Functional characterisation of the barley ZIP7 zinc transporter

Jingwen Tiong

B. Science (Hons), The University of Adelaide

A thesis submitted for the degree of

Doctor of Philosophy

The University of Adelaide

Faculty of Sciences

School of Agriculture, Food & Wine

Waite Campus

February 2012

Contents

List of figures

List of tables

Abstract

Zinc (Zn) is an essential micronutrient for the function of many biological processes. Zn deficiency therefore affects normal growth and development in all living organisms. Many cropping soils are low in Zn, leading to widespread Zn deficiency in cereal crops. This reduces crop yield and grain nutrition value, which increases the risk of Zn deficiency-related health problems, especially for women and children in developing countries. Increasing Zn density in cereal grains (biofortification) is a potentially effective strategy to alleviate widespread Zn malnutrition in humans. However, grain Zn biofortification is restricted by the translocation of Zn from roots to shoots and from shoots to grains. So far, little is known about the metal transporters involved in these two limiting processes. Identification and characterisation of these transporters are crucial for Zn biofortification. The Zn regulated, Iron regulated-like Protein (ZIP) family is a group of transporters controlling cellular Zn influx, and could be important for Zn uptake and translocation in plants. In this study, barley (*Hordeum vulgare* L.) was used as a model plant to identify and characterise new ZIP transporters. Nine new HvZIP members were identified, and transcript analyses of 13 *HvZIP* genes revealed that eight of them were induced in Zn-deficient plants. *HvZIP7* was further characterised as there is no available functional information for this transporter. *HvZIP7* is primarily expressed in vascular tissues of roots and shoots and localises in the plasma membrane when expressed in onion epidermal cells. The over-expression of *HvZIP7* resulted in a specific increase of Zn translocation from roots to shoots and Zn accumulation in shoots and grains when Zn is abundant in the growth media, suggesting that HvZIP7 mediates Zn translocation and/or retranslocation. The enhanced Zn accumulation in plants did not affect plant growth or grain yield. Furthermore, the over-expression of *HvZIP7* did not enhance Cd accumulation in shoots, which differs from the over-expression of heavy metal ATPases. In addition, transgenic *HvZIP7* plants could achieve 30% higher Zn concentration in grains than

that of controls when grown with a low dose of Zn fertilisers under conditions similar to the field, indicating that our findings would have direct applications to Zn biofortification and the improvement of plant Zn nutrition in cereals.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Jingwen Tiong and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, *via* the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

…… ……

Jingwen Tiong Date

Acknowledgements

First and foremost, I am most grateful to my principal supervisor Dr Chunyuan Huang. Your dedication, vast knowledge and unwavering patience have helped guided me to completing the PhD. Thank you for all your support during both good and bad times. To my co-supervisor, Assoc. Prof Glenn McDonald, thank you for all your guidance. Your knowledge and ideas have been extremely pivotal to the completion of my project.

I gratefully acknowledge the financial support provided during my candidature from the University of Adelaide through the provision of the Adelaide Graduate Fee Scholarship, and The Australian Centre for Plant Functional Genomics (ACPFG), along with its funding bodies (the Grains Research and Development Corporation, the Australian Research Council, the University of Adelaide and the South Australian Government) for research and financial support.

Many people in the ACPFG and Waite Campus have supported me with advice and services, and have thus made this project an enjoyable experience. I am extremely grateful to Prof Peter Langridge for intellectual inputs to the project. I am also thankful to Dr Yusuf Genc for his contribution in the project; your knowledge input, advice, friendship and much-needed manpower were vital in inspiring me to complete my PhD. I like to extend my sincere thanks also to Dr Rohan Singh for producing transgenic *HvZIP7* plants, Dr Neil Shirley, Mr Stephen Fletcher and Ms Yuan Li for qRT-PCR analyses, Mr John Toubia for phylogenetic analyses, Dr Gwen Mayo and Dr Simon Conn for their excellent guidance for *in situ* PCR work, Dr Serik Eliby, Dr Ainur Ismagul and Mr John Harris for assistance in the biolistic particle bombardment, Dr Juan Juttner for providing the *HvTrx5::GFP* construct in the onion epidermis transient assay, Dr Pai Pedas and Dr Julie Hayes for yeast complementation experiments, Dr Ursula Langridge for her excellent assistance in growing transgenic plants,

Assoc. Prof James Stangoulis and Ms Bianca Kyriacou for allowing me to use the grain miller, Mrs Teresa Fowles and Mr Lyndon Palmer for ICP analyses, and Ms Margaret Pallotta for assistance in Southern blotting and proof-reading thesis chapters.

The PhD journey has been mostly a lonely one, but the safe, helpful and friendly nature in the centre has helped a lot in making the experience less painful. I would like to thank both past and present Honours, Masters and PhD students and the administration team of ACPFG for organising fun and helpful events, and also for lending an ear whenever I needed to relax or vent out.

On a more personal note, I would like to thank my friends in Adelaide who have helped made my life as a PhD student less empty. To my dear friends Hong-Yau, Kevin, Kelvin and Wan Chin and, you have helped get me through extremely tough times, and I am forever indebted to you guys.

Finally, I would like to thank my Mum, Dad and brother Jingren for their support. Although not being physically present here with me, I know that they have always been cheering me on.

Glossary of abbreviations

Abbreviation	Full term
β	Beta
°C	Degree Celsius
µg	Microgram(s)
µM	Micromolar
µmol	Micromole
ATP	Adenosine triphosphate
At	*Arabidopsis thaliana*
BAC	Bacterial artificial chromosome
BLAST	Basic Local Alignment Search Tool
Bd	*Brachypodium distachyon*
C-terminal	Carboxyl terminal
CA	Carbonic anhydrase
CaMV	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
CDF	Cation diffusion facilitator
CoT	Cobalt transporter
cv.	Cultivar
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriaminepentaacetic acid
dUTP	Deoxyuridine triphosphate
DM	Dry matter
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
FAA	Formalin-acetic acid-alcohol
FAOSTAT	Food and Agriculture Organisation Statistical Database
g	Gram(s)
GFP	Green fluorescent protein
Gm	*Glycine max*
GUS	Beta-glucuronidases
HEDTA	Hydroxyethyl ethylenediamine triacetic acid
HMA	Heavy metal ATPase
Hr	Hour(s)

Hv	*Hordeum vulgare*
IAR	Indole-3-acetic acid resistant
IBSC	International Barley Genome Sequencing Consortium
ICP-OES	Inductively coupled plasma optical emission spectrometry
IRT	Iron-regulated transporter
kg	Kilogram(s)
Le	*Lycopersicon esculentum*
LSD	Least significant difference
M	Molar
m	Meter(s)
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram(s)
min	Minute(s)
MSU	Michigan State University
Mt	*Medicago truncatula*
MTP	Metal tolerance protein
N-terminal	Amino terminal
NADPH	Nicotinamide adenine dinucleotide phosphate
NAS	Nicotianamine synthase
NCBI	National Center for Biotechnology
OPT	Oligopeptide transporter
Os	*Oryza sativa*
OX	Over-expressing
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PM	Plasma membrane
PT	Phosphate transporter
Pv	*Phaseolus vulgaris*
RGAP	Rice Genome Annotation Project
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcript polymerase chain reaction
s	Second(s)
SE	Standard error
SOD	Superoxide dismutase

ssp.	Sub-species
Ta	*Triticum aestivum*
TAIR	The Arabidopsis Information Resource
Tc	*Thlaspi caerulescens*
Td	*Triticum turgidum*
Tg	*Thlaspi goesingense*
Tj	*Thalspi japonicum*
TM	Trans-membrane
Tr	Transgenic
Trx	Thioredoxin
U	Unit(s)
WT	Wildtype
YS	Yellow strip
YSL	Yellow stripe-like
ZIP	Zinc-regulated, iron-regulated transporter-like protein
ZnT	Zinc transporter
ZRC	Zinc resistant conferring
ZRT	Zinc regulated transporter
ZTP	Zinc transporter protein

Chapter 1: Literature Review

1.1 Introduction

Zinc (Zn) is an important micronutrient for maintaining normal growth and reproduction processes in plants, humans and animals. As Zn is an important co-factor for the function of a myriad of essential enzymes, Zn deficiency affects numerous metabolic processes in living organisms. Zn deficiency is also one of the most widespread abiotic stresses in agriculture (Broadley et al., 2007; Grotz et al., 1998; Hacisalihoglu et al., 2004). If Zn deficiency in crop plants is not corrected, the productivity and nutritional value of food crop will be severely hampered. This is a bleak outcome in the effort to support the dietary needs for the ever growing human population, which is expected to reach 10 billion by the end of this century (Lee, 2011).

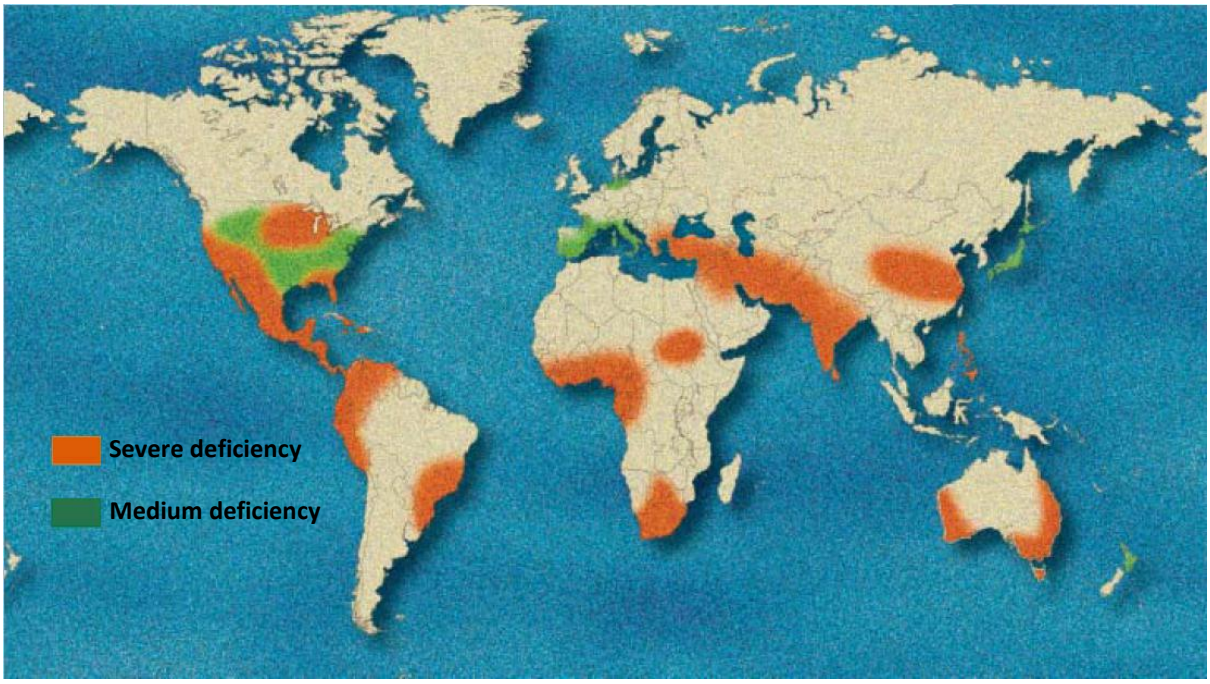

Figure 1.1. Global distribution of major cropping soils affected by Zn deficiency (Alloway, 2001).

Zinc deficiency in crops is widespread as a large part of the world's major cropping soils are deficient in Zn (Figure 1.1; Alloway, 2001). Soils low in available Zn are also widespread in the agricultural zones of Australia (Alloway, 2001). Despite various efforts to enhance the availability of Zn in soils, such as by the addition of fertilisers, crops are still not effectively utilising the added Zn to sustain normal growth (Cakmak, 2008). This may be due to the selection of cultivars that are poor in taking up Zn (Genc et al., 2002; Graham et al., 1992; Hacisalihoglu & Kochian, 2003), the inefficient use of Zn fertilisers or having problematic soils (in both physical and chemical terms) that restricts the mobilisation of Zn to plant roots (Cakmak, 2008). This problem of low accessibility of Zn to crops and pastures is a major limiting factor for the sustainable production of crops (Grewal & Graham, 1998).

Symptoms of severe Zn deficiency in plants include chlorosis (which may develop into interveinal necrosis), stunted growth and deformed leaves (Alloway, 2004; Hacisalihoglu et al., 2003). Although symptoms of severe Zn deficiency are easily diagnosed, plants experiencing moderate Zn deficiency are difficult to identify due to the lack of obvious symptoms (Alloway, 2004) and moderate deficiency can lead to significant reductions in vegetative growth and grain yield. Crops with moderate deficiency may go through crucial growth stages such as tillering and booting without displaying any symptoms, thus not giving any indication that a remedy is needed. By the time yield has begun to form, any remedial application of Zn will be too late. Moderate or severe Zn deficiency during crucial growth stages can reduce crop yield by at least 40% if left undiagnosed and untreated (Alloway, 2004).

Wheat, rice, maize, and barley are the main staple foods consumed by humans (http://faostat.fao.org/). However, a significant amount of cereal-based foods consumed is likely to have inherently low concentrations of Zn due to widespread Zn deficiency in soils (Ozturk et al., 2006). Consumption of these foods can cause Zn deficiency in humans,

resulting in health problems such as diarrhoea, retarded mental development and anorexia (Cunningham-Rundles et al., 2005; Hotz et al., 2004). Clearly, there is a need to improve Zn nutrition in cereal-based foods to help alleviate health problems associated with Zn deficiency in humans.

1.2 Improvement of plant Zn nutrition and biofortification

The widespread occurrence of Zn deficiency in human populations has resulted in considerable efforts to overcome nutrient deficiencies and to improve the nutrient density of essential food crops. The average grain Zn concentrations of wheat, rice and barley are similar at around 25 mg kg^{-1} DW (Graham et al., 1992; Lombi et al., 2011; Ramesh et al., 2004; Rengel et al., 1999; Ruel & Bouis, 1998). For wheat, this concentration is deemed inadequate for human consumption (Cakmak et al., 2010). Biofortification programs in cereals aim to increase the concentration of Zn (as well as other micronutrients) in the grain to increase the micronutrient intake of consumers (Welch, 2005). The Harvestplus program, an international initiative to reduce micronutrient malnutrition by improving the micronutrient content of staple foods, suggested that successful wheat and rice grain Zn biofortification should be targeted at 33 and 24 mg kg^{-1} DW respectively, although no such target was assigned to barley grains (www.harvestplus.org). Different approaches have been deployed for improvement of plant Zn nutrition and biofortification. They include agronomic approaches, genetic approaches, or a combination of both.

1.2.1 Application of Zn fertilisers as an agronomic approach

Zn availability in soil is affected by several factors, such as pH, drought and organic matter (Genc, 1999, Alloway, 2004; Broadley et al., 2007). For example, high soil pH (due to high $CaCO_3$ content) causes the formation of insoluble calcium zinctate or Zn salts, such as

hydroxide, oxide, carbonate or phosphate salts (Genc 1999), which decreases Zn desorption from soil and limits Zn absorption by roots (Lindsay, 1991; Marschner, 1993). Agronomic approaches involve the application of fertilisers to increase the availability of micronutrients in the soil, enhancing the uptake of the essential micronutrients by the plants. Applying Zn fertilisers either in the solid granular form or by foliar spray is a common practice to boost the intake of Zn by plants (Cakmak, 2008). After the application of Zn fertilisers, the grain Zn concentrations from a variety of wheat and barley genotypes could be increased from 9-12 mg kg^{-1} DW to 20-30 mg kg^{-1} DW (Graham et al., 1992). However, the distribution of Zn in soils when added as fertilisers is uneven, limiting the uptake of Zn by plants (Alloway, 2004; Broadley et al., 2007). Zn fertilisers are generally distributed in the top soil with little delivered into the subsoil (Alloway, 2004; Broadley et al., 2007). Consequently, Zn uptake by roots in the subsoil is limited as topsoil drying reduces the solubility and leaching of Zn into the subsoil (Marschner, 1993; Rattan & Deb, 1981; Wang et al., 2006).

In contrast to soil-applied Zn, foliar Zn application primarily relies on the remobilisation of the sprayed Zn from the leaves into other parts of the plant including the grains, and largely bypasses the factors in soils that limit Zn uptake. Furthermore, foliar Zn spray can lead to a portion of the Zn being applied to reach the soil surface, which promotes the leaching of Zn into the subsoil due to its soluble nature. Therefore, foliar Zn fertilisers usually have a more immediate effect than granular Zn fertilisers. However, multiple fertiliser applications are necessary to keep up with the demand for Zn during crop growth (Cakmak, 2008). The need for a repeated supply of fertilisers is expensive for farmers, especially in areas where soil pH is high.

1.2.2 Breeding and genetic engineering for improving Zn nutrition

An alternative to agronomic approaches for improving Zn nutrition and grain Zn loading is the genetic approach. Genetic variation in Zn efficiency has been found in cereals such as barley and wheat (Graham et al., 1992). Zn efficiency refers to a plant's ability to grow better and maintain yields under low Zn conditions (Broadley et al., 2007; Graham & Rengel, 1993; Hacisalihoglu & Kochian, 2003; Ramesh et al., 2004), and is therefore sustainable in cereal growing regions with low soil Zn availability (Genc et al., 2006; Graham et al., 1992). The selection and breeding of Zn-efficient cereal varieties is thus the most common genetic approach implemented. However, there is little correlation between Zn efficiency and grain Zn content; many studies have reported very little differences in shoot and grain Zn content between cereal lines with different Zn efficiency when grown under controlled conditions (Chen et al., 2010; Genc et al., 2009; Kalayci et al., 1999). Even if lines with different Zn efficiency display genotypic variations in grain Zn content, it is often associated with negative correlations with grain yield and size (McDonald et al., 2008). Therefore, while the selection and breeding of Zn efficient lines may be helpful for maintaining the growth of crops in Zn deficient soils, it may not be an appropriate approach for increasing Zn grain accumulation.

However, genotypic variation in grain Zn content does exist in cereal lines, thus presenting the opportunity to breed for lines with high Zn grains (Cakmak, 2008; Lonergan et al., 2009). For example, accessions of wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) have been reported to have high grain Zn concentrations (up to 139 mg kg^{-1} DW) (Peleg et al., 2008). Even in some modern wheat cultivars, grain Zn concentrations of up to 85 mg kg^{-1} DW have been found (Welch, 2001). Variations in grain Zn levels also exist in germplasms of rice (up to 45 mg kg^{-1} DW) (Wissuwa et al., 2008), and has been exploited in breeding programs (Graham et al., 1999). However, breeding applications for achieving Zn

biofortification has its limitations, most notably due to the long timeline requirement in identifying useful genetic variations, crossing and back-crossing activities, and measuring the stability of the target traits (Cakmak, 2008). Moreover, many cropping soils are deficient in Zn; the low level of Zn in the supply pool prevents Zn from being absorbed and transported into the grains. This masks the high grain Zn content traits in cereal cultivars that are supposed to display this trait. Thus, improvements in both Zn efficiency and Zn loading to the grain are complementary goals for increasing grain Zn concentrations in Zn deficient soils.

Genetic engineering is another form of the genetic approach, usually involving the generation and utilisation of transgenic plants. This strategy includes introducing single or multiple Zn homeostasis genes, whose expression profiles have been manipulated, into the genome of a targeted plant. If the integration of these transgenes can lead to improvements in plant Zn nutrition and grain Zn loading, this strategy can save significant amounts of time and money relative to breeding strategies. However, a detailed understanding of the mechanism of Zn homeostasis is necessary to identify crucial components of the mechanism. Identifying these crucial components permits the selection of candidate genes for application in the transgenic approach. More of this will be discussed in later sections.

The transgenic approach has been successfully used to overcome another micronutrient problem in rice – the low levels of vitamin A – through the development of the β-carotene-fortified ‘Golden Rice’ (Lucca et al., 2006). Ye et al. (2000) targeted the expression of a daffodil and a soil bacterium β-carotene (the precursor of vitamin A) biosynthesis gene in rice (cv. Japonica) endosperm to enable an increase in β-carotene biosynthesis within the endosperm. This resulted in increased levels of β-carotene, indicated by the yellow colour of the endosperm that is otherwise white in wildtype rice. A subsequent modification of this technique by another group (Paine et al., 2005) further increased the levels of β-carotene by approximately 23-fold compared to that achieved by Ye et al. (2000).

Although the β-carotene-biofortified rice is so far not available yet for human consumption, it illustrates the ability of genetic manipulation to increase essential micronutrient levels in cereal crops, especially within the grain. Increasing Zn content in cereal crops by genetic manipulation could assist in increasing the nutritional quality of cereal crops and overcoming low Zn content in staple diets.

1.3 Zinc homeostasis in plants

1.3.1 Zinc uptake and translocation in the plant

Zinc is taken up by plants as the divalent cation, Zn^{2+} (Broadley et al., 2007; Hacisalihoglu et al., 2001; Lasat et al., 1996). In soils, Zn^{2+} ions can also bind with ligands such as citrate, malate, oxalate and phytosiderophores (Suzuki et al., 2008) which assist Zn to move towards root hairs. After Zn^{2+} ions are taken up by root epidermal cells, Zn is first transported through the cortex *via* symplastic and apoplastic pathways until it reaches the endodermis (Figure 1.2).

In the endodermis, the movement of Zn *via* the apoplastic pathway is terminated by the Casparian band, preventing direct entry of Zn from the apoplastic pathway to the stele (Figure 1.2). To pass the Casparian band, the apoplastic Zn needs to enter the symplastic pathway, which guides the cell-to-cell movement of Zn to cross the Casparian band. This process is mediated by Zn transporters embedded in plasma membranes of endodermal cells. Once Zn^{2+} ions pass the Casparian band and endodermis, they move into xylem parenchyma cells which subsequently load Zn into the xylem (Figure 1.2 and see section 1.5.2 for more details). Through the xylem, Zn is translocated from roots to shoots in a chelated form (see below) *via* the transpiration stream (Curie et al., 2009). The rates of Zn translocation from roots to shoots positively correlates with the level of Zn present in roots (Hart et al., 1998).

Therefore, if Zn uptake from soil is restricted, less Zn will be accumulated in the above-ground organs of the plant, which could lead to Zn deficiency.

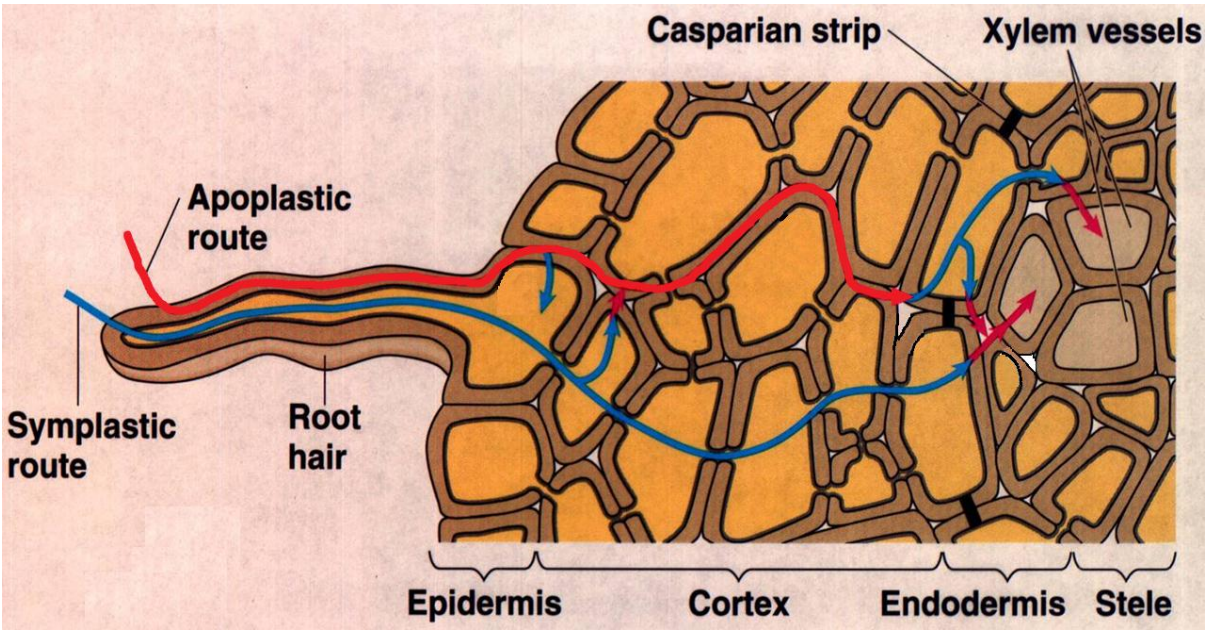

Figure 1.2. Lateral transport of Zn from root hairs to the xylem.
Diagram modified from Campbell et al. (2009).

The transport of Zn from roots to shoots is mediated by low molecular weight ligands in the xylem sap (Kramer et al., 1996; Van Belleghem et al., 2007). These ligands include organic acids, nicotianamine and phytosiderophores that form a Zn chelating complex (Clemens et al., 2002; Suzuki et al., 2008; von Wiren et al., 1999; White et al., 1981). It has also been suggested that phytochelatin present in the xylem is involved in the transport of Zn (Chen et al., 2006; Mendoza-Cozatl et al., 2008). Zn transported in the xylem from the roots is usually deposited in leaves or loaded into the phloem (Palmgren et al., 2008; Patrick & Offler, 2001). The loading of Zn into the phloem is mediated by metal transporters within the xylem network (Palmer & Guerinot, 2009). Alternatively, phloem Zn loading could also occur in vegetative tissues such as leaves (Wu et al., 2010). The transport of Zn within the phloem has been shown to be mediated by nicotianamine (Nishiyama et al., 2012). Phloem Zn loading is important as the transport of Zn into grains of barley and wheat occurs only through

the phloem (Patrick & Offler, 2001). In rice, however, Zn can be loaded into grains directly *via* the xylem as the xylem is continuous between the roots and panicle (Stomph et al., 2009).

1.3.2 Cellular distribution of Zn

Within a plant cell, the vacuole normally contains more than 50% of the total cellular Zn content (Yang et al., 2006), while approximately 40% gets bound to insoluble components and around 5% are found in the cytosol (White & Gadd, 1986). The vacuole also plays a part in the regulation of the cytoplasmic Zn concentration (MacDiarmid et al., 2000). Free Zn^{2+} within the cytosol is readily utilised by metabolic processes, and when the level of Zn^{2+} is depleted, the vacuole can transport Zn into the cytoplasm to replenish the depleted Zn (MacDiarmid et al., 2000). In Zn hyperaccumulator plant species, Zn concentrations in the vacuole are usually at least five-fold more than those of non-hyperaccumulator species (Küpper et al., 1999; Yang et al., 2006), indicating that the vacuole is also essential to prevent Zn toxicity.

1.3.3 Deposition of Zn and Fe in grains

Within the grain, Zn is deposited in the embryo, aleurone and endosperm. In wheat and barley grains, higher Zn concentrations are normally observed in the embryo and aleurone compared to the endosperm (Mazzolini et al., 1985; Ozturk et al., 2006; Palmgren et al., 2008). The Zn content in the endosperm of wheat and barley grains accounts for approximately one third of the total grain Zn (Eđed & Rengel, 2011; Lombi et al., 2011; Pieczonka & Rosopulo, 1985). Iron (Fe) distribution in the grains of wheat and barley is similar to that of Zn, and a similar percentage of the total grain Fe content is found in the endosperm (Lombi et al., 2011; Ozturk et al., 2009; Welch & Graham, 2004). In contrast, approximately 75% of total grain Zn content is deposited in the endosperm of rice grains, but

Fe content in the endosperm accounts only for approximately 10% of the total grain Fe content (Jiang et al., 2008; Lombi et al., 2009).

The aleurone also contains high concentrations of phytic acid, leading to a high degree of complexion of phytate with Zn and Fe in the aleurone (Borg et al., 2009; Persson et al., 2009). High deposition of metals into the aleurone and embryo is widely viewed as a hindrance to the improvement of nutrition value of cereal grains (Palmgren et al., 2008; Wirth et al., 2009). This is because most cereal grains are polished or milled to remove the bran before being processed into cereal-based foods. This bran-removal process usually diminishes the whole aleurone and embryo. In rice grains, removal of the bran by polishing is essential as the bran contains oil which can become rancid, leading to the deterioration in quality. Even if the grains are not polished, the abundance of phytate within the aleurone which binds to these metals restricts their absorption by human gut cells.

The consumption of nutritionally-poor cereal based products by humans will lead to health complications related to micronutrient malnutrition (Hotz et al., 2004). Much effort has been invested into generating crops capable of storing more of these essential metals into the grains, especially in the endosperm, to minimise their loss as a result of polishing (Masuda et al., 2009; Wirth et al., 2009). Although Zn concentration is high in the embryo and aleurone, the endosperm contains a significant proportion of total grain Zn. Therefore, while the increment of grain Zn accumulation will inevitably increase the Zn in the aleurone and embryo, it could also lead to higher endosperm Zn accumulation.

1.3.4 Relationship between plant Zn efficiency and Zn nutrition

A plant's Zn efficiency may influence the distribution of Zn in plant tissues (Hacisalihoglu et al., 2004). Although most studies have shown that there is no positive

correlation between Zn efficiency and seed Zn content (as mentioned in Section 1.2.2), there are some reports showing that Zn efficient cultivars possess higher seed Zn. For example, higher Zn content in grains of Zn-efficient rice and common bean cultivars relative to Zn-inefficient cultivars have been observed when grown under controlled conditions (Hacisalihoglu et al., 2004; Rengel, 2001). Zn-efficient cultivars are also able to sustain normal growth under Zn-deficient conditions for a significantly longer period of time than inefficient cultivars. This suggests that Zn-efficient cultivars have better Zn uptake and storage capacity, which could be contributed by increased activities of metal uptake transporters or possessing better Zn utilisation systems (Hacisalihoglu et al., 2004; Hacisalihoglu & Kochian, 2003). Zn efficiency may also be associated with root-structure traits which enhance Zn retrieval and/or the release of more Zn-binding ligands such as phytosiderophores (Hacisalihoglu & Kochian, 2003).

Based on currently available information regarding Zn movement and storage in plants, there exist a few prominent bottlenecks which can limit the utilisation of soil Zn for biofortification (Palmgren et al., 2008). These bottlenecks include the transport of Zn through the root-to-shoot barrier, phloem Zn loading and unloading, and grain Zn loading. Later in this review, the transporter components involved in Zn transport through these checkpoints will be discussed to provide an overview of the possible solutions towards increasing Zn loading for biofortification.

1.4 Physiological functions of Zn in plants

Zinc is an important co-factor of many enzymes (Coleman, 1998) and plays a structural role in various proteins, which include thousands of transcription factors (Hershfinkel et al., 2007; Krämer & Clemens, 2006). Physiological processes in which Zn is involved include photosynthesis, protein synthesis, fertility and seed production, growth

regulation and defence against diseases. When plants experience Zn deficiency, these physiological processes are affected, causing impaired health and productivity.

Plants carry out photosynthesis to synthesise carbohydrates from CO_2, using ATP and NADPH generated in the light reaction. A key step in photosynthesis is the initial conversion of CO_2 to carbonic acid by the carbonic anhydrase (CA) enzyme, whose catalytic activity is Zn-dependent (Lindskog, 1997). Under Zn deficiency, reduction in CA activity has been observed (Ohki, 1976). The formation of photosynthetic pigments such as chlorophyll is also Zn dependent. When the plant is deficient in Zn, some of the pigments are degraded, resulting in chlorosis (Alloway, 2004). Reduced photosynthetic rates due to Zn deficiency have also been related to decreased stomatal conductance (Sharma et al., 1995). Adequate Zn is required to maintain high levels of potassium in guard cells for stomatal conductance (Sharma et al., 1995). When Zn supply is low, stomatal conductance is reduced, leading to lower intake of CO_2 which is required for photosynthesis.

Copper/Zn-superoxide dismutase (SOD) is an enzyme important in protecting plants against oxidative damage catalysed by reactive oxygen species (ROS), free radicals and peroxides (Cakmak & Marschner, 1993). As Zn is a co-factor of SOD, Zn deficiency can inhibit SOD activities, leading to oxidative damage to membrane lipids, proteins, chlorophyll, and nucleic acids (Cakmak, 2000; Chen et al., 2008b; Yu & Rengel, 1999) The oxidative stress induced by Zn deficiency can also destroy the integrity of the membrane surrounding the thylakoid (where the light-dependent reactions of photosynthesis occur) (Ohki, 1978; Randall & Bouma, 1973; Sasaki et al., 1998). Damage to thylakoids further contributes to reduction in photosynthesis rates under Zn deficient conditions.

Furthermore, Zn is vital for the synthesis and metabolism of carbohydrates in plants. The synthesis of starch is mediated by starch synthase enzymes, whose activity is Zn-

dependent. Zn deficiency has been shown to reduce the activity of starch synthases (Alloway, 2004). Growth of roots and shoots is also Zn-dependent as the synthesis of auxin, a class of plant growth hormones, requires Zn (Broadley et al., 2007). The development of reproductive organs is also affected by low Zn concentrations, resulting in small anthers and deformed pollen grains. Lower grain set and smaller grains are often observed in Zn deficient plants (Alloway, 2004). In addition, Zn plays a structural role in many Zn finger DNA-binding proteins, such as the plant-specific WRKY protein family, which regulates a large number of plant-defence related genes (Dong et al., 2003). The various important roles that Zn is involved in emphasise the necessity to maintain adequate cellular Zn concentrations.

1.5 Zinc transporters in plants

Zinc uptake and homeostasis in plants is mediated by a number of cellular Zn transporters (Eide, 2006). Several transporter families have been shown to be involved in Zn homeostasis within higher plants: (1) Zinc-regulated transporter, Iron-regulated transporter-like Proteins (ZIP) (Eide, 2006; Grotz et al., 1998; Guerinot, 2000), (2) Cation Diffusion Facilitator (CDF), often known as Metal Tolerance Proteins (MTP) (Arrivault et al., 2006; Eide, 2006; Kobae et al., 2004), (3) P-type ATPases, particularly the Heavy Metal ATPase (HMA) subgroup (Eide, 2006; Eren & Arguello, 2004; Hussain et al., 2004) and (4) Oligopeptide Transporters (OPT) (Palmer & Guerinot, 2009).

1.5.1 General functions of different Zn transporter families

Members of the *ZIP* family are regarded as the main transporters controlling plant Zn uptake. It has been shown that plant ZIP transporters are involved in Zn uptake at the cellular level when tested in yeast complementation assays (Grotz et al., 1998; Guerinot, 2000). ZIP transporters could therefore play an important role in controlling Zn influx from the external

environment into the cytoplasm. Members of the CDF/MTP transporter family are known to be involved in sequestering Zn from the cytoplasm into organelles such as vacuoles (Eide, 2006). The expression pattern of CDF/MTP transporters such as AtMTP1 and AtMTP3 in *Arabidopsis thaliana* is positively correlated with enhanced tolerance to Zn deficiency, likely due to having higher reserves of vacuolar Zn (Arrivault et al., 2006; Kobae et al., 2004). The expression of AtMTP3 was also highly induced in the root epidermal and cortex layers in the presence of high external Zn concentrations, indicating that AtMTP3 has a role in restricting Zn mobilisation from roots to shoots to prevent Zn toxicity in above-ground organs.

HMA transporters (a subgroup of the P-type ATPase family) have been shown to be involved in Zn efflux from the cytoplasm to the extracellular environment (Eren & Arguello, 2004; Hussain et al., 2004). Two Arabidopsis HMA transporters, AtHMA2 and AtHMA4, regulate the Zn translocation from roots to shoots (Eren & Arguello, 2004; Hussain et al., 2004). By knocking out the function of AtHMA2, mutant *athma2* lines accumulated more Zn in roots compared to wildtype plants (Eren & Arguello, 2004), likely due to the disruption of Zn efflux activities. The double knockout mutant of *athma2* and *athma4* accumulated less shoot Zn compared to the wildtype (Hussain et al., 2004), most likely due to the elimination of Zn translocation activity from roots to shoots in mutant lines. The localisation of AtHMA2 and AtHMA4 within the xylem parenchyma further suggests that these two HMA transporters are responsible for translocating Zn from roots to shoots.

