The Physiological Basis for Variable Cadmium Accumulation in Rice: Interaction of Environmental and Genetic Factors

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Abstract

Human exposure to elevated levels of Cd in the environment is known to lead to accumulative toxicity and rice is a key pathway of entry for communities exposed to elevated levels of Cd in Asia. Rice cultivars vary in the degree to which they accumulate Cd in their grain, and the mechanisms responsible for genotypic variation in Cd accumulation are not fully understood. What follows is a study of the physiological mechanisms involved in Cd accumulation in rice and the factors responsible for genotypic differences in Cd accumulation between rice cultivars.

This research included a study of the timing of Cd loading into grain over the rice lifecycle, which demonstrated that post-flowering Cd uptake contributed 40% of grain Cd in hydroponically grown rice plants. Grain Cd is therefore not just the product of shoot accumulation of Cd prior to flowering.

There was also an attempt to contrast naturally occurring variation in grain Cd accumulation with root expression of genes potentially involved in Cd uptake and translocation. A selection of germplasm from an international rice genebank, representing a large degree of the diversity in Cd accumulation in modern rice varieties, was used for this study. There was not a consistent pattern between expression of these candidate genes and Cd accumulation characteristics, although there were some general trends that received further study, including higher root expression of Fe/Cd transporters, including OsNRAMP1, in the high Cd accumulating *indica* varieties.

Other nutritional factors were examined alongside this work for their role in influencing Cd uptake. Silicon supply decreased the accumulation of Cd in rice, most likely through the physical blocking of transpiration pathways in shoot tissue. Growing rice without Fe led to a large increase in the accumulation of Cd, showing evidence of the link

between Cd uptake and Fe nutrition. However, Fe deficiency response, which in other plant species has been shown to have a large positive effect on Cd uptake, was found to have a smaller effect than competition with Fe^{2+} ions. In fact, Fe deficiency response only led to small increases in shoot Cd concentration, with no accompanying increase in root Cd concentration or overall plant Cd uptake.

It has previously been postulated that Fe deficiency could play a role in Cd accumulation in field conditions, and this was tested with rice plants grown under variable flooding regimes in potted paddy soil. Changes in redox conditions and Cd availability were contrasted with Fe deficiency response during the growth of the plants. Upregulation of Fe-deficiency-responsive genes was observed in some plants grown in aerobic soils, especially *indica* varieties, but this was not directly associated with an increase in Cd accumulation.

OsNRAMP1 and its effect on Cd translocation were studied further using transgenic rice lines with manipulated expression of this gene. Despite reports to the contrary, there was a lack of evidence for an important role for OsNRAMP1 in the accumulation of Cd by rice plants. Large differences in OsNRAMP1 expression did not correlate well with Cd accumulation. The main observable effect of OsNRAMP1 was increased shoot Fe content in over-expressing plants, but this only occurred with the co-upregulation of other genes during minus Fe conditions. OsNRAMP1 plays a significant role in the pathway of movement of Fe from root to shoot, but this was not demonstrated for Cd at physiologically relevant concentrations.

This research is a step towards a better understanding of the physiological and molecular regulation of Cd uptake in rice, and higher plants in general.

Extended Synopsis

Cd is a toxic heavy metal element that occurs in some agricultural soils and is known to be taken up by plants, including the edible portions of crops. Human exposure to elevated levels of Cd in the environment is known to lead to accumulative toxicity. In terms of the risk of Cd to human health, the consumption of contaminated rice as a part of subsistence diets is of particular concern because of the relatively low levels of Zn, Fe and Ca in rice grains, a nutrient imbalance that increases the absorption of Cd (Simmons *et al.* 2003). It has been seen that rice is a key pathway of exposure for communities in parts of Asia subject to elevated levels of Cd.

Rice plants vary in the degree to which they accumulate Cd in their grain, but the nature of genotypic variation in Cd accumulation and the molecular mechanisms responsible are not fully understood. Recently, significant progress has been made in rice with the discovery of a Zn membrane transporter, OsHMA3, which is critical for the root vacuolar storage of Cd. Nevertheless, this gene is not the basis of all genotypic variation in rice grain Cd accumulation, and there is also significant genotype by environmental interaction in the Cd accumulation of rice genotypes, the reasons for which have not been fully elucidated.

What follows is a study of the physiological mechanisms involved in Cd accumulation in rice and also the factors responsible for genotypic differences in Cd accumulation between rice cultivars. This research included, firstly, a study of the timing of Cd loading into the grain over the rice lifecycle, comparing accumulation before and after flowering. Post-flowering Cd was found to contribute 40% of grain Cd in hydroponically grown rice plants, showing that grain Cd is not just the product of shoot accumulation of Cd prior to flowering. Secondly, there was an attempt to contrast naturally occurring variation in Cd uptake with genes thought to be involved in plant Cd uptake. A selection of germplasm,

which represented a large degree of the diversity in Cd accumulation in modern rice varieties, was used in analysis of the genotypic expression of membrane transporters putatively involved in Cd uptake and translocation. These varieties were characterised for Cd accumulation under different conditions, including under hydroponics and flooded paddy soil, and this was compared with patterns in gene expression. A significant aim was to apply much of the published research on Cd transport to the question of why rice cultivars differ in Cd uptake. There was not a consistent pattern between expression of these candidate genes and Cd accumulation characteristics, and it is likely that the molecular bases of Cd accumulation differences are not the same in all cultivars. There were some general trends that received further study, including the higher root expression of the Fe/Cd transporter OsNRAMP1 in high Cd accumulating varieties. Particularly there were similarities between varieties of the *indica* and *japonica* subspecies of rice in terms of gene expression.

Other nutrition factors, including Fe and Si, were examined alongside this work for their role in influencing Cd uptake. Silicon supply was confirmed to decrease the accumulation of Cd in rice, and this seemed to be because of a mechanism associated with the accumulation of Si in shoot tissues. Growing rice without Fe led to a great increase in the accumulation of Cd, showing evidence of the link between Cd uptake and Fe nutrition. Iron deficiency response in other plants has been seen to have a large effect on Cd uptake, and so its role in the accumulation of Cd was examined. The regulation of Fe/Cd membrane transporters during the development of Fe deficiency was studied and compared with the influx of Cd accumulation that occurred concomitantly. Surprisingly, the effect of Fe deficiency (approximate 20% increase in shoot Cd) was found to be smaller than the effect of competition with Fe^{2+} ions in solution, but this was not seen for Fe(III) supplied in a chelated form. It has previously been postulated that Fe deficiency could play a role in Cd accumulation in field-grown plants, and so this hypothesis was tested with rice grown

under variable irrigation regimes, including continuous and intermittent flooding over the growing season. Analysis of the availability of Cd, Fe and Mn under these conditions, led to novel observations of the fluctuation in the availability of these metals with changes in soil redox conditions. Changes in redox conditions and Cd availability were contrasted with Fe deficiency response during the growth of the plants and importantly during the development of the rice grain. Upregulation of iron-deficiency responsive (IDR) genes was observed in some plants grown in aerobic soils, especially in *indica* varieties, but this was not found to be associated with a specific increase in Cd accumulation.

Finally, on the basis of results of earlier experiments and reports in the recent literature, OsNRAMP1 and its effect of Cd translocation were further studied using transgenic rice varieties with manipulated expression of this membrane transporter. Despite reports to the contrary, no evidence could be found for a role for OsNRAMP1 in the accumulation of Cd by rice plants. Over-expression produced large differences in the number of OsNRAMP1 transcripts but this did not result in a significant increase in Cd accumulation. The main observable effect of OsNRAMP1 was increased shoot Fe content relative to WT plants, but this was only found to occur with the co-upregulation of other IDR genes under minus Fe conditions. OsNRAMP1, therefore, seems to play a role in the pathway of movement of Fe from root to shoot, but not for Cd at physiologically relevant concentrations.

This research is a step towards a better understanding of the physiological and molecular regulation of Cd uptake in rice, and higher plants in general. Specifically it has provided a better understanding of the way in which the nutritional factors Fe and Si influence Cd uptake. It has also given clarification of the role of the Fe transporter OsNRAMP1, showing its limited effect on Cd accumulation in rice.

Signed declaration for thesis submission

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*__Rodda MS, Li G, Reid RJ__ (2011) The timing of grain Cd accumulation in rice plants: the relative importance of remobilisation within the plant and root Cd uptake post-flowering. Plant and Soil **347:** 105-114

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Abbreviations

^{109}Cd	Radioactive Cd isotope (atomic mass 109)
ABC	ATP-binding-cassette
ANOVA	Analysis of variance
ATPase	Enzyme that catalyses the hydrolysis of ATP to ADP
BSO	Buthionine sulfoximine, a chemical inhibitor of glutathione
CAX	Cation/H+ exchanger (gene family)
CDS	Coding sequence (of a gene)
DAS	Days after sowing
DAT	Days after (beginning of) treatment
DGT	Diffusive gradient in thin films
DTPA	Diethylene triamine pentaacetic acid
EDTA	Ethylene diamine tetraacetic acid
GSH	Glutathione
HMA	Heavy-metal associated (gene family)
HMW	High molecular weight
ICP-MS	Inductively coupled plasma - mass spectrometry
ICP-OES	Inductively coupled plasma - optical emission spectrometry
IDR	Iron-deficiency responsive
IRT	Iron-regulated transporter
L.S.D.	Least significant difference
LMW	Low molecular weight
MLs	Maximum (allowable) levels
NA	Nicotianamine
NAS	Nicotianamine synthase
NRAMP	Natural resistance-associated macrophage protein (gene family)
ORF	Open reading frame (of a gene)
OsLsi	*O. sativa*, low silicon (gene family)
OX	Over-expression
PC	Phytochelatin
PCS	Phytochelatin synthase (enzyme)
PM	Plasma membrane

QTL	Quantitative trait locus
RNAi	RNA-interference (method of gene knockdown)
RT-qPCR	Reverse transcriptase quantitative (real-time) polymerase chain reaction
spAc	Specific activity
UTR	Untranslated region at the 5’ or 3’ end of a gene
WT	Wild-type (untransformed) plant
YSL	Yellow-strip like protein (gene family)
ZIP	ZRT (Zinc-regulated transporters)-IRT-like protein (gene family)

Statement of authorship

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Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation):

Matthew S. Rodda (candidate)

Designed the experiment, carried out experimental work, coordinated analysis of samples, interpreted and analysed data, wrote manuscript and acted as corresponding author

Certification that the statement of contribution is accurate

Signed …… Date ……

Gang Li (co-author)

Contributed to planning of elemental analysis, assisted in sample preparation and analysis, and provided critical evaluation of manuscript

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed …… …… Date *22-04-12*

Robert J. Reid (co-author)

Supervised development of experimental work, advised on data interpretation, assisted in manuscript preparation and critical evaluation

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Chapter 1

1.1: Literature Review

1. *Cadmium in our environment*

Cadmium is a heavy metal trace element, present at low concentrations in rock and sediment worldwide. While some trace elements, such as zinc (Zn) and manganese (Mn), are essential nutrients required in low amounts in biological systems, others, like cadmium (Cd), lead (Pb), mercury (Hg) and the metalloid, arsenic (As), are non-essential and are toxic to most organisms at a certain level of exposure. For humans, Cd is most commonly an issue where long-term exposure occurs. The toxin can accumulate in our bodies from even low levels of contamination in drinking water, food and our living environment. In some parts of the world, uncontrolled industrial development and unchecked exploitation of mineral resources are increasing exposure to toxic trace elements and the contamination of agricultural land and agricultural produce. As food is known to be a significant pathway of uptake of Cd (World Health Organisation 1992; Nordberg 2003), this is a concern for human health.

1.1. *Occurrence of cadmium in agricultural soils*

Depending on their parent material, soils will normally have some amount of Cd, but human activity is increasing the amount of heavy metals found in soils. Phosphate fertilisers are known to be a significant source of Cd contamination in agricultural soils, because of the natural association of Cd with phosphate rock. Indeed, in Australia, impurities in phosphatic fertilisers are the principal source of Cd in soils (McLaughlin *et al.*

1996). In general, the anthropogenic sources of Cd in agricultural soils also include short and long range atmospheric deposition (predominantly from industrial sources); agricultural application of sewage, biosolids or urban composts; and contamination by the waste water or runoff from non-ferrous (especially Zn) mines and smelters (Alloway 1990; Chen *et al.* 1999; Helmke 1999; Kijne 2006).

Cd is generally found at low levels in agricultural soils, in the range of 0.01−1.0 mg kg^{-1} (McLaughlin *et al.* 1999; Wang *et al.* 2001; Zarcinas *et al.* 2004a; 2004b), or in molecular terms, in the nanomolar range (Nolan *et al.* 2003). However, locations that are affected by 'point-source' heavy metal pollution like sewage or mine waste water can have soil Cd concentrations much higher than common levels (Cui *et al.* 2004; Lee *et al.* 2004; Liu *et al.* 2005b; Yang *et al.* 2006; Zhai *et al.* 2008). Increasing water scarcity (Kijne 2006) and the high cost of chemical fertilisers means that poorer regions are more likely to apply sewage, waste water and/or biosolids to agricultural land. In addition, soils can be contaminated by deposition from smelting works from many kilometres away (Alloway 1990; Cui *et al.* 2004). In countries like China, where environmental regulations are not well enforced, pollution from heavy metals is a widespread problem (Zhang *et al.* 1998; Wang *et al.* 2001; Cheng *et al.* 2006a).

1.2. Cadmium in food crops and the threat to human safety

Cd is highly toxic to plants and animals, but at low levels most plants are able to detoxify and safely sequester Cd in their cells. For this reason, Cd is one of only a small group of elements that can be a threat to human health through accumulation in food before growth-inhibiting phytotoxicity occurs (Chaney 1980, as cited by McLaughlin and Singh 1999).

During the 20th century, Cd contamination of food was made well known by experiences in Japan, where paddy rice in the Jinzu valley was irrigated with water polluted by a Pb/Zn mine, during and after the Second World War. Rice produced in this valley at this time contained Cd concentrations around 0.7 mg kg^{-1}, which is much higher than normal (Codex Alimentarius 2007). Total Cd intake by the local people, including all sources of Cd, was found to be 10 times the maximum tolerable limit. Elderly women were particularly affected by a condition named Itai-Itai (or "ouch-ouch") disease, associated with kidney damage, skeletal weakness/deformations, which lead to eventual death in many (Alloway 1990; Nordberg 2003). Investigations following this mass poisoning revealed subsequently that approximately 9.5% of all paddy soils in Japan produced rice with unsafe levels of Cd (Asami 1984, as cited by Alloway 1990).

As well as leading to chronic renal failure and bone density problems, Cd is recognised to be carcinogenic, affecting many other tissues and organs (Nordberg 2003; Satarug *et al.* 2003; Nordberg 2004). Following medical studies that occurred in Japan, and now also in China, there is irrefutable evidence of the link between long-term consumption of Cd-contaminated rice and human disease (Ishihara *et al.* 2001; Kobayashi *et al.* 2002; Nordberg 2003; Simmons *et al.* 2009).

Cd contamination in food is an ongoing problem in many parts of the world, especially in Asia (Simmons *et al.* 2005; Bandara *et al.* 2008). But, while the toxic effects of a high level of acute cadmium poisoning are well documented, the safety of small amounts in everyday food is harder to determine (McLaughlin *et al.* 1999). It is known that Cd accumulates in the organs of the body, specifically the kidneys, where it has a half-life of at least decades (Alloway 1990).

The Codex Alimentarius is a joint FAO-WHO agency (Food & Agriculture Organisation and World Health Organisation, respectively) that sets international trade

standards for additives, residues and contaminants in food. Based on the accepted safety standard for Cd of 1 μg kg^{-1} (body weight) per day, the maximum levels (MLs) for Cd in wheat and rice have been set at 0.2 and 0.4 mg kg^{-1}, respectively (Codex Alimentarius 2007). It has been argued by many that these levels have only a small safety margin, given the uncertainty, and are not adequate protection for all, especially high rice consumers (Satarug *et al.* 2003). For instance, Ishihara *et al.* (2001) found a higher incidence of mortality in Japanese villages that grew and ate rice with Cd levels above 0.3 mg kg^{-1}. Many people in Asia eat an average of 320-380 g rice per day (Li *et al.* 2003, as cited by Cheng *et al.* 2006a; FAO 2006, as cited by Bandara *et al.* 2008), and so these MLs will not keep all consumers below the safety limit. The government of China has approved a standard of 0.2 mg kg^{-1} Cd for rice (Li *et al.* 2005; Cheng *et al.* 2006a), which is more fitting for its high rice consuming population. Unfortunately, in many parts of China, rice producers have difficulty achieving these low Cd levels in rice, especially in the southern regions where a large amount of mining activity occurs alongside rice growing areas (Williams *et al.* 2009; Zhang *et al.* 2009b). The percentage of Chinese rice that does not meet the safety standards for heavy metal content has been estimated between 10-60%, (Zhang *et al.* 2009b).

The issue of human toxicity is complicated because other factors, such as human nutrition, will affect the absorption of cadmium in the digestive tract. It has been found in medical studies that mammalian toxicity of Cd is increased by micronutrient malnutrition. Specifically, Fe, Ca and Zn deficiencies are known to increase Cd absorption (Chaney *et al.* 2004). This is very relevant for populations that have rice as the staple food within a subsistence diet, because rice is a low accumulator of micronutrients like Zn and Fe (Simmons *et al.* 2003). For example, in Bangladesh, paddy rice makes up 73% of most of the population's calorific intake (del Ninno and Dorosh 2001). In polluted locations, if rice is not sufficiently supplemented with other foods, the Cd present in the grain will have an amplified impact.

In comparison with other food crops, rice is not the highest accumulator of Cd. Foods such as leafy vegetables and root vegetables will grow with a higher Cd content at a given soil concentration (Brown *et al.* 1996). The issue with rice is that it makes up such a large proportion of the diet of a multitude around the globe.

2. Physiology of uptake and accumulation of cadmium in plants

Of concern for human toxicity from food, the accumulation of Cd in the edible plant parts is of importance; for the most part, this will be in the shoots. Although some heavy metal contamination, such as Pb, has been found to primarily be from deposition of dust (Williams *et al.* 2009), the main entry of Cd into plants is by root uptake.

Although plant uptake of solutes from the rhizosphere is somewhat selective, Cd is able to be taken up by plant roots. Subsequently, translocation of Cd ions to the shoots occurs through both radial symplastic passage in the root and active loading into the xylem (Colangelo and Guerinot 2006). For plants where the edible portion is the seed, there is movement via the phloem into the developing seed. In this section, the mechanisms involved in, (1) the uptake of Cd from soil by roots, (2) the fate of Cd inside the root symplasm, including detoxification, sequestration and translocation, and (3) remobilisation by the phloem and movement to the grain, will be discussed from the perspective of the current knowledge.

2.1. Uptake of Cd from the soil

With the exception of the newly emerged zone around the root tip, plant uptake of nutrients and other solutes from soil solution is selective. The structure of plant roots

ensures that all solutes which enter the plant other than at the root tip must, at some point, cross the selectively-permeable plasma membrane of root cells. Extracellular passage of solutes can occur via the cell wall space (the apoplasm) but only until the Casparian Strip, which blocks the apoplastic pathway into the root stele and the vascular pathways.

Non-essential trace metals such as Cd are understood to cross the plasma membrane via low-specificity transporters of other cations, such as Cu^{2+}, Zn^{2+}, and Fe^{2+} (Clemens 2006). This is borne out experimentally by demonstrated competitive uptake of Cd with other cations in rice, wheat, barley and *Arabidopsis* (Hart *et al.* 2002; Chen *et al.* 2007a; Cui *et al.* 2008; Van de Mortel *et al.* 2008). For this reason, the uptake of soil Cd by plants is governed by the operation of the relevant membrane transporters and also the availability of both Cd and competitive cations. The regulation of plant membrane transporters is affected by plant development and internal nutrient status, most notably, nutrient deficiency. The available concentration of a cation is determined by both the bulk concentration and also a complex combination of factors including soil pH, redox, and chelating compounds.

A factor which affects Cd availability in soil is pH. Under lower soil pH, Cd is more labile and hence, phyto-available, most likely because of decreased sorption to soil particles (Helmke 1999). The application of lime is a management option to reduce plant accumulation of Cd from soils with Cd contamination (Maier *et al.* 2003; Lee *et al.* 2004).

In the rice system, it is well known that Cd uptake is affected by redox conditions, with availability being considerably higher in ‘upland’ or ‘drained’ conditions compared to flooded paddy rice (Arao and Ishikawa 2006). In fact, even within a single growing season, when rice paddies are drained of water close to grain filling to increase yield and improve harvest operations, it is known that more soil Cd becomes available and is taken up by the

plant (Iimura 1981 and Chaney et al. 1996, as cited by Simmons *et al.* 2008). It has been argued that this is related to pH, which is most often higher in the anaerobic conditions of flooded paddy soil (Masui *et al.* 1971, as cited by Arao and Ishikawa 2006). More likely, however, it is the result of the precipitation of Cd under low redox conditions, through the formation of CdS (de Livera *et al.* 2011b) or $CdCO_3$ (Khaokaew *et al.* 2011).

Another commonly encountered environmental variable is salinity. It is known that increases in soil salinity cause the amount of available soil Cd to increase, because of the complexation of Cd by chloride ions, which leads to a greater proportion in soil solution (McLaughlin *et al.* 1994; McLaughlin *et al.* 1997b).

Cd is likely to move through a range of metal transporters whose main substrates are essential cations (Clemens 2006), but when considering root uptake from the apoplast, there are only a limited number of candidate membrane transporters that have been proposed as responsible for plant uptake of Cd from soil. In dicots and rice, an Fe transporter called IRT1 has been identified as mediating Cd transport across the plasma membrane (Cohen *et al.* 1998; Korshunova *et al.* 1999; Nakanishi *et al.* 2006). The effect of Fe deficiency on the uptake of Cd is often discussed and used as evidence for the role of IRT1 in Cd uptake in dicot species. *Arabidopsis thaliana, Thlaspi caerulescens* and pea (*Pisum sativum*) show a strong increase in the uptake of Cd and other divalent cations because of Fe deficiency (Cohen *et al.* 1998; Korshunova *et al.* 1999; Connolly *et al.* 2002; Lombi *et al.* 2002; Vert *et al.* 2002).

A number of studies have shown that application of Si can reduce rice plant uptake of Cd and also protect from toxicity symptoms (Shi *et al.* 2005; Zhao and Masaihiko 2007; Nwugo and Huerta 2008; Liu *et al.* 2009). Si is ubiquitous but not an essential element for plants, although it has been demonstrated to have a beneficial effect on the growth of some plants, especially rice. In rice, it is known to be actively accumulated at tissue levels

as high as other macronutrients (Ma and Yamaji 2006; Ma *et al.* 2007; Yamaji and Ma 2007). Si supply has also been found to decrease accumulation of Na and Ca (Ma and Takahashi 1993; Yeo *et al.* 1999). The mechanism by which Si reduces Cd uptake has not been confirmed, but there is good evidence that in rice, Si, as polymerised silica sol and/or gel, accumulates in the apoplasm of roots and shoots; reducing both the rate of transpiration (Ma and Takahashi 1993; Agarie *et al.* 1998; Gao *et al.* 2005; Ueno and Agarie 2005; Gao *et al.* 2006) and apoplastic bypass-flow around the Casparian strip (Yeo *et al.* 1999; Shi *et al.* 2005; Gong *et al.* 2006). This mechanism is thought to relate not just to a physical blocking of the transport pathways, but primarily to the strong and unique affinity for cations like Cd that the deposited extracellular silica matrix has (Iler 1979; Wang *et al.* 2000).

2.2. The fate of Cd inside the plant

2.2.1. Plant strategies to deal with heavy metals in the cytosol - detoxification

Plants have evolved mechanisms to deal with non-essential trace elements to prevent them interfering with cellular functioning. There is a class of thiol compounds, including glutathione and phytochelatins, which are produced in plants to bind to toxic solutes that are inadvertently taken up, such as of Cd and As (Cobbett 2000). These 'non-protein thiols' (i.e. peptides containing '-SH' groups and not the product of translation) are complexing agents and are an important component of the detoxification pathways of plants, in both metal-hyperaccumulator species and those with a normal (or basal) level of heavy metal tolerance (Clemens 2006).

Glutathione (GSH; γ-Glu-Cys-Gly) is an important molecule in cells with a number of different roles; therefore plant cells will have an available pool of GSH in the absence of

heavy metals. Phytochelatins (PCs) are more specialised, synthesised from multiple GSH groups by the action of PC-synthase when needed (Howden *et al.* 1995b). PCs have also been described as 'cadmium-binding peptides,' and are analogous to another class of metal-binding ligands, metallothioneins, that are more common in animals (Grill *et al.* 1985; Grill *et al.* 1987). Different length PCs are found in plants and these are classified according to the number of GSH subunits they contain, according to the formula (γ-Glu-Cys)$_n$-Gly, where 'n' generally = 2-5 (Cobbett 2000).

It has been shown experimentally that Cd uptake induces GSH and PC synthesis in plants (Ernst *et al.* 2008). Semane *et al.* (2007) demonstrated the ability of Cd to induce both GSH and PC synthase genes in the model plant *A. thaliana*. Sulphate assimilation is also upregulated in plants under these conditions in response to the need for increased GSH production (Herbette *et al.* 2006; Nocito *et al.* 2006). Gupta *et al.* (2004) found that in chickpea, Cd and As were the only metals to stimulate PC synthase genes, and in addition, although some other metals induced GSH production, Cd exposure caused the greatest increase in GSH content. It has repeatedly been found in plant studies that Cd is the most potent activator of PC synthesis (Clemens 2006).

Analysis of the plant tissues following Cd uptake shows that GSH and PCs do not conjugate all cellular Cd (Wojcik and Tukendorf 1999; Marentes and Rauser 2007). Organic ligands, like citrate, are alternative binding agents commonly suggested (Wagner 1993; Lugon-Moulin *et al.* 2004; Marentes and Rauser 2007). Nevertheless, it is clear that thiols play an important role, as plants deficient in thiol biosynthesis are highly sensitive to Cd (Howden *et al.* 1995a; Howden *et al.* 1995b; Cobbett *et al.* 1998).

2.2.2. Discrimination of Cd movement in and from the root

In order for Cd taken up from the soil to reach the shoot it must travel through the root symplasm and then be transported across the plasma membrane (PM) into the xylem. The fate of Cd which enters the root cell is determined by the operation of both chelating agents and membrane transporters for which Cd is a substrate. The chelating agents can include the detoxifying thiols already mentioned, but can also include other ligands which bind aspecifically with Cd forming complexes of relatively low thermodynamic stability (Nocito *et al.* 2011). Whereas the primary destination of Cd-thiol conjugates seems to be the vacuole or efflux, loosely bound or free Cd in the cytosol can be transported away by cation transporters at either the tonoplast or the plasma membrane of cells adjacent to the xylem (xylem loading).

2.2.3. Efflux or vacuolar compartmentalisation

Plants not only take up Cd, but are also capable of releasing Cd back into the soil via cation-efflux transporters. Lindberg *et al.* (2007) and Kim *et al.* (2008) both found evidence for transporters capable of Cd efflux in wheat that were upregulated by the presence of Cd. Burzynski *et al.* (2005) elucidated that Cd^{2+} efflux in cucumber roots occurred via a metal/H^+ antiporter. Thiol-conjugated forms of Cd can also be transported. Li *et al.* (2002) and Kim *et al.* (2007) demonstrated the role of ABC-type-transporters in the efflux of Cd across the plasma membrane. Given that ABC transporters generally carry large molecules (Martinoia *et al.* 2002), the substrates of these ABC-transporters are likely to be Cd-thiol conjugates.

If it is not extruded, Cd bound by thiol compounds in the cytosol is not stored there. In order to maintain conditions in the cytosol, Cd must be sequestered into the vacuole (Vögeli-Lange and Wagner 1990; Van Belleghem *et al.* 2007). At moderate to high external concentrations of Cd, there is evidence that Cd-thiol conjugates can be transported whole into the vacuole. The mechanism of this has not been fully elucidated, but it most likely by ATP-Binding Cassette (ABC) type transporters on the tonoplast (Martinoia *et al.* 2002). ABC-type transporters have stress-related functions in cells (Martinoia *et al.* 2002; Frelet-Barrand *et al.* 2008; Gaillard *et al.* 2008), and in yeast, these transporters have been shown to move both GSH-Cd (Li *et al.* 1996; 1997b; Tommasini *et al.* 1998; Song *et al.* 2003) and PC-Cd complexes (Ortiz *et al.* 1995). For plants, studies have demonstrated that a Mg^{2+} and ATP-dependent transporter is responsible for this compartmentation in *A. thaliana* (Li *et al.* 1995; Salt and Rauser 1995). In line with this, it has been shown that genes for a number of different of ABC-type transporter proteins are induced by the presence of Cd in plants (Bovet *et al.* 2003; 2005; Fusco *et al.* 2005; Kim *et al.* 2006; Gaillard *et al.* 2008), including rice (Aina *et al.* 2007).

Nevertheless, although conjugation with thiols can occur in the cytosol and Cd-thiol compounds then accumulate inside the vacuole, there is actually more evidence for the importance of transport of Cd^{2+} ions across the tonoplast than there is for transport of whole Cd-thiol complexes. This is especially true at lower concentrations of Cd. A number of membrane proteins have been characterised in plants that are capable of conveying Cd^{2+} across the tonoplast (Salt and Wagner 1993; Koren'kov *et al.* 2007a; 2007b; Berezin *et al.* 2008). These include cation/H^+ antiporters of the CAX family, which have been found to influence tolerance and accumulation of the heavy metals Cd, Zn and Mn in dicots (Hirschi *et al.* 2000; Cheng *et al.* 2002; Pittman *et al.* 2004; Koren'kov *et al.* 2007a; Koren'kov *et al.* 2007b). In rice, the Zn transporter OsHMA3, which is characterised by the presence of

heavy-metal binding domains, has been demonstrated to move Cd into the vacuole (Ueno *et al.* 2010; Miyadate *et al.* 2011).

CAX transporters in rice have been found to have a broad range of substrates and functions, and the gene of this family in rice with the highest tissue expression in all organs, OsCAX1a, is a confirmed vacuolar transporter of Ca and Mn (Kamiya *et al.* 2005; Kamiya *et al.* 2006). In barley, a close protein homologue of OsCAX1a was found to be upregulated by 20 µM Cd treatment. High Cd treatment (100-200 µM), in barley and rice, did not cause CAX1a to be upregulated in the same way (Kamiya *et al.* 2006; Schneider *et al.* 2009).

Once inside the vacuole, complexation of Cd^{2+} could occur because other proteins are capable of transporting glutathione across the tonoplast (Bogs *et al.* 2003; Zhang *et al.* 2004b). Indeed, it has been shown that inside the vacuole, low-molecular-weight (LMW) PC-Cd complexes eventually combine with sulphide ions and possibly more Cd^{2+} ions to form high-molecular-weight (HMW) PC-Cd complexes (Rauser 1995). Rauser (2003) showed in maize that the proportions of PC complexes change from the LMW type to the HMW type in the days following the beginning of Cd exposure. The ratio of HMW to LMW Cd-binding compounds also depends on the concentration of Cd supplied to plants. Nocito *et al.* (2011) demonstrated that after rice was supplied with 0.1 µM Cd for two weeks, there were equal proportions of LMW and HMW compounds, whereas at 1 µM Cd, HMW compounds clearly dominated. It therefore appears that at low concentrations of Cd, PCs are less important than GSH in the binding and sequestration of Cd ions (Wagner 1993; Vögeli-Lange and Wagner 1996).

The production of HMW PCs inside root vacuoles is an important part of the long-term Cd tolerance strategy in plants (Howden *et al.* 1995b). This pathway is highly dependent on S nutrition, and S deficiency will limit Cd accumulation in plants, but will quickly lead to Cd toxicity when exposure is prolonged (Astolfi *et al.* 2012). Conversely, high

S supply to rice has been shown to increase root accumulation of Cd and decrease transfer to the grain (Fan *et al.* 2010). An unknown part of the sequestration pathway is the accessibility of Cd which is stored in the vacuole. Clemens (2006) commented that we do not actually know a lot about the long-term metabolism of PC complexes after their initial compartmentalisation in vacuoles. The questions that remain unanswered include: Are the HMW PC-Cd complexes the final state? And, is it possible for Cd sequestered in the vacuole to be remobilised? At many other points in the plant, Cd moves via pathways designed for essential nutrients so it is conceivable that Cd could be released from vacuoles via transporters utilised for the maintenance of Fe or Zn homeostasis.

2.2.4. Translocation to the shoot: xylem loading and transport

In arabidopsis, a P_{1B}-ATPase, or heavy metal-transporting ATPase (AtHMA4) has been shown to transport Cd^{2+} across the PM, primarily in the root tissue adjacent to the vascular vessels (Mills *et al.* 2003; Verret *et al.* 2004; Mills *et al.* 2005). This Zn transporter, and its homologues, are good candidates for the site of competition of Cd and Zn ions (Nocito *et al.* 2011).

The speciation of Cd during its translocation from the roots to shoots in plants, and the membrane transporters involved, have not been fully characterised (Nakamura *et al.* 2008). Given the acidic nature of xylem sap, there are a number of compounds that could possibly form complexes with Cd in the xylem, including thiol ligands, organic acids and amino acids (Welch and Norvell 1999). Salt *et al.* (1995) reported for Indian mustard, that Cd moving in the xylem was coordinated predominantly with oxygen or nitrogen ligands, such as hydrated cations or small organic acids.

Although the picture is not completely clear, it seems that thiol complexes are also involved in the long distance transport of Cd in plants. PC-Cd (and GSH-Cd) complexes have been found to travel in both the xylem (Gong *et al.* 2003) and the phloem (Raab *et al.* 2005; Chen *et al.* 2006; Mendoza-Cózatl *et al.* 2008). Li *et al.* (2006) induced leaf tissue-specific expression of EC-synthetase (the enzyme of the precursor to GSH) and found that it resulted in increased phloem transport of As/Hg. Given that they are formed in the cytosol, their presence in the vascular tissues most likely implies the existence of a Cd-conjugate transporter at the PM.

Nocito *et al.* (2011) demonstrated in rice that the Cd concentration in the xylem reached a point of saturation at 0.1 µM Cd^{2+} (external concentration). They concluded that root retention, at higher Cd concentrations, is influenced by the plant's ability to move Cd in the xylem. Following saturation of the xylem transport pathway, there would then be the gradual saturation of cellular sites with potential for Cd adsorption, in turn stimulating PC biosynthesis and Cd sequestration in Cd-binding complexes (Nocito *et al.* 2011).

2.3. Remobilisation of Cd and movement to the seed

In order for Cd to reach the seed of any plant, it must travel through the vascular system. Higher plants have two main vascular transport systems: xylem, which transports water and solutes from roots to shoots; and phloem, for transporting the products of photosynthesis from leaves to carbohydrate sinks, such as roots and seeds. Cd is transportable within both the xylem and phloem of plants (Reid *et al.* 2003; Riesen and Feller 2005; Mendoza-Cózatl *et al.* 2008; Uraguchi *et al.* 2009).

As developing grains are carbohydrate sinks and non-transpiring organs, the phloem is the main vascular pathway to them. There are numerous types of organic ligands

which could complex Cd in the phloem to enable movement (Welch and Norvell 1999). In wheat, it has been shown that the amount of Cd reaching the developing grain is significantly reduced when the phloem is interrupted (Riesen and Feller 2005). The phloem is also implicated in the movement of Zn to wheat grains (Pearson & Rengel 1995).

Tanaka *et al.* (2007) estimated that 91-100% of the rice grain Cd entered the developing caryopsis via the phloem. This is compared to around 11% for the Cd content of the glumes (grain husk), which was predominantly transported directly from the root via the xylem. They calculated these estimates using a mathematical model based on the measured Cd movement to parts of the panicle 7-8 days after anthesis; however, the findings do not mean that all grain Cd is remobilised from the leaves. During seed maturation, redistribution of nutrients does occur within the plant and Cd can move from leaf/stem to grain (Harris and Taylor 2001; Chan and Hale 2004; Chen *et al.* 2007b), however, nutrient exchange from the xylem to the phloem within the stem is a recognised phenomenon (Grusak *et al.* 1999). There is good evidence from other cereals that xylem-to-phloem transfer in the rachis or head is a major mechanism in the transfer of micronutrients like Zn and Mn to the grain (Wolswinkel 1999). In experiments with detached, cultured wheat ears, Pearson *et al.* (1995, 1996) found that the xylem was crucial in transporting Zn and Mn to the spikelet despite the phloem being the point of entry into the caryopsis. They saw that when transpiration in the ear was inhibited by a relative humidity of 100%, Zn and Mn transport into the grain was almost completely blocked. So, Cd reaching the grain by the phloem is not necessarily remobilised Cd but could have come from uptake by the roots.

Consistent with this understanding, Sankaran & Ebbs (2008) found in Indian mustard that leaf-stored Cd was relatively immobile and that the majority of seed Cd was translocated from the roots during seed-set. They found little evidence of sequestered-Cd

being remobilised from leaves and moving to the grain. Florijn & Van Beusichem (1993a) also found that redistribution of Cd by the phloem was of minor importance in the Cd distribution process in maize. In contrast to these results, however, Chan & Hale (2004) found using stable ^{106}Cd isotopes in durum wheat that there was no direct (<24 h) root to grain Cd transport during the ripening phase. As the experimental methods varied, it is difficult to ascertain whether these results contradict or accurately show the mechanisms of different species.

In rice, the stem nodes and the discriminatory region at the base of the stem have been shown to be important in the distribution of Cd (Fujimaki *et al.* 2010; Ishikawa *et al.* 2011), and these are likely points at which transfer to the phloem could occur (Yamaguchi *et al.* 2012).

Although the phloem is the primary pathway of Cd movement to grain (Hart *et al.* 1998), there are two main possible pathways of root to grain movement of Cd: (1) Cd is taken up by actively transpiring parts and then transferred to the phloem and then the grain; or (2) Cd could be taken up directly through the xylem to the developing grain. To some degree, it is likely that both mechanisms occur in rice. Unlike wheat and barley, the pericarp of the immature rice grains is green tissue (Oparka and Gates 1984) and there is xylem continuity into the caryopsis (Zee 1972; Krishnan and Dayanandan 2003). Therefore, direct xylem uptake into the grain is possible (Stomph *et al.* 2009). It seems that this is limited to when the developing seed is exposed to the air to allow transpiration (Zee 1972), or when transpiration of glumes is slowed by high humidity (>85%; Oparka and Gates 1984). Thus, early in seed development when the glumes are not tight against the developing grain, transpiration of the green seed tissue could occur.

3. Genotypic variation in plant cadmium accumulation

3.1. Plant tolerance to heavy metals

The characteristics of metal tolerance and accumulation can vary both between plant species and within them. Different types of tolerances to heavy metals have been defined for plants. There are three main categories, although different labels are used for them:

1. Metal hypersensitive or hypotolerant
2. Basal metal tolerance (sometimes, non-tolerant)
3. Metal tolerant, hypertolerant or resistant

Tolerance is not necessarily related to the level of accumulation that will be observed in a plant, particularly in the edible portion. Nevertheless, plants that accumulate high amounts of an element, the 'hyperaccumulators,' are by definition hypertolerant. In the context of heavy metal contamination in food crops, it is basal metal tolerance that is most relevant because in the majority of cases the level of soil contamination is not high enough to cause toxicity. In order to reduce metal pollutants in the grain, the interest is not in breeding on the basis of tolerance, per se, but rather is primarily focussed on the characteristics of metal translocation and accumulation that occur with concentrations of Cd that are relevant to field conditions; i.e. at levels of basal tolerance (Clemens 2006; Ernst *et al.* 2008).

Although heavy metal tolerance and low accumulation are not normally tightly linked, there are exceptions to this rule. A physiological example of this is the gene OsLCD. A reverse genetic screen of T-DNA mutants identified a rice line which had increased tolerance to Cd supply in nutrient solution and also reduced grain Cd accumulation when grown in the field (Shimo *et al.* 2011). This gene has no homology to known plant proteins and represents a novel type of transporter.

3.2. Cd accumulation in food crops

There is great variability in the degree to which crop species and cultivars accumulate essential and non-essential trace elements like Cd (Grant *et al.* 2008). This section will summarise some recent studies into genotypic variation of Cd accumulation in agricultural crops, beginning with cereals and other crops that shed light on the mechanisms determining the distribution of Cd in plants. The next subsection will look specifically at rice.

There are many studies which have shown that cereal crops vary in the way they distribute Cd around the plant. Cieslinski *et al.* (1996) saw genotypic variation in grain-Cd accumulation between durum wheat cultivars to the order of 20-fold. Clarke *et al.* (2002) also demonstrated genotypic trends in a range of durum cultivars, which were consistent regardless of environmental location; there was variation in the degree of difference between high and low accumulators, depending on the Cd availability at the site, but cultivar differences were strong, as high as 15-fold. Chen *et al.* (2007a) trialed 600 barley genotypes at one site and found grain Cd content ranged between 0 to 1.21 mg/kg DW, with a mean of 0.16. Eurola *et al.* (2003) found about 10-fold variation in Cd content in six varieties of oats (*Avena sativa*).

A lot of research has been done on wheat trying to elucidate the processes involved in Cd accumulation in the grain. Genetic variation in this crop shows up in different ways. Wolnik *et al.* (1983, as cited by Li *et al.* 1997a) found that durum wheat grains consistently had more Cd than bread wheat on soil with low levels of Cd. Stolt *et al.* (2003) compared spring bread wheat (*Triticum aestivum*) and durum wheat (*T. turgidum* var. *durum*), and found that the main difference in grain Cd accumulation between the two

species came about because of differences in overall uptake of Cd by the plant. However, when they compared durum wheat cultivars, they found evidence for variable allocation of Cd within the plant; with similar plant Cd uptake but different accumulation in the grain.

