofortified material for advanced nutritional analysis

Bread wheat cultivar (cv.) Bobwhite transformants with constitutive expression (CE) of the rice nicotianamine synthase 2 (*OsNAS2*) gene were generated through biolistic transformation of a cassette containing the *OsNAS2* gene under regulatory control of the maize ubiquitin 1 promoter (UBI-1) (Figure 1a). Six independent CE-*OsNAS2* transformation events termed CE-1, CE-5, CE-7, CE-8, CE-13 and CE-15 were regenerated from tissue culture and Southern blot analysis showed that insert copy number ranged from 1 to 7 among the six events (Figure 1b). Automated imaging facilities at The Plant Accelerator (Adelaide, Australia) were used to phenotype T₁ progeny of the six events; two events (CE-1 and CE-5) showed no phenotypic differences from a null segregant (NS) line derived from CE-1 nor wild-type (WT) wheat with respect to shoot area, plant height, total grain number and thousand grain weight (Figure 1c–g). Elemental analysis showed that four of the CE-*OsNAS2* events (CE-1, CE-8, CE-13, CE-15) produced T₂ grain with significantly increased Fe and Zn concentrations relative to NS and WT and one CE-*OsNAS2* event (CE-5) produced T₂ grain with significantly increased Zn concentration relative to NS and WT (Figure 1h,i). Wild-type plants did not differ from NS plants for any trait measured and

had mean values of 1465 cm² shoot area, 65 cm plant height; 375 total grain number, 38 g thousand grain weight, 40 µg/g DW grain Fe and 78 µg/g DW grain Zn.

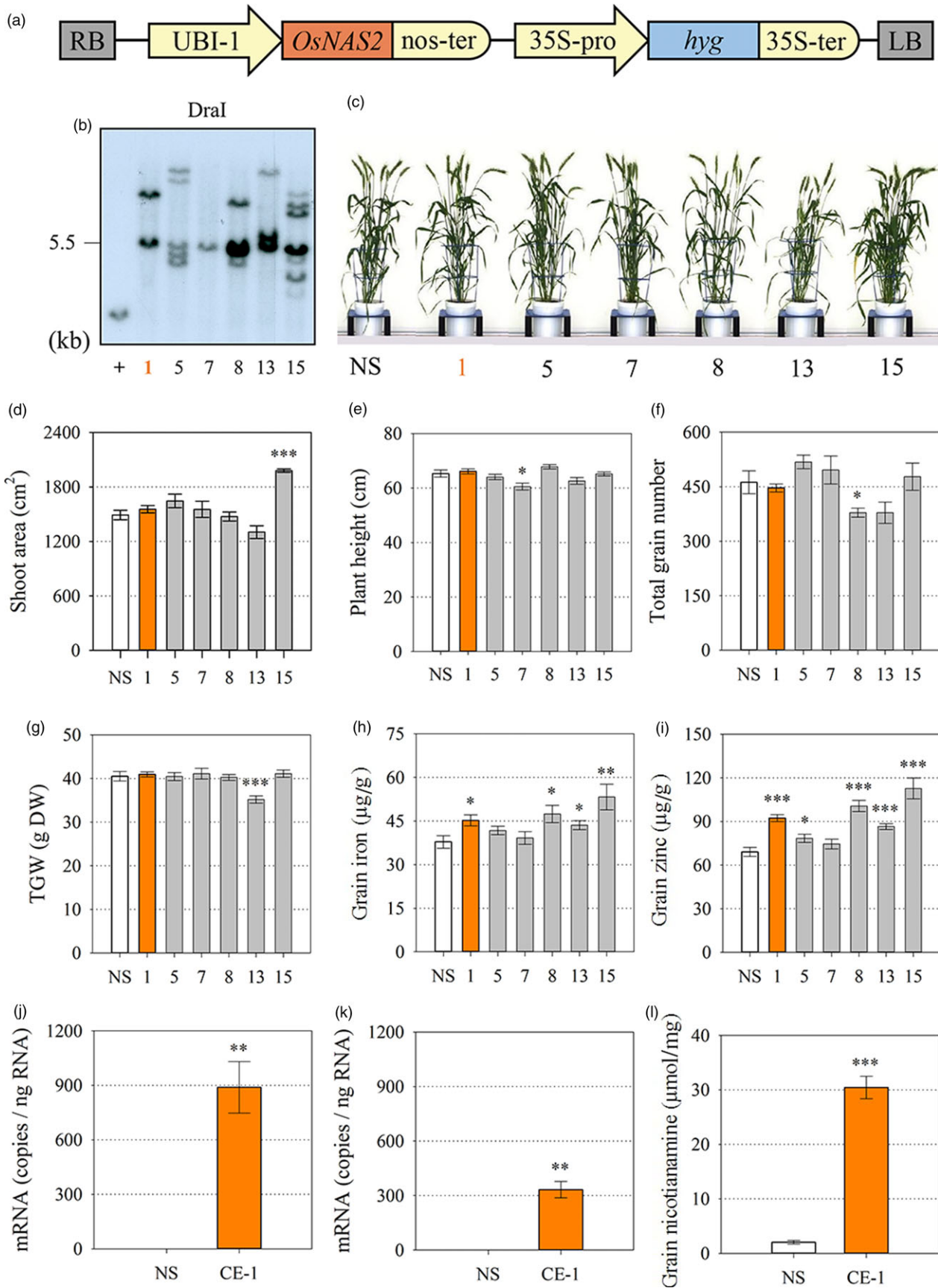
Based on low insert copy number, no difference in plant phenotype, and increased grain Fe and Zn concentrations from the T₀ to T₂ generations, homozygous progeny of the double-insert event CE-1 and corresponding NS line were selected for a range of additional analyses from the T₃ to T₆ generation as depicted in Figure S1. Glasshouse-grown CE-1 seedlings displayed high *OsNAS2* expression in roots and shoots (Figure 1j,k). Expression of a range of endogenous *TaNAS*, *TaNAAT* and *TaDMAS* genes involved in NA and DMA biosynthesis was not significantly different between CE-1 and NS seedlings, however, a trend towards slightly reduced expression was detected in CE-1 (Figure S2). The CE-1 and NS seedlings did not differ with respect to shoot Fe, Zn and DMA concentration while shoot NA concentration was 1.3-fold higher in CE-1 seedlings (Figure S3). Nicotianamine concentration was 15-fold higher in CE-1 mature grain relative to NS (Figure 1l).