OPT family members such as the Yellow Stripe-Like (YSL) protein transport metals complexed with phytosiderophores and regulate phloem loading and unloading of these phytosiderophore-bound metals (Koike et al., 2004; Le Jean et al., 2005; Schaaf et al., 2005; von Wiren et al., 1996; Waters et al., 2006). In addition to transporting phytosiderophore-bound Fe complexes, YSL proteins can also transport Zn complexes, as well as complexes of other divalent metals such as Mn, Cu, Ni and Co (Schaaf et al., 2005; von Wiren et al., 1996).

Several YSL proteins such as AtYSL1 and AtYSL3 (Waters et al., 2006) are important in seed Zn loading, and the rice OsYSL2 is expressed in the plant vasculature system (Koike et al., 2004). These findings suggest that some YSL proteins are important in Zn translocation and loading into grains.

Although ZIP transporters are generally involved in controlling cellular Zn influx, MtZIP2 in *Medicago truncatula* was found to facilitate the efflux of Zn from the cytoplasm, suggesting that it is important in preventing cellular Zn toxicity (Burleigh et al., 2003). Furthermore, although ZIP proteins generally transport Zn and Fe, they can also transport elements with similar cationic affinity such as Mn, Cu, Ni and the toxic element Cd (Küpper & Kochian, 2010; Mizuno et al., 2005; Pedas et al., 2008; Stephens et al., 2011). Therefore, ZIP family members may also contribute to the homeostasis of other metals in addition to Zn.

1.5.2 Manipulation of Zinc transporters for improving plant Zn nutrition

The existence of several gene families for Zn transport provides a good range of candidates to select for genetic manipulation to improve plant Zn uptake and accumulation. The pathways of Zn trafficking from soil to grains, and the different transporters that are involved, are illustrated in Figure 1.3. For the effective utilisation of internal Zn, and also to maintain adequate levels of Zn in all tissues, it is important that the plant is able to take up a sufficient amount of Zn from soils. Generating plants with increased Zn uptake from external Zn sources is therefore important for increasing Zn concentration in vegetative tissues. This could also be achieved by increasing the capacity of Zn loading into the xylem/phloem pathway, leading to increased redistribution of Zn within different plant tissues, including grains.

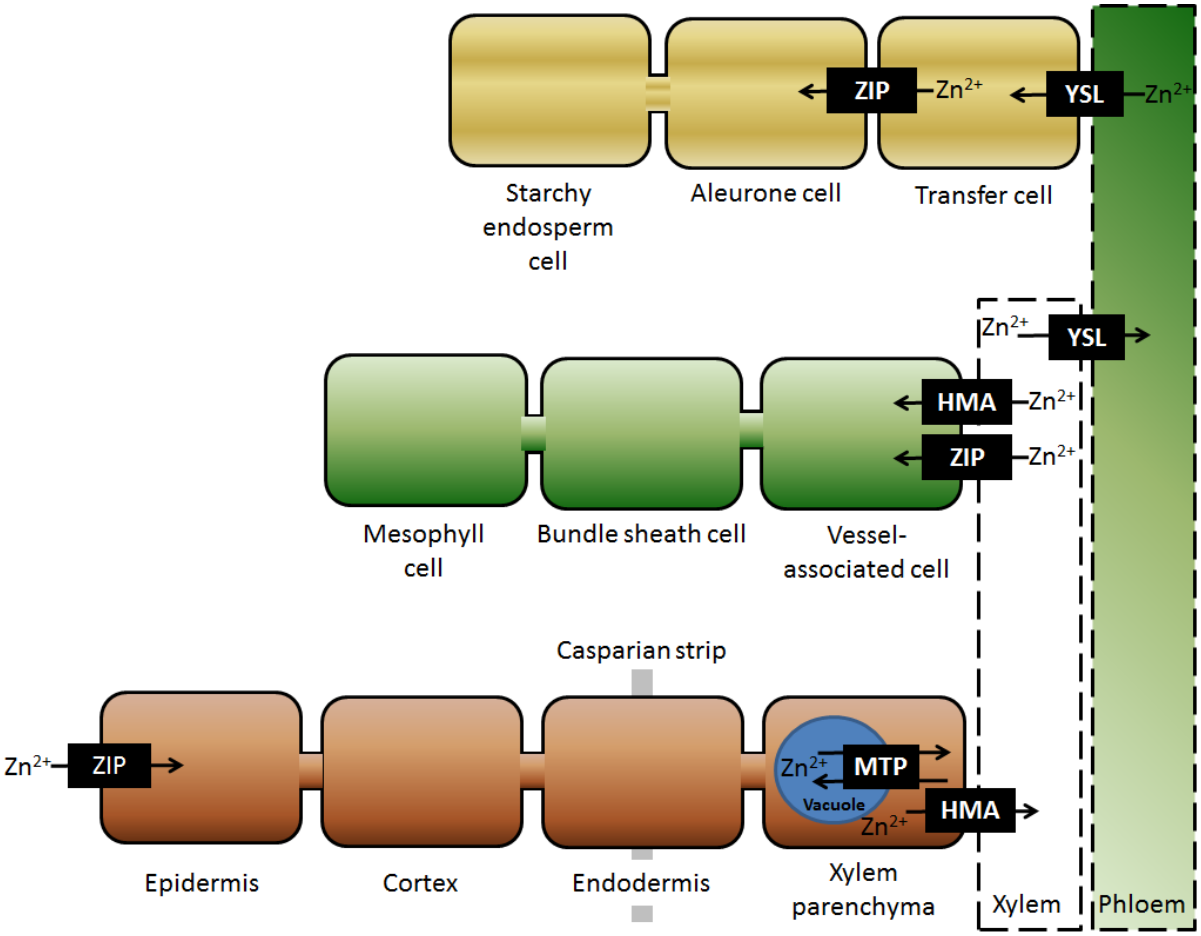

Figure 1.3. Overview of the transport pathway of Zn cation from soil to grain.
Different families of Zn transporters that are involved in the Zn trafficking process, along with their speculative tissue localisation in the plant (Palmer & Guerinot, 2009; Palmgren et al., 2008; Waters & Sankaran, 2011).

So far, there is no evidence showing direct involvement of Zn transporter activity in enhancing shoot and grain Zn accumulation. However, there are examples of increases in activity of related transporters increasing the uptake of other nutrients. For instance, increased barley Mn transporter activity (HvIRT1, a member of the ZIP family of transporters) correlated with increased Mn uptake in Mn-efficient barley lines (Pedas et al., 2008). Also, phosphate-efficient common bean lines have shown positive correlation between increased phosphate transporter activity (PvPT1) and increased phosphate accumulation (Tian et al., 2007). These findings provide insights into the relationship between nutrient transporter activity and nutrient uptake, and that increasing the activity of Zn transporters may contribute to increased Zn uptake for better plant nutrition and grain Zn loading.

Some members of HMA such as AtHMA2, AtHMA4 and OsHMA2 are involved in Zn translocation from roots to shoots, making them potential candidates for increasing Zn accumulation in above ground organs (Hanikenne et al., 2008; Hussain et al., 2004; Satoh-Nagasawa et al., 2011). However, these HMA proteins can transport the toxic Cd *in planta* in addition to Zn (Hanikenne et al., 2008; Hussain et al., 2004; Satoh-Nagasawa et al., 2011). Furthermore, the over-expression of *AtHMA4* increases the accumulation of both Zn and Cd in shoots (Verret et al., 2004). Increasing HMA activity for Zn biofortification may therefore inadvertently increase Cd accumulation within grains, which is detrimental for human health.

Transcriptions of *ZIP* genes are observed in roots during Zn uptake from the rhizosphere, in shoots during Zn distribution into leaves and in transfer cells during Zn loading into grains (Palmgren et al., 2008; Tauris et al., 2009; Waters & Sankaran, 2011), all of which are processes that could restrict plant Zn uptake, tissue Zn distribution and grain Zn biofortification (Section 1.3.4). The involvement of ZIP transporters in these processes makes them suitable candidates for genetic manipulation to increase shoot and grain Zn accumulation. Furthermore, most ZIP proteins show specificity for Zn ion transport (see Section 1.6.2) unlike HMA. The Zn ion-specificity of most ZIP transporters is therefore an important characteristic to successfully achieve Zn biofortification. Increasing Zn uptake and accumulation in cereals by manipulating ZIP transporters is therefore the central theme of this thesis.

Successful manipulation of Zn uptake processes by over-expressing *ZIP* genes has been reported in human, animal and yeast cells (Franklin et al., 2003; Huang et al., 2002; Kumanovics et al., 2006; MacDiarmid et al., 2000). Maintaining healthy growth under low Zn conditions is achieved in these *ZIP* over-expressing cells. It is thus possible that application of this technology in agricultural crops, particularly cereals, will be similarly beneficial. Several ZIP transporters that are involved in Zn homeostasis in rice has been identified and

characterised (Ishimaru et al., 2005; Ramesh et al., 2003; Yang et al., 2009). There exists potential for manipulating the expression of these ZIP transporters towards improving Zn uptake and accumulation in rice. However, little work has so far been done in identifying Zn transporters from the two other major cereal crops, barley and wheat. Over-expression of the Arabidopsis *AtZIP1* in barley has been described, showing some success in terms of increasing short term whole plant Zn uptake and grain Zn accumulation relative to controls, but not enhancing Zn content in shoots and roots (see Section 1.7.1.1 for more details) (Ramesh et al., 2004). There is an urgent need to identify all key ZIP transporters in cereal crops and to investigate their role in Zn uptake and homeostasis. More details about this family of transporters will be discussed in greater detail in the subsequent sections to further support the selection of ZIP transporters as tools for increasing Zn uptake and accumulation.

1.6 Importance of ZIP transporters in Zn homeostasis

ZIP family members have been isolated from a diverse range of organisms including bacteria, fungi, plants and human (Gaither & Eide, 2001; Grotz & Guerinot, 2006). ZIP family members have been reported in plant species with fully sequenced genomes such as Arabidopsis (Grotz & Guerinot, 2006) and rice (Chen et al., 2008a). The presence of ZIP transporters in such a diverse range of organisms indicates that the ZIP transporter family play a significant role in Zn homeostasis in both prokaryotes and eukaryotes. The highest number of *ZIP* genes identified so far from a single plant species is 16 in Arabidopsis (Grotz & Guerinot, 2006), closely followed by rice (14) (Chen et al., 2008a; Narayanan et al., 2007). Most studies on *ZIP* gene functions in Zn homeostasis thus far are therefore from Arabidopsis and rice.

1.6.1 Structure of ZIP transporters

The analyses of ZIP transporter protein sequences from different species have revealed several highly conserved regions (Guerinot, 2000). Eight conserved trans-membrane (TM) domains have been detected, with both the N- and C- termini residing in the extracellular region (Figure 1.4). Another highly conserved property of ZIP is the presence of a large cytoplasmic loop between TMIII and TMIV. This cytoplasmic loop is approximately 60 to 100 amino acid residues in length. Although the existence of this loop region is conserved among ZIP transporters, its amino acid sequence is highly variable among the members of the ZIP family (Guerinot, 2000). Therefore, this cytoplasmic loop commonly represents a signature motif to distinguish different members of the ZIP family.

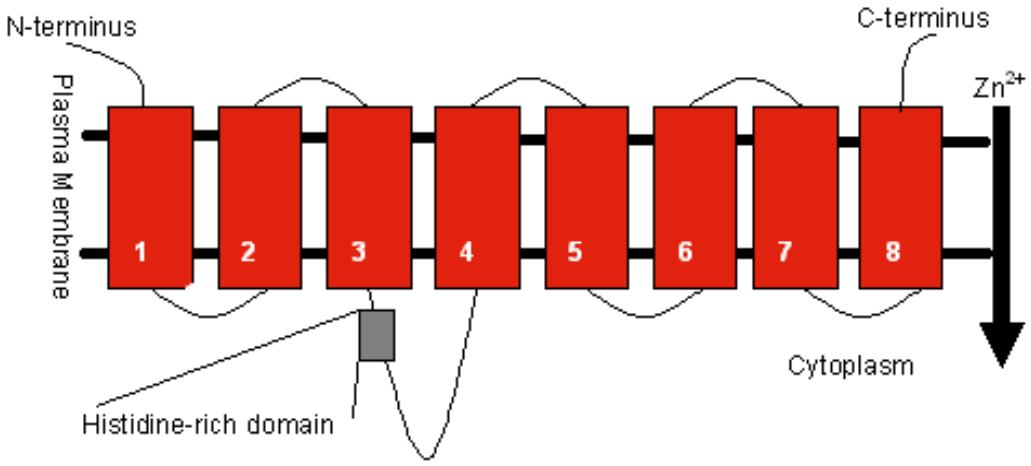

Figure 1.4. Predicted topology of human hZIP2 transporter (Franklin et al., 2003).
The variable region is located within the cytoplasmic loop between TM III and TM IV.

The metal ion-specificity of ZIP proteins is likely conferred by a histidine-rich region residing within the large cytoplasmic loop of the protein (Figure 1.4). Site directed mutagenesis of the histidine-rich region of the human hZIP1 resulted in reduction of Zn transport activity (Milon et al., 2006). There are also studies suggesting that the amino acid

sequence of the extracellular N-terminal of ZIP transporters could be responsible for metal selectivity (Nishida et al., 2011). Site directed mutagenesis of the N-terminal of the *Thlaspi japonicum* ZNT2 (TjZNT2) resulted in a change of ion-selectivity from Mn to Zn (Nishida et al., 2011). This is not unlike the Arabidopsis AtHMA2 transporter, whose N- and C- termini were shown to be essential for ion selectivity and transporter function (Wong et al., 2009).

The unique nature of the ZIP protein's large cytoplasmic loop has allowed specific functional characterisation of ZIP transporters. Antibodies capable of binding to the unique amino acid sequence of the loop permit various molecular biology applications such as measuring protein expression levels by Western blot analysis or by enzyme-linked immunosorbent assay (ELISA) analysis (Franklin et al., 2003; Huang et al., 2005; Moreau et al., 2002). The antibodies can also be used to investigate cellular and tissue localisation of ZIP transporters *via* immuno-histolocalisation (Huang et al., 2005; MacDiarmid et al., 2000; Milon et al., 2006).

1.6.2 Functional characterisation of plant ZIP transporters

With cereal crops being the central theme of this review, it is interesting to note that, besides rice, few ZIP transporters have been described from important cereal crops such as wheat and barley. As the manipulation of ZIP transporters is a strong candidate approach to increase Zn uptake and translocation, greater effort should be invested into identifying more ZIP transporters from other cereal crops.

Functional characterisation of ZIP transporters is conducted mostly by use of heterologous yeast systems. More infrequently, functional characterisation is further extended to *in planta* studies. In the subsequent sections, efforts in investigating the functions of ZIP

transporters will be briefly summarised to deliver a snapshot of the typical role these transporters have at the cellular and whole plant level.

1.6.2.1 Characterisation of ZIP *genes in the yeast system*

Only a limited number of ZIP transporters has been functionally characterised *in planta*. Most investigations on the functions of ZIP transporters have been conducted using yeast complementation (Grotz & Guerinot, 2006). This usually involves transforming a ZIP transporter of interest into mutant yeast strains with defects in Zn (or other metal) transport. Yeast transformants which display the ability to grow and survive under low Zn conditions, or are able to accumulate more Zn than the untransformed mutant controls indicate a Zn transport role for the introduced gene. The results of yeast complementation studies of ZIP members from Arabidopsis and rice will be described in more detail below.

Among the 16 Arabidopsis ZIP members identified so far, AtZIP1, AtZIP2, AtZIP3 and AtZIP4 are capable of complementing the growth of yeast strains defective in Zn uptake (Grotz & Guerinot, 2006). AtIRT1 and AtIRT2, also belonging to the ZIP family, are capable of complementing the growth of yeast strains defective in Fe uptake (Grotz et al., 1998; Vert et al., 2001; Vert et al., 2002), whereas AtIRT3 is capable of complementing the growth of mutant yeast cells defective in the uptake of both Fe and Zn (Lin et al., 2009). In rice, OsZIP1, OsZIP3, OsZIP4, OsZIP5 and OsZIP8 were shown to complement yeast strains defective in Zn uptake (Ishimaru et al., 2005; Lee et al., 2010a; Lee et al., 2010b; Ramesh et al., 2003; Yang et al., 2009). OsZIP7a and OsIRT1 were able to rescue the growth of yeast strains defective in Fe uptake (Bughio et al., 2002; Yang et al., 2009).

So far, only four ZIP transporters from barley have been identified and characterised. HvZIP3, HvZIP5 and HvZIP8 restored the growth of yeast strains defective in Zn uptake

(Pedas et al., 2009) while HvIRT1 is capable of restoring growth of yeast defective in Mn, Zn and Fe uptake (Pedas et al., 2008). From wheat and its close relatives, only one ZIP from the tetraploid emmer wheat, TdZIP1, has so far been found. TdZIP1 could complement a yeast mutant defective in Zn transport (Durmaz et al., 2010). The expression of *TdZIP1* in yeast cells resulted in higher Zn accumulation.

While yeast studies provide evidence that these ZIP transporters are capable of transporting Zn at the single-cell level, they do not provide information on what roles these transporters play in whole plant Zn homeostasis. Functional characterisation of ZIP transporters *in planta* is therefore necessary to determine their role at the multicellular level. Determining the expression profiles of *ZIP* genes in different plant tissues and in response to different Zn levels in the growth media can help uncover their roles in Zn homeostasis. Furthermore, investigating *ZIP* expression levels in a cell type-specific manner, determining cellular ZIP localisation and studying the effect of *ZIP* over-expression or knock-out in the plant will provide further functional information of ZIP transporters *in planta*.

1.6.2.2 Characterisation of ZIP *genes* in planta

The expression of *AtZIP1*, *AtZIP3* and *AtZIP4* was induced under Zn deficiency, with *AtZIP1* and *AtZIP3* being highly expressed in the roots whereas *AtZIP4* was highly expressed in both roots and shoots (Grotz et al., 1998). AtZIP1 and AtZIP3 might therefore be responsible for Zn uptake during Zn deficiency, while AtZIP4 could be responsible for Zn translocation under low Zn conditions. The expression of both *AtIRT1* and *AtIRT2* was highly induced in the roots under low Fe, suggesting that these two ZIP transporters are involved in Fe uptake during Fe deficiency (Zhao & Eide, 1996). Furthermore, *AtIRT1*-knockout Arabidopsis mutants (*atirt1*) accumulated less Fe compared to control lines (Vert et al., 2001). The *atirt1* findings are further supported by Henriques et al. (2002), who showed reductions

in the accumulation of radioactively-labelled ^{59}Fe and ^{65}Zn in *atirt1* mutant lines. Characterisation of AtZIP1 by heterologous over-expression in barley resulted in increased short term (24 hr) Zn uptake and translocation, along with higher grain Zn concentration and content (Ramesh et al., 2004). More of this work will be discussed in Section 1.7.1.1.

In rice, *OsZIP1* transcript levels were up-regulated when the plant was deprived of Zn (Ramesh et al., 2003). Furthermore, the up-regulation of *OsZIP1* by Zn deficiency was higher in roots than in shoots, suggesting that OsZIP1 may be primarily involved in Zn uptake under low Zn conditions. The authors also showed that *OsZIP3* expression is constitutive in both roots and shoots regardless of Zn nutritional status, suggesting that OsZIP3 may be involved in overall plant Zn homeostasis (Ramesh et al., 2003). Ishimaru et al. (2005) showed that the expression of *OsZIP4* is induced in both roots and shoots under low Zn conditions, indicating that OsZIP4 may be involved in Zn uptake by roots and translocation in shoots during Zn deficiency. Yang et al. (2009) found that *OsZIP7a* is induced only in roots under low Fe conditions, whereas *OsZIP8* is induced in both roots and shots under low Zn conditions. Characterisation of *OsZIP4*, *OsZIP5* and *OsZIP8* by over-expression resulted in increased Zn accumulation in roots but lowered shoot Zn levels (Ishimaru et al., 2007; Lee et al., 2010a; Lee et al., 2010b). These over-expression studies will be further discussed in Section 1.7.1.3.

Pedas et al. (2009) showed that the expression of the barley *HvZIP3, HvZIP5* and *HvZIP8* genes were induced in the roots under low Zn conditions, suggesting that these three ZIP transporters may be involved in Zn uptake under low Zn conditions. The expression level of *HvIRT1*, also a ZIP transporter gene, is correlated with Mn efficiency in barley (Pedas et al., 2008). The Mn-efficient barley cultivar, Vanessa, displayed higher Mn uptake under Mn-deficient conditions relative to the Mn-inefficient cultivar, Antonia. The expression level of *HvIRT1* in Vanessa is higher than that of Antonia, indicating that HvIRT1 could be important in Mn uptake. In the tetraploid emmer wheat, *TdZIP1* expression levels in the roots increased

under low external Zn supply, suggesting that TdZIP1 may be important in Zn uptake during Zn deficiency (Durmaz et al., 2010).

The ability of ZIP to transport Zn and/or other metals in yeast, in combination with its gene expression profiles in roots and shoots having varying Zn nutritional status, provides an indication of roles these ZIP transporters may have in Zn homeostasis. ZIP members from different plant species which have so far been identified and characterised are summarised in Table 1.1. These functional data can be used in the selection of suitable ZIP transporter candidates for genetic manipulation to increase plant Zn uptake and biofortification.

Table 1.1: Identified and characterised ZIP transporters from different plant species

Plant	Transporter	Metal(s) trafficked	Tissue localisation	Source(s)
Arabidopsis thaliana	AtZIP1	Cd, Fe, Mn	Roots	Grotz et al. 1998; Rogers et al. 2000
	AtZIP2	Fe, Zn	Roots	Grotz et al. 1998; Vert et al. 2001
	AtZIP3	Zn	Roots	Grotz et al. 1998
	AtZIP4	Zn	Shoots, Roots	Grotz et al. 1998
	AtIRT1	Fe, Zn	Roots	Henriques et al. 2002
	AtIRT2	Fe, Zn	Roots	Henriques et al. 2002
	AtIRT3	Fe, Zn	N/A	Lin et al. 2009
Soya bean (*Glycine max*)	GmZIP1	Zn	Roots (nodules)	Moreau et al. 2002
Barley (*Hordeum vulgare*)	HvIRT1	Mn	Roots	Pedas et al. 2008
	HvZIP3	Zn	Roots	Pedas et al. 2009
	HvZIP5	Zn	Roots	Pedas et al. 2009
	HvZIP8	Zn	Roots	Pedas et al. 2009
Tomato (*Lycopersicon esculentum*)	LeIRT1	Fe	Roots	Eckhardt et al. 2001
	LeIRT2	Fe	Roots	Eckhardt et al. 2001
Medicago truncatula	MtZIP1	Zn	Leaves, Roots	Lopez-Millan et al. 2004
	MtZIP2	Zn	Shoots, Roots	Burleigh et al. 2003
	MtZIP3	Zn, Fe	Leaves, Roots	Lopez-Millan et al. 2004
	MtZIP4	Zn, Mn	Leaves, Roots	Lopez-Millan et al. 2004
	MtZIP5	Zn, Mn	Leaves	Lopez-Millan et al. 2004
	MtZIP6	Zn	N/A	Lopez-Millan et al. 2004
	MtZIP7	Mn	N/A	Lopez-Millan et al. 2004
Thlaspi caerulescens	TcMTP1	Cd, Zn	Leaves	Kupper et al. 2010
	TcIRT1	Fe	Roots	Lombi et al. 2002
	TcZNT1	Cd, Zn	Shoots, Roots	Lasat et al. 1996; Kupper et al. 2010
	TcZNT5	Cd, Zn	Leaves	Kupper et al. 2010
Thlaspi japonicum	TjZNT1	Cd, Mn, Zn	N/A	Mizuno et al. 2005
	TjZNT2	Mn, Zn	N/A	Mizuno et al. 2005
Rice (*Oryza sativa*)	OsZIP1	Zn	Roots	Ramesh et al. 2003
	OsZIP3	Zn	Shoots, Roots	Ramesh et al. 2003
	OsZIP4	Zn	Shoots, Roots	Ishimaru et al. 2005
	OsZIP5	Zn	N/A	Lee et al. 2010a
	OsZIP7	Fe	Roots	Yang et al. 2009
	OsZIP8	Zn	Shoots, Roots	Yang et al. 2009
Wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*)	TdZIP1	Zn	Roots	Durmaz et al. 2010

1.6.3 Cellular and tissue localisation of ZIP transporters

Knowledge of cellular and tissue localisation of ZIP transporters is important for deciphering ZIP functions *in planta*. This information can help reveal possible roles of ZIP transporters in Zn homeostasis at the cellular level (Franklin et al., 2003; Ishimaru et al., 2005; Milon et al., 2006) and at the whole plant level (Ishimaru et al., 2005; Lee et al., 2010a; Lee et al., 2010b; Ramesh et al., 2003; Vert et al., 2001; Vert et al., 2002).

ZIP transporter proteins are usually embedded in the plasma membrane (Burleigh et al., 2003; Ishimaru et al., 2005; Pedas et al., 2008; Vert et al., 2002), indicating that they are predominantly involved in cellular Zn influx or efflux. A common method to determine the cellular localisation of ZIP transporters is by transient or stable expression of a ZIP transporter gene tagged with a reporter such as Green Fluorescent Protein (GFP) (Burleigh et al., 2003; Ishimaru et al., 2005; Pedas et al., 2008; Vert et al., 2002). The location of the reporter gene product, shown as a fluorescence signal, indicates the cellular compartments to which the ZIP transporter is targeted to. An example of a typical transient assay result is illustrated in Figure 1.5.

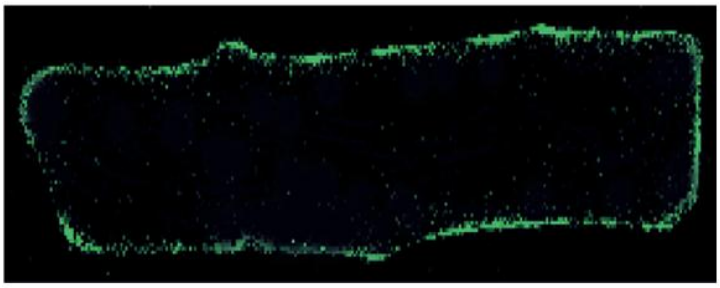

Figure 1.5. Localisation of OsZIP4::GFP on onion epidermal cells.
Visualisation using confocal laser scanning microscopy (Ishimaru et al., 2005).

The localisation of ZIP transporters can also be investigated *via* immuno-histolocalisation analyses. By utilising antibodies specific against the unique loop region of a ZIP transporter protein (see Section 1.6.1), the localisation of a ZIP transporter of interest can be assessed at the cellular and whole organism level (Huang et al., 2005; MacDiarmid et al., 2000; Milon et al., 2006). Immuno-histolocalisation has been used to show localisation of ZIP transporters to cellular plasma membranes and vacuolar membranes (Huang et al., 2005; MacDiarmid et al., 2000; Milon et al., 2006), supporting the likely role of ZIP transporters in cellular Zn influx or efflux. Using specific antibodies, the root localisation of HvYS1 and HvYSL5 from the OPT group of metal transporters were also determined (Murata et al., 2006;

Zheng et al., 2011), thus displaying the ability of antibodies to reveal the tissue localisation of membrane-bound transporters.

Besides aiding in investigating the cellular localisation of ZIP transporters, reporter constructs have also been used to determine in which tissue or organ within a plant the ZIP transporter is predominantly localised. This method usually involves introducing a gene construct consisting of a *ZIP* promoter fused with a reporter tag into the organism of interest at an early stage of the organism's life cycle (usually during the callus stage in the case of transgenic cereal plants). By studying the location pattern of the reporter gene's translated product, one can determine where within the organism the ZIP transporter primarily expresses, and this can help determine what Zn homeostasis role(s) the ZIP transporter may have. Other than GFP, β-glucuronidase (GUS) is also commonly used as a reporter tag (Vert et al., 2001; Vert et al., 2002). So far, most studies have reported that the expression of *ZIP* genes is predominantly restricted to the roots and is induced by either Zn or Fe deficiency, indicating that ZIP transporters play important roles in metal uptake from the external environment and also in remobilisation into shoots under low Zn or Fe conditions.

1.7 Genetic manipulation of ZIP transporters for improvement of Zn uptake and translocation

The over-expression of either an endogenous or heterologous metal transporter driven by, for example, a CaMV 35S promoter can result in the constitutive expression of the transporter in all tissues (Odell et al., 1985). This differs from the manner of a regulated, unmodified transporter, whose expression is usually tissue-specific and tightly controlled by the plant nutritional status (Guerinot, 2000; Moreau et al., 2002; Ramesh et al., 2003).

The constitutive expression of *ZIP* genes may therefore lead to higher Zn uptake, regardless of the amount of Zn present in the external environment or the plant Zn nutrition status. For example, the over-expression of Arabidopsis *ZIP* genes endogenously and heterologously (in barley) has resulted in increased Zn uptake (Lin et al., 2009; Ramesh et al., 2004). Increased Zn uptake means more Zn could be available for translocation from roots to shoots, which in turn may lead to increased grain Zn loading. This could contribute to better crop Zn nutrition and enhanced grain Zn accumulation. However, the over-expression of *ZIP* genes in rice has so far shown increases only in root Zn content while reducing the shoot and grain Zn content (Ishimaru et al., 2007; Lee et al., 2010a; Lee et al., 2010b). These rice ZIP transporters may have a very specific function, restricting their phenotype to a particular locality. Studies in investigating the effect of over-expressing plant ZIP transporters will be discussed in the sections below.

1.7.1 Over-expression of ZIP transporters in plants

1.7.1.1 Over-expression of AtZIP1 *in barley*

The Arabidopsis *AtZIP1* is highly induced in roots under low Zn conditions, suggesting that it could be involved in Zn uptake under low Zn conditions (Grotz et al., 1998). Ramesh and co-workers (2004) over-expressed *AtZIP1* in barley to investigate its role in Zn homeostasis and explore the possibility of increasing Zn in grains. Under Zn deficiency, short-term Zn uptake and translocation rates in *AtZIP1* over-expressing lines were higher than control lines (over a 24 hr period). However, after shifting the plants from Zn deficient to sufficient conditions, the Zn uptake rate of both transgenic and control plants over a period of 120 min were reduced (Ramesh et al., 2004), indicating that a regulatory mechanism exists to supress the activity of the transgene despite its constitutive nature. Furthermore, there were no

differences in shoot and root Zn accumulation between transgenic and control plants grown under both low and adequate Zn conditions for 44 days.

The grains of the *AtZIP1* over-expressing barley lines showed higher Zn concentration and content than control lines. However, the grain sizes of the transgenic lines were significantly smaller, and the effect of the transgene on yield production was not reported (Ramesh et al., 2004). Therefore, the overall effect of AtZIP1 on total grain Zn content is unknown. It is not clear whether the increase in grain Zn was due to higher translocation of Zn from the over-expression of *AtZIP1*, or resulted from lower total grain yield (McDonald et al., 2008).

1.7.1.2 Over-expression of AtIRT3 *in Arabidopsis*

AtIRT3 was capable of complementing the growth of mutant yeast cells defective in both Fe and Zn uptake (Lin et al., 2009). Transgenic Arabidopsis lines over-expressing *AtIRT3* accumulated more Zn in the shoots and more Fe in the roots relative to control lines. Furthermore, the content of other metals such as Mn, Cu, Mo, Mg and calcium were not affected in the transgenic lines. The results indicate the specificity of AtIRT3 in transporting Fe and Zn, and that over-expressing *AtIRT3* can increase plant Zn uptake under adequate Zn conditions (Lin et al., 2009).

1.7.1.3 Over-expression of OsZIP4, OsZIP5 *and* OsZIP8 *in rice*

Rice *ZIP* genes have also been over-expressed to analyse their function and to test their potential for increasing Zn uptake in rice (Ishimaru et al., 2007; Lee et al., 2010a; Lee et al., 2010b). The *OsZIP* genes selected for over-expression were based on their enhanced expression levels in both roots and shoots under low Zn conditions (Ishimaru et al., 2005;

Yang et al., 2009), as well as their capability to transport Zn in yeast strains defective in Zn uptake (Ishimaru et al., 2005; Lee et al., 2010b; Yang et al., 2009). Increased Zn uptake by over-expressing *OsZIP4* (Ishimaru et al., 2007), *OsZIP5* (Lee et al., 2010a) and *OsZIP8* (Lee et al., 2010b) in rice was observed, whereby all three transgenic lines displayed higher root Zn content but lower Zn content in shoots and grains. In lines over-expressing *OsZIP5* and *OsZIP8*, reduced shoot biomass and grain yield were also observed. The higher root Zn accumulation from over-expressing *OsZIP4*, *OsZIP5* and *OsZIP8* suggests that these OsZIP transporters are involved in Zn uptake from the external environment. However, as the shoot Zn concentration was reduced in the transgenic lines, the over-expression of these *OsZIP* genes alone could not confer higher translocation of Zn from roots to shoots. This shows that coordinated expression of other Zn transporters involved in Zn translocation from roots to shoots is required to increase Zn accumulation in shoots and grains.

1.7.2 Molecular manipulation of ZIP transporters for the improvement of Zn nutrition in temperate cereals

Barley and bread wheat are the most nutritionally and economically important winter cereal crops. Only a few *ZIP* genes have so far been identified in barley (Pedas et al., 2009; Pedas et al., 2008), but none has been described in bread wheat except for *TdZIP1* from its close relative, the wild emmer wheat (Durmaz et al., 2010). Although HvZIP3, HvZIP5 and HvZIP8 can transport Zn in yeast (Pedas et al., 2009), their functions *in planta* remains unclear. Only *AtZIP1* has been tested in barley as described above in detail (see Section 1.7.1.1), but no work on over-expressing native *ZIP* genes in either barley or wheat has been reported.

1.8 Research questions

Zinc is an essential micronutrient for normal growth and development of plants and humans. Zn deficiency in crop plants is recognised as the most common micronutrient deficiency worldwide. The consumption of foods derived from cereal grains low in Zn exacerbates Zn deficiency in humans. The improvement of Zn nutrition in cereal crops will reduce Zn deficiency, leading to better plant nutrition and higher grain Zn content. Grains with high Zn content could help alleviate health problems associated with Zn deficiency in humans. However, increasing Zn content in grains is restricted by two processes, which are i) Zn translocation from roots to shoots, and ii) Zn loading into grains. It is crucial to identify the genes that play important roles in these limiting processes, and then use these genes and knowledge of their functions to improve plant Zn nutrition and grain Zn content through genetic manipulation.

Zinc transporters such as ZIP are known to play a significant role in Zn uptake and translocation within the plant. The expression of some *ZIP* genes is induced under Zn deficiency and correlates with increased rates of Zn uptake and accumulation. We hypothesised that the increased expression of some of these Zn deficiency-inducible ZIP transporters could enhance Zn uptake and accumulation, which can lead to higher grain Zn content. To test this hypothesis, identifying members of the ZIP family from a target crop is needed. Barley is a good model plant for other temperate cereals and is a close relative of the nutritionally and economically important bread wheat. Unlike bread wheat, barley has a diploid genome and a genome sequence close to completion, making the identification of ZIP orthologs easier. Furthermore, transformation of barley plants with a gene of interest for functional characterisation is a relatively simpler task compared to transforming wheat. Knowledge gained from the identification and characterisation of *ZIP* genes in barley can then be applied in other important cereal crops.

The available barley genome sequence was used to identify additional *ZIP* genes. Some characterisation of these newly-identified barley *ZIP* genes is necessary prior to selection of potential candidates for further functional characterisation. This project will detail the function of an important barley *ZIP* gene in Zn homeostasis, and its potential role in improving plant Zn nutrition and grain Zn content. In addition, the barley *ZIP* genes identified and knowledge generated from their functional analysis could contribute to reducing crop Zn deficiency, and ultimately reduce human Zn malnutrition.