Hart *et al.* (1998) investigated why durum wheat accumulated more Cd in the grain than bread wheat. Unlike Stolt *et al.* (2003), they found that the root uptake of Cd was not significantly different to bread wheat and also that bread wheat, the lower accumulator, actually had higher root to shoot xylem translocation of Cd than durum. It was therefore concluded that the higher grain accumulation was due to greater shoot to root, downwards, phloem transfer, with associated compartmentalisation in root cells. In the same way, the work of Greger & Löfstedt (2004) did not identify different root uptake by the different wheat species, but clearly showed variable plant distribution of Cd between different cultivars. It also seemed likely from their results that more than one mechanism was responsible for the differences.

Harris & Taylor (2004) examined Cd distribution in near isogenic lines of durum and concluded that there was good evidence for a physiological mechanism in the root controlling grain Cd content. On the same basis, Florijn & Van Beusichem (1993b) identified different maize cultivars as either shoot-excluders or non-shoot-excluders. Hart *et al.* (2006) and Stolt *et al.* (2006) also concluded that the same applied to wheat.

A large number of studies have looked at the uptake of Cd into crop plants. In addition to those of wheat and maize, notable studies have been conducted with legumes (Bell *et al.* 1997; McLaughlin *et al.* 2000; Gupta *et al.* 2002; Pajuelo *et al.* 2007; Sugiyama *et al.* 2007), oilseeds (Li *et al.* 1997a; Brennan and Bolland 2005), potato (Dunbar *et al.* 2003) and tobacco (Bovet *et al.* 2006). Different plant species take up and/or accumulate higher levels of Cd in the edible portions of the plant than in other tissues (Grant *et al.* 2008). For example, Brennan & Bolland (2005) found that canola took up more cadmium from the soil

than spring wheat. In addition to this, the extent of intra-specific variation in Cd accumulation varied between different crops (Welch and Norvell 1999). Li *et al.* (1997a) found a 3-fold variation between durum wheat cultivars, a 4-fold range between sunflower genotypes, and a 10-fold range between flax (linseed) lines. Evidence for genetic variability in the degree to which cultivars accumulate Cd in the shoots and seed is seen in all crops tested.

The majority of genetic variability in Cd accumulation is the result of plant internal distribution of Cd rather than uptake. For example, Gupta *et al.* (2002) found faster transport of Cd to the shoots in a high Cd-accumulating chickpea cultivar than the low-accumulator. Often the genotypic differences observed involve decreased root-shoot translocation of Cd because of retention in the roots (McLaughlin *et al.* 2000; Dunbar *et al.* 2003; Reid *et al.* 2003). Sugiyama *et al.* (2007) demonstrated this clearly in soybean using grafting experiments; there was a strong mechanism controlling Cd accumulation in the roots of that species. Cd accumulation characteristics where shown to be transferrable by grafting the shoots of high-Cd accumulators onto the roots of low-Cd genotypes or vice versa.

3.3. Studies of variation in Cd accumulation in rice

As there is good evidence of large cultivar differences in Cd accumulation, several research groups have aimed at selecting/breeding low Cd-accumulating rice varieties. Zeng *et al.* (2008) screened 138 *japonica* rice varieties under field conditions and found 9-14 fold variation in Cd content between maximum and minimum grain accumulators. Liu *et al.* (2007b) found genetic variation in the order of 5-8 times between the lowest and highest Cd accumulators in their study. Studies of the causes of differential accumulation of Cd in

rice genotypes have produced a range of conclusions (Grant *et al.* 2008). Some researchers found that the main mechanism driving rice grain accumulation was shoot to grain translocation (Arao and Ishikawa 2006; Cheng *et al.* 2006b; Liu *et al.* 2007b); others concluded that it was more closely related to total plant uptake into above-ground tissues, or root to shoot translocation (Kashiwagi *et al.* 2009), because of strong correlations between shoot Cd content and seed Cd content (Cheng *et al.* 2004; He *et al.* 2006). In reality, there is genetic variability in both the translocation from root to shoots, and also, transport from shoots to grain (Liu *et al.* 2005a; 2007b).

The mechanism responsible for differential grain accumulation in rice has been shown to vary depending on the cultivars tested and conditions used. In most studies, there were only a limited number of cultivars tested, often less than a dozen. In addition, there are results that suggest probable genotype and environment interaction (GxE) in rice varieties. In a study of 11 rice varieties, Ishikawa *et al.* (2005b) found varying genotypic differences of grain-Cd accumulation in four different soils. Zeng *et al.* (2008) also found significant GxE interaction for rice in three different field soils. They observed that the relationship between shoot and grain Cd was not consistent, with grain-Cd more highly variable than that of the leaves/stem.

Much research effort has been placed recently on discovering the genetic basis for differential Cd accumulation in rice cultivars. Numerous Quantitative Trait Locus (QTL) studies have been published using populations from crosses of high and low Cd accumulators (Ishikawa *et al.* 2005b; Xue *et al.* 2008; Kashiwagi *et al.* 2009; Ueno *et al.* 2009a; Ueno *et al.* 2009b; Ishikawa *et al.* 2010; Tezuka *et al.* 2010). From this research, the gene that has so far been discovered to have the strongest effect on Cd accumulation in rice plants is OsHMA3 (Ueno *et al.* 2010; Miyadate *et al.* 2011; Ueno *et al.* 2011). This P_{1B}-type ATPase transporter is located on the tonoplast and mediates vacuolar storage of Cd.

Plants that have a naturally occurring single-base-pair mutation have a non-functional version of the protein. This has been found in some *indica* rice cultivars, which have higher shoot accumulation of Cd as a result of reduced root storage.

The QTL that included the OsHMA3 gene also included the OsNRAMP1 protein (Ueno *et al.* 2009b). This Fe and Cd transporter (Takahashi *et al.* 2011) is another good candidate for controlling Cd accumulation in rice.

4. Factors responsible for genotypic differences in plant distribution of cadmium

There is evidence in the literature for some plant species having a greater tendency to take up Cd from the soil than others, and also clear variation in the way different genotypes distribute Cd around the plants. Given the range of mechanisms cited as controlling Cd translocation, it seems likely that more than one physiological mechanism is important in determining grain Cd (Van der Vliet *et al.* 2007). In this section we will explore some of the findings that have improved our knowledge of the precise factors determining genotypic differences in Cd partitioning. These will be presented according to the four main steps at which differential Cd movement to the grain could occur: root uptake; vacuolar sequestration; root to shoot translocation by the xylem (loading and transport); and transport via the phloem.

4.1. Root uptake

Plants are able to exert an influence on the rhizosphere, which affects the soil mobility of and hence plant availability of Cd. Liu *et al.* (2007c) found that high Cd-accumulating rice cultivars had higher exudation of low molecular weight organic acids (LMWOAs) than

lower-accumulating varieties. Supporting this, in durum wheat, Cieslinski *et al.* (1998) found that grain Cd accumulation was proportional to root production of LMWOAs. The release of these exudates is unlikely to be due to the presence of Cd, but rather a nutritional response caused by lack of an essential nutrient. Other non-essential elements can also be mobilised by organic acids. Zhang *et al.* (2005) observed the release of bound-up arsenate/arsenite in soils because of LMWOAs; and Wang *et al.* (2007) found that organic acids increased uptake of Pb in wheat roots. In contrast to these findings, phytosiderophores, which plants exude for Fe uptake, have not been shown to increase Cd uptake and accumulation (Shenker *et al.* 2001; Hill *et al.* 2002; Meda *et al.* 2007).

Cd, like other cations, has a strong tendency to bind to anions like phosphate groups on the cell walls of plants (Van de Mortel *et al.* 2008). This binding in the apoplastic space is often cited as a point at which genotypic variation in Cd movement could arise (Rauser 1999; Cakmak *et al.* 2000). The results of Rauser (1987) give an example of the degree to which binding in the root apoplast can vary. He found that in maize, 4-7% of tissue Cd was associated with the cell walls, compared with 11-15% in *Agrostis gigantea*. The relative importance that is attributed to Cd bound in the extra-cellular space will depend upon experimental methodology, particularly the technique used to desorb plant roots prior to analysis of the tissue (Hart *et al.* 2006).

Some morphological and physiological studies have uncovered factors associated with the ability of different plant genotypes to take up Cd from the soil. Berkelaar & Hale (2000) studied durum wheat cultivars that varied in Cd accumulation and found that one low grain-Cd accumulating cultivar, Arcola, took up more Cd from the soil than the high accumulating type. It seems that this was associated with the significantly greater root surface area and number of root tips of Arcola, which led it to have greater concentrations of Cd per g of root. Nevertheless, this characteristic is not true of all high-root accumulating

cultivars; in maize, Florijn *et al.* (1993b) could find no relationship between morphological characteristics and Cd distribution.

As rice plants are normally grown in flooded paddy soil, the distribution of roots in the soil could also affect the plants access to Cd. Close to the soil surface Cd availability is higher because the soil conditions are less reduced (Roberts *et al.* 2011), therefore cultivars that have a tendency to grow a matt of roots at the soil surface may accumulate more than the plants that send their roots deeper. In rice plants, the radial oxygen loss by roots in low-redox soil is also an important determinant of the geochemical composition of the rhizosphere (Wang *et al.* 2011). It is theoretically possible that genotypic differences in release of oxygen would lead to different rates of root Cd uptake.

4.2. Discrimination of Cd movement at the root

Given the strong evidence in wheat and soybean for a root mechanism controlling Cd distribution, it seems likely that this is a factor affecting grain-Cd accumulation in rice. Other physiological mechanisms notwithstanding, it may be that the root to shoot translocation is what separates some of the cultivars of rice in terms of partitioning characteristics. Clemens *et al.* (2002) summarised the four phases in the movement of Cd in roots that could determine translocation to the aerial parts of the plant. These are: (1) the activity of metal-sequestering pathways in root cells (i.e. PC synthesis and/or vacuolar transport); (2) the degree of accessibility and mobilisation of sequestered metal; (3) the efficiency of radial symplastic passage through the root and across the endodermis; and (4) the xylem loading activity (i.e. the efflux activity from xylem parenchyma cells into the xylem). Of these options, those most commonly supported in the literature are the first and

the fourth. This sub-section will discuss the findings relevant for the first two of these options, and the third and fourth will be considered under the following heading.

The sequestration of Cd involves the detoxification mechanism by which cellular Cd is conjugated and moved into vacuoles to prevent toxicity. Sequestered Cd is then unavailable for transport to the shoot while it remains in the vacuole, and so, this pathway could potentially reduce seed Cd. Harris & Taylor (2004) favoured variable vacuolar sequestration as the key factor driving root to shoot transport in durum wheat, because the trait has been demonstrated to be the result of a single gene (Clarke *et al.* 1997), as it is in oats (Tanhuanpää *et al.* 2007). Their argument was that loading and/or chelation in the vascular system would most likely involve more than one gene.

Given the role of thiols in complexation and tonoplast transport, it is possible that variable sequestration of Cd occurs because of a genotype's ability to produce the enzymes required in the glutathione (GSH) and phytochelatin (PC) biosynthetic pathway. In support of this, there is considerable variation between plant species in their tendency to upregulate synthesis of glutathione and phytochelatins in response to Cd (Wojcik and Tukendorf 1999; Souza and Rauser 2003; Marentes and Rauser 2007). Sun *et al.* (2007) demonstrated how two different populations of *Sedum alfredii* varied considerably in both the degree of metal tolerance as well as the actual mechanism of that tolerance. Hernández-Allica *et al.* (2006) found that the two ecotypes ('Ganges' and 'Prayon') of the metal hyperaccumulator *Thlaspi caerulescens* have different Cd tolerance mechanisms; one seemed to rely solely on detoxification by thiols and the other did not. It appears that cereals have a greater propensity to complex Cd with PCs in their roots than other plants (Marentes and Rauser 2007).

In order to test the importance of GSH/PC genes, a number of research groups have tried overexpressing genes encoding the enzymes involved in this biosynthetic

pathway. Many of these groups were trying to improve metal accumulation ability for the purposes of phytoremediation of contaminated soil. Clemens *et al.* (1999) cloned the wheat PC synthase gene (TaPCS1) and demonstrated in *Saccharomyces cerevisiae* that it led to Cd tolerance and accumulation. In contrast, Lee *et al.* (2003) and Li *et al.* (2004) both found that overexpression of PCS genes led to Cd hypersensitivity. The explanation for this seems to be that the overproduction of PCs caused the plants to exhaust the supply of GSH. This was supported by the results of Pomponi *et al.* (2006), where a supply of GSH increased the tolerance and accumulation of Cd. Wawrzynski *et al.* (2006) had more success in manipulating an increase in thiol production by overexpressing multiple genes in the PC synthesis pathway. However, they still found that another factor was controlling shoot accumulation of Cd as there was not an increase in the rate of xylem translocation.

Researchers looking at Cd in durum wheat have not favoured the capacity to produce PCs as the basis of a mechanism for variable grain accumulation (Stolt *et al.* 2003). The reason for this is that PCs are commonly present in excess of chelation needs and so are not limiting (Hart *et al.* 2006). It is possible that the factor which distinguishes some cultivars is the variable capacity to transport those Cd complexes into the vacuole (Hart *et al.* 1998; Clemens 2006). Supporting this is the fact that many low-accumulating cultivars have the ability to store high concentrations of Cd in their roots (Chan and Hale 2004). We now have strong evidence for differential root vacuole accumulation of Cd in rice varieties by the action of OsHMA3. The functionality, or not, of OsHMA3 in the different cultivars of rice is an exemplar for the way vacuolar storage can affect shoot Cd accumulation (Ueno *et al.* 2010; Miyadate *et al.* 2011).

Results from non-crop plants have shed some light on transporters potentially involved in xylem loading. Verret *et al.* (2004) found that overexpression of AtHMA4 led to increased root to shoot translocation of Cd and increased Cd tolerance. The importance of

AtHMA4 was also demonstrated by Courbot *et al.* (2007) using QTL mapping of the first backcross population from the cross of *Arabidopsis halleri* (Cd tolerant) and *A. lyrata* ssp. *Petraea* (Cd intolerant). They found that the QTL for Cd tolerance co-localised with the gene AhHMA4, the homologue of the *A. thaliana* gene. Unexpectedly, this gene was down-regulated by the presence of Cd in *A. thaliana* (Verret *et al.* 2004), so its exact function remains uncertain.

Chan & Hale (2004) found that the low grain-Cd cultivar they studied had lower shoot Cd accumulation and actually ceased root to shoot translocation of Cd during flowering and grain ripening. Hart *et al.* (2006) studied near-isogenic lines (NILs) of durum wheat and concluded that the root mechanism controlling Cd translocation to shoots was most likely a difference in xylem-loading capability of the studied genotypes. They suggested that this could be the activity of a PM transporter similar to AtHMA4. A gene of this family in rice, OsHMA2, has recently been characterised as an important Zn and Cd transporter, critical for root to shoot translocation of Zn. This gene has some homology to OsHMA3 but is located on the plasma membrane rather than the tonoplast. Unlike arabidopsis, in which two Zn transporters of the HMA family appear to have redundant functions (Hussain *et al.* 2004), in rice, only one gene is responsible for the xylem loading of Zn (Satoh-Nagasawa *et al.* 2012).

Tonoplast metal/proton antiporters of the “NRAMP” family have been found to be responsible for the remobilisation of the nutrient elements Fe, Mn and Zn stored in the vacuole (Thomine *et al.* 2000; Thomine *et al.* 2003), and also transport of Cd (Lanquar *et al.* 2005; Clemens 2006). In rice, Aina *et al.* (2007) found that a transporter from this protein family, OsNRAMP1, was upregulated by the presence of Cd. However, this expression was tested at 10 µM Cd, and so this result could be a combination of the plant response to Cd and induced Fe deficiency that high levels of Cd are known to cause (Siedlecka and

Baszynaski 1993; Hodoshima *et al.* 2007). It has since been shown that OsNRAMP1, unlike the gene family members in arabidopsis, is located on the plasma membrane and is likely involved in xylem loading of Fe and Cd (Takahashi *et al.* 2011).

4.3. Transport from root to shoot - Cd in the xylem

A number of studies on crop cultivars have found that high shoot/grain accumulating varieties have higher concentrations of Cd in the xylem sap. Harris & Taylor (2004) demonstrated that a high grain-Cd accumulating cultivar had both reduced root Cd concentration and increased Cd concentration in xylem sap, and that these differences were independent of transpirational flow. This could very well be related to a plant's ability to complex Cd in the xylem. Senden *et al.* (1995) demonstrated with tomato that a supply of citric acid increased both the root uptake and xylem translocation of Cd.

An interesting but complicated aspect of the control of Cd movement in plants is the upregulation of genes in response to Cd exposure. Whether they are for transporter proteins or complexation mechanisms, expression of the genes that determine uptake and translocation of Cd can change over time. A number of studies have looked at the effect of plant pre-exposure to Cd on the movement of Cd in the plant. Larsson *et al.* (2002) observed that pre-exposed *Arabidopsis thaliana* exhibited increased root to shoot translocation and also total Cd uptake. Van der Vliet *et al.* (2007) found that low and high Cd-accumulating wheat varieties responded differently to Cd pre-exposure. The low accumulator increased root accumulation and decreased translocation to the shoot, while the high accumulator increased xylem translocation of Cd to the shoots during tillering. It may be that this related to the induction of PC synthesis during Cd pre-treatment because a

phytochelatin-deficient mutant was seen to translocate less ^{109}Cd from root to shoot than the wild type (Larsson *et al.* 2002).

Some have concluded that PC production in the root can determine the capacity to transport Cd from root to shoot (Florijn *et al.* 1993a; Guo and Marschner 1996). However, the results of Salt *et al.* (1995) led them to conclude that translocation of Cd in the xylem is independent of PC production in the roots, and that PCs play no direct role in speciation in the sap or translocation to the shoot. It is possible that the chelation requirements in the xylem depend on the Cd concentration in question. The mechanisms and transporters relevant to Cd partitioning at low Cd levels will be different to those at very high concentrations, as toxicity effects overwhelm the patterns of Cd translocation that occur at plant tolerant levels (Yu *et al.* 2006). On the basis of the results for rice of Nocito *et al.* (2011), a possible scenario could be that above external Cd concentrations around 0.1 µM, specific detoxifying chelators are needed to enable higher root to shoot accumulation.

4.4. Phloem transport to the grain

The phloem is the main route of Cd to the grain, and so, Cd phloem mobility in a plant genotype would affect the amount amassed during grain maturation. Cakmak *et al.* (2000) demonstrated genotypic variability in the translocation of Cd isotopes applied to the leaves of durum wheat. Using a similar method, Harris & Taylor (2001) demonstrated a genetic difference of about 1.5–2 fold between near-isogenic durum wheat lines. These results strongly suggest there is greater phloem mobility of Cd in high accumulating cultivars. However, as is repeatedly seen, Harris & Taylor (2001) concluded that this variation probably only explains part of the differences in accumulation in the grain.

In rice, a membrane transporter controlling leaf to grain remobilisation of Cd has been functionally analysed. Uraguchi *et al.* (2012) found that OsLCT1 is involved in the regulation of phloem translocation, especially during the reproductive stage. This protein is expressed in the cells surrounding the vascular bundles of leaves, stems and nodes. RNAi-mediated knockdown of OsLCT1 reduced phloem-mediated Cd transport and Cd accumulation in the grain.

As the phloem facilitates carbohydrate translocation to the roots, phloem mobility will also affect Cd movement back to the roots. Chan & Hale (2004) found evidence for increased shoot to root transport of Cd in a low grain-Cd accumulating variety. They observed that although low and high cultivars transported similar amounts to the shoots during the vegetative growth phase, some cultivars sent more of it back down to the roots. These cultivars did not also have differences in leaf to grain translocation and so must use a slightly different mechanism.

5. Conclusion

Current research into Cd accumulation in food crops is revealing a great diversity in the patterns of Cd translocation and accumulation (Clemens 2006). We have considerable knowledge of the general physiological pathways that are involved in Cd detoxification and transport, and in recent times, considerable advances have been made for rice in terms of elucidating the genetic mechanisms controlling Cd translocation to the shoot, particularly the role of OsHMA3 in vacuolar sequestration (Ueno *et al.* 2010; Miyadate *et al.* 2011) and OsHMA2 in xylem loading (Satoh-Nagasawa *et al.* 2012). It seems that OsIRT1 plays a large role in the uptake of Cd from the soil (Nakanishi *et al.* 2006) and that OsNRAMP1 also operates in the root and can transport Cd across plasma membranes. Fe nutrition is

thought to have a large effect on the uptake of Cd by rice plants, but the degree to which this is important has not been fully demonstrated. In addition, there are good theories for the reasons behind differential Cd uptake in flooded and aerobic soil, but they remain to be proven more fully (de Livera *et al.* 2011b). More research is needed on the nature and basis of genotypic differences in Cd accumulation, as well as the factors behind genotype by environment interaction.

1.2: Contextual Statement

Since the 1970s, when the harmful effects of Cd in rice in parts of Japan became known, much research in the fields of toxicology and plant science has focussed on Cd toxicity in food, especially rice. Much is known about the physiological basis of Cd accumulation in plants and the environmental factors that influence its uptake. But, as Clemens (2006) concluded in a review of toxic metal accumulation in plants,

> What we are lacking … is a detailed and quantitative understanding of Cd accumulation in plants. … The same is true for the molecular mechanisms underlying the considerable diversity found among crop plants with respect to Cd translocation and accumulation. Finding the responsible genes will be important also because low Cd content of edible plant parts might well become a target of future crop breeding programs. Towards this end there is a need for more studies on effects of low-level toxic metal exposure to get closer to real field situations.

Plant science is beginning to see the fruits of research into genotypic variation in Cd accumulation in rice. Many quantitative trait locus (QTL) studies have been conducted, but until recently the genes that were responsible for differences in the Cd accumulation trait were unknown (Ueno *et al.* 2010; Miyadate *et al.* 2011). Nevertheless, there is evidence that the genes identified so far are not the basis of all genotypic variation in grain Cd accumulation (Takahashi *et al.* 2011). In addition, it is known that environmental factors can have a big impact on the uptake of Cd. Particularly, in rice, it is known that soil redox can cause differences of grain accumulation of more than an order of magnitude. The interaction of rice genotypes and environmental conditions has been shown to be significant but the reasons for it are unclear (Ishikawa *et al.* 2005a).

The body of work that follows incorporates a number of areas of investigation relevant to the physiological basis of variable grain Cd accumulation in rice. It begins with a study on the timing of grain Cd accumulation, comparing the roles of remobilisation of

stored shoot Cd, from prior to flowering, and the more direct uptake, after flowering, when the grain is beginning to form. Much of the physiological research on Cd has focussed on the mechanism of phloem remobilisation from leaves to grain, whereas the conclusions from agronomic research into Cd in paddy rice often emphasise soil uptake during the period late in grain maturation (post yellow-grain stage) when paddy fields are often drained of water. The exact timing of grain Cd accumulation is not known, but it is important to understand for the purpose of strategies to decrease rice Cd.

For this research, rice genotypes were selected that have previously been found to have significant differences in grain Cd accumulation. These included four Chinese *japonica* rice varieties from a breeding program in China, as well as selected germplasm from the worldwide rice collection held at the National Institute of Agrobiological Sciences, Tsukuba, Japan. The latter group represented a large degree of the diversity in modern rice varieties in terms of Cd uptake. The cultivars were grown under various conditions, such as under hydroponics for radioactive-Cd influx studies, or with high and low Fe and/or Si supply. Some of the genotypes were also tested under varying flooding regimes in paddy soil in later experiments. Gene expression analysis was carried out on roots of these rice lines to search for cultivar specific patterns which correlate with Cd uptake characteristics. This investigation aimed to apply some of the published research on Cd transport by contrasting naturally occurring variation in Cd uptake with the expression of membrane transporters thought to be involved in plant Cd uptake. These included OsIRT1, OsNRAMP1, OsHMA2, OsHMA3 and others.

Following on from this, the role of Fe deficiency response in Cd uptake was investigated through a number of experiments. In the Literature, Fe deficiency has been shown to greatly increase Cd uptake in dicotyledonous plants. In order to compare the importance of different Fe deficiency responsive genes on Cd accumulation, Cd uptake and

gene expression during the development of Fe deficiency in rice were studied in hydroponically grown seedlings. Through further experiments, the varying role of Fe deficiency response and competition with Fe was also elucidated. The observations taken from hydroponic culture were tested in paddy soil grown rice with manipulated flooding conditions. In this way, changes in redox conditions and Cd availability were compared with Fe deficiency response during the growth of the plants and importantly during the development of the rice grain.

Lastly, the role of the OsNRAMP1 transporter in Cd uptake under field conditions was investigated. OsNRAMP1 has been found to be an Fe and Cd transporter and is upregulated during Fe deficiency (Takahashi *et al.* 2011). Transgenic plants were acquired with altered expression of OsNRAMP1 (both knockdown by RNAi and overexpression) and tested for Cd uptake under hydroponic and potted-soil conditions. Cd, Fe and Mn accumulation was studied particularly in these plants as these cations have been shown to share similar uptake and chelation pathways in plants.

Genotypic differences in Cd accumulation are complex and varied. In line with the comments of Clemens (2006), this work has contributed to our understanding of, 'the molecular mechanisms underlying the considerable diversity found among crop plants with respect to Cd translocation and accumulation.' In addition, the roles of Si and Fe nutrition in rice Cd uptake have been clarified. Throughout this investigation, experiments were conducted at low Cd concentrations as the mechanisms of Cd transport relevant to the real field conditions have been in view. This work has contributed to our understanding of the physiological mechanisms of Cd accumulation in rice and hopefully assists, where necessary, in the aim of growing rice with lower concentrations of Cd.

Chapter 2

The timing of grain Cd accumulation in rice plants: the relative importance of remobilisation within the plant and root Cd uptake post-flowering

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Published journal article (*Plant and Soil* **347**: 105–114), beginning on the following page

Rodda, M.S., Li, G. & Reid, R.J. (2011) The timing of grain Cd accumulation in rice plants: the relative importance of remobilisation within the plant and root Cd uptake post-flowering. *Plant and Soil, v. 347 (1-2), pp. 105 -114*

Chapter 3

Genotypic variation in Cd uptake and accumulation by four Chinese rice varieties: interaction with nutritional factors known to affect Cd movement into rice plants

Introduction

For the purposes of this study, the seeds of four varieties of Chinese rice were acquired from the breeding lines of Zhejiang University, China, which have been screened for grain Cd accumulation. Two of these varieties have been designated low-Cd accumulating lines, and the other two, high-Cd accumulating. The different lines were characterised based on their accumulation of Cd from paddy soil. Table 3.1 shows a summary of the results of the screening of six varieties for accumulation of Cd. These rice varieties were sourced from recent breeding trials and so represent realistic accumulation of Cd in modern rice. From communication with the breeder the results below are representative of the results for these cultivars over multiple crop seasons. Unfortunately statistical analysis of this data is not available, and so these results were only regarded as indicative of their probable characteristics.

Table 3.1: Grain Cd content of white (polished) rice (mg kg^{-1}) of six Chinese breeding lines that differ in Cd accumulation. The varieties used in the present study were N07-60, 6, 38 and 63, shown below in bold font, as they were most consistent in both spiked and control soil.

		Control soil (Chinese paddy soil)	Spiked paddy soil (+ 2 mg Cd kg^{-1})	Ratio of Cd accumulation in spiked to unspiked soil
Low Cd	**N07—60**	0.106	0.164	1.54
Low Cd	**N07—6**	0.129	0.218	1.69
	N07—5	0.120	0.223	1.86
	N07—11	0.203	0.241	1.19
High Cd	**N07—38**	0.161	0.368	2.28
High Cd	**N07—63**	0.138	0.393	2.85

Accumulation of Cd can be variable and dependent on the environmental conditions, especially soil Cd form and availability (Zeng *et al.* 2008; Zhang *et al.* 2008b). This small set of results demonstrates this to some degree; it can be seen that the ranking of the varieties for accumulation of Cd differed in the control soils (with a DTPA extractable Cd level of 0.062 mg kg^{-1}) and the spiked soil. For example, cultivar N07-63 is highest in spiked soil but intermediate in the control.

To begin, it was necessary to confirm the Cd accumulation characteristics of four of these varieties. Chapter 3.1 reports on the study of plant influx experiments using seedlings of these rice lines in nutrient solution. Chapter 3.2 presents the results of a whole lifecycle experiment in potted soil, including grain Cd accumulation. Thirdly, Chapter 3.3 covers a hydroponic experiment testing the Cd uptake of these varieties with the interaction of Fe and Si nutrition. In the final sections, 3.4-3.6, follow-up experiments are reported on that pursued explanations for some of the observations in this series of experiments. This included examination of thiol production in these cultivars (3.4), a comparison of Cd uptake in the absence of Fe and Mn (3.5) and a study of the effect of Si supply on root hydraulic conductivity in rice, a possible mechanism by which Si affects Cd uptake.

3.1: Cd accumulation in rice seedlings: short-term radioinflux uptake experiments

Introduction

The initial step in characterising these varieties was to investigate whether the grain Cd differences came about because of varying rates of Cd uptake from the soil, or differences in plant partitioning. There is evidence from a number of other crops that cultivars vary in the extent to which they accumulate Cd in their roots and shoots (Florijn and Van Beusichem 1993a; Dunbar *et al.* 2003; Chan and Hale 2004; Ishikawa *et al.* 2005a; Bovet *et al.* 2006; Hart *et al.* 2006; He *et al.* 2006). In rice, the association between plant Cd uptake, shoot accumulation, and grain Cd is variable, depending on the cultivars tested and environmental conditions (Cheng *et al.* 2005; Liu *et al.* 2005a; Arao and Ishikawa 2006; He *et al.* 2006; Liu *et al.* 2007b). The following experiments were used to identify whether the observed differences were the result of root uptake ability, i.e. overall plant accumulation, or are due to genotypic differences in root to shoot translocation.

In order to test accumulation and translocation in these cultivars, Cd influx experiments were carried out using the radioisotope ^{109}Cd, which enabled rapid detection of Cd accumulation in the plant tissues. The methods used in this experiment were similar to those used elsewhere, except the concentration of Cd used in the nutrient solution (at 50 nM) was much lower than many other studies of plant Cd uptake, for example, 50 µM used in similar rice experiments by Zhang & Duan (2008).

Materials and Methods

Plant culture

Twenty seeds of each of the four rice varieties, N07- 60, 6, 38 and 63, were surface sterilised with 1.5% hypochlorite bleach solution for 20 min. The seeds were then rinsed four times with reverse-osmosis (RO) water before being placed out on separate trays with paper towel moistened with deionised water (dH_2O). The trays were stored in the dark in a plant growth room for eight days (20-28^oC).

The germinated seeds were transferred to truncated 1.5 ml tubes (Eppendorf) suspended in the lid of rectangular plastic tubs (see Figure 3.1 below). The seedlings of two varieties were grown in a single tub, with approximately 15- 18 seeds of each variety, i.e. 3 L of nutrient solution per 30-35 plants. Prior to the uptake experiments the solution was not aerated, as it was not necessary given rice's ability to emit oxygen into the rhizosphere. The nutrient solution was completely replaced after one week, and then as required.

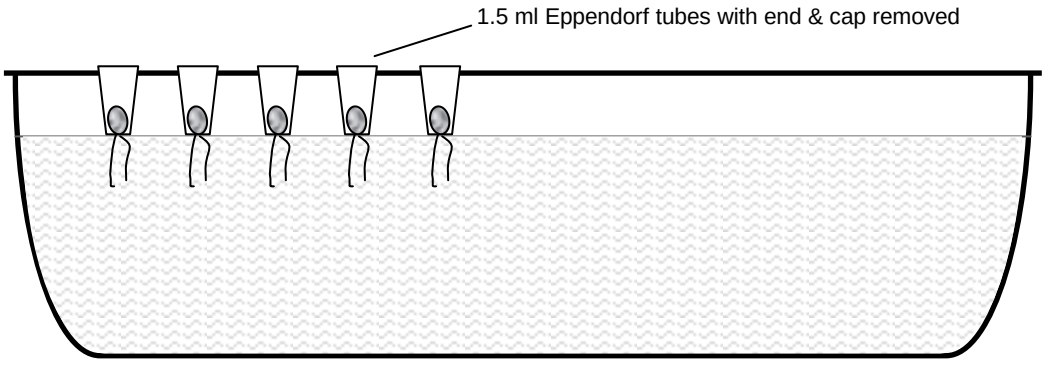


Figure 3.1: Diagram of hydroponic setup for seedling growth and influx experiment

^{109}Cd influx experiment - 24 h root and shoot accumulation study

The plants used in this experiment were 28 days old when used and had been pre-treated for seven days with a basic nutrient solution plus 1 mM SiO_2, as follows: 1.5 mM NH_4NO_3; 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.1 mM KH_2PO_4; 50 µM FeNaEDTA; 5 µM $ZnSO_4$; 0.5 µM $CuSO_4$; 5 µM $MnCl_2$; 50 µM H_3BO_3; and 0.1 µM MoO_4. The solution was adjusted back to pH 5.0 after sodium silicate was added. Both nitrate and ammonium forms of nitrogen were applied (Yoshida 1976), and this was found to balance the pH of the solution. Prior to the uptake experiment the plants were grown at room temperature.

For the radioactive Cd uptake experiments the plants were transferred to a controlled temperature growth room: 12/12 h, 23^{o}C dark, 27±2^{o}C light; illuminated at an approximate flux density of 400 µmol m^{-2} s^{-1}. To maintain uniform conditions for the cultivars, large replicate tubs were used with constant aeration for mixing, with 16 plants per 15 L of nutrient solution (half the plants of each genotype in each). Eight plants per cultivar were tested, with two plants combined for each sample. During analysis, two samples were averaged for each replicate (n=2; total of four plants per replicate). In regards to Si, in order to reduce potential variation and the need to dramatically adjust pH, Si was not added to the uptake solution. For Fe nutrition, 20 µM $Fe(II)SO_4$ was added at the beginning (and once midway through) in lieu of FeNa-EDTA, as EDTA is known to increase the uptake of Cd by plants and also chelate Cd radioisotopes (McLaughlin *et al.* 1997a). Ferrous Fe has been validated in other studies as suitable Fe supply for hydroponically grown plants (Florijn and Van Beusichem 1993b; 1993a).

^{109}Cd for use in this experiment was sourced from Eckert & Ziegler Isotope Products (Valencia, California); the activity at production reference date was 7.4 MBq ml^{-1}. For this experiment, $^{109}Cd/CdCl_2$ was added from a pre-prepared diluted stock solution, to enable adjustment of solution Cd concentration without changing the specific activity of ^{109}Cd. The

stock consisted of 5 mM $CdCl_2$, of which ≥70% (w/w) was non-radioactive, with a specific activity (spAc) of 6.7 cpm $pmol^{-1}$ Cd (in 15-100 keV range of the scintillation counter). This enabled 150 µl of stock to be used to make up 15 L of nutrient solution to 50nM Cd.

At the conclusion of the influx period, two final samples of the influx solution were taken to observe any change in solution Cd concentration. The plant roots were rinsed according to the following procedure to remove extracellular Cd:

1. The roots were rinsed for 2 s in dH_2O.
2. The tub lid, holding the seedlings, was then transferred for 15 min to another plastic tub filled with a rinse solution of 5 mM $CaCl_2$, 0.5 mM citric acid, and 10 µM $CdCl_2$ (non-radioactive), which had been adjusted to pH 3.5 with NaOH. The roots and solution were agitated at regular intervals.
3. Step 2 was repeated twice, so that there were three successive rinses of 15 min each.

Following rinsing, roots were excised just below the seed and blotted dry with paper towel. Tissue samples were then immediately weighed out in separate polypropylene tubes for roots and shoots. To each tube, a dilute solution of 0.1 M HCl and 1 mM $CaCl_2$ was added and the tubes (with loosened lids) were then placed in boiling water for at least 30 min to partially digest the tissue and release the ^{109}Cd. Following digestion, the tubes were individually weighed to calculate moisture loss during boiling. A 1 ml aliquot of each digest solution was transferred to a scintillation vial, with 4 ml of Ultima Gold® scintillation fluid (Perkin Elmer Life Sciences) and the vials were shaken vigorously for 10 s before being placed in the scintillation counter (Packard Tri Carb 2100TR, Canberra Packard).

Data analysis

Blank vials containing 1 ml dH_2O and 4 ml scintillation fluid were added to the scintillation counter on each counting occasion to detect background fluorescence. The Cd content of the root tissue or leaf digest sample was calculated using the known specific activity of the

solution, that is, the ratio of ^{109}Cd to total Cd. The scintillation counter was calibrated to express measured fluorescence according to three regions of the light spectrum. The third region, in the range 15-100 keV was used for these ^{109}Cd-labelled samples.

The Genstat statistical package (2007, Lawes Agricultural Trust; VSN International Ltd) was used to perform analysis of variance (ANOVA). Outliers were removed which did not conform (from residual values) to the normality assumptions of the ANOVA model. The mean values for each of the cultivars, within a treatment, were compared using the least significant difference value (L.S.D., 5% significance level).

Results

In this experiment, N07-38 plants had the greatest shoot and root weight (Table 3.3). N07-60 was found to have the greatest rate of Cd influx, both to roots and shoots (Table 3.4). The root to shoot translocation in N07-60, as measured by influx into shoots, was more than double that of N07-6 and N07-38, and also significantly greater than N07-63. N07-60 also partitioned a greater proportion of the plant Cd into the shoots than the other varieties.

Table 3.3: Plant growth characteristics of seedlings of four Chinese rice varieties used in 24 h Cd uptake experiments. The adjacent lettering represents means that are significantly different, within a single column, by ANOVA and L.S.D. (1% level).

	24 h uptake expt		(n=2, 4 plants/rep)
Plant	Root fresh weight (mg plant^{-1})	Shoot fresh weight (mg plant^{-1})	Shoot-weight: root-weight ratio
(Low accumulators)			
N07—6	113 *a*	480 *a*	4.3
N07—60	105 *a*	470 *a*	4.5
(High accumulators)			
N07—63	114 *a*	448 *a*	4.0
N07—38	163 *b*	644 *b*	4.0

Table 3.4: Cd uptake in rice seedlings of four rice cultivars grown in Cd^{109}-radiolabelled nutrient solution over 24 h (50 nM Cd). The adjacent lettering represents means that are significantly different, within a single column, by ANOVA and L.S.D. (5% level).

	Roots		**Shoots**		(n=2, 4 plants/rep)	
Plant	Average root influx (nmol g_{FW}^{-1} h^{-1})	Total root Cd (nmol $plant^{-1}$)	Average shoot influx (pmol g_{FW}^{-1} h^{-1})	Total shoot Cd (pmol $plant^{-1}$)	Shoot Cd as $prop^{n}$ of total plant Cd	Total plant uptake (pmol g^{-1} $root_{FW}$)
(Low accumulators)						
N07—6	0.47 *a*	1.27 *a*	16 *a*	190 *a*	0.13 *b*	13
N07—60	0.82 *d*	2.05 *c*	39 *c*	458 *c*	0.18 *c*	24
(High accumulators)						
N07—63	0.69 *c*	1.87 *b*	26 *b*	274 *b*	0.13 *b*	19
N07—38	0.59 *b*	2.31 *d*	17 *a*	266 *b*	0.10 *a*	16

Discussion

This experiment revealed that the differences in grain Cd accumulation characteristics seen in the paddy soil experiments are not due to general trends in overall plant Cd uptake. Apart from N07-6, the relative root and shoot Cd accumulation of these rice cultivars did not match the grain Cd patterns found in previous trials.

In both influx experiments, N07-60, a reported low-Cd accumulator, had the greatest uptake and translocation of Cd. N07-6, which is a low grain-Cd variety, showed lowest, or equal lowest, influx of Cd. Neither of these results could be accounted for by plant size, because in both experiments they were not significantly smaller or larger than at least some of the other varieties.

A comparison with other published results from studies of rice suggests that the results produced here may be due to the inherent differences between soil and solution culture. Arao & Ae (2003) found that even varieties which exhibited large differences in grain Cd in soil were not significantly different in Cd uptake in solution culture (to shoots or

grain). For other elements this result has also been seen. Wissuwa *et al.* (2006) found that the ranking of rice cultivars for Zn efficiency in solution culture did not match the ranking of cultivars in field conditions; they concluded that soil rhizosphere effects had a greater impact on plant Zn uptake than inherent Zn efficiency.

Another possibility is that there are significant differences between patterns of Cd accumulation between seedlings and mature plants. Li *et al.* (2011) found, with two rice cultivars which varied in grain Cd accumulation, that Cd uptake of the seedlings in hydroponic or soil culture was opposite to that of the genotypes at later stages and the final grain accumulation.

3.2: Comparison of Chinese rice varieties grown to maturity in potted soil

Introduction

In order to test the Cd accumulation characteristics of the four rice cultivars being examined here, potted plants were grown in a controlled environment growth chamber and analysed for shoot and grain Cd accumulation. This was an observational experiment, with the only treatment being a Cd spike for all the plants. There were four replicate pots per variety, with two plants per pot: one plant was harvested just prior to flowering and the other at final maturity.

Materials and Methods

Plant material and growth conditions

The growth medium used was University of California (UC) mix (Barker *et al.* 1998), Waite Campus version (University of Adelaide). This comprises 400 L of coarse washed sand, 300 L of peatmoss, 700 g calcium hydroxide, 480 g calcium carbonate and 600 g *Nitrophoska* 15:4:12 (BASF, Cheshire, UK), with pH (H_2O) of 5.5.