Constitutive *OsNAS2* expression alters Fe and Zn accumulation in grain, bracts, rachis and flag leaf at multiple points during grain filling

The main stem flag leaf, rachis, bracts and grain of glasshouse-grown CE-1 and NS plants were harvested at five timepoints post anthesis to determine Fe and Zn accumulation patterns in vegetative and grain tissues during grain filling. The CE-1 grain had significantly higher Fe content at 5–8 days after anthesis (DAA) and 19–21 DAA relative to NS grain ($P = 0.006$ and $P = 0.046$; respectively) and showed non-significant trends towards higher grain Fe and Zn content at maturity (Figure 2a, b). Bracts of CE-1 plants had significantly higher Fe and Zn contents at 12–15 DAA relative to NS ($P = 0.013$ and $P \leq 0.001$; respectively) but did not differ from NS bracts at maturity (Figure 2c,d). Rachis of CE-1 plants had significantly higher Fe content at 5–8 DAA ($P = 0.012$), and significantly higher Zn contents at 12–15 DAA and 19–21 DAA ($P = 0.014$ and $P = 0.020$, respectively), but did not differ from NS rachis at maturity (Figure 2e,f). Flag leaf of CE-1 and NS plants contained similar Fe content throughout grain filling. Conversely, CE-1 flag leaf contained significantly lower Zn content at 19–21 DAA and 26–29 DAA relative to NS ($P = 0.009$ and $P = 0.038$, respectively) but did not differ from NS flag leaf at maturity (Figure 2g,h,i).

Iron and Zn concentration data followed similar trends to the content data although more significant differences were detected (Table S1). Vegetative and grain tissue biomass was largely unchanged between CE-1 and NS plants throughout anthesis

Figure 1 Generation and characterization of independent bread wheat transformation events constitutively expressing the rice nicotianamine synthase 2 (*OsNAS2*) gene. (a) Schematic representation of the T-DNA construct. RB and LB: right and left borders, respectively; UBI-1: maize ubiquitin 1 promoter; *OsNAS2*: rice nicotianamine synthase 2 gene (LOC_Os03 g19420); nos-ter: nopaline synthase terminator; 35S-pro: dual promoter of 35S cauliflower mosaic virus gene; *hyg*: hygromycin phosphotransferase gene; 35S-ter: terminator of 35S cauliflower mosaic virus gene. (b) Southern blot analysis of T₁ wheat events to determine T-DNA insertion number. Dral: restriction endonuclease; + indicates positive control. (c) Representative plants of null segregant (NS) and the 6 transformation events (CE-*OsNAS2*) 100 days after sowing. (d–g) Projected shoot area (cm²), plant height (cm), total grain number and thousand grain weight (TGW) of NS (white), leading CE-*OsNAS2* event (CE-1, orange) and other CE-*OsNAS2* events (grey) at the T₁ generation. Bars represent mean $\pm$ SEM of at least 7 biological replicates. (h, i) Iron and zinc concentration (µg/g DW) in T₂ whole grain of NS, CE-1 and other CE-*OsNAS2* events. Bars represent mean $\pm$ SEM of at least seven biological replicates. (j, k) Relative quantification of *OsNAS2* transcript levels in NS and CE-1 shoots and roots. Bars represent mean $\pm$ SEM of three bulked biological replicates, each with three technical replicates of quantitative RT-PCR. (l) Nicotianamine concentration (µmol/mg) in whole grain of NS and CE-1 plants at the T₂ generation. Bars represent mean $\pm$ SEM of three biological replicates. Asterisks denote the significance between NS and CE-*OsNAS2* events for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's *t*-test. Wild-type plants did not differ from NS plants for any trait measured and therefore only NS data is presented.



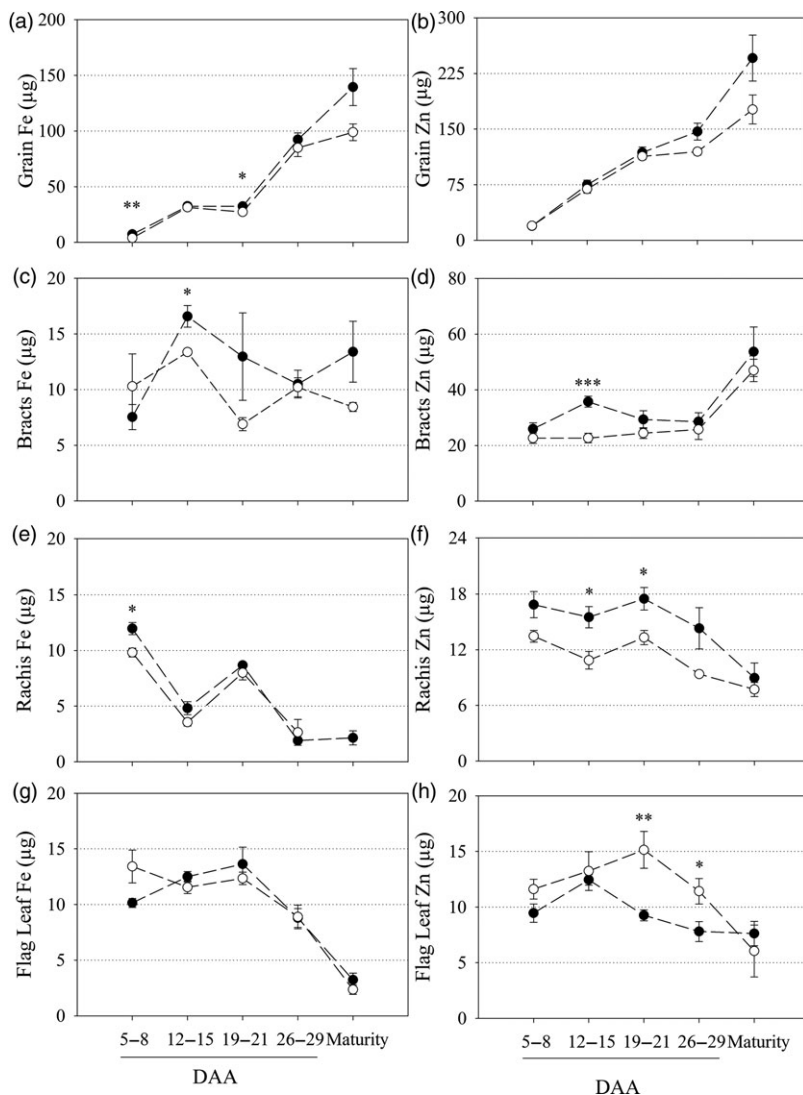


Figure 2 Fe and Zn content in vegetative and grain tissues during grain filling of CE-OsNAS2 and NS wheat lines. Fe and Zn content (µg) in NS (open circles) and CE-1 (closed circles) plant tissues between 5–8, 12–15, 19–21 and 26–29 days after anthesis (DAA) as well as at maturity. (a, b) grain; (c, d) bracts; (e, f) rachis; and (g, h) flag leaf tissues were sampled for Fe and Zn content, respectively. Each symbol represents mean $\pm$ SEM of at least 3 biological replicates. Asterisks denote the significance between NS and CE-1 for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's *t*-test.

and was not significantly different for any tissue at maturity (Table S2).