Chapter 2: Zinc deficiency modulates expression of *ZIP* family genes in barley (*Hordeum vulgare*)

2.1 Introduction

Zinc (Zn) is an important micronutrient for plants and other organisms as it is involved in many diverse and essential processes. All organisms must maintain an adequate intracellular concentration of Zn for normal growth and development. Zn deficiency reduces not only cereal yields, but also the nutritional value of the grains harvested for human consumption (Graham et al., 2001). Understanding the physiological and molecular responses of cereal crops to low Zn availability is essential to improve Zn nutrition of cereal crops and food-based strategies for human health (Hirschi, 2009; Mayer et al., 2008).

Large areas of cereal growing regions in the world have soils low in available Zn (White & Zasoski, 1999). Soil Zn availability is often found below micromolar concentrations (Broadley et al., 2007) and fluctuates considerably within a growing season due to a range of factors including pH, organic matter and temperature (Alloway, 2009). Moreover, the distribution of Zn in soils is heterogeneous. Even following the addition of Zn fertiliser, a high degree of spatial and temporal variation in Zn availability can occur (Genc, 1999; Graham et al., 1992). Consequently, roots are exposed to varying concentrations of available Zn, from inadequate to sufficient, as they grow through the soil profile. To cope with this variability, higher plants adjust uptake capacity and translocation of Zn to maintain an adequate Zn level for normal growth.

Zinc transporters are responsible for Zn uptake and translocation within the plant. The ZRT/IRT-like protein (ZIP) family is the primary group of transporters controlling Zn influx

into the cytoplasm (Grotz et al., 1998; Ishimaru et al., 2005; Ramesh et al., 2003; Stephens et al., 2011). The cation diffusion facilitator (CDF) family confers heavy metal tolerance by facilitating efflux of Zn from the cytoplasm to organelles or out of the cell (Desbrosses-Fonrouge et al., 2005; Drager et al., 2004), especially in high Zn conditions (Shahzad et al., 2010). The heavy metal P-type ATPase (HMA) family is involved in Zn efflux into xylem, and plays a unique role in Zn translocation from roots to shoots (Hussain et al., 2004).

There are 18 *ZIP* genes in the Arabidopsis genome (Grotz & Guerinot, 2006), seven in *Medicago truncatula* (López-Millán et al., 2004; Stephens et al., 2011) and 16 in the rice genome (Chen et al., 2008a; Narayanan et al., 2007). However, only a small number of *ZIP* genes have so far been identified from other nutritionally and economically important cereal crops such as barley and wheat. Four *ZIP* family genes in barley have been described. HvIRT1 restores the growth of a yeast mutant with defects in Mn-, Fe- and Zn-uptake (Pedas et al., 2008). HvZIP3, HvZIP5, and HvZIP8 could complement a yeast mutant defective in Zn uptake (Pedas et al., 2009). It is not clear what functions these three *HvZIP* genes have in Zn homeostasis of the plant. Only one *ZIP* gene from the tetraploid emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) has been found so far that could complement a yeast mutant defective in Zn uptake (Durmaz et al., 2010). No functional roles in Zn homeostasis have been characterised for any *ZIP* genes in bread wheat (*Triticum aestivum* L.).

In this chapter, the sequences of *ZIP* genes from Arabidopsis and rice were used to identify new *ZIP* genes from barley, wheat and Brachypodium, a model for temperate grass species (Brkljacic et al., 2011). The inclusion of Brachypodium and wheat sequences not only improves the robustness of phylogenetic relationships, but also determines the relatedness of HvZIP family proteins to the other species in the tree. The responsiveness of all *HvZIP* genes identified to Zn deficiency will also be determined for potential functions in Zn-deficiency responses.

2.2 Materials and methods

2.2.1 Plant growth in nutrient solution

Seeds of barley (Hordeum *vulgare* L. cv. Lofty Nijo) were used. Plant growth and conditions were similar to those described by Genc et al. (2007). Briefly, seeds with emerged radicles were transferred into a seedling cup which was placed in the lid of a black plastic container. Each container contained two plants. The basal nutrients were as follows (in µM); $Ca(NO_3)_2$, 1000; $NH_4H_2PO_4$, 100; $MgSO_4$, 250; KCl, 50; H_3BO_3, 12.5; Fe-HEDTA, 10; $MnSO_4$, 0.4; $CuSO_4$, 0.1; $NiSO_4$, 0.1 and MoO_3, 0.1. 2-[N-morpholino] ethane-sulfonic acid-KOH of 2 mM was used to buffer pH to 6.0. There were two Zn treatments, -Zn (0.005 µM Zn supplied as $ZnSO_4$) and +Zn (0.5 µM Zn). Macronutrients and micronutrients were supplied at half and full strength, respectively until D10, and full strength of all nutrients thereafter. The plants were harvested at D13. The nutrient solution was aerated continuously and replaced at D10. The pH of the nutrient solution was constant at pH 6.0 during the experiment.

Plants were grown in a growth room at 20/15 ºC day/night temperature and a photoperiod of 14 hr day/10 hr night at 300 µmol m^{-2} s^{-1} photon flux intensity at the plant level. At harvest, both sets of plants were removed from each container. One set was separated into roots and shoots, frozen immediately in liquid nitrogen and stored at -80 °C for the transcript analysis. The other set was used to measure dry weights and nutrient concentrations. The roots were rinsed briefly in deionised water, excess water was blotted on fresh laboratory tissues, roots and shoots were separated, and subsequently oven-dried at 80 ºC for 48 hr.

2.2.2 Mineral element analysis

Dry plant samples and grain were used for mineral element analysis by inductively coupled plasma optical emission spectrometry (ICP-OES) (Wheal et al., 2011).

2.2.3 Phylogenetic analysis

Sequences of *ZIP* family members from rice (*Oryza sativa* L.) and Arabidopsis (*Arabidopsis thaliana* L.) (Table 2.1) were used to search for homologous sequences in barley, Brachypodium and wheat. Rice and Arabidopsis *ZIP* family members were retrieved from the Rice Genome Annotation Project Database release 6.1 [http://rice.plantbiology.msu.edu/ at MSU (Michigan State University)] and from the Arabidopsis genome annotation database release 9 [http://www.arabidopsis.org/ at TAIR (The Arabidopsis Information Resource)]. Similarity searches were performed primarily by BLASTp (Altschul et al., 1997; Altschul et al., 2005) querying the JGI v1.0, 8x assembly of Brachypodium at Phytozome, version 7 (http://www.phytozome.net/) and the non-redundant database of proteins at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for barley and wheat. Similarity searches were also performed by BLASTn querying the barley BAC sequences of *HvZIP1* with *TaZIP1* nucleotide sequence at http://mips.helmholtz-muenchen.de/plant/barley/bacs/searchjsp/index.jsp and then the HvZIP1 protein sequence was obtained by identification of intron-exon positions using the mRNA sequence of *TaZIP1*. ZIP protein sequences were aligned by standalone MAFFT v6.846b using the L-INS-I method with associated default parameters (Katoh et al., 2009), and imported into ClustalW2 v2.0.12 (Larkin et al., 2007). The unrooted tree was generated in ClustalW2 using the neighbour-joining method with all parameters set to default. One thousand bootstrap datasets were generated to estimate the confidence limits of nodes. The tree was visualised using the Molecular Evolutionary Genetics Analysis (MEGA) package version 5 (Tamura et al., 2011).

Table 2.1. Identifiers and annotations for ZIP proteins of Arabidopsis and rice.

Protein Symbol	TAIR/RGAP Annotation	Accession no. TAIR/RGAP	Accession no. UniProtKB	Amino acids
Arabidopsis ZIP Family				
AtIAR1	ZIP metal ion transporter family	AT1G68100.1	Q9M647	469
AtIRT1	iron-regulated transporter 1	AT4G19690.2	Q38856	347
AtIRT2	iron regulated transporter 2	AT4G19680.2	O81850	350
AtIRT3	iron regulated transporter 3	AT1G60960.1	Q8LE59	425
AtZIP1	zinc transporter 1 precursor	AT3G12750.1	O81123	355
AtZIP2	ZRT/IRT-like protein 2	AT5G59520.1	Q9LTH9	353
AtZIP3	zinc transporter 3 precursor	AT2G32270.1	Q9SLG3	339
AtZIP4	zinc transporter 4 precursor	AT1G10970.1	O04089	408
AtZIP5	zinc transporter 5 precursor	AT1G05300.1	O23039	360
AtZIP6	ZIP metal ion transporter family	AT2G30080.1	O64738	341
AtZIP7	zinc transporter 7 precursor	AT2G04032.1	Q8W246	365
AtZIP8	zinc transporter 8 precursor	AT5G45105.2	Q8S3W4	299
AtZIP9	ZIP metal ion transporter family	AT4G33020.1	O82643	344
AtZIP10	zinc transporter 10 precursor	AT1G31260.1	Q8W245	364
AtZIP11	zinc transporter 11 precursor	AT1G55910.1	Q94EG9	326
AtZIP12	zinc transporter 12 precursor	AT5G62160.1	Q9FIS2	355
AtZTP29	ZIP metal ion transporter family	AT3G20870.1	Q940Q3	276
AtPutZnT	ZIP metal ion transporter family	AT3G08650.2	Q9C9Z1	619
Rice ZIP Family				
OsIRT1	metal cation transporter	LOC_Os03g46470.1	Q75HB1	374
OsIRT2	metal cation transporter	LOC_Os03g46454.1	Q6L8G1	370
OsZIP1	metal cation transporter	LOC_Os01g74110.1	Q94DG6	352
OsZIP2	metal cation transporter	LOC_Os03g29850.1	Q852F6	358
OsZIP3	metal cation transporter	LOC_Os04g52310.1	Q7XLD4	364
OsZIP4	metal cation transporter	LOC_Os08g10630.1	Q6ZJ91	396
OsZIP5	metal cation transporter	LOC_Os05g39560.1	Q6L8G0	353
OsZIP6	metal cation transporter	LOC_Os05g07210.1	Q6L8F9	395
OsZIP7	metal cation transporter	LOC_Os05g10940.1	Q6L8F7	384
OsZIP8	metal cation transporter	LOC_Os07g12890.1	A3BI11	390
OsZIP9	metal cation transporter	LOC_Os05g39540.1	Q0DHE3	362
OsZIP10	metal cation transporter	LOC_Os06g37010.1	Q5Z653	404
OsZIP11	metal cation transporter	LOC_Os05g25194.1	Q5W6X5	577
OsZIP13	metal cation transporter	LOC_Os02g10230.1	Q6H7N1	276
OsZIP14	metal cation transporter	LOC_Os08g36420.5	Q6YSC5	498
OsZIP16	metal cation transporter	LOC_Os08g01030.1	Q6Z1Z9	289

Provided are the TAIR/RGAP annotation and identifier, UniprotKB identifier for each *ZIP* gene. Amino acid length of each protein is also provided. Protein symbols for all Arabidopsis sequences were derived from TAIR, and those for rice sequences, OsIRT1, and OsIRT2 as well as OsZIP1 to OsZIP10 were derived from UniProKB. Protein symbols for OsZIP11, OsZIP14 and OsZIP16 were from Chen et al. (2008a).

2.2.4 Quantitative real-time RT-PCR

Briefly, total RNA from roots was prepared using Trizol reagent according to the manufacturer's instructions (Invitrogen), and treated with DNase I (Ambion, Austin, TX, USA). Two micrograms of total RNA from roots was used to synthesise cDNA with SuperScriptTM III reverse transcriptase (Invitrogen). The transcript levels of four control genes (barley α-tubulin, heat shock protein 70, glyceraldehyde-3-phosphate dehydrogenase and cyclophilin) were determined for all cDNA samples, and the most similar three of these four genes were used as normalisation controls. PCR products were amplified from cDNA using primer pairs, and then the product was isolated and sequenced to confirm that the primer pairs were specific for the targeted gene sequence. Following confirmation of PCR products, they were used as standards for calibration curves and determination of absolute copy number and identity (melt curve) of transcripts. PCR amplification efficiency for each primer set ranged from 0.9 to 1. Three biological replicates were used for transcript analysis. Three technical replicates were conducted for each cDNA sample. Normalisation was carried out as described by Vanesompele et al. (2002) and Burton et al. (2008) for the transcript levels of all cDNA samples. The normalised copies μg^{-1} RNA were used to represent transcript levels. The primer sequences for all genes determined are listed in Table 2.

Table 2.2. Primer sequences used for quantitative real-time RT-PCR analysis of transcript levels.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	GenBank accession no
HvZIP1	TTCATCTATGTCGCCATCAACCACCTC	CTATGCCAGTGAGCACACCGAAGAAC	109	Contig_2164938
HvZIP2	CGTGGCCGACTGGATCTTC	CTCGTGCATCCAACCCACAC	215	AK253136
HvZIP3	CATATCTTGCACTTTTCCTAGGTG	ATGGTCAATTCTTCACCAAGG	178	FJ208991
HvZIP5	ATCATCGGCATGTCCTTGGG	AAGAAAGACTTGTGGCGAAACC	143	FJ208992
HvZIP6	GCGAGGAAGAAGCAGAAGATGG	AAGACCTGGTGGAAGGAGAGC	146	AK369168
HvZIP7	TGGAAGGCATCCTCGACTCTG	CAATCAGATGGACACAGGCACAT	281	AM182059
HvZIP8	CGCTCTTCTTCTCACTCACC	GGAGAGGTTTACGATGACCTG	224	FJ208993
HvZIP10	GACCTCATTGCTGCTGATTT	AGCTAGGCAACAGGTCGTAGT	188	AK363919
HvZIP11	CTTGCGAAGATTGGCTATGA	AGACCCTCTCTTACGGTTTCAGT	297	AK249581
HvZIP13	GCTCGGCATCAACATCTCC	GTTGTAGGCTTGCGGCTAG	82	AK360012
HvIZIP14	CACAGATGCACGATCAGAGAAC	CCACAATATCCACGGAACTCATA	207	AK249958
HvIZIP16	TGCATCAGTTGGTGGTGTTA	TCTCAGGCTTCAGAGGACACT	229	AK251124
HvIRT1	CATCCTCCAGGCCGAGTA	TCAACGAGGGCCATGTAGT	196	EU545802
HvMTP1	CGCAGGATGTGGATGCTGAT	CTCCAGCACCAAAGGCAACA	223	AM286795

2.2.5 Statistical analysis

Experiments were set up as a completely randomised block design with four replications. Data of plant growth and metal nutrition were analysed using the Genstat Statistical Program (version 11.1, VSN International Ltd). The Least Significant Difference (LSD) at P=0.05 was used for comparisons of means.

2.3 Results

2.3.1 Effect of Zn deficiency on plant growth and uptake of Zn

Plants had reached Zn deficiency status after 13 days (D13) of growth with 0.005 µM of Zn (-Zn). At this stage, the Zn concentration in shoots of –Zn treated plants was 6 µg g^{-1} DM (Figure 2.1b), which is well below the concentration of 20 µg g^{-1} DM required for normal plant growth (Genc et al., 2002). There was a significant difference in shoot DW between –Zn and +Zn treated plants, but no difference in root DW (Figure 2.1a). The Zn concentration in roots of the –Zn treated plants at D13 was 11 µg g^{-1} DM (Figure 2.1b),

which was higher than that in the shoots. The concentrations of Fe, Mn and Cu in shoots were adequate for plants in both Zn treatments (Figure 2.2). The concentration of Cu in the roots of–Zn treated plants was higher than that in the +Zn treated plants (Figure 2.2c)

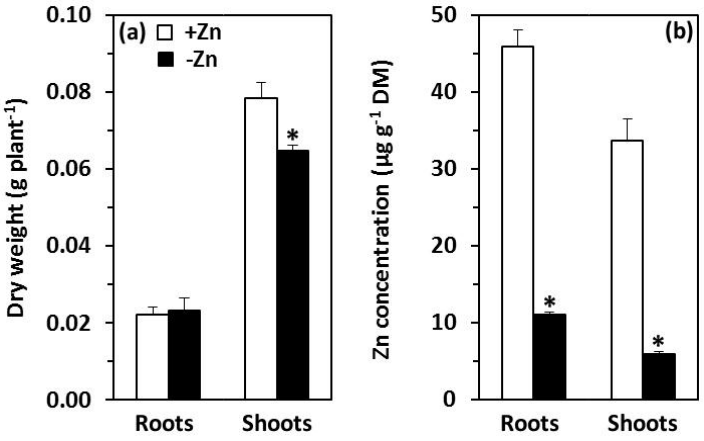

Figure 2.1. Effect of Zn supply on growth and Zn concentrations of plants grown in nutrient solution with two rates of Zn.
(a) Dry weight of roots and shoots. (b) Zn concentrations of roots and shoots. Barley seedlings were grown in nutrient solution with two rates of Zn (0.005 µM and 0.5 µM, referred to as –Zn and +Zn, respectively) for thirteen days. Means and SE values of four replicates are presented. Asterisks indicate a significant difference at $P<0.05$ between two Zn treatments using Student's *t*-test.

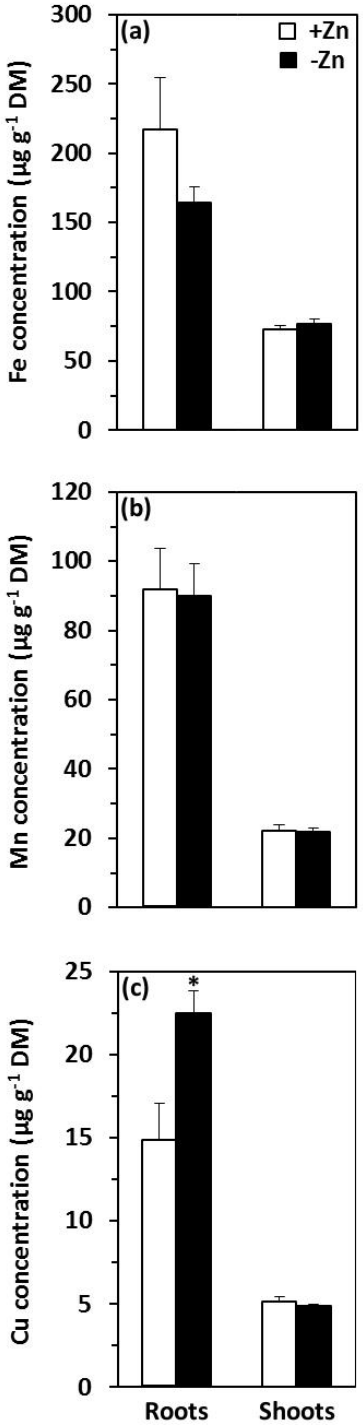

Figure 2.2. Effect of Zn supply on Fe, Mn and Cu concentrations of plants grown in nutrient solution with two rates of Zn.

(a) Fe concentrations of roots and shoots. (b) Mn concentrations of roots and shoots. (c) Cu concentrations of roots and shoots. Barley seedlings were grown in nutrient solution with two rates of Zn (0.005 µM and 0.5 µM, referred to as –Zn and +Zn, respectively) for thirteen days. Means and SE values of four replicates are presented. The Cu concentration in roots of plants grown in -Zn was significantly higher than that of plants grown in +Zn. Asterisks indicate a significant difference at $P<0.05$ between two Zn treatments using Student's *t*-test.

2.3.2 Identification of additional members of the ZIP family from the barley genome

Sequences of 16 ZIP family members of rice and 18 members of Arabidopsis (Table 2.1) were used to search for candidate members in Brachypodium (*Brachypodium distachyon* L.), barley and wheat (*Triticum aestivum* L.). Nine new HvZIP proteins (HvZIP1, HvZIP2, HvZIP6, HvZIP7, HvZIP10, HvZIP11, HvZIP13, HvZIP14 and HvZIP16) were identified in addition to the four HvZIP proteins (HvIRT1, HvZIP3, HvZIP5 and HvZIP8) reported by Pedas et al. (2009; 2008). Phylogenetic analyses showed that HvZIP3 formed a distinct clade together with OsZIP3 and OsZIP4, which did not contain any closely related homologs of Arabidopsis (Figure 2.3). HvZIP5 and HvZIP8, which were 86% similar in protein sequence, were grouped with OsZIP9 and OsZIP5 (Figure 2.3). HvZIP5 and HvZIP8 were also closely related to HvZIP13, which is grouped with OsZIP8 (Figure 2.3). There were no closely related ZIP homologs of Arabidopsis found in this monocotyledonous clade containing HvZIP5, HvZIP8 and HvZIP13 (Figure 2.3). HvZIP1 and the partial sequence of HvZIP2 (249 amino acid residues) were clustered with OsZIP1, OsZIP2, AtZIP2 and AtZIP11 (Figure 2.3). HvZIP6 clustered with OsZIP6 and AtZIP6 (Figure 2.3). HvZIP7 was closely related to OsZIP7, and formed a unique clade with HvZIP10 and OsZIP10. Three AtZIP proteins (AtIRT3, AtZIP4 and AtZIP9) were grouped to the HvZIP7 and OsZIP7 clade (Figure 2.3). HvZIP11, HvZIP14 and HvZIP16 formed a distinct clade with OsZIP11, OsZIP14, OsZIP13 and OsZIP16, respectively. Each of these three clades contained one Arabidopsis homolog, AtZnT, AtIAR1 and AtZTP29, respectively (Figure 2.3).

Sixteen members of the ZIP family were identified in the fully sequenced genome of Brachypodium. Almost all distinct ZIP family clades of Brachypodium and rice contain at least one homolog of barley (Figure 2.3), indicating that barley homologs are represented in the major clades of the ZIP family in monocotyledonous species. At least seven wheat ZIP (TaZIP) proteins were found in distinct homologous groups of HvZIP proteins (Figure 2.3).

The results indicate that the ZIP family proteins are relatively conserved in monocotyledonous lineages. By comparison with Arabidopsis, some members of the AtZIP family, such as AtZIP1, AtZIP3, AtZIP5 and AtZIP12, branched separately from all other sequences in the tree with low bootstrap values (Figure 2.3), suggesting that sequence divergence occurs between monocotyledonous and dicotyledonous species for some members of the ZIP family.

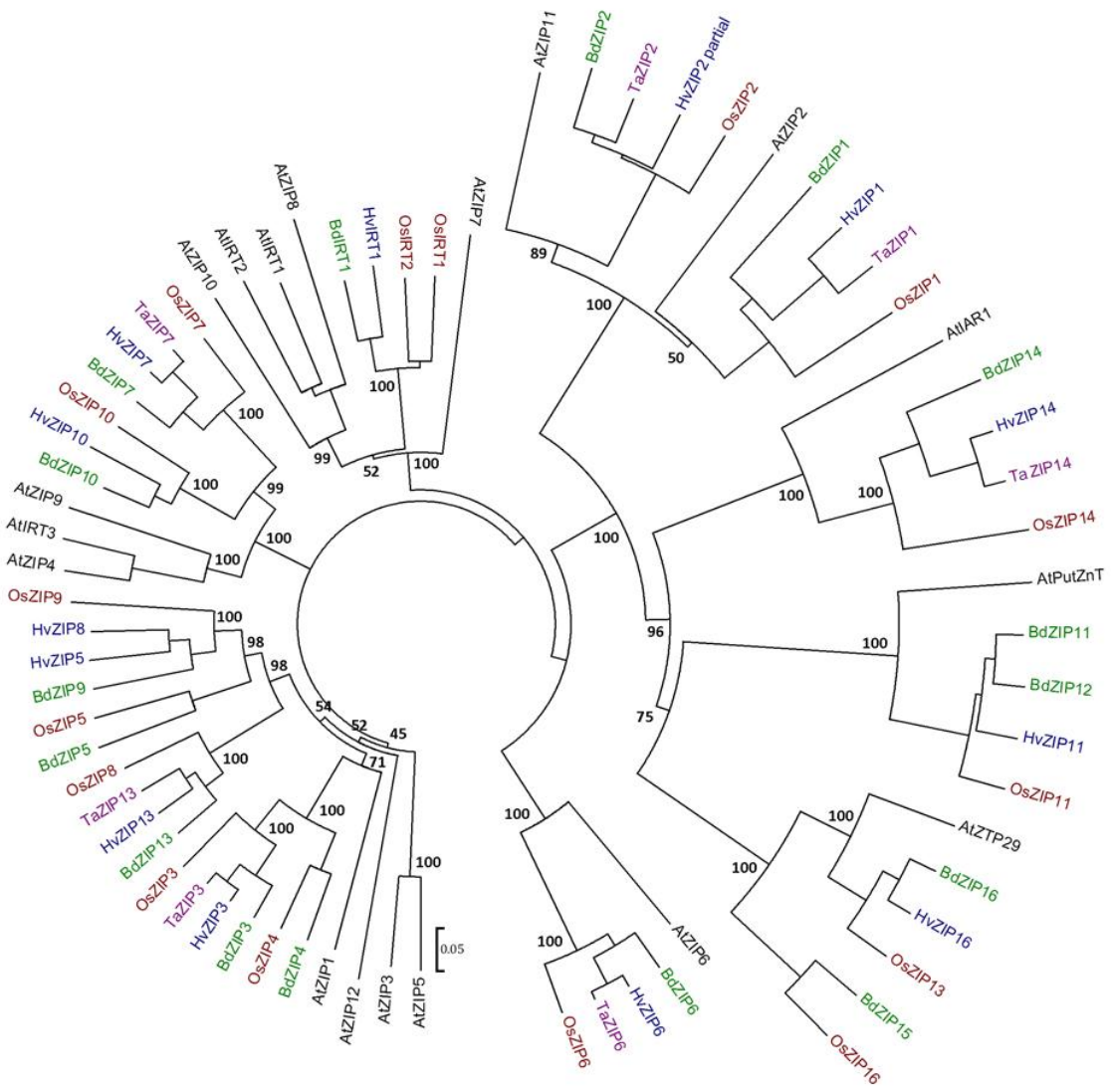

Figure 2.3. Phylogenetic tree of ZIP proteins in Arabidopsis, rice, Brachypodium, barley and wheat.
The unrooted tree was generated using the neighbour-joining method of ClustalW after sequence alignment with MAFFT (see materials and methods). Bootstrap values from 1000 replicates were used to estimate the confidence limits of nodes. The distance scale represents a 5% estimated amino acid substitution per residue. Species colour designation and corresponding accession numbers: *Arabidopsis thaliana* in black (AtIAR1: AT1G68100.1, AtIRT1: AT4G19690.2, AtIRT2: AT4G19680.2, AtIRT3: AT1G60960.1, AtZIP1: AT3G12750.1, AtZIP2: AT5G59520.1, AtZIP3: AT2G32270.1, AtZIP4: AT1G10970.1,

AtZIP5: AT1G05300.1, AtZIP6: AT2G30080.1, AtZIP7: AT2G04032.1, AtZIP8: AT5G45105.2, AtZIP9: AT4G33020.1, AtZIP10: AT1G31260.1, AtZIP11: AT1G55910.1, AtZIP12: AT5G62160.1, AtZTP29: AT3G20870.1, AtZnT: AT3G08650.2); *Oryza sativa* in red (OsIRT1: LOC_Os03g46470.1, OsIRT2: LOC_Os03g46454.1, OsZIP1: LOC_Os01g74110.1, OsZIP2: LOC_Os03g29850.1, OsZIP3: LOC_Os04g52310.1, OsZIP4: LOC_Os08g10630.1, OsZIP5: LOC_Os05g39560.1, OsZIP6: LOC_Os05g07210.1, OsZIP7: LOC_Os05g10940.1, OsZIP8: LOC_Os07g12890.1, OsZIP9: LOC_Os05g39540.1, OsZIP10: LOC_Os06g37010.1, OsZIP11: LOC_Os05g25194.1, OsZIP13: LOC_Os02g10230.1, OsZIP14: LOC_Os08g36420.5, OsZIP16: LOC_Os08g01030.1), *Brachypodium distachyon* in green (BdIRT1: Bradi1g12860.1 , BdZIP1: Bradi2g04020.1 , BdZIP2: Bradi1g60110.1, BdZIP3: Bradi5g21580.1, BdZIP4: Bradi3g17900.1, BdZIP5: Bradi2g22520.1, BdZIP6: Bradi2g34560.1, BdZIP7: Bradi2g33110.1, BdZIP9: Bradi2g22530.1, BdZIP10: Bradi1g37670.1, BdZIP11: Bradi1g76820.1, BdZIP12: Bradi2g31260.1, BdZIP13: Bradi1g53680.1, BdZIP14: Bradi3g37910.2, BdZIP15: Bradi3g12850.1, BdZIP16: Bradi3g07080.1), *Hordeum vulgare* in blue (HvIRT1: ACD71460.1, HvZIP1: Contig_2164938, HvZIP2_partial: AK253136 and GH225540, HvZIP3: ACN93832.1, HvZIP5: ACN93833.1, HvZIP6: BAK00370.1, HvZIP7: CAJ57719.1, HvZIP8: ACN93834.1, HvZIP10: BAJ95122.1, HvZIP11: BAJ85740.1, HvZIP13: BAJ91221.1, HvZIP14: BAJ85144.1, HvZIP16: AK251124), *Triticum aestivum* in purple (TaZIP1: ABF55691.1, TaZIP2: CAJ19368.1, TaZIP3: AAW68439.1, TaZIP6: AK333945, TaZIP7: ABF55692.1, TaZIP13: ABF55690.1, TaZIP14: AK331623).

2.3.3 Effect of Zn deficiency on transcript levels of 13 *HvZIP* genes

Transcript profiles of 13 *HvZIP* genes were determined in both roots and shoots for plant response to Zn deficiency using quantitative real-time RT-PCR. The transcript levels of *HvZIP5*, *HvZIP7*, *HvZIP8*, *HvZIP10* and *HvZIP13* in roots of Zn-deficient plants were increased by at least three-fold while *HvZIP2* was increased by 1.5-fold (Figure 2.4). The expression of *HvZIP1*, *HvZIP3*, *HvZIP6*, *HvZIP11*, *HvZIP14*, *HvZIP16* and *HvIRT1* in roots did not respond to Zn deficiency (Figure 2.4 and 2.5). In the shoots, the transcript levels of *HvZIP1*, *HvZIP2*, *HvZIP3*, *HvZIP5*, *HvZIP7*, *HvZIP8*, *HvZIP10* and *HvZIP13* were enhanced by at least five-fold in the Zn-deficient plants (Figure 2.4), while the expression of *HvZIP6*, *HvZIP11*, *HvZIP14*, *HvZIP16* and *HvIRT1* in shoots did not respond to Zn deficiency (Figure 2.5). In addition, the transcript levels of a metal tolerance protein gene, *HvMTP1* were determined. *HvMTP1* belongs to the *CDF* gene family, and it shares high homology with *AtMTP* genes (Desbrosses-Fonrouge et al., 2005; Kobae et al., 2004). The transcript levels of *HvMTP1* were not responsive to Zn deficiency (Figure 2.5f).

The transcript abundance of three *HvZIP* genes in roots varied greatly from that in shoots. *HvZIP1* was predominantly expressed in roots, whereas *HvZIP3* and *HvZIP13* were predominantly expressed in shoots (Figure 2.4a, c, h). The transcript abundance of *HvZIP10* in both roots and shoots of Zn-deficient plants was the highest among Zn-inducible *HvZIP* genes (Figure 2.4h), whereas the transcript abundance of *HvZIP5* was the lowest (Figure 2.4d). Although *HvZIP1* transcripts in the roots of Zn-deficient plants were not enhanced, it was approximately 15-fold higher than in the shoots, where it was induced by Zn-deficiency (Figure 2.4a). *HvZIP3* was induced by Zn deficiency only in the shoots (Figure 2.4c) while *HvZIP5* was evenly induced in both roots and shoots under Zn-deficiency (Figure 2.4d). The transcripts of *HvZIP2*, *HvZIP8*, *HvZIP7*, *HvZIP10* and *HvZIP13* were all induced by low Zn in both roots and shoots, but their transcripts were predominant in the shoots (Figure 2.4b, e, f, g, h).

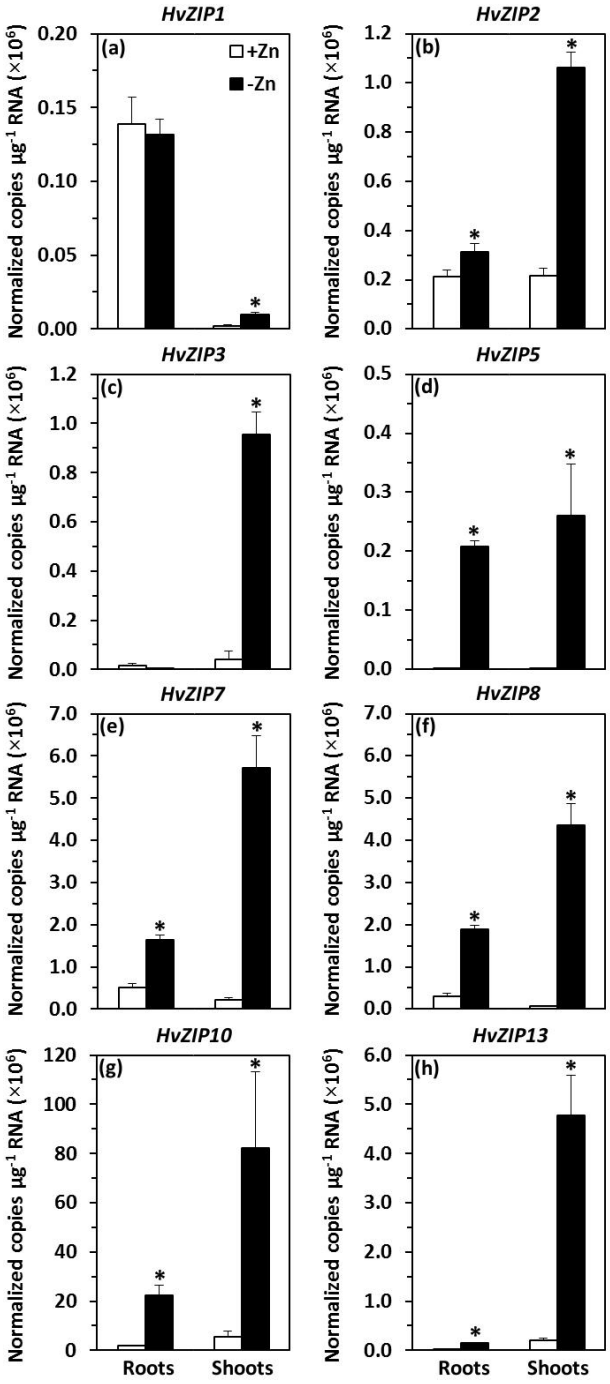

Figure 2.4. Transcript levels of eight Zn-deficiency inducible *HvZIP* genes in roots and shoots of the plants grown with two rates of Zn.

Transcript levels of *HvZIP1* (a), *HvZIP2* (b), *HvZIP3* (c), *HvZIP5* (d), *HvZIP7* (e), *HvZIP8* (f), *HvZIP10* (g) and *HvZIP13* (h) in roots and shoots. Barley seedlings were grown in nutrient solution with two rates of Zn (0.005 µM and 0.5 µM, referred to as –Zn and +Zn, respectively) for thirteen days. Quantitative real-time RT-PCR was used to determine transcript levels. The means and SE of three biological replicates are presented as normalised copies μg^{-1} RNA. Asterisks indicate a significant difference at $P<0.05$ between two Zn treatments using Student's *t*-test.