Pots were prepared 46 d before use. Rectangular 10 L pots were used with 11 kg of potting mix per pot. The mix was spiked with 1 mg kg^{-1} Cd, added as $CdCl_2$ salt, and mixed thoroughly before potting. The next day the soil was flooded with reverse-osmosis (RO) water. To allow for cation exchange and equilibrium of dissolved Cd content, the pots were stored at room temperature, with complete soil flooding, for 45 d prior to planting rice.

Rice seedlings of four different Chinese rice cultivars (from Chapter 3.1) were germinated in the dark on moist tissue paper (28^{o}C). Seven days after soaking (DAS) seedlings were transferred to floating nets in tubs of 20% modified Hoagland's Solution

(see Chapter 3.1). Two weeks later (21 DAS), the seedlings were transplanted into the prepared potting mix, with two plants per pot. The pots were kept in a plant growth chamber, with 12 h day/night, at 22°C/28°C respectively, with a flux density of approximately 400 µmol m^{-2} s^{-1}.

The soil was kept flooded with approx. 5 cm of standing water from transplanting until full flower. At the beginning of full flower the pots were allowed to dry out and had dried to non-waterlogged conditions 10 d later (45 d prior to harvest). Aerobic soil conditions were then maintained until harvest.

Plant harvest

At 83 DAS, just prior to the beginning of flowering, one plant from each of the pots was completely harvested. The whole plant was dried for six days at 65°C. Plants were weighed and then ground to <2 mm with a mechanical grinder. These samples will be referred to as 'Pre-flowering Shoot Harvest'.

At 149 DAS, the rice grain had fully matured and the remaining plants were harvested. The plants were separated into shoots and grain/panicles and were dried for three days at 77°C. The grain was then dehusked to obtain brown rice. The shoot samples were weighed and then ground to <2 mm with a mechanical grinder. These samples will be referred to as 'Final Shoot Harvest'.

Prior to analysis the shoot material was sieved using a no. 60 sieve (0.3 mm) to obtain very fine material. The brown rice samples were ground to a powder using an electric household grinder. Following this the plant material was analysed for Cd concentration as per the method outlined in Chapter 2; using the same equipment and reference standards.

Results

There were significant differences in plant size seen in this experiment. At the final harvest, N07-60 and N07-63 were significantly smaller than N07-6 and N07-38 (Fig. 3.2). N07-6 had greater shoot biomass under these conditions but much lower grain yield.

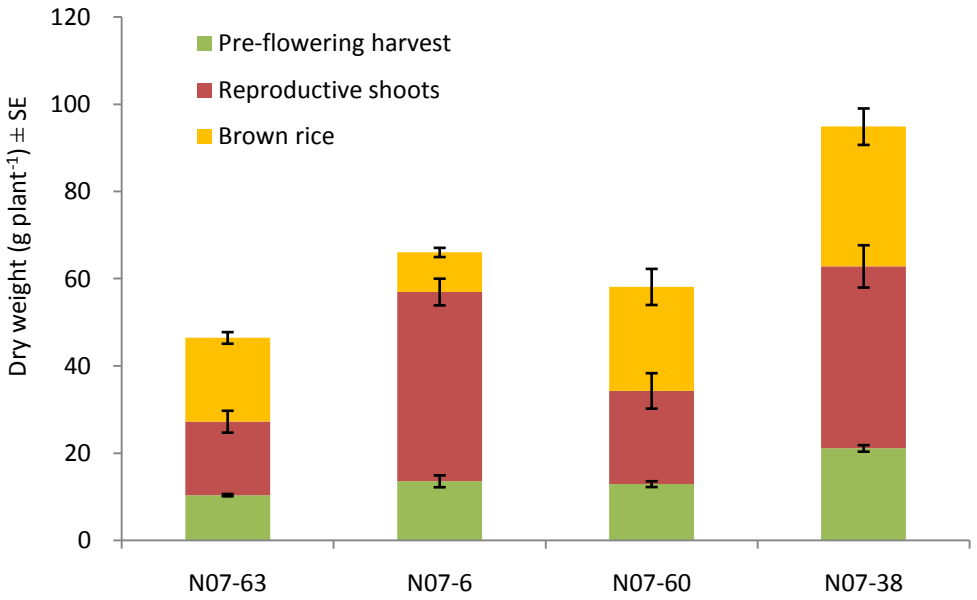


Figure 3.2: Plant dry matter of replicate soil-grown plants (n=4) of four rice varieties. Average weights from plants harvested just prior to flowering and additional plants harvested at maturity. Reproductive shoots = average shoot dry weight at final harvest minus average weight at pre-flowering harvest.

There was a difference between the cultivars in terms of shoot Cd accumulation post-flowering (Fig. 3.3). The Cd concentration of the shoot increased in both the N07-60 and N07-63 rice lines, whereas the concentration in N07-6 and N07-38 decreased, or stayed the same. Another obvious difference between these cultivars can be seen in Fig. 3.3: N07-60 and N07-63 invested less plant resources in growing stems and leaves post-flowering and more into grain development. Nevertheless, there seems to have been an increase in the rate of Cd uptake during the reproductive phase of the lifecycle in these two cultivars, because they also accumulated the highest grain Cd concentrations (Fig. 3.4).

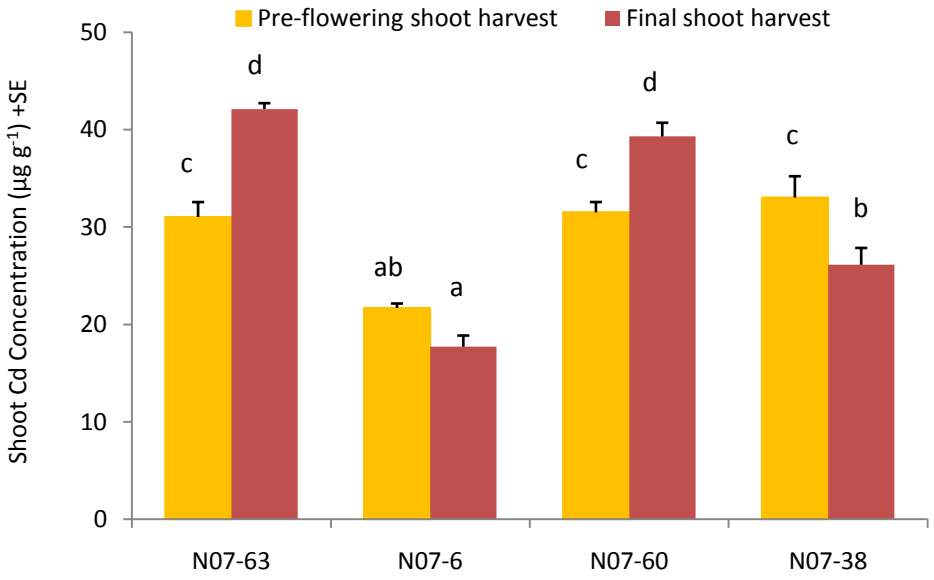


Figure 3.3: The average shoot Cd concentration of rice plants grown in potting mix inside a controlled growth chamber; harvested at two time points: Pre-flowering: just prior to anthesis (83 DAS); Final harvest: at plant maturity (149 DAS). Different letters above columns represent significantly different means (n=4) by ANOVA and L.S.D. 5% level.

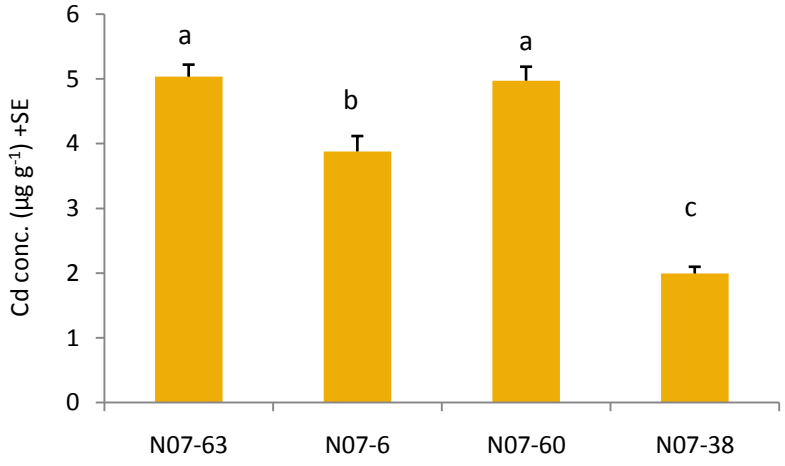


Figure 3.4: Mean Cd concentration in brown rice of four Chinese rice varieties grown in potting mix spiked with 1 mg kg^{-1} Cd. Different letters above columns represent significantly different means (n=4) by ANOVA and L.S.D. 5% level.

There were also differences in the plant partitioning of Cd in this experiment. Table 3.6 displays the average grain Cd content of these plants expressed relative to the total shoot Cd content and shoot Cd concentration. As seen above (Fig. 3.2), N07-6 had a low grain yield, and so the grain Cd was a low percentage of the above-ground Cd, at 3%. Conversely,

N07-63 and N07-60 had high grain yield, relative to their final shoot harvest, and so their grain made up more than 7% of the Cd content above ground (seen graphically in Fig. 3.5).

The low proportion of grain Cd in N07-38 is not explained by a low grain yield; in fact, this variety had the largest yield of brown rice (Fig. 3.2). With the exception of N07-60, there was a negative relationship between plant biomass at harvest and the Cd accumulation in the grain (Fig. 3.6). N07-38, which was significantly larger, had the lowest grain Cd concentration despite the fact that it was previously reported as a high-Cd accumulator.

In a similar way, when the Cd concentration of shoot and grain is compared (Table 3.6), N07-6 had a much higher relative grain concentration. This can be interpreted as a measure of translocation of plant Cd to the grain, coming from the roots and/or shoots. This result suggests that decreased number of rice grains and the large proportion of shoot dry matter above ground led to a higher concentration of Cd accumulating in the rice.

Table 3.6: Grain Cd content of four rice lines grown in potting mix relative to the shoot Cd accumulation of those plants. Mean values are shown (n=4).

		Grain Cd content, as % of total above-ground Cd **(Partitioning)**		Grain Cd conc. relative % to shoot Cd conc. **(Remobilisation)**	
		Mean	SE	Mean	SE
N07-6	Low accumulators	3.1%	1.4%	20%	0.2%
N07-60		7.1%	0.7%	11%	0.1%
N07-63	High accumulators	7.4%	0.6%	10%	0.6%
N07-38		3.9%	0.9%	7%	0.6%

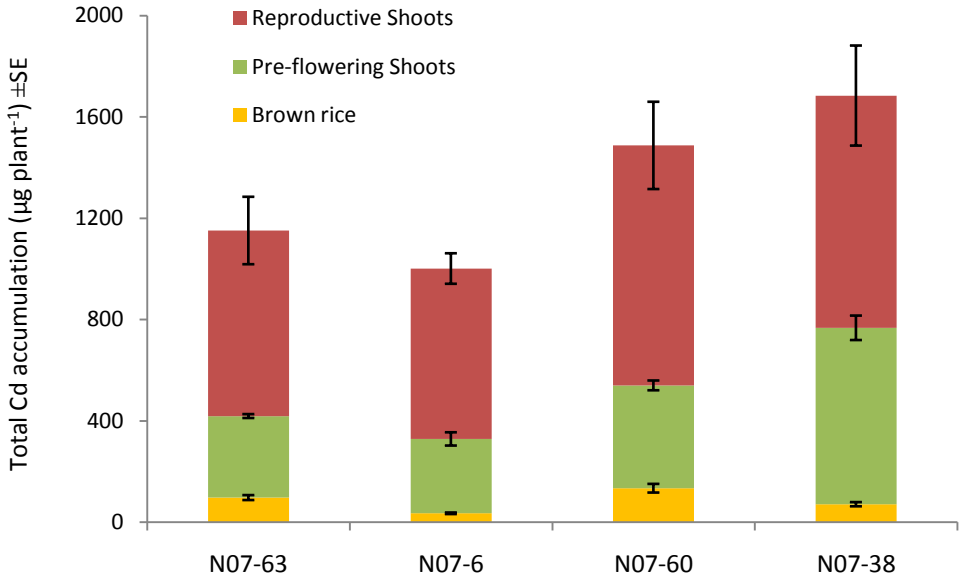


Figure 3.5: Average total plant Cd accumulation of replicate plants (n=4) of four rice cultivars grown in soil. Pre-flowering Shoots = whole plant harvest just prior to anthesis (83 DAS); Reproductive shoots = calculated total shoot Cd at final shoot harvest (149 DAS) minus average "Pre-flowering Shoot" totals.

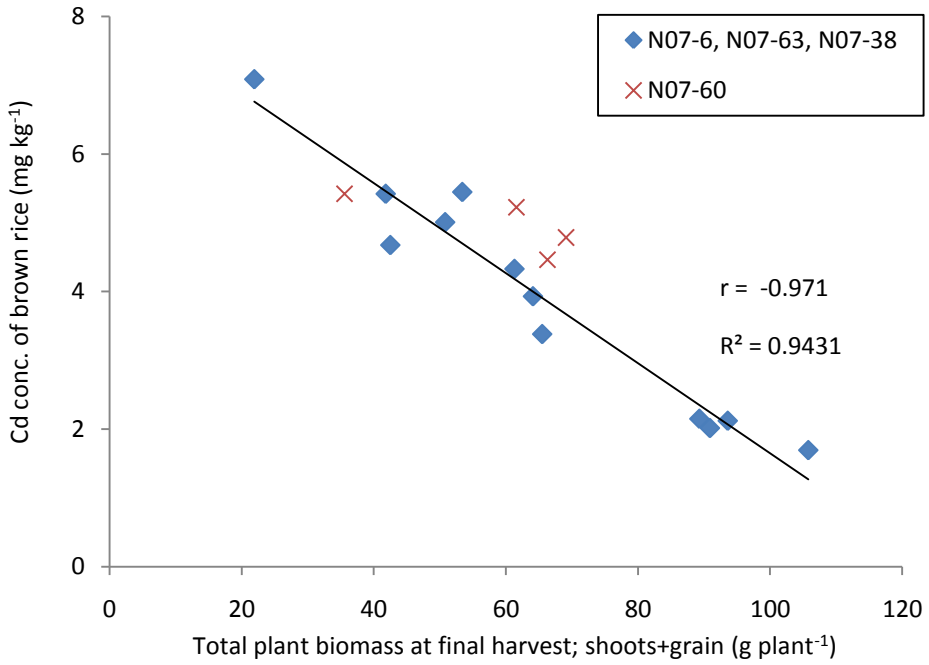


Figure 3.6: Negative relationship between plant biomass and grain Cd concentration observed in rice lines grown in potting mix spiked with 1 mg kg^{-1} Cd. R^2 value is shown for the linear trendline displayed, not including N07-60 cultivar, for which the trend did not well fit. Pearson's correlation coefficient (r) for this data is also shown.

Discussion

Significant differences in Cd accumulation were seen in this experiment, which continue some of the trends seen in Chapter 3.1, if only for N07-6 and N07-60. The results here showed that N07-60 and N07-63 had the highest shoot and grain Cd accumulation at final harvest (Fig. 3.3-4). N07-38 displayed the lowest Cd accumulation, most likely because of its large plant size in this potted situation, with a limited supply of Cd.

The plant tissue Cd concentrations in this experiment were very large. The values of 2–5 mg kg^{-1} for grain Cd are much higher than food safety standards, and would be a health concern, as Cd concentrations below 1 mg kg^{-1} are known to lead to accumulative toxicity (Codex Alimentarius 2007). The Cd availability in the soil medium of this experiment is the likely cause of this unexpectedly high Cd accumulation. Although only 1 mg kg^{-1} Cd was added to the soil, Cd bioavailability is known to be higher when it is added as a soluble salt compared to pre-existing contamination or through the addition of contaminated biosolids (McLaughlin *et al.* 2006), so a high proportion of the added Cd may have been available in soil solution as free Cd ions.

The soil used here was allowed to dry to aerobic conditions from flowering onwards. This would have led to an increase in Cd supply to the roots during post-flowering Cd uptake (see Chapter 2). A 'drainage' timing later in the lifecycle, e.g. a few weeks after heading, would have led to lower grain Cd content.

N07-6 was confirmed here as a low shoot Cd accumulator. The physiological cause of this is not clear, but there were significant morphological differences between these plants. The large shoot biomass may have led to the low Cd accumulation in the brown rice of this variety.

Morphological changes were noticed in these plants in response to the controlled growth room conditions, compared to the growth habits seen in a glasshouse with a lower temperature and humidity and with natural light conditions. N07-38 exhibited the greatest differences. When grown in a greenhouse, this plant had a smaller plant size and matured the fastest; in the growth chamber, these plants grew much taller than the other three varieties and the grain were slower to mature (see Fig. 3.2). In the growth room, N07-6 was a smaller plant and was the fastest to come to head. These differences are likely the result of photoperiod differences in the controlled temperature room, and need to be taken into consideration when comparing other studies.

Conclusion

It is widely documented that grain Cd accumulation between genotypes varies with growing and soil conditions (Zhang *et al.* 2008b). Indeed, in this experiment the reported Cd-accumulation characteristics of these varieties were hard to verify. The "low-Cd" accumulator N07-6, did display the lowest shoot Cd concentrations, and the "high-Cd" variety N07-63 had the equal highest shoot and grain Cd concentrations. However, for N07-60 and N07-38, the grain Cd characteristics found in trials in paddy soil were not reproduced. Previously, in both spiked and unspiked paddy soil, N07-60 had the lowest grain Cd concentration of six varieties tested (Table 3.1), but under hydroponic conditions (Chapter 3.1) and in this soil experiment, N07-60 had equal highest shoot and grain Cd concentration.

In Chapter 4, these rice lines are studied further. Their Cd accumulation characteristics are considered in comparison with gene expression data for membrane transporters potentially involved in the movement of Cd within rice plants.

3.3: Genotypic characteristics of Cd accumulation under differing Fe and silicon nutrition status

Introduction

Previous studies have shown that Cd accumulation characteristics of rice cultivars and the ranking of Cd accumulators often vary between different soils and between soil and hydroponic conditions (Arao and Ae 2003; Ishikawa *et al.* 2005a; Zeng *et al.* 2008). The aim of this experiment was to examine genotypic characteristics of Cd accumulation under different nutritional treatment conditions. This was achieved by subjecting four Chinese cultivars (from Chapter 3.1-3.2) to four different treatment combinations of plus and minus Fe and plus and minus Si. The plants were grown in a hydroponic nutrient solution using radiolabelled Cd to measure Cd accumulation in plant roots and shoots.

Fe was included in this experiment as a treatment because of the large effect of Fe nutrition on Cd uptake. Numerous studies have demonstrated how Fe deficiency increases Cd accumulation in rice and most dicots (e.g. Cohen *et al.* 1998; Shao *et al.* 2007), likely caused by the large up-regulation of membrane transporters, such as IRT1, under Fe deficiency (Connolly *et al.* 2002; Ishimaru *et al.* 2006; Nakanishi *et al.* 2006). Even under normal conditions, the evidence points to Fe transporters, like OsIRT1, as being the most important for root uptake of Cd in rice. It has been postulated that late-season periods of Fe deficiency in paddy fields (because of field drainage) are associated with the majority of the Cd uptake in field grown rice (Nakanishi *et al.* 2006). It is not known, however, the extent to which the effect of Fe deficiency is consistent between rice varieties. In an experiment with two genotypes of the dicot *Thlaspi caerulescens*, Lombi *et al.* (2002) found that the increase in Cd uptake associated with IRT1 only occurred in one of the genotypes.

Therefore, in the context of genotypes which vary in Cd accumulation characteristics, it is relevant to compare Cd uptake with cultivar responses to Fe deficiency.

Si is an important element in rice as it is known to be actively accumulated in its tissues at levels as high as other macronutrients (Ma and Yamaji 2006; Ma *et al.* 2007; Yamaji and Ma 2007). A number of studies have shown that application of Si can reduce rice plant uptake of Cd and also protect it from toxicity symptoms (Shi *et al.* 2005; Zhao and Masaihiko 2007; Nwugo and Huerta 2008; Liu *et al.* 2009). Si is a ubiquitous element in soil but is not often added to nutrient solutions, and so Si nutrition is a common difference between soil and hydroponic culture. It is possible that Si accumulation characteristics are one of the causes of different rice cultivar rankings for Cd accumulation between soil and hydroponics.

The effect of Si on Cd in rice has been studied before, but all of the published studies have used levels of Cd which are associated with toxic effects to the plant. The lowest level of Cd used in a Si/Cd study in the current literature was 2.5µM (Nwugo and Huerta 2008). This level is known to be orders of magnitude higher than plants commonly experience in the soil solution (Zarcinas *et al.* 2004a; 2004b), and given the importance of soil solution concentration on mineral partitioning in plants, it is of questionable application to the accumulation of Cd in food crops (Welch and Norvell 1999). The concentration of Cd used here was close to environmentally-relevant levels, 50nM (McLaughlin *et al.* 1998), which is fifty times lower than the supposed 'low-level cadmium' used by Nwugo and Huerta (2008).

Recent reports in the literature have thrown into doubt the validity of using chelator-buffered nutrient solutions to study plant uptake of nutrients and non-essential elements (McLaughlin *et al.* 1997a; Degryse *et al.* 2006). In order to avoid this uncertainty, this experiment utilised a large volume of nutrient solution in order to maintain a more stable, but still low, concentration of Cd in solution. This was achieved by having a single tub for

each treatment with 15 L of nutrient solution per 24 seedlings (four rice varieties with six replicates each). As well as aiding stability, this ensured the Cd concentration experienced by the cultivar replicates was consistent. In addition, Fe was added daily as unchelated $Fe(II)SO_4$ to avoid possible effects of EDTA on Cd uptake. This technique has been validated in other studies as suitable Fe nutrition for hydroponically grown plants (Florijn and Van Beusichem 1993b; 1993a).

Materials and Methods

Rice genotypes

The four Chinese rice varieties used in this experiment have been introduced in Chapter 3.1. They were sourced from Professor Wang-Da Cheng, from the Jiaxing Academy of Agricultural Sciences, Zhejiang, China. Repeated pot trials of these varieties with paddy soil have shown N07-6 and N07-60 to be low Cd-accumulating varieties and N07-63 and N07-63 to be higher-accumulating varieties. N07-60 has been seen to have the most consistently low grain Cd concentration. The results found in Chapters 3.1-2 deviated from this, and will be discussed later.

Plant culture

Seeds of each of the rice varieties were surface sterilised with 1.5% hypochlorite bleach solution for 20 min, rinsed four times with reverse-osmosis (RO) water and placed out on separate trays with paper towel moistened with deionised water (dH_2O). The seeds were germinated in the dark in a plant growth room for four days (25-31^oC). The germinated seeds were transferred to truncated 1.5 ml tubes suspended in the lid of rectangular plastic tubs (see Chapter 3.1). The tubs were blackened over the entire outer surface and the

plants were grown for 14 days in nutrient solution (as per Table 3.5, but with FeNaEDTA for Fe (at 50 µM) and without Si added). Nutrient solution was replaced weekly initially.

Experimental treatments

At 28 DAS, the plants were moved to the larger tubs (15 L of nutrient solution per 24 plants) and the different ±Fe and ±Si treatments were begun, without Cd added. Growing all the genotypes in one large container for each treatment combination was chosen above the option of individual experimental units because of the need to control Cd concentration in the solution, by monitoring and adjusting it twice daily. The shared nutrient solution provided greater confidence that the replicate plants were experiencing a very similar concentration of Cd throughout the experiment. However, the plants did have a shared root environment and a drawback of this design was that the effect of differing root exudation of organic and inorganic compounds was ignored. This decision was made on the basis of the assumption that as this was a hydroponic setup, the effect of root compounds on Cd uptake in this volume of nutrient solution would have been diluted and diminished.

Five days after beginning the treatments (33 DAS) the nutrient solutions were completely refreshed and radiolabelled Cd was added. At this point, FeNaEDTA was replaced with $Fe(II)SO_4$ as well. The nutrient solution used is itemised in Table 3.5, with Fe and Si added according to treatment combinations. All treatments received a final concentration of 50 nM radiolabelled Cd, which was approximately 90% non-radioactive Cd. The Cd in this experiment had a specific activity of 10.1 cpm $pmol^{-1}$ Cd (in 4-100 keV range of the scintillation counter). ^{109}Cd for use in this experiment was sourced from Eckert & Ziegler Isotope Products (Valencia, California); activity at production reference date was 7.4 MBq ml^{-1}.

Table 3.5: Rice hydroponic culture solution (pH adjusted to 5)

	Salt added	**Solution concentration**	
Macronutrients (mM)	NH_4NO_3	1.5	
	KNO_3	1	
	$Ca(NO_3)_2$	1	
	$MgSO_4$	1	
	KH_2PO_4	0.1	
Micronutrients (μM)	$ZnSO_4$	5	
	$CuSO_4$	0.5	
	$MnCl_2$	5	
	H_3BO_3	50	
	MoO_4	0.1	
Treatments	SiO_2 (as Na_2SiO_3)	0.83 mM	(50 mg kg^{-1} SiO_2)
	Fe (as $Fe(II)SO_4$)	20 μM	(added daily)
Cadmium	$CdCl_2$	0.05 μM	

During preparation the solutions were adjusted to pH 5.0 using H_2SO_4. Nitrogen was supplied in the growth solution as both ammonium and nitrate in order to prevent large pH changes in only one direction (Yoshida 1976). This was tested in previous experiments using this nutrient solution for rice plants and significant pH drift was not found to occur. The hydroponic solutions used were analysed in GEOCHEM-PC (Parker *et al.* 1995) and no significant Cd precipitation was found in the analyses. During the experiment a precipitate did form in the '+Si +Fe' solution, but this was checked in the scintillation counter and was not found to contain more Cd than the rest of the solution; hence, Cd was not precipitating out.

During the experiment, a 1 ml sample of the solution of each tub was taken daily and mixed with 4 ml of scintillation fluid (Ultima Gold®) and analysed in a scintillation counter. To maintain the initial (T_o) Cd concentration of the growth solutions, they were topped up as required with a Cd stock solution of the same specific activity. The solutions were not

aerated but were stirred twice daily. The nutrient solutions were topped up with dH_2O as necessary and then completely replaced after 7 d. The plants were harvested after 13 d.

Plant harvest

After 13 d the plant roots were rinsed according to the procedure outlined in Chapter 3.1, to remove extracellular Cd, with the only difference that the rinse times were 20 min each. Following rinsing, roots were excised just below the seed and blotted dry with paper towel. Root and shoot samples were then cut up and weighed out in separate plastic digestion tubes. Depending on the amount of plant tissue present, a volume of dilute acid and calcium solution was added to each tube (0.1 M HCl + 1 mM $CaCl_2$). The plant material was then digested in a 100^{o}C water bath and samples analysed in the scintillation counter as per Chapter 3.1. ANOVA (Genstat) was used to test the data set, comparing the cultivars by analysing each treatment grouping separately.

Results

The most influential treatment on plant Cd accumulation was Fe. The figures below show the large increase in both total Cd uptake (Fig. 3.8) and concentration (Fig. 3.9, 3.11) caused by -Fe treatment. Lack of Fe increased not only total plant Cd uptake but also root to shoot translocation. In Figure 3.13, it can be seen that it brought about an average increase in shoot Cd concentration of 13-fold in +Si treatments and 17-fold in -Si treatments. Rice roots not supplied with Fe also accumulated more Cd (Fig. 3.11), but it was less of an increase, with around a 5-fold increase in Cd concentration in +Si treatments and a median 6.3-fold increase in -Si treatments (see Fig. 3.13). For this reason, Fe deficiency caused changes in the plants' distribution of Cd, with the proportional shoot Cd ratio notably increased in -Fe plants (see Table 3.6). In the +Fe treatments, shoot Cd was

12% and 14% of total plant Cd for +Si and -Si, respectively, whereas in the -Fe treatments it was 24% and 32% of total plant Cd, respectively.

Figure 3.14 shows the changes in total plant accumulation across all the treatments. There was greatly increased total Cd uptake despite a large reduction in plant size because of deficiency (seen in Fig. 3.7). Total shoot Cd uptake was in the order of 8 and 9-fold higher, and total root Cd content per plant increased by 3-fold due to Fe-deficiency.

In both the Fe sufficient and Fe deficient conditions, lack of Si caused an increase in the plant uptake of Cd (Figures 3.10, 3.12). Under normal, "+Fe", conditions, removing Si caused an average increase in the rate of Cd uptake of 1.5-1.7 fold; and under Fe deficiency, an increase of 2.0-2.7 fold (Fig. 3.15). From this it can be seen that lack of Si magnified the extent to which Fe deficiency increased Cd influx (Fig. 3.13). Silicon also caused a significant increase in plant size (Fig. 3.8), but this effect does not account for the increase in Cd concentration observed because lack of Si caused an overall increase in Cd uptake per plant (Fig. 3.15).

Despite variability in plant growth as a result of the treatments, there were notable differences in Cd accumulation-characteristics in the four varieties tested here. In general terms, cultivars N07-60 and N07-63 consistently had the highest tissue Cd concentration in both roots and shoots. Across all four treatments, cultivar N07-6 accumulated a lower concentration of Cd than these two varieties. Although the concentration of Cd in N07-38 was often not significantly different to N07-6, this variety grew much more quickly and larger (Fig. 3.8), and so accumulated much more Cd in terms of total uptake (Fig. 3.9). In all the treatments except '-Fe-Si', N07-38 had a higher total shoot Cd uptake than all three other varieties. In terms of total root accumulation, this variety always had the highest amount of Cd.

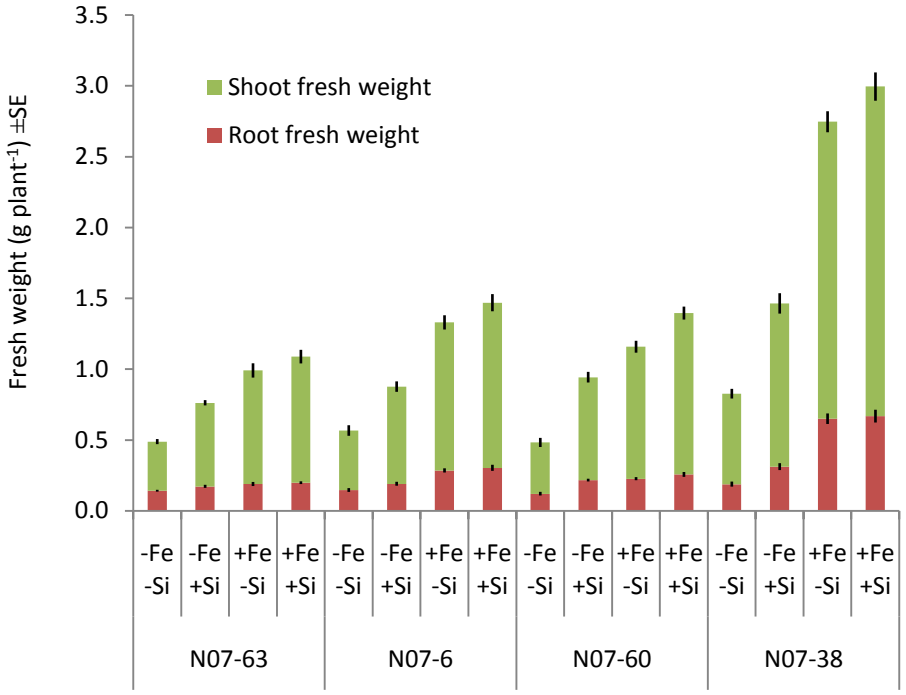


Figure 3.7: Effect of experimental treatments on the growth of rice seedlings. Plants harvested at 46 days old, 18 d after the beginning of treatments: 0 or 20 µM Fe(II)SO_4 daily; and 0 or 0.83 mM SiO_2.

Table 3.6: Shoot Cd content as a proportion of total plant Cd. Average ratio for each cultivar under each treatment combination (n=6). Different letters in adjacent indicate means which are significantly different according to Fisher's Protected L.S.D. (5% level) across all genotype by treatment combinations (because the interaction of the factors was significant, F pr. <0.001). The treatment means are also displayed.

	N07-63		N07-6		N07-60		N07-38		Mean
-Fe -Si	0.32	*h*	0.35	*h*	0.39	*i*	0.25	*g*	0.32
-Fe +Si	0.24	*g*	0.25	*g*	0.25	*g*	0.22	*f*	0.24
+Fe -Si	0.13	*d*	0.14	*d*	0.16	*e*	0.11	*b*	0.14
+Fe +Si	0.12	*c*	0.14	*d*	0.13	*d*	0.09	*a*	0.12

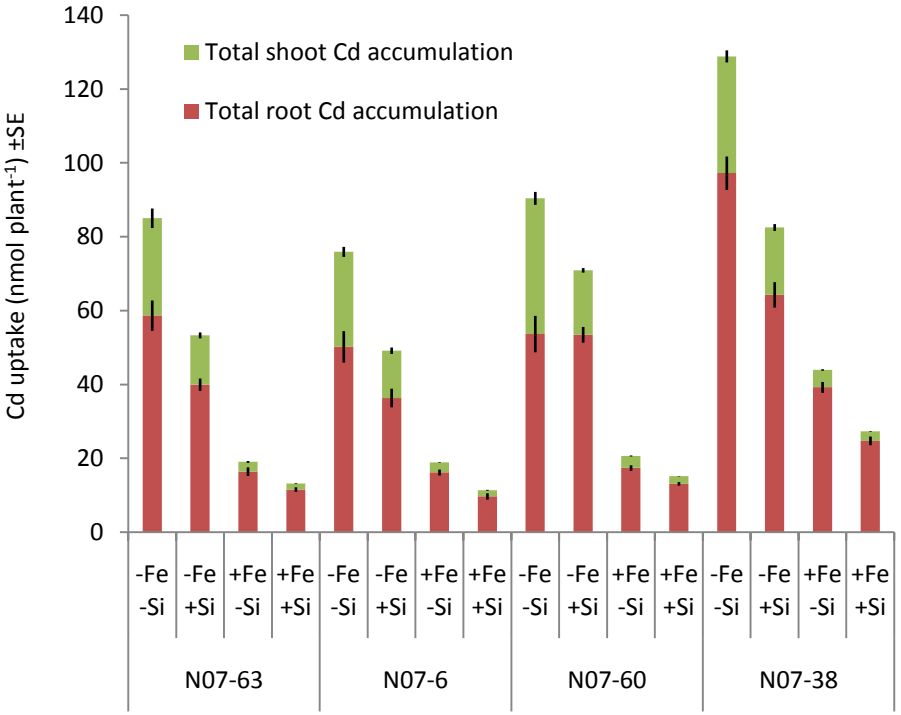


Figure 3.8: Total Cd accumulation in roots & shoots of four rice culivars, under four different Fe and Si treatment combinations, grown for 13 days in nutrient solution containing 50nM radiolabelled Cd.

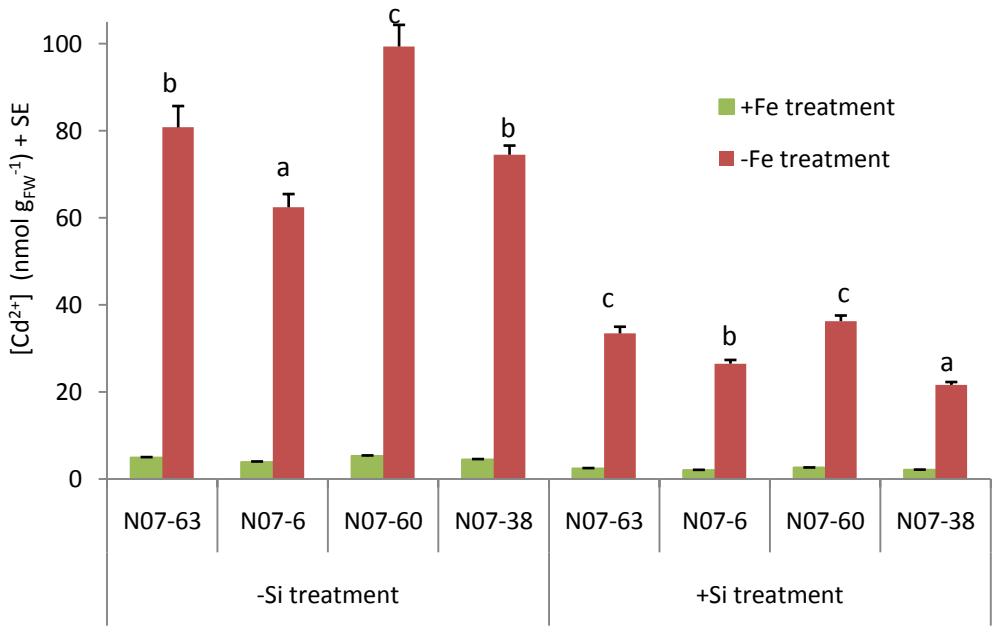


Figure 3.9: Shoot Cd concentration of rice plants during ±Fe and ±Si treatment. Average Cd concentration (n=6) for four rice cultivars grown for 13 days in nutrient solution with or without 50 ppm SiO_2 and 20 µM $Fe(II)SO_4$, respectively. Different letters above columns (within a single treatment) represent rice cultivar means which are significantly different using the L.S.D., 5% level.

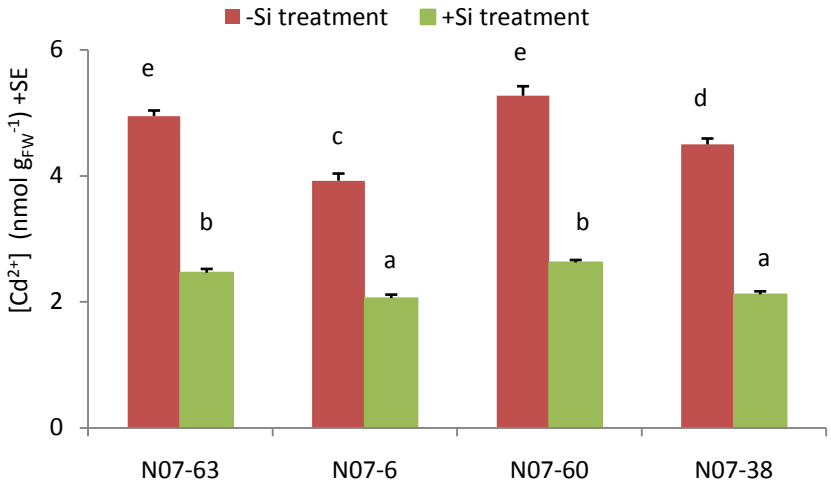


Figure 3.10: Shoot Cd concentration of plants with ±Si supply. Average Cd concentration (n=6) for four rice cultivars grown for 13 days in nutrient solution with or without 50 ppm Si. Different letters above columns represent means which are significantly different using the L.S.D., 5% level.

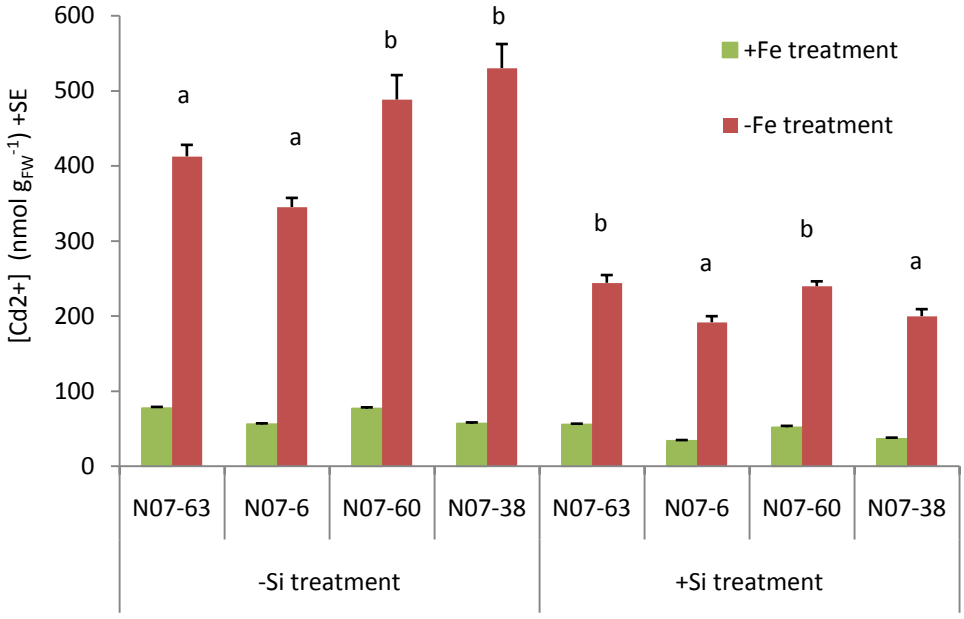


Figure 3.11: Root Cd concentration of rice plants during ±Fe and ±Si treatment. Average Cd concentration (n=6) for four rice cultivars grown for 13 days in nutrient solution with or without 50 ppm SiO_2 and 20 µM $Fe(II)SO_4$, respectively. Different letters above columns (within one treatment group) represent rice cultivar means which are significantly different using the L.S.D., 5% level.