Constitutive *OsNAS2* expression increases Fe accumulation in grain endosperm tissue and Fe and Zn accumulation within the crease region

Elemental maps of Fe and Zn in transverse cross-sections of two representative CE-1 and NS grain showed that CE-1 grain had higher Fe signal intensities in all grain tissue types relative to NS with the largest difference detected in endosperm tissues (Figure 3c,d). The Zn signal in both CE-1 and NS grain was localized to the aleurone and crease regions and was not detectable in the endosperm. Line scans across the mid-section demonstrated that CE-1 grain had higher Fe signal intensities relative to NS in all regions of the grain, particularly in the endosperm, while CE-1 grain had slightly higher Zn signal intensities in the aleurone cells relative to NS (Figure 3e,f). The line scans also revealed different Fe and Zn distribution patterns within the crease region of NS and CE-1 grain, with Zn signals appearing as two distinct peaks on either side of the crease while Fe signals clustered into one central peak. Tri-colour elemental maps of Fe, Zn and Cu distribution within the crease region

demonstrated that these distinct peaks result from extensive Zn localization in modified aleurone cells bordering the crease in contrast to prominent Fe localization in the centrally located nucellar projection (Figure S4). While CE-1 and NS grain showed similar Zn signal intensities in the modified aleurone cells of the crease, the CE-1 grain had slightly higher Zn signal intensity in the nucellar projection relative to NS (Figure 3f). The CE-1 and NS grain had similar distribution and signal intensities for Cu and Mn (Figure S5).

Tri-colour elemental maps of Fe, Zn and phosphorus (P) near the grain edge of one representative CE-1 and NS grain demonstrated that the Fe signal in CE-1 grain was enhanced within pericarp, aleurone and endosperm regions relative to NS grain (Figure 4c,d). The Zn signal in CE-1 grain was enhanced primarily within the pericarp and aleurone cells, relative to the NS, and co-localized with Fe in that region. The P signal in both NS and CE-1 grain did not differ in intensity nor distribution and was localized to the aleurone cells. The trends observed in CE-1 and NS tri-colour maps were further confirmed by line scans (Figure 4e,f). In both CE-1 and NS grain, P was localized exclusively to the aleurone cells with equal signal intensity, indicating that the significant enrichment of Fe in CE-1

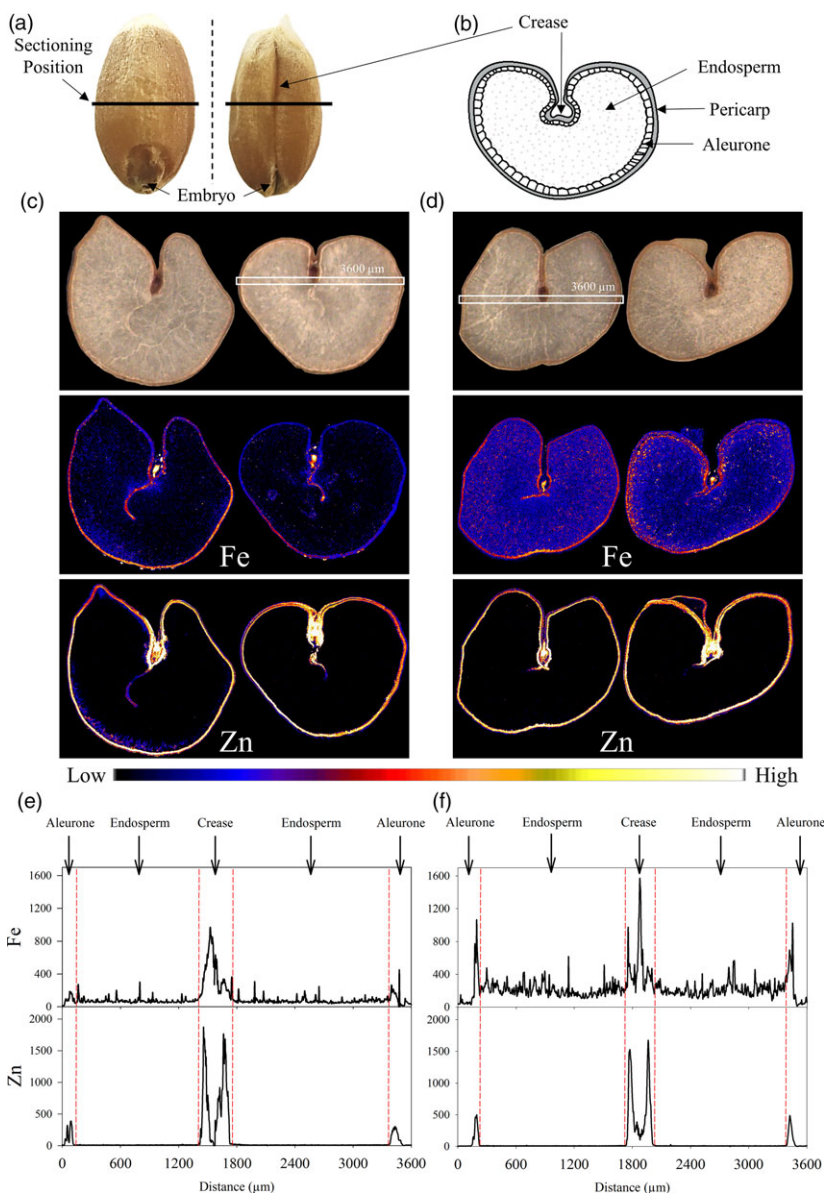


Figure 3 Distribution of Fe and Zn in CE-*OsNAS2* and NS wheat grain. (a) Grain position where transverse cross-sections were made. (b) Location of major tissue types in transverse section of wheat grain. (c) Bright field images of two representative NS grain sections and corresponding XFM elemental maps of Fe and Zn. (d) Bright field images of two representative CE-1 grain sections and corresponding XFM elemental maps of Fe and Zn. Colour bar represents high (white) and low (black) elemental concentration. White boxes in the bright field images represent areas in two grain sections (one each for NS and CE-1) used to generate line scans. (e) Line scans showing the distribution and signal intensity of Fe and Zn across NS grain. (f) Line scans showing the distribution and signal intensity of Fe and Zn across CE-1 grain. Units for the y-axis are elemental counts per pixel.

endosperm was not associated with phytic acid (a P containing compound). Sulphur (S) distribution and intensity (a proxy for protein) appeared slightly reduced in the CE-1 aleurone, relative to NS, but similar to NS in the endosperm (Figure 4e,f).

Constitutive *OsNAS2* expression does not alter the phenotype of field-grown plants and increases Fe, Zn, NA and DMA concentrations in grain and white flour

Replicated plots of three CE-1 sibling lines designated CE-1.1, CE-1.2 and CE-1.3 and the NS were evaluated in confined field trials at Katanning and Merredin, Western Australia in 2015. With the exception of CE-1.1 at Merredin, phenotype of the CE-1 sibling lines and NS did not differ with respect to plant height, spike number, biomass, thousand grain weight (TGW) and grain yield (Table S3). Whole grain Fe concentrations were significantly higher for CE-1.1 at both Katanning ($P = 0.042$) and Merredin ($P = 0.002$) relative to NS (Figure 5a). Whole grain Zn and P concentrations did not differ between the three CE-1 sibling lines and NS at both Merredin and Katanning. All lines (CE-1 and NS)

had higher whole grain Zn and P concentrations at Katanning relative to Merredin, possibly due to lower TGW at Katanning (Figure 5b,c, Table S3). Analysis of NA and DMA showed that whole grain NA concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P = 0.005$ and $P \leq 0.001$, respectively), and for all three CE-1 sibling lines at Merredin ($P = 0.004$, $P = 0.008$ and $P = 0.016$, respectively), relative to NS. Whole grain DMA concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P = 0.014$ and $P = 0.045$, respectively), and CE-1.2 and CE-1.3 at Merredin ($P = 0.022$ and $P \leq 0.001$, respectively), relative to NS (Figure 5d,e).