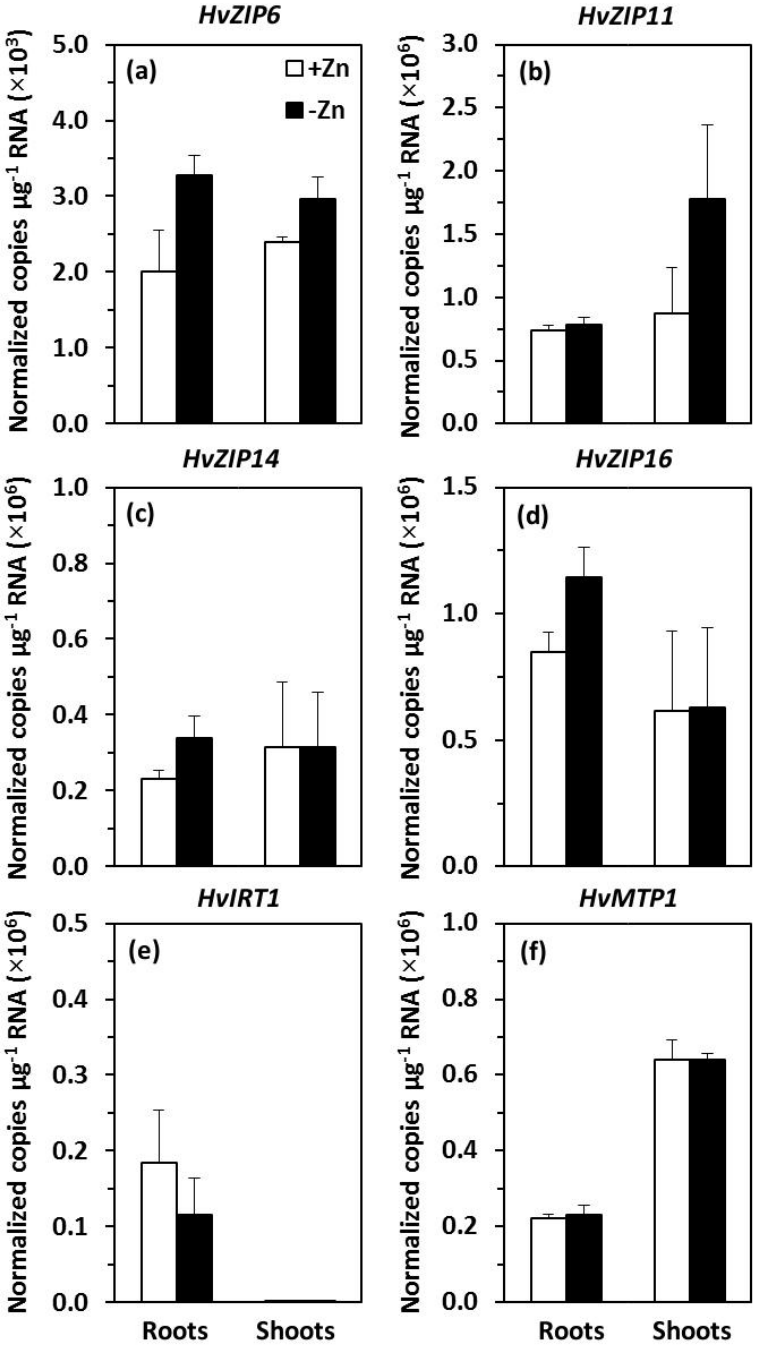

Figure 2.5. Transcript levels of five Zn-deficiency non-inducible *HvZIP* genes and *HvMTP1* in plants grown in nutrient solution with two rates of Zn.
Transcript levels of *HvZIP6* (a), *HvZIP11* (b), *HvZIP14* (c), *HvZIP16* (d), *HvIRT1* (e) and *HvMTP1* (f) in roots and shoots. Note the difference in scale at the vertical axis of (a) from (b) to (f). Barley seedlings were grown in nutrient solution with two rates of Zn (0.005 µM and 0.5 µM, referred to as –Zn and +Zn, respectively). Thirteen days after seed imbibition (D13), both –Zn and +Zn treated plants were harvested. Quantitative real-time RT-PCR was used to determine transcript levels. The means and SE of three biological replicates are presented as normalised copies μg^{-1} RNA.

2.4 Discussion

The barley genome sequencing is close to completion, but HvZIP members have not been fully identified. By using three fully sequenced plant species, Arabidopsis (dicotyledonous), rice and Brachypodium (monocotyledonous), nine new *HvZIP* genes were identified. The responsiveness of 13 *HvZIP* genes to Zn deficiency reveals that at least eight *HvZIP* genes are highly induced by Zn deficiency. Distinct transcript profiles displayed by these Zn-inducible *HvZIP* genes suggest that they play different roles in Zn-deficiency induced responses.

2.4.1 Identification of nine new *HvZIP* genes

Nine new *HvZIP* genes were identified by similarity searches of various databases, using *OsZIP* and *AtZIP* genes as queries (Table 2.1). Phylogenetic analysis allows for the classification of a large gene family into clades which may possess distinct functions. In addition to the gene sequences of barley, we have also included the sequences of Brachypodium and wheat for the analysis. The dicotyledonous genome of Arabidopsis was used because it has the most number of identified *ZIP* genes relative to other plant species (Grotz & Guerinot, 2006). Phylogenetic analysis of AtZIP family members has therefore resulted in the formation of many distinct clades, which helps categorise ZIP members from other species into different clades. According to the phylogenetic tree (Figure 2.3), barley ZIP members are represented in each of the monocotyledonous clades consisting of the ZIP family members of two completed monocotyledonous genome sequences (rice and Brachypodium), suggesting that they have different roles from each other in Zn homeostasis (see below for more discussion). The phylogenetic analysis also revealed new ZIP members from wheat, which were represented in most of the monocotyledonous clades (Figure 2.3). These results

can assist in the identification and selection of ZIP transporters from cereals for Zn biofortification purposes.

2.4.2 Eight *HvZIP* genes are induced by Zn-deficiency

The responsiveness of all 13 *HvZIP* genes to Zn deficiency were determined in both roots and shoots to identify *HvZIP* genes that could be involved in plant adaptation to low Zn conditions. Six *HvZIP* genes (*HvZIP2*, *HvZIP5*, *HvZIP7*, *HvZIP8*, *HvZIP10* and *HvZIP13*) were largely induced in both roots and shoots of Zn-deficient plants, whereas *HvZIP1* and *HvZIP3* were induced only in the shoots of Zn-deficient plants (Figure 2.4). HvZIP1 is closely related to HvZIP2 and forms a unique clade with OsZIP1 and OsZIP2 (Figure 2.3). This clade also contains AtZIP2 and AtZIP11. However, the transcript profile of *HvZIP1* is different from that of *HvZIP2*. *HvZIP1* was inducible only in the shoots of Zn-deficient plants, and its transcript level in roots was much higher than that in shoots (Figure 2.4a, b). The role of OsZIP1, the rice homolog of HvZIP1, is still unclear. *OsZIP1* is induced in both roots and shoots of Zn-deficient plants (Chen et al., 2008a; Ramesh et al., 2003) while other studies have shown that *OsZIP1* is induced only by Cu deficiency in the roots (Ishimaru et al., 2005). Our results suggest that HvZIP1 could play a role in Zn uptake under both Zn-sufficient and Zn-deficient conditions, and also have a minor role in shoot Zn retranslocation under Zn-deficient conditions. The rice homolog of HvZIP2, *OsZIP2*, has been shown to be induced in both roots and shoots of Zn-deficient plants (Chen et al., 2008a), which is similar to the expression profile of *HvZIP2* (Figure 2.4b). HvZIP2 might therefore be influential in Zn uptake and Zn translocation/retranslocation under Zn deficiency.

Similar to *HvZIP1*, *HvZIP3* was also induced only in the shoots by Zn deficiency, but the transcript level of *HvZIP3* in shoots was much higher than that in roots (Figure 2.4c). HvZIP3 belongs to another unique monocotyledonous clade, which includes OsZIP3 and

OsZIP4 (Figure 2.3). These two *OsZIP* genes are induced in both roots and shoots by Zn deficiency (Ishimaru et al., 2005; Ramesh et al., 2003). HvZIP3, OsZIP3 and OsZIP4 could restore growth of a yeast mutant with defects in Zn uptake (Ishimaru et al., 2005; Pedas et al., 2009; Ramesh et al., 2003). *OsZIP4* expresses particularly in phloem cells of shoots and roots, suggesting that OsZIP4 is involved in Zn retranslocation in Zn-deficient plants (Ishimaru et al., 2005). Therefore, similar to OsZIP4, HvZIP3 could promote Zn retranslocation in shoots but may also play a small role in Zn uptake in conditions of both deficient and adequate Zn supply (Figure 2.4c).

HvZIP5, HvZIP8 and HvZIP13 form a unique monocotyledonous clade with OsZIP5, OsZIP8 and OsZIP9 (Figure 2.3). The expression of these three *HvZIP* genes in both roots and shoots was induced by Zn-deficiency, but differences in expression profiles were observed between HvZIP5/HvZIP8 and HvZIP13 (Figure 2.4d, f, h). The transcript levels of *HvZIP5* and *HvZIP8* were relatively equal between roots and shoots of Zn-deficient plants, while *HvZIP13* was predominantly expressed in shoots of Zn-deficient plants (Figure 2.4d, f, h). In comparison to *HvZIP8*, *HvZIP5* was highly sensitive to Zn deficiency and its transcripts were hardly detectable in either roots or shoots of Zn-sufficient plants (Figure 2.4d). Both *HvZIP5* and *HvZIP8* are able to complement a yeast mutant defective in Zn uptake (Pedas et al., 2009). Interestingly, the rice homologs (*OsZIP5* and *OsZIP8*) of *HvZIP5*, *HvZIP8* and *HvZIP13* are also induced by Zn deficiency and expressed in both shoots and roots (Lee et al., 2010a; Lee et al., 2010b). Over-expression of *OsZIP5* and *OsZIP8* in rice increases Zn accumulation in roots but reduces Zn accumulation in shoots (Lee et al., 2010a; Lee et al., 2010b). These results suggest that *HvZIP5*, *HvZIP8* and *HvZIP13* could be involved in Zn uptake by roots and Zn translocation in shoots. As HvZIP5 and HvZIP8 are closely related in protein sequence, and relatively similar in transcript profiles, they may play partially redundant roles in plant Zn homeostasis. HvZIP13 could be involved in Zn translocation, primarily within shoots under low Zn conditions.

HvZIP7 and *HvZIP10* are two other *HvZIP* genes for which expression in both roots and shoots was highly induced by Zn deficiency (Figure 2.4 e, g). In comparison to *HvZIP7*, the transcript levels of *HvZIP10* were very high (Figure 2.4g). HvZIP7 and HvZIP10 form a distinct phylogenetic clade together with OsZIP7 and OsZIP10, and also AtIRT3, AtZIP4 and AtZIP9 (Figure 2.3). There is no functional information available for either *OsZIP7* or *OsZIP10* in Zn homeostasis. However, *AtZIP4* and *AtIRT3* of Arabidopsis from the same clade are able to complement the yeast mutant strain *zrt1zrt2* (Guerinot, 2000; Lin et al., 2009). Over-expression of *AtIRT3* in Arabidopsis increases Zn accumulation in shoots (Lin et al., 2009). Therefore, it is possible that both HvZIP7 and HvZIP10 are involved in Zn uptake and translocation, and that they may play partially redundant roles in plant Zn homeostasis.

These eight *HvZIP* genes (*HvZIP1*, *HvZIP2*, *HvZIP3*, *HvZIP5*, *HvZIP7*, *HvZIP8*, *HvZIP10* and *HvZIP13*) belong to four different clades, two of which are monocotyledon-specific (Figure 2.3). The enhanced transcript levels of these *HvZIP* genes in Zn-deficient plants indicate that multiple *HvZIP* genes contribute to enhanced uptake and translocation of Zn in Zn-deficient plants (Suzuki et al., 2006). These Zn-inducible *HvZIP* genes may play an important role in adaption to low Zn environments in the field.

2.4.3 Five *HvZIP* genes and *HvMTP1* show little response to Zn deficiency

Of the 13 *HvZIP* genes tested, five (*HvZIP6*, *HvZIP11*, *HvZIP14*, *HvZIP16* and *HvIRT1*) were not responsive or only marginally responsive to Zn deficiency (Figure 2.5). The homologs of these five HvZIP proteins were highly conserved between Arabidopsis and monocotyledonous species, and they belong to five different clades (Figure 2.3). *AtZIP6* and *OsZIP6* are close homologs of *HvZIP6* (Figure 2.3), and both are constitutively expressed in shoots and roots (Widodo et al., 2010; Wu et al., 2009), suggesting that they may play little or

no role in physiological responses to Zn deficiency. There is no functional information available from either Arabidopsis or monocotyledonous homologs for HvZIP11 and HvZIP16 although AtZTP29, an Arabidopsis homolog of HvZIP16 is involved in salinity tolerance (Wang et al., 2010). AtIAR1, an Arabidopsis homolog of HvZIP14 (Figure 2.3), participates in auxin metabolism, in which the transport of Zn^{2+} or Cu^{2+} out of endoplasmic reticulum may be required (Lasswell et al., 2000). The non-responsiveness of *HvZIP14* to Zn deficiency suggests that the HvZIP14 function in Zn homeostasis (if any) is independent of plant Zn status, and does not contribute to the physiological response induced by Zn deficiency. Despite the non-responsiveness of *HvZIP6*, *HvZIP11*, *HvZIP14* and *HvZIP16* to Zn deficiency, it is possible that these four *HvZIP* genes play roles in plant Zn homeostasis.

Despite its different nomenclature, *HvIRT1* is also a member of the *HvZIP* gene family and was previously described by Pedas et al. (2008) as a Mn transporter. OsIRT1 and AtIRT1 are close homologs of HvIRT1 (Figure 2.3). *OsIRT1* and *AtIRT1* are expressed predominantly in roots of rice and Arabidopsis, and are highly inducible by Fe deficiency (Eide et al., 1996; Ishimaru et al., 2006). *HvIRT1* was also predominantly expressed in roots, but did not respond to Zn deficiency (Figure 2.5e). Although HvIRT1 is capable of transporting Mn^{2+}, Zn^{2+} and Fe^{2+} in a yeast complementation assay (Pedas et al., 2008), it may play only a small role in the physiological responses induced by Zn deficiency.

HvMTP1 belongs to a different family of Zn transporters which transports Zn from the cytoplasm to the vacuole for compartmentation (Eide, 2006). The *MTP* genes of Arabidopsis were shown to be responsive to elevated concentrations of Zn (Arrivault et al., 2006; Desbrosses-Fonrouge et al., 2005; Kobae et al., 2004). HvMTP1 is highly similar to OsMTP1 (GenBank accession number: AY266290) and AtMTP proteins. *OsMTP1* is the only *MTP* gene present in the rice genome. Likewise, *HvMTP1* is the only *MTP* gene in barley. Zn deficiency had no effect on the expression of *HvMTP1* in either shoots or roots (Figure 2.5f).

These results suggest that enhanced sequestration of Zn into the vacuole *via* HvMTP1 may not be critical when a sub-micromolar concentration of Zn is available in growth media.

In summary, thirteen *HvZIP* genes have been identified, and they are represented in each of the phylogenetic groups of the ZIP family in monocotyledonous species. At least eight *HvZIP* genes are highly responsive to Zn deficiency, and display distinct transcript profiles in response to Zn deficiency, suggesting that they play different roles in plant Zn homeostasis. Therefore, these Zn-inducible *HvZIP* genes may be critical in plant adaption to low Zn environments. Our results provide new insights into plant responses to low Zn conditions in a temperate cereal. These findings can serve as a basis for further functional characterisation of *ZIP* genes in barley and other economically and nutritionally important cereals such as wheat. *HvZIP7* was selected for detailed studies (see Chapter 3) because it belongs to a phylogenetic clade different from those (*OsZIP4*, *OsZIP5*, *OsZIP8* and *AtZIP1*) which have been previously studied in cereals.

Chapter 3: Over-expression of *HvZIP7* in barley increases specific Zn translocation from roots to shoots and promotes Zn loading into grains.

3.1 Introduction

Zinc (Zn) is an essential micronutrient for plants, animals and humans. It is involved in a variety of biochemical processes (Welch & Shuman 1995; Maret 2004), and all organisms must maintain adequate intracellular concentrations of Zn to support their normal growth and development. Zn deficiency in cereal crops is widespread in many regions of the world (Hacisalihoglu et al., 2004; Broadley et al., 2007), and the consumption of low Zn cereals exacerbates Zn malnutrition in humans (Cunningham-Rundles et al., 2005; Hotz et al., 2005). Approximately one third of the world's population is affected by Zn deficiency (Alloway 2009), with pregnant women and pre-school children living in developing countries being at most risk to Zn deficiency (Welch & Graham 2004). The improvement of Zn nutrition in cereal crops is not only necessary for high crop yield, but also for human health. The alleviation of human Zn malnutrition was identified as the most important global challenge at the Copenhagen Consensus 2008 conference (www.copenhagenconsensus.com). Biofortification of cereal crops, especially of rice and wheat, is one of the most effective strategies to combat human Zn deficiency (Cakmak 2008; Palmgren et al., 2008).

Biofortification will require a better understanding and enhancement of physiological processes which limit Zn accumulation in grains (Waters & Sankaran 2011). There are two processes regarded as bottlenecks for Zn accumulation in grains. One is Zn loading into the xylem of roots. Higher Zn loading into the root xylem can increase Zn accumulation in shoots, making more Zn available for high Zn concentrations in cereal grains. The other

process which limits grain Zn accumulation is Zn loading from maternal tissues into the grain. The phloem appears to be the only path for Zn transport from maternal tissues into wheat grains (Patrick & Offler 2001). In the process of grain loading, Zn ions that are transported through the phloem stream first enter the grain transfer cells and are then unloaded into the apoplastic space of the grain. However, little is known about the Zn transporters involved in these processes (Palmgren et al., 2008).

Several Zn transporter families are involved in plant Zn homeostasis. These are the Zn-regulated, Iron-regulated transporter-like Proteins (ZIP) (Grotz et al., 1998; Guerinot 2000; Eide 2006), Metal Tolerance Proteins (MTP) (Kobae et al., 2004; Arrivault et al., 2006; Eide 2006), and Heavy Metal ATPases (HMA), a subgroup of the P-type ATPases (Eren & Arguello 2004; Hussain et al., 2004; Eide 2006). The ZIP transporter family is considered to be the primary group of transporters controlling plant Zn uptake and translocation (Gaither & Eide 2001; Palmgren et al., 2008).

There are 18 members of the ZIP gene family identified in the Arabidopsis genome (Grotz & Guerinot 2006), and 16 have been identified in the rice genome (Narayanan et al., 2007; Chen et al., 2008). So far, only four *ZIP* genes from barley (*HvIRT1*, *HvZIP3*, *HvZIP5* and *HvZIP8*) (Pedas et al., 2008; 2009) and one from the tetraploid emmer wheat, *TdZIP1* (Durmaz et al., 2010) have been described. To date, most studies on *ZIP* gene functions have focused on the genes from Arabidopsis and rice. The over-expression of *OsZIP4*, *OsZIP5* and *OsZIP8* resulted in higher Zn accumulation in roots but lower Zn accumulation in shoots and grains (Ishimaru et al., 2007; Lee et al., 2010a; 2010b), suggesting that these three genes are involved mainly in Zn uptake. The over-expression of *AtZIP1* in barley increased short-term Zn uptake (over 24 hrs) and the grain Zn concentration, but reduced the grain size (Ramesh et al., 2004). It is not clear whether the over-expression of *AtZIP1* in barley could increase Zn

uptake over a longer growth period, and whether the higher grain Zn concentration resulted from increased Zn uptake and translocation or a lower grain yield (McDonald et al., 2008).

In this chapter, *HvZIP7* was selected for detailed studies because it belongs to a different phylogenetic clade from those (*OsZIP4*, *OsZIP5*, *OsZIP8* and *AtZIP1*) which have been previously studied in cereals (Remash et al., 2004; Ishimaru et al., 2004; Lee et al., 2010a; Lee et al. 2010b).

3.2 Materials and methods

3.2.1 Semi-quantitative and quantitative real-time RT-PCR analysis

Barley (*Hordeum vulgare* L. cv. Lofty Nijo) seedlings used for examining effects of Zn deficiency on transcript levels of *HvZIP7* were grown in hydroponics with 0.005 or 0.5 μM Zn for 14 days. Plant growth conditions were similar to those described by Genc et al. (2007). Plants (*H. vulgare* L. cv. Golden Promise) used for examining transcript levels of endogenous *HvZIP7* and the transgene were grown in soil supplemented either with no Zn or with 0.5 mg Zn kg^{-1} soil. Plant growth conditions were similar as those described below for Zn and Cd uptake in soil-grown plants.

RNA isolation and cDNA synthesis were conducted as described in Section 2.2.4. For semi-quantitative RT-PCR, transgene-specific transcripts were amplified from cDNA using the primer pair of 5'-GCTGGTTTGTCCTCATTTTACG-3' and 5'-ATGATAATCATCGCAAGACCG-3', and the transcripts of the barley glyceraldehydes-3-phosphate dehydrogenase (*HvGAPDH*) gene were also amplified as a loading control using the primer pair of 5'-GTGAGGCTGGTGCTGATTACG-3' and 5'-TGGTGCAGCTAGCATTTGACAC-3'.

The quantitative real-time RT-PCR analysis was conducted as described in Section 2.2.4. The primer sequences found in Table 2.2 were used for endogenous *HvZIP7*, and the primers (5'- TACTCATATACATGGCGCTGGT-3' and 5'- TTTATTGCCAAATGTTTGAACG-3') were used for the *HvZIP7* transgene. The normalised copies μg^{-1} RNA were used to represent transcript levels.

3.2.2 *In situ* PCR analysis

For *in situ* PCR analysis, plants (cv. Golden Promise) were grown in hydroponics with no Zn addition. Plant growth conditions were the same as those described above for transcript analysis. Roots and leaves were collected from 16 day-old plants. *In situ* PCR was performed using the method described by Koltai and Bird (2000) with a few modifications. Roots and leaves were fixed in FAA (63% ethanol, 5% acetic acid and 2% formaldehyde), embedded in 5% agarose in 1×PBS, and then sectioned using the Leica VT1200S microtome. The sections were treated with DNAse (Ambion), followed by heat-inactivation. cDNA synthesis was made using Superscript III RT (Invitrogen) with a gene specific primer (5'- CACGTTATCTGATGTATGTATG-3'). PCR reactions were performed in a volume of 50 µl containing 1×PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 nmol digoxigenin-11-dUTP (Roche), 1.5 U Taq polymerase (Invitrogen) and 150 ng of both forward and reverse primers (5'-ACCAGGTTCTACGAGACCAAG-3' and 5'-TTAGGAGCGCACGTGTC-3') for the variable region of the *HvZIP7* coding sequence.

3.2.3 Subcellular localisation of HvZIP7

HvZIP7 fragments containing the full-length open reading frame were amplified from barley cDNA by PCR with the primer pair of 5´- TCAGGGCATGATGATCGGTGTA-3´ and

5´-CAATCAGATGGACACAGGCACAT-3´. The *HvZIP7* fragments were cloned into a pGEM®T-easy vector (Promega) and sequenced. The coding sequence of *HvZIP7* without the stop codon was amplified from pGEM®T-easy*HvZIP7* plasmid using the primer pair of 5´-ATGATGATCGGTGTAGCAGGCTTC-3´ and 5´-GGCCCAGACTGCAAGCAT-3´. The PCR fragment was ligated into the pCR8-GW-TOPO vector (Invitrogen) and transferred into *pMDC83* containing the *mGFP* gene (Curtis and Grossniklaus 2003). The resulting plasmid (*pMDC83HvZIP7*::*GFP*) places *HvZIP7* upstream of *mGFP*. Transient expression of *HvZIP7::GFP* in onion epidermal cells and visualisation of GFP in subcellular locations were conducted as described by Preuss et al. (2010). The plasmolysis of onion epidermal cells was performed by immersion in 1 M sucrose for one min before confocal image analysis.

3.2.4 Generation of *HvZIP7* over-expressing barley plants

The full-length open reading frame of *HvZIP7* was amplified from the *pGEM®T-easyHvZIP7* plasmid by PCR using the primer pair of 5´-CTGGTACCATGGTGATCGGTGTAGCAG-3´ and 5´-TACTCGAGTTCAGGCCCAGACTGC-3´. The PCR product was ligated into *pCR8-GW-TOPO* vector and then transferred into the *pMDC32* vector (Invitrogen) by LR reaction according to the manufacturer's instructions. The resulting *pMDC32HvZIP7* plasmid was used for barley (*Hordeum vulgare L.* cv. Golden Promise) transformation using an *Agrobacterium*-mediated method described by Tingay et al. (1997) and Matthews et al. (2001). The constitutive expression of *HvZIP7* was driven by a double 35S promoter. The number of transgenic loci was determined by standard Southern hybridisation.

3.2.5 Zinc and Cd uptake in soil-grown plants

Zn-deficient siliceous sand (DTPA-extractable Zn=0.07 mg kg^{-1} soil) was used in soil experiments. Basal nutrients and plant growth conditions were as described by Genc et al. (2007). Two barley grains with emerged radicles were planted into each pot containing one kg soil. Plants were thinned to one per pot nine days after imbibition (D9). Plants were grown in a growth room at 20/15 ºC day/night temperature and a photoperiod of 14 hr day/10 hr night at 300 µmol m^{-2} s^{-1} photon flux intensity at the plant level. At D28, the plants were harvested. The roots were washed free of soil, briefly rinsed in deionised water and excess water was blotted on fresh laboratory tissues. The roots and shoots were then oven-dried at 80 ºC for 48 hr for nutrient analysis.

For Cd uptake, the experimental setup was similar to that of the Zn uptake experiment. Two rates of Cd (3 and 10 mg Cd kg^{-1} soil) were added into the soil with basal nutrients. At D26, shoots were harvested and oven-dried at 80°C for 48 hr for nutrient analysis.

3.2.6 Zinc accumulation in grains

Plants were grown either in an 8-inch pot with 4.5 kg of a low Zn soil mix (University of California soil mix) containing 1.44 mg Zn, or in a 6-inch pot with 1 kg of a high Zn soil mix (coco peat soil mix) containing 17.8 mg Zn. Two barley seeds with emerged radicles were planted into the 8-inch pots, and one was planted into the 6-inch pots. The plants were grown to maturity. Deionised water was used for watering during the experiments. For the low dose of Zn treatment, Zn solution (1 mg Zn in 20 ml water) was added into the 8-inch pots for three times during anthesis. Plants were grown in a greenhouse with approximately 24/13 °C day/night temperature and 12 hr day/12 hr night cycle. Shoots and grains were harvested at maturity and oven-dried at 80 °C for 48 hr. For grain polishing, ten barley grains

for each sample were dehusked manually, loaded into a Kett® Pearlest miller and milled for seven min.

3.2.7 Mineral element analysis

Mineral element analysis was conducted as described in Section 2.2.2.

3.2.8 Statistical analysis

All experiments were set up as a completely randomised block design with three to four replications. The data were analysed using the Genstat Statistical Program (version 11.1, VSN International Ltd), and pair-wise comparisons of the means were made using Least Significance Difference (LSD) test at P=0.05. To overcome the problem of non-homogeneity of variances, log-transformed data were used for the analysis of variance.

3.3 Results

3.3.1 Zinc deficiency increases expression of *HvZIP7* in both roots and shoots

Transcript levels of *HvZIP7* were low in both roots and shoots of Zn-adequate plants (Figure 2.4e). Zn deficiency largely increased the transcript levels of *HvZIP7* in both roots and shoots (Figure 2.4e). To find out in which cell types *HvZIP7* is specifically expressed, *in situ* RT-PCR was performed in cross sections of roots and leaves of the Zn-deficient plants. *HvZIP7* transcripts were detected mainly in epidermal cells and the cells within the vascular bundle of roots (Figure 3.1c), while in the cross section of leaves *HvZIP7* transcripts were found primarily in the cells of the vascular bundle (Figure 3.1d). There were no *HvZIP7* transcripts detected in the no-RT controls of either roots or leaves (Figure 3.1a, b). These

results suggest that HvZIP7 is involved mainly in Zn translocation of roots and Zn retranslocation of leaves by mediating Zn loading into the xylem of roots and unloading into the phloem of shoots.

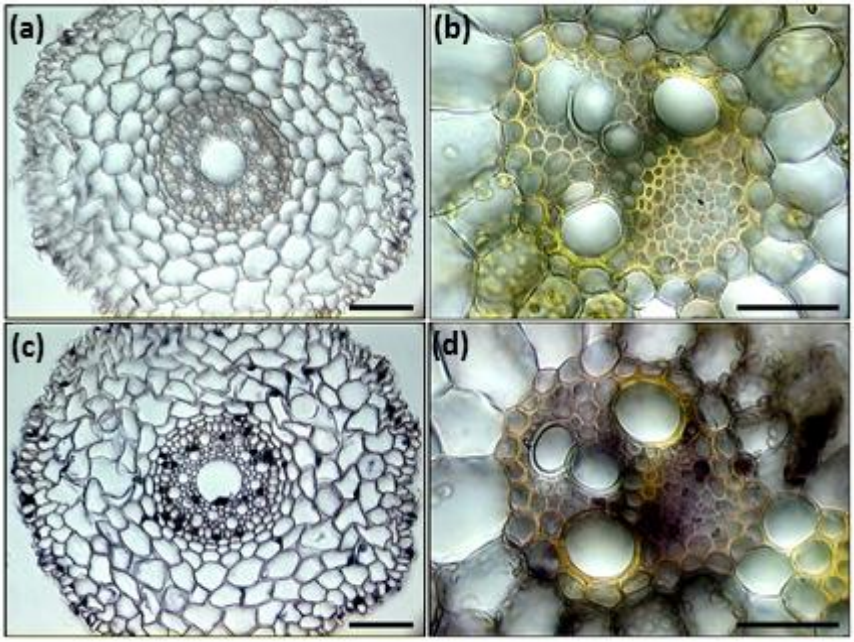

Figure 3.1. Zn deficiency-induced expression of *HvZIP7* in roots and leaves.
(a) and (b), no RT control in cross sections of roots and leaf tissues, respectively. (c) and (d), *in situ* RT-PCR detection of *HvZIP7* transcripts (purple staining) in cross sections of roots and leaf tissues, respectively. Abbreviations: ep, epidermis; xy, xylem cells; ph, phloem cells; en, endodermis; co, cortex; bs, bundle sheath cells; ms, mesophyll cells containing chloroplasts. Scale bars = 100 µM in (a) and (c), and 50 µM in (b) and (d). For *in situ* RT-PCR, barley seedlings were grown in nutrient solution with no Zn addition for 16 days after seed imbibition.

3.3.2 Subcellular localisation of HvZIP7

To determine the subcellular localisation of HvZIP7, a *HvZIP7::GFP* construct was generated for transient expression in onion epidermal cells. The green fluorescence of HvZIP7::GFP appeared in the location of the plasma membrane (PM) (Figure 3.2a), whereas the green fluorescence of the organelle control, barley thioredoxin 5::GFP (HvTrx5::GFP) was scattered in the cell (Figure 3.2b). To distinguish PM from the vacuolar membrane, the onion epidermal cell which expressed HvZIP7::GFP was plasmolysed for visualisation of the Hechtian strands connecting PM to the cell wall (Figure 3.2c, d). A close-up view shows

adhesion of the green fluorescent strands of PM to the cell wall (Figure 3.2e, f), confirming that the fluorescent signal derived from HvZIP7::GFP is associated with PM.

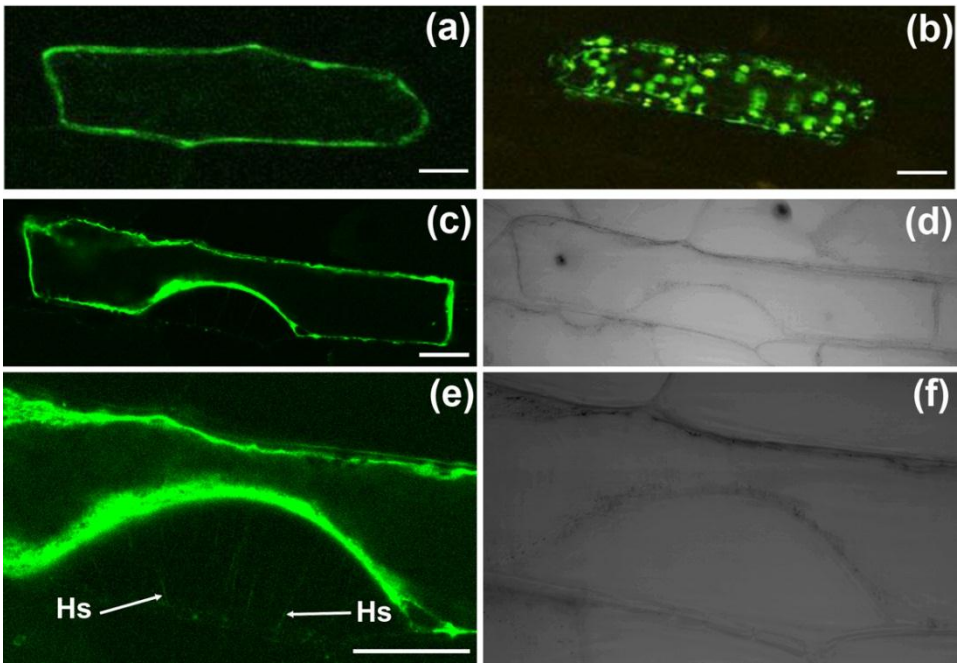

Figure 3.2. Subcellular localisation of HvZIP7::GFP.
(a), an onion epidermal cell expressing HvZIP7::GFP. (b), an onion epidermal cell expressing HvTrx5::GFP. (c) and (d), an onion epidermal cell expressing HvZIP7::GFP after partial plasmolysis under UV or bright light, respectively. (e) and (f), a close-up view of Hechtian strands (Hs) from the same cell shown in (c) under UV or bright light, respectively. Arrows indicate the Hechtian strands with GFP fluorescence. Scale bars = 50 µM.

3.3.3 Higher translocation of Zn from roots to shoots in *HvZIP7* over-expressing plants

Attempts were made to complement yeast strains involved in Zn homeostasis (*zrt1zrt2* mutant defective in Zn uptake and *zrc1cot1* mutant with a Zn sensitive phenotype) with *HvZIP7* to no success. Therefore, transgenic lines over-expressing *HvZIP7* were generated in barley for functional characterisation of *HvZIP7* in Zn homeostasis of plants. Transgenic plants with a single locus insert of the *HvZIP7* transgene were identified by Southern blot analysis and selected for propagation. Homozygous progenies of three independent, single locus-insert lines were used for further molecular and physiological analyses. Semi-quantitative RT-PCR analysis showed that the *HvZIP7* transgene was expressed in the leaves

from all three independent transgenic lines, while there were no transcripts detected in either the null progenies or wildtype (Figure 3.3).

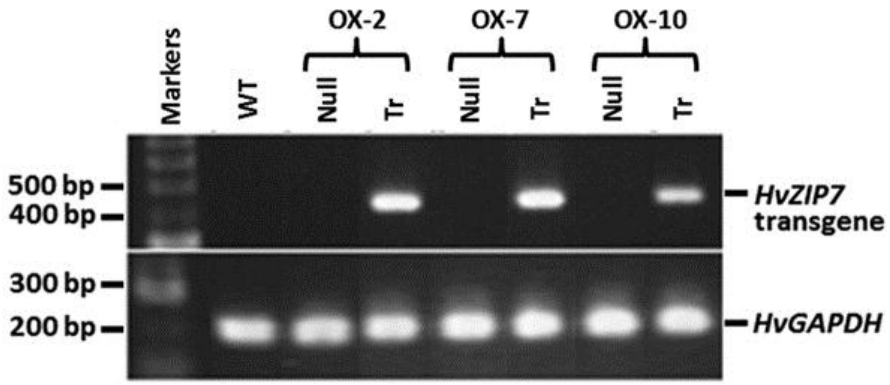

Figure 3.3. Semi-quantitative RT-PCR analysis of the *HvZIP7* transgene in three independent transgenic lines.
Total RNA of leaves from three independent, over-expressing *HvZIP7* (OX) lines, null lines and the wildtype (WT) were used in RT-PCR amplification of the *HvZIP7* transgene and *HvGAPDH* (a loading control). Amplified PCR products were run in agarose gel and stained with ethidium bromide. Molecular sizes (markers) are shown on the left.