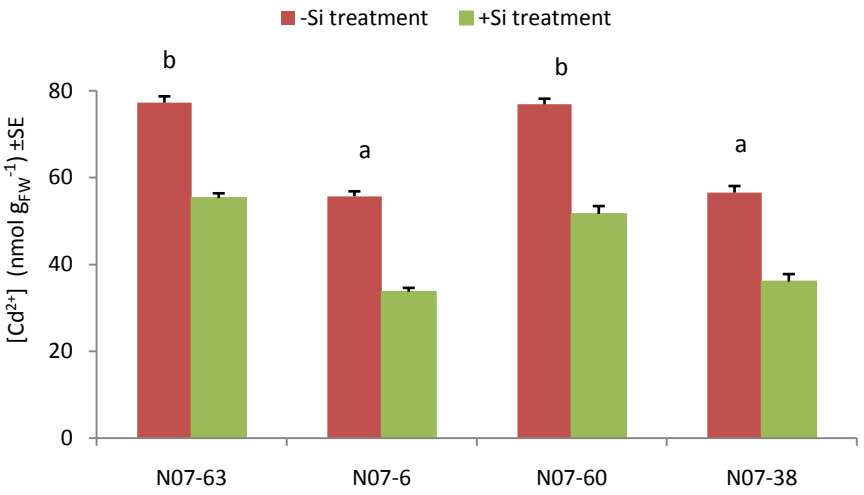


Figure 3.12: Root Cd concentration of plants (n=6) of four rice cultivars grown for 13 days in the nutrient solution, with sufficient Fe supply, with or without 50 ppm Si . Different letters above columns represent rice cultivar means that are significantly different, for both of the treatments, using the L.S.D., 5% level.

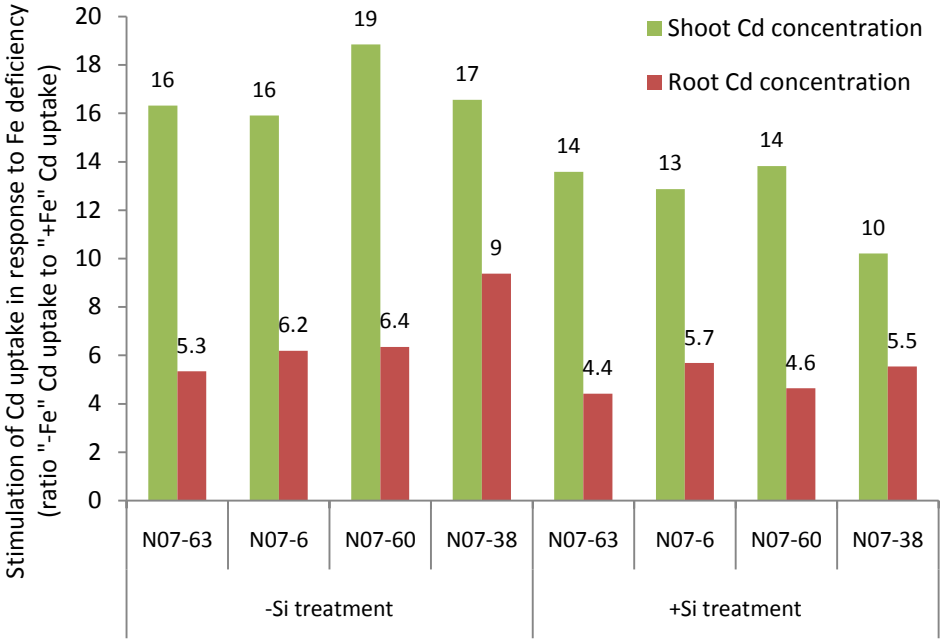


Figure 3.13: Proportional increase in plant Cd accumulation caused by Fe deficiency. Values shown are the average difference in Cd concentration in four different rice cultivars. ±Si treatment shows the influence of Si nutrition on the Fe deficiency effect observed.

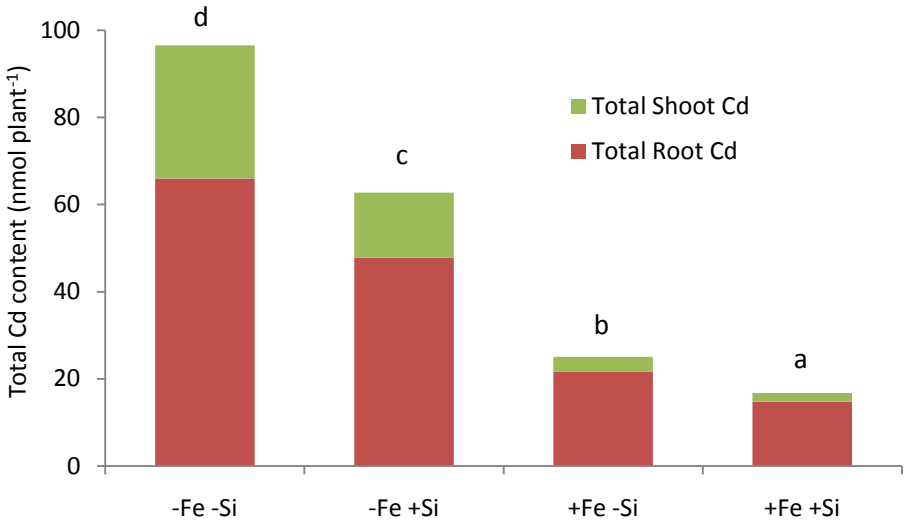


Figure 3.14: Total accumulation of Cd of rice plants grown for 13 days in combinations of treatments of ±Fe and ±Si. Mean values for four different rice genotypes are shown. Cd uptake measured using solution labelled with ^{109}Cd. All means significantly different by L.S.D. 5% level.

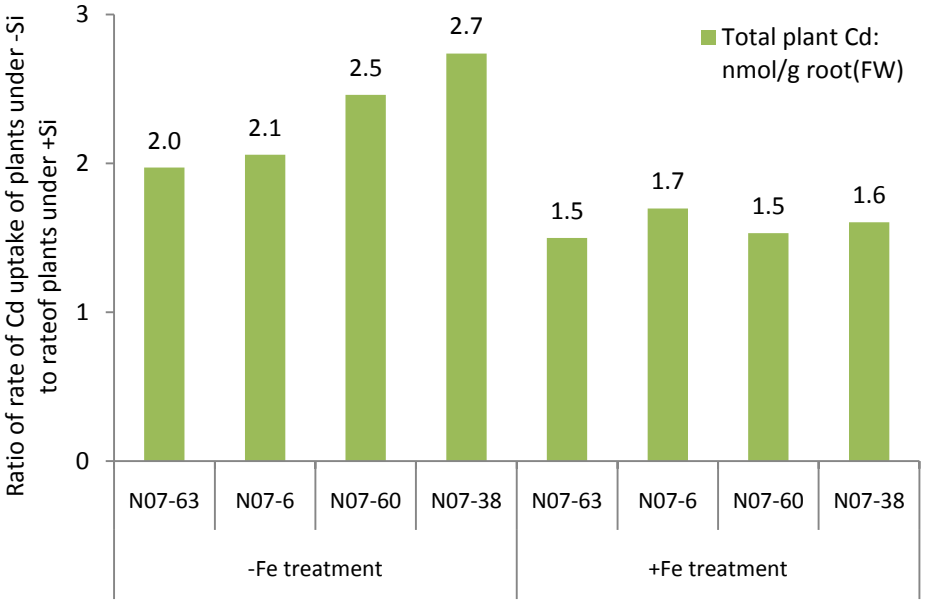


Figure 3.15: Effect of the absence of added Si in the nutrient solution on Cd uptake by rice seedlings with and without Fe deficiency. Values displayed are the average proportional difference in Cd content caused by lack of Si on total plant Cd uptake, per g of root.

Discussion

This experiment confirmed the large effect of Fe nutrition on the accumulation of Cd in plants. Despite a strong negative effect on the growth of plants, Fe deficiency and lack of competition from Fe led to increases in both the concentration (Fig. 3.9, 3.11) and total amount (Fig. 3.8) of Cd accumulated in the rice seedlings. Fe deficiency response increased the root to shoot translocation of Cd, with larger increases in shoot concentration than root concentration (Fig. 3.13). This resulted in a greater proportion of plant Cd found in the shoot (Fig. 3.14; Table 3.6).

Silicon supply in the nutrient solution decreased the Cd accumulation of all the rice seedlings, in plus or minus Fe conditions (Fig. 3.10, 3.12). The effect of added Si was much less than that of Fe, but did have slightly more of an effect under Fe deficiency than +Fe conditions.

Four rice genotypes were tested in four treatment combinations of Fe and Si. Genotypic differences in Cd accumulation were found to be fairly consistent in all of the treatments. In terms of Cd concentration, N07-60 and N07-63 had the highest accumulation of root and shoot Cd in all treatment combinations, except '-Fe-Si', where N07-63 was reduced to the second highest. N07-38 was a faster growing plant, with a larger plant size, and so had the highest total Cd accumulation. The cultivar, N07-6, was found to be the lowest, or equal lowest accumulator, of Cd in roots and shoots.

The genotypic differences seen in this experiment were not explained purely by plant growth characteristics but there were obvious differences in the level of Cd accumulation. However, when compared to results for grain Cd accumulation in paddy soil, the differences between the varieties do not closely match the trends found previously.

Fe deficiency

The magnitude of the effect of Fe deficiency seen in this experiment was greater than that previously recorded for rice in the literature. For example, Nakanishi *et al.* (2006) conducted an uptake experiment over only eight hours and was unable to show a significant increase in Cd uptake under a "-Fe treatment" similar to that applied here. They did, however, demonstrate an increased shoot Cd concentration when plants, pre-treated without Fe, were resupplied with Fe while Cd was added. Kudo *et al.* (2007) saw only small increases in Cd uptake in response to Fe deficiency in barley. In another study, with wheat grown in a chelator-buffered nutrient solution, no apparent effect at all was seen (Shenker *et al.* 2001).

Nevertheless, the differences found in this experiment are consistent with the influx kinetics demonstrated by Cohen *et al.* (1998) using pea seedlings; they found that, "the maximum initial velocity for Cd^{2+} uptake in Fe-deficient seedlings was nearly 7-fold higher than that in Fe-grown seedlings."

A weakness identified after the conclusion of this experiment was the large effect that the presence of Fe^{2+} had on the uptake of Cd (see Chapter 3.5). The magnitude of this effect was not expected and so this experiment made no comparison of Cd influx of Fe deficient plants in identical conditions to Fe sufficient plants. In Chapter 6.1, the effects of Fe competition and Fe deficiency were compared in rice seedlings.

The translocation of Cd to the shoot (Table 3.6) is possibly an indicator of the upregulation of Fe-deficiency responsive (IDR) genes. In a follow-up experiment, where non Fe-deficient plants were grown in $\pm FeSO_4$, the Fe(II) in the nutrient solution was seen to decrease the overall plant uptake of Fe but not change the proportion of plant Cd found in the shoot (see Chapter 3.5). In contrast, large differences in the proportional shoot Cd were seen in this experiment (Table 3.6).

For rice, Fe deficiency responses in the plant are known to include (1) the exudation of phytosiderophores to increase Fe chelation, and (2) the up-regulation of membrane transporter genes, such as IRT1. In the case of the former, there is no evidence that phytosiderophores increase the uptake of Cd by Fe-deficient plants (Shenker *et al.* 2001; Hill *et al.* 2002; Meda *et al.* 2007), although it is possible that, in soil, phytosiderophores could assist in the release of immobile Cd into the rhizosphere (Kudo *et al.* 2007). Another valid hypothesis for these results is that synthesis of the phytosiderophore precursor nicotianamine (NA) was upregulated in response to Fe deficiency. NA is known to chelate Cd and could facilitate greater transport from root to shoot (Kim *et al.* 2005; Ishimaru *et al.* 2010). This mechanism will be explored in further detail in Chapter 4 and Chapter 6.1.

The Fe(II) transporter IRT1 (and IRT2 in rice) has been shown repeatedly to be associated with an increase in Cd uptake in dicots and rice (Cohen *et al.* 1998; Lombi *et al.* 2002; Nakanishi *et al.* 2006). The level of upregulation of OsIRT1 seen in rice roots under Fe deficiency, in the order of 40 to 75 times the number of transcript copies (Ishimaru *et al.* 2006; Yokosho *et al.* 2009), is consistent with the magnitude of differences in accumulation seen here.

The partitioning of Cd between roots and shoots in the Fe deficiency treatment provides evidence for the upregulation of membrane transporters responsible for translocation of Cd from roots to shoots. At these relatively low levels of Cd supply, the plant roots have a large ability to sequester and detoxify Cd. To a certain extent, as Cd supply increases, the roots normally actively contain more of it in their vacuoles. Nocito *et al.* (2011) found that as Cd supply to rice roots increased from 0.01 µM to 1 µM, the percentage of Cd retained in the roots increased. In contrast to that, in this study, Fe-deficient plants increased in the proportion of plant Cd found in the shoot (approximately double). Fe-deficient plants must then have increased expression of genes which, not only

increase Cd uptake, but also facilitate/increase root to shoot translocation. Candidate genes for this mechanism are studied in Chapters 4 and 6.

Iron plaque

Some minor (i.e. very faint) Fe plaque was formed on the seedling roots in the two +Fe treatments in this experiment. This surface coating of precipitated Fe^{3+} has been found not to have an effect on Cd uptake in rice plants (Liu *et al.* 2007a; Liu *et al.* 2008). Liu *et al.* (2008) demonstrated that although added soil Fe can decrease Cd uptake, the evidence points to this being because of the root Fe status, rather than the direct effect of Fe plaque binding Cd or impeding its movement into roots.

The desorption-solution used here (with a pH of 3.5) would have removed external Cd, whether bound to Fe plaque or not (although the efficiency of this removal was not directly checked), so the root Cd content measured should not have been affected by root Fe plaque.

The silicon effect

Some studies have raised doubts as to whether the effect of Si on Cd is only a dilution effect caused by the impact of Si on plant growth and total mass (Zhang *et al.* 2008a). However, the results presented here show that there is a 'Si effect' that is more than just an increase in plant mass or an improved tolerance to Cd toxicity. In a similar way to what has been observed here for Cd, Si nutrition has been found to decrease accumulation of Na and Ca in rice (Ma and Takahashi 1993; Yeo *et al.* 1999). The mechanism by which Si reduces Cd uptake will be studied more closely in Chapters 3.5-3.6.

Genotypic characteristics

In comparison to what has been established about the grain Cd accumulation of these rice varieties in previous trials, the results of this experiment are notably different. Repeated pot trials in paddy soil, found N07-6 and N07-60 to be low Cd-accumulating varieties, with N07-60 having the most consistently low grain Cd concentration (Table 3.1; pers. comm. 7/11/08, Wang-Da Cheng, Jiaxing Academy of Agricultural Sciences, Zhejiang, China). The results of this experiment and those of a pot trial conducted here in Australia, which was continued to grain (Chapter 3.2), have shown no evidence that N07-60 is a low Cd accumulating variety.

The differences in Cd accumulation between these rice varieties must be significantly influenced by environmental effects on uptake. This experiment has indicated that the primary environmental-interaction effects are not related to Fe or Si nutrition.

3.4 Mechanisms behind genotypic variation in Cd accumulation: comparison of root thiol levels in Chinese rice lines in response to Cd supply

Introduction

Plants tolerate the presence of toxic elements like Cd^{2+} by the production of a class of compounds called non-protein thiols (NPTs). These enzymatically produced molecules are formed in plants, and among a range of functions, chelate toxic elements to render them safe for metabolic function in the cell.

Buthionine sulfoximine (BSO) is a chemical inhibitor of glutathione (GSH) biosynthesis that has been used in plant studies to detect the role of phytochelatins in Cd accumulation (Howden and Cobbett 1992; Lindberg *et al.* 2007). By virtue of the fact that GSH is a precursor to the phytochelatins (PCs), in the presence of a toxic agent like Cd, the presence of BSO inhibits the production of PCs which are necessary for detoxification mechanisms.

Materials and Methods

Thiols in Chinese rice cultivars

Root thiol content in four rice varieties was tested following exposure to hydroponically fed $CdCl_2$, at 1 µM, for 8 d. Plants were grown in the following nutrient solution: 1.5 mM NH_4NO_3; 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.1 mM KH_2PO_4; 50 µM FeNaEDTA; 5 µM $ZnSO_4$; 0.5 µM $CuSO_4$; 5 µM $MnCl_2$; 50 µM H_3BO_3; and 0.1 µM MoO_4. The solution, including added Cd, was refreshed every few days.

Soluble thiols were extracted and analysed based on the method described in Wawrzynski *et al.* (2006). Approximately 100 mg_{FW} of root material was ground in 1 ml 0.1

M HCl/1 mM EDTA then centrifuged at 12,000 g for 5 min. An aliquot of the supernatant (200 µl) was mixed with 120 mM K_2HPO_4/6 mM EDTA pH 7.8 (700 µl) and 6 mM 5,5′-dithio-2-nitrobenzoic acid (DTNB) (100 µl) and the absorbance read at 412 nm. Correction was made for the background absorbance of the sample by subtracting the absorbance of a duplicate sample without DTNB. Thiol content was then calculated and expressed as cysteine equivalents (µmol g^{-1}).

Effect of BSO treatment on Cd in uptake in rice

An uptake experiment was designed to measure the effect of BSO on the rate of uptake and translocation of Cd in rice plants. The difference between this experiment and others published is the low concentration of Cd that was used, 50 nM, as opposed to 1 µM or more. The Australian rice cultivar 'Amaroo' (*O. sativa* subsp. *japonica*), rather than the previous Chinese cultivars, was used for its faster growth. It has similar Cd uptake characteristics to low Cd accumulating *japonica* varieties (see Table 5.2, p 161).

Seedlings were planted in nutrient solution 5 d after germination, and grown in a controlled temperature growth room (as previously). One week later BSO was added to the solution to a final concentration of 250 µM (concentrations between 0.1 and 1 mM have been used in previous plant studies (Howden and Cobbett 1992; Lindberg *et al.* 2007)). This solution was replaced 2.5 d later, and then 4 d after beginning of BSO treatment, radio-labelled Cd was added at a concentration of 50 nM for a 45 h uptake experiment.

The plant material was analysed for ^{109}Cd content as per the previous section, except that 3 desorption rinses of 20 min each were used, and then both root and shoot material was digested under dilute acid before scintillation counting.

Results

Thiols in Chinese rice cultivars

Small but significant differences were found between the rice cultivars in terms of non-protein thiol content of the roots (Fig. 3.16). N07-63 had the highest average concentration and N07-60 had a significantly lower concentration than both N07-63 and N07-38.

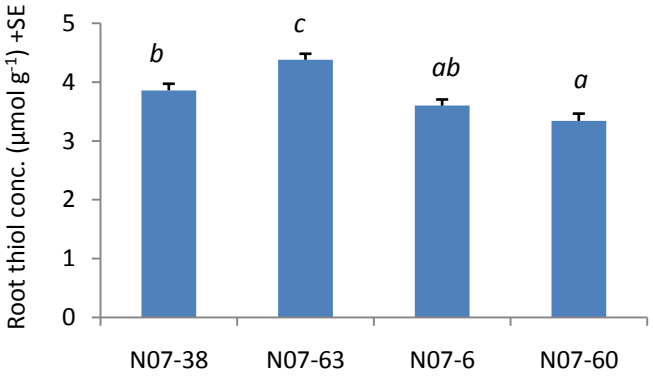


Figure 3.16: Root thiol content in four rice varieties following exposure to hydroponically fed $CdCl_2$, at 1 µM, for 8 d. Different letters above columns represent significantly different means (n=5; ANOVA, LSD 5% level).

Effect of BSO treatment on Cd in uptake in rice

BSO treatment had a significant, negative effect on the Cd accumulation ability of rice roots, but did not have a significant effect on shoot Cd accumulation (Table 3.7, Fig. 3.17). Despite there being a significant increase in proportional shoot Cd content under +BSO, the difference in plant Cd distribution seen between these treatments was explained by concomitant differences in root to shoot ratio of plant tissue. BSO treatment significantly reduced shoot fresh weight, associated with a visible minor phytotoxic effect.

Table 3.7: Effect of 4 d BSO pre-treatment on the root and shoot accumulation of Amaroo rice seedlings over 2 d.

	Root fresh weight		Shoot fresh weight		Root [Cd]		Shoot [Cd]		Shoot Cd as a proportion of total plant Cd	
	mg plant^{-1}	*ns*	mg plant^{-1}	**	nmol g^{-1}	*	nmol g^{-1}	*ns*		*
-BSO	104	*a*	378	*b*	44.8	*b*	3.7	*a*	0.21	*a*
+BSO	97	*a*	295	*a*	38.2	*a*	4.0	*a*	0.24	*b*

N.B. Different letters, within a single column, indicate means which are significantly different by ANOVA-L.S.D. (*5% sig. level, ** 1% sig. level).

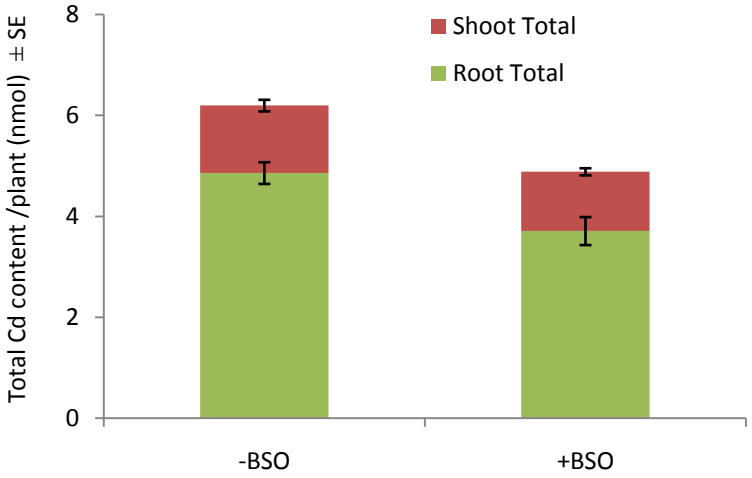


Figure 3.17: Effect of BSO treatment on the root and shoot accumulation of Amaroo rice seedlings over 2 d. Shoot Cd content was not significantly different between the treatments.

Discussion

When contrasted with the results for cultivar-specific Cd accumulation (Chapter 3.1-3), the root thiol content found in this experiment did not show a clear correlation with either elevated or decreased Cd content. These results provide no support for the conclusion, made elsewhere, that NPTs are an important determiner of genotypic root to shoot translocation of Cd (Li *et al.* 2011). BSO treatment reduced the ability of rice seedlings to accumulate Cd in their roots and this had the effect of decreasing overall plant Cd

accumulation. It is possible that the reduced thiol accumulation in the roots of N07-60 is part of the reason for increased root to shoot translocation in this cultivar, but that is only conjecture and not supported by the pattern of results for the other cultivars.

Two of these same cultivars, N07-6 and N07-63, were studied in another recent report (Wang *et al.* 2010). In agreement with the results here, those researchers found that Cd induced greater increases in NPTs content in N07-63 than in N07-6. However, they also found that less Cd was translocated from root to shoot in N07-63 than in N07-6, opposite to the result found consistently here (this Chapter). The reason for the difference may have something to do with the concentration of Cd used in their experiments: 50 µM, a thousand-fold higher than the concentration used in the Cd uptake experiments here. At a high Cd concentration, it is possible that Cd would be transported in the xylem in inorganic forms, as Ueno *et al.* (2008) found for *Arabidopsis halleri* supplied with 35 µM $Cd(NO_3)_2$. At a low concentration, the presence of organic chelators may play more of a role in controlling Cd translocation.

3.5 Cd in uptake in rice: competitive interaction with Fe and Mn

Introduction

In the previous chapter, the interaction of Mn and Fe with Cd uptake in soil-grown plants was discussed as a partial explanation for the differences in Cd accumulation between flooded and unflooded soil. In order to further test this hypothesis, an uptake experiment in solution culture was used to observe the competitive interaction between Cd and these two essential nutrient metals. Cd uptake was measured using radioactive Cd-109 in nutrient solutions containing either full nutrient solution, minus Fe or minus Mn. To further detect environmental interaction effects, rice seedlings used in the experiment were pre-treated with ±Si for 12 d prior to 24 h exposure to 50 nM Cd.

Materials and Methods

Plant material and culture

The Australian rice cultivar Amaroo (*O. sativa* subsp. *japonica*) was used in this experiment because it was found to grow more rapidly and easily. This variety has similar Cd uptake characteristics to relatively low Cd accumulating (*japonica*) varieties such as Nipponbare (see Table 5.2, p 161). Seeds were germinated on moist paper towel and six days later, seedlings were transferred to 10% modified Hoagland's solution (as per Chapter 3.1). After 5 d growth at 23^{o}C ±2^{o}C, the plants were transferred to two different treatments of ±Si. For these treatments, the following nutrient solution was used: 1.5 mM NH_4NO_3; 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.1 mM KH_2PO_4; 50 µM FeNaEDTA; 5 µM $ZnSO_4$; 0.5 µM $CuSO_4$; 5 µM $MnCl_2$; 50 µM H_3BO_3; and 0.1 µM MoO_4. The +Si treatment included 1 mM SiO_2 added as sodium silicate solution (Sigma), and the solution was adjusted back to pH 5.0 with HCl after sodium silicate was added. Plants were grown in a temperature controlled

growth room, 12 h day/night, 23ºC night, 27ºC ±2ºC day. Overhead lamps gave a flux density of approximately 400 µmol m^{-2} s^{-1}.

Plants were grown in Si treatments for 12 d prior to a 24 h uptake experiment. Before being used, the plants were rinsed and then transferred to a nutrient solution without Fe and Mn, to desorb Mn and Fe on the outside of the roots. After 2 h this solution was discarded and the plants were put onto the three experimental treatments: -Mn, -Fe, and Full nutrient solution. For each treatment, a large tub with 15 L of nutrient solution was used for ten plants, five pre-treated with Si and five without. For the Full nutrient solution and -Mn treatment, ferrous Fe (Fe(II)) was added in lieu of Fe-EDTA. Solid $FeSO_4 \cdot 7H_20$ was added to bring the solution to 20 µM Fe.

To each tub, the equivalent of 50 nM Cd radiolabelled with ^{109}Cd was added to initiate the 24 h of uptake. For this experiment, $^{109}Cd/CdCl_2$ was added from a pre-prepared diluted stock solution, to enable adjustment of solution Cd concentration without changing the specific activity of ^{109}Cd. The stock consisted of 5 mM $CdCl_2$, of which ≥70% (w/w) was non-radioactive, with a specific activity (spAc) of 6.7 cpm $pmol^{-1}$ Cd (in 15-100 keV range of the scintillation counter): 150 µl of stock was added to make up 15 L of nutrient solution to 50nM Cd. To maintain uniform conditions the tubs were mixed by constant aeration. Samples were taken during the 24 h experiment to check for changes in the Cd content of the solution, small adjustments were made but large changes were not seen because of the large volume of solution used: 1 plant per 1.5 L of nutrient solution.

Analysis of Cd content

At the conclusion of this uptake period, the plant roots were rinsed and desorbed as per the protocol outlined in Chapter 3.1: three desorption rinses, of 15 min each, with a

solution of 5 mM $CaCl_2$, 0.5 mM Citric Acid, and 10 µM $CdCl_2$ (non-radioactive), pH 3.5. The roots and shoots of each plant were then harvested into separate tubes. The roots were placed directly into scintillation fluid and incubated for >24 h prior to counting. Samples of up to 100 mg were placed in 6 ml of scintillation fluid (Ultima Gold). Root samples larger than 110 mg were divided into two vials to be combined arithmetically after analysis. The shoots were digested in a dilute solution of 0.1 M HCl and 1 mM $CaCl_2$, inside a boiling water bath, as per the method of Chapters 3.1 & 3.3.

Results

Both Fe(II) and Mn, at concentrations found in regular nutrient solutions, had a significant effect on the plant accumulation of Cd (Table 3.8). At these concentrations, Fe(II) had a very large effect on the root and shoot uptake of Cd. Mn did not influence the shoot concentration of Cd, but had a significant effect on total plant Cd content and root content in both plus and minus Si pretreated plants (Fig. 3.18).

Table 3.8: Cd accumulation over 24 h, as measured by Cd-109, for Amaroo rice under three different nutrient solution treatments: Full nutrient solution, -Mn and -Fe nutrient solution. Plants were tested with and without 12 d of Si pre-treatment (n=5). Cd was supplied at a concentration of 50 nM.

		Root weight (g $plant_{FW}^{-1}$)	Shoot weight (g $plant_{FW}^{-1}$)	Root Cd conc (nmol g^{-1})	Shoot Cd conc (nmol g^{-1})	Total plant Cd (nmol $plant^{-1}$)	Proportion of plant Cd found in the shoot
+ Silicon pre-treatment	Full	0.20 *b*	0.73 *a*	9.8 *a*	0.4 *a*	2.2 *a*	0.12 *ab*
	-Mn	0.24 *c*	0.90 *b*	11.2 *a*	0.4 *a*	3.0 *b*	0.11 *a*
	-Fe	0.22 *bc*	0.86 *b*	36.0 *d*	1.5 *c*	9.2 *e*	0.14 *b*
- Silicon pre-treatment							
	Full	0.22 *bc*	0.79 *ab*	14.3 *b*	1.0 *b*	4.0 *c*	0.19 *c*
	-Mn	0.22 *bc*	0.91 *b*	21.7 *c*	1.1 *b*	5.3 *d*	0.18 *c*
	-Fe	0.17 *a*	0.79 *ab*	66.8 *e*	3.4 *d*	14.0 *f*	0.19 *c*

N.B. Different letters, within a single column, indicate means which are significantly different by ANOVA-L.S.D. (5% sig. level, of the log-transformed values, except for last column for which there was equal variance in the untransformed data).

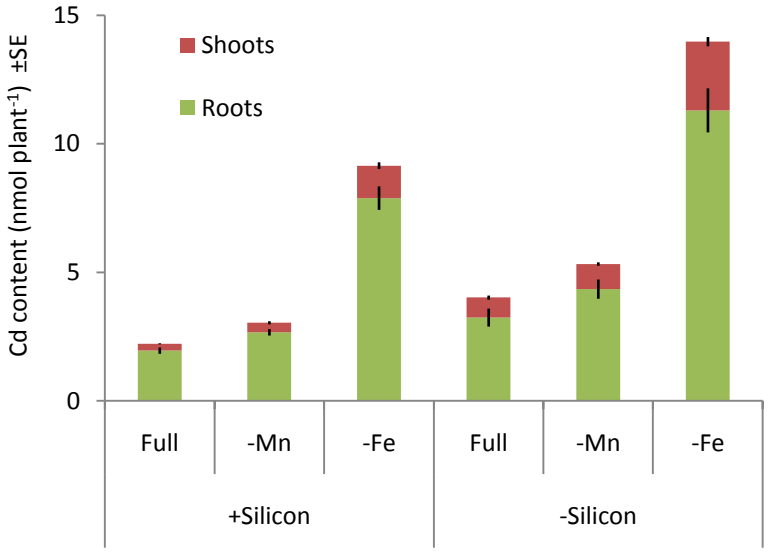


Figure 3.18: Total average Cd content of rice seedlings after 24 h uptake in 50 nM Cd nutrient solution. Plants were prepared on full nutrient solution ±Si, and then transferred to three different nutrient solution treatments during uptake. In terms of combined root + shoot totals, all means are significantly different (by L.S.D., 5% level).

Discussion

In this experiment, Si and Fe were both again shown to affect Cd uptake and translocation in rice. The important difference in this experiment was that the effect of Si was tested without the presence of Si in the nutrient solution as prior, and the competitive effect of Fe^{2+} was investigated in Fe sufficient plants.

Pre-treatment with Si for 12 d was found to reduce shoot Cd concentration by 50%, in a similar way to the result of the experiment in Chapter 3.3. Root Cd concentration was also reduced across the treatments by 30-50%. Si also had a significant effect on the partitioning of Cd, with more plant Cd found in the shoots. Rice roots are known to have a tendency to incorporate large amounts of Si during growth (Zhang *et al.* 2008a), and the observation of increased root Cd accumulation to a greater degree than shoots under -Si treatment has been noted in previous studies (Wang *et al.* 2000).

Minus Fe conditions led to a 3.5 to 4 fold increase in root and shoot Cd concentration compared to nutrient solution with Fe^{2+} added. The large effect that supply of (only transiently available) Fe(II) had on Cd uptake was surprising, and shows there was considerable competition between Fe^{2+} and Cd. This result has not been reported previously because Fe is not commonly supplied as unchelated Fe(II) in nutrition studies. It seems that chelated Fe(II) does not produce the same result, as Cheng *et al.* (2007) did not find a significant effect of 125 mM EDTA-Fe(II) on uptake of Cd from nutrient solution with 1 μM $CdSO_4$.

It was assumed that Fe deficiency responsive (IDR) gene expression was not driving the observed effect of -Fe treatment on Cd in these plants. Although the regulatory mechanisms, including Fe responsive transcription factors, begin to be upregulated just after the onset of Fe starvation (Kobayashi *et al.* 2009), it has repeatedly been found that it takes a few days for transporters such as IRT1 to increase in expression (Ueno *et al.* 2008; Yokosho *et al.* 2009).

Interestingly, when these Fe-sufficient rice plants were grown under -Fe conditions, there was a large increase in Cd uptake, but this was not associated with a significant increase in the ratio of Cd found in the shoot (Table 3.8). The lack of competitive interaction between Fe and Cd at the site of Fe membrane transporters presumably led to an overall increase in Cd uptake from the external medium when Fe was absent. Once inside the plant, this Cd would be available to internal transport mechanisms so that shoot Cd content increased as well. However, as the plants still had available internal stores of Fe, the expression of transporters for Fe at the vascular bundle was most likely not increased. The distribution ratio of Cd between the root and shoot therefore remained similar. The effect of Fe deficiency and competition between Fe and Cd were further investigated in Chapter 6.1.

When tested here, Mn supply reduced root accumulation of Cd but not shoot content (Table 1). This could have been the result of increased apoplastic Cd because of the reduced deposition of Mn plaque. The desorption method used would have removed the majority of apoplastic Cd, although some could have still remained. As it is, this result is inconclusive of competition between Mn and Cd. When tested the other way around in *Thlaspi caerulescens*, high Cd supply reduced shoot Mn accumulation (Papoyan *et al.* 2007), but the substrate specificity of TcIRT1 is not necessarily the same as OsIRT1. Interaction between Fe, Mn and Cd is discussed further in Chapters 5 and 6.

3.6: Study of the mechanisms responsible for Si-mediated reduction of Cd uptake in rice: effect of Si on root hydraulic conductivity

Introduction

In the hydroponic experiment of Chapter 3.3, on average, Si was found to have an approximate 2-fold inhibitory effect on Cd uptake in rice plants. The mechanism by which Si decreased Cd uptake was not conclusively proven. In this section, a follow-up experiment was designed to test the nature of the effect of Si on Cd uptake. The prevailing opinion is that silicon influences the uptake of cations such as Na^+, Ca^{2+} and Cd^{2+} through a reduction of transpirational flow and/or apoplastic bypass flow (Ma and Takahashi 1993; Agarie *et al.* 1998; Yeo *et al.* 1999; Gao *et al.* 2005; Shi *et al.* 2005; Ueno and Agarie 2005; Gao *et al.* 2006; Gong *et al.* 2006). The following experiments examined the effect of Si on the root hydraulic conductivity of rice seedlings.

Materials and Methods

Seedlings of the commercial Australian rice variety, Amaroo, were used in this experiment (as per the previous section). Seeds were germinated in the dark at 25^{o}C and four days later planted in ±Si hydroponic treatments. The following nutrient solution was used to grow the plants: 1.5 mM NH_4NO_3; 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.1 mM KH_2PO_4; 50 µM FeNaEDTA; 5 µM $ZnSO_4$; 0.5 µM $CuSO_4$; 5 µM $MnCl_2$; 50 µM H_3BO_3; and 0.1 µM MoO_4. The +Si treatment included 1 mM SiO_2 added as sodium silicate solution (Sigma), and the solution was adjusted back to pH 5.0 with HCl after sodium silicate was added. Plants were grown under fluorescent lights at 23^{o}C ±2ºC. Plants were used at 14 d.

Hydraulic conductivity, or the permeability of the roots to water, was measured according to the protocol outlined in Pitman and Wellfare (1978). A capillary tube was

attached to the stem of a rice seedling decapitated 0.5 cm above the seed. The rate of exudation from the seedling was first measured with the roots in nutrient solution, as above, and then the solution around the roots was changed to 300 mM mannitol for a short period, inducing a reverse flow of water out of the seedling which could be determined from the level in the capillary.

Hydraulic conductivity (L_p) was estimated using the initial rate of exudation and the back-flow of exudate according to the equation outlined below. Measurements of the rate of loss to mannitol, $J_{v,m}$, were made over the initial 2-3 min when the rate of flow was approximately constant.

$$L_p = (J_v + J_{v,m}) / (\pi_m - \pi_0)$$

J_v = rate of xylem exudate flow

$J_{v,m}$ = rate of loss to mannitol

L_p = hydraulic conductivity

π_m = osmotic pressure in mannitol solution

π_0 = osmotic pressure in nutrient solution (±Si)

Results

The addition of Si to the nutrient solution significantly reduced the hydraulic conductivity of the rice seedling root systems (Fig. 3.19). This effect was seen regardless of Si pre-treatment, which was found not to have a significantly greater effect on hydraulic conductivity than Si applied shortly before measurement.

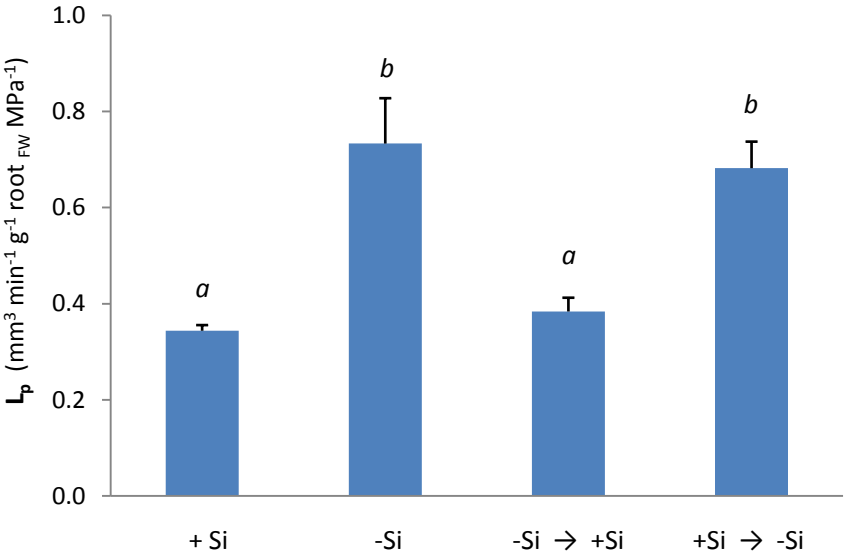


Figure 3.19: Measured root hydraulic conductivity of 14 d old rice seedlings pre-treated with 8 d of ± 1 mM Si, measured in that same nutrient solution, or transferred for 3-4 h to the opposite solution and measured. Means are shown + SE (n=5); different letters above columns represent significantly different means (ANOVA, LSD 5% level).

Discussion

Contrary to expectation, the effect of Si was not found to be associated with the gradual accumulation of Si gel in the transpiration stream in the root apoplast. Pre-treatment with +Si, for more than a week, did not result in a significant decrease in root hydraulic conductivity, as shown when tested in -Si solution. In the same way, plants tested in +Si solution had reduced hydraulic conductivity whether or not they had had a week of +Si pre-treatment. Therefore, there are inherent differences in the physical qualities of the +Si and -Si nutrient solutions which result in varying hydraulic conductivity.

It is assumed that silica that accumulates in the apoplast of the plant is not easily lost to the surrounding nutrient solution without the specific pH conditions that would dissolve it. If Si pre-treatment had had a significant effect on root hydraulic conductivity, then the +Si plants that were tested in -Si solution would have shown a different result to -Si plants tested in -Si. There is no evidence here that differences in root hydraulic conductivity are

the result of anything more than differences in the physical properties of the nutrient solution itself.

It is possible that part of the mechanism by which Si reduces Cd uptake in hydroponic rice plants is by its direct effect on the physical characteristics of the nutrient solution. Experiments in Chapter 3.3 showed an approximately 2-fold decrease in Cd uptake brought about by the addition of Si to the nutrient solution. That result matches a 2-fold decrease in root hydraulic conductivity caused by 1 mM Si in the hydroponic solution. However, Si pre-treatment was also found to have an approximate 2-fold effect on Cd uptake in the previous section (3.5), therefore, it seems unlikely that root apoplast is the main site for the reduction in Cd movement caused by Si.

Transpirational flow to the shoots of plants is more than just a product of root permeability. The rate of water movement out of transpiring leaves and stems is also of importance. Other reports have favoured leaf and stomatal conductance as the point at which Si reduces transpirational flow (Ma and Takahashi 1993; Agarie *et al.* 1998; Gao *et al.* 2006).

The findings of this experiment are contrary to the idea that the action of Si is by blocking apoplastic bypass-flow around the Casparian strip (Yeo *et al.* 1999; Shi *et al.* 2005; Gong *et al.* 2006). In view of the evidence, Si deposition in the shoot is likely more important, than deposition in roots, for reduction of Cd uptake by rice seedlings grown under hydroponic conditions. However, the ionic affinity of deposited silica for cations such as Cd and Mn, and the likelihood of being immobilised in Cd-Si complexes, is also a possible reason for the reduction in plant accumulation of Cd (Wang *et al.* 2000).

Chapter 4

Genetic basis of rice cultivar differences in grain Cd accumulation: gene expression analysis of candidate membrane transporters

Introduction

Much of the work done on Cd in the field of molecular biology has focused on what could be categorised as toxicity studies. Plant toxicity-stress responses and phytochelatins (PCs) are important for tolerance of heavy metals and metalloids when they are at high concentrations (Semane *et al.* 2007). The general theory is that PCs complex excess heavy metals in the cytosol, or vacuole, and are then stored in the vacuole and over time low-molecular-weight (LMW) PCs are converted into high-molecular-weight (HMW) PCs (Clemens 2006). However, there is a growing opinion in the literature that complexation of Cd in plants, at the low levels commonly seen in Cd-polluted soils, is not associated with PCs, but more likely associated with organic acids or the basic thiol unit glutathione, GSH (Chaney *et al.* 1999; Rauser 1999).