White flour Fe concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P \leq 0.001$ and $P = 0.012$, respectively) relative to NS (Figure 6a) but did not differ between any line at Merredin. White flour Zn concentrations were significantly higher for all three CE-1 sibling lines at Katanning ($P = 0.002$, $P = 0.010$ and $P = 0.036$, respectively) relative to NS (Figure 6b) but did not differ between any line at Merredin. White flour P concentration did not differ between any line at both Merredin

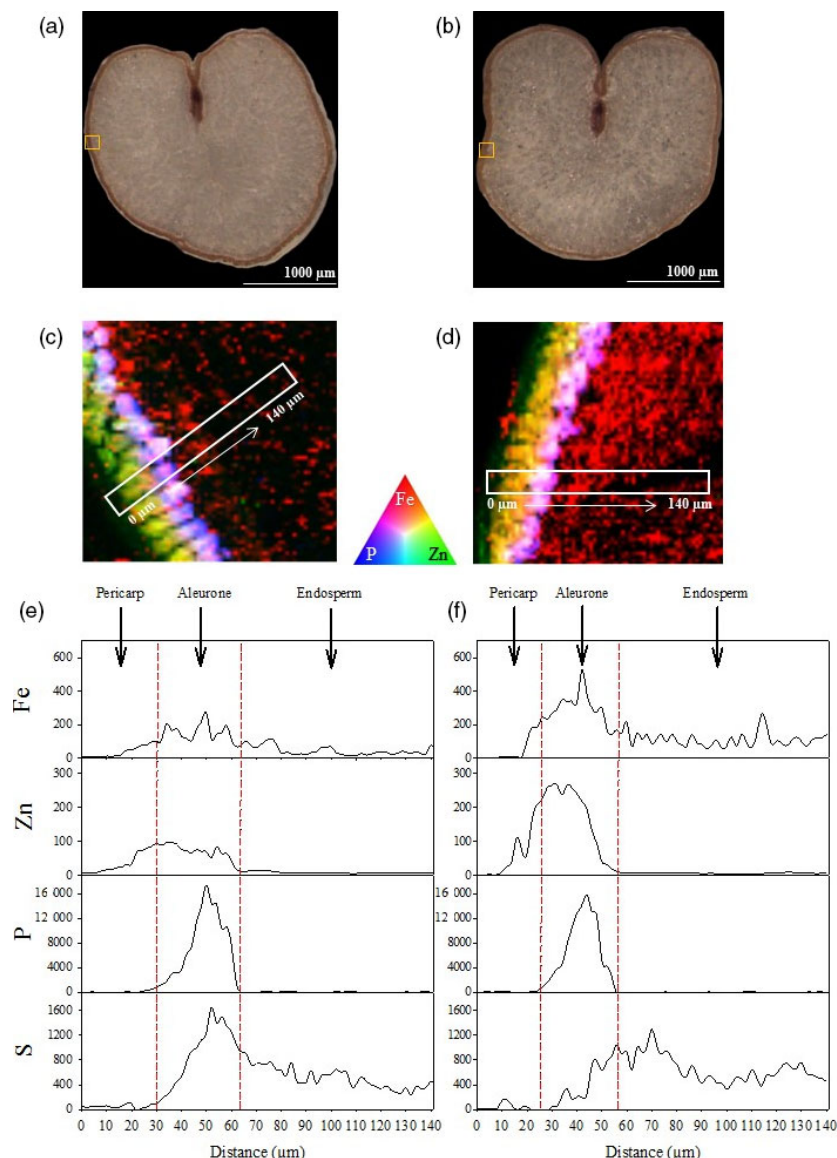


Figure 4 Distribution of Fe, Zn and P in CE-OsNAS2 and NS wheat grain. (a, b) Bright field images of NS and CE-1 grain sections, respectively. Yellow boxes represent areas used to generate tri-colour elemental maps. (c) Tri-colour XFM elemental map of Fe (red), Zn (green) and P (blue) in NS grain. White box represents the area used to generate line scans. (d) Tri-colour XFM elemental map of Fe (red), Zn (green) and P (blue) in CE-1 grain. White box represents the area used to generate line scans. (e) Line scans showing the distribution and signal intensity of Fe, Zn, P and S in NS grain. (f) Line scans showing the distribution and signal intensity of Fe, Zn, P and S in CE-1 grain. Units for the y-axis are elemental counts per pixel.

and Katanning (Figure 6a–c). White flour NA concentrations were significantly higher for CE-1.1 and CE-1.2 at Katanning ($P = 0.004$ and $P \leq 0.001$, respectively) and for all three CE-1 sibling lines at Merredin ($P \leq 0.001$, $P = 0.002$ and $P = 0.002$, respectively), relative to NS (Figure 6d). White flour DMA concentrations were significantly higher for all three CE-1 sibling lines at both Katanning ($P = 0.010$, $P = 0.020$ and $P = 0.035$, respectively) and Merredin ($P = 0.006$, $P \leq 0.001$ and $P = 0.002$, respectively) relative to NS (Figure 6e).

Iron bioavailability is increased in white flour containing increased concentrations of NA and DMA

Iron bioavailability in CE-1.1, CE-1.2, CE-1.3 and NS whole grain and white flour produced from field-grown grain was determined through ferritin assays of *in vitro* Caco-2 cells incubated with flour digests. The levels of bioavailable Fe in CE-1.1, CE-1.2, CE-1.3 and NS whole grain flour were negligible and did not differ significantly; a result likely due to phytic acid and other inhibitory compounds in the outer layers of wheat grain (Figure 5f). By contrast, levels of bioavailable Fe were significantly increased in CE-1.1, CE-1.2, CE 1.3 white flour digests from Katanning

($P = 0.005$, $P = 0.009$ and $P = 0.045$, respectively), and CE-1.1 and CE-1.2 white flour digests from Merredin ($P = 0.050$ and $P = 0.010$, respectively) relative to NS (Figure 6f). White flour Fe bioavailability was significantly correlated with NA concentration ($r = 0.711$, $P = 0.048$) and DMA concentration ($r = 0.812$, $P = 0.014$). White flour Fe bioavailability was not significantly correlated with concentrations of Fe ($r = 0.242$, $P = 0.563$), Zn ($r = -0.132$, $P = 0.755$) and P ($r = -0.433$, $P = 0.284$) (Table 1).