Zn uptake experiments were conducted by growing one of the single-insert lines, OX-10 (Figure 3.3) in soil supplemented with a range of Zn (from zero to 12.5 mg Zn kg^{-1} soil). There were no significant differences in dry weight of shoots or roots between the transgenic and null or wildtype plants after 28 days of growth in any of the four rates of Zn supplement (Figure 3.4a, b). The Zn concentrations of shoots in all three genotypes rose from approximately 10 to 500 µg Zn g^{-1} DW or more as the Zn supplement rates increased. The shoot Zn concentration of 10 µg Zn g^{-1} DW is below the critical level required for normal plant growth (Genc et al., 2002), while the shoot Zn concentration of 500 to 700 µg Zn g^{-1} DW is close to the toxic concentration for barley (Aery & Jagetiya 1997). However, there was no significant growth reduction in shoots observed for nil Zn or the highest Zn supplement. This could be due to the appearance of Zn deficiency or toxicity towards the end of the experiment, and the short period of Zn deficiency or toxicity had not resulted in a measurable growth difference (Figure 3.4a, b). There was no significant difference in shoot Zn concentrations between transgenic and null or wildtype plants when grown with no Zn

supplement (Figure 3.4c). However, significant differences in shoot Zn concentrations were observed between transgenic and null or wildtype plants when grown with Zn supplement (Figure 3.4c). The transgenic plants had at least 60% higher Zn concentrations than those of the null or wildtype (Figure 3.4c). The transgenic plants had also higher shoot Zn content than the null or wildtype plants when grown with Zn supplement (Figure 3.4e).

Zn concentrations in roots of all three genotypes also rose as Zn supplement rates increased (Figure 3.4d). There were no significant differences in root Zn concentration and content between the transgenic and null or wildtype plants when grown either with no Zn supplement or with 12.5 mg Zn kg^{-1} soil (Figure 3.4d). However, the transgenic plants had a significantly lower Zn concentration and content in roots than the null or wildtype when grown with 1.0 and 5.0 mg Zn kg^{-1} soil (Figure 3.4d, f). Furthermore, the transgenic plants had a higher shoot:root ratio of Zn content than the null or wildtype when grown with Zn supplement (Figure 3.5b) despite that the total plant Zn content was not significantly different (P=0.59) between the transgenic and null or wildtype (Figure 3.5a). Zn leaching from the roots during root washing and soil Zn contamination could affect Zn concentrations in roots, but if any leaching and contamination occurred in the roots, they would have similar effects on both the null and transgenic plants. Therefore, the results indicate that the increasing Zn accumulation in shoots of the *HvZIP7* over-expressing line is a result of enhanced translocation of Zn from roots to shoots, and this enhanced translocation is Zn concentration-dependent.

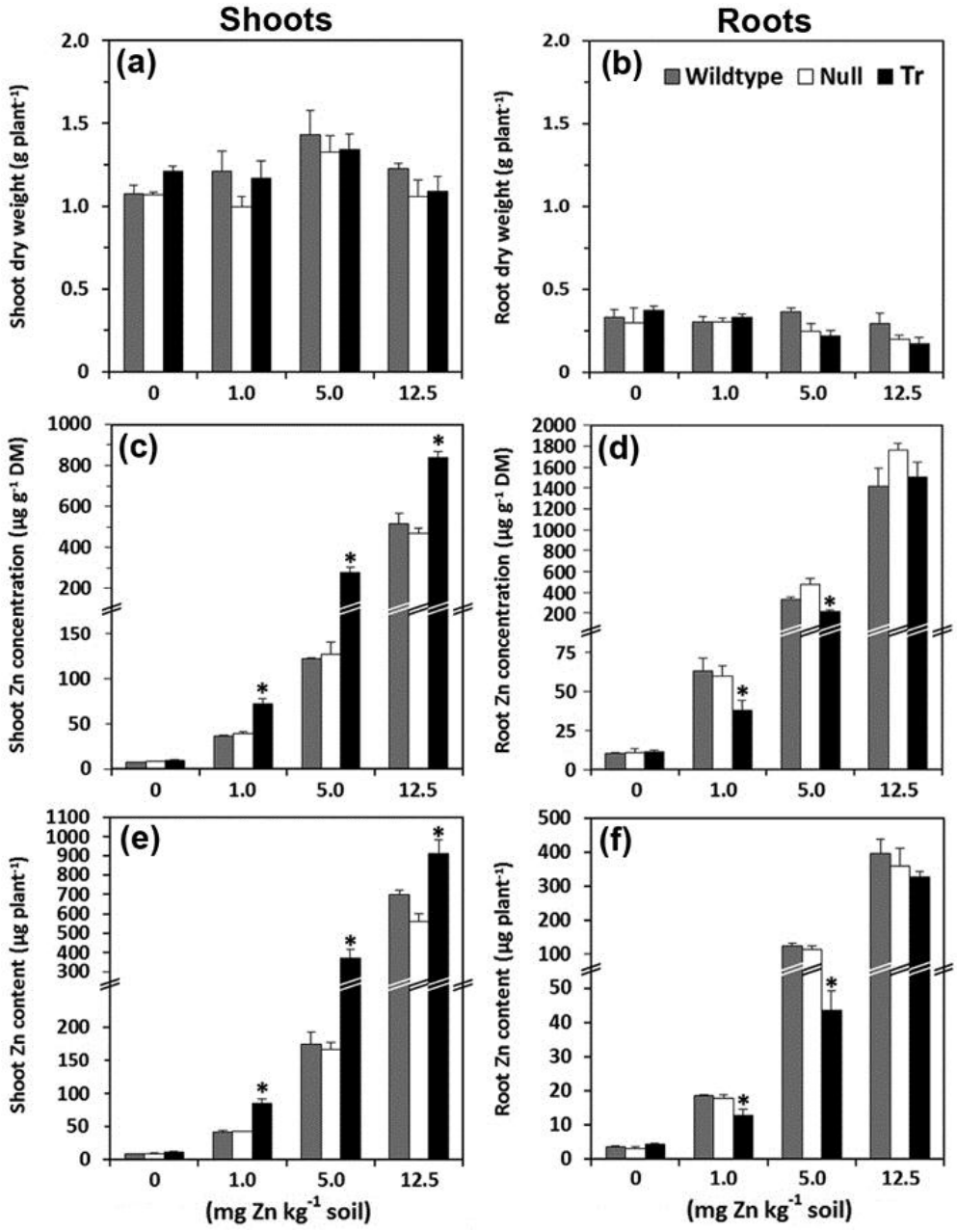

Figure 3.4. Effect of Zn supplement rates on growth, Zn concentration and Zn content of soil-grown plants.

Dry weight of shoots (a) and roots (b). Zn concentration of shoots (c) and roots (d). Zn content of shoots (e) and roots (f). Barley seedlings of the OX-10 line (Tr), null and wildtype (WT) were grown in soil supplemented with four Zn rates. Plants were harvested 28 days after seed imbibition. Means and SE values of three replicates are presented. Asterisks indicate the least significant differences at $P<0.05$.

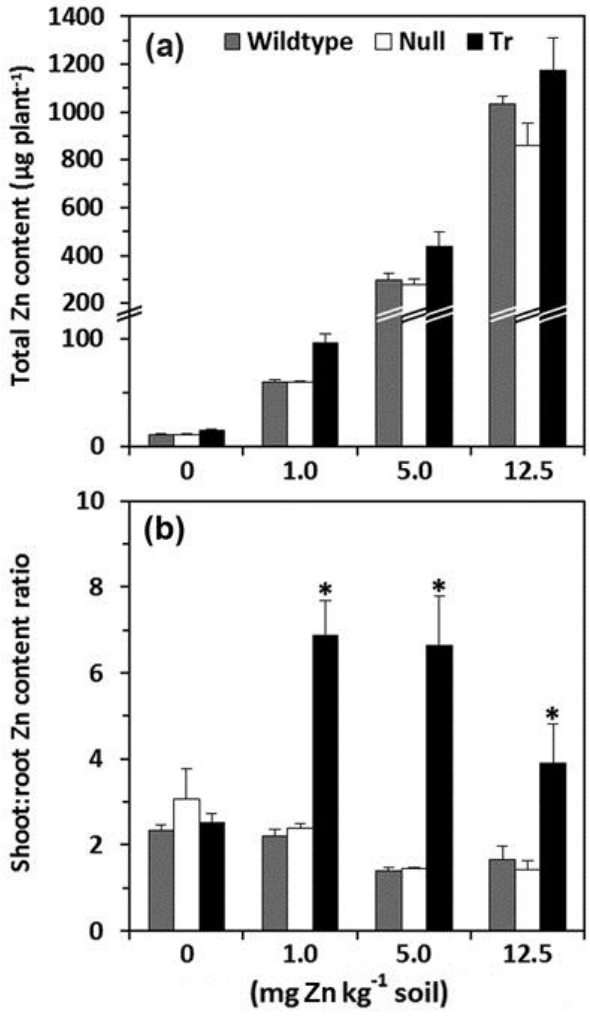

Figure 3.5. Effect of Zn supplement rates on total Zn uptake and shoot:root ratios of Zn content in soil-grown plants.
Total Zn content of plants grown in soil (a). Shoot:root ratios of Zn content in plants grown in soil (b). Barley seedlings of the OX-10 line (Tr), null and wildtype (WT) were grown in soil supplemented with four Zn rates. Plants were harvested 28 days after seed imbibition. Means and SE values of three replicates are presented. Asterisks indicate the least significant differences at $P<0.05$.

The different rates of Zn supplement to soil also changed the concentrations of Fe, Mn and Cu in both shoots and roots of all three genotypes (Figure 3.6). Generally, Fe concentrations in both shoots and roots were reduced with an increase in Zn supplement (Figure 3.6,a, b), whereas Mn and Cu concentrations in both shoots and roots were enhanced with an increase in Zn supplement (Figure 3.6, c to f). Metal imbalance associated with changes in Zn concentrations is a common phenomenon (Cakmak et al., 1994; Walter et al., 1994). It is not clear how the increasing rates of Zn supplement have different effects on different metals. However, over-expression of *HvZIP7* had a little effect on the concentration

of Fe, Mn or Cu in both shoots and roots relative to the null or wildtype plants despite that the concentrations of Fe, Mn and Cu in roots were higher than those in shoots (Figure 3.6). These results indicate that the over-expression of *HvZIP7* is specific for Zn translocation from roots to shoots.

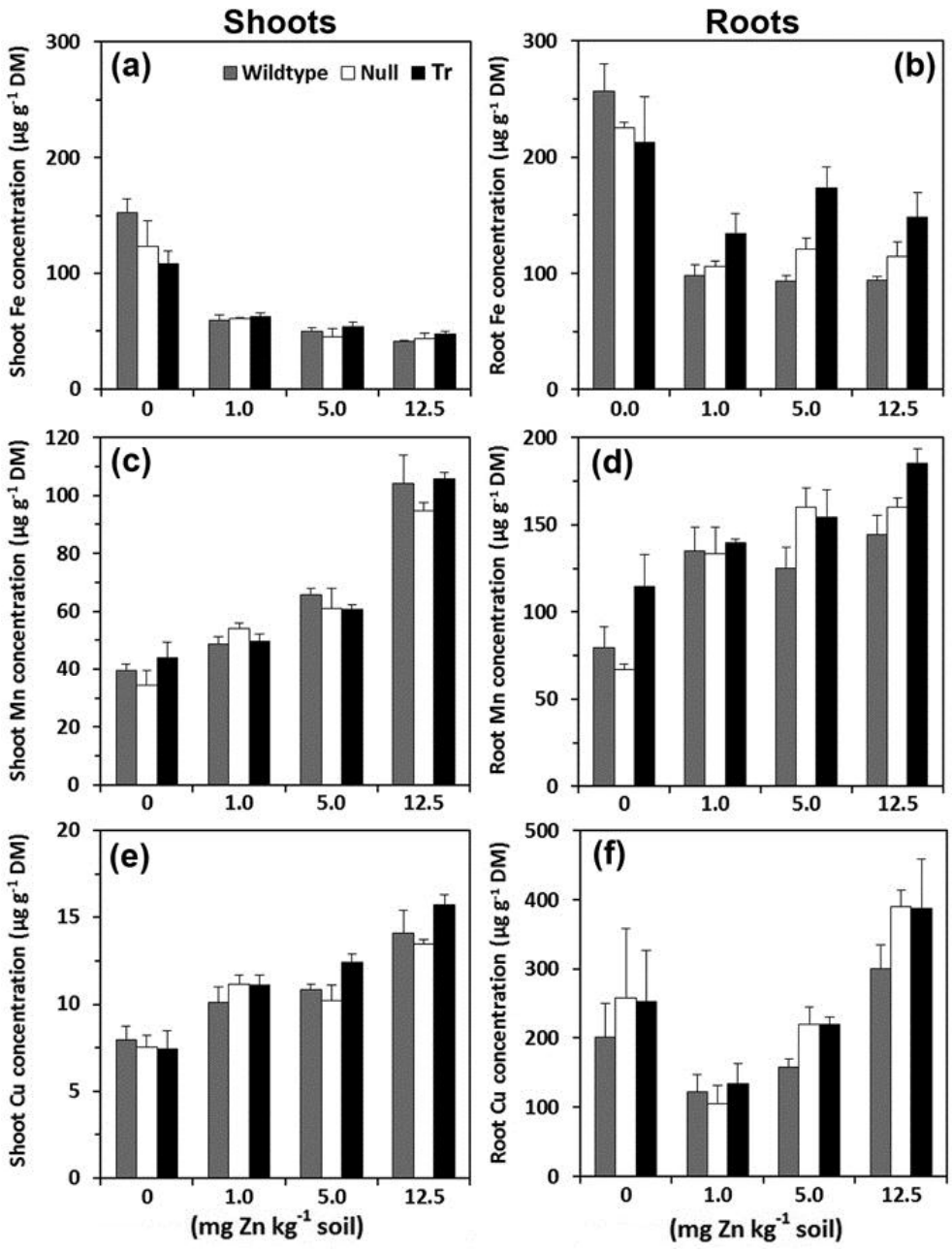

Figure 3.6. Effect of Zn supplement rates on Fe, Mn and Cu concentrations of soil-grown plants.
Fe concentrations of shoots (a) and roots (b). Mn concentrations of shoots (c) and roots (d). Cu concentrations of shoots (e) and roots (f). Barley seedlings of the OX-10 line (Tr), null and wildtype (WT) were grown in soil supplemented with four Zn rates. Plants were harvested 28 days after seed imbibition. Means and SE values of three replicates are presented.

3.3.4 Transcript levels of endogenous *HvZIP7* and the transgene in transgenic plants

To examine whether lack of difference in Zn translocation from roots to shoots under the low Zn conditions between transgenic and control plants is due to altered expression levels of either endogenous *HvZIP7* and/or the transgene, the transcript levels of both endogenous *HvZIP7* and transgene were determined for the OX-10 transgenic plants grown in soil supplemented with no Zn (-Zn) or 0.5 mg Zn kg^{-1} soil (+Zn). The Zn concentration of shoots in the -Zn treatment was approximately 12 µg Zn g^{-1} DW, which is below the critical level (Genc et al., 2002), whereas the Zn concentration of shoots in the +Zn treatment was above the critical level (Figure 3.7a). The transcript levels of endogenous *HvZIP7* in both roots and shoots of all three genotypes were very low (approximately 0.05 $\times 10^6$ copies μg^{-1} RNA or less) in the +Zn treatment, but were strongly enhanced in the -Zn treatment to approximately 0.4 $\times$ 10^6 copies μg^{-1} RNA or more in both roots and shoots of all three genotypes (Figure 3.7c, d). The expression of the transgene had no effects on the transcript levels of endogenous *HvZIP7* in either roots or shoots under the -Zn treatment despite a small reduction in the shoots of the transgenic plants treated with +Zn (Figure 3.7c, d). Regardless of the Zn status of the plants, the transcript levels of the transgene in both shoots and roots of the transgenic plants were comparable to those of endogenous *HvZIP7* in the -Zn treatment (Figure 3.7e, f).

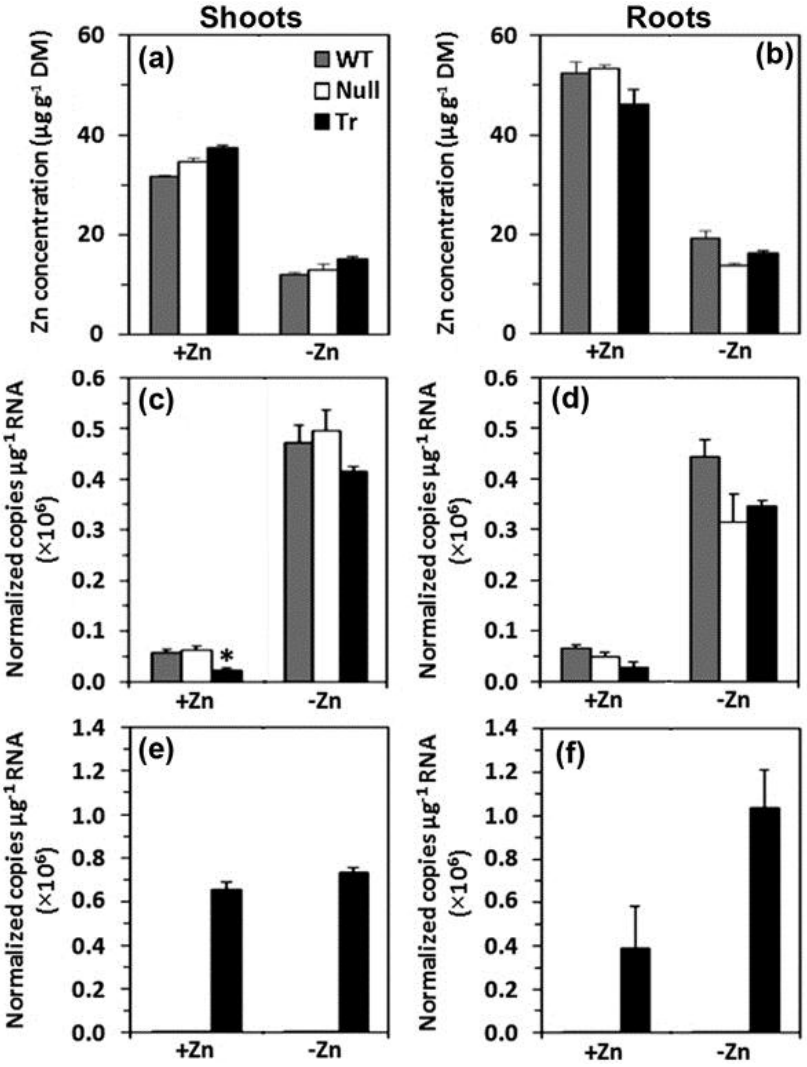

Figure 3.7. Transcript levels of endogenous *HvZIP7* and the transgene in soil-grown plants.
Zn concentrations in shoots (a) and roots (b). Transcript levels of endogenous *HvZIP7* in shoots (c) and roots (d). Transcript levels of the *HvZIP7* transgene in shoots (e) and roots (f). Seedlings of the OX-10 line (Tr), null and wildtype (WT) were grown in soil supplemented either with no Zn (-Zn) or with 0.5 mg Zn kg^{-1} soil (+Zn). Plants were harvested 28 days after seed imibition. Quatitative real-time RT-PCR was used to determine transcript levels of endogenous and transgenic *HvZIP7*. The means and SE of four biological replicates are presented as normalised copies μg^{-1} RNA. Asterisks indicate the least significant differences at $P<0.05$.

3.3.5 *HvZIP7* over-expressing plants increases Zn accumulation in grains when grown in a low Zn potting mix

To test the ability of *HvZIP7* over-expressing plants in grain Zn accumulation, the transgenic lines of OX-10 were grown to maturity in a low Zn potting mix with no additional Zn supplement. There were no significant differences in grain Zn concentration or content

between the transgenic and null plants grown with no additional Zn supplement (-Zn) (Figure 3.8a, b). The grain Zn concentrations were approximately 23 µg Zn g^{-1} DW for both transgenic and null lines of OX-10 (Figure 3.8a). When a small amount of Zn (3 mg Zn pot^{-1}) was supplemented to the potting mix (+Zn) during anthesis, the grain Zn concentration was significantly increased to 31 µg Zn g^{-1} DW in the null line, and the increase was even higher in the transgenic line (Figure 3.8a). An increase of approximately 30% in the grain Zn concentration and content was observed in the transgenic line relative to the null (Figure 3.8a, b). The grain Zn concentrations of the null plants from this pot experiment are similar to those (21-31 µg Zn g^{-1} DW) of the field-grown plants in a low Zn soil with Zn fertilisers (Graham et al., 1992). These results indicate that the transgenic *HvZIP7* plants can increase grain Zn accumulation in a low Zn condition with Zn fertilisation during anthesis.

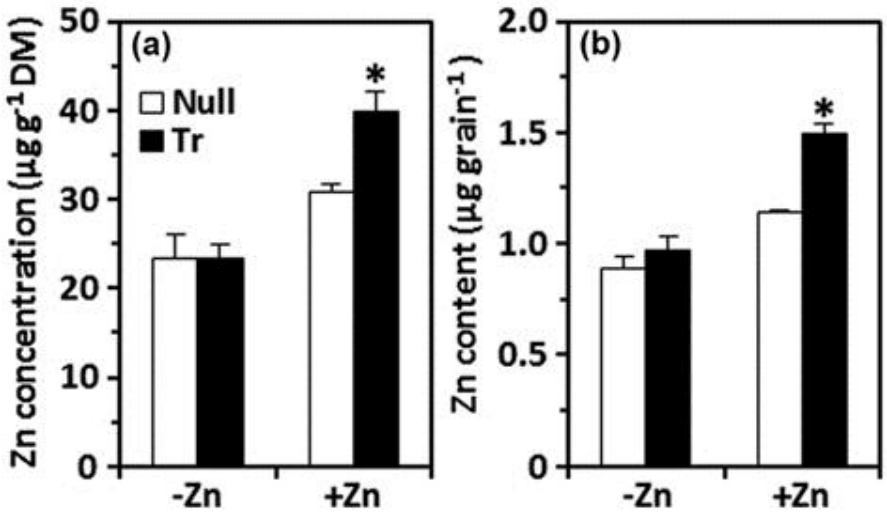

Figure 3.8. Effect of a low dose of Zn supplement on grain Zn concentration and content of plants grown in a low Zn potting mix.
Grain Zn concentration (a) and content (b). Transgenic OX-10 (Tr) and null plants were grown to maturity in a 8-inch pot containing a low Zn potting mix (1.44 mg Zn pot^{-1}) either with no Zn supplement (-Zn) or with 3 mg Zn per pot during anthesis (+Zn). Five grains were analysed for each sample. Means and SE values of four replicates are presented. Asterisks indicate the least significant differences at $P<0.05$.

In contrast to Zn, the concentrations and content of Fe, Mn and Cu in the grains of the transgenic line (OX-10) were similar to those of the null in the low Zn potting mix with or without Zn supplement (Figure 3.9).

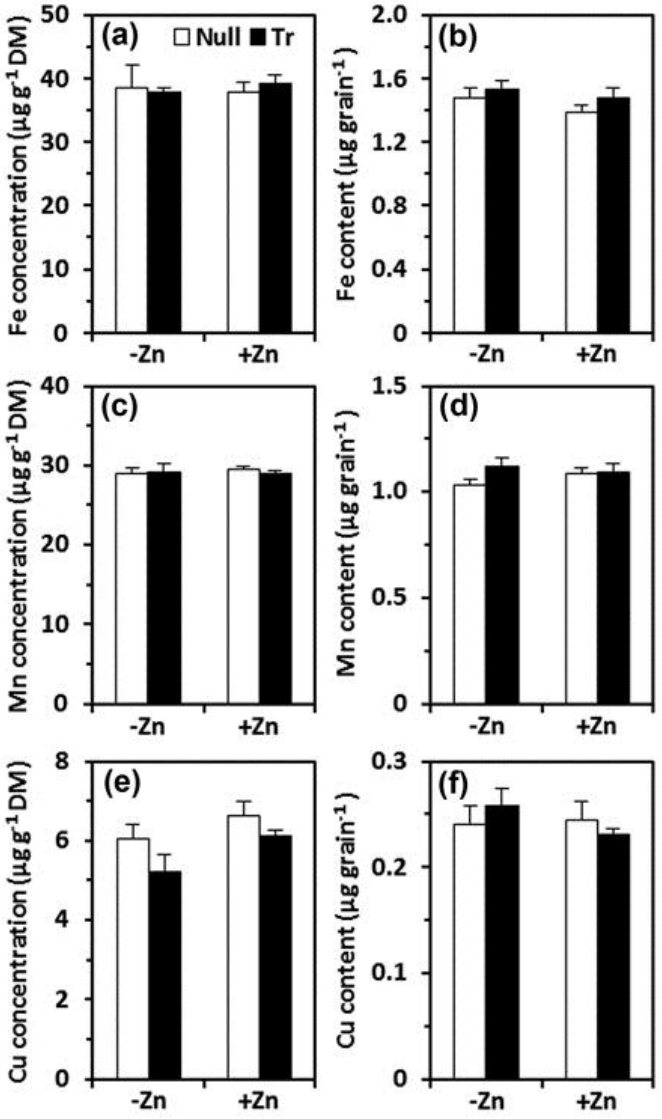

Figure 3.9. Effect of low Zn supplement on grain Fe, Mn and Cu concentration and content of plants grown in a low Zn potting mix.
Fe concentration (a) and content (b). Mn concentration (c) and content (d). Cu concentration (e) and content (f). Transgenic OX-10 (Tr) and null plants were grown to maturity in a 8-inch pot containing a low Zn potting mix (1.44 mg Zn pot^{-1}) either with no Zn supplement (-Zn) or with 3 mg Zn per pot during anthesis (+Zn). Five grains were analysed for each sample. Means and SE values of four replicates are presented.

3.3.6 Grain yield and grain Zn content of *HvZIP7* over-expressing plants grown in a high Zn potting mix

Grain Zn concentrations have been shown to be negatively correlated with grain yield (McDonald et al., 2008). Therefore, it is possible that the higher Zn concentration in the grains of the transgenic plants could result from lower grain yield. To assess this possibility,

three independent transgenic lines, OX-2, OX-7 and OX-10 were grown to maturity in a high Zn potting mix. There were no significant differences in total shoot dry weight, grain yield (Figure 3.10a, b) or 1000-grain weight between the transgenic and null or wildtype lines. The 1000-grain weight of the wildtype, OX-2, OX-7 and OX-10 were 41.9, 39.4, 39.6 and 40.3 g, respectively. These results show that the over-expression of *HvZIP7* does not have negative impacts on plant growth and grain yield.

Grain Zn concentrations of all three transgenic lines were significantly higher than those of the null or wildtype (Figure 3.10c). The average grain Zn concentration of the three transgenic lines was approximately 110 mg kg^{-1} DW and was at least 60% higher than that of the null or wildtype (Figure 3.10c). The total grain Zn content of all three transgenic lines was also significantly higher than that of the null or wildtype (Figure 3.10d), which excludes the possibility that the higher grain Zn concentration in the transgenic plants results from the lower grain yield. Furthermore, the higher Zn accumulation in the grains of the transgenic plants did not have significant impacts on the concentrations of Fe, Mn or Cu in the grain (Figure 3.12a, c, e).

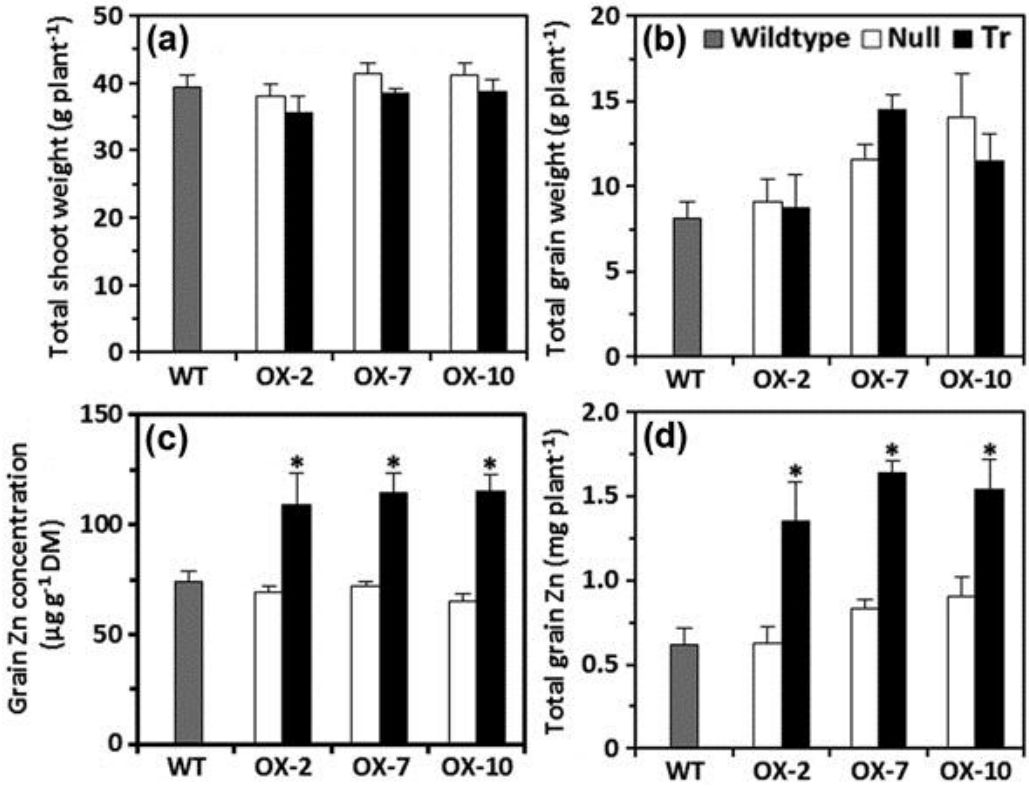

Figure 3.10. Total shoot weight, total grain weight, grain Zn concentration and total grain Zn content of plants grown in a high Zn potting mix.
Total shoot weight (a), total grain weight (b), grain Zn concentration (c) and total grain Zn content (d). Transgenic *HvZIP7* (Tr), null and wildtype (WT) plants were grown to maturity in a 6-inch pot filled with a high Zn potting mix (17.8 mg Zn pot^{-1}). Five grains were analysed for each sample. Means and SE values of four replicates are presented. Asterisks indicate the least significant differences at $P<0.05$. There are no significant differences in shoot dry weight and total grain weight between transgenic and null lines or wildtype ($P=0.52$ and 0.23, respectively).

In addition, we investigated the effect of polishing on the reduction of grain Zn content. Polishing reduced dry weight of the grains by approximately 50% in all genotypes (Figure 3.11a), and a similar reduction in the average Zn concentration was also observed (Figure 3.11b). In contrast, average Zn content was reduced by approximately 70% (Figure 3.11c) because higher Zn concentrations are present in the embryo and aleurone tissues (Lombi et al., 2011). However, the proportion of Zn retained in the polished grains of the transgenic lines was either similar or slightly higher than those of the null or wildtype (Figure 3.11b, c). This indicates that the Zn distribution in the grains of the transgenic plants is similar to that of the null or wildtype.

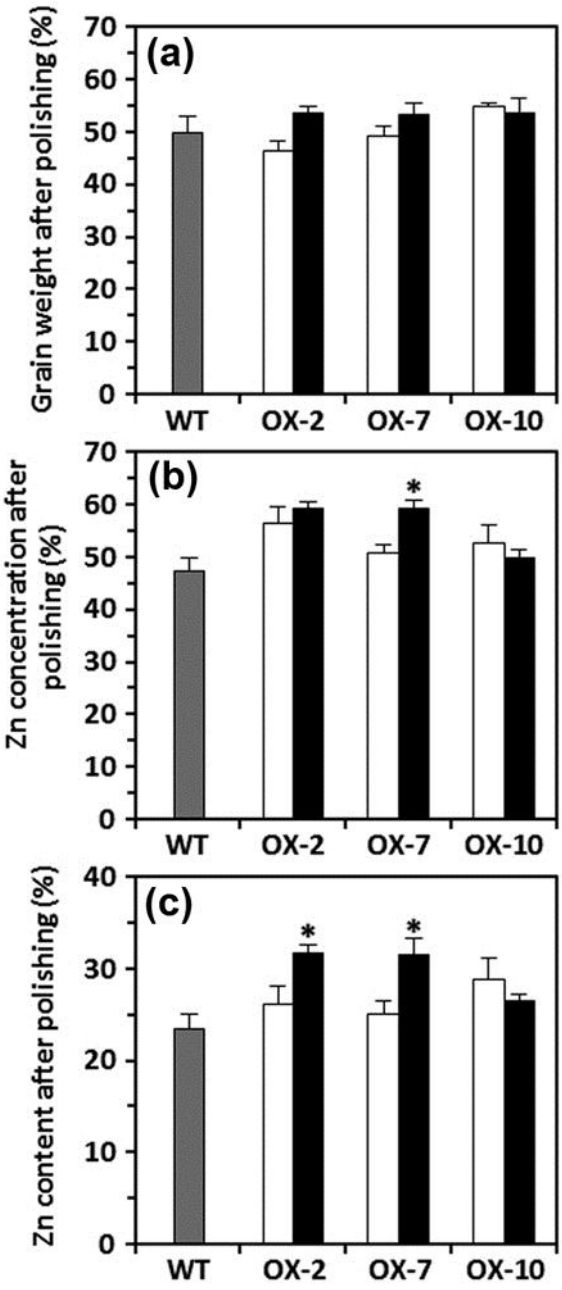

Figure 3.11. Effect of polishing on weight, Zn concentration and Zn content of barley grains.

Percentage of grain weight after polishing (a), percentage of Zn concentration in grains after polishing (b) and percentage of Zn content in grains after polishing (c) relative to that of unpolished grains. Transgenic *HvZIP7* (Tr), null and wildtype (WT) plants were grown to maturity in a 6-inch pot filled with a high Zn potting mix (17.8 mg Zn pot^{-1}). Ten grains from each sample were used for polishing and Zn analysis. Means and SE values of four replicates are presented. Asterisks indicate the least significant differences at $P<0.05$.

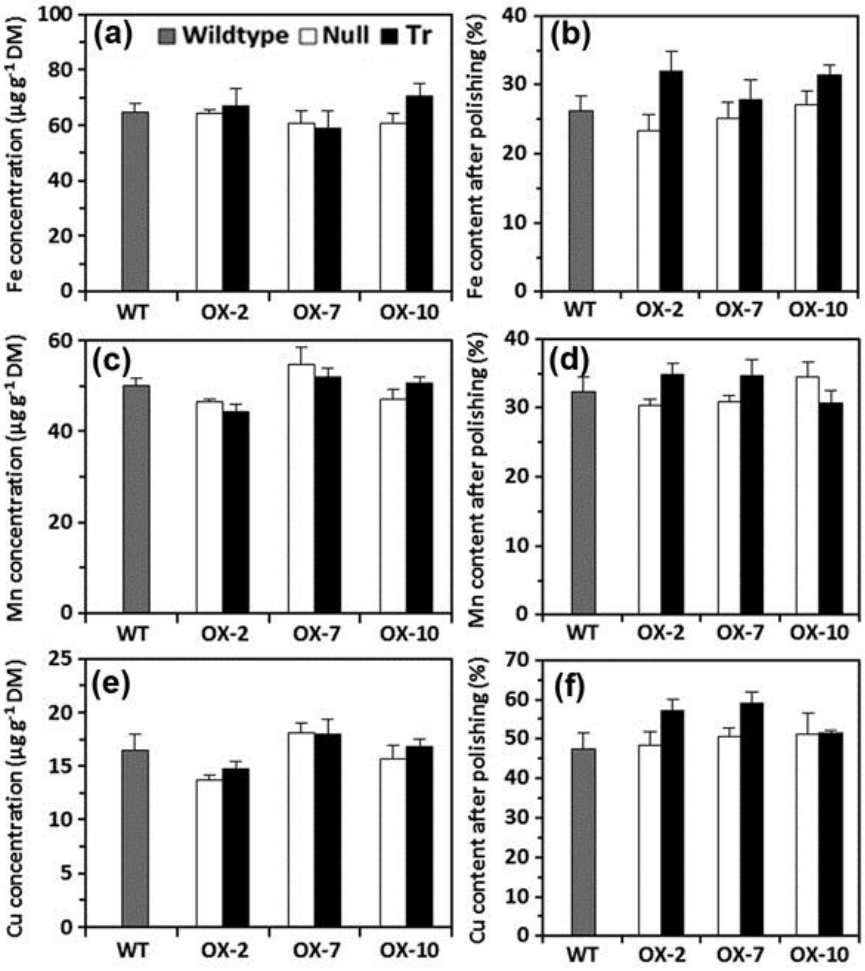

Figure 3.12. Effect of polishing on grain Fe, Mn and Cu concentration and content of barley grains.
Grain Fe (a), Mn (c) and Cu (e) concentrations before polishing. Percentage of grain Fe (b), Mn (d) and Cu (f) content after polishing relative to unpolished grains. Plants of three independent transgenic lines (OX-2, OX-7 and OX-10), null lines and wildtype (WT) were grown until maturity in 6-inch pots with a high Zn potting mix (17.8 mg Zn pot^{-1}). Ten grains were used for polishing and metal analysis. Polishing was performed in a Kett® Pearlest miller for seven min. Means and SE values of four replicates are presented.