Aina *et al.* (2007) studied thiols in rice and observed no change in the concentrations of γ-EC (i.e. γ-Glu-Cys), GSH or PCs by two weeks of 0.1 µM Cd treatment. In contradiction to this, another study of rice, Nocito *et al.* (2011), did find significant increases of non-protein thiols (of which phytochelatins are a large constituent) after ten days of 0.1 µM Cd treatment, with HMW and LMW compounds present in equal proportions. Nevertheless, while Cd can be found to occur at high concentrations in some heavily polluted soils, the majority of agricultural soils where Cd is present are not polluted enough to induce a special tolerance response to the metal in the plant. In terms of the general threat to food

safety, the focus must be restricted to the physiological mechanisms that are relevant to realistic levels of Cd in the environment.

The predominant mechanisms for the uptake of non-essential elements are the membrane transporters constitutively expressed in plants for the uptake of basic nutrients. In the case of Cd, it has been found that this element is readily taken up and translocated by low-specificity transporters for essential cations, namely, Fe^{2+}, Zn^{2+} and Ca^{2+} (reviewed by Clemens 2006). In addition to uptake, the compartmentation of Cd in the vacuole is an important determinant of shoot accumulation (e.g. Ueno *et al.* 2010). Therefore, metal transport proteins active on the tonoplast are also of interest. An overview of these transporters in rice will be presented below.

There is considerable evidence that Cd uptake in many plants is facilitated by Fe transporters at the plasma membrane. The IRT1 gene (iron-regulated transporter 1), in a number of plant species, has been shown to transport a range of metals including Zn^{2+}, Cd^{2+} and Fe^{2+}. The evidence also points to IRT1 being responsible, to a large extent, for the increase in Cd uptake during Fe deficiency (Cohen *et al.* 1998; Korshunova *et al.* 1999; Bughio *et al.* 2002; Connolly *et al.* 2002; Lombi *et al.* 2002; Kudo *et al.* 2007). The rice version of the gene, OsIRT1, was found to facilitate Cd transport when expressed in yeast and is hypothesised to be a key part of the uptake of Cd in rice plants under common field conditions (Ishimaru *et al.* 2006; Nakanishi *et al.* 2006). Lee and An (2009) over-expressed the OsIRT1 gene in rice, causing increased Fe and Zn content, as well as increased sensitivity to excess Zn and Cd.

The involvement of a metal/H^+ antiporter that facilitates the vacuolar sequestration of Cd^{2+} has been well established in physiological studies (Salt and Wagner 1993; Koren'kov *et al.* 2002). One such transporter gene, AtCAX2, of the cation-exchanger family, mediates transport of Mn^{2+}, Zn^{2+} and Cd^{2+} into the vacuole in arabidopsis (Hirschi *et al.* 2000; Pittman

et al. 2004; Koren'kov *et al.* 2007a; Koren'kov *et al.* 2007b). The closest known rice homologues are OsCAX2 & OsCAX3 (Kamiya *et al.* 2005). The OsCAX2 gene, on chromosome 3 (Genbank Acc. No. AB112772.1), is located within one of the quantitative trait loci (QTL) for rice grain Cd accumulation which was discovered by Ishikawa *et al.* (2005b). The exact function of this putative cation transporter has not been demonstrated, but it was found to be expressed in the roots and shoots of rice plants and is likely to work cooperatively with the OsCAX3, with which it shares a high similarity (Kamiya *et al.* 2005). In barley, the homologue of OsCAX1a was found to be expressed in response to Cd stress (Schneider *et al.* 2009). It is possible that this CAX gene also transports Cd.

The NRAMP family of genes consists of a range of functional types. Initially, the presence of efflux transporters on the tonoplast was the most well known type of NRAMP, e.g. AtNRAMP3 that facilitated Fe^{2+} and Cd^{2+} efflux (Thomine *et al.* 2000; Thomine *et al.* 2003). In a proteomic study in barley, the only known NRAMP transporter in that species was found to be upregulated under mild Cd stress (Schneider *et al.* 2009), but this could have been the result of induced Fe deficiency. More recently, in rice, the gene OsNRAMP1 has been found to transport Cd^{2+} across the plasma membrane and increase shoot uptake of Cd (Takahashi *et al.* 2009; Takahashi *et al.* 2011).This gene is also located within a reported QTL for grain Cd accumulation (Ueno *et al.* 2009b). OsNRAMP1 is also a candidate for the increased transport of Cd (and Fe) under Fe deficiency stress.

Apart from the membrane transporters, a potentially important molecule in the movement and accumulation of Cd in plants is the amino acid nicotianamine (NA). There is now good evidence for the role of NA in the long distance transport of the nutrient metals Fe (II), Zn, Mn, and Cu in plants (Ishimaru *et al.* 2010). For example, Kim *et al.* (2005) demonstrated that NA-synthase (HvNAS1) overexpression in arabidopsis and tobacco resulted in increased metal tolerance and accumulation, with tobacco plants producing

increases in seed metal content of 6.6-fold Fe, 3.2-fold Zn, 2-fold Cu and 1.8-fold Mn. Accumulation of Cd was not measured by Kim *et al.* (2005), but AtNAS-overexpressing *Arabidopsis thaliana* showed increased tolerance to Cd in addition to these other metals. Importantly, the current evidence also points to NA playing a large role in some naturally occurring differences in heavy metal accumulation characteristics. The Zn and Cd hyperaccumulator, *A. halleri*, has been found to exhibit 70-fold higher root expression of NAS than the non-metallophyte *A. thaliana* (Weber *et al.* 2004).

The key difference in the role of NA between dicots and graminaceous plants is that dicots do not utilise NA for phytosiderophore production, but rather, it is used in plant metal homeostasis internally. In a similar way to its Fe uptake strategies, rice appears to have a combination of dicot and graminaceous mechanisms at work as rice plants upregulate NAS and accumulate NA in shoots as well as roots during Fe deficiency, unlike the other grasses (Higuchi *et al.* 2001). Consistent with the experiments in dicots, NAS overexpression in rice also resulted in increased Zn and Fe content in shoot and grain (Masuda *et al.* 2009; Zheng *et al.* 2010).

It is well known that Cd can move in plants via a shared pathway with Zn. It has been found repeatedly that application of Zn reduces the accumulation of Cd in cereal plants (Hart *et al.* 2005; Wu *et al.* 2005). Hart *et al.* (2002) concluded that Zn and Cd share a common plasma membrane (PM) transporter, which could be one of the ZIP-family genes, of which IRT1 is one. In soil-grown rice, Ishikawa *et al.* (2005a) found that shoot Cd was correlated with Zn and Mn concentration in a large range of genotypes.

In a number of model plant species, the expression of P_{1B}-ATPase (or HMA) genes has been found to be associated Zn/Cd hyperaccumulation. One of the key genes in this family, designated HMA4 in most species (i.e. AhHMA4, AtHMA4, TcHMA4), has been localised on the PM adjacent to the xylem and is probably involved in xylem loading of metals (Mills *et*

al. 2003; Papoyan and Kochian 2004; Verret *et al.* 2004; Courbot *et al.* 2007). There are three highly similar genes of this grouping in arabidopsis that are putative Zn/Cd/Pb/Co transporters: AtHMA 2; 3; & 4. The evidence points to AtHMA2 being located on the plasma membrane like AtHMA4, and together with that gene, seems to account for nearly all root to shoot Cd translocation in arabidopsis (Wong and Cobbett 2009). AtHMA3 is found on the tonoplast and mediates vacuolar sequestration of the Zn/Cd/Pb/Co (Gravot *et al.* 2004; Williams and Mills 2005; Morel *et al.* 2009). In the rice genome, the most homologous genes to these transporters are OsHMA 2 and 3 (Baxter *et al.* 2003; Williams and Mills 2005).

Until recently, the only HMA genes in rice that had been studied were the Cu-transporting members of this family, OsHMA 4-9 (Lee *et al.* 2007). However, since OsHMA3 was found within a quantitative trait locus (QTL) for Cd accumulation (Ueno *et al.* 2009b), a lot more attention has been given to this family of genes in rice. It has been found to be a constitutively expressed tonoplast transporter in roots, highly selective for Cd sequestration to the root vacuoles (Ueno *et al.* 2010). The reason for this gene's location within a QTL for Cd accumulation is that the version of the OsHMA3 gene present in some *indica* cultivars is non-functional, leading to reduced Cd storage in roots and greater translocation to the shoots (Ueno *et al.* 2010; Miyadate *et al.* 2011). The evidence for the role of OsHMA2 in Cd accumulation is also building. Nocito *et al.* (2011) demonstrated, using heterologous expression in yeast, that OsHMA2 transports Cd. Satoh-Nagasawa *et al.* (2012) have now shown that OsHMA2 is a Zn transporter that is also able to facilitate root to shoot translocation of Cd.

The mechanism controlling Cd accumulation is to some degree independent of uptake and homeostasis of other metals in the plant. Despite the fact that Cd is found to be taken up by transporters for metals such as Zn, Mn, Cu and Fe, when high and low Cd

accumulators are tested in QTL studies, these other metals rarely also differ to the same degree as Cd (Ueno *et al.* 2009a; Ueno *et al.* 2009b; Ishikawa *et al.* 2010). The functionality of OsHMA3 *in planta* demonstrated this selectivity: overexpression caused large differences in Cd translocation; this was not the case for other nutrient metals (Ueno *et al.* 2010).

Si supply is another determinant of Cd uptake in rice plants (Chapter 3.3). Rice cultivars can differ in the expression of genes which have been shown to cause root Si accumulation (Ma *et al.* 2007). Therefore, theoretically, the expression of Si uptake genes could be a cause of genotypic differences in Cd accumulation.

The aim of this section was to assess the genotypic differences in the expression of genes that potentially determine the accumulation of Cd in rice grain. A number of genes were selected, which are all very likely to transport Cd in rice plants or influence its accumulation; these were: OsIRT1, OsIRT2, OsHMA2, OsHMA3, OsCAX2, OsCAX1a, OsNRAMP1 OsLsi1, OsLsi2 and OsNAS1. Real-time RT-PCR (reverse-transcriptase polymerase chain reaction) was used to assess the expression of these particular genes in rice varieties which differ in their accumulation of Cd.

It would be useful to know which genes are critical in determining the ability of the roots to store Cd in the vacuole or influence the rate of Cd movement from root to shoot. In this section, the results of two phases of a gene expression study of rice cultivars are presented. The first section covers analysis of the four Chinese rice lines studied in Chapter 3. The second section presents the study of eight different rice cultivars with larger differences in grain Cd accumulation characteristics. Finally, the last section presents the results of an investigation into potential genetic differences between Cd transporters in the *japonica* and *indica* subspecies.

4.1 Gene expression analysis of four Chinese rice cultivars previously characterised for Cd uptake

Introduction

Despite what was found in previous published trials in paddy soil concerning the Cd accumulation characteristics of the four Chinese rice cultivars studied here, repeated testing under hydroponic culture and in potting mix showed that N07-60 and N07-63 were the highest Cd accumulators (see Chapter 3). This was found for both shoot and grain, and although it is still possible that under field conditions the ranking is different, for the purposes of this study, the results gained in these experiments have to be taken into account. It is likely that genotype by environment (GxE) interactions are influencing the performance of these cultivars.

For N07-38 and N07-6, the results were more variable, but there were significant morphological differences between these two cultivars, with the tall and fast-growing N07-38, and the slow-growing and smaller N07-6 variety. N07-6 was most consistently the lowest shoot Cd accumulator in both solution culture and potting mix. N07-38 had the greatest rice yield when tested in a growth room with potted soil (Chapter 3.2) and when harvested pre-maturity, prior to flowering, N07-38 had a shoot Cd concentration not significantly different to N07-60 and N07-63.

In this section, gene expression of putative Cd transporters was studied in rice seedlings grown under hydroponic conditions. For simplicity, gene expression was tested in the absence of Cd. An assumption was made that the membrane transporters important in allowing Cd uptake into plants are those needed for other nutrient metals, and are not expressed in response to low concentrations of Cd (nearly all of these genes were

examined again in the experiments of Chapter 6.1, where gene expression was measured in ±Cd ±Fe, and this assumption was proven to be correct).

Materials and Methods

Plant material

Twenty seeds of each of the four rice varieties were surface sterilised with 1.5% hypochlorite bleach solution for 20 min. The seeds were then rinsed four times with reverse-osmosis (RO) water before being germinated in sealed trays, on paper towel moistened with deionised water (dH_2O). The trays were stored in the dark in a plant growth room for seven days (night and day temperatures of 20^oC and 28^oC respectively). The seedlings were then transferred to floating plastic mesh in containers of 20% modified Hoagland's solution, containing: 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.4 mM $MgS0_4$; 0.2 mM KH_2PO_4; 20 µM FeNa-EDTA; 9 µM H_3BO_3; 1.82 µM $MnCl_2$; 0.22 µM Na_2MoO_4; 0.25 µM $ZnSO_4$; 0.06 µM $CuSO_4$. The plants were grown under fluorescent lights (flux density of 150 µmol m^{-2} s^{-1}) at room temperature (approx. 20-25^oC), with 16 hours light and 8 hours darkness. The nutrient solution was topped up with dH_2O to account for transpiration and the nutrient solution was completely replaced after one week. Ten days after transfer to nutrient solution (17 DAS), the plants were harvested for RNA extraction.

RNA extraction and RT

The entire root systems of the 17-day old plants were harvested, with three plants used for each replicate of the cultivars. Approximately 80-100 mg (fresh weight) made up each pooled sample. Each sample was immediately frozen in liquid nitrogen and then stored at -80ºC prior to RNA extraction. Following grinding in liquid nitrogen, Trizol® reagent (Invitrogen) was used to extract RNA according to the manufacturer's instructions. Sample

concentration was quantified using a UV spectrophotometer; and then every RNA sample was treated with RNase-free DNase I (NEB) to remove any contaminating genomic DNA. To satisfactorily remove gDNA, 2 units of DNase (1 µl) were used per 5 µg of RNA (100 ng μl^{-1} RNA concentration), and samples incubated for 30 min at 37^{o}C. The DNase was inactivated according to the manufacturer's instructions.

First-strand cDNA was generated from approximately 400 ng of RNA template using oligo-dT primers (to select for mRNA) and Omniscript® Reverse Transcriptase (Qiagen) according to the manufacturer's instructions.

Gene-specific primers

Genes known or suspected to transport Cd were selected for gene expression analysis. For simplicity, in the case of genes for which there are published quantitative PCR studies, the primers used in those studies were employed. Two internal control genes, 'Ubiquitin-conjugating enzyme E2' and 'Eukaryotic elongation factor 1-alpha,' were also analysed for normalisation of the expression data (see Table 4.1). These two genes were selected for normalisation based on their validation in rice as very stably expressed genes, compared to a range of other house-keeping genes (Jain *et al.* 2006). Primer pairs of genes for which there were no published qPCR studies were designed using the PerlPrimer software program, v1.1.14 (Marshall 2004). These primers were tested and annealing temperatures optimised with standard PCR using Taq polymerase (Fisher Biotech). The reaction products were visualised using 1.5% agarose gel electrophoresis and stained with ethidium bromide.

The sequence fidelity of the OsHMA 2 & 3 primers was confirmed by sequencing of the isolated PCR gel-product. The product was cut from the gel and after purification, was ligated into the pGEM®-T Easy Vector (Promega) and then inserted into DH5α (chemically-

competent) *E. coli* cells. Successfully transformed cultures were selected from overnight cultures grown on LB media agar plates treated with 100 µg/ml ampicillin. These colonies were then cultured in ampicillin-treated LB broth overnight and the pGEM-T plasmid DNA was recovered with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). The purified plasmids were then sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Capillary sequencing was carried out by the South Australian Institute of Medical and Veterinary Science (IMVS) Sequencing Centre.

Gene expression

Real-time quantitative PCR (qPCR) was performed with the Rotor-Gene™ 6000 (Corbett Research). Reactions were performed in a volume of 10 µl with the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). Each cDNA sample was run in duplicate. Gene quantification was performed using standard curves for each primer pair, from a serial dilution of cDNA template, over more than three orders of magnitude. The amount of sample cDNA to be used per reaction was selected to enable quantification based on Ct values in the range 24-29 cycles. Transcript level was then expressed as an amount relative to the cDNA pool used for the standard curve. These expression levels were then normalised against the geometric mean of the two internal controls.

Results

Small differences in gene expression were found between the four varieties for some of the genes tested. Table 4.2 presents the results from the seedlings grown in nutrient solution under control conditions. Of the target genes tested, only OsIRT1 and OsHMA2 expression in roots produced highly significant differences between the cultivar means (i.e. ANOVA F

pr. < 0.01). Figure 4.1 displays these two results graphically. The low-Cd accumulator, N07-6 had the lowest expression of both of these genes.

Table 4.1: Primer pairs used for RT-qPCR reactions to measure gene expression.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Ampl-icon length (bp)	Single/ Mult. exons	Aneal-ing temp. used	Primer Reference; or Rice Ensembl Gene ref. for designed primers
OsIRT1	CGTCTTCTTCTTCTCCACCACGAC	GCAGCTGATGATCGAGTCTGACC	357	M	64^{o}C	(Ishimaru *et al.* 2006)
OsIRT1	GATGTGCTTCCACCAGATGTTC	GAAGAAGACGAGCACCGA	97	S	63^{o}C	Os03g46470
OsIRT2	GTCATTGTGCAGGTTCTCG	CGAACATCTGGTGGAAGC	129	M	60^{o}C	Os03g46454
OsCAX2	GATGTTGTTGTACTGATGCC	CTGGCACCGTTTGGAAATGT	103	S	60^{o}C	(Kamiya *et al.* 2005)
OsCAX1a	TCAAGAACAAGCTGGACATCAC	GCAGCTTGAAGTCAAGATCC	131	M	61^{o}C	Os01g37690
OsHMA2	TCTCCCAATCCCAAATCGTC	CCAGAAGAAGTCCGCAGAG	134	M	60^{o}C	*PlantsT* - 64490
OsHMA3	CAAATCCATCCAACCAAACC	GCCCTTGATCATCTCACC	169	M	60^{o}C	Os07g12900
OsNRAMP1	AGGAATGAAGGATGTCTGTAG	AGCATCTTCTGGTGAAAGG	136	M	59^{o}C	Os07g15460
OsNRAMP2	CTGGAGAACTATGGAGTGAGA	GAACCACCAAACCAATCAGA	133	M	60^{o}C	Os03g11010
OsNAS1	ATCGAAAGGCGCACTATATTATGG	TGGCATGTTCCTCGTTTACAC	227	M	63^{o}C	Os03g19427
OsLsi1	CGGTGGATGTGATCGGAACCA	CGTCGAACTTGTTGCTCGCCA	255	M	64^{o}C	(Ma *et al.* 2007)
OsLsi2	ATCTGGGACTTCATGGCCC	ACGTTTGATGCGAGGTTGG	101	?	61^{o}C	(Ma *et al.* 2007)
Os*e*EF-1α	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA	103	M	60^{o}C	(Jain *et al.* 2006)
OsUBC	CCGTTTGTAGAGCCATAATTGCA	AGGTTGCCTGAGTCACAGTTAAGTG	76	M	65^{o}C	(Jain *et al.* 2006)

Table 4.2: Relative gene expression of membrane transport protein in roots of four rice cultivars. Mean normalised expression in cDNA, against a geometric mean of the expression of OsUBC and Os*e*EF1α. Letters within a single column (and tissue type) show significant differences by L.S.D. 5% level. ANOVA result, F pr., is indicated by */** or *n.s.* Bold numbering indicates significantly higher or lower expression of that gene transcript relative to the majority of the other cultivars.

	OsHMA2	OsHMA3	OsIRT1	Os-NRAMP1	Os-NRAMP2	OsCAX2	OsLsi1	OsLsi2
Root cDNA (n=4)	**	(F pr. 0.1)	**	*	*	(*n.s.*)	*	(*n.s.*)
Low grain Cd accumulators								
N07-6	2.5 *a*	1.2 *ab*	4.0 *a*	1.18 *a*	2.4 *a*	3.4	2.9 *ab*	2.8
N07-60	4.6 *b*	1.5 *ab*	6.7 *ab*	1.64 *a*	2.3 *a*	1.9	3.0 *ab*	3.7
High grain Cd accumulators								
N07-63	4.7 *b*	0.9 *a*	**10.5** *c*	2.67 *ab*	1.9 *a*	3.5	1.9 *a*	3.0
N07-38	**8.4** *c*	1.8 *b*	6.9 *b*	**5.54** *b*	3.3 *b*	2.6	5.0 *b*	4.7

Shoot cDNA (n=3)	*	(*n.s.*)	(*n.s.*)
Low grain Cd accumulators			
N07-6	0.00 *a*	0.02	0.2
N07-60	0.10 *b*	0.02	0.3
High grain Cd accumulators			
N07-63	0.13 *b*	0.00	0.2
N07-38	0.24 *b*	0.02	0.4

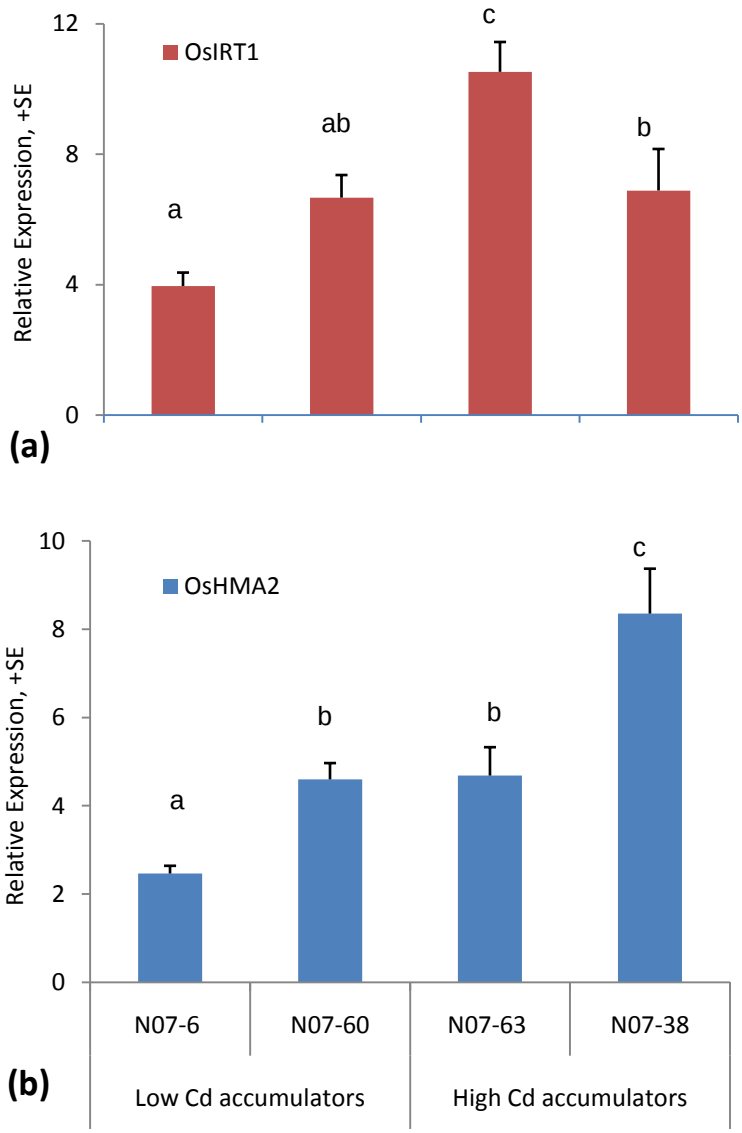


Figure 4.1: Average relative root expression of two putative Cd transporters (**a**, OsIRT1, **b**, OsHMA2) in four rice varieties which differ in grain Cd accumulation characteristics. cDNA synthesised on mRNA templates from rice seedlings grown in nutrient solution, under control conditions. Values shown are normalised against the geometric mean of relative expression of two house-keeping genes. Different letters indicate means which are significantly different, by L.S.D. 5%.

Discussion

These experiments did not demonstrate any strong trend between the expression of cation transporters and grain Cd-accumulation characteristics seen in plants grown in paddy soil or nutrient solution (Chapter 3.1-3.2). For both IRT1 and HMA2, there was significantly greater expression in at least one of the higher Cd accumulating cultivars. It is not clear whether this pattern of expression is related to the accumulation of Cd for the varieties, but it is possible that the mechanism responsible for high grain Cd in one variety is not the same in all high accumulators.

The OsIRT1 gene can facilitate Cd movement across the root plasma membrane; therefore this gene could contribute to the higher Cd content of N07-63.

Nocito et al (2011) demonstrated that OsHMA2 transports Cd, but concluded that it was involved in sequestration in root vacuoles. The greatest expression in this experiment was seen in a high-Cd accumulator variety, and the lowest was seen in a low-Cd accumulator. Nevertheless, the association between the Cd accumulation of N07-38 and OsHMA2 warrants further exploration.

The results of this section of the study were fairly equivocal; therefore, a new group of rice cultivars with greater variation in Cd accumulation was sourced from a Japanese germplasm bank to further test the hypothesis that expression of these genes is related to Cd accumulation characteristics, as described in Chapter 4.2.

4.2 Screening of additional germplasm for expression of genes putatively involved in Cd uptake and accumulation

Introduction

There have been many studies published comparing genotypic variation in Cd accumulation in cereal crops. For rice, there have been some comprehensive studies using Asian rice varieties (Liu *et al.* 2005a; He *et al.* 2006; Yu *et al.* 2006) and worldwide rice collections (Arao & Ae 2003; Ueno *et al.* 2009a). For the following study, cultivars were selected from the research of Arao and Ae (2003) and Ueno *et al.* (2009a) to further analyse the variation in gene expression. Varieties were selected that have exhibited large differences in Cd accumulation, with preference given to germplasm for which more in-depth studies exist that confirm the Cd accumulation characteristics. Table 4.2.1 summarises the varieties used.

Germplasm was sourced from the National Institute of Agrobiological Sciences (NIAS; Tsukuba, Japan). Three low grain Cd accumulators were selected, Shwe War, Nipponbare, and Koshihikari; one intermediate Cd accumulator, Kasalath; and three high Cd accumulators, Badari Dhan, Habataki, and Milyang23. In addition, Lac23 was tested, which is a unique cultivar, found to have high shoot Cd accumulation, but low grain accumulation; presumably because of reduced translocation to the grain (Ishikawa *et al.* 2005a).

Table 4.3: Rice germplasm, with variable Cd accumulation, used here to study the genetic basis of differences in Cd accumulation.

Name	Grain Cd accumulation	Subspecies	Country of Origin	References
Shwe War	Low	*Japonica*	Myanmar	(Ueno *et al.* 2009a)
Nipponbare	Low	*Japonica*	Japan	(Kashiwagi *et al.* 2009; Ueno *et al.* 2009b)
Koshihikari	Low	*Japonica*	Japan	(Ishikawa *et al.* 2005b)
Lac23	Low	*Indica*	Sierra Leone (upland)	(Ishikawa *et al.* 2005a)
Kasalath	Medium	*Indica*	India	(Ishikawa *et al.* 2005b; Kashiwagi *et al.* 2009)
Badari Dhan	High	*Indica*	Nepal	(Ueno *et al.* 2009a)
Habataki	High	*Indica*	Japan	(Uraguchi *et al.* 2009; Ishikawa *et al.* 2010)
Milyang23	High	*Indica* cross	Korea	(Arao and Ae 2003; Murakami *et al.* 2008; Yan *et al.* 2010a);

Materials and Methods

Plant material

Seeds of each of the rice varieties were surface sterilised with 1.5% hypochlorite bleach solution for 20 min, rinsed with RO water and placed out on separate trays with paper towel moistened with deionised water (dH_2O). The seed were germinated in a dark incubator for six days (29^oC). Six days after sowing (DAS) the seedlings were transferred to truncated 1.5 ml Eppendorf tubes, suspended in the lid of blackened plastic tubs (see Figure 1, in Chapter 1). The plants were grown for 15 days on a basic rice nutrient solution (1.5 mM NH_4NO_3; 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.1 mM KH_2PO_4; 50 µM FeNaEDTA; 2.5 µM $ZnSO_4$; 0.25 µM $CuSO_4$; 2.5 µM $MnCl_2$; 25 µM H_3BO_3; 0.05 µM MoO_4). At 21 DAS, plant roots were harvested for RNA extraction. Up to 100 mg was sampled from individual plants (n=4). Extraction, cDNA synthesis and qPCR were performed as per Chapter 4.1.

Iron deficiency experiment

At 22 DAS, after rinsing with dH_2O, the remaining plants were transferred to the ±Fe treatments. At 34 DAS, 12 d after the beginning of ±Fe, roots were again harvested for RNA extraction (n=4). Only four of the varieties, Shwe War, Nipponbare, Badari Dhan and Habataki, were processed for RNA extraction, based on the expression results of the first harvest date. In addition to the membrane transporters mentioned above, expression of nicotianamine synthase 1 (OsNAS1) was also examined as an additional indicator of plant Fe deficiency response.

Results

Given the number of cultivars tested here, and the inherent variability in qPCR, L.S.D. values at a 1% level of significance were used to compare the gene expression results from this study. Table 4.4 is a summary of all the results, from which selected cultivars are displayed graphically in Figures 4.2 and 4.3. The CAX genes tested here are not displayed graphically, because apart from Lac23, the differences between the cultivars were not significant (Table 4.4).

The most obvious differences between the high and low Cd accumulators were for the expression of Fe-nutrition-related genes. Overall, except for Milyang23, the high Cd accumulating varieties showed greater root expression of these genes than the low-accumulators. There were some exceptions, but there was a pattern of difference between the two groups. The results for Kasalath, the purported ‘intermediate accumulator‘ (Arao and Ae 2003), were more variable, but were not meaningfully different to the high-Cd accumulators.

The differences in average expression for the HMA genes were not large (Fig. 4.2). For OsHMA2, Badari Dhan had the lowest expression and Milyang23 and Shwe War had the greatest. For OsHMA3, apart from Nipponbare and Koshihikari, there was not a significant difference between any of the cultivars. For these two genes, there were no logical patterns of expression differences that related to Cd-accumulating characteristics.

Table 4.4: Relative gene expression of membrane transport proteins (and OsNAS1) in roots of eight rice cultivars. Mean normalised expression in root cDNA (n=4), against a geometric mean of the expression of OsUBC and Os*e*EF1α. Letters within a single column (and tissue type) show significant differences by L.S.D. 1% level. ANOVA result, F pr., is indicated by **/***, or *n.s.* Bold numbering indicates significantly higher or lower expression of that gene transcript relative to the majority of the other cultivars.

Cultivar details			OsHMA2		OsHMA3		OsIRT1		Os-NRAMP1		OsNAS1		OsCAX1a		OsCAX2
subsp.	**Grain Cd accum.**	**Name**	***		**		**		**		***		***		(*n.s.*)
Ind	High	Badari Dhan	**0.6**	*a*	0.4	*a*	**0.9**	*c*	**5.0**	*b*	4.1	*bc*	1.6	*ab*	0.7
Ind	High	Habataki	1.1	*b*	0.6	*ab*	0.3	*a*	**4.6**	*b*	6.8	*bc*	1.8	*ab*	1.0
Ind	Med	Kasalath	1.4	*bc*	0.5	*ab*	0.7	*bc*	3.1	*ab*	**8.1**	*c*	1.7	*ab*	0.9
Ind	Low	Lac23	1.4	*bc*	0.6	*ab*	0.5	*ab*	2.5	*ab*	5.8	*bc*	**6.0**	*c*	0.7
Cross	High	Milyang23	**2.3**	*d*	0.5	*ab*	0.3	*a*	1.0	*a*	**1.7**	*a*	2.3	*b*	0.7
Jap	Low	Shwe War	**1.7**	*c*	0.4	*a*	0.5	*ab*	1.1	*a*	**0.9**	*a*	2.1	*ab*	0.7
Jap	Low	Nipponbare	1.1	*ab*	**1.1**	*c*	0.3	*a*	2.1	*ab*	4.0	*b*	1.3	*ab*	0.8
Jap	Low	Koshihikari	1.0	*ab*	0.9	*bc*	0.3	*a*	1.0	*a*	**1.6**	*a*	**1.2**	*a*	0.7

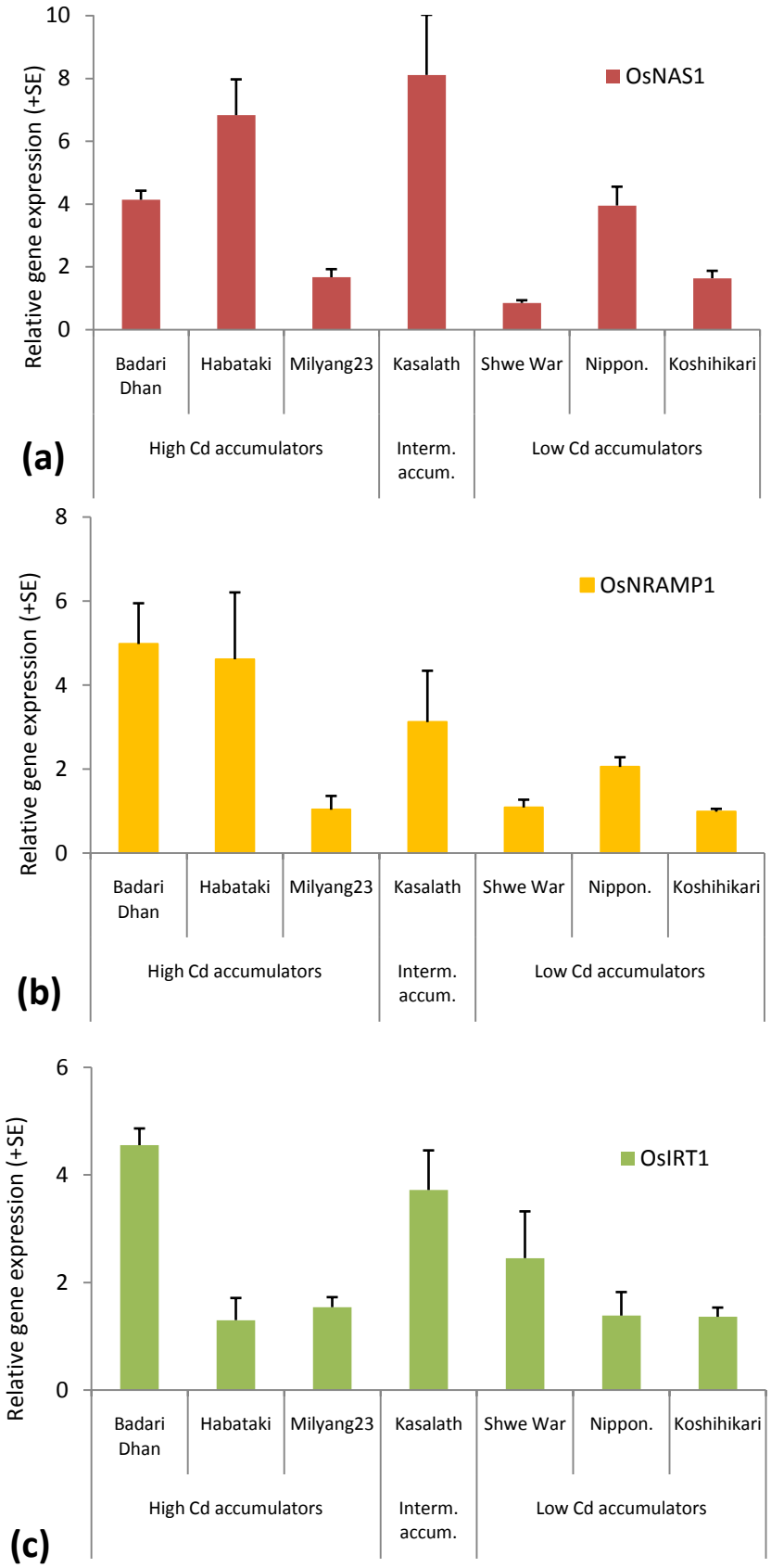


Figure 4.2: Expression of selected iron-nutrition-related genes (**a**, OsNAS1; **b**, OsNRAMP1, **c**, OsIRT1), under control conditions, in roots of rice cultivars which differ in grain Cd accumulation characteristics. Values shown are mean expression in root cDNA (n=4) relative to a single cDNA pool, normalised against two house-keeping genes. Significant differences between means is indicated in Table 4.4.

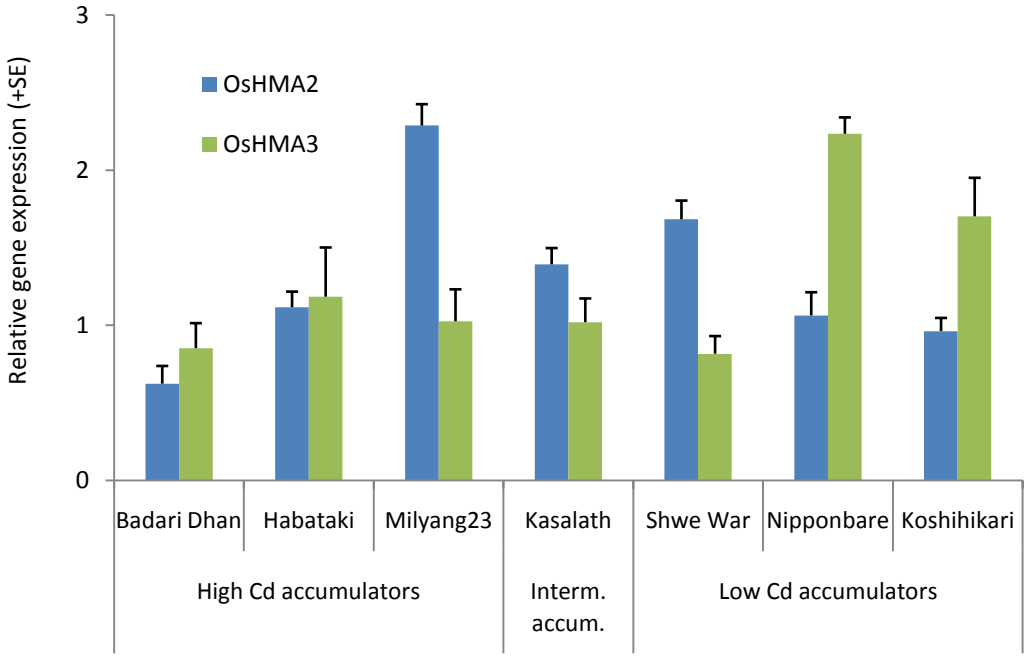


Figure 4.3: Expression of two P_{1B}-ATPases, of the HMA gene family, in roots of rice cultivars which differ in grain Cd accumulation characteristics. Values shown are mean expression in root cDNA (n=4), relative to a single cDNA pool, normalised against two house-keeping genes. Significant differences between means are indicated in Table 4.4.

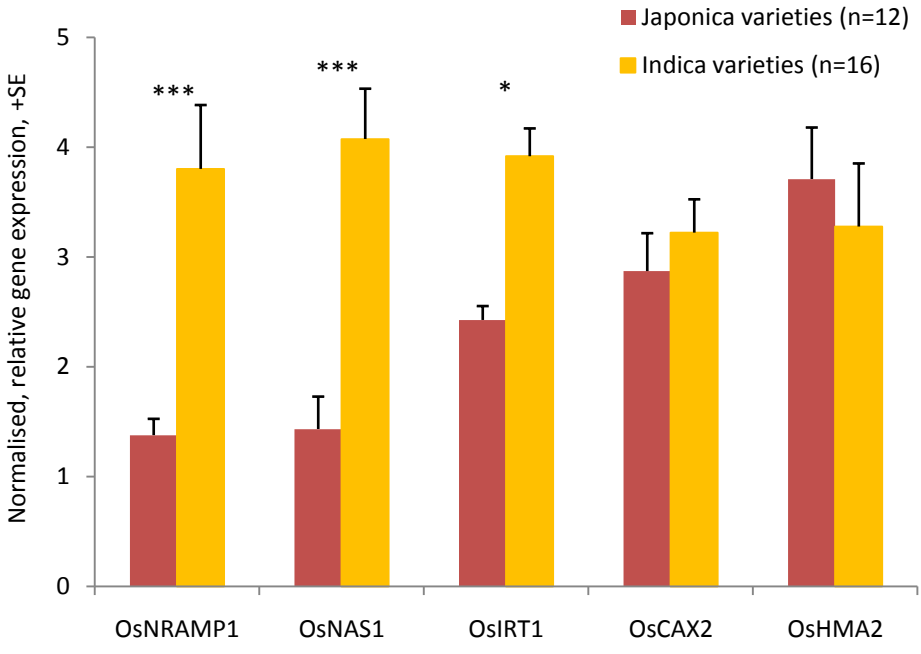


Figure 4.4: A comparison of root expression, between the subspecies of rice, of genes potentially involved in the uptake and translocation of Cd in rice plants. The overall mean relative expression from cDNA of four *indica* varieties and three *japonica* varieties (Milyang23 excluded). Asterisks indicate means, for individual genes, which are significantly different by ANOVA and L.S.D.

Iron deficiency experiment

Four of the cultivars tested above were continued in ±Fe treatment for a further 12 d. Figures 4.5 and 4.6 display the Fe-deficiency responses of these cultivars, indicated by the expression of the Fe-nutrition-related genes OsNAS1 and OsNRAMP1. It can be seen that the responses of Badari Dhan and Habataki to 12 days of -Fe treatment were greater than that of Shwe War and Nipponbare. Under -Fe, there was higher expression of these genes and also greater upregulation relative to the respective +Fe control.

For an unexplained reason, the level of IRT1 expression in these samples was found to be too low for reliable quantification. This could have been a problem with RNA quality or the RT reaction, but the results for all other genes exhibited good replicate variability.