Discussion

S-adenosylmethionine (SAM) is an ubiquitous cosubstrate involved in the production of polyamines, ethylene, biotin, NA and DMA in plants (Mao *et al.*, 2015; Roeder *et al.*, 2009). Because increased *NAS* gene activity diverts more endogenous SAM towards NA and DMA production, it is important to carefully phenotype Fe and Zn biofortified plants with constitutive *NAS* expression to identify any pleiotropic effects that may negatively impact on plant phenotype. In our study we analyzed multiple CE-OsNAS2 transformation events in several glasshouse experiments, and the lead CE-1 event in multi-location field trials

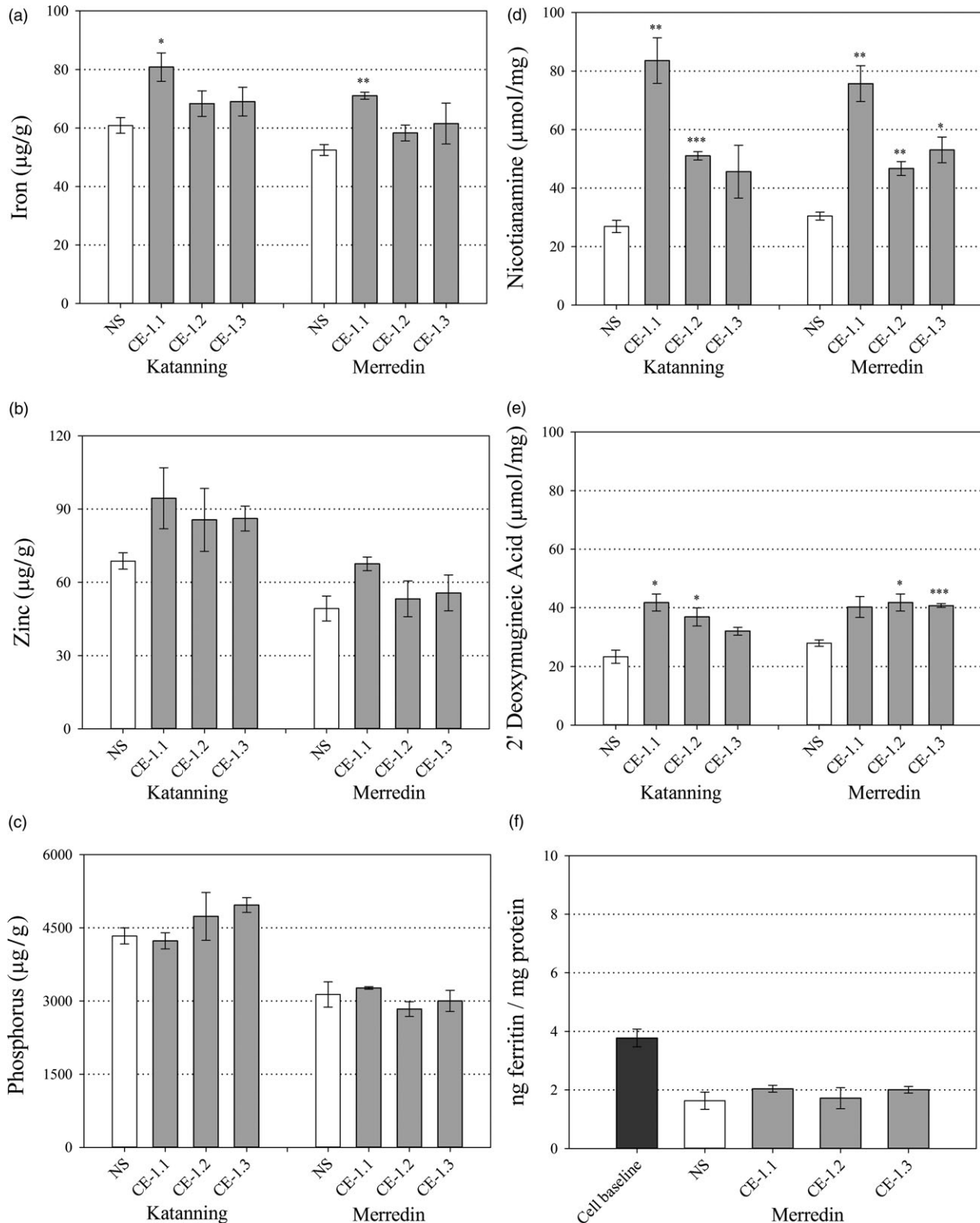


Figure 5 Whole grain nutrition of field grown CE-1 and NS wheat lines. Nutrient and metabolite concentrations in whole grain samples of NS (white) and three CE-1 sibling lines (CE-1.1, 1.2 and 1.3, grey) at the T_6 generation. (a–c) Whole grain Fe, Zn and P concentrations (µg/g) of NS and CE-1 plants grown at Katanning and Merredin field sites. (d, e) Whole grain NA and DMA concentrations (µmol/mg) of NS and CE-1 plants grown at Katanning and Merredin field sites. (f) Whole grain Fe bioavailability of NS and CE-1 plants grown at Merredin field site. Bars represent mean $\pm$ SEM of 3 biological replicates. Asterisks denote the significance between NS and each CE-1 wheat line for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's *t*-test.