3.3.7 Effect of over-expressing *HvZIP7* on Cd accumulation in plants

The over-expression of *HvZIP7* increased Zn translocation in the barley plants (Figure 3.5b), but not Fe, Mn or Cu (Figure 3.6). However, it is not known whether HvZIP7 increases translocation of the toxic Zn analogue, Cd. To test the ability of over-expressing *HvZIP7* plants in Cd transport, plants were grown in the same soil as the plant Zn uptake experiment (Figure 3.4) with two rates of Cd supplement (3 and 10 mg Cd kg^{-1} soil). There were no significant differences in plant growth either between the two rates of Cd supplement or

between genotypes. Concentrations and content of Cd in shoots were increased with the rates of Cd supplement (Figure 3.13a, b). However, there were no significant differences in shoot Cd concentration or content between the transgenic and null lines in either rate of Cd supplement (Figure 3.13a, b). These results indicate that the over-expression of *HvZIP7* in barley does not increase Cd accumulation in shoots.

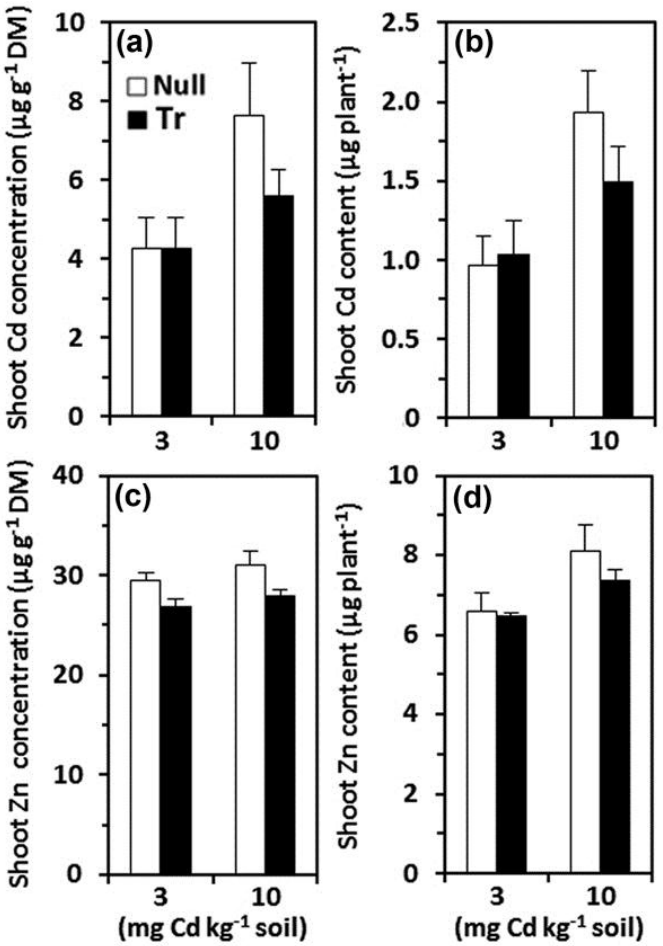

Figure 3.13. Effect of two rates of Cd supplement on shoot Cd and Zn concentration and content of soil-grown plants.
Shoot Cd concentration (a) and content (b). Shoot Zn concentration (c) and content (d). Plants of OX-10 (Tr) and null lines were grown in soil and supplemented with two rates of Cd. Plants were harvested 17 days after seed imbibition. Means and SE values of four replicates are presented.

3.4 Discussion

3.4.1 HvZIP7 mediates Zn translocation from roots to shoots

HvZIP7 shares a high sequence identity with OsZIP7 and TaZIP7, and differs from other ZIP members (OsZIP4, OsZIP5, OsZIP8 and AtZIP1) that have been functionally characterised (Chen et al., 2008; Pedas et al., 2009). HvZIP7 expression was highly induced by Zn deficiency in both roots and shoots (Figure 3.1a), and *in situ* RT-PCR analysis revealed that *HvZIP7* was expressed mainly in vascular tissues (Figure 3.1d, e). The expression of *HvZIP7::GFP* in onion epidermal cells showed that HvZIP7 was localised in the plasma membrane (Figure 3.2a, c), suggesting that HvZIP7 is involved in Zn transport at the plasma membrane of the plant cell. Unlike other ZIP proteins, HvZIP7 was unable to restore the growth of the *zrt1zrt2* yeast mutant defective in Zn uptake, whereas OsZIP4, OsZIP5, OsZIP8, AtZIP1, NcZNT1 and AtIRT3 are able to restore the growth of the zrt1zrt2 mutant defective in Zn uptake (Grotz et al., 1998; Ishimaru et al., 2005; Lee et al., 2010a; Lee et al., 2010b; Milner et al., 2012). OsZIP7a, a close homologue of HvZIP7 was unable to complement the zrt1zrt2 mutant, either (Yang et al., 2009). It is worthwhile mentioning that different yeast expression vectors (pFL61 and pYES2) and yeast strains (ZHY3 and BY4741) have been used in the expression of HvZIP7 and OsZIP7a. These data show that HvZIP7 and OsZIP7a are unable to fully complement mutations in the yeast Zn transport system. Other plant ion transporters are also reported to be unable to complement yeast mutants, such as HvPHT1,1, HvPHT1;6 and OsPHT1;2, but they are able to transport ions in plants (Rae et al., 2003; Ai et al., 2009). Nevertheless, the over-expression of HvZIP7 in barley plants led to decreased Zn accumulation in roots and increased Zn accumulation in shoots in a Zn concentration-dependent manner (Figure 3.4), which demonstrates that HvZIP7 is involved in the translocation of Zn from roots to shoots. Zn translocation from roots to shoots has been considered a bottleneck for increasing grain Zn content (Palmgren et al., 2008). This is

evident in the studies using over-expression of OsZIP4, OsZIP5 or OsZIP8 (Ishimaru et al., 2007; Lee et al., 2010a; 2010b). More Zn is accumulated in rice roots but less Zn is in rice shoots. In contrast, the over-expression of *HvZIP7* can overcome the restriction on Zn translocation from roots to shoots when Zn in roots is above adequacy (Figure 3.4), and leads to high Zn accumulation in shoots. The high Zn accumulation in shoots can lead to more Zn being loaded into grains (see below for more discussion).

3.4.2 HvZIP7 shows specificity for Zn translocation

HvZIP7 has a unique property in metal transport. Many ZIP family proteins can transport other metals such as Fe and Cd in addition to Zn (Connolly et al., 2002; Ishimaru et al., 2007; Lin et al., 2009), but in this study, we have shown that the over-expression of HvZIP7 did not increase the concentrations of Fe, Mn, Cu or Cd in shoots (Figures 3.6 and 3.13) nor in grains (Figures 3.9 and 3.12). This indicates that HvZIP7 is highly selective for Zn ions. This ion selectivity is crucial for cereal biofortification because the accumulation of other undesired metals such as Cd will adversely affect human health. AtHMA2, AtHMA4 and OsHMA2 are also involved in Zn translocation from roots to shoots, but they transport Cd *in planta* as well (Hussain et al., 2004; Hanikenne et al., 2008; Satoh-Nagasawa et al., 2011). The over-expression of AtHMA4 increases the accumulation of both Zn and Cd in shoots (Verret et al., 2004). AtIRT1, a member of the ZIP family, is capable of transporting Cd as well (Connolly et al., 2002). The apparent metal ion specificity of HvZIP7 is a desirable characteristic for Zn biofortification of cereals.

3.4.3 The over-expression of *HvZIP7* promotes Zn loading into grains

Transgenic *HvZIP7* plants grown in a low Zn potting mix had 30% higher grain Zn concentrations and content than null plants when a low dose of Zn was applied during

anthesis (Figure 3.8). The grain Zn concentration of the null plants (31 μg Zn g^{-1} DW) is equivalent to that (21-31 μg Zn g^{-1} DW) observed from the field-grown barley plants with Zn fertilisation (Graham et al., 1992). The results suggest that the transgenic HvZIP7 plants have the potential for a 30% increase in grain Zn content when grown in the field with standard Zn fertiliser applications.

With a further increase in Zn fertiliser rates, transgenic plants could achieve grain Zn concentrations approximately 60% higher than those of the controls (Figure 3.10c). Moreover, the large increase in grain Zn concentration of the transgenic lines did not affect plant growth or grain yield (Figure 3.10a, b). In contrast, over-expression of *AtZIP1* in barley increased grain Zn concentrations but reduced grain sizes (Ramesh et al., 2004). It is not clear whether the higher grain Zn concentration in the transgenic *AtZIP1* plants results from reduced grain yield or increased Zn uptake and translocation. It has been shown that high grain Zn concentration is negatively correlated with grain yield (McDonald et al., 2008). The protein sequence and expression pattern of AtZIP1 is different from HvZIP7, OsZIP4, OsZIP5 and OsZIP8 (Grotz et al., 1998; Ishimaru et al., 2005; Lee et al., 2010a; 2010b), suggesting that AtZIP1 has a different function in plant Zn homeostasis from HvZIP7 and three rice ZIP proteins.

The increased Zn accumulation in the grains of the transgenic plants (Figure 3.10d) could be due to the enhanced translocation of Zn from roots to shoots (Figure 3.4) or enhanced retranslocation of Zn from maternal tissues to grains, or both. HvZIP7 is also expressed in tissues other than roots (Figure 3.1). Tauris et al. (2009) showed that in a microarray analysis of the developing barley grain, HvZIP7 is highly expressed in transfer cells of the grain relative to other grain tissues, which suggests that HvZIP7 could be involved in grain Zn loading. Zn loading into grains has been considered as another bottleneck for Zn biofortification (Palmgren et al., 2008; White & Broadley 2011). Little is known about the

transporters that are involved in grain Zn loading. In this study, we were unable to separate the effects of the higher Zn accumulation in shoots on grain loading from the potential involvement of HvZIP7 in Zn remobilisation within shoots owing to the constitutive expression of *HvZIP7* driven by the CaMV 35S promoter. It remains to be established whether the higher expression of *HvZIP7* could also increase the amount of phloem-mobile Zn and/or Zn retranslocation from maternal tissues into the grain. Future experiments with shoot- or transfer cell-specific promoters to drive expression of *HvZIP7* in combination with the root-specific expression could shed new light on the involvement of HvZIP7 in grain Zn loading.

In this study, barley was used as a model for wheat and other temperate cereals. For Zn biofortification of cereals, distribution of Zn in cereal grains is another important factor. Cereal grains such as wheat and rice are milled or polished to remove the bran before being used for human consumption. These processes reduce total grain Zn content because of the removal of the Zn-rich embryo and aleurone tissues (Palmgren et al., 2008; Wirth et al., 2009). The polishing process did not reduce the proportion of Zn in the grains of the transgenic lines relative to the null or wildtype in spite of the difference in total Zn content (Figure 3.12b, c). If these transgenic grains were milled into flour for human consumption, more Zn would be available for dietary Zn intake, assuming that the bioavailability of Zn in the transgenic grain is similar to that of the wildtype.

In conclusion, HvZIP7 appears to be a Zn-specific transporter which controls Zn translocation from roots to shoots in a Zn concentration-dependent manner. The over-expression of *HvZIP7* not only increases the specific accumulation of Zn in shoots when Zn in roots is above adequacy, but also enhances grain Zn content without yield penalty. Plants over-expressing *HvZIP7* with Zn fertilisation could achieve 30% higher grain Zn content in the low Zn conditions similar to those found in the field. Our findings could have direct

applications to the improvement of Zn fertiliser use efficiency and Zn biofortification in cereals.

Chapter 4: Effects of *HvZIP7* transgene expression on transcript profiles of other *HvZIP* members

4.1 Introduction

Enhancing Zn uptake in plants *via* genetic engineering for biofortification purposes requires not only the identification of genes crucial in Zn homeostasis, but also the functional characterisation of these genes in a target plant by transgenic approaches. The phenotype of transgenic plants can provide insights into the function of the gene of interest and determine whether the gene is suitable for Zn biofortification. Using transgenic approaches, phenotypes such as increased Zn accumulation can be obtained. For example, the heterologous expression of *Arabidopsis halleri AhHMA4* in the model tobacco plant increased Zn accumulation in the leaves of plants cultivated in low Zn (Barabasz et al., 2010). However, when cultivated in adequate Zn, transgenic plants had lower Zn accumulation in the shoots (Barabasz et al., 2010). Such a Zn supply-dependent Zn accumulation in transgenic plants could be related to changes of other metal homeostasis genes in the transgenic plants which did not occur in wildtype.

Plant Zn status regulates transcriptome profiles of a number of genes related to Zn homoeostasis (Talke et al., 2006; van de Mortel et al., 2006). The expression of a Zn transporter transgene alters Zn homeostasis of the host plants, and thus may also influence the transcript profiles of native genes involved in Zn homeostasis. Changes in the transcript profiles of native genes may in turn affect Zn homeostasis of the plant. Therefore, the modified expression of native genes could play a role in generating the phenotype of transgenic plants (Barabasz et al., 2012).

The over-expression of *HvZIP7* resulted in increased Zn accumulation in shoots and decreased Zn accumulation in roots when the plants were grown in Zn-abundant conditions (Chapter 3). HvZIP7 appears to be a specific Zn transporter which mediates Zn translocation from roots to shoots, and its over-expression leads to more Zn accumulation in shoots in a Zn concentration-dependent manner. However, it is not clear whether native genes are contributing to the phenotype observed in the transgenic plants. In Chapter 2, nine new members of the HvZIP family were identified, bringing the total number of HvZIP family members to 13. Transcript levels of eight *HvZIP* genes (*HvZIP1, HvZIP2, HvZIP3, HvZIP5, HvZIP7, HvZIP8, HvZIP10* and *HvZIP13*) were highly induced in roots and/or shoots of plants with Zn deficiency, while five of them (*HvZIP6*, *HvZIP11*, *HvZIP14*, *HvZIP16* and *HvIRT1*) were not responsive to plant Zn status.

In this chapter, the effects of over-expression of *HvZIP7* on the transcript profiles of the *HvZIP7* transgene and all 13 native *HvZIP* genes (including *HvZIP7*) were determined in both roots and shoots of Zn-sufficient and -deficient transgenic barley plants. Transcript levels of the only known barley MTP gene, *HvMTP1*, were also analysed. The results of the transcript analyses will provide evidence of whether the phenotype observed in transgenic plants was a direct consequence of transgene expression or due to the changes in the expression of both transgene and native *HvZIP* genes.

4.2 Materials and methods

4.2.1 Plant growth

The experimental setup is similar to that described in Chapter 3 with several modifications. Two seeds of *Hordeum vulgare* L. cv. Golden Promise (from transgenic *HvZIP7*, null and wildtype lines) with emerged radicles were planted in each pot containing

one kg dry sand (Zn-deficient siliceous sand, DTPA-extractable Zn=0.07 mg kg^{-1} soil). There were two Zn treatments, -Zn (no additional Zn) and +Zn (0.5 mg Zn kg^{-1} soil supplied as $ZnSO_4$). Plants were grown in a glasshouse with approximately 24/13 °C day/night temperature and 12 hr day/12 hr night cycle. After 28 days of growth, plants were removed from the pots. One of the plants was used to measure dry weight and nutrients, while the other was used for transcript profile analysis. Samples for transcript analyses were snap-frozen in liquid nitrogen and stored at -80 °C.

4.2.2 Mineral element analysis

Mineral element analysis was conducted as described in Section 2.2.2.

4.2.3 Quantitative real-time RT-PCR

RNA isolation and quantitative real-time RT-PCR analysis of transcripts were conducted as described in Section 2.2.4. The primer sequences for all genes analysed are also listed in Table 2.2.

4.2.4 Statistical analyses

The experiment was set up as a completely randomised block design with four replications. Data for plant growth, metal nutrition and transcript profiles were analysed using the Genstat Statistical Program (Version 11.1, VSN International Ltd). To overcome the problem of non-homogeneity of variances, log-transformed data were used for the analysis of variance. The Least Significant Difference (LSD) at P=0.05 was used for comparisons of means.

4.3 Results

4.3.1 Plant growth and Zn nutrition of *HvZIP7* over-expressing plants

Biomass of shoots and roots of transgenic plants were not significantly different to those of the null or wildtype (Figure 4.1a, b) when grown either in +Zn or -Zn, indicating that the transgene did not negatively affect plant growth. Zn concentrations in the shoots of all plants grown in –Zn was below 20 µg g^{-1}, which is lower than the critical level required for normal plant growth (Genc et al., 2002), while Zn concentrations in the shoots of plants grown in +Zn were well above the critical level (Figure 4.1c). Despite the low Zn concentrations, shoot and root biomass were not affected (Figure 4.1a, b), suggesting that at this growth stage the low Zn concentration had not yet resulted in significant growth reductions. Zn concentrations in both shoots and roots of transgenic plants were not significantly different to those of the null or wildtype in both Zn treatments (Figure 4.1c, d). In addition, there were no significant differences in Fe, Mn or Cu concentrations in either shoots or roots between the three genotypes when the plants were grown either in +Zn or –Zn (Figure 4.2). These results indicate that the transgene has no effect on the plant accumulation of Zn, Fe, Mn or Cu when grown either in Zn-sufficient or -deficient conditions.

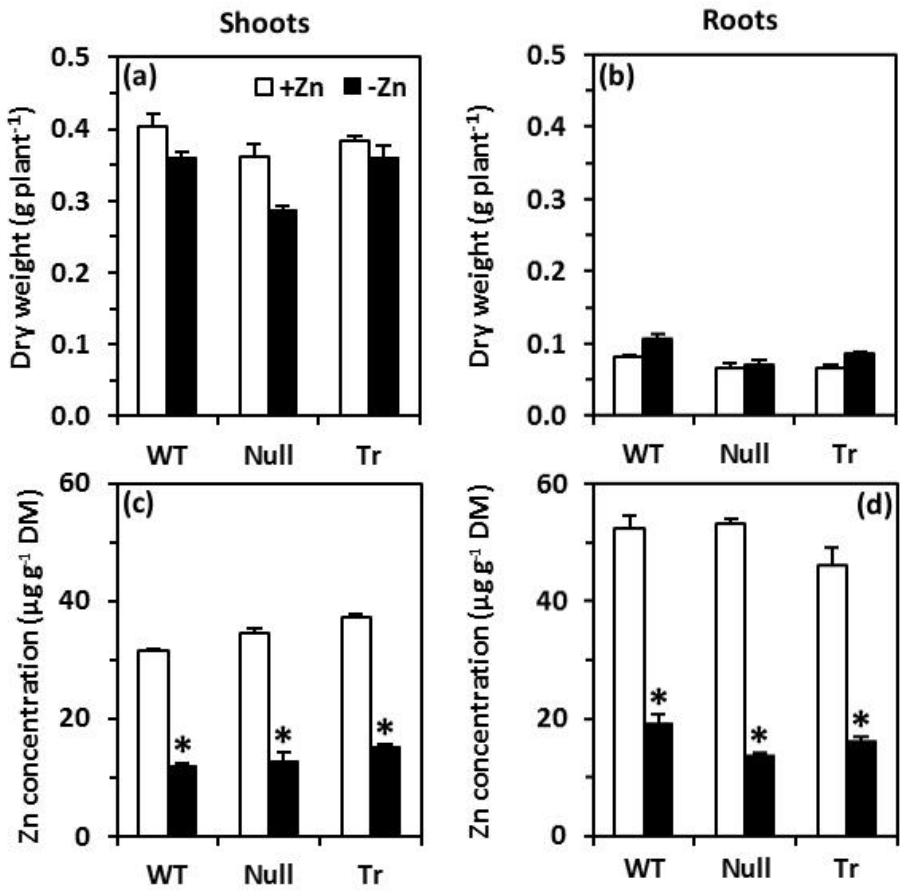

Figure 4.1. Effect of two Zn rates on growth, and Zn concentration and content of transgenic *HvZIP7* (Tr), null and wildtype plants grown in soil.

Dry weight of shoots (a) and roots (b). Zn concentration of shoots (c) and roots (d). Plants were grown either in 0.5 mg Zn kg^{-1} soil (referred to as +Zn) or without additional Zn (referred to as –Zn) and harvested 28 days after seed imbibition. Means and SE values of four replicates are presented. The shoot and root Zn concentrations of plants grown in -Zn were significantly lower than those grown in +Zn (P<0.001).

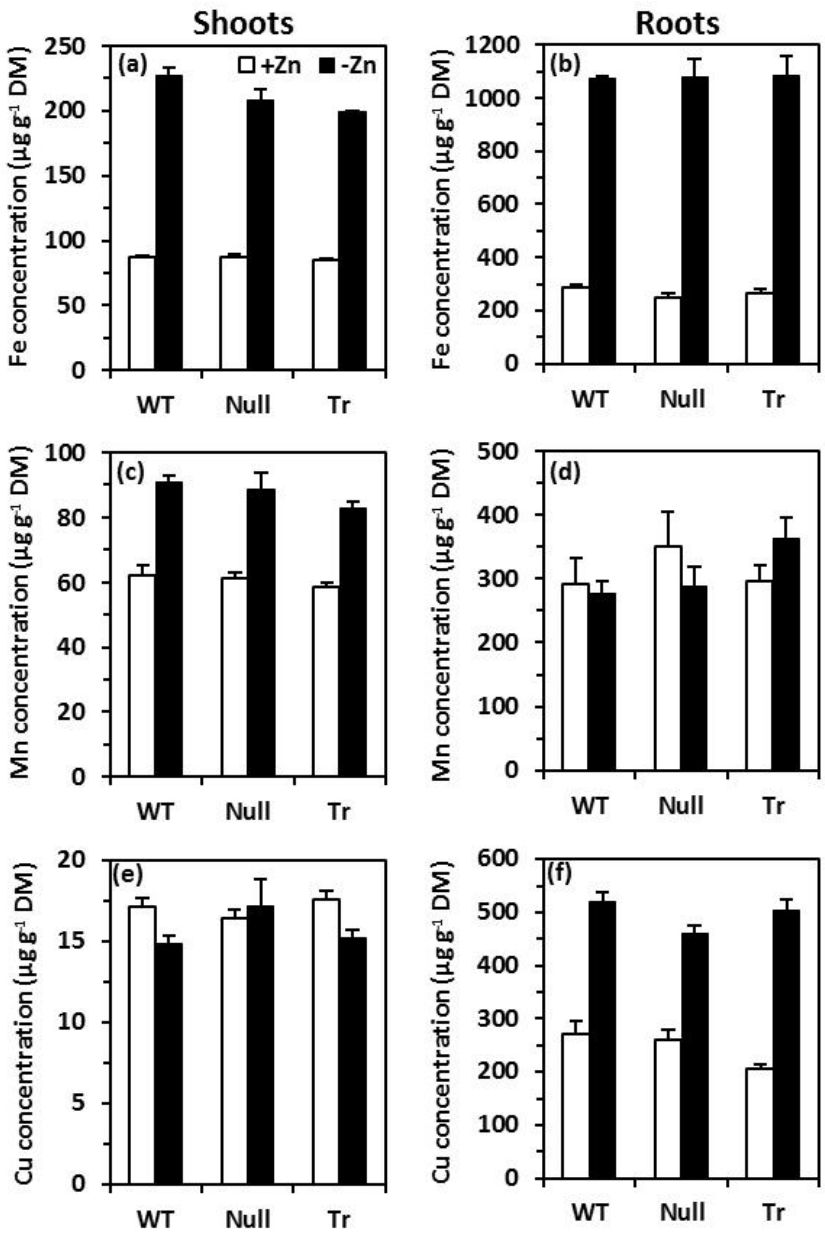

Figure 4.2. Effect of two Zn rates on Fe, Mn and Cu concentrations of transgenic *HvZIP7* (Tr), null and wildtype plants grown in soil.

Fe concentration of shoots (a) and roots (b). Mn concentration of shoots (c) and roots (d). Cu concentration of shoots (e) and roots (f). Plants were grown either in 0.5 mg Zn kg^{-1} soil (referred to as +Zn) or without additional Zn (referred to as –Zn) and harvested 28 days after seed imbibition. Means and SE values of four replicates are presented.

4.3.2 Transcript profiles of *HvZIP7* transgene and eight Zn deficiency-inducible native *HvZIP* genes in both roots and shoots

The expression of the *HvZIP7* transgene (*TxZIP7*) was not detected in either roots or shoots of null and wildtype plants (Figure 4.3a). *TxZIP7* transcripts were detected in both roots and shoots of transgenic plants grown in +Zn and –Zn (Figure 4.3a). Regardless of the Zn status of the plants, the transcript level of *TxZIP7* was either similar to or higher than that of the native *HvZIP7* gene (Figure 4.3a, b). Interestingly, *TxZIP7* expression in the roots of plants grown in –Zn was significantly higher ($P<0.05$) than that in roots of plants grown in +Zn (Figure 4.3a).

In the roots of Zn-deficient plants, transcript levels of the native *HvZIP7* gene and six other *HvZIP* genes (*HvZIP1*, *HvZIP3*, *HvZIP5*, *HvZIP8*, *HvZIP10* and *HvZIP13*) were increased by two-fold or more relative to Zn-sufficient plants (Figure 4.3b, c, e, f, g, h, i). *HvZIP2* transcripts in the roots of Zn-deficient plants were also marginally increased (1.4-fold) relative to Zn-sufficient plants (Figure 4.3d). The expression levels of these eight Zn-deficiency inducible *HvZIP* genes in the roots of transgenic plants were not significantly different to those of the null or wildtype when grown either in +Zn or –Zn.

In the shoots of Zn-deficient plants, transcript levels of the native *HvZIP7* gene and seven other *HvZIP* genes (*HvZIP1*, *HvZIP2*, *HvZIP3*, *HvZIP5*, *HvZIP8*, *HvZIP10* and *HvZIP13*) were increased by two-fold or more relative to Zn-sufficient plants (Figure 4.3b, c, d, e, f, g, h, i). The transcript levels of *HvZIP7* and *HvZIP10* in the shoots of transgenic plants grown in +Zn were significantly lower by approximately 50% compared to the null or wildtype (Figure 4.3b, h), indicating that the expression of the transgene had some effects on the expression of the native *HvZIP7* and *HvZIP10* genes only in the shoots of Zn-sufficient plants. In addition, the transcript levels of *HvZIP2* in the shoots of both transgenic and null

plants were not increased by Zn deficiency despite its two-fold increase in the shoots of Zn-deficient wildtype plants (Figure 4.3d). This lower *HvZIP2* expression in transgenic and null plants could be due to effects from the *HvZIP7* transgene-transformation event. Transcript levels of other Zn-deficiency inducible *HvZIP* genes in the shoots of transgenic plants were not significantly different from those of the null or wildtype when grown either in +Zn or -Zn (Figure 4.3).

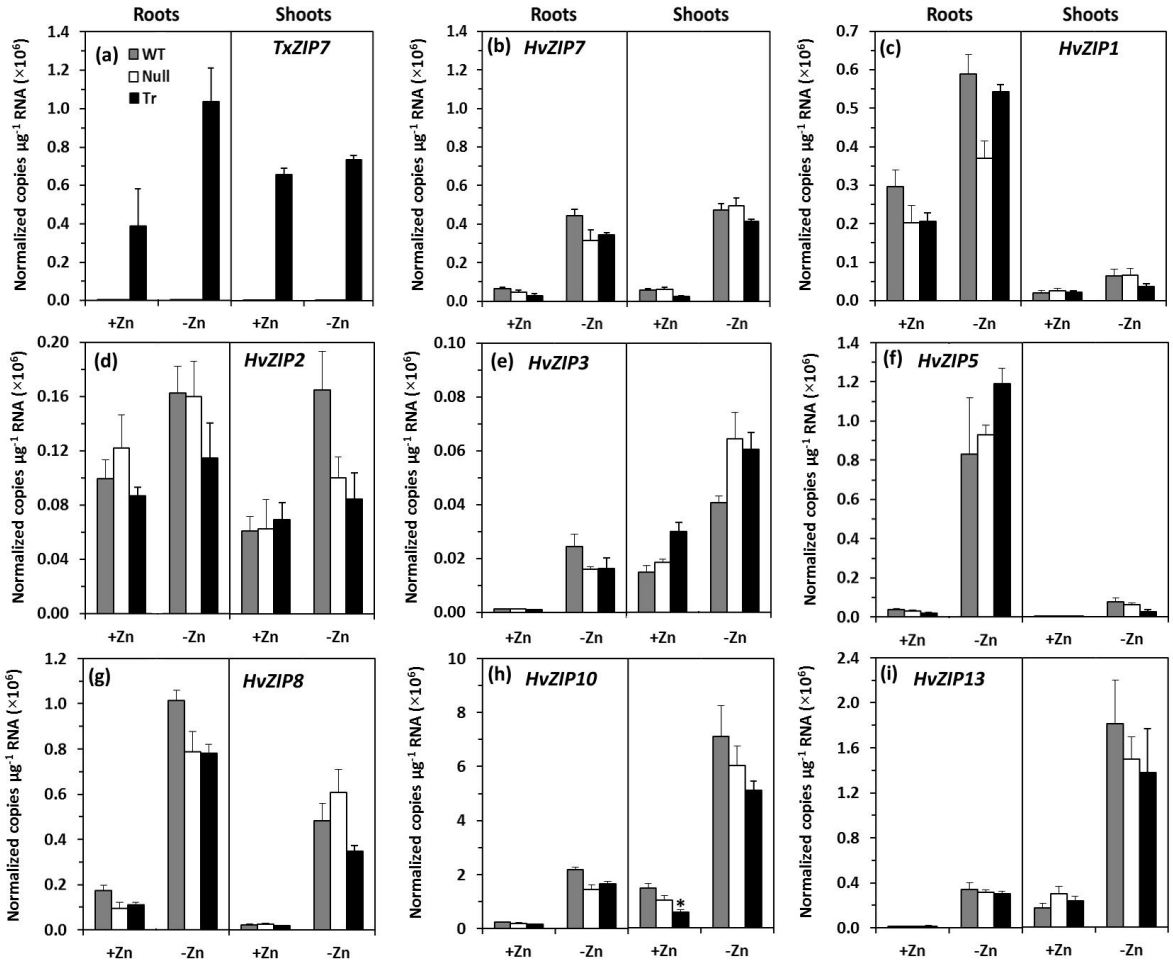

Figure 4.3. Transcript levels of the *HvZIP7* transgene (*TxZIP7*), endogenous *HvZIP7* and seven other Zn-deficiency inducible *HvZIP* genes in transgenic *HvZIP7* (Tr), null and wildtype plants grown in soil.

Transcript levels of *TxZIP7* (a), *HvZIP7* (b), *HvZIP1* (c), *HvZIP2* (d), *HvZIP3* (e), *HvZIP5* (f), *HvZIP8* (g), *HvZIP10* (h) and *HvZIP13* (i) in roots and shoots. Plants were grown either in 0.5 mg Zn kg^{-1} soil (referred to as +Zn) or without additional Zn (referred to as –Zn), and harvested 28 days after seed imbibition. Quantitative real-time RT-PCR was used to determine transcript levels. Log-transformed data were used in the statistical analyses for comparisons of means in (b), (c), (e), (f), (g), (h) and (i). The means and SE of four biological replicates are presented as normalised copies μg^{-1} RNA. The *TxZIP7* expression level in roots of plants grown in –Zn is significantly higher ($P<0.05$) than that of plants grown in +Zn (Student's *t*-test). There are also significant differences in shoot transcript levels of *HvZIP7* and *HvZIP10* for interactions of Zn supply rates × genotypes ($P<0.04$). Asterisks indicate the least significant difference at $P \leq 0.05$ (LSD 0.05).

4.3.3 Transcript profiles of five Zn-deficiency non-inducible *HvZIP* genes and *HvMTP1*

Transcript levels of *HvZIP6*, *HvZIP11*, *HvZIP14* and *HvIRT1* in the roots of Zn-deficient plants were not significantly different to those of Zn-sufficient plants (Figure 4.4a, b, c, e). *HvZIP16* and *HvMTP1* transcripts in the roots of Zn-deficient plants were marginally increased (1.3- and 1.2-fold, respectively at $P<0.01$) relative to Zn-sufficient plants (Figure 4.4d, f). However, the transcript levels of these five *HvZIP* genes and *HvMTP1* in the shoots of Zn-deficient plants were not significantly different to those of Zn-sufficient plants (Figure 4.4). Transcript levels of these Zn-deficiency non-inducible *HvZIP* genes and *HvMTP1* in the roots and shoots of transgenic plants were not significantly different to those of the null or wildtype when grown either in +Zn or -Zn (Figure 4.4).

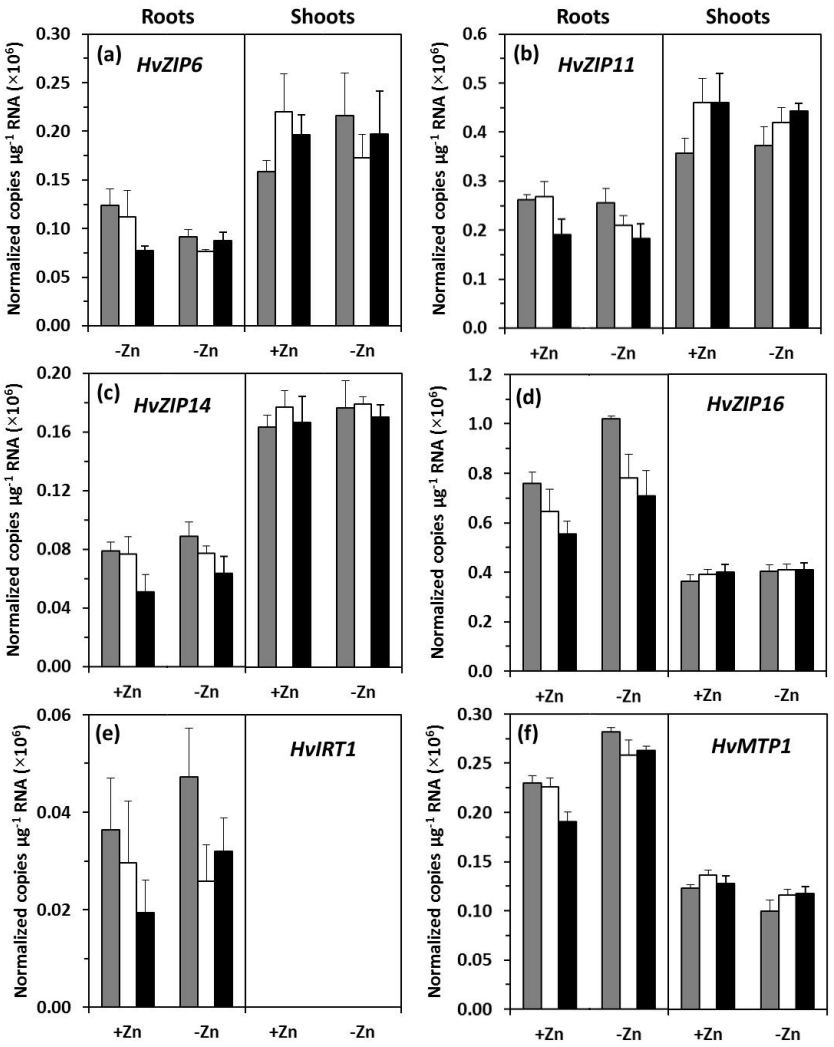

Figure 4.4. Transcript levels of the five Zn-deficiency non-inducible *HvZIP* genes and *HvMTP1* in transgenic *HvZIP7* (Tr), null and wildtype plants grown in soil.
Transcript levels of *HvZIP6* (a), *HvZIP11* (b), *HvZIP14* (c), *HvZIP16* (d), *HvIRT1* (e) and *HvMTP1* (f) in roots and shoots. Plants were grown either in 0.5 mg Zn kg^{-1} soil (referred to as +Zn) or without additional Zn (referred to as –Zn) and harvested 28 days after seed imbibition. Quantitative real-time RT-PCR was used to determine transcript levels. The means and SE of four biological replicates are presented as normalised copies μg^{-1} RNA.