The HMA genes had previously been found to be unaffected by Fe deficiency (see Chapter 5.1), and so were not examined here.

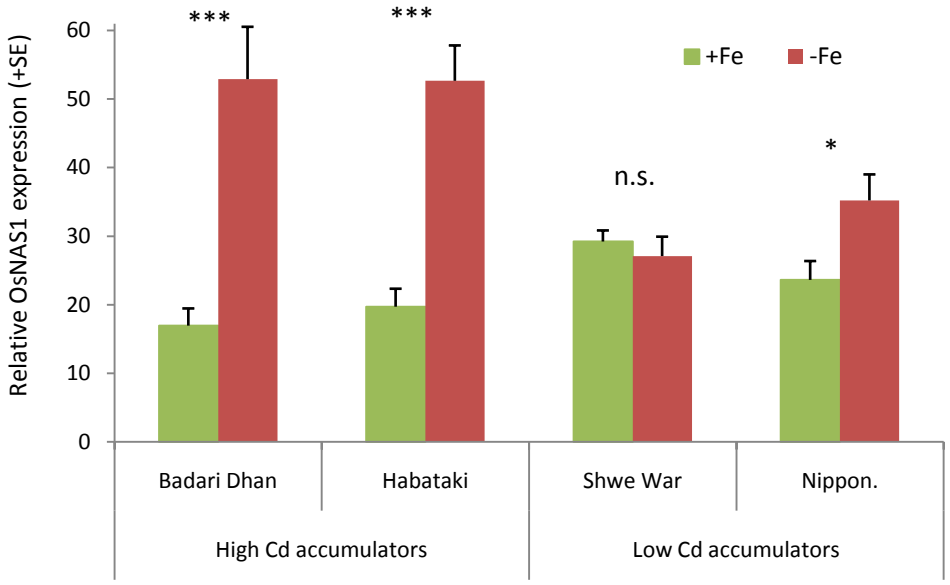


Figure 4.5: Average relative OsNAS1 expression in the roots of four rice cultivars after 12 d of ±Fe treatment in nutrient solution (34 DAS). Asterisks indicate treatment means, *for individual cultivars*, which are significantly different by ANOVA and L.S.D., of log-transformed values.

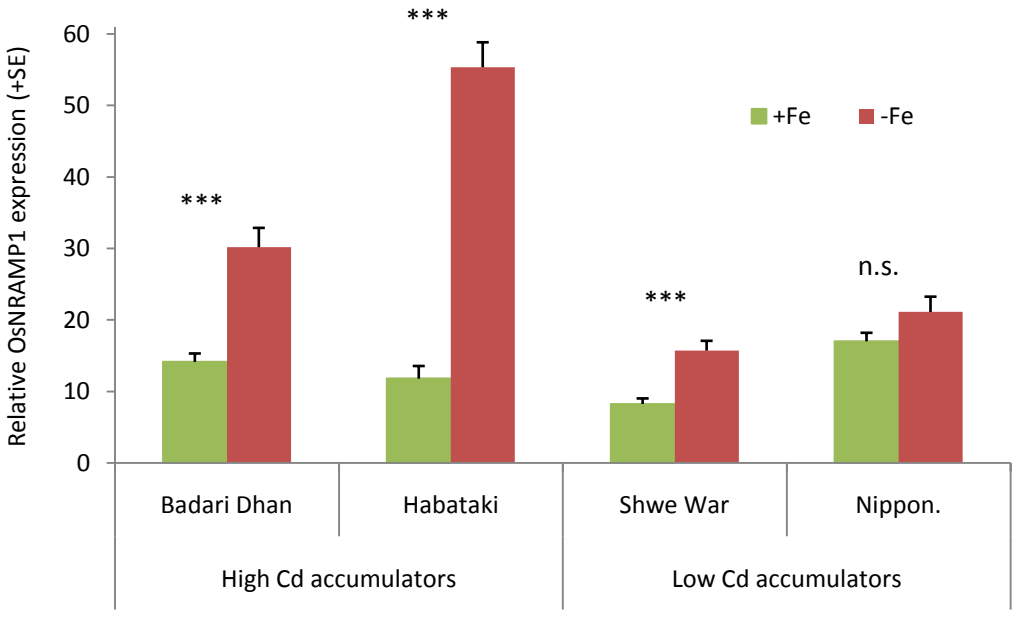


Figure 4.6: Average relative OsNRAMP1 expression in the roots of four rice cultivars after 12 d of ±Fe treatment in nutrient solution (34 DAS). Asterisks indicate treatment means, *for individual cultivars*, which are significantly different by ANOVA and L.S.D., of log-transformed values.

Discussion

Much larger variation in gene expression was seen in these cultivars than the four Chinese *japonica* lines compared in Chapter 4.1. Not all of the genes showed significant difference, but in general, the high Cd accumulators showed higher expression for genes related to Fe-nutrition.

The rice subspecies, *indica* and *japonica*, characteristically differ in their ability to accumulate Cd (Yan *et al.* 2010b). With the exception of Milyang23, an *indica-japonica* cross, all of the high-Cd accumulators tested here are also *indica* varieties, and conversely, all of the low-accumulators are *japonica*. Another exception was Lac23, but Lac23 is known to have paradoxically high shoot Cd but low grain Cd. When all samples of the cultivars of the two subspecies were compared, excluding the 'cross' Milyang23, a clear difference due to Fe nutrition was seen between them under these conditions (Fig. 4.4). The explanation for this must be either that *indica* rice varieties have a higher constitutive baseline regulation of the Fe-uptake pathway or that these rice lines were experiencing some degree of Fe stress in this nutrient solution. Either way, this could indicate a higher Fe requirement in the *indica* varieties.

The propensity to greater Fe stress was seen also in the Fe deficiency experiment. The two *indica* cultivars, which are also high-Cd accumulators, displayed greater upregulation of Fe-deficiency-responsive genes than the low-Cd, *japonica* varieties (Figs 4.5 & 4.6).

In addition to the larger differences in root gene expression, larger morphological differences were also present between these cultivars. For example, there were some notable differences in plant size, with Badari Dhan being the largest and fastest growing, and Nipponbare the smallest and slowest growing. To some degree, these morphological

differences could partially explain the magnitude of the response to -Fe treatment in Badari Dhan, as the faster growing plants would experience deficiency more quickly.

The root expression of the OsCAX1a gene did not differ substantially between these varieties. The exception was Lac23, which stood out with much stronger OsCAX1a expression than all the others. The exact mechanism of CAX1a is not identified here, but it is upregulated by Cd toxicity and is probably located on the tonoplast (Schneider *et al.* 2009).

In this experiment, differences were seen between cultivars of the two rice subspecies, *indica* and *japonica*, in terms of expression of genes related to Fe nutrition. Given that Fe deficiency caused increased expression of these genes and also increased shoot Cd uptake (see Chapter 6.2), it is possible that this is a mechanism by which *indica* cultivars accumulate higher concentrations of above-ground Cd. The differences in gene expression presented here need to be validated under conditions other than those used for confirmation of the genotypic differences. Nevertheless, despite differences in morphology and growth rate, the differences seen under control conditions and under minus Fe treatment, especially for OsNRAMP1, are worthy of further investigation.

4.3 Genetic analysis of selected candidate genes: OsNRAMP1 and OsHMA3

Introduction

There are a number of mechanisms by which genotypic difference in Cd accumulation could be related to a particular membrane transporter protein. These include: (1) differences in a gene promoter or regulatory sequence (often occurring in the UTR of the gene transcript), meaning that a gene is differently expressed under the same conditions; (2) functionality of the gene itself, because of amino acid sequence differences caused by mutation in the gene sequence (e.g. nucleotide substitution); and (3) indirect physiological differences in the genotypes, i.e. the expression/translation of the gene/protein in question is influenced by another gene product or physiological/morphological difference in the plant.

Investigation of the sequences of some of the above membrane transporters found in online databases identified some potential differences in gene sequence between *indica* and *japonica* rice lines. As Cd accumulation characteristics seemed to segregate commonly on subspecies lines, gene sequence differences between them could be a source of the physiological differences in the functioning of these genes in Cd accumulation.

OsNRAMP1 – sequence analysis

Two slightly different versions of the OsNRAMP1 gene were available on the GenBank database. In one *indica* version of the gene (L41217.1 or S81897.1 from *O. sativa*, cv. IR36), 30-40 bp downstream from the start-codon of the OsNRAMP1 ORF, three base pairs are missing that are present in the available *japonica* sequence (in multiple accessions, NM_001065850.1 from *O. sativa*, cv. Nipponbare and DQ431468.1 from *O. sativa*, cv. JingDao21), see below. If this is a real variant sequence, it would result in the loss of one

amino acid, plus alteration of the next two codons, and could potentially alter the functioning of this gene.

```
                    ....|....| ....|....| ....|....| ....|....| ....|
                              1       10        20        30        40
"Japonica version"  ATGGGGGTGA CGAAGGCGGA GGCGGTTGCC GGCGACGGCG GGAAG
"Indica version"    ATGGGGGTGA CGAAGGCGGA GGCGGTTGCC G-CGACGG-- GGAAG
```

When these contrasting sequences were tested in the SOSUI protein prediction software, version 1.11 (Hirokawa *et al.* 1998; http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html), the "*japonica*" sequence resulted in 11 transmembrane domains, whilst the "*indica*" sequence only had 10, and so the two ends of the protein were predicted to be on the same side of the membrane.

OsHMA3 – 3' RACE

Using the *Gramene* database, a comparison of the *indica* and *japonica* genomic sequences for the OsHMA3 gene showed that there was a significant sequence difference 370-420 bp downstream from the stop-codon of OsHMA3. The available CDS data for OsHMA3 all terminated at the stop-codon and so information about the 3' UTR was unavailable on *GenBank* and *Gramene*. The sequence in question is displayed below. In order to investigate whether there was a difference in the 3' UTR of the *indica* and *japonica* versions of the OsHMA3 gene, 'classic 3' RACE' was performed using cDNA generated from the preceding experiments.

```
                |....|....| ....|....| ....|....| ....|....| ....|....|
                             360       370       380       390       400
Indica           CTATCTATTC TATTATATAC TAAAAGTCCA TTATACTTCC TACAAACGCT
Japonica         CTATCTATTC TATTCTATTC TA-------- TTATA--TAC TA-AAAGTCC

                |....|....| ....|....| ....|....| ....|....| ....|....|
                             410       420       430       440       450
Indica           CCTAAGCCAC CACGTGGCAC TCCTACAAAC GCTCCTAAGC CACCACGTGG
Japonica         ATTAA----- -ACTT----- -CCTACAAAC GCTCCTAAGC CACCACGTGG
```

Materials and Methods

OsNRAMP1 – sequence analysis

Using primers designed in PerlPrimer (Marshall 2004), the ORF of OsNRAMP1 was cloned from Habataki (*indica*), Badari Dhan (*indica*) and Nipponbare (*japonica*). The primers used were: Forward, 5' -> 3', (including *Kpn*I restriction site) GAGA(GGTACC) TGAGAGGCAAGGCAAAC, and Reverse, 5' -> 3', (including *Eco*RI site), GAGA(GAATTC) AGCCAACACCGGTAAC. The PCR to clone this ORF was carried out using high fidelity Taq (Taq Hi-Fi® Invitrogen); the product was ligated into the pGEM®-T Easy Vector (Promega); and sequenced as per the method in Chapter 4.1. The gene sequence of each of the three clones was compared to check for presence of this mutation.

OsHMA3 – 3' RACE

The 3' ends of OsHMA3 were isolated and sequenced using a published protocol (Scotto-Lavino *et al.* 2006). Reverse-transcription reactions were carried out using the Omniscript® RT-Kit (Qiagen), the Q_T primer (see Table 4.3.1), and four different RNA samples: two from *indica* cultivars, Habataki and Badari Dhan, and two from *japonica* cultivars, Nipponbare and Shwe War. Two successive rounds of nested PCR were performed, according to the protocol, with the Phusion Hot Start II™ - HF DNA Polymerase (Finnzymes, Thermo Fisher

Scientific). The resulting PCR products were cut from the agarose gel, purified using PCR Cleanup Kit (MO BIO Laboratories) and sequenced directly with Big Dye Terminator v3.1 (Applied Biosystems).

Table 4.5: Primers used for amplification of the 3' End of OsHMA3 (Classic RACE). Gene-specific primers (GSP) and Sequencing Primer designed using PerlPrimer. RACE primers taken from 3' RACE protocol, Scotto–Lavino *et al.* (2006). The Q_T primer was HPLC-purified prior to use for fidelity. Note, V = A, C or G; N = random base.

Primer name	Sequence (5' > 3')
3'RACE - RT Pr - Q_T	CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTVN
3'RACE - Outer Pr - Q_O	CCAGTGAGCAGAGTGACG
3'RACE - Inner Pr - Q_I	GAGGACTCGAGCTCAAGC
OsHMA3 GSP1 - pos2795	GATGTGGTGCCTCCAAGAG
OsHMA3 GSP2 - pos2846	GTGAAGGCGGTACCAATGG
OsHMA3 Seq Pr -pos2860	AATGGTGTTGGTCGTTGCTG

Results and Discussion

OsNRAMP1 – sequence analysis

The gene of two *indica* cultivars (from Japan and Nepal) and one *japonica* cultivar were cloned and sequenced. The gene sequence for the section of interest was found to be identical between the two subspecies. These two high Cd-accumulating *indica* varieties did not have the missing nucleotides reported to be present in some *indica* cultivars. Whether or not there is a difference in this gene in any *indica* varieties cannot be stated, but this putative amino acid deletion is not causing the higher Cd accumulation characteristics of these varieties. Takahashi *et al.* (2011) recently published their study of OsNRAMP1 and also did not find sequence variants between *indica* and *japonica*.

In Chapter 4.2, genotypic differences between rice cultivars were found in the constitutive expression of OsNRAMP1 and also its upregulation in response to Fe deficiency. These have most likely arisen from a regulatory mechanism related to OsNRAMP1 expression or other physiological differences between the cultivars, rather than a difference of the gene functionality.

OsHMA3 – 3' RACE

The hypothesis of this analysis was also proven incorrect. Of the sequenced 3' ends from four OsHMA3 gene transcripts, none of the 3' UTR continued further than 205 bp from the stop-codon. The genomic sequence differences found after 370 bp are not likely to affect translation of this gene directly.

Ueno *et al.* (2010) found a single amino acid mutation in the *indica* cultivar, Anjana Dhan, to be responsible for the loss of function of this gene in rice and Miyadate *et al.* (2010) repeated this result with another *indica* cultivar Cho-Ko-Koku. These research groups found strong evidence that it is the functionality of OsHMA3 which leads to the large differences in Cd accumulation between *indica* and *japonica* rice. Nevertheless, this amino acid mutation was not found in the Japanese *indica* variety, Habataki, which is also a high Cd accumulator (Takahashi *et al.* 2011, REF), so although OsHMA3 functionality has a large affect on Cd accumulation, it is not the cause of increased shoot Cd in every high accumulator.

Chapter 5

Genotypic variation in Cd accumulation in soil grown plants: investigation of the interaction with redox conditions and iron-nutrition-related genes

Introduction

Cd uptake in soil-grown rice plants

Many investigations over the past decade have focused on the genotypic variation in Cd accumulation in rice and wheat cultivars. Across the *indica* and *japonica* subspecies of rice, there exists a large range in Cd accumulation characteristics. Differences in grain accumulation characteristics between rice cultivars typically fall within a range of one order of magnitude under a single set of environmental conditions (Arao and Ae 2003; Codex Alimentarius 2007; Zeng *et al.* 2008). Agronomic conditions, however, are known to play a considerable role in determining the grain accumulation of Cd in rice. It has long been known that key soil factors influence the accumulation of Cd by rice plants. Soil pH, redox, and Fe supply to the roots are the most easily identifiable.

A factor which affects Cd availability under most soil conditions, not just in rice paddies, is pH. Cd becomes more available at low pH, such that the application of lime is a management option to reduce plant accumulation of Cd from soils with Cd contamination (Maier *et al.* 2003; Lee *et al.* 2004). Conversely, in solution culture, some have found that Cd uptake increased with a pH change from 5 to 7 (Hatch *et al.* 1988), however, this could have been caused by changes in the relative availability of competing nutrient metals such as Fe, which is less available at higher pH (Ishimaru *et al.* 2007).

Soil redox is the single greatest factor affecting Cd uptake, which, among the cereals, is unique to rice because of its ability to grow under flooded conditions. Under low-redox conditions, Cd availability to plants is reduced because of precipitation of Cd compounds (Arao *et al.* 2009; de Livera *et al.* 2011b). In low-to-moderately contaminated soil this can mean 20-25 fold less grain Cd accumulated under continuous flooding than under aerobic conditions (Ishikawa *et al.* 2005b). There are a number of factors that potentially contribute to the changes in Cd availability that occur in response to redox changes. The dominant cause has not yet been identified conclusively, but it has long been held to be due to the formation of cadmium sulphide, which precipitates and becomes unavailable to plants (de Livera *et al.* 2011b). This may depend on soil type, however, as it is possible that Cd can also become bound to other minerals like carbonates (Khaokaew *et al.* 2011). Nevertheless, regardless of the mineral precipitates that form, the reduction in soil solution Cd content during continuous flooding has been convincingly documented (Arao *et al.* 2009; de Livera *et al.* 2011b).

Rice paddies are very different from regular aerobic soils in their availability of Fe. Under aerobic conditions, Fe is primarily present as Fe(III), which reacts and precipitates readily in the absence of chelating molecules. Therefore, although Fe is often present in large quantities in soil, plants require special mechanisms to take it up. In dicots, this involves a ferric reductase enzyme and then uptake by Fe(II) transporters, while in grasses, phytosiderophores are utilised for chelation and uptake of Fe(III). These are the Strategy I and Strategy II Fe uptake systems, respectively. Rice also uses Strategy II for Fe uptake when grown in aerobic soil (Cheng *et al.* 2007), however under the anaerobic, low redox conditions that occur when soils are flooded, soil Fe(III) is reduced to Fe(II) and rice is able to utilise Fe(II) directly through OsIRT1 (Ishimaru *et al.* 2006).

The relevance of this redox effect to field grown rice is that it is common agronomic practice to drain excess water from paddy fields at flowering to improve grain quality. Therefore, rice plants commonly experience a period of late season aerobic conditions, which is known to cause increased Cd uptake (Iimura 1981; Chaney *et al.* 1995; Simmons *et al.* 2008). Many have speculated that the change in Cd accumulation with redox conditions is predominantly associated with differences in Cd availability in these soils because of sulphide precipitation (Chaney *et al.* 1995). However, in addition to cadmium availability, one of the most significant things that coincide with redox changes is the availability of Fe in soil solution. Nearly all Fe is soluble Fe(II) under reduced conditions, but is precipitated as Fe(III) when aerobic (de Livera *et al.* 2009).

Soil redox could potentially affect Cd accumulation by rice plants for two reasons, (1) changes in soluble Cd present in soil solution, and (2) changes in root Fe supply, which could influence Cd uptake by reduced competition for common transporters. Nakanishi *et al.* (2006) hypothesised that Fe deficiency response in plants, brought about by mid-season changes to paddy drainage (from flooded to drained), could stimulate Cd accumulation through membrane transporters such as OsIRT1. This theory is supported by findings from a recent geochemical study, which showed that paddy soil oxidation resulted in fast changes in Fe availability, with Fe quickly becoming unavailable in solution as conditions went from flooded to aerobic (de Livera *et al.* 2011b). Conversely, that study found a lack of evidence for fast increases in Cd availability after recovery from low redox conditions, with limited release from sulphide minerals (de Livera *et al.* 2011a).

Plant roots in anoxic soil experience an excess of free Fe(II), which would be toxic if most of it was not kept outside of the plant. Rice roots possess aerenchyma, which transport air down to the roots, where some is released into the rhizosphere through radial oxygen loss. For this reason, the surface of the rice root is more oxic than the bulk soil,

leading to the oxidation and precipitation of a proportion of the Fe in the soil solution. This leads to the formation of so-called 'iron plaque' on the surface of rice roots. While Fe plaque has not been found to affect Cd uptake by rice plants (Liu *et al.* 2007a; Liu *et al.* 2008) the Fe status of rice plants does affect Cd uptake. Fe status could influence Cd uptake in two ways, (1) direct competition between Cd and Fe for uptake and translocation (Liu *et al.* 2008), or (2) the increase in Cd uptake because of the plant's response to Fe nutrition changes (Nakanishi *et al.* 2006).

Ishikawa *et al.* (2005b), while examining the effects of a QTL for Cd uptake in rice, also demonstrated the commonly observed facts: that growing rice under aerobic conditions greatly increased grain Cd content (in this case, to the order of 20 times); and also, changing redox conditions could significantly change the Cd-accumulation rankings of the different rice genotypes. The greenhouse pot trial and associated analysis reported in this chapter were designed to test the hypotheses that:

Hypothesis 1

Iron-deficiency-responsive (IDR) genes play a role in the increased Cd uptake that occurs when rice is grown in oxic soil condition, and OsNRAMP1 is one of the membrane transporters involved in this phenomenon

Hypothesis 2

The degree of change in Cd accumulation brought about by altered soil redox conditions depends on plant genotype

Experimental overview

Eight rice cultivars were chosen, with a large degree of genotypic variation in Cd accumulation, and were grown under three different irrigation treatments in potted paddy soil. The plants were grown until maturity and the shoots and brown rice were analysed for Cd and other nutrients. To relate soil conditions to plant IDR gene expression, during the

growth of these plants, leaf samples were taken to measure the expression of selected IDR genes. Although the membrane transporters that could increase Cd translocation are necessarily expressed in the roots, repeated evidence has shown that upregulation of IDR genes in roots coincides with upregulation in shoot tissue (Kobayashi *et al.* 2009; Takahashi *et al.* 2011; and my unpublished data). Sampling of root tissue for RNA extraction would have involved destructive sampling of the plants so samples were instead taken of leaf tissue.

DGT

In order to check and contrast the roles of soil-redox driven changes in Cd availability and the effect of Fe deficiency response on plant Cd uptake, a method of determining Cd availability in the soil was required. In addition to direct methods of soil pore-water sampling, an emerging technique in environmental geochemistry was utilised in this study called Diffusive-Gradient in Thin-films, or DGT™ (DGT Research; Zhang *et al.* 2001).

DGT is a technique used to estimate the biologically-available component of metals in water or soil by measuring the amount of a metal which diffuses across a defined thin-film gel to bind to a strong-binding resin, such as Chelex®. DGT devices have been tested in a variety of situations and, for soil, have been found to give a more accurate measure of plant available metal content, compared with pore-water sampling or soil-extraction techniques (Davison *et al.* 2000; Zhang *et al.* 2001). The reason for DGT's superior ability to predict plant metal content is because the technique estimates the mean flux of labile species into the device from the soil (Zhang *et al.* 2001). This is made up of two components, (1) the concentration in soil solution, at the surface of the device, and (2) the rate of resupply of species from the labile pool in the soil's solid phase. The DGT device acts

as a sink for metals, as plant roots are a sink, and so it measures the *effective concentration of labile metal*, or C_E (Zhang *et al.* 2001).

The developers/manufacturers of the DGT devices have a standardised bench-top protocol for use with soil of controlled water content, but, if sufficient soil moisture content is available (80-100% water-holding capacity, WHC) DGT devices can be placed directly in soil to compare available metal content *in situ*. (pers. comm. Hao Zhang, 3rd November 2010). In the following experiment, the DGT technique was used to measure plant available Cd under different irrigation regimes by placing the units directly in the pots, allowing a direct comparison of the conditions of the growing plants.

Materials and Methods

Plant material and experimental setup

Rice germplasm was selected from the collection at the Japanese National Institute of Agrobiological Sciences Genebank that represented a broad range in grain Cd accumulation characteristics. The rice varieties used were, Habataki, Badari Dhan, Shwe War, Kasalath, Nipponbare, Koshihikari, Milyang 23, Lac 23 and Amaroo (abbreviated to HBK, BDN, SWE, KAS, NIP, KOSH, MLY, LAC and AMR, respectively). These cultivars are those described and referenced in Chapter 4.2. The last rice variety listed is an Australian rice variety, Amaroo, grown for comparison, the same as that used in Chapter 6.1.

Seeds were surface sterilised and germinated on perlite. At 16 d after sowing, the seedlings were transferred to hydroponic nutrient solution. The plants were grown in a greenhouse, with natural light conditions, supplemented with overhead lamps during low-light conditions and to ensure a 12 h day. The plants were grown over autumn/winter and average temperatures in the greenhouse varied between 15-32^{o}C, with an average temperature of 24^{o}C.

The paddy soil used for this experiment, and that of Chapter 6.3, was sourced from a rice-growing area near Xiamen, Fujian province, China. It was a sandy-loam (66% sand, 29% silt, 5% clay), with pH 6.09 (2.5:1 water-soil ratio). The background Cd concentration was measured to be 0.07 mg kg^{-1}. Prior to use in the pot experiment, the soil was passed through a 2 mm sieve, with large clods broken up. Individual pots were prepared with 3 kg of soil each and the soil was spiked with $Cd(NO_3)_2$, at a rate of 0.4 mg kg^{-1} Cd, applied as a diluted solution used to saturate the soil with minimal excess. After one day, the soil was completely flooded for one week prior to transplanting of the 44 d old rice seedlings. The seedlings were planted one to a pot, with four replicate pots for three different irrigation regimes. Nitrogen was supplied as urea during the pre-heading 'extension' stage. The irrigation treatments were as follows:

A. Aerobic soil conditions: Shallow-flooding of soil until late-tillering stage, followed by a week to allow the soil to dry to below field capacity (equivalent to the mid-summer drainage period, used in Japan (Sasaki *et al.* 1998; Saito *et al.* 2005)). For the rest of the season, watering was continued every few days until maturity, keeping the soil dry to damp but not saturated.

B. Intermittent flooding: Shallow-flooding of soil until late-tillering stage, followed by a week to allow the soil to dry to below field capacity, and then alternate flooding/drying until maturity; i.e. pots were watered to > 5 cm of standing surface water, which was allowed to dry to less than saturated before water was re-applied.

C. Continuous flooding: Shallow-flooding of soil until late-tillering stage, followed by continuous, deep flooding (10 cm of standing surface water) until maturity.

DGT: in situ and bench-top

'Loaded DGT soil deployment units for metals' were purchased from DGT Research Ltd (Lancaster, UK). They are small round units 2.5 cm in diameter, consisting of a plastic mount, with plastic cover enclosing a round window of 2.54 cm^2 surface area. Inside the unit is a disc of Chelex® resin-gel (0.4 mm thick) underneath an open-pore diffusive gel (0.78 mm thick) and covered by a filter membrane at the surface (0.14 mm). The units were prepared in small 'snap-lock' polypropylene bags with a few drops of 0.01 M NaCl solution (in lieu of $NaNO_3$) to minimise changes to redox when placed in soil.

At three times over the growing season, DGT units were placed in the soil of pots for 24 h to measure plant available Cd under the three treatments. At each time point four replicate units for each treatment were placed in separate pots of the Amaroo variety. The same pots were sampled each time, but a different section of soil was disturbed each time. The units for treatments B and C were placed in a hole approximately 10 cm deep, pushed gently into the soil and then buried. The units for treatment A were buried near the top of the soil (within 5 cm) because the soil was much firmer. For the A and B treatments, the DGT were placed in the soil at least one day following a watering to ensure adequate soil moisture. Additional deployments were also made in treatment B at various stages in the flooding/drying cycle, i.e. 1, 2, 3 or 4 days after soil flooding.

For comparison, aerobic soil DGT deployments were also repeated using the standardised bench-top protocol recommended by the manufacturers (*Practical Guide for using DGT in Soils*, unpublished). In this method, replicate soil samples from this pot experiment were wetted to 60% of maximum water-holding capacity (WHC), mixed and left for two days. Further deionised water was then added to bring the soil to 97% WHC (80-100% required), and mixed to a smooth paste and left to equilibrate for a further 24 h. To

begin the deployment, a lump of soil paste was pressed gently onto each DGT unit, which was then placed inside a moistened polypropylene bag and stored in the dark for 24 h.

Soil temperature was monitored over the 24 h greenhouse deployment using electronic data loggers thermocouple probes (EL-USB-TC-LCD; Lascar Electronics, HK), which were inserted into the soil of two representative pots. During lab deployment, the room temperature was recorded alongside the DGT units.

Retrieval of the Chelex® resin-gel from the DGT units was performed as per the manufacturer's instructions and the gel was placed in separate acid-washed 1.5 ml tubes for elution with 0.8 ml of 1 M HNO_3. The gel was allowed to elute for at least 48 h before a 5x dilution was made to be analysed by ICP-MS for Fe, Cd, Mn and Zn.

The mass of each metal eluted from the resin gel was used to calculate the effective concentration of metal measured at the interface of the DGT unit. This was performed according to the published equations of the standard soil DGT technique, using an elution factor of 0.8 and diffusion coefficients based on the average temperature during deployment (Zhang *et al.* 2001).

Soil solution sampling

In addition to DGT, soil solution samples were taken from each treatment at late-tillering stage, after the soil of treatments A and B were rewet following their first drying period. Approximately 35-40 ml of soil was taken from below the soil surface and placed in centrifuge tubes and spun at 3500 *g* for 15 min. The resulting supernatant was immediately filtered using a syringe-driven 0.22 µm filter and acidified with HNO_3 to a final acid concentration of 0.1 M.

Soil redox

The redox conditions of the flooded soil were confirmed using a combined ORP probe, with a platinum ring electrode and internal reference electrode (Nengshi Analytical Sensor Co., China), inserted into the soil. After allowing time for the reading to stabilise, the ORP millivolt measurement (Eh_m) was used to calculate the soil redox potential (Eh) by the addition of the potential of the reference electrode (Eh_{ref}, e.g. +205 mV, at 30^{o}C). The redox in treatment A or B pots was taken by inserting the ORP probe into moist, recently watered soil. The change in redox in selected pots of treatment B was measured by keeping the probe in place over the cycle of flooding, drying and re-flooding.

Leaf sampling for RNA extraction and cDNA synthesis

Leaf samples were taken at three times over the growth of these plants: late-tillering, early-flowering, and two-weeks after flowering. Up to 0.1 g of leaf material was collected from the youngest expanded leaf (at tillering) or the flag-leaf opposite the most-recently flowering panicle. These samples were collected at a similar time each day of sampling. The samples were placed in individual 1.5 ml tubes and frozen in liquid nitrogen, and stored at -85^{o}C prior to processing.

The leaf tissue was ground to powder under liquid N, and then RNA was extracted using TRIzol® Reagent (Invitrogen). Precipitated RNA was stored in 75% ethanol at -20^{o}C until being dissolved in DEPC-treated water prior to quantification and use in the reverse-transcription reaction.

All RNA samples were first treated with RNase-free DNase I (Fermentas) for 30 min at 37C, and then denatured at 70^{o}C for 10 min, following the addition of EDTA to 4.5 mM final concentration. cDNA was generated from these treated RNA samples using oligo-dT primers and RevertAid^{TM} First Strand cDNA Synthesis Kit (M-MuLV RT enzyme; Fermentas),

as per the manufacturer's instructions, but in a half reaction of 10 µl. Finally, the first-strand cDNA was treated with RNase H (Fermentas) for 20 min at 37°C. The resultant cDNA product was diluted to 1 ng/µl in TE for use in real-time PCR.

Quantitative PCR

Real-time PCR was performed with an Applied Biosystems 7500 Real-Time PCR System using 96-well plates. Duplicate reactions of 20 µl for each biological replicate (n=4) were performed using hot-start SYBR-green master mixes with ROX™ Reference Dye: KAPA SYBR® FAST qPCR Master Mix or TaKaRa SYBR® *Premix Ex Taq*^{TM} (Perfect Real Time). Primers used were as per Chapter 4, plus a primer pair for the Fe-deficiency transcription factor IDEF1 (Kobayashi *et al.* 2009): F pr. ATGGACGACATGGTGCTCC, R pr. CTAGGGATTTGTTGTCTGCT, annealing temperature 64°C.

Gene expression was quantified based on the standard curve of a serial dilution of a pooled cDNA sample of all eight cultivars (treatments A and B) at tillering stage (not including Lac 23, which did not mature). In this way, relative expression was calculated based on average expression in this pooled cDNA sample. The standard curve was included in triplicate in each qPCR run. The target genes were normalised for variability in mRNA content by dividing by relative expression of *e*EF-1α in each of the diluted cDNA samples.

Plant harvest and analysis

Once the rice panicles had reached maturity the pots were allowed to dry to facilitate harvest. Rice panicles were harvested when the plant was dry. Shoots were cut 6 cm from the base of the plant to avoid soil contamination from the section that was submerged when flooded. The bottom 6 cm of shoot was also harvested to include in total shoot weight.

All plant material was dried completely at 70°C. Rice husks were removed using a bench-top dehulling machine (Chinese manufacturer). The rice was not polished because studies have found that the Cd concentration in rice and bran is similar (Masironi *et al.* 1977; Williams *et al.* 2009). Brown rice samples were ground first in an electric grinder (Midea, China) and then to a fine powder with a mortar and pestle. Plant shoots were ground in an electric grinder and then passed through a 2 mm sieve.

The plant material was re-dried in the oven before weighing out 0.3 g for grain and 0.2 g for shoots into acid-washed quartz-glass digestion tubes. To each tube, 5 ml of concentrated HNO_3 was added and samples were left overnight to pre-digest. The following morning, 2 ml of 30% H_2O_2 was added to each tube before placing tubes randomly in a block digester. The samples were digested according to the following program: temperature was raised initially to 75°C, and then gradually to 120 °C, held for 100 min, and then raised to 140°C and held for 100 min. After the digests had cooled, a further 1 ml of H_2O_2 was added to ensure the breakdown of all organic matter.

All the plant digests were transferred to 50 ml plastic centrifuge tubes and diluted to 50 ml. The final weight of the dilution was recorded and then aliquots were taken and, using a syringe, were passed through a 0.22 µm filter. The digests were analysed by ICP-OES for macronutrients and ICP-MS for micronutrients, As and Cd.

Replicated digestion blanks, spiked samples and certified reference material (GBW10010, rice flour, Cd concentration = 0.087 mg kg^{-1}) were run alongside every digestion batch for quality control, ensuring reproducibility of results.

Statistical analysis

Data were analysed by ANOVA using the GenStat program (11th Edn, VSN International). Where appropriate, data was log transformed to comply with assumptions of equal

variance. Means were compared, following ANOVA, by the Least Significant Difference (L.S.D.) method.

Results

Effect of soil redox on availability of Cd, Fe and Mn in soil: DGT

Soil redox differed markedly under the three irrigation regimes. Aerobic soil (treatment A) had redox potentials in the range 250–500 mV when measured during the unflooded period. Under intermittent flooding (treatment B), soil redox potential fluctuated during the cycle of flooding and drying conditions. The redox potential, over the days of the cycle, varied in the range -50 mV to +450 mV (see Fig 5.1). The continuous flooding of treatment C took some time to reach minimum redox. During tillering-phase, the redox at a depth of 4-5 cm was only reduced to around 0–50 mV. By flowering, the soil redox in treatment C pots was less than 0 mV and this value decreased with depth. Eventually, even near the soil surface, the redox potential dropped to -200mV ± 50 mV. The values observed in this pot trial are similar in range to that of published field studies from paddy soil (Sasaki *et al.* 1998).

DGT analysis of this soil, under the variable redox conditions imposed, revealed distinct patterns of elemental availability. Figures 5.2 to 5.5 present the results of the DGT analysis for Cd, Fe and Mn over the growing season. Although DGT units were deployed at three times during the season, there was no significant variability within treatments over time. For this reason, the results presented below are means of all three sampling times. The exception to this was treatment B, for which redox conditions were constantly changing in response to flooding conditions. Where significant differences were found, the results from treatment B pots have been grouped and averaged according to the number of days of flooding.

Cd availability was higher in aerobic, non-flooded soil (A) than in continuously flooded soil conditions (C). The average difference between these two treatments was a 3.7-fold increase in A (Fig. 5.2). Cd availability also varied in real-time as redox conditions fluctuated in the intermittent flooding treatment (B). This became significantly different to treatment A after three days of flooding. On average, the pots were watered again after five days.

The *effective concentration* (C_E) for Cd calculated by DGT in aerobic soil conditions was not significantly different to the result found using the bench-top DGT method (Table 5.1). The DGT results for the three irrigation treatments were also supported by soil pore water samples that were taken (Table 5.1). For Cd, the actual concentration in the soil solution differed from that estimated by DGT, but the pattern of difference between A and C was similar (4.1-fold difference compared to 3.7-fold).

Mn availability also varied in real-time as redox conditions fluctuated, but in the opposite direction to Cd (Fig. 5.3). Mn was most available when flooded (C) and lowest in aerobic soil (A). The availability under treatment A was higher than that found in the lab DGT (Table 5.1), but was still much lower than in flooded conditions. During the five day cycle of wet and dry in treatment B, the Mn concentration in soil solution varied from the same level as A, to a concentration approaching that of C.

Fe availability was very high in flooded soil and much lower in the more aerobic conditions of treatments A and B (Fig. 5.4). There was a significant difference between A and B also, with B treatment higher on average. In the same way as Mn (see comparison in Fig. 5.5), Fe availability did increase in response to the short term flooding in treatment B, but when compared to the much higher concentration found in treatment C, this was to a much smaller degree, relatively. The Fe availability in potted soil, under any of the treatments, was higher than that found using the highly aerated soil mixture used in the lab

DGT test (Table 5.1). Inexplicably, the pore water samples that were taken (by centrifuge) from the 'lab DGT' soil had a much higher Fe content than was found by the DGT test (504 µg kg^{-1} compared with 2.6 µg kg^{-1}). This is could have been the result of the difference in filtering used. The DGT technique only allows ions and very small molecules to cross the selectively permeable membrane, whereas the soil pore water was taken up through a 0.22 µm filter, which may have let up larger particles containing Fe.

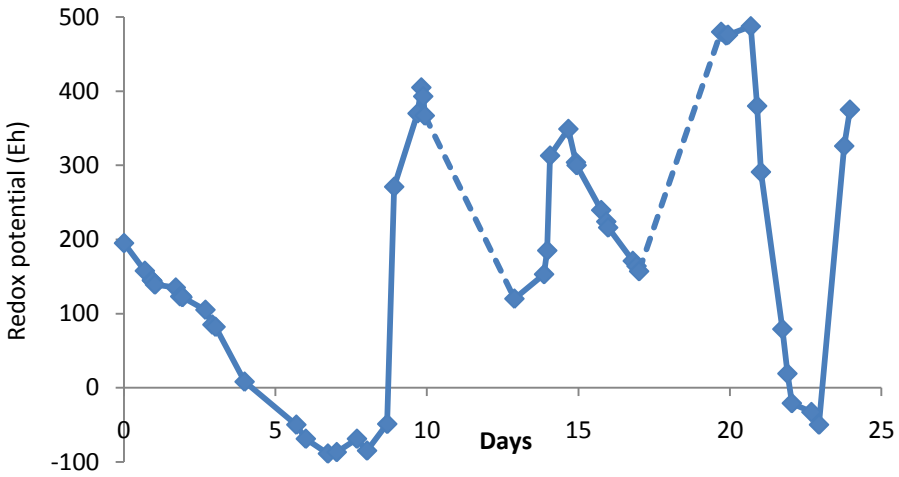


Figure 5.1: Redox variation (at soil depth of 5 cm) in a single pot of treatment B (intermittent flooding) over 24 d (during the later, post-flowering period of shorter flooding cycles). Spikes in soil redox occurred in response to dry conditions and/or an irrigation. Dashed lines indicate gaps of 2-3 d with no reading taken.

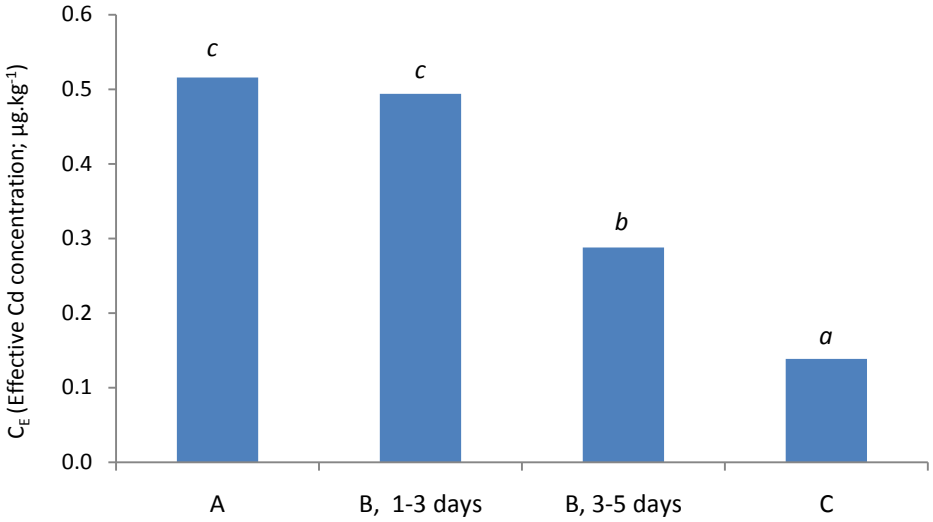


Figure 5.2: Effective Cd concentration in soil solution (measured by DGT technique) of potted paddy soil spiked with 0.4 mg.kg^{-1} Cd ; A = non-flooded soil; B = intermittent flooding (showing number of days since soil was flooded); C = continuous flooding. Different letters above columns indicate means which are significantly different by ANOVA-L.S.D., (5% level; log-transformed data; n= 7, 10, 5, 9, respectively).