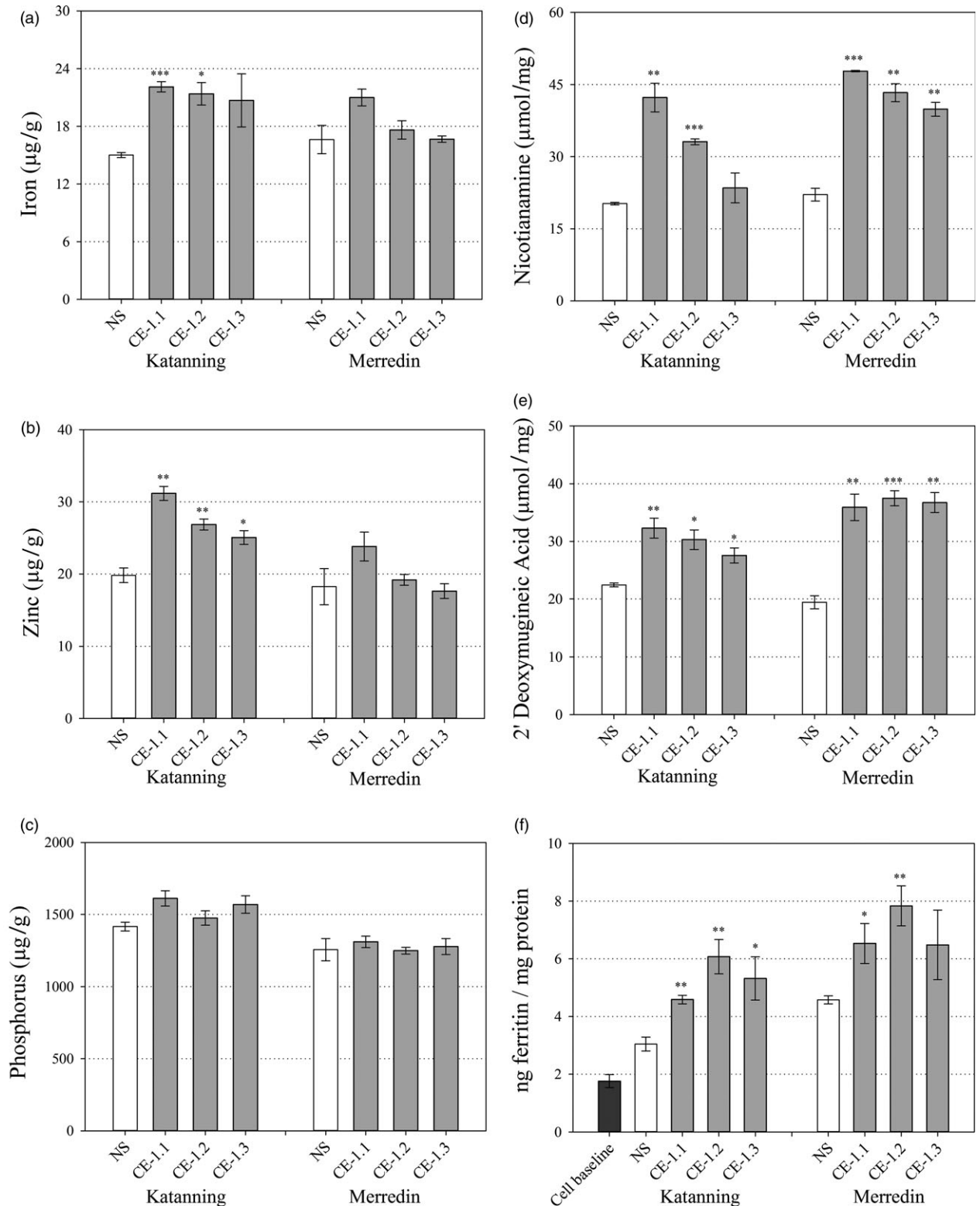


Figure 6 White flour nutrition of field grown CE-1 and NS wheat lines. Nutrient and metabolite concentrations in white flour samples of NS (white) and three CE-1 sibling lines (CE-1.1, 1.2 and 1.3, grey) at the T_6 generation. (a–c) White flour Fe, Zn and P concentrations ($\mu\text{g/g}$) of NS and CE-1 plants grown at Katanning and Merredin field sites. (d, e) White flour NA and DMA concentrations ($\mu\text{mol/mg}$) of NS and CE-1 plants grown at Katanning and Merredin field sites. (f) White flour Fe bioavailability of NS and CE-1 plants grown at Merredin and Katanning field sites. Bars represent mean $\pm$ SEM of 3 biological replicates. Asterisks denote the significance between NS and each CE-1 wheat line for $P < 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***) as determined by student's t -test.

Table 1 Pearson's correlation analysis of NA, DMA, Fe, Zn and P concentrations in NS and CE-1 white flour and ferritin production in Caco-2 cells

	DMA ($\mu\text{mol}/\text{mg}$)	Fe ($\mu\text{g}/\text{g}$)	Zn ($\mu\text{g}/\text{g}$)	P ($\mu\text{g}/\text{g}$)	Ferritin (ng/mg protein)
NA	0.912**	0.440	0.239	-0.180	0.711*
($\mu\text{mol}/\text{mg}$)	0.002	0.276	0.569	0.670	0.048
DMA		0.355	0.132	-0.145	0.812*
($\mu\text{mol}/\text{mg}$)		0.389	0.755	0.732	0.014
Fe ($\mu\text{g}/\text{g}$)			0.893**	0.627	0.242
			0.003	0.096	0.563
Zn ($\mu\text{g}/\text{g}$)				0.854**	-0.132
				0.007	0.755
P ($\mu\text{g}/\text{g}$)					-0.433
					0.284

Bold and non-bold numbers represent correlation coefficients and P values, respectively. Asterisks denote significant correlation between factors for $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

in Western Australia, and found that important agronomical traits such as shoot area, plant height, total grain number and TGW did not differ from NS in most cases (Figure 1, Tables S2 and S3). These results are similar to those reported for glasshouse and field-grown 35S::OsNAS2 rice (Johnson *et al.*, 2011; Trijatmiko *et al.*, 2016), as well as glasshouse grown UBI-1::OsNAS2 wheat (Singh *et al.*, 2017), and demonstrate that constitutive expression of the OsNAS2 gene to biofortify bread wheat with Fe and Zn does not negatively impact on plant phenotype and yield. Determining whether the changes observed in Fe and Zn accumulation patterns during grain filling (Figure 2) relate to increased remobilization of Fe and Zn from vegetative to grain tissues in CE-OsNAS2 plants will require further analysis using radioactive or stable Fe and Zn isotopes (Pottier *et al.*, 2014).

The most striking result from our synchrotron X-ray fluorescence microscopy (XFM) analyses was the increased Fe signal in endosperm tissue of CE-1 grain relative to NS (Figures 3d and 4d). Line scans showed that the increased Fe signal extended throughout CE-1 endosperm and did not co-localize with P (Figures 3f and 4f), indicating that Fe is not associated with phytic acid in this tissue nor the inhibitory effects that phytic acid has on mineral absorption in humans (Hurrell, 2003). By contrast, both CE-1 and NS grain showed high Zn signal co-localized with P in aleurone cells and essentially no Zn signal in the endosperm (Figure 3f and 4f). The observed differences in grain Fe and Zn localization demonstrate that wheat white flour contains Zn that is derived almost entirely from outside of the endosperm. We postulate that the wheat crease, which is difficult to remove through milling (Campbell, 2007), represents the major source of Zn in white flour. Line scans across the crease region showed similar Zn signal intensities in CE-1 and NS modified aleurone cells yet higher Zn signal intensity in the CE-1 nucellar projection (Figure 3e–f; Figure S4), a tissue which has been shown to contain NA-bound Fe (De Brier *et al.*, 2016), and this difference likely accounts for the observed Zn concentration increase in CE-1 white flour (Figure 6b). Interestingly, synchrotron XFM studies of rice grain reveal a radically different Zn distribution pattern that extends throughout the endosperm and shows no obvious

barrier to endosperm loading (Johnson *et al.*, 2011; Kyriacou *et al.*, 2014). Determining whether differences in transport mechanisms or sink strength account for the Zn distribution differences between wheat and rice grain merits further investigation.

While flour milled from CE-1 grain contained significantly higher concentrations of NA and DMA at both Merredin and Katanning field sites, relative to NS, while white flour Fe and Zn concentrations only differed from NS at Katanning (Figure 6a–e). The NS and CE-1 wheat plants were larger and higher yielding at the Merredin field site (Table S3), which may have had a dilution effect on grain micronutrient concentrations and thereby minimized differences between the genotypes. This result demonstrates the importance of multi-location field trials for accurate assessment of wheat Fe and Zn biofortification traits, yet also uniquely enabled us to examine the effects of NA and DMA as promoters of Fe bioavailability (Eagling *et al.*, 2014b; Glahn *et al.*, 1998; Lee *et al.*, 2012; Tako *et al.*, 2016) under differing Fe concentrations. We used the *in vitro* Caco-2 cell line model to measure Fe bioavailability in NS and CE-1 white flour produced at both field sites and found that white flour NA and DMA concentrations were significantly and positively correlated with Fe bioavailability at both sites while white flour Fe concentration was not significantly correlated with Fe bioavailability (Figure 6, Table 1). This result was most evident at the Merredin field trial, where white flour Fe concentrations of two CE-1 sibling lines (CE-1.2 and CE-1.3) were virtually identical to that of NS, however, CE-1.2 white flour contained 1.7-fold more bioavailable Fe than NS (Figure 6a and f). In addition to Fe, white flour P concentrations were unchanged between NS and CE-1 sibling lines, indicating that altered white flour phytate/Fe ratios are not likely to be responsible for the increase in Fe bioavailability (Figure 6c). These results reinforce the finding that NA and/or DMA bind Fe in a readily bioavailable form within wheat white flour (Eagling *et al.*, 2014a) and suggests that NA and/or DMA mediate increased uptake of Fe into human cells independent of endosperm Fe concentration. As such, NA and DMA can be viewed as important phytonutrients that increase Fe bioavailability in plant foods (Martin and Li, 2017; Nozoye, 2018), and metabolic engineering strategies focused on increasing their concentrations could help offset the effects of decreased mineral concentrations forecast for C₃ grains by mid-century.