4.4 Discussion

4.4.1 Lack of Zn translocation from roots to shoots in transgenic lines under low Zn is neither due to low transgene expression levels nor the altered expression of native *HvZIP* genes

The expression of the *HvZIP7* transgene in both roots and shoots is comparable to the induced level of native *HvZIP7* by Zn deficiency, regardless of the Zn status of the plants. Furthermore, the transcript levels of native *HvZIP* genes (except for *HvZIP2*, *HvZIP7* and *HvZIP10* in the shoots) and also *HvMTP1* in roots and shoots of Zn-deficient transgenic plants were similar to those of the null or wildtype (Figures 4.3 and 4.4). These results indicate that the transgene had only minimal effects on the expression profile of native *HvZIP* genes and *HvMTP1* under low Zn conditions. Therefore, the lack of Zn translocation from roots to shoots in transgenic plants under low Zn conditions (Chapter 3) is not due to low transgene expression levels or alterations in native *HvZIP* genes or *HvMTP1* expression profiles. This suggests that it is the low Zn concentration in the roots rather than native transporter activities which caused the lack of increased Zn translocation from roots to shoots in transgenic plants. The expression of the transgene did not affect the transcript levels of the 13 native *HvZIP* genes and *HvMTP1* in the roots of either Zn-deficient or -sufficient transgenic plants (Figure 4.4).

4.4.2 *HvZIP7* transgene expression can affect transcript levels of closely related native *HvZIP* genes (*HvZIP7* and *HvZIP10*)

The transcript levels of native *HvZIP7* and *HvZIP10* were significantly reduced only in the shoots of Zn-sufficient transgenic plants (Figure 4.3b). This indicates that the effect of the transgene on the expression profile of native genes is not only dependent on the plant Zn

nutrition status but also on which tissues the genes are expressed in. It is interesting to note that HvZIP10 is a close homolog of HvZIP7, as they are placed in the same phylogenetic clade (Chapter 2). The transgene only reduces the expression of genes closely related in sequence, but not of other genes in the HvZIP family. It is not clear what mechanisms are responsible for the reduction in the transcript levels of native *HvZIP7* and *HvZIP10*. However, it is possible that RNA interference mechanisms induced by the *HvZIP7* transgene may have led to the degradation of RNA with sequences identical or with high similarity to the transgene such as *HvZIP7* and *HvZIP10* (Fire et al., 1998; Plasterk, 2002).

Despite the small reduction in transcript levels of native *HvZIP7* and *HvZIP10* in the shoots of Zn-sufficient transgenic plants, the levels of the native *HvZIP7* and *HvZIP10* may still be sufficient for Zn homeostasis. Alternatively, the transcript level of the *HvZIP7* transgene could compensate for the reduced expression levels of native *HvZIP7* and *HvZIP10*. As a result, no changes in Zn accumulation were observed in the transgenic plants (Figure 4.1c, d).

4.4.3 Plant Zn status could influence the expression of the *HvZIP7* transgene

The higher transcript level of the *HvZIP7* transgene in the roots of Zn-deficient plants relative to Zn-sufficient plants indicates that the expression of a CaMV 35S-driven gene in the roots could be regulated by plant Zn status. It is interesting to note that plant Zn status does not affect the expression of the transgene in shoots, suggesting that root-specific mechanisms could be involved in the regulation of the transgene. It is not known whether the difference in the transgene expression resulted from transcriptional regulation, post-transcriptional regulation, or both. Obviously, further work is needed to reveal the underlying mechanisms causing the regulation of the transgene.

In conclusion, the *HvZIP7* transgene had minimal effects on native *HvZIP* genes and *HvMTP1* in both roots and shoots of Zn-sufficient and -deficient plants. Despite small alterations in the transcript levels of native *HvZIP7* and *HvZIP10* genes, there were no significant impacts on Zn accumulation in either shoots or roots of the transgenic plants. The increased translocation from roots to shoots in Zn-abundant conditions (Chapter 3) is therefore likely due to over-expression of *HvZIP7* alone.

Chapter 5: General discussion

5.1 Review of thesis aims

Zinc deficiency in soils is widespread, leading to a reduction in productivity and nutrition value of cereal grains. Consumption of these cereal foods with low Zn can increase the risk of Zn deficiency in humans. Improving dietary Zn intake by increasing Zn density in grains (Zn biofortification) is potentially an effective way to alleviate Zn deficiency in humans. However, increasing grain Zn content is restricted by limitations in Zn translocation from roots to shoots and Zn loading into grains. Little is known about the Zn transporters involved in these processes, thus identifying and understanding these Zn transporters is pivotal for Zn biofortification. This thesis is focused on i) the identification of HvZIP family transporters as they are the primary group of transporters controlling plant Zn uptake and translocation within the plant, and ii) the characterisation of HvZIP7 for its role in plant Zn homeostasis.

5.2 Identification and characterisation of new *HvZIP* genes responsive to Zn deficiency

Most efforts towards identifying and characterising plant ZIP transporters have so far been made in Arabidopsis, *Medicago truncatula* and rice. There is very little information available on the functions of ZIP transporters in temperate cereals. So far, only four *ZIP* genes from barley (Pedas et al., 2009; Pedas et al., 2008) and one from the wild tetraploid emmer wheat (Durmaz et al., 2010) have been described. The responses of these five *ZIP* genes to Zn, Fe, Mn and Cu deficiencies were investigated, but none have been functionally characterised *in planta*. In this project, barley was used as a model plant to identify ZIP

transporters for wheat and other temperate cereals because barley is a close relative of wheat, without the complications of multiple genomes.

Nine *HvZIP* genes, in addition to the four *HvZIP* genes described in the literature, were identified by sequence similarity searches (Chapter 2). The transcript profile analysis of all 13 *HvZIP* genes showed that the expression of at least eight of them was highly induced by Zn deficiency in either roots, shoots or both (Chapter 2). The results suggest that multiple *HvZIP* genes are involved in plant responses to low Zn conditions. Therefore, a coordinated expression of these Zn deficiency-inducible genes is required for the observable changes in Zn uptake, translocation and/or retranslocation in Zn deficient plants (Suzuki et al., 2006). The rice orthologs of some of these Zn deficiency inducible *HvZIP* genes are also responsive to Zn deficiency, and were shown to be involved in Zn transport when functionally characterised *in planta* (Ishimaru et al., 2007; Lee et al., 2010a; Lee et al., 2010b). These observations suggest that some of these Zn deficiency-inducible HvZIP members could have direct roles in transporting Zn and could contribute to the Zn status of Zn-deficient plants.

These results not only serve as a basis for further functional characterisation of *ZIP* genes in barley, but also for the identification and characterisation of *ZIP* genes in other economically and nutritionally important cereals such as wheat for Zn biofortification and enhancing plant Zn nutrition. HvZIP7 was selected for further functional characterisation as its phylogenetic position is different from other functionally characterised ZIP members of rice and Arabidopsis (Chapter 2). Furthermore, *HvZIP7* is expressed in both roots and shoots (Chapter 2), implying that HvZIP7 is likely involved in Zn uptake, translocation and/or retranslocation.

The identification of 13 *ZIP* genes in barley and 16 *ZIP* genes in Brachypodium (Chapter 2) can help identify additional *ZIP* genes in wheat. Wheat is the second most

consumed cereal grain (behind rice), and due to low availability of Zn in most of the world's major cropping soils, most wheat grains do not have adequate Zn levels for human consumption. The knowledge of *ZIP* genes in wheat could help improve grain Zn content.

5.3 HvZIP7 regulates Zn translocation from roots to shoots

To further characterise the function of HvZIP7, plants over-expressing *HvZIP7* were generated. The over-expression of *HvZIP7* in barley resulted in increased Zn content in shoots but lowered Zn content in roots when Zn in plants is above the adequate level (Chapter 3). In addition, HvZIP7 was found to be localised in the plasma membrane, and, in wildtype plants, is expressed mainly in vascular bundles of roots and leaves. These results suggest that HvZIP7 mediates Zn translocation from roots to shoots. Analyses of xylem exudates of transgenic plants can provide further evidence on whether the over-expression of *HvZIP7* increases Zn translocation from roots to shoots. The higher Zn accumulation in shoots of transgenic plants shows that the over-expression of *HvZIP7* can overcome the bottleneck restricting Zn movement through the root-to-shoot barrier. As more Zn accumulates in the shoots, more Zn is then available for transport to grains.

The higher Zn accumulation in shoots was observed only in Zn-abundant conditions, indicating that Zn translocation mediated by HvZIP7 from roots to shoots is Zn concentration-dependent. This Zn concentration-dependent characteristic of HvZIP7 suggests that this transporter possesses two important physiological functions in wildtype barley. Firstly, when external Zn supply is low, the transcript level of *HvZIP7* is enhanced, but due to the low Zn concentration in the roots, Zn translocation from roots to shoots is limited. Consequently, the plant is able to retain Zn in roots for root functions. Secondly, when external Zn supply is above adequacy, the expression of *HvZIP7* is repressed, leading to a restriction in Zn

translocation from roots to shoots. This would prevent Zn toxicity in the shoots if elevated Zn is present in the plant growth media.

Our results indicate that HvZIP7 plays an important role in regulating plant Zn homeostasis. It would be interesting to investigate the effects of knocking out or knocking down the expression of *HvZIP7* on Zn homeostasis of the plant *via* methods such as T-DNA insertional mutagenesis and RNA interference (Ayliffe & Pryor, 2011). Loss of function or reduced expression of *HvZIP7* could provide further insights into the functions of HvZIP7 in the plant. For example, Henriques et al. (2002) showed that by knocking out the *IRT1* transporter gene in Arabidopsis, *irt1* mutants displayed reduced overall Fe and Zn accumulation, indicating that IRT1 is an important Fe and Zn uptake transporter in Arabidopsis. From our results, it is possible that *hvzip7* mutants could translocate less Zn from roots to shoots, provided that there are no functionally redundant genes of the *HvZIP* family or other gene families involved in Zn translocation from roots to shoots.

In situ RT-PCR analysis showed that *HvZIP7* expression is localised in the vascular bundle of roots and leaves (Chapter 3). However, a relatively low resolution was obtained for the difference in the *HvZIP7* transcript levels among different cell types using this technique. Better results in cell-specific expression may be obtainable using native *HvZIP7* promoter::reporter constructs (Johansen, 1997). The isolation of the *HvZIP7* promoter sequence from a Morex BAC library was attempted. Unfortunately, the isolation of BAC clones containing *HvZIP7* genomic sequences was not successful. Recently, the International Barley Genome Sequencing Consortium (IBSC) has made some barley genomic sequences available for the barley research community. The promoter sequence of *HvZIP7* has been identified from the released sequences and thus can be used for future studies to determine the tissue- and cell type-specificity of *HvZIP7* expression.

5.4 Over-expression of *HvZIP7* has minimal effects on plant growth and the expression of native *HvZIP* genes

Pleiotropic side effects from the over-expression of *ZIP* genes *in planta*, including reductions in grain size and plant growth have been previously described (Ishimaru et al., 2007; Lee et al., 2010a; Lee et al., 2010b; Ramesh et al., 2004). The utilisation of the CaMV 35S promoter to drive the constitutive expression of a gene of interest could interfere with normal functions of the plant and therefore produce side effects such as reduced growth of transformed plants (Rees et al., 2009). In this study, we have shown that the over-expression of *HvZIP7* specifically increases Zn translocation from roots to shoots without affecting growth (Chapter 3). Furthermore, the increase in Zn accumulation in shoots occurred only when abundant Zn was available. The phenotype of the *HvZIP7* transgene is therefore being regulated by the Zn status of the plant. These are novel findings, as no work has so far reported such substantial and specific increase in shoot Zn content by over-expressing a single *ZIP* gene. The lack of any growth penalty with the over-expression of *HvZIP7* also makes a transgenic approach suitable for applications such as the production of cereal-based foods with high Zn content. In addition, the Zn concentration-dependent increase in Zn translocation suggests that the over-expression of *HvZIP7* can be applied to improve Zn fertiliser use efficiency to increase Zn accumulation in shoots and grains (see Section 5.5).

The *HvZIP7* transgene could potentially influence the expression profile of native *HvZIP* genes, which in themselves could contribute to the phenotype observed in transgenic plants. Therefore, the expression of all native *HvZIP* genes in transgenic plants was examined (Chapter 4). We found that the transcript levels of *HvZIP2*, *HvZIP7* and *HvZIP10* were slightly reduced in the shoots of Zn-adequate transgenic plants. However, these changes did not alter the Zn, Fe, Mn and Cu concentrations in the shoots (Chapter 4). These results

indicate that small changes in the expression profile of native *HvZIP* genes do not constitute part of the mechanism contributing to the phenotype of the transgenic plants. The increased translocation from roots to shoots in Zn-abundant plants is therefore likely due to over-expression of *HvZIP7* alone. However, we cannot discount that other components of Zn homeostasis may be affected by the transgene and play a role in increasing the shoot Zn accumulation of transgenic plants. Therefore, interactions between the *HvZIP7* transgene and native Zn transporters of other family genes such as HMAs should be examined in the future.

5.5 Over-expression of *HvZIP7* contributes to Zn loading into grains

The over-expression of *HvZIP7* promotes an increase of Zn concentration in grains when Zn in roots is above adequacy (Chapter 3). As more Zn is accumulated in the shoots, more Zn could be available as phloem-mobile Zn (Herren & Feller, 1994), contributing to the higher grain Zn content by overcoming the bottleneck of Zn loading into grains. Similar to shoot growth, the over-expression of *HvZIP7* had minimal effect on total grain yield (Chapter 3). Consequently, transgenic plants have higher total grain Zn content due to the higher Zn concentration in grains.

Transgenic *HvZIP7* plants had 30% higher grain Zn concentration compared to controls when grown in a low Zn potting mix with a low dose of Zn applied during anthesis. This indicates that the transgenic plants have the potential for a 30% increase in grain Zn content if grown in field conditions with standard Zn fertiliser applications, similar to those described by Graham et al. (1992). This also demonstrates that genetic engineering in combination with agronomic Zn fortification could further increase grain Zn content for Zn biofortification in cereals. In addition, higher Zn content in seeds could improve the establishment of seedlings in soils with low available Zn (Genc et al., 2000) and therefore could lead to higher plant production and yield.

The mechanisms underlying high grain Zn content due to the over-expression of *HvZIP7* is not known at this stage. It is possible that HvZIP7 could mediate Zn retranslocation from maternal tissues into the phloem (as it expresses in the vascular tissues of shoots, Chapter 3) and Zn unloading from the phloem stream into grain transfer cells (Figure 5.1). In a microarray analysis of Zn loading in the developing barley grain, Tauris et al. (2009) showed that *HvZIP7* is expressed specifically in transfer cells of the grain, which suggests that HvZIP7 could be involved in grain Zn loading. It is also possible that HvZIP7 could be involved in loading more Zn into the grain apopolastic space (Figure 5.1). In the *HvZIP7* over-expressing plants, due to the constitutive nature of *HvZIP7* driven by the CaMV 35S promoter, we were unable to separate the effect of higher shoot Zn accumulation from the potential involvement of HvZIP7 in the retranslocation of Zn into grains. Future experiments with tissue- or cell type-specific promoters to drive shoot-specific expression and transfer cell-specific expression of *HvZIP7* in combination with the root-specific expression could shed more light on the specific involvement of HvZIP7 in grain Zn loading.

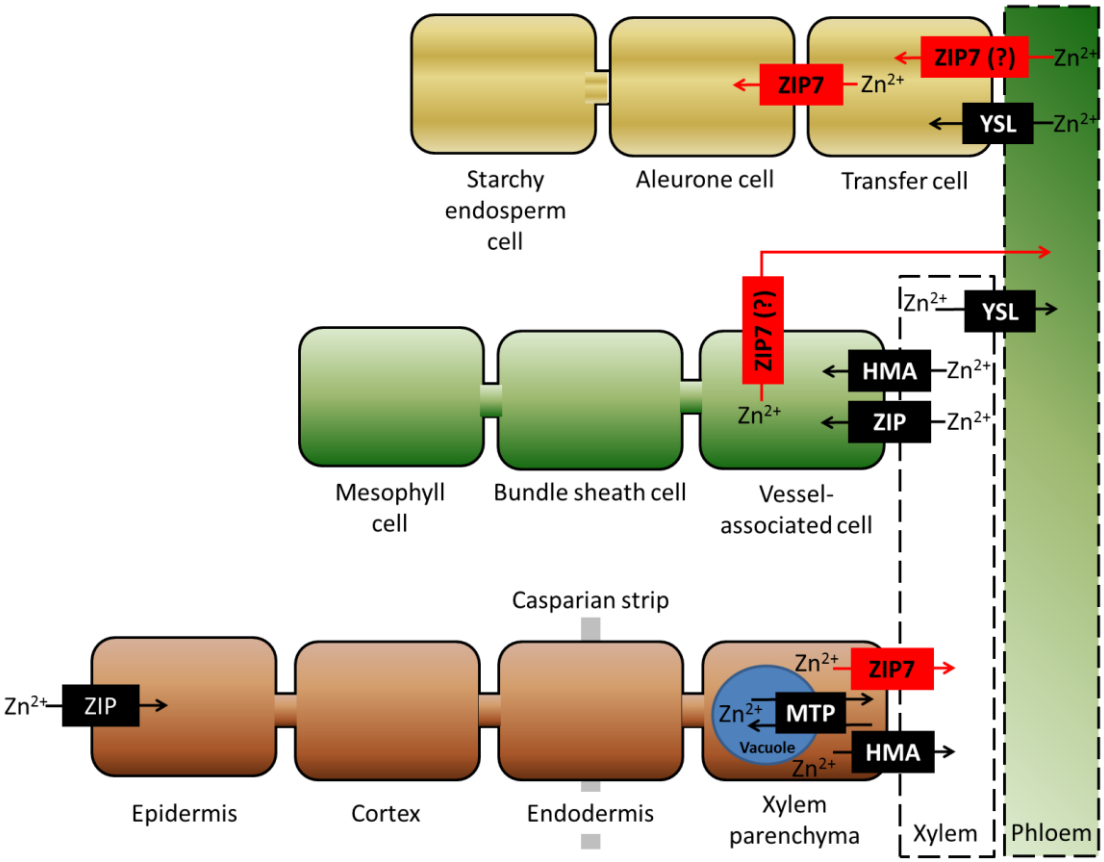

Figure 5.1. The potential localisation of HvZIP7 in plant tissues and its role in Zn trafficking in the plant.

An alternative approach to investigate Zn movement *in planta* is the use of Zn isotopes as a tracer. Zn remobilisation experiments using either radioactive (^{65}Zn) or stable (^{66}Zn) Zn isotopes could provide evidence of increased Zn translocation, retranslocation and/or grain loading (if present) in *HvZIP7* transgenic plants under Zn-abundant conditions. If HvZIP7 is indeed involved in retranslocation within shoots, we would expect to see a larger proportion of Zn in younger tissues than older tissues (relative to control plants), which indicates that more Zn might also get retranslocated from older tissues into grains. Alternatively, applying foliar Zn to the plants during anthesis could also help determine whether transgenic lines have increased Zn retranslocation and loading into grains as this method bypasses the process of Zn translocation from roots to shoots.

5.6 Zinc distribution in transgenic grains with high Zn

Preparation of cereal-based foods requires the removal of the husk and external part of seeds *via* polishing or milling. In wheat and barley, the majority of Zn in grains is localised in the aleurone layer and embryo. Polishing removes a large proportion (70%) of barley grain Zn from both transgenic plants and controls, but a similar percentage of Zn was retained in the polished grains of both transgenic plants and controls (Chapter 3). These results indicate that the distribution of Zn in the grains of the transgenic plants is similar to that of the wildtype despite being higher in total Zn content. These results provide an estimation of the Zn distribution pattern in the transgenic grains with high Zn content. The high grain Zn content retained in the polished transgenic grains would make more Zn available for human consumption (see Section 5.9).

5.7 HvZIP7 is specific for Zn transport

In addition to transporting Zn, ZIP transporters have also been shown to transport other metal ions such as Fe and Cd (Connolly et al., 2002; Lin et al., 2009). The over-expression of *HvZIP7* did not increase the accumulation of Fe, Mn, Cu or Cd in shoots nor the accumulation of Fe, Mn or Cu in the grain, indicating that HvZIP7 is a transporter that specifically translocates Zn from roots to shoots and into grains (Chapter 3). P-type ATPases such as AtHMA2, AtHMA4 and OsHMA2 are also involved in Zn translocation from roots to shoots (Hanikenne et al., 2008; Hussain et al., 2004; Satoh-Nagasawa et al., 2011). Unlike HvZIP7 however, these HMA proteins can transport Cd *in planta* in addition to Zn (Hanikenne et al., 2008; Hussain et al., 2004; Satoh-Nagasawa et al., 2011). Furthermore, the over-expression of *AtHMA4* increases the accumulation of both Zn and Cd in shoots (Verret et al., 2004). Therefore, for biofortification, HvZIP7 is a suitable candidate transporter for genetic engineering of cereal crops.

It would be beneficial to investigate the structural properties of the HvZIP7 protein. This information could potentially be used to alter the physical properties of other metal transporters to either increase the uptake of a metal of interest, or to eliminate the ability to take up undesired heavy metals. The ZIP transporter consists of eight trans-membrane anchoring domains (TM) and a large cytoplasmic loop between the third and fourth TM (Guerinot, 2000). This large cytoplasmic loop present in most ZIP proteins is rich in histidine residues and is thought to be responsible for metal binding and transport (Guerinot, 2000). Site directed mutagenesis of this histidine-rich loop of the human hZIP1 protein resulted in the reduction of Zn transport activity (Milon et al., 2006). It is therefore possible that the histidine-rich region in the large cytoplasmic loop determines the Zn ion selectivity of HvZIP7. Metal ligands present within this cytoplasmic loop have also been suggested to confer metal selectivity. Site directed mutagenesis of protein residues which act as potential

metal ligands in AtZIP1 resulted in the abolishment of Zn, Fe or Mn transport (Rogers et al., 2000). Therefore, site directed mutagenesis can be used to investigate and identify the protein residues conferring the Zn selectivity of HvZIP7.

5.8 Field testing of transgenic *HvZIP7* lines

Transgenic barley lines can increase Zn accumulation in shoots and grains relative to control lines when grown in a low Zn controlled environment with Zn fertilisers (Chapter 3). This shows the potential of transgenic plants for improving plant Zn nutrition and grain Zn content when grown in the field. However, growth conditions in the field such as Zn availability, water availability, temperature and radiation are very different from those in the controlled environments. Therefore, it will be essential to test the performance of the transgenic plants in the field.

To test this, the *HvZIP7* transgene needs to be transferred into elite barley cultivars. The transgene is currently in Golden Promise, a standard cultivar that is highly amenable to transformation but grows poorly in South Australian field conditions. Elite barley cultivars are usually not chosen for transformation due to their low transformation rates (Han et al., 2011). Crossing may therefore be a more suitable method to transfer the transgene from Golden Promise into an elite line. Another benefit of backcrossing the transgenic line with elite lines is that genetic alterations which can result from the transformation event could be 'cleaned up' (David, 2007). Multiple backcrossing will produce near-isogenic elite lines suitable for field trials to assess the performance of the *HvZIP7* transgene in terms of plant Zn nutrition, grain yield and grain Zn content. The outcome of the field trials will determine whether the transgenic lines are suitable for release or whether further optimisation of *HvZIP7* expression is required.

5.9 Bioavailability of higher grain Zn content from transgenic plants in humans

The higher Zn content in the polished grains of transgenic plants needs to be tested for bioavailability in humans. This is because bioavailability of Zn in humans is affected by a number of factors including phytate content, which reduces Zn bioavailability (Hotz et al., 2004), and the forms of Zn present in the grain. Some forms of Zn, such as Zn glycinate (Schlegel & Windisch, 2006), are more easily absorbed than others such as Zn oxide (Allen, 1998). The most common method to test the bioavailability of nutrients from plant materials is *via* mice feeding trials (Jeong & Guerinot, 2008). Alternatively, the Caco-2 cell line derived from the human gut could be used to test the bioavailability of Zn in the grains. The Caco-2 cell line is widely used in the pharmaceutical industry as an *in vitro* model to test for the absorption of orally administered drugs (Artursson & Karlsson, 1991). By milling the grains to flour and adding digestive enzymes to break down proteins and carbohydrates, the human gut cell line can be used to determine if the consumption of transgenic grains could increase Zn absorption. The outcome from the Caco-2 model could lead to the consideration of human clinical trials for high dietary intake of Zn derived from the transgenic grains. Obviously, bioavailability tests of transgenic grains with high Zn content is beyond the scope of this PhD project.

5.10 Summary

Eight members of the *HvZIP* gene family are up-regulated under Zn deficiency, and the coordinated expression of these Zn-deficiency inducible *HvZIP* genes is expected to play an important role for plant adaption to low Zn environments. One of these Zn-inducible *HvZIP* genes, *HvZIP7*, plays a role in mediating specific Zn translocation from roots to

shoots. The over-expression of *HvZIP7* could overcome the bottleneck restricting Zn translocation from roots to shoots when Zn is abundant in roots, and consequently increases Zn accumulation in the shoots and Zn loading into grains. Importantly, the increase in Zn accumulation in both shoots and grains did not have negative effects on the growth or yield of the plants. These results demonstrate the potential of HvZIP7 in Zn biofortification and improvement of Zn fertiliser usage.

The world population is increasing at an alarming rate, and superior cereal lines will need to be generated not only to meet increasing food demands, but also to possess high nutritional quality for the well-being of human health. The latter is easily overlooked as low nutritional values of staple foods are usually compensated by the consumption of fortified foods and supplements. However, more than half of the world's population resides in developing countries where artificial supplementation of foods is difficult to achieve. Their heavy reliance on cereal-based staple foods for dietary needs causes health problems associated with micronutrient malnutrition. Further increasing Zn in staple foods using transgenic lines in combination with fertiliser application could help those at risk of Zn deficiency.

References

Aery, N. C., & Jagetiya, B. L. (1997). Relative toxicity of cadmium, lead, and zinc on barley. *Communications in Soil Science and Plant Analysis, 28*(11-12), 949-960.

Ai, P. H., Sun, S. B., Zhao, J. N., Fan, X. R., Xin, W. J., Guo, Q., Yu, L., Shen, Q. R., Wu, P., Miller, A. J., & Xu, G. H. (2009). Two rice phosphate transporters, OsPht1;2 and OsPht1;6, have different functions and kinetic properties in uptake and translocation. *Plant Journal, 57*(5), 798-809.

Allen, L. (1998). Zinc and micronutrient supplements for children. *The American Journal of Clinical Nutrition, 68*(2), 495S-498S.

Alloway, B. J. (2001). *Zinc - The vital micronutrient for healthy, high-value crops*. Brussels: IZA Publications.

Alloway, B. J. (2004). *Zinc in soils and crop nutrition*. Brussels: IZA Publications.

Alloway, B. J. (2009). Soil factors associated with zinc deficiency in crops and humans. *Environmental Geochemistry and Health, 31*(5), 537-548.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389-3402.

Altschul, S. F., Wootton, J. C., Gertz, E. M., Agarwala, R., Morgulis, A., Schaffer, A. A., & Yu, Y. K. (2005). Protein database searches using compositionally adjusted substitution matrices. *FEBS Journal, 272*(20), 5101-5109.

Arrivault, S., Senger, T., & Kramer, U. (2006). The *Arabidopsis* metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *Plant Journal, 46*(5), 861-879.

Artursson, P., & Karlsson, J. (1991). Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochemical and Biophysical Research Communications, 175*(3), 880-885.

Ayliffe, M. A., & Pryor, A. J. (2011). Activation tagging and insertional mutagenesis in barley *Plant Reverse Genetics* (Vol. 678, pp. 107-128).

Barabasz, A., Krämer, U., Hanikenne, M., Rudzka, J., & Antosiewicz, D. M. (2010). Metal accumulation in tobacco expressing *Arabidopsis halleri* metal hyperaccumulation gene depends on external supply. *Journal of Experimental Botany, 61*(11), 3057-3067.

Barabasz, A., Wilkowska, A., Ruszczyńska, A., Bulska, E., Hanikenne, M., Czarny, M., Krämer, U., & Antosiewicz, D. M. (2012). Metal response of transgenic tomato

plants expressing P1B-ATPase. *Physiologia Plantarum*, DOI: 10.1111/j.1399-3054.2012.01584.x.

Borg, S., Brinch-Pedersen, H., Tauris, B., & Holm, P. (2009). Iron transport, deposition and bioavailability in the wheat and barley grain. *Plant and Soil, 325*(1), 15-24.

Brkljacic, J., Grotewold, E., Scholl, R., Mockler, T., Garvin, D., Vain, P., Brutnell, T., Sibout, R., Bevan, M., Budak, H., Caicedo, A., Gao, C., Gu, Y., Hazen, S., Holt, B. r., Hong, S., Jordan, M., Manzaneda, A., Mitchell-Olds, T., Mochida, K., Mur, L., Park, C., Sedbrook, J., Watt M, Zheng, S., & Vogel, J. (2011). Brachypodium as a model for the grasses: Today and the future. *Plant Physiology*, DOI:10.1104pp.111.179531.

Broadley, M. R., White, P. J., Hammond, J. P., Zelko, I., & Lux, A. (2007). Zinc in plants. *New Phytologist, 173*(4), 677-702.

Bughio, N., Yamaguchi, H., Nishizawa, N. K., Nakanishi, H., & Mori, S. (2002). Cloning an iron-regulated metal transporter from rice. *Journal of Experimental Botany, 53*(374), 1677-1682.

Burleigh, S. H., Kristensen, B. K., & Bechmann, I. E. (2003). A plasma membrane zinc transporter from *Medicago truncatula* is up-regulated in roots by Zn fertilization, yet down-regulated by arbuscular mycorrhizal colonization. *Plant Molecular Biology, 52*(5), 1077-1088.

Burton, R. A., Jobling, S. A., Harvey, A. J., Shirley, N. J., Mather, D. E., Bacic, A., & Fincher, G. B. (2008). The genetics and transcriptional profiles of the cellulose synthase-like *HvCslF* gene family in barley. *Plant Physiology, 146*(4), 1821-1833.

Cakmak, I. (2000). Possible roles of zinc in protecting plant cells from damage by reactive oxygen species. *New Phytologist, 146*(2), 185-205.

Cakmak, I. (2008). Enrichment of cereal grains with zinc: Agronomic or genetic biofortification? *Plant and Soil, 302*(1-2), 1-17.

Cakmak, I., & Marschner, H. (1993). Effect of zinc nutritional-status on activities of superoxide radical and hydrogen peroxide scavenging enzymes in bean leaves. *Plant and Soil, 156*, 127-130.

Cakmak, S., Gülüt, K. Y., Marschner, H., & Graham, R. D. (1994). Efect of zinc and iron deficiency on phytosiderophore release in wheat genotypes differing in zinc efficiency. *Journal of Plant Nutrition, 17*(1), 1-17.

Cakmak, I., Pfeiffer, W. H., & McClafferty, B. (2010). Review: Biofortification of durum wheat with zinc and iron. *Cereal Chemistry Journal, 87*(1), 10-20.

Campbell, N. A., Reece, J. B., Meyers, N., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. V., & Jackson, R. B. (2009). *Biology* (8th ed.): Benjamin Cummings.

Chen, A., Komives, E. A., & Schroeder, J. I. (2006). An improved grafting technique for mature *Arabidopsis* plants demonstrates long-distance shoot-to-root transport of phytochelatins in *Arabidopsis*. *Plant Physiology, 141*(1), 108-120.

Chen, W., Feng, Y., & Chao, Y. (2008a). Genomic analysis and expression pattern of *OsZIP1*, *OsZIP3*, and *OsZIP4* in two rice (*Oryza sativa* L.) genotypes with different zinc efficiency. *Russian Journal of Plant Physiology, 55*(3), 400-409.

Chen, W., Yang, X., He, Z., Feng, Y., & Hu, F. (2008b). Differential changes in photosynthetic capacity, 77 K chlorophyll fluorescence and chloroplast ultrastructure between Zn-efficient and Zn-inefficient rice genotypes (*Oryza sativa*) under low zinc stress. *Physiologia Plantarum, 132*(1), 89-101.

Chen, Z. H., Tian, X. H., Yang, X. W., Lu, X. C., Mai, W. X., Gale, J. W., & Cao, Y. X. (2010). Comparison of zinc efficiency among winter wheat genotypes cultured hydroponically in chelator-buffered solutions. *Journal of Plant Nutrition, 33*(11), 1612-1624.

Clemens, S., Palmgren, M. G., & Kramer, U. (2002). A long way ahead: Understanding and engineering plant metal accumulation. *Trends in Plant Science, 7*(7), 309-315.

Coleman, J. E. (1998). Zinc enzymes. *Current Opinion in Chemical Biology, 2*(2), 222-234.

Connolly, E. L., Fett, J. P., & Guerinot, M. L. (2002). Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell Online, 14*(6), 1347-1357.

Cunningham-Rundles, S., McNeeley, D. F., & Moon, A. (2005). Mechanisms of nutrient modulation of the immune response. *Journal of Allergy and Clinical Immunology, 115*(6), 1119-1128.

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., Misson, J., Schikora, A., Czernic, P., & Mari, S. (2009). Metal movement within the plant: Contribution of nicotianamine and yellow stripe 1-like transporters. *Annals of Botany, 103*(1), 1-11.

Curtis, M. D., & Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiology, 133*(2), 462-469.

David, W. (2007). GM maize from site-specific recombination technology, what next? *Current Opinion in Biotechnology, 18*(2), 115-120.

Desbrosses-Fonrouge, A. G., Voigt, K., Schroder, A., Arrivault, S., Thomine, S., & Kramer, U. (2005). *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar

membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS Letters, 579*(19), 4165-4174.

Dong, J., Chen, C., & Chen, Z. (2003). Expression profiles of the *Arabidopsis* WRKY gene superfamily during plant defense response. *Plant Molecular Biology, 51*(1), 21-37.

Drager, D. B., Desbrosses-Fonrouge, A. G., Krach, C., Chardonnens, A. N., Meyer, R. C., Saumitou-Laprade, P., & Kramer, U. (2004). Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *Plant Journal, 39*(3), 425-439.

Durmaz, E., Coruh, C., Dinler, G., Grusak, M., Peleg, Z., Saranga, Y., Fahima, T., Yazici, A., Ozturk, L., Cakmak, I., & Budak, H. (2010). Expression and cellular localization of *ZIP1* transporter under zinc deficiency in wild emmer wheat. *Plant Molecular Biology Reporter*, 1-15.

Eckhardt, U., Mas Marques, A., & Buckhout, T. J. (2001). Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. *Plant Molecular Biology, 45*(4), 437-448.

Eđed, A., & Rengel, Z. (2011). *Distribution of zinc in barley grain*. Paper presented at the 3rd International Zinc Symposium, Hyderabad, India.

Eide, D., Broderius, M., Fett, J., & Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences of the United States of America, 93*(11), 5624-5628.

Eide, D. J. (2006). Zinc transporters and the cellular trafficking of zinc. *Biochimica Et Biophysica Acta-Molecular Cell Research, 1763*(7), 711-722.

Eren, E., & Arguello, J. M. (2004). *Arabidopsis* HMA2, a divalent heavy metal-transporting P-IB-type ATPase, is involved in cytoplasmic Zn^{2+} homeostasis. *Plant Physiology, 136*(3), 3712-3723.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., & Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature, 391*(6669), 806-811.