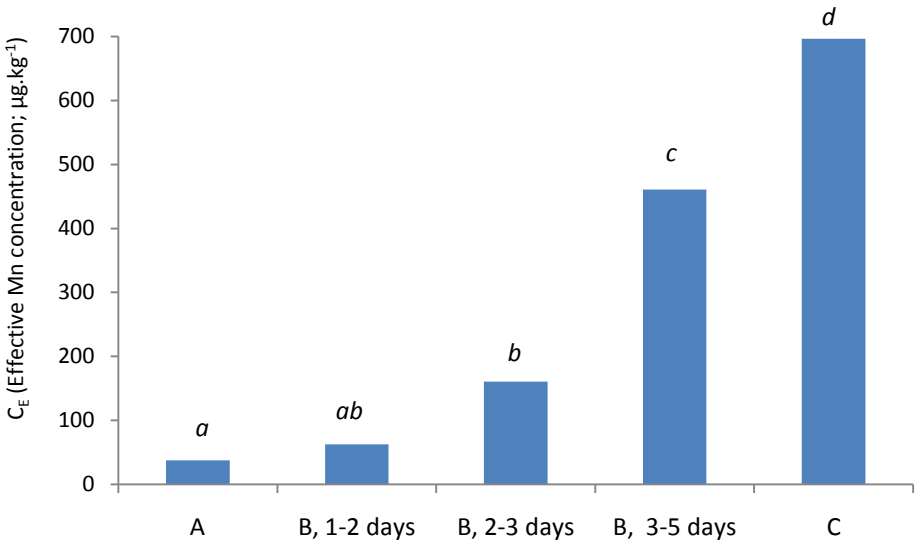


Figure 5.3: Mn concentration in soil solution (calculated by DGT); A = aerobic soil; B = intermittent (showing no. of days of flooding); C = continuously flooded. Different letters above columns indicate means which are significantly different (ANOVA-L.S.D., 5% level; n = 8, 6, 4, 4, 12, respectively).

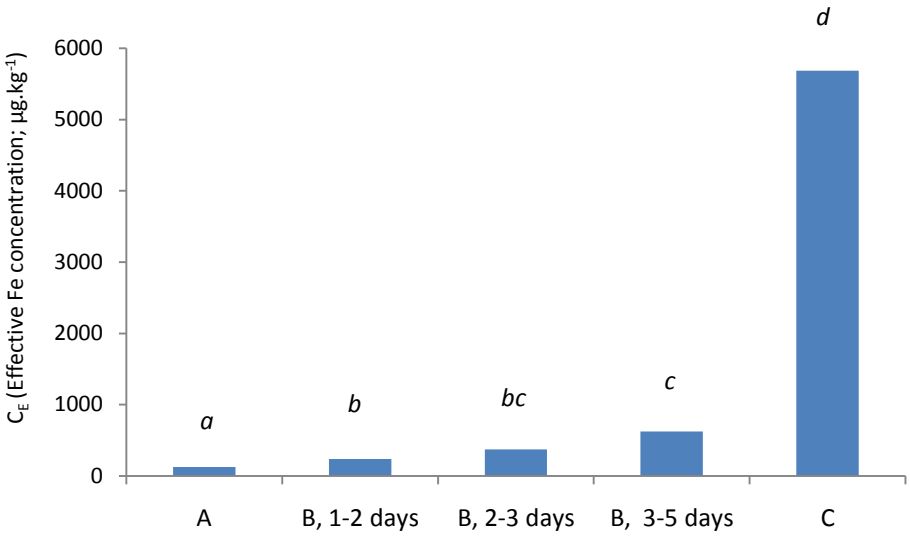


Figure 5.4: Fe concentration in soil solution (calculated by DGT); A = aerobic soil; B = intermittent (showing no. of days of flooding); C = continuously flooded. Different letters above columns indicate means which are significantly different (ANOVA-L.S.D., 5% level; n = 9, 7, 4, 4, 12, respectively)

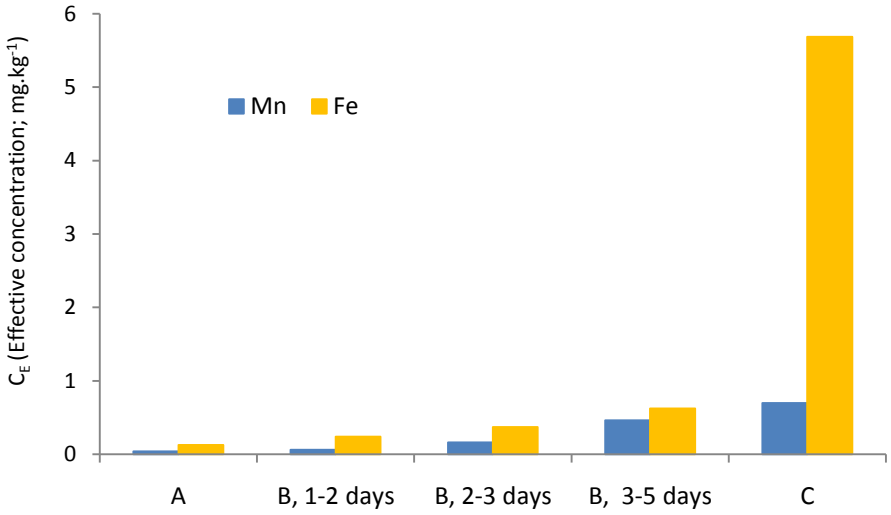


Figure 5.5: Comparison of Mn & Fe concentration in soil solution (calculated by DGT); A = aerobic soil; B = intermittent (showing no. of days of flooding); C = continuously flooded. Darker (red) bars represent Mn conc and light (blue) bars represent Fe concentration.

Table 5.1: Comparison of soil pore water sampling (centrifuge method) and DGT for measuring available elemental concentrations in soil solution. 'Lab test' refers to soil subsamples removed from the experimental pots for use in the bench-top DGT protocol, and also pore water sampling of the prepared soil at water-holding capacity. "*b.d.*" = below detection limit.

		Total soil conc (mg kg^{-1})	Soil pore water - Lab test (µg kg^{-1})	Soil pore water - (A) Aerobic (µg kg^{-1})	Soil pore water - (C) Flooded (µg kg^{-1})	DGT - Lab test (µg kg^{-1})	DGT - (A) Aerobic (µg kg^{-1})	DGT - (C) Flooded (µg kg^{-1})
Cd Spiked soil	Cd	0.43	0.87	1.40	0.34	0.48	0.52	0.14
Unspiked soil	Cd	0.07	0.11	-	-	0.10	-	-
	Fe	5729	504	354	7873	2.6	124	5685
	Mn	43	40	121	1698	5.6	37	697
	Zn	31	4	*b.d.*	*b.d.*	3.9	20	9

Gene expression analysis: role of OsNRAMP1 in Cd accumulation under soil conditions

At three sampling times over the growing season, selected leaves were sampled for expression of a number of Fe-deficiency-responsive genes. OsNRAMP1, OsIRT1, and the transcription factor IDEF1 (Kobayashi *et al.* 2009) were successfully measured in shoots. OsNRAMP1 was the only gene for which significant differences in expression were seen between the treatments.

Figures 5.6 to 5.8 show the normalised, relative expression results of OsNRAMP1 for the three sampling points. At the late-tillering sampling, the conditions of treatments A and B had been identical to that point, so B was omitted. A comparison of the three graphs showed that a number of the cultivars, HBK, BDN, MLY, KAS and SWE, had repeatedly higher leaf expression in aerobic conditions (A) than in flooded conditions (C). BDN, HBK and SWE did not show upregulation at every sampling point, whereas MLY and KAS did and had the greatest upregulation of OsNRAMP1 at the late-flowering stage (most likely to be associated with grain Cd accumulation).

Plants in treatment B did not show significantly higher expression of OsNRAMP1 than treatment C, except for HBK at the late-flowering stage.

With the exception of SWE, the cultivars which show increased OsNRAMP1 expression in treatment A are *indica* varieties, or *indica*-cross, and are also high Cd accumulators. The OsNRAMP1 upregulation in SWE was also not as great as those other varieties, with a lower significance level at late-tillering and late-flowering, and no significant difference at the early-flowering phase.

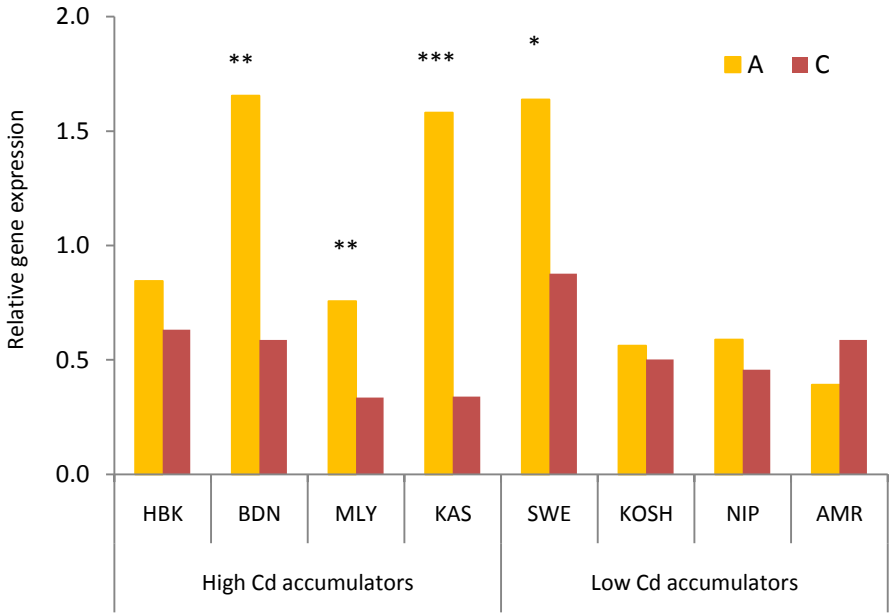


Figure 5.6: Expression of OsNRAMP1 in newly-expanded leaves at late-tillering stage from rice varieties which vary in grain Cd accumulation. Samples taken at the conclusion of a week of "drainage" (from flooded to dry conditions) in treatment A and B, while treatment C remained continuously flooded. Gene expression was calculated relative to a combined cDNA pool, and normalised against expression of *e*EF1α. Asterisks indicate means which are significantly greater than treatment C for that cultivar (ANOVA-L.S.D.; * 5%, ** 1% or *** 0.1% significance level) .

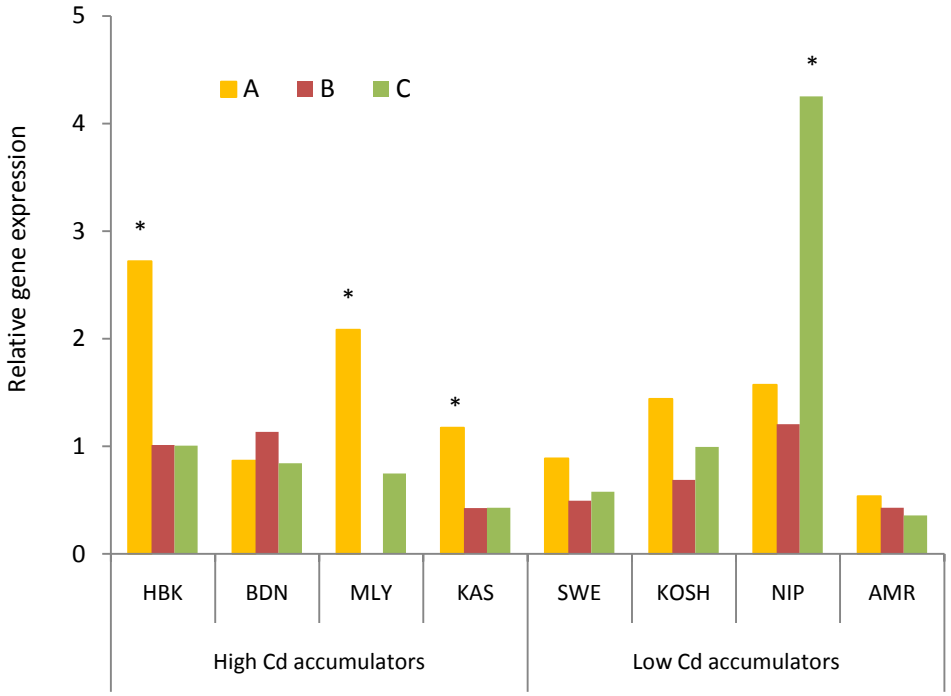


Figure 5.7: Expression of OsNRAMP1 in selected flag-leaves at early-flowering stage from rice varieties which vary in grain Cd accumulation, under three different irrigation regimes; A = aerobic soil; B = intermittent flooding; C = continuously flooded. Gene expression was calculated relative to a combined cDNA pool, and normalised against expression of *e*EF1α. Asterisks indicate means which are significantly greater than the other treatments for that cultivar (L.S.D. 5% level) .

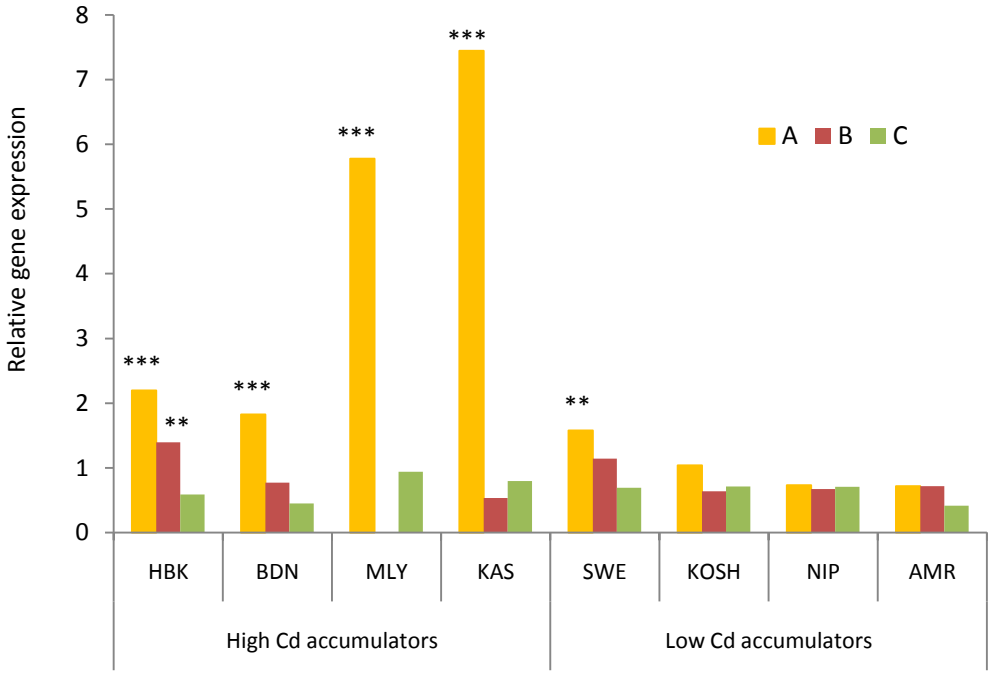


Figure 5.8: Expression of OsNRAMP1 in selected flag-leaves at late-flowering stage from rice varieties which vary in grain Cd accumulation, under three different irrigation regimes; A = aerobic soil; B = intermittent flooding; C = continuously flooded. Gene expression was calculated relative to a combined cDNA pool, and normalised against expression of *e*EF1α. Asterisks indicate means which are significantly greater than treatment C for that cultivar (ANOVA-L.S.D.; ** 1%, or, *** 0.1% level) .

Elemental analysis: accumulation of Cd, Fe and Mn

Clear differences in plant Cd accumulation were seen between the cultivars tested in this experiment (Table 5.2). The genotypic accumulation characteristics were also consistent with that previously reported (outlined in Chapter 4.2). Regardless of the treatment, BDN and HBK had the highest grain and shoot Cd accumulation. MLY and KAS had intermediate levels of accumulation, and were somewhat more variable. The results and ranking of the four *japonica* lines varied with the treatments, especially for shoot Cd concentration. For grain Cd, they were consistently the lowest Cd accumulators, with no significant differences between SWE, KOSH, NIP and AMR for all of the treatments (L.S.D. at 1% level).

The high grain Cd accumulation of BDN and HBK coincided with significantly higher grain to shoot Cd ratios than those of the low Cd accumulators (Table 5.2). These plants had increased shoot accumulation overall but also increased partitioning of plant Cd to the grain. BDN also displayed increased Fe and Mn concentration in shoots and grain (Table 5.3). This was not the case for HBK, which had low shoot Fe concentration and an average shoot Mn concentration that was not significantly increased.

Treatment B resulted in the highest Cd and Mn accumulation, in grain and shoot, for all the cultivars, and except for SWE, treatment C resulted in the lowest grain Cd accumulation and the highest shoot Fe concentration (Table 5.3).

Elemental analysis: As and Zn

In addition to the elements in Table 5.3, As concentrations also significantly differed between the treatments. As expected, the highest As content was found in plants under the continuous flooded soil conditions. There was not a significant genotype effect for

these cultivars and the average grain As concentration in brown rice was 0.06 µg g^{-1}, 0.11 µg g^{-1} and 0.24 µg g^{-1} for treatments A, B and C, respectively.

No significant differences in Zn content were seen between the treatments or cultivars.

Table 5.2: Analysis of grain and shoot material from final plant harvest, showing (a), biomass data and Cd concentration of eight rice varieties which vary in grain Cd accumulation, grown under three different irrigation regimes; A = aerobic soil; B = intermittent flooding; C = continuously flooded. Oven-dried grain and shoot tissue was ground to fine powder and digested by block digestion with concentrated HNO_3 and H_2O_2. Diluted, filtered samples were analysed by ICP-MS. No plants were grown under treatment B for MLY.

(a) **Cult.**	Reported Grain Cd accumul-ation	Sub-sp.	Grain Dry Wt (g plant^{-1})		Total Shoot Dry Wt (g plant^{-1})		Grain Cd conc. (µg g^{-1})						Shoot Cd conc. (µg g^{-1})						Ratio of Grain:Shoot Cd conc.					
			Combined avg. (A,B,C)				**A**		**B**		**C**		**A**		**B**		**C**		**A**		**B**		**C**	
HBK	High	Ind	4.2	*ab*	18	*d*	0.90	*e*	0.95	*c*	0.60	*c*	1.4	*d*	2.1	*d*	1.7	*d*	0.6	*e*	0.5	*d*	0.4	*c*
BDN	High	Ind	8.4	*d*	16	*c*	0.30	*cd*	0.58	*bc*	0.50	*c*	1.2	*d*	1.7	*d*	1.6	*d*	0.2	*bc*	0.4	*cd*	0.3	*c*
MLY	High	Cross	5.9	*c*	13	*ab*	0.39	*d*			0.11	*b*	1.0	*cd*			0.7	*bc*	0.4	*d*			0.2	*b*
KAS	Med	Ind	5.5	*bc*	19	*d*	0.18	*bc*	0.40	*b*	0.10	*b*	0.6	*bc*	1.0	*c*	0.4	*ab*	0.3	*cd*	0.3	*c*	0.2	*b*
SWE	Low	Jap	3.5	*a*	17	*cd*	0.07	*a*	0.15	*a*	0.09	*ab*	0.4	*a*	0.5	*a*	0.8	*c*	0.2	*a*	0.3	*bc*	0.2	*b*
KOSH	Low	Jap	8.6	*e*	12	*a*	0.10	*ab*	0.15	*a*	0.09	*ab*	0.6	*bc*	0.8	*bc*	0.5	*ab*	0.2	*a*	0.2	*ab*	0.2	*b*
NIP	Low	Jap	3.9	*a*	14	*b*	0.10	*ab*	0.14	*a*	0.06	*ab*	0.6	*ab*	0.9	*c*	0.6	*bc*	0.2	*ab*	0.2	*a*	0.1	*a*
AMR		Jap	10.3	*f*	12	*a*	0.08	*a*	0.14	*a*	0.05	*a*	0.5	*ab*	0.5	*ab*	0.3	*a*	0.2	*ab*	0.3	*bc*	0.2	*b*

N.B. Different letters, within a single column, indicate which cultivar means (adjacent) are significantly different by ANOVA-L.S.D. (1% sig. level). These differences only relate to differences between cultivars within a single treatment. The analysed statistical differences between the treatments, incorporating genotype by treatment interaction, are shown in Table 5.3.

Table 5.3: Analysis of grain and shoot material from final plant harvest, showing (a), biomass data and Cd concentration, and (b), Fe and Mn accumulation, of six rice varieties which vary in grain Cd accumulation, grown under three different irrigation regimes; A = aerobic soil; B = intermittent flooding; C = continuously flooded. Samples were analysed by ICP-MS, or ICP-OES (shoot Fe and Mn). Data for Cd is as per Table 5.2, except that cultivars SWE and AMR are omitted for clarity, and statistical analysis was performed by treatment and cultivar. Different letters, within a single analyte and tissue type, indicate which treatment combination means are significantly different by ANOVA-L.S.D. (5% sig. level).

(a)	Reported Grain Cd accumul-ation	Subsp.	Grain Cd conc (µg g^{-1})						Redox effect on Cd uptake	Shoot Cd conc (µg g^{-1})						Redox effect on Cd uptake	Ratio of Grain:Shoot Cd conc.					
			A		**B**		**C**		**= B/C**	**A**		**B**		**C**		**= B/C**	**A**		**B**		**C**	
HBK	High	Ind	0.90	*gh*	0.95	*h*	0.60	*fg*	1.6	1.4	*fgh*	2.1	*i*	1.7	*ghi*	*n.s.d.*	0.6	*g*	0.5	*fg*	0.4	*ef*
BDN	High	Ind	0.30	*e*	0.58	*fg*	0.50	*f*	*n.s.d.*	1.2	*fg*	1.7	*hi*	1.6	*ghi*	*n.s.d.*	0.2	*cde*	0.4	*ef*	0.3	*e*
MLY	High	Cross	0.39	*ef*			0.11	*bc*		1.0	*ef*			0.7	*cd*		0.4	*ef*			0.2	*b*
KAS	Med	Ind	0.18	*d*	0.40	*ef*	0.10	*bc*	4.1	0.6	*bcd*	1.0	*ef*	0.4	*a*	2.4	0.3	*de*	0.3	*e*	0.2	*b*
KOSH	Low	Jap	0.10	*bc*	0.15	*cd*	0.09	*ab*	1.7	0.6	*bcd*	0.8	*cde*	0.5	*ab*	1.6	0.2	*b*	0.2	*bcd*	0.2	*bc*
NIP	Low	Jap	0.10	*bc*	0.14	*cd*	0.06	*a*	2.4	0.6	*abc*	0.9	*de*	0.6	*bcd*	*n.s.d.*	0.2	*bc*	0.2	*b*	0.1	*a*

(b)	Grain Fe conc (µg g^{-1})				Shoot Fe conc (µg g^{-1})				Grain Mn conc (µg g^{-1})						Shoot Mn conc (µg g^{-1})					
	A & B		**C**		**A & B**		**C**		**A**		**B**		**C**		**A**		**B**		**C**	
HBK	16	*abc*	18	*c*	139	*a*	203	*cde*	15	*bc*	27	*gh*	23	*ef*	390	*abc*	797	*f*	538	*cd*
BDN	17	*bc*	17	*bc*	178	*bc*	357	*f*	16	*bc*	29	*h*	21	*de*	572	*de*	1193	*g*	874	*fg*
MLY	15	*ab*	17	*bc*	174	*bc*	238	*e*	14	*b*			15	*bc*	377	*ab*			526	*bcd*
KAS	13	*a*	13	*a*	151	*ab*	211	*de*	10	*a*	25	*fg*	18	*cd*	320	*a*	784	*ef*	382	*ab*
KOSH	17	*bc*	16	*bc*	143	*a*	178	*bc*	15	*bc*	24	*efg*	16	*bc*	523	*bcd*	763	*ef*	369	*a*
NIP	18	*c*	15	*ab*	181	*bcd*	224	*e*	15	*b*	23	*ef*	16	*bc*	419	*abcd*	818	*f*	516	*bcd*

Discussion

Genotypic differences in grain Cd accumulation were seen over an order of magnitude in this experiment. The ranking of the cultivars for grain Cd accumulation was also matched by their shoot Cd accumulation. These genotypic Cd characteristics were much larger than the treatment effect associated with the different flooding conditions. They also were unique to Cd, and did not follow trends in accumulation for As, Fe or Mn, except for BDN which was also a high Fe and Mn accumulator (Table 5.3).

Iron

Large differences in soil chemistry exist between anaerobic soil and aerobic soil. The concentration of Fe in soil solution is one of the most significant differences, where under flooded conditions, Fe availability was around 6-7 mg kg^{-1}, compared to 0.1-0.5 mg kg^{-1} in aerobic soil (Table 5.1). The switch in redox potential caused by microbial-assisted reduction during extended soil flooding (Zhang *et al.* 2009a), led to the transformation of Fe(III) to Fe(II), which resulted in a 45-fold increase in the effective concentration in soil solution.

The in situ DGT deployment showed that intermittent flooding did not cause Fe availability to reach the concentration found in continuously flooded soil. This is contrary to Mn and Cd, where proportionally large changes in availability were seen under treatment B. DeLivera *et al.* (2011) demonstrated that the precipitation of Fe following oxidation of paddy soil was rapid, so it is likely that the Fe redox transformations are slow when changing from aerobic to anaerobic, but rapid once oxygen is reintroduced. The results showed that to achieve the high level of Fe availability seen in treatment C, the reduced conditions must be continuous, and intermittent flooding does not allow enough drop in redox potential for complete transformation of Fe(III) to Fe(II). This confirms earlier reports

that demonstrated Fe oxides and hydroxides precipitate at relatively low redox potentials (St-Cyr and Crowder 1990; Crowder and St-Cyr 1991; Christensen and Sand-Jensen 1998).

The lack of large variation in grain Fe concentrations between treatments in this experiment is indicative of the ability of rice plants to maintain internal Fe homeostasis and control grain Fe levels. For all of these cultivars, there was only small variation in grain Fe content in response to the large changes in Fe availability (Fig. 5.4) and even shoot Fe concentration. The concentration of Fe accumulated in brown rice under treatment C was often not significantly different to that of the A and B treatments (which are shown as an average of the two because they did not differ greatly; Table 5.3).

Cadmium

Paradoxically, although treatment A had the highest average available-Cd concentration in the soil (Fig. 5.2), it did not result in the highest shoot or grain Cd concentration in any of the cultivars. Treatment B plants had the highest grain Cd in every cultivar tested.

The differences in Cd availability in this experiment were not as large as have been found in other reported studies. At environmentally realistic Cd levels, Cd availability in soil pore water has been shown to drop to undetectable levels (Arao *et al.* 2009). In this experiment, Cd in soil solution was always detectable by ICP-MS, and the DGT devices enabled measurement of Cd at low soil solution concentrations. Nevertheless, the availability of Cd in treatment C did not drop as much as expected, and therefore, the differences in plant Cd uptake between aerobic and anaerobic soil conditions were smaller in this study than those reported elsewhere; for example, 20-25x less Cd accumulated under continuous flooding in moderately contaminated soil (Ishikawa *et al.* 2005b). The effect of irrigation treatment on shoot Cd accumulation in this study was also not

consistent between the cultivars, and some even showed increased Cd uptake under flooded conditions.

It is not likely that this was a problem with a failure to achieve reduced conditions in the flooded soil, because the differences in redox potential were reflected in the grain and shoot As concentrations of these plants. There was a significant and consistent difference that followed the well-known phenomenon that As is more plant-available under anaerobic soil conditions than aerobic soil (Arao *et al.* 2009). The As concentration of treatment C plants was, on average, 4.5x higher than treatment A plants, in both shoots and grain (As data is found listed in the text above).

The magnitude of the 'redox effect' could be related to the form of Cd used. The Cd salt that was used to spike the soil in this experiment would have been more available in the soil than Cd from long-term field contamination (Zhang *et al.* 2004a). It is possible that under flooded soil conditions this also affected the amount of the Cd that was bound or precipitated with other minerals. The soil used in this pot trial was also sandy, with low clay content, and so this may also have contributed to the higher Cd availability.

The lower plant Cd content in A compared with B could have arisen because of the physical conditions of the soil. For DGT soil deployment a high level of soil moisture is required. For this reason, the DGT tests in treatment A were always performed the day following a watering (so that the soil was close to water-holding capacity). Therefore, the DGT results have a bias towards the wettest period in the watering cycle of treatment A. It is possible that while the soil dried between watering, the bulk flow of Cd to the roots was restricted and the Cd available to the roots would have been reduced.

It is also possible that this result is an artefact of the potted conditions used for this experiment. Under flooded conditions, potted soil swells as the soil becomes saturated.

Therefore, after the initial flooded phase, the soil in treatment A pots shrank away from the edges of the pots, leaving soil and roots exposed down the side of the pot. Given the warm conditions of the greenhouse, these plants may have taken up the fresh irrigation water more rapidly than the soil pore water into which soil minerals would have dissolved.

In general, except for MLY, the ranking of the cultivars in this experiment was consistent in each of the treatments. This is the same as has been found in other studies with rice under submerged and drained soil treatments (Xu *et al.* 2009).

Manganese

Large differences in soil solution Mn content were found between flooded and unflooded soil (Fig. 5.3). These were consistent with field studies in paddy soil, which have shown that Mn availability is lowest near the oxic soil surface, and increased with depth in flooded soil (Roberts *et al.* 2011). Intermittent flooding (treatment B) also produced very responsive changes in Mn availability as well, but always less than in continuously flooded soil.

The high shoot concentrations of Mn in treatments B and C are therefore indicative of the high Mn availability in the flooded soil. These levels are high, but in the sub-toxic range for mature rice plants (Reuter and Robinson 1997, p 191). Plants lack effective mechanisms of restricting Mn uptake and limiting translocation to the shoot (Cailliatte et al 2010), but nevertheless, as these plants were growing in soil, Si was available to them and deposition of silica would have occurred in the shoot apoplasm. The formation of complexes between Mn and Si would have immobilised a significant portion of this Mn preventing its movement into the cytoplasm and hence toxic effects (Wang *et al.* 2000).

Despite the higher soil Mn availability in treatment C, the highest shoot and grain content was found in treatment B (Table 5.3). There was 50 to 100% greater shoot Mn concentration in treatment B plants than in treatment C, which is remarkable. The

difference in shoot Mn content between B and C could be explained by the Fe status of the roots of those plants. Although treatment C soil had higher Mn availability, it also displayed a 20-40-fold increase in Fe availability in soil solution, and thus, the difference in Mn availability between B and C was eclipsed by the changes that occurred in soil solution Fe as a result of this redox change (Fig. 5.5). The excess Fe in treatment C would have been present as external root plaque because of oxidation at the root surface (Liu *et al.* 2007a; Liu *et al.* 2008) and Mn, which also precipitates on root surfaces because of radial oxygen release (Liu *et al.* 2005c), may have been precipitated on/with the Fe plaque, thereby inhibiting plant Mn uptake. Another possibility is that the increased plant Fe, which in the root would have been both apoplastic and symplastic, would have competitively reduced Mn uptake. If these metals shared membrane transporters, such as an NRAMP gene or ZIP protein (like OsIRT1), then the Fe content could have restricted uptake and translocation of Mn competitively.

Recent evidence suggests that the site of competition between Mn and Fe for membrane transport is different in rice than in other plants. The arabidopsis gene, AtIRT1, transports Fe(II), Mn, Zn and Co (Korshunova *et al.* 1999), and an IRT1 homologue in barley transports Mn (Pedas *et al.* 2008), but OsIRT1 and OsIRT2 have been shown not to transport Mn and Cu (Ishimaru *et al.* 2006; Lee and An 2009). OsNRAMP1, also, has been tested for Mn transport in a heterologous system and was not able to recover a Mn-deficient yeast mutant (Takahashi *et al.* 2011). The remaining possibility, from current knowledge, is OsYSL2, which is known to be Fe-NA and Mn-NA transporter involved in phloem transport of these metals (Koike *et al.* 2004; Ishimaru *et al.* 2010). Although the Strategy II uptake system, involving the release of DMA, would not be functional under flooded conditions, NA is an internal chelator of Fe and Mn, and is critical for their translocation to aerial plant parts (Ishimaru *et al.* 2010). The supply of NA and competition for OsYSL2 could be behind reduced Mn uptake at high exogenous Fe supply.

Gene expression analysis: role of OsNRAMP1 in Cd accumulation under soil conditions

Shoot expression analysis for OsNRAMP1 was used in this study as a substitute for root sampling and analysis because of the known link between root and shoot expression of OsNRAMP1. While that root and shoot expression would not exactly match, upregulation in response to Fe deficiency would occur in both roots and shoots (Takahashi *et al.* 2011).

In a number of the cultivars tested here, OsNRAMP1 was repeatedly upregulated under the aerobic soil conditions of treatment A, compared to flooded soil. Except for SWE, this effect was restricted to the *indica* rice varieties, rather than the *japonica* lines. However, although these *indica* varieties are also high Cd accumulators, the pattern of OsNRAMP1 upregulation did not coincide with high grain Cd content; MLY and KAS showed the most consistently high OsNRAMP1, but were not the highest Cd accumulators. In addition, there was no strong evidence for upregulation of OsNRAMP1 under treatment B, which was the treatment which resulted in the highest Cd accumulation.

What seems clear is that the OsNRAMP1 expression was a plant response to drier soil conditions. This could have been related to the physical soil characteristics, or more likely, was a response to substrate metal availability. Under aerobic conditions, ferrous iron, Fe(II), becomes unavailable in soil solution because of the high redox potential. The DGT results (Fig. 5.4) showed that some Fe is still present in the labile phase, but it is likely that the Strategy II system of Fe(III) uptake would need to be operational under these dryland conditions (Ishimaru *et al.* 2006). Given that OsNRAMP1 is one of the first genes to respond to low Fe supply (see Chapter 6.1-2), the upregulation of expression found here could logically indicate that the soil-grown plants are responding to Fe status in this situation.

The dry, aerobic soil also had lower Mn availability. To test the theory that OsNRAMP1 may respond to Mn status, colleagues at the University of Tokyo tested cDNA

from rice grown under -Mn conditions for expression of OsNRAMP1. It was found that shoot expression of OsNRAMP1 was significantly increased in -Mn plants compared to Control or -Zn plants. The difference in root expression was not significant, so it is doubtful that OsNRAMP1 is driving Mn uptake in rice roots (pers. comm. Ryuichi Takahashi, September 12th 2011). Nevertheless, it is likely that the increased OsNRAMP1 shoot expression in aerobic soil is in response to reduced Fe and/or Mn supply.

Conclusion

Within the rice species, strong genotype effects influence Cd accumulation. Between the eight cultivars tested here, genotype was a stronger factor in determining Cd accumulation than irrigation regime.

This pot trial did not provide support for the conclusion that varying grain Cd accumulation under differing soil redox conditions is caused by anything other than changes in Cd availability in the soil solution. The degree to which soil Cd availability varied between the predominantly aerobic, intermittent flooding (B) and anaerobic, continuous flooding (C) treatments matched that of the shoot and grain Cd content. However, the reason that treatment A, which had highest Cd availability, did not have the highest Cd accumulation is not explained.

As in solution culture (Section 4.2; Takahashi *et al.* 2011), the *indica* rice varieties displayed a greater propensity for OsNRAMP1 upregulation than *japonica* rice. This could be associated with the higher genotypic Cd accumulation in *indica varieties*, but the effect cannot be linked directly to the treatments used here because the intermittent flooding treatment, which produced the greatest plant Cd accumulation, did not have increased expression of OsNRAMP1.

In the following chapter the function of OsNRAMP1 will be examined in more detail, to clarify what role this gene plays in the accumulation of Cd by rice plants.

Chapter 6

The relevance of iron-deficiency-responsive genes to Cd accumulation in rice

Introduction

The phenomenon of Fe-deficiency-stimulated increase in Cd uptake has been repeatedly documented in the plant nutrition literature. This effect is most often linked to the role of the IRT1 (iron-regulated transporter 1) and its homologues, as these genes are greatly upregulated under Fe-deficiency (Cohen *et al.* 1998; Korshunova *et al.* 1999; Connolly *et al.* 2002; Lombi *et al.* 2002; Vert *et al.* 2002), and have a broad substrate range for metals, including Zn^{2+}, Cd^{2+} and Fe^{2+}. Primarily this role of IRT1 in Cd transport has been examined in dicots, but in rice OsIRT1 has also been found to transport Cd (Nakanishi *et al.* 2006).

The Strategy II Fe acquisition systems –secretion of phytosiderophores (mugineic and deoxymugineic acid, MA and DMA) for the uptake of Fe(III)– have not been strongly implicated in Cd uptake in grasses. Although phytosiderophores can mobilise Cd in the soil, it seems that these Cd-MA complexes are not readily taken up into the plant (Shenker *et al.* 2001; Hill *et al.* 2002; Nakanishi *et al.* 2006; Kudo *et al.* 2007). In fact, Meda *et al.* (2007) demonstrated in maize that secretion of phytosiderophores provided a level of tolerance to Cd because of increased uptake of Fe(III) for which they have more affinity. However, in addition to the mugineic acids, their key precursor, nicotianamine (NA), is potentially important in the movement of Cd within plants independent of phytosiderophore secretion from the roots. NA is known to form conjugates with metal cations within the plant, and is implicated in long-distance transport of Fe and other metals around the plant (including Zn, Mn, and Cu) (Kim *et al.* 2005). Nicotianamine synthase (NAS) genes are highly expressed in

rice roots under Fe deficiency (Inoue *et al.* 2003; Curie *et al.* 2009), and so may play a key role in the increased Cd uptake that occurs under Fe deficiency.

More recently another Fe transporter, OsNRAMP1, has been found to also transport Cd (Takahashi *et al.* 2011). OsNRAMP1 has a number of identified characteristics that make it a candidate for the increase in Cd accumulation under Fe deficiency: (1) it is Fe-deficiency responsive (Cheng *et al.* 2007); (2) it is expressed primarily in roots (Belouchi *et al.* 1997); (3) it localises to the plasma membrane (Takahashi *et al.* 2011) and (4) it is able to transport Cd in heterologous expression systems (Takahashi *et al.* 2011). Finally, the OsNRAMP1 gene is of interest for genotypic variation in Cd accumulation because it is located within a quantitative trait locus (QTL) for root-to-shoot Cd translocation in soil-grown rice plants (Ueno *et al.* 2009b). Chapter 5 showed that some upregulation of OsNRAMP1 occurred when rice plants were grown in aerobic soil.

The experiments in this chapter were designed to test the hypothesis that OsNRAMP1 is an important membrane transporter associated with the uptake of Cd during Fe deficiency in rice. This necessarily involved a comparison with other IDR genes also potentially affecting Cd uptake. The first section describes a study that aimed to distinguish the Fe-deficiency-responsive transporter genes that correlate well with the known deficiency-induced increase in Cd uptake. As a part of this, the changes in Cd uptake during the development of Fe deficiency in hydroponically grown rice plants' uptake were contrasted with the concomitant expression of IDR genes relevant to Cd uptake in plants grown under the same conditions. For the final two sections, to further test the role of OsNRAMP1, transgenic rice germplasm was acquired with (a) overexpression of the OsNRAMP1 gene and (b) suppression of OsNRAMP1 expression by RNA-interference. These lines were tested for Cd, Fe and Mn uptake in both hydroponic and soil conditions.

6.1: The development of Fe deficiency in rice: dynamics of stress responsive gene expression and associated Cd uptake

Introduction

This study aimed to determine which Fe-deficiency-responsive transporter genes in rice correlate well with the known deficiency-induced increase in Cd uptake. Part a, describes preliminary results comparing expression of OsIRT1 and OsNRAMP1 during Fe deficiency; part b, details an investigation into the dynamics of Fe-deficiency-responsive (IDR) genes and concomitant uptake rates of Cd during the development of Fe deficiency in rice.

a. Comparison of the upregulation of OsNRAMP1 and OsIRT1 during early Fe deficiency

It had been noticed in preliminary experiments, with rice plants growth in hydroponic conditions, that there was a phase in early Fe deficiency during which OsNRAMP1 was significantly upregulated but OsIRT1 was not. After this phenomenon was observed in rice roots on more than one occasion, an experiment was planned to test the timing of upregulation of OsNRAMP1 and OsIRT1 during the development of Fe deficiency in rice.

b. Effect of ±Fe pre-treatment on the uptake of Cd by rice plants and related expression of iron-deficiency-responsive genes

In order to contrast Fe-deficiency-driven Cd uptake and IDR gene expression, plus and minus Fe pre-treated rice plants were tested for their rate of Cd uptake using ^{109}Cd radioinflux experiments at three different time points after the beginning of Fe deficiency. At each of these time points, roots of plants pre-treated in the same way were analysed by reverse transcription, real-time PCR (RT-qPCR) to correlate changes in plant gene

expression with increases in Cd uptake rates. To distinguish the effect of Fe competition and Fe deficiency, the plants were tested in nutrient solutions both with and without Fe added.

In hydroponics, Fe is generally supplied in an artificially chelated form because of the precipitation of Fe^{3+} that occurs in unchelated conditions. In this study, when Cd influx was measured in the presence of Fe, Fe(III)NaEDTA was replaced with Fe(II)SO_4 (added twice a day) to avoid possible confounding effects of EDTA on Cd uptake from solution. It is known that ferrous Fe is soluble in water but, in the presence of oxygen, it is relatively quickly oxidised to Fe(III). Despite this, it has been shown empirically that regular (e.g. daily) addition of ferrous Fe can supply sufficient Fe for the growth of plants (1993a; 1993b). The rate at which Fe(II) is oxidised is actually highly pH dependent and at pH values below 6 (common for nutrient solutions), the half-life of Fe(II) is in hours not minutes, especially when anions such as sulphates and nitrates are present in the solution (Tamura *et al.* 1976; Davison and Seed 1983; Kirby *et al.* 1999; Cornell and Schwertmann 2003). Although not directly measured in this experiment, a realistic estimate of the survival of the Fe(II) supplied at 20 µM is a half-life of 6-12 h (see Davison and Seed 1983).