Methods

Vector construction and generation of wheat transformation events

The full-length coding sequence of OsNAS2 (LOC_Os03g19420) was PCR amplified from rice (*Oryza sativa* L.) cv. Nipponbare genomic DNA (Johnson *et al.*, 2011). Recombination into a modified pMDC32 vector (Curtis and Grossniklaus, 2003) with the hygromycin phosphotransferase plant-selectable marker gene placed OsNAS2 under transcriptional control of the maize (*Zea mays* L.) ubiquitin 1 promoter. Particle bombardment of the construct into immature wheat (*Triticum aestivum* L.) cv. Bobwhite embryos (1.0–1.5 mm in length) was performed at the University of Adelaide (Adelaide, Australia) using established protocols (Kovalchuk *et al.*, 2009). Plants were grown in glasshouse conditions (12 h photoperiod, 23 °C day/12 °C night, 50% humidity) in soil (coconut peat and sand mixture) with complete fertilizers.

Insert copy number analysis

Genomic DNA (10 µg) was isolated from CE-*OsNAS2* leaf tissue and digested with *DraI* and *HindIII* restriction enzymes. Restriction fragments were separated by gel electrophoresis (0.8% agarose) alongside a positive barley control and blotted to a nylon membrane. Two independent hybridizations of a ³²P-labelled probe to both nopaline synthase terminator and dual 35S promoter were performed using established protocols (Pallotta *et al.*, 2014).

Automated phenotyping

Grain were sown in white plastic pots (14 × 19 cm) containing 2.5 kg of soil mixture (equal parts clay-loam soil and coconut peat) and Osmocote® fertilizer. Plants were maintained under glasshouse conditions (12 h photoperiod, 24 °C day/18 °C night, 50%–90% humidity) in the phenotyping platform of The Plant Accelerator (Adelaide, Australia). Projected shoot area and plant height were measured 100 days after sowing using the conveyor automated imaging system (Berger *et al.*, 2012). Grain number and TGW were manually determined at harvest.

Inductively coupled plasma optical emission spectrometry (ICP-OES)

Plant tissues were submerged in 0.1% Tween 20 solution, rinsed with dH₂O and oven dried for 48 h at 60°C before grinding to a powder using an IKA tube mill (www.ika.com). Inductively coupled plasma optical emission spectrometry (ICP-OES) analysis was conducted at Waite Analytical Services (Adelaide, SA, Australia), the Robert W. Holley Centre for Agriculture and Health (USDA-ARS, Ithaca, NY) and the CSBP Soil and Plant Analysis Laboratory (Perth, WA, Australia).

Quantitative reverse transcription PCR (qRT-PCR)

Shoot and root tissues (without the crown) of 4-week-old plants were separated, cleaned with dH₂O and snap frozen. Three plants of each genotype (representing one biological replicate) were combined and total RNA was extracted from pulverized frozen plant tissue (100–150 mg) using TRIzol Reagent (Life Technologies, Carlsbad, CA) and a commercial kit (Direct-zol™; Zymo-Research, Irvine, CA). Genomic DNA was removed from RNA (2 µg) using a DNase I treatment (Promega, Madison, WI) and reverse transcription was performed using a commercial kit (Bioline).

Consensus primers were designed to amplify homeologous groups of *TaNAS*, *TaNAAT* and *TaDMAS* gene families using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0>) software. Each biological replicate was analyzed in triplicate and transcripts were quantified against four replicates of 10-fold serial dilutions (10²–10⁸) for each purified PCR template (DNA Clean & Concentrator™-5; ZymoResearch). Expression levels of *OsNAS2*, *TaNAS*, *TaNAAT* and *TaDMAS* were measured in root and shoot tissues using qRT-PCR analysis (CFX384-BioRad). The geometric mean expression of three housekeeping genes: *TaCyclophilin*, *TaGAPDH* and *TaELF* and *TaGAPDH*, *TaActin* and *TaELF*, was used to normalize *OsNAS2*, *TaNAS*, *TaNAAT* and *TaDMAS* gene expression within shoot and root tissues, respectively (Schreiber *et al.*, 2009; Vandesompele *et al.*, 2002). All primers had annealing temperatures between 61–65 °C and primer sequences and efficiencies are provided (Table S4).

Quantification of NA and DMA

Quantification of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatized NA in whole grain was performed via liquid chromatography-mass spectrometry (LC-MS) using established protocols (Callahan *et al.*, 2007; Johnson *et al.*, 2011). Quantification of 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl) derivatized NA and DMA in whole grain (Figure 5d,e) and white flour (Figure 6d,e) was performed via RP LC-MS on an 1290 Infinity II and 6490 Triple Quadrupole LC/MS system (Agilent Technologies Inc., Santa Clara, CA) using established protocols (Selby-Pham *et al.*, 2017). Briefly, sequential methanol (100%) and deionized H₂O (18MΩ) extractions of pulverized wheat grain or white flour (25 mg) were combined and added (5 µL) to sodium borate buffer (pH = 8, 1 M, 10 µL), EDTA buffer (pH = 8, 50 mM, 10 µL) and fresh FMOC-Cl solutions (50 mM, 40 µL). After incubation (60 °C, 700 rpm, 15 min), the derivatization reaction was quenched via the addition of formic acid (FA; pH = 4, 5%, 8.9 µL). A Zorbax Eclipse XDB-C18 Rapid Resolution HS 2.1x100 mm, 1.8 µm particle size column (Agilent Technologies Inc.) was used during chromatography with aqueous (0.1% v/v FA in dH₂O) and organic (0.1% v/v FA in acetonitrile) mobile phases. For quantification, a stock aqueous solution of NA and DMA (Toronto Research Chemicals, Toronto, ON, Canada) was prepared at 750 µM and a calibration set was generated in the range of 0.005 to 75 µM.

Analysis of Fe and Zn accumulation post anthesis

Plants were grown in glasshouse conditions (12 h photoperiod, 18 °C day/13 °C night, 40%–80% humidity) in Hortico® potting mix with Osmocote® fertilizer at The University of Melbourne (Victoria, Australia). The main stem flag leaf, rachis, bracts and grain were harvested at 5–8 DAA (days after anthesis), 12–15 DAA, 19–21 DAA, 26–29 DAA and maturity. Samples were washed, oven dried for 48 h at 60°C and ground to a powder before analysis by inductively coupled plasma mass spectrometry (ICP-MS) at the Environmental Analysis Laboratory (Lismore, NSW, Australia).