Franklin, R. B., Ma, J., Zou, J., Guan, Z., Kukoyi, B. I., Feng, P., & Costello, L. C. (2003). Human ZIP1 is a major zinc uptake transporter for the accumulation of zinc in prostate cells. *Journal of Inorganic Biochemistry, 96*(2-3), 435-442.

Gaither, L. A., & Eide, D. J. (2001). Eukaryotic zinc transporters and their regulation. *Biometals, 14*(3-4), 251-270.

Genc, Y. (1999). *Screening for zinc efficiency in barley*. Adelaide: The University of Adelaide.

Genc, Y., Huang, C. Y., & Langridge, P. (2007). A study of the role of root morphological traits in growth of barley in zinc-deficient soil. *Journal of Experimental Botany, 58*(11), 2775-2784.

Genc, Y., McDonald, G. K., & Graham, R. D. (2000). Effect of seed zinc content on early growth of barley *Hordeum vulgare* under low and adequate soil zinc supply. *Australian Journal of Agricultural Research, 51*(1), 37-46.

Genc, Y., McDonald, G. K., & Graham, R. D. (2002). Critical deficiency concentration of zinc in barley genotypes differing in zinc efficiency and its relation to growth responses. *Journal of Plant Nutrition, 25*(3), 545-560.

Genc, Y., McDonald, G. K., & Graham, R. D. (2006). Contribution of different mechanisms to zinc efficiency in bread wheat during early vegetative stage. *Plant and Soil, 281*(1-2), 353-367.

Genc, Y., Verbyla, A. P., Torun, A. A., Cakmak, I., Wilsmore, K., Wallwork, H., & McDonald, G. K. (2009). Quantitative trait loci analysis of zinc efficiency and grain zinc concentration in wheat using whole genome average interval mapping. *Plant and Soil, 314*(1-2), 49-66.

Graham, R. D., Ascher, J. S., & Hynes, S. C. (1992). Selecting zinc-efficient cereal genotypes for soils of low Zn status. *Plant and Soil, 146*(1-2), 241-250.

Graham, R. D., & Rengel, Z. (1993). *Genotypic Variation in Zn Uptake and Utilization by Plants.* Paper presented at the Zinc in Soils and Plants Conference, The University of Western Australia.

Graham, R. D., Senadhira, D., Beebe, S., Iglesias, C., & Monasterio, I. (1999). Breeding for micronutrient density in edible portions of staple food crops: Conventional approaches. *Field Crops Research, 60*(1-2), 57-80.

Graham, R. D., Welch, R. M., & Bouis, H. E. (2001). Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: Principles, perspectives and knowledge gaps. *Advances in Agronomy 70*, 77-142.

Grewal, H. S., & Graham, R. D. (1998). Residual effects of subsoil zinc and oilseed rape genotype on the grain yield and distribution of zinc in wheat. *Plant and Soil, 207*(1), 29-36.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M. L., & Eide, D. (1998). Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proceedings of the National Academy of Sciences of the United States of America, 95*(12), 7220-7224.

Grotz, N., & Guerinot, M. L. (2006). Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochimica Et Biophysica Acta-Molecular Cell Research, 1763*(7), 595-608.

Guerinot, M. L. (2000). The ZIP family of metal transporters. *Biochimica Et Biophysica Acta-Biomembranes, 1465*(1-2), 190-198.

Hacisalihoglu, G., Hart, J. J., & Kochian, L. V. (2001). High- and low-affinity zinc transport systems and their possible role in zinc efficiency in bread wheat. *Plant Physiology, 125*(1), 456-463.

Hacisalihoglu, G., Hart, J. J., Vallejos, C. E., & Kochian, L. V. (2004). The role of shoot-localized processes in the mechanism of Zn efficiency in common bean. *Planta, 218*(5), 704-711.

Hacisalihoglu, G., Hart, J. J., Wang, Y. H., Cakmak, I., & Kochian, L. V. (2003). Zinc efficiency is correlated with enhanced expression and activity of zinc-requiring enzymes in wheat. *Plant Physiology, 131*(2), 595-602.

Hacisalihoglu, G., & Kochian, L. V. (2003). How do some plants tolerate low levels of soil zinc? Mechanisms of zinc efficiency in crop plants. *New Phytologist, 159*(2), 341-350.

Han, Y., Jin, X., Wu, F., & Zhang, G. (2011). Genotypic differences in callus induction and plant regeneration from mature embryos of barley (*Hordeum vulgare* L.). *Journal of Zhejiang University - Science B, 12*(5), 399-407.

Hanikenne, M., Talke, I. N., Haydon, M. J., Lanz, C., Nolte, A., Motte, P., Kroymann, J., Weigel, D., & Kramer, U. (2008). Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature, 453*(7193), 391-395.

Hart, J. J., Norvell, W. A., Welch, R. M., Sullivan, L. A., & Kochian, L. V. (1998). Characterization of zinc uptake, binding, and translocation in intact seedlings of bread and durum wheat cultivars. *Plant Physiology, 118*(1), 219-226.

Henriques, R., Jasik, J., Klein, M., Martinoia, E., Feller, U., Schell, J., Pais, M. S., & Koncz, C. (2002). Knock-out of *Arabidopsis* metal transporter gene *IRT1* results in iron deficiency accompanied by cell differentiation defects. *Plant Molecular Biology, 50*(4-5), 587-597.

Herren, T., & Feller, U. (1994). Transfer of zinc from xylem to phloem in the peduncle of wheat. *Journal of Plant Nutrition, 17*(9), 1587-1598.

Hershfinkel, M., Silverman, W. F., & Sekler, I. (2007). The zinc sensing receptor, a link between zinc and cell signaling. *Molecular Medicine, 13*(7-8), 331-336.

Hirschi, K. D. (2009). Nutrient biofortification of food crops. *Annual Review of Nutrition, 29*, 401-421.

Hotz, C., DeHaene, J., Villalpando, S., Rivera, J., Woodhouse, L., & King, J. C. (2004). Comparison of fractional zinc absorption (FAZ) from ZnO, ZnO + Na_2EDTA, $Na_2ZnEDTA$, and $ZnSO_4$, when added as fortificants to a maize tortilla-based meal. *FASEB Journal, 18*(4-5), Abst. 129.125.

Hotz, C., DeHaene, J., Woodhouse, L. R., Villalpando, S., Rivera, J. A., & King, J. C. (2005). Zinc absorption from zinc oxide, zinc sulfate, zinc oxide + EDTA, or sodium-zinc EDTA does not differ when added as fortificants to maize tortillas. *Journal of Nutrition, 135*(5), 1102-1105.

Huang, L., Kirschke, C. P., Zhang, Y., & Yu, Y. Y. (2005). The *ZIP7* gene (Slc39a7) encodes a zinc transporter involved in zinc homeostasis of the Golgi apparatus. *Journal of Biological Chemistry, 280*(15), 15456-15463.

Huang, L. P., Kirschke, C. P., & Gitschier, J. (2002). Functional characterization of a novel mammalian zinc transporter, ZnT6. *Journal of Biological Chemistry, 277*(29), 26389-26395.

Hussain, D., Haydon, M. J., Wang, Y., Wong, E., Sherson, S. M., Young, J., Camakaris, J., Harper, J. F., & Cobbett, C. S. (2004). P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. *Plant Cell, 16*(5), 1327-1339.

Ishimaru, Y., Masuda, H., Suzuki, M., Bashir, K., Takahashi, M., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2007). Overexpression of the OsZIP4 zinc transporter confers disarrangement of zinc distribution in rice plants. *Journal of Experimental Botany, 58*(11), 2909-2915.

Ishimaru, Y., Suzuki, M., Kobayashi, T., Takahashi, M., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2005). OsZIP4, a novel zinc-regulated zinc transporter in rice. *Journal of Experimental Botany, 56*(422), 3207-3214.

Ishimaru, Y., Suzuki, M., Tsukamoto, T., Suzuki, K., Nakazono, M., Kobayashi, T., Wada, Y., Watanabe, S., Matsuhashi, S., Takahashi, M., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2006). Rice plants take up iron as an Fe^{3+}-phytosiderophore and as Fe^{2+}. *Plant Journal, 45*(3), 335-346.

Jeong, J., & Guerinot, M. L. (2008). Biofortified and bioavailable: The gold standard for plant-based diets. *Proceedings of the National Academy of Sciences, 105*(6), 1777-1778.

Jiang, W., Struik, P. C., Van Keulen, H., Zhao, M., Jin, L. N., & Stomph, T. J. (2008). Does increased zinc uptake enhance grain zinc mass concentration in rice? *Annals of Applied Biology, 153*(1), 135-147.

Johansen, B. (1997). *In situ* PCR on plant material with sub-cellular resolution. *Annals of Botany, 80*(5), 697-700.

Kalayci, M., Torun, B., Eker, S., Aydin, M., Ozturk, L., & Cakmak, I. (1999). Grain yield, zinc efficiency and zinc concentration of wheat cultivars grown in a zinc-deficient calcareous soil in field and greenhouse. *Field Crops Research, 63*(1), 87-98.

Katoh, K., Asimenos, G., & Toh, H. (2009). Multiple alignment of DNA sequences with MAFFT. In D. Posada (Ed.), *Bioinformatics for DNA Sequence Analysis* (Vol. 537, pp. 39-64): Humana Press Inc, 999 Riverview Dr, Ste 208, Totowa, Nj 07512-1165 USA.

Kim, D., Gustin, J. L., Lahner, B., Persans, M. W., Baek, D., Yun, D.-J., & Salt, D. E. (2004). The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. *Plant Journal, 39*(2), 237-251.

Kobae, Y., Uemura, T., Sato, M. H., Ohnishi, M., Mimura, T., Nakagawa, T., & Maeshima, M. (2004). Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant and Cell Physiology, 45*(12), 1749-1758.

Koike, S., Inoue, H., Mizuno, D., Takahashi, M., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2004). OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *Plant Journal, 39*(3), 415-424.

Koltai, H., & Bird, D. M. (2000). High throughput cellular localization of specific plant mRNAs by liquid-phase *in situ* reverse transcription-polymerase chain reaction of tissue sections. *Plant Physiology, 123*(4), 1203-1212.

Krämer, U., & Clemens, S. (2006). Functions and homeostasis of zinc, copper, and nickel in plants. In M. Tamas & E. Martinoia (Eds.), *Molecular Biology of Metal Homeostasis and Detoxification* (Vol. 14, pp. 216-271): Springer Berlin / Heidelberg.

Kramer, U., CotterHowells, J. D., Charnock, J. M., Baker, A. J. M., & Smith, J. A. C. (1996). Free histidine as a metal chelator in plants that accumulate nickel. *Nature, 379*(6566), 635-638.

Kumanovics, A., Poruk, K. E., Osborn, K. A., Ward, D. M., & Kaplan, J. (2006). *YKE4* (YIL023C) encodes a bidirectional zinc transporter in the endoplasmic reticulum of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 281*(32), 22566-22574.

Küpper, H., Jie Zhao, F., & McGrath, S. P. (1999). Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology, 119*(1), 305-312.

Küpper, H., & Kochian, L. V. (2010). Transcriptional regulation of metal transport genes and mineral nutrition during acclimatization to cadmium and zinc in the Cd/Zn hyperaccumulator, *Thlaspi caerulescens* (Ganges population). *New Phytologist, 185*(1), 114-129.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and clustal X version 2.0. *Bioinformatics, 23*(21), 2947-2948.

Lasat, M. M., Baker, A. J. M., & Kochian, L. V. (1996). Physiological characterization of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of *Thlaspi*. *Plant Physiology, 112*(4), 1715-1722.

Lasswell, J., Rogg, L. E., Nelson, D. C., Rongey, C., & Bartel, B. (2000). Cloning and characterization of *IAR1*, a gene required for auxin conjugate sensitivity in *Arabidopsis*. *Plant Cell, 12*(12), 2395-2408.

Le Jean, M., Schikora, A., Mari, S., Briat, J. F., & Curie, C. (2005). A loss-of-function mutation in AtYSL1 reveals its role in iron and nicotianamine seed loading. *Plant Journal, 44*(5), 769-782.

Lee, R. (2011). The outlook for population growth. *Science, 333*(6042), 569-573.

Lee, S., Jeong, H. J., Kim, S. A., Lee, J., Guerinot, M. L., & An, G. (2010a). OsZIP5 is a plasma membrane zinc transporter in rice. *Plant Molecular Biology, 73*(4-5), 507-517.

Lee, S., Kim, S. A., Lee, J., Guerinot, M. L., & An, G. (2010b). Zinc deficiency-inducible *OsZIP8* encodes a plasma membrane-localized zinc transporter in rice. *Molecules and Cells, 29*(6), 551-558.

Lin, Y.-F., Liang, H.-M., Yang, S.-Y., Boch, A., Clemens, S., Chen, C.-C., Wu, J.-F., Huang, J.-L., & Yeh, K.-C. (2009). *Arabidopsis* IRT3 is a zinc-regulated and plasma membrane localized zinc/iron transporter. *New Phytologist, 182*(2), 392-404.

Lindsay, W. L. (1991). Inorganic equilibria affecting micronutrients in soils. In J. J. Mortvedt, F. R. Cox, L. M. Schuman & R. M. Welch (Eds.), *Micronutrients in Agriculture* (pp. 89-112). Madison, Wisconsin, USA: Soil Science Society of America Book Series.

Lindskog, S. (1997). Structure and mechanism of carbonic anhydrase. *Pharmacology and Therapeutics, 74*(1), 1-20.

Lombi, E., Scheckel, K. G., Pallon, J., Carey, A. M., Zhu, Y. G., & Meharg, A. A. (2009). Speciation and distribution of arsenic and localization of nutrients in rice grains. *New Phytologist, 184*(1), 193-201.

Lombi, E., Smith, E., Hansen, T. H., Paterson, D., de Jonge, M. D., Howard, D. L., Persson, D. P., Husted, S., Ryan, C., & Schjoerring, J. K. (2011). Megapixel imaging of (micro)nutrients in mature barley grains. *Journal of Experimental Botany, 62*(1), 273-282.

Lombi, E., Tearall, K. L., Howarth, J. R., Zhao, F.-J., Hawkesford, M. J., & McGrath, S. P. (2002). Influence of iron status on cadmium and zinc uptake by different ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology, 128*(4), 1359-1367.

Lonergan, P. F., Pallotta, M. A., Lorimer, M., Paull, J. G., Barker, S. J., & Graham, R. D. (2009). Multiple genetic loci for zinc uptake and distribution in barley (*Hordeum vulgare*). *New Phytologist, 184*(1), 168-179.

López-Millán, A.-F., Ellis, D., & Grusak, M. (2004). Identification and characterization of several new members of the ZIP family of metal ion transporters in *Medicago truncatula*. *Plant Molecular Biology, 54*(4), 583-596.

Lucca, P., Poletti, S., & Sautter, C. (2006). Genetic engineering approaches to enrich rice with iron and vitamin A. *Physiologia Plantarum, 126*(3), 291-303.

MacDiarmid, C. W., Gaither, L. A., & Eide, D. (2000). Zinc transporters that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*. *Embo Journal, 19*(12), 2845-2855.

Maret, W. (2004). Zinc and sulfur: A critical biological partnership. *Biochemistry, 43*(12), 3301-3309.

Marschner, H. (1993). Zinc uptake from soils. In A. D. Robson (Ed.), *Zinc in Soils and Plants* (Vol. 55, pp. 59-77). Dordrecht: Kluwer Academic Publ.

Masuda, H., Usuda, K., Kobayashi, T., Ishimaru, Y., Kakei, Y., Takahashi, M., Higuchi, K., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2009). Overexpression of the barley nicotianamine synthase gene *HvNAS1* increases iron and zinc concentrations in rice grains. *Rice, 2*(4), 155-166.

Matthews, P. R., Wang, M.-B., Waterhouse, P. M., Thornton, S., Fieg, S. J., Gubler, F., & Jacobsen, J. V. (2001). Marker gene elimination from transgenic barley, using co-transformation with adjacent `twin T-DNAs' on a standard *Agrobacterium* transformation vector. *Molecular Breeding, 7*(3), 195-202.

Mayer, J. E., Pfeiffer, W. H., & Beyer, P. (2008). Biofortified crops to alleviate micronutrient malnutrition. *Current Opinion in Plant Biology, 11*(2), 166-170.

Mazzolini, A. P., Pallaghy, C. K., & Legge, G. J. F. (1985). Quantitative microanalysis of Mn, Zn and other elements in mature wheat seed. *New Phytologist, 100*(4), 483-509.

McDonald, G. K., Genc, Y., & Graham, R. D. (2008). A simple method to evaluate genetic variation in grain zinc concentration by correcting for differences in grain yield. *Plant and Soil, 306*(1-2), 49-55.

Mendoza-Cozatl, D. G., Butko, E., Springer, F., Torpey, J. W., Komives, E. A., Kehr, J., & Schroeder, J. I. (2008). Identification of high levels of phytochelatins, glutathione and cadmium in the phloem sap of *Brassica napus*. A role for thiol-peptides in the long-distance transport of cadmium and the effect of cadmium on iron translocation. *Plant Journal, 54*(2), 249-259.

Milner, M. J., Craft, E., Yamaji, N., Koyama, E., Ma, J. F., & Kochian, L. V. (2012). Characterization of the high affinity Zn transporter from *Noccaea caerulescens*, NcZNT1, and dissection of its promoter for its role in Zn uptake and hyperaccumulation. *The New Phytologist, 195*(1), 113-123.

Milon, B., Wu, Q., Zou, J., Costello, L. C., & Franklin, R. B. (2006). Histidine residues in the region between transmembrane domains III and IV of hZip1 are required for zinc transport across the plasma membrane in PC-3 cells. *Biochimica Et Biophysica Acta-Biomembranes, 1758*(10), 1696-1701.

Mizuno, T., Usui, K., Horie, K., Nosaka, S., Mizuno, N., & Obata, H. (2005). Cloning of three ZIP/Nramp transporter genes from a Ni hyperaccumulator plant *Thlaspi japonicum* and their Ni^{2+}-transport abilities. *Plant Physiology and Biochemistry, 43*(8), 793-801.

Moreau, S., Thomson, R. M., Kaiser, B. N., Trevaskis, B., Guerinot, M. L., Udvardi, M. K., Puppo, A., & Day, D. A. (2002). *GmZIP1* encodes a symbiosis-specific zinc transporter in soybean. *Journal of Biological Chemistry, 277*(7), 4738-4746.

Murata, Y., Ma, J. F., Yamaji, N., Ueno, D., Nomoto, K., & Iwashita, T. (2006). A specific transporter for iron(III)–phytosiderophore in barley roots. *The Plant Journal, 46*(4), 563-572.

Narayanan, N. N., Vasconcelos, M. W., & Grusak, M. A. (2007). Expression profiling of *Oryza sativa* metal homeostasis genes in different rice cultivars using a cDNA macroarray. *Plant Physiology and Biochemistry, 45*(5), 277-286.

Nishida, S., Morinaga, Y., Obata, H., & Mizuno, T. (2011). Identification of the N-terminal region of TjZNT2, a Zrt/Irt-like protein family metal transporter, as a novel functional region involved in metal ion selectivity. *FEBS Journal, 278*(5), 851-858.

Nishiyama, R., Kato, M., Nagata, S., Yanagisawa, S., & Yoneyama, T. (2012). Identification of Zn-nicotianamine and Fe-2'-deoxymugineic acid in the phloem sap from rice plants (*Oryza sativa* L.). *Plant and Cell Physiology, 53*(2), 381-390.

Odell, J. T., Nagy, F., & Chua, N. H. (1985). Identification of DNA-sequences required for activity of the cauliflower mosaic virus-35S promoter. *Nature, 313*(6005), 810-812.

Ohki, K. (1976). Effect of zinc nutrition on photosynthesis and carbonic anhydrase activity in cotton. *Physiologia Plantarum, 38*(4), 300-304.

Ohki, K. (1978). Zinc concentration in soybean as related to growth, photosynthesis, and carbonic anhydrase activity. *Crop Science, 18*(1), 79-82.

Ozturk, L., Altintas, G., Erdem, H., Gokmen, O. O., Yazici, A., & Cakmak, I. (2009). *Localization of iron, zinc, and protein in seeds of spelt (*Triticum aestivum *ssp.* spelta*) genotypes with low and high protein concentration*. Paper presented at the International Plant Nutrition Colloquium XVI, The University of California, Davis.

Ozturk, L., Yazici, M. A., Yucel, C., Torun, A., Cekic, C., Bagci, A., Ozkan, H., Braun, H. J., Sayers, Z., & Cakmak, I. (2006). Concentration and localization of zinc during seed development and germination in wheat. *Physiologia Plantarum, 128*(1), 144-152.

Paine, J. A., Shipton, C. A., Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S. Y., Hinchliffe, E., Adams, J. L., Silverstone, A. L., & Drake, R. (2005). Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nature Biotechnology, 23*(4), 482-487.

Palmer, C. M., & Guerinot, M. L. (2009). Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nature Chemical Biology, 5*(5), 333-340.

Palmgren, M. G., Clemens, S., Williams, L. E., Kraemer, U., Borg, S., Schjorring, J. K., & Sanders, D. (2008). Zinc biofortification of cereals: Problems and solutions. *Trends in Plant Science, 13*(9), 464-473.

Patrick, J. W., & Offler, C. E. (2001). Compartmentation of transport and transfer events in developing seeds. *Journal of Experimental Botany, 52*(356), 551-564.

Pedas, P., Schjoerring, J. K., & Husted, S. (2009). Identification and characterization of zinc-starvation-induced ZIP transporters from barley roots. *Plant Physiology and Biochemistry, 47*(5), 377-383.

Pedas, P., Ytting, C. K., Fuglsang, A. T., Jahn, T. P., Schjoerring, J. K., & Husted, S. (2008)**.** Manganese efficiency in barley: Identification and characterization of the metal ion transporter HvIRT1. *Plant Physiology, 148*(1), 455-466.

Peleg, Z., Saranga, Y., Yazici, A., Fahima, T., Ozturk, L., & Cakmak, I. (2008). Grain zinc, iron and protein concentrations and zinc-efficiency in wild emmer wheat under contrasting irrigation regimes. *Plant and Soil, 306*(1-2), 57-67.

Persson, D. P., Hansen, T. H., Laursen, K. H., Schjoerring, J. K., & Husted, S. (2009). Simultaneous iron, zinc, sulfur and phosphorus speciation analysis of barley grain tissues using SEC-ICP-MS and IP-ICP-MS. *Metallomics, 1*(5), 418-426.

Pieczonka, K., & Rosopulo, A. (1985). Distribution of cadmium, copper, and zinc in the caryopsis of wheat (*Triticum aestivum* L.). *Fresenius' Journal of Analytical Chemistry, 322*(7), 697-699.

Plasterk, R. H. A. (2002). RNA silencing: The genome's immune system. *Science, 296*(5571), 1263-1265.

Preuss, C. P., Huang, C. Y., Gilliham, M., & Tyerman, S. D. (2010). Channel-Like characteristics of the low-affinity barley phosphate transporter PHT1;6 when expressed in *Xenopus oocytes*. *Plant Physiology, 152*(3), 1431-1441.

Rae, A. L., Cybinski, D. H., Jarmey, J. M., & Smith, F. W. (2003). Characterization of two phosphate transporters from barley; evidence for diverse function and kinetic properties among members of the Pht1 family. *Plant Molecular Biology, 53*(1), 27-36.

Ramesh, S. A., Choimes, S., & Schachtman, D. P. (2004). Over-expression of an *Arabidopsis* zinc transporter in *Hordeum vulgare* increases short-term zinc uptake after zinc deprivation and seed zinc content. *Plant Molecular Biology, 54*(3), 373-385.

Ramesh, S. A., Shin, R., Eide, D. J., & Schachtman, D. P. (2003). Differential metal selectivity and gene expression of two zinc transporters from rice. *Plant Physiology, 133*(1), 126-134.

Randall, P. J., & Bouma, D. (1973). Zinc deficiency, carbonic anhydrase, and photosynthesis in leaves of spinach. *Plant Physiology, 52*(3), 229-232.

Rattan, R. K., & Deb, D. L. (1981). Self-diffusion of zinc and iron in soils as affected by pH, $CaCO_3$, moisture, carrier and phosphorus levels. *Plant and Soil, 63*(3), 377-393.

Rees, J. D., Ingle, R. A., & Smith, J. A. C. (2009). Relative contributions of nine genes in the pathway of histidine biosynthesis to the control of free histidine concentrations in *Arabidopsis thaliana*. *Plant Biotechnology Journal, 7*(6), 499-511.

Rengel, Z. (2001). Genotypic differences in micronutrient use efficiency in crops. *Communications in Soil Science and Plant Analysis, 32*(7-8), 1163-1186.

Rengel, Z., Batten, G. D., & Crowley, D. E. (1999). Agronomic approaches for improving the micronutrient density in edible portions of field crops. *Field Crops Research, 60*(1–2), 27-40.

Rogers, E. E., Eide, D. J., & Guerinot, M. L. (2000). Altered selectivity in an *Arabidopsis* metal transporter. *Proceedings of the National Academy of Sciences, 97*(22), 12356-12360.

Ruel, M., & Bouis, H. (1998). Plant breeding: A long-term strategy for the control of zinc deficiency in vulnerable populations. *The American Journal of Clinical Nutrition, 68*(2), 488S-494S.

Sasaki, H., Hirose, T., Watanabe, Y., & Ohsugi, R. (1998). Carbonic anhydrase activity and CO_2-transfer resistance in Zn-deficient rice leaves. *Plant Physiology, 118*(3), 929-934.

Satoh-Nagasawa, N., Mori, M., Nakazawa, N., Kawamoto, T., Nagato, Y., Sakurai, K., Takahashi, H., Watanabe, A., & Akagi, H. (2011). Mutations in rice (*Oryza sativa*) heavy metal ATPase 2 (OsHMA2) restrict the translocation of Zn and Cd. *Plant and Cell Physiology*, DOI: 10.1093/pcp/pcr166.

Schaaf, G., Schikora, A., Haberle, J., Vert, G., Ludewig, U., Briat, J. F., Curie, C., & von Wiren, N. (2005). A putative function for the *Arabidopsis* Fe-phytosiderophore transporter homolog AtYSL2 in Fe and Zn homeostasis. *Plant and Cell Physiology, 46*(5), 762-774.

Schlegel, P., & Windisch, W. (2006). Bioavailability of zinc glycinate in comparison with zinc sulphate in the presence of dietary phytate in an animal model with ^{65}Zn labelled rats. *Journal of Animal Physiology and Animal Nutrition, 90*(5-6), 216-222.

Shahzad, Z., Gosti, F., Frerot, H., Lacombe, E., Roosens, N., Saumitou-Laprade, P., & Berthomieu, P. (2010). The five AhMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. *Plos Genetics, 6*(4), e1000911.

Sharma, P. N., Tripathi, A., & Bisht, S. S. (1995). Zinc requirement for stomatal opening in cauliflower. *Plant Physiology, 107*(3), 751-756.

Stephens, B. W., Cook, D. R., & Grusak, M. A. (2011). Characterization of zinc transport by divalent metal transporters of the ZIP family from the model legume *Medicago truncatula*. *Biometals, 24*(1), 51-58.

Stomph, T. J., Jiang, W., & Struik, P. C. (2009). Zinc biofortification of cereals: rice differs from wheat and barley. *Trends in Plant Science, 14*(3), 123-124.

Suzuki, M., Takahashi, M., Tsukamoto, T., Watanabe, S., Matsuhashi, S., Yazaki, J., Kishimoto, N., Kikuchi, S., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2006). Biosynthesis and secretion of mugineic acid family phytosiderophores in zinc-deficient barley. *Plant Journal, 48*(1), 85-97.

Suzuki, M., Tsukamoto, T., Inoue, H., Watanabe, S., Matsuhashi, S., Takahashi, M., Nakanishi, H., Mori, S., & Nishizawa, N. K. (2008). Deoxymugineic acid increases Zn translocation in Zn-deficient rice plants. *Plant Molecular Biology, 66*(6), 609-617.

Talke, I. N., Hanikenne, M., & Krämer, U. (2006). Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology, 142*(1), 148-167.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony methods. *Molecular Biology and Evolution*, DOI:10.1093.

Tauris, B., Borg, S., Gregersen, P. L., & Holm, P. B. (2009). A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. *Journal of Experimental Botany, 60*(4), 1333-1347.

Tian, J., Venkatachalam, P., Liao, H., Yan, X. L., & Raghothama, K. (2007). Molecular cloning and characterization of phosphorus starvation responsive genes in common bean (*Phaseolus vulgaris* L.). *Planta, 227*(1), 151-165.

Tingay, S., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S., & Brettell, R. (1997). *Agrobacterium tumefaciens*-mediated barley transformation. *Plant Journal, 11*(6), 1369-1376.

Van Belleghem, F., Cuypers, A., Semane, B., Smeets, K., Vangronsveld, J., d'Haen, J., & Valcke, R. (2007). Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytologist, 173*(3), 495-508.

van de Mortel, J. E., Almar Villanueva, L., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P. D., Ver Loren van Themaat, E., Koornneef, M., & Aarts, M. G. M. (2006). Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology, 142*(3), 1127-1147.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., & Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology, 3*(7), research0034.0031-research0034.0011.

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., Vavasseur, A., & Richaud, P. (2004). Overexpression of *AtHMA4* enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letter, 576*(3), 306-312.

Vert, G., Briat, J. F., & Curie, C. (2001). *Arabidopsis IRT2* gene encodes a root-periphery iron transporter. *Plant Journal, 26*(2), 181-189.

Vert, G., Grotz, N., Dedaldechamp, F., Gaymard, F., Guerinot, M. L., Briat, J. F., & Curie, C. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *Plant Cell, 14*(6), 1223-1233.

von Wiren, N., Marschner, H., & Romheld, V. (1996). Roots of iron-efficient maize also absorb phytosiderophore-chelated zinc. *Plant Physiology, 111*(4), 1119-1125.

Walter, A., Römheld, V., Marschner, H., & Mori, S. (1994). Is the release of phytosiderophores in zinc-deficient wheat plants a response to impaired iron utilization? *Physiologia Plantarum, 92*(3), 493-500.

Wang, J. P., Raman, H., Zhang, G. P., Mendham, N., & Zhou, M. X. (2006). Aluminium tolerance in barley (*Hordeum vulgare* L.): Physiological mechanisms, genetics and screening methods. *Journal of Zhejiang University-SCIENCE B, 7*(10), 769-787.

Wang, M. Y., Xu, Q. Y., Yu, J. J., & Yuan, M. (2010). The putative *Arabidopsis* zinc transporter ZTP29 is involved in the response to salt stress. *Plant Molecular Biology, 73*(4-5), 467-479.

Waters, B. M., Chu, H. H., DiDonato, R. J., Roberts, L. A., Eisley, R. B., Lahner, B., Salt, D. E., & Walker, E. L. (2006). Mutations in *Arabidopsis* Yellow Stripe-Like1 and Yellow Stripe-Like3 reveal their roles in metal ion homeostasis and loading of metal ions in seeds. *Plant Physiology, 141*(4), 1446-1458.

Waters, B. M., & Sankaran, R. P. (2011). Moving micronutrients from the soil to the seeds: Genes and physiological processes from a biofortification perspective. *Plant Science, 180*(4), 562-574.

Welch, R. M. (2001). Micronutrients, agriculture and nutrition; linkages for improved health and well bing. In K. Singh, Mori, S., Welch, R. M. (Ed.), *Perspective on the micronutrient nutrition of crops* (pp. 247-289). Jodhpur: Scientific Publishers.

Welch, R. M. (2005). Biotechnology, biofortification, and global health. *Food and Nutrition Bulletin, 26*(4), 419-421.

Welch, R. M., & Graham, R. D. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany, 55*(396), 353-364.

Welch, R. M., & Shuman, L. (1995). Micronutrient nutrition of plants. *Critical Reviews in Plant Sciences, 14*(1), 49-82.

Wheal, M. S., Fowles, T. O., & Palmer, L. T. (2011). A cost-effective acid digestion method using closed polypropylene tubes for inductively coupled plasma optical

emission spectrometry (ICP-OES) analysis of plant essential elements. *Analytical Methods, 3*(12), 2854-2863.

White, C., & Gadd, G. M. (1986). Uptake and cellular distribution of copper, cobalt and cadmium in strains of *Saccharomyces cerevisiae* cultured on elevated concentrations of these metals. *FEMS Microbiology Letters, 38*(5), 277-283.

White, J. G., & Zasoski, R. J. (1999). Mapping soil micronutrients. *Field Crops Research, 60*(1-2), 11-26.

White, M. C., Baker, F. D., Chaney, R. L., & Decker, A. M. (1981). Metal complexation in xylem fluid. *Plant Physiology, 67*(2), 301-310.

White, P. J., & Broadley, M. R. (2011). Physiological limits to zinc biofortification of edible crops. *Frontiers in Plant Science, 2*(80), DOI: 10.3389/fpls.2011.00080.

Widodo, B., Broadley, M. R., Rose, T., Frei, M., Pariasca-Tanaka, J., Yoshihashi, T., Thomson, M., Hammond, J. P., Aprile, A., Close, T. J., Ismail, A. M., & Wissuwa, M. (2010). Response to zinc deficiency of two rice lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates, and not by zinc-transporter activity. *New Phytologist, 186*(2), 400-414.

Wirth, J., Poletti, S., Aeschlimann, B., Yakandawala, N., Drosse, B., Osorio, S., Tohge, T., Fernie, A. R., Gunther, D., Gruissem, W., & Sautter, C. (2009). Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnology Journal, 7*(7), 631-644.

Wissuwa, M., Ismail, A., & Graham, R. (2008). Rice grain zinc concentrations as affected by genotype, native soil-zinc availability, and zinc fertilization. *Plant and Soil, 306*(1), 37-48.

Wong, C. K. E., Jarvis, R. S., Sherson, S. M., & Cobbett, C. S. (2009). Functional analysis of the heavy metal binding domains of the Zn/Cd-transporting ATPase, HMA2, in *Arabidopsis thaliana*. *New Phytologist, 181*(1), 79-88.

Wu, C. Y., Lu, L. L., Yang, X. E., Feng, Y., Wei, Y. Y., Hao, H. L., Stoffella, P. J., & He, Z. L. (2010). Uptake, translocation, and remobilization of zinc absorbed at different growth stages by rice genotypes of different Zn densities. *Journal of Agricultural and Food Chemistry, 58*(11), 6767-6773.

Wu, J., Zhao, F. J., Ghandilyan, A., Logoteta, B., Guzman, M. O., Schat, H., Wang, X. W., & Aarts, M. G. M. (2009). Identification and functional analysis of two ZIP metal transporters of the hyperaccumulator *Thlaspi caerulescens*. *Plant and Soil, 325*(1-2), 79-95.

Yang, X., Huang, J., Jiang, Y., & Zhang, H.-S. (2009). Cloning and functional identification of two members of the ZIP (Zrt, Irt-like protein) gene family in rice (*Oryza sativa* L.). *Molecular Biology Reports, 36*(2), 281-287.

Yang, X., Li, T. Q., Yang, J. C., He, Z. L., Lu, L. L., & Meng, F. H. (2006). Zinc compartmentation in root, transport into xylem, and absorption into leaf cells in the hyperaccumulating species of *Sedum alfredii* Hance. *Planta, 224*(1), 185-195.

Ye, X. D., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P., & Potrykus, I. (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science, 287*(5451), 303-305.

Yu, Q., & Rengel, Z. (1999). Micronutrient deficiency influences plant growth and activities of superoxide dismutases in narrow-leafed lupins. *Annals of Botany, 83*(2), 175-182.

Zhao, H., & Eide, D. (1996). The *ZRT2* gene encodes the low affinity zinc iransporter in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 271*(38), 23203-23210.

Zheng, L., Fujii, M., Yamaji, N., Sasaki, A., Yamane, M., Sakurai, I., Sato, K., & Ma, J. F. (2011). Isolation and characterization of a barley Yellow Stripe-like gene, *HvYSL5*. *Plant and Cell Physiology*, *52*(5), 765-774.