Synchrotron X-ray fluorescence microscopy (XFM)

Elemental X-ray fluorescence (XRF) maps of Fe, Zn, Cu and Mn in transverse cross-sections of two representative CE-1 and NS grain (four sets of maps total) were collected at the XFM beamline at the Australian Synchrotron (Melbourne, Australia) as previously described (Van Malderen *et al.*, 2017). Briefly, the beam energy was set at 15.6 keV and the beam focused to approximately 2 × µm² using Kirkpatrick-Baez mirrors. Samples were analyzed continuously in the horizontal direction with a sampling interval of 4 µm and a step size of 4 µm in the vertical direction (pixel transit time was set at 5.2 ms). The XRF signal from the 80 µm transverse grain sections was collected using a 384-element Maia detector system. Tri-colour elemental maps showing the distribution of Fe, Zn and P near the grain edge of one representative CE-1 and NS grain (two sets of maps total; different grain from those used with the Maia detector) were collected using a separate Vortex-EM detector. The tri-colour maps were used as guides to select rectangular areas of approximately 14 × 140 µm near the grain edge for the generation of Fe, Zn, P and S line scans. Elemental maps were generated using GeoPIXE (<http://nmp.csiro.au/GeoPIXE.html>) software. The NS grain contained 38 µg/g DW Fe, 71 µg/g DW Zn, 4700 µg/g DW P and 1630 µg/g

DW S while CE-1 grain contained 69 µg/g DW Fe, 122 µg/g DW Zn, 5300 µg/g DW P and 2100 µg/g DW S (Table S5).

Confined field trials

Confined field trials were conducted in Western Australia from June to December 2015 at the New Genes for New Environment facilities located in Merredin (31.4837° S, 118.2771° E) and Katanning (33.6894° S, 117.5551° E). Grain were sown in 2 m² plots with three replicate plots per genotype and arranged in a randomized block design at each site. Rows were spaced at 30 cm and grain were sown at a rate of 60 kg/ha. At maturity, average plant height was determined from three representative measurements per plot and spike number, total biomass and TGW were determined from 0.15 m² subsamples per plot (Table S3). Grain yield was calculated from the amount of grain harvested per 2 m² plot and extrapolated to kg/ha. Soil properties of both field sites are provided in Table S6.

Production of white flour

Whole grain samples harvested at Merredin and Katanning were conditioned to 13% moisture content for 24 h prior to milling. Each sample was milled using a Quadrumat Junior laboratory mill (Brabender, Duisburg, Germany) at constant temperature and run through a 280 µm sieve to isolate the white flour fraction. Average flour extraction for all lines from Merredin and Katanning was 71.5 ± 0.2%.

Caco-2 cell culture assessment of Fe bioavailability

Whole grain and white flour samples were digested for Caco-2 cell Fe-bioavailability analysis as previously described (Glahn *et al.*, 1998; Trijatmiko *et al.*, 2016). The Caco-2 cells were maintained in supplemented Dulbecco's modified Eagle medium (DMEM) for 11 days post-seeding and replaced with supplemented minimum essential media (MEM) solution 48 h prior to the experiment. On the experiment day, gastric-digested samples (1.5 mL) were added to cylindrical Transwell inserts (Corning Life Sciences, Corning, NY) fitted with a semipermeable (15 000 Da MWCO) basal membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA). The inserts were placed within wells containing Caco-2 cell monolayers and incubated for 2 h (37 °C), after which the inserts were removed and additional MEM (1 mL) added to the cells before incubation for 22 h (37 °C). After incubation, growth medium was removed by aspiration and the Caco-2 cells were washed twice with a solution (pH = 7.0) containing NaCl (140 mmol/L), KCl (5 mmol/L) and PIPES (10 mmol/L) and harvested with the addition of dH₂O (1.5 mL) and brief sonication (Lab-Line Instruments, Melrose Park, IL). In an aliquot of the Caco-2 cell solution, ferritin content was determined using an immunoradiometric assay (FER-IRON II Ferritin Assay, Ramco Laboratories, Houston, TX) and total protein content was determined using a colorimetric assay (Bio-Rad DC Protein Assay, Bio-Rad, Hercules, CA). As Caco-2 cells synthesize ferritin in response to intracellular Fe, we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as an index of cellular Fe uptake.

Statistical analysis

All graphs and statistical analyzes were generated using Minitab® 17 Statistical Software, Minitab, State College, PA and SigmaPlot v13, Systat Software Inc., San Jose. Data are presented as mean ± SEM with biological replicate numbers noted in table and figure legends. Student's *t*-test was used to determine

significant differences between means. Data are available upon request to the corresponding author of this paper.

Acknowledgements

This work was supported by grants from the HarvestPlus Challenge program to A.A.T.J. We thank Bettina Berger at The Plant Accelerator (Adelaide, Australia), Chris Ryan at the Australian Synchrotron (Victoria, Australia) and Adrian Cox, Leigh Smith, Shahajahan Miyan and Ed Barrett-Lennard at the Department of Primary Industries and Regional Development Western Australia (Western Australia, Australia) for excellent technical assistance related to the phenotyping, XFM and field trial components of our research. We also thank Lorraine Carruthers, Margie Pallotta, Jan Nield, Mary Bodis, Yongpei Chang and Shree Giri for invaluable help provided throughout the project. Analysis of NA and DMA was carried out at Metabolomics Australia (School of BioSciences, The University of Melbourne, Australia), an NCRIS initiative under Bioplatforms Australia Pty Ltd.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Flowchart detailing the analyses performed for each generation of the Ubi::OsNAS2 lead event (CE-1) from transformation T₀ to T₆ generation.

Figure S2 Relative quantification of DMA biosynthetic gene transcript levels in NS and CE-1 shoots (left) and roots (right).

Figure S3 Concentrations of Fe, Zn, NA and DMA in NS and CE-1 seedling shoot tissue.

Figure S4 Tri-colour elemental maps of Fe, Zn and Cu in transverse cross-sections of one representative NS and CE-1 grain.

Figure S5 Elemental maps of Cu and Mn in transverse cross-sections of two representative NS and CE-1 grain.

Table S1 Iron and zinc concentrations ($\mu\text{g/g DW}$) in NS and CE-1 plant tissues at 5–8, 12–15, 19–21 and 26–29 days after anthesis (DAA) as well as maturity.

Table S2 Biomass (mg DW) of NS and CE-1 plant tissues at 5–8, 12–15, 19–21 and 26–29 days after anthesis (DAA) as well as maturity.

Table S3 Agronomic performance of NS and CE-1 sibling lines grown at the Katanning and Merredin field sites.

Table S4 Wheat genes and primers used for quantitative reverse transcription PCR (qRT-PCR) analysis of CE-1 and NS seedling shoot and root tissue.

Table S5 Elemental concentrations ($\mu\text{g/g DW}$) of T_3 grain harvested from 10 NS and 9 CE-1 plants including the two batches used for synchrotron XFM analysis (in bold).

Table S6 Soil properties of Katanning (K) and Merredin (M) field sites.