Materials and Methods

Plant Culture

Rice seeds (*O. sativa* subsp. *japonica*) were surface sterilised and placed on moist paper towel to germinate (in dark for 4 or 5 d at 28^{o}C). Germinated seedlings were planted in a hydroponic setup with the following nutrient solution: 1.5 mM NH_4NO_3; 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.5 mM $MgSO_4$; 0.1 mM KH_2PO_4; 50 µM FeNaEDTA; 5 µM $ZnSO_4$; 0.5 µM $CuSO_4$; 5 µM $MnCl_2$; 50 µM H_3BO_3; and 0.1 µM MoO_4. Plants were grown in a controlled temperature

growth room on a 12 h day/night cycle, 28^{o}C and 25^{o}C respectively, ± 2^{o}C. Overhead lamps gave a flux density of approximately 400 µmol m^{-2} s^{-1}.

a. Comparison of the upregulation of OsNRAMP1 and OsIRT1 during early Fe deficiency

For the first experiment, the expression of OsIRT1 and OsNRAMP1 in rice (Chinese cultivar 'N07-63') during the development of Fe deficiency was compared in three different treatments, +Fe (including 50 µM FeNaEDTA), -Fe, and -Fe+Cd (50 nM Cd). At 13 DAS, plants were transferred to three different nutrient solutions: (1) nutrient solution including 50 µM FeNaEDTA; (2) nutrient solution without Fe; and (3) nutrient solution without Fe, with 50 nM $CdCl_2$ added (four biological replicates per treatment). These solutions were refreshed every 5 days, and then roots were harvested at 6 d and 14 d (DAT) for RT-qPCR.

b. Effect of ±Fe pre-treatment on the uptake of Cd by rice plants and concomitant expression of iron-deficiency-responsive genes

Eleven day old rice seedlings (Australian cultivar 'Amaroo') were subjected to treatment combinations of ±Fe (50 µM FeNaEDTA) and ±Cd (50 nM). At 2, 5 and 10 days after treatment (DAT), the root expression of a number of IDR genes was measured in these plants relative to an internal control gene (see below). OsIRT1, OsNRAMP1, OsNAS1, OsNAS2, and OsYSL2 were tested, as well as OsCAX1a (a membrane transporter which should be unaffected by Fe deficiency signalling).

At each of these time points, the rate of uptake of ^{109}Cd (50 nM Cd) by roots and shoots, over 24 h, was measured for ±Fe (+Cd) pre-treated plants, in two different nutrient solutions (± 20 µM Fe(II)SO_4). Hence, there were four treatment combinations. For each

solution, a large tub containing 15 L of nutrient solution was used for ten seedlings, five pre-treated in Fe deficient conditions and five with Fe supplied. Growing all the plants in one large container for each nutrient solution was chosen above the option of individual experimental units because of the need to control and monitor the Cd concentration in the solution. The shared nutrient solution provided greater confidence that the replicate plants were experiencing a very similar concentration of Cd throughout the experiment. However, the plants did have a shared root environment and a drawback of this design was that the effect of differing release of compounds such as phytosiderophores was ignored. This decision was made on the basis of the assumption that as this was a hydroponic setup, the effect of root compounds on Cd uptake would have been diluted and diminished. In addition, iron phytosiderophores have not been found to increase plant Cd uptake (see Introduction, this chapter), and principally, this experiment aimed to study the effect of root expression of membrane transporters on Cd uptake rather than the operation of exudates in the rhizosphere.

At 10 DAT, the uptake of Cd from an additional treatment containing 20 µM Fe(III) was contrasted. In addition, radioactive ^{55}Fe (in the form of chelated FeEDTA; New England Nuclear) was added to this solution. The accumulation of ^{55}Fe could be measured simultaneously in plant tissues because of its comparatively lower energy (0-4 keV range) than ^{109}Cd (which was measured in 15-100 keV range of the scintillation counter).

Cd radioinflux experiments: setup, harvest and scintillation counting

For these experiments, radiolabelled $^{109}Cd/CdCl_2$ was added from a prepared diluted stock, to enable adjustment/maintenance of solution Cd concentration without changing the specific activity of ^{109}Cd. The activity of ^{109}Cdat the production reference date was 7.4 MBq ml^{-1}, for a 5.1 mM Cd solution (Eckert & Ziegler Isotope Products; Valencia, California). The

diluted stock consisted of 5 mM $CdCl_2$, of which ≥70% was non-radioactive: 150 µl of stock was added to make up 15 L of nutrient solution to 50 nM Cd. To maintain uniform conditions the tubs were mixed by constant aeration. Samples were taken during the 24 h experiment to check for changes in the Cd content of the solution, small adjustments were made (for losses of up to 20% of the initial Cd) but large changes did not occur because of the large volume of solution used: 1 plant per 1.5 L of nutrient solution.

After 24 h, the plant roots were rinsed in deionised water and desorbed in three 15 min successive rinses with a solution of 5 mM $CaCl_2$, 0.5 mM Citric Acid, and 10 µM $CdCl_2$ (non-radioactive), adjusted to pH 3.5 with NaOH. The roots and shoots of each plant were then harvested into separate tubes, with fresh weight recorded. The roots were placed directly into scintillation fluid (Ultima Gold, Perkin Elmer Life Sciences) and incubated for >24 h prior to scintillation counting (Packard Tri Carb 2100TR, Canberra Packard). The shoots were digested in plastic centrifuge tubes in a dilute solution of 0.1 M HCl and 1 mM $CaCl_2$, at 100^{o}C for 40 min. After weighing the tubes to determine final volume of digest solution, a 1 ml aliquot was mixed with scintillation fluid for counting.

Gene expression analysis: RNA extraction and RT-qPCR

Plant tissue was ground to a powder in liquid nitrogen, and TRIzol® reagent (Invitrogen) was used to extract RNA according to the manufacturer's instructions. First-strand cDNA was generated using Omniscript® Reverse Transciptase (Qiagen) and oligo-dT primers to create a cDNA pool for use with gene-specific primers. RT-qPCR primers were designed using the PerlPrimer software program, v1.1.14 (Marshall 2004). Rather than DNase I treatment of RNA prior to RT, where possible, primers were designed to have at least one primer overlapping an exon junction, and hence, specific to cDNA copies of the genes (primer sequences can be found in Table 4.1). Nevertheless, non-RT controls were

performed for all primer pairs. The reaction products of the qPCR reaction were checked by melt-curve analysis and visualisation on 1.5% agarose gel.

Real-time quantitative PCR (qPCR) was performed with the Rotor-Gene™ 6000 (Corbett Research). Reactions were performed in a volume of 10 µl with the KAPA™ SYBR® FAST qPCR Master Mix (Kapa Biosystems), with each cDNA sample run in duplicate. Standard curves for each primer pair were generated from a pooled sample of cDNA from plants under control conditions (+Fe). Amplification efficiency was checked and conditions optimised to be within 91-105% reaction efficiency for all genes quantified. The validated internal control gene, eukaryotic Elongation Factor-1 alpha (*e*EF-1α) was analysed for normalisation of expression data (Jain *et al.* 2006).

Results

a. Comparison of the upregulation of OsNRAMP1 and OsIRT1 during early Fe deficiency

This experiment confirmed the observation that OsNRAMP1 was upregulated earlier than OsIRT1 in response to Fe deficiency stress in rice roots. Figure 6.1 displays the results of the comparison of the regulation of OsIRT1, OsNRAMP1 and OsNRAMP2 in response to Fe deficiency. Of these three genes, only OsNRAMP1 and OsIRT1 were responsive to -Fe treatment. OsNRAMP1 was shown to respond to Fe deficiency signals faster than OsIRT1, and at 6 DAT the significant upregulation of OsNRAMP1 under -Fe was not matched by OsIRT1. There was, however, evidence of Cd-induced OsIRT1 expression under -Fe conditions. This was observable in the earlier stimulation of OsIRT1 expression when Cd was present (Fig. 6.1a). In contrast, at 14 DAT, low Cd treatment appeared to reduce IDR gene expression. This RT-qPCR result was supported by visual observations of the +Cd plants at that sampling date: 14 DAT the -Fe plants were stunted and had chlorotic new leaves, while the -Fe+Cd plants were looking relatively healthy and similar in height to the +Fe control plants.

b. Effect of ±Fe pre-treatment on the uptake of Cd by rice plants and concomitant expression of iron-deficiency-responsive genes

Gene expression analysis was performed on rice plants 2, 5 and 10 days after the beginning of Fe deficiency treatment (Table 6.1). These results are displayed visually in Fig. 6.2, for the ±Fe+Cd treatments only. Upregulation of some IDR genes were seen from 2 DAT. OsNRAMP1 and the OsNAS genes (panel B and C, respectively) displayed the most rapid increase in expression in response to lack of Fe. Visual yellowing symptoms were beginning to be seen at 5 DAT, but negative effects on plant growth were first seen at 10 DAT, with obvious stunting (Fig. 6.3).

Plants grown and treated in exactly the same way as the ±Fe +Cd treatments were tested in a Cd uptake experiment (Table 6.2). Fe deficiency had a relatively small (approximately 20%) but significant effect on the shoot Cd accumulation of the seedlings at 2 d and 10 d after the beginning of -Fe treatment (Fig. 6.4). No evidence for Fe deficiency induced upregulation of Cd was seen at 5 DAT. Although Fe pre-treatment had a significant effect on root to shoot translocation at 2 and 10 DAT, Fe deficiency did not cause an increase in root Cd concentration (Table 6.2) or total Cd uptake (Fig. 6.5) at any time-point. The Fe deficiency effect on Cd uptake was overshadowed by the large change in Cd uptake caused by Fe(II) competition. At 2 and 10 DAT, where plants were tested in a solution with 20 µM $FeSO_4$, there was a 6-fold and 4-fold effect, respectively, on Cd uptake. At 5 DAT, when a 2 µM $FeSO_4$ uptake solution was compared, the effect of Fe(II) competition was only seen in shoot Cd concentration.

At 10 DAT, Fe(III) uptake was also measured. Fe deficiency increased the translocation of Fe from root to shoot as well (2-fold increase, Table 6.2), but did not lead to an increase in root Fe accumulation. Root Fe concentration was three orders of magnitude higher than the shoot accumulation after the 24 h duration of this experiment (mean root Fe content was 13,600 nmol g^{-1} for Fe sufficient plants and 9600 nmol g^{-1} for Fe deficient plants). However, large variability was seen in the root Fe content, and the desorption protocol used here was optimised for Cd not apoplastic Fe.

Despite the large effect of the presence of Fe^{2+} on Cd uptake, Fe(III) supplied chelated with EDTA did not restrict the uptake of the divalent Cd^{2+}. There was not a significant difference to the plants tested under -Fe conditions (Table 6.2).

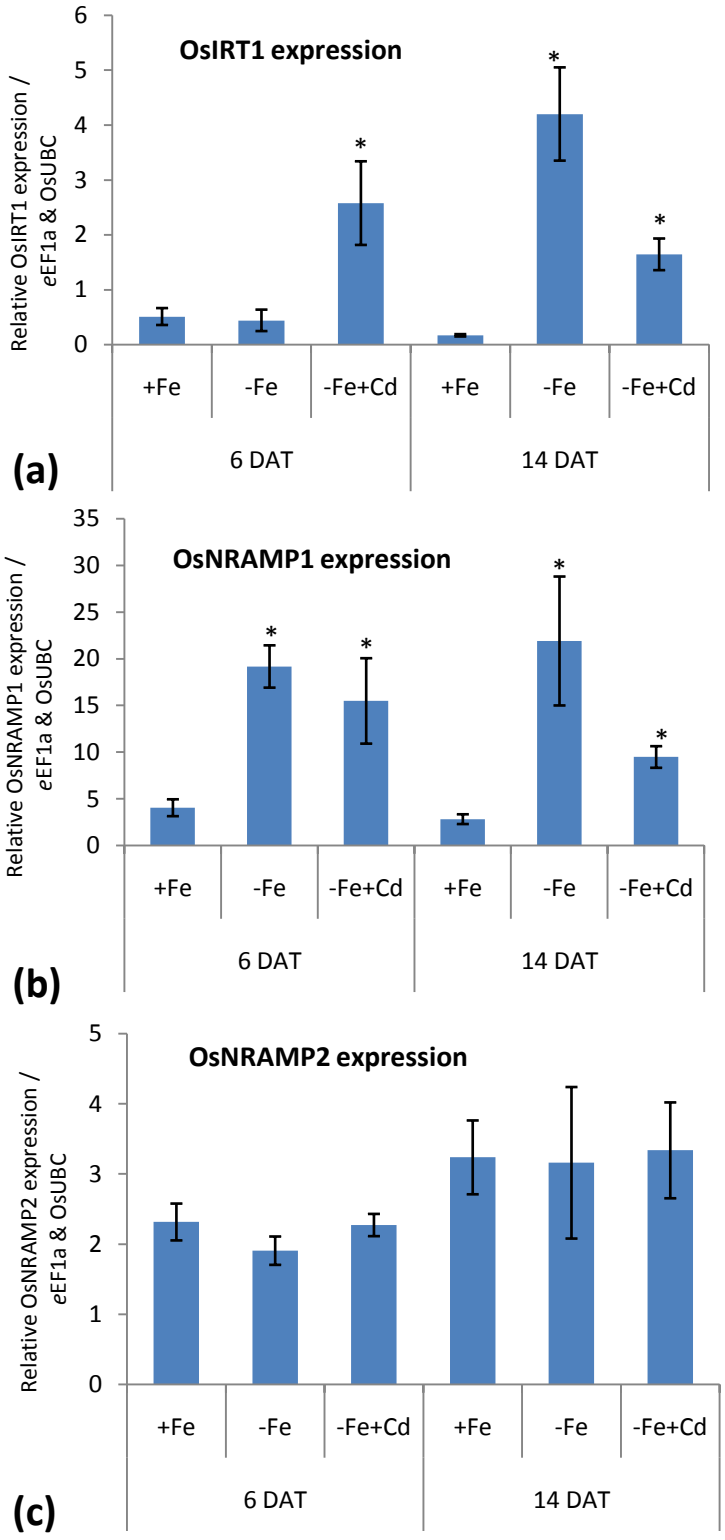


Figure 6.1: Expression of three different iron-deficiency-responsive genes (OsIRT1, **a**; OsNRAMP1, **b**; OsNRAMP2, **c**) in roots of rice plants at two points after the beginning of -Fe treatment: 6 DAT and 14 DAT. Rice seedlings were grown in three different nutrient solution treatments: +50 µM FeEDTA, -Fe, and -Fe +50 nM $CdCl_2$. Gene expression was measured by RT-qPCR, and is expressed relative to constitutive expression from a pooled control sample, and then normalised against the (geometric mean) expression of two control genes: OsUBC and Os*e*EF1α. Asterisks above columns indicate means which are significantly different (L.S.D., 5% level).

Table 6.1: The measured relative root expression of iron-deficiency-responsive genes. Results of quantitative RT-qPCR using a cDNA pool reverse-transcribed from root RNA of plants grown under four different treatment conditions, sampled at 2, 5 and 10 DAT. Expression data is relative to the gene expression of plants under control conditions (pooled +Fe cDNA sample) and is normalised against the expression of the *e*EF-1α gene.

			CAX1a		NRAMP1		IRT1		NAS1		NAS2		YSL2	
2 DAT	-Cd	+Fe	0.9	*ab*	0.6	*a*	0.7	*a*	1.1	*ab*	0.4	*a*	0.3	*ab*
	+Cd	+Fe	0.8	*a*	0.7	*a*	1.2	*bc*	1.9	*b*	0.8	*bc*	0.6	*abc*
	-Cd	-Fe	0.8	*a*	3.0	*e*	1.6	*c*	8.2	*ef*	3.5	*d*	0.5	*bc*
	+Cd	-Fe	0.9	*ab*	2.0	*d*	1.6	*c*	6.0	*de*	2.6	*d*	0.3	*a*
5 DAT	-Cd	+Fe	0.9	*abc*	1.2	*c*	1.1	*bc*	2.5	*c*	1.1	*c*	0.4	*ab*
	+Cd	+Fe	1.0	*abc*	1.1	*bc*	0.6	*a*	2.7	*c*	1.1	*c*	0.4	*ab*
	-Cd	-Fe	1.2	*c*	4.9	*f*	3.7	*d*	18.2	*g*	8.7	*f*	0.5	*ab*
	+Cd	-Fe	1.2	*c*	5.1	*f*	4.1	*de*	12.9	*fg*	6.6	*ef*	0.5	*ab*
10 DAT	-Cd	+Fe	1.0	*abc*	0.8	*ab*	0.8	*ab*	1.1	*a*	0.8	*bc*	0.5	*ab*
	+Cd	+Fe	1.1	*bc*	0.7	*a*	0.8	*ab*	0.8	*a*	0.6	*ab*	0.5	*ab*
	-Cd	-Fe	0.9	*ab*	5.2	*f*	6.2	*f*	4.0	*cd*	2.3	*d*	1.2	*d*
	+Cd	-Fe	1.0	*abc*	4.1	*ef*	5.6	*ef*	6.0	*de*	3.9	*de*	1.0	*cd*

N.B. Letters in adjacent columns indicate statistical significance of log transformed values; different letters (within a single column) are significantly different according to L.S.D. (5% level; ANOVA).

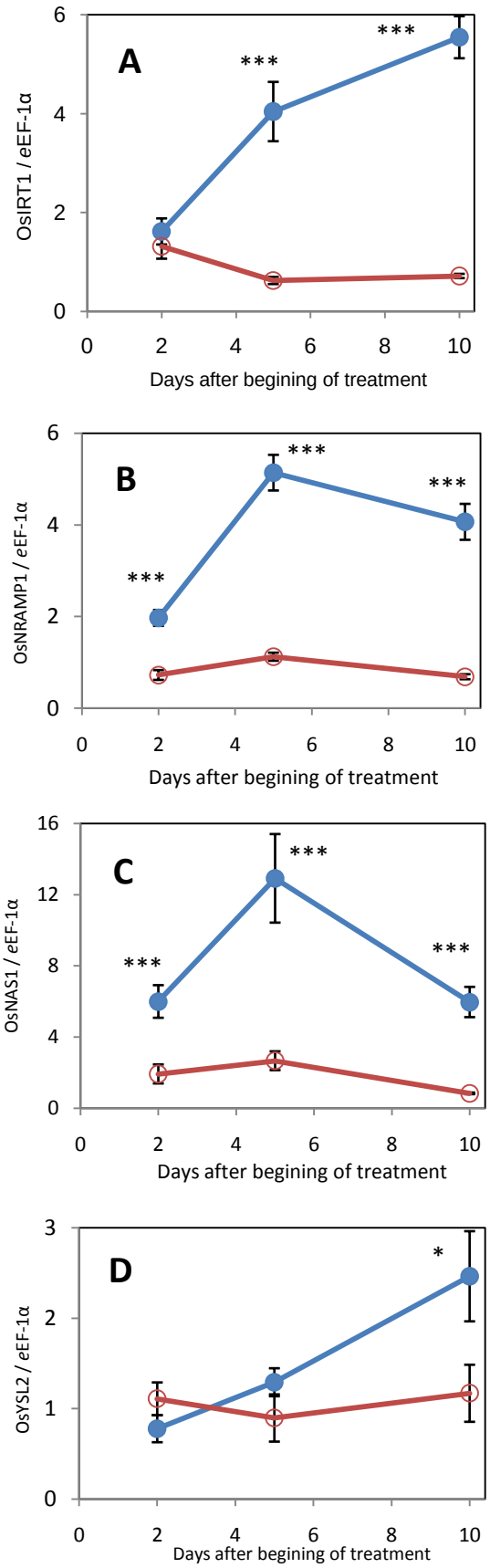


Fig. 6.2: The measured relative root expression of four iron-deficiency-responsive genes, OsIRT1 (**A**), OsNRAMP1 (**B**), OsNAS1 (**C**), and OsYSL2 (**D**). Results of quantitative RT-PCR using a cDNA pool reverse-transcribed from root RNA of plants grown under two different treatment conditions: -Fe+Cd (closed circles), and +Fe+Cd (open circles); sampled at 2, 5 and 10 DAT. Expression data is relative to the gene expression of plants under control conditions (pooled +Fe cDNA sample) and is normalised against the expression of the *e*EF-1α gene. Error bars display SE of the mean at each sampling date (n=4). Expression at 0 DAT is that of plants under +Fe-Cd treatment. Asterisks indicate the first instance of statistical significance (***L.S.D., 0.1% level; *L.S.D., 5% level).

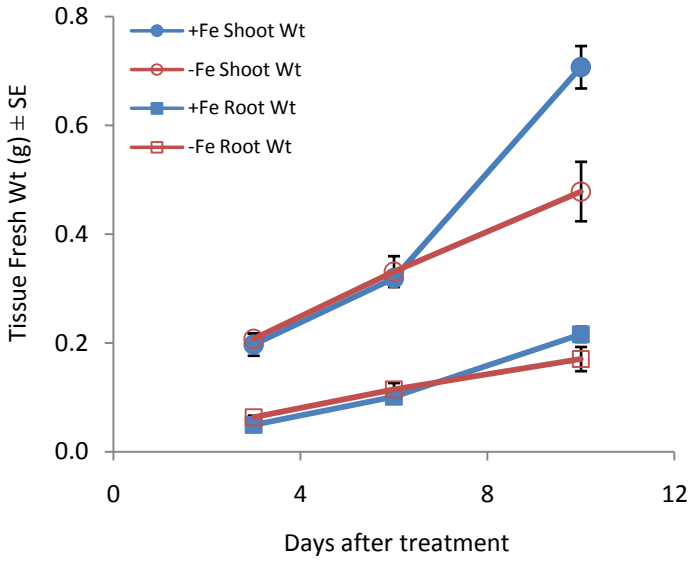


Fig. 6.3: Comparison of growth of rice seedlings (root and shoot fresh weight) following application of ±Fe treatment. Closed circles and squares represent shoot and root weight in +Fe conditions. Open circles and squares represent shoot and root weight in -Fe conditions, respectively.

Table 6.2: Cd accumulation in rice over 24 h, at 2, 5, and 9-10 d after the beginning of minus Fe treatment. Uptake was measured by radiotracer ^{109}Cd and Fe(III) accumulation in shoots measured by ^{55}Fe. Roots were desorbed with a Ca-citrate solution, pH 3.5.

	Fe pre-treatment	Nutrient soln	Root Cd conc (nmol g^{-1} d^{-1})		Shoot Cd conc (nmol g^{-1} d^{-1})		Shoot Cd as a prop^{n} of plant Cd	Shoot Fe conc (nmol g^{-1} d^{-1})	
2 DAT (n=5)	+Fe	+Fe(II) 20 µM	17	*b*	1.4	*a*	0.22		
	-Fe		17	*b*	1.7	*b*	0.23		
	+Fe	-Fe	73	*g*	8.1	*f*	0.29		
	-Fe		61	*f*	9.5	*g*	0.34		
5 DAT (n=5)	+Fe	+Fe(II) 2 µM	55	*de*	7.9	*f*	0.32		
	-Fe		56	*ef*	8.0	*f*	0.34		
	+Fe	-Fe	56	*ef*	10.2	*g*	0.33		
	-Fe		51	*de*	10.1	*g*	0.36		
9-10 DAT (n=4)	+Fe	+Fe(II) 20 µM	16	*ab*	1.3	*a*	0.20		
	-Fe		15	*a*	1.7	*b*	0.23		
	+Fe	-Fe	55	*de*	5.4	*c*	0.25		
	-Fe		44	*c*	6.5	*de*	0.28		
	+Fe	+Fe(III)	57	*ef*	6.2	*cd*	0.26	10.4	*a*
	-Fe		49	*cd*	7.4	*ef*	0.28	20.3	*b*

N.B. Different letters in the column adjacent indicate means which are significantly different by log-transformed ANOVA, L.S.D. 5% level.

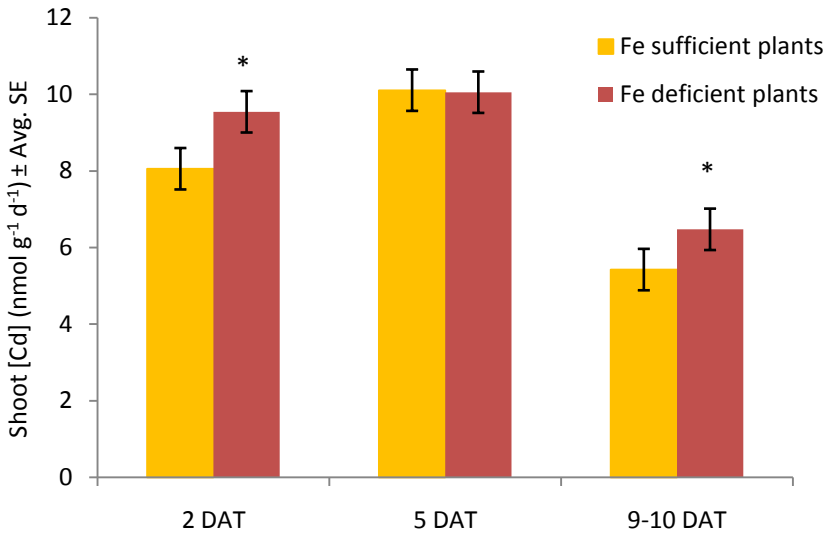


Fig. 6.4: Shoot Cd uptake of rice seedlings at three time points after beginning of -Fe treatment, tested in nutrient solution without $FeSO_4$ added. Data presented is a subset of that of Table 6.2. Asterisks represent statistical differences between treatment means by ANOVA, L.S.D. 5% level.

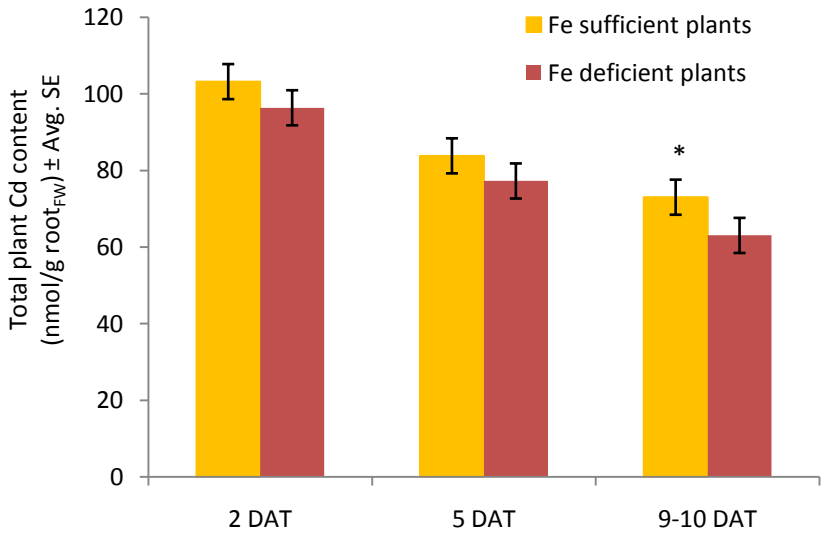


Fig. 6.5: Comparison of plant Cd uptake, from solutions without $FeSO_4$, expressed as total plant Cd content per g $root_{(FW)}$. Asterisk represents statistical differences between treatment means by ANOVA, L.S.D. 5% level. Differences in uptake between sampling dates are all significantly different.

Discussion

Iron deficiency effect

In contrast to the results that have been reported in dicot plants, the Fe-deficiency-induced increase in Cd uptake in rice was relatively minor. In these experiments, at 2 d and 10 d after the removal of Fe supply, there was a small (ca. 20%) but significant increase in shoot Cd content in the Fe deficient plants (Table 6.2). Surprisingly, this effect was not seen at 5 DAT, and also, Fe deficiency did not lead to an overall increase in plant Cd content or root Cd concentration. Nevertheless, there was a consistently strong effect of supplementation with Fe(II) on Cd uptake. This is most likely the result of competition between Fe^{2+} and Cd^{2+} at the site of a transporter/s that the two have in common, and Fe(III) did not have the same effect on Cd uptake. The small (or absent) effect of Fe deficiency on Cd uptake in rice seen here is entirely consistent with results published previously for rice by (Nakanishi *et al.* 2006) (despite the research article title suggesting otherwise).

During Fe deficiency development, OsNRAMP1 was upregulated at a similar rate to OsNAS1 and OsNAS2, but more quickly than both OsIRT1 and OsYSL2 (Table 6.1; Fig. 6.2) At 2 DAT, when a 20% increase in Cd translocation (i.e. to the shoot) occurred in Fe deficient plants, there was not a significant upregulation of OsIRT1 transcription. At this time there was a significant increase in some of the IDR genes, including OsNRAMP1. The increase in Cd uptake was not found to be correlated with the timing of the increase in OsIRT1 expression. At 10 DAT, when then were large increases in expression of both OsNRAMP1 and OsIRT1, the proportional increase in Cd translocation was still only approximately 20%.

This study has demonstrated that OsNRAMP1 and OsIRT1 are regulated differently by Fe deficiency, either independently or by different degrees of deficiency. Under these conditions, OsNRAMP1 was repeatedly stimulated before OsIRT1, in both a Chinese and an

Australian *japonica* rice cultivar. Yokosho (2009) found that OsIRT1 was easily detected after a few days of -Fe treatment, i.e. 5-6 d.

The transcription factor that controls OsIRT1 expression, IDEF1, was found to be upregulated within one day of a change to -Fe conditions (Kobayashi *et al.* 2009). There is clearly a relatively rapid regulatory response to the lack of Fe. OsNRAMP1 has been found to be Fe deficiency responsive in a number of other studies with rice (Cheng *et al.* 2007; Zheng *et al.* 2009; Ishimaru *et al.* 2010). However, the transcription factors involved in its regulation have not been identified.

In this experiment, OsNRAMP2 was not found to be responsive to Fe deficiency. The function of OsNRAMP2 has not been fully resolved (Narayanan *et al.* 2007), but it is reported to be primarily expressed in leaves (Belouchi *et al.* 1997) and it is in a gene family in which Mn transport is common. Heterologous expression of OsNRAMPs 2, 3, 4 and 8 restored the growth of the *smf* yeast mutant, which has impaired Mn transport activity (Naryanan *et al.* 2006).

Iron supply and Cd uptake

Given the strong effect that the presence of Fe(II) had on the accumulation of Cd, even at 2 µM $FeSO_4$ (Table 6.2), it is possible that the effect of Fe deficiency seen in this experiment is the result of differences in internal plant Fe (from that supplied previously in the growth nutrient solution). The +Fe pre-treatment (for Fe sufficient plants) in this experiment was most likely a combination of the transiently available free Fe^{2+}, and also the plant access to deposited Fe(III) on the surface of the roots. Rice roots release oxygen into the rhizosphere via aerenchyma and so oxidised Fe can precipitate at the roots. This deposited Fe plaque has been found not to influence the uptake of Cd in rice plants (Liu *et al.* 2007a; Liu *et al.* 2008). But the overall plant Fe content could directly affect Cd uptake (Shao *et al.* 2007).

For a similar reason, the lack of an Fe deficiency effect at 5 DAT could have been due to the large increase in OsNAS expression that occurred at this time (Fig. 6.2). This is fairly consistent with the result of Mori *et al.* (1991), who showed a peak in MA secretion at 2-3 d after the beginning of -Fe treatment, and then secretion dropping off to nothing at 7 DAT. So, in this experiment, the high level of NA present in the roots at 5 DAT would have led to a significant increase in mobilisation of stored root Fe and more chelation of Fe(II) in the phloem, because it is known that the internal transport of Fe is limited by the need for a specific chelator (Grusak *et al.* 1999). This increased mobilisation of Fe would have caused greater internal competition for Cd transport across membranes, and is a plausible explanation for why Cd translocation was not increased in these Fe deficient plants relative to the Fe sufficient plants.

The higher root Cd uptake (Table 6.2) and total plant accumulation (Fig. 6.5) seen in Fe-sufficient plants could have been the result of remnant EDTA molecules present in the roots from the growth solution. In a similar way, Nakanishi *et al.* (2006) observed that Fe (Fe-EDTA) resupply to Fe-deficient plants increased Cd uptake and translocation, compared with Fe-deficient plants tested under -Fe. It is known that EDTA can influence the influx of Cd (McLaughlin *et al.* 1997a) and so could have facilitated entry. Despite this, there was still a significant increase in the partitioning of Cd into the shoot, possible evidence for increased activity of IDR membrane transporters leading to greater Cd translocation.

OsIRT1

At 10 DAT there was increased root to shoot translocation of Fe, as measured by radioactive ^{55}Fe. This was likely the result of increased expression of a membrane transporter that can facilitate phloem loading. This could have been OsIRT1, because unlike the dicot versions, OsIRT1 has been found to be also operational at the companion cells

adjacent the phloem (Ishimaru *et al.* 2006), not just in the epidermal layers of the root (Morrissey and Guerinot 2009). No evidence was found for increased Fe uptake under conditions of Fe deficiency, as is the case in *A. thaliana* (Vert *et al.* 2002).

There is wide support in the literature for the role of IRT1 in Cd uptake. Primarily this has can be found in studies with arabidopsis. Most compellingly, when AtIRT1 was knocked-out, the Fe-deficiency-driven increase in Mn, Zn, Co and Cd uptake in *A. thaliana* did not occur (Vert *et al.* 2002). However, the conditions under which the plants of Vert *et al.* (2002) were growing differed greatly from this uptake experiment, with 20 µM Cd used, which is 400 times the concentration used here. Although IRT1 can and does facilitate Cd uptake during Fe deficiency, its importance has not been tested at environmentally-relevant concentrations of Cd.

In this experiment, no evidence was seen for the role of OsIRT1 in increasing the root uptake rates of Cd, as has been postulated (Nakanishi *et al.* 2006). There was no doubt that Fe deficiency was occurring; at 10 DAT there were obvious visual symptoms, which were confirmed by elevated expression of OsIRT1, but nevertheless the plants had reduced total Cd accumulation.

Arabidopsis plants overexpressing AtIRT1 have been shown to become sensitive to Cd levels as low as 0.01 µM (Connolly *et al.* 2002). However, this was in plants tested under strongly Fe deficient conditions (Ferrozine added), and toxicity was significantly less when Fe was present. When the affinity of the pea IRT1 homologue (called RIT1) was studied, it was found to have very high affinity for Fe, moderate affinity for Zn and low affinity for Cd (Cohen *et al.* 2004). While the K_m for Fe^{2+} was in the nanomolar range, for Cd it was 100 µM, a completely unrealistic environmental concentration of Cd for rice growth.

Barley also contains an IRT1 homologue (Pedas *et al.* 2008), but the increase in Fe transport that occurs under Fe deficiency in barley is much lower than in dicots (Zaharieva and Römheld 2000). This could presumably be because of the lack of functional ferric-chelate-reductase in the strategy II grasses (Ishimaru *et al.* 2006). Zaharieva & Römheld (2000) found that, whereas the presence of Cd was found to inhibit Fe^{2+} uptake in Fe-deficient cucumber plants, Fe^{2+} transport was markedly less affected (only by 12%) in Fe deficient barley plants, even at 100-fold excess of Cd . This also indicates a low affinity for Cd.

Lee *et al.* (2009) overexpressed OsIRT1 in rice and found increased accumulation of Fe and Zn, but not Cu, Mn or Co. They did see increased sensitivity to Cd, but only at high concentrations. In contrast, when the over-expressing plants were grown in the field (with a much lower concentration of Cd), Cd accumulation in shoots and seeds was not increased.

It is likely that OsIRT1 has similar functionality to the barley version of the gene, with which it has high sequence similarity (Pedas *et al.* 2008). Apparently this does not include Mn transport because HvIRT1 has been shown to respond to Mn deficiency and also to transport Mn, whereas OsIRT1 does neither (Ishimaru *et al.* 2006).

6.2: An investigation into the functional role of OsNRAMP1 in Cd uptake in rice, especially during low Fe conditions

Introduction

In order to further test the hypothesis that OsNRAMP1 is involved in Cd uptake in rice plants, rice germplasm was acquired with (a) overexpression of the OsNRAMP1 gene (OX) and (b) suppression of OsNRAMP1 expression by RNA-interference (RNAi). Following on from the experiments of Chapter 6.1, the uptake of Cd during the development of Fe deficiency in rice seedlings was analysed in these transgenic varieties. These plants with manipulated expression of OsNRAMP1 were compared with the untransformed (WT) plant at two and ten days after the beginning of ±Fe treatments. Two uptake experiments were conducted over 24 h and are designated 2 DAT and 10 DAT below.

Materials and Methods

Germplasm: OX and RNAi varieties

The transgenic rice varieties used in this study were sourced from the Laboratory of Plant Biotechnology at the Graduate School of Agricultural and Life Sciences, the University of Tokyo, Japan. The wild-type (WT) variety used for creation of the transgenics was the Japanese rice cultivar Tsukinohikari (*O. sativa* subsp. *japonica*). OsNRAMP1 over-expressing plants were created as outlined in Takahashi *et al.* (2011). The OsNRAMP1-ORF was cloned and overexpressed on the WT background, driven by the CaMV-35S promoter. RNA-interference lines for OsNRAMP1 were generated according to the method of Ishimaru *et al.* (2010), using a 300 bp interfering fragment located on the 3' UTR of OsNRAMP1, with the following primers, forward: (CACC)TACCCTTCCAGACCGGTTACC, reverse:

GATTGTCCTTGTCCTCGCATC. RNAi lines were selected for use based on OsNRAMP1 suppression, checked using RT-qPCR normalised against α-tubulin expression (Fig. 6.6).

Plant culture

T1 seeds of two RNAi lines, designated RNAi 1 & 10; two OX lines, OX-5 & 6; and the WT were surface-sterilised and placed on MS medium to germinate. For all lines except the WT, the MS medium was supplemented with Hygromycin B (Roche) at 50 mg L^{-1}, to select for transgenics containing the genes of interest. The seeds were incubated in the dark in a 25^{o}C incubator for germination. OX-6 was not used in the hydroponic experiment but was included in the latter potted soil experiment (Chapter 6.3).

The seeds were incubated in the dark at a warm room temperature (24-27^{o}C) for seven days. The plates were then moved to a 28^{o}C light/dark incubator for a further two days, until enough growth had occurred to facilitate selection. Nine days after sowing (DAS), suitable plants were transferred to a basic nutrient solution: 1.26 mM NH_4NO_3; 1 mM KNO_3; 1 mM $Ca(NO_3)_2$; 0.4 mM $MgSO_4$; 0.165 mM KH_2PO_4; 50 µM FeNaEDTA; 1 µM $ZnSO_4$; 1 µM $CuSO_4$; 5 µM $MnCl_2$; 10 µM H_3BO_3; 0.2 µM $CoSO_4$; 0.5 µM MoO_4 and 0.1 mM NaCl. The plants were suspended above PVC pots with 6.7 L of nutrient solution.

After 6 six days of growth, the seedlings (15 DAS) were transferred from a nutrient solution with Fe-EDTA for Fe nutrition, to one with 20 µM $Fe(II)SO_4$ added daily. Four days later 19 DAS seedlings were transferred to ± Fe treatments, with approximately 35-40 seedlings per 6.7 L of nutrient solution. The nutrient solution of these pots was changed frequently, at least twice per week, and again 24 h before each of the two uptake experiments. $FeSO_4$ was added daily to the +Fe treatment throughout. After the first uptake experiment, the number of plants remaining was 17-25 seedlings per pot.

Cd uptake experiments

At 2 and 10 DAT, a 24 h Cd uptake experiment was performed on ±Fe seedlings. The two experiments were essentially the same, except for frequency of solutions changes; refreshed once during the first experiment, and changed twice during the latter.

Individual seedlings were suspended from the lids of custom-made 0.5 L PVC pots, with 460 ml of nutrient solution. Nutrient solution was prepared in bulk, for consistency, as per the above composition but with added $Cd(NO_3)_2$ at a concentration of 0.1 µM. For each rice line, eight replicate pots were used, except for RNAi 6, for which only six pots were used (because of low germination rates). Plants were grown in a glasshouse with natural light, with a daily temperature range between 24-34^{o}C.

The 2 DAT experiment began late morning, with a full nutrient solution change for all pots after 9 h. At this nutrient solution change, the -Fe treatment was replaced with 110% Cd concentration to account for increased Cd uptake under Fe deficiency (as per Chapter 6.1). The 10 DAT experiment began 1 pm to 2 pm, with a solution change at 7 h and 20 h. For the first two periods, the -Fe treatment had 110% Cd added, and 105% Cd at T_{20}.

After 24 h of uptake, the plant roots were rinsed with dH_2O and then desorbed with three successive 15 min rinses of Ca-citrate solution (5mM $CaCl_2$ and 0.5 mM Citric Acid; pH 3.5). The shoots were also rinsed in dH_2O. For analysis, the plants of every two replicate pots were pooled to make four biological replicates of roots and shoots, except RNAi 6, which had only three.

Tissue sampling for RNA extraction and cDNA synthesis

At the same time as the Cd uptake experiment, additional plants were transferred from the bulk pots to two treatments of ±Fe. All five rice lines were placed in a single pot per treatment, with a solution of the same composition as the uptake experiment (+Cd ±Fe).

The plants were harvested 4-5 h later for RNA extraction. At 2 DAT, two plants were pooled for each root and shoot replicate, with up to 0.1 g_{FW} of root tissue and up to 0.1 g_{FW} of shoot material from the youngest expanded leaves. At 10 DAT, 1 to 2 plants were used as they were much larger.

The samples were collected at a similar time each day of sampling and placed in individual 1.5 ml tubes, frozen in liquid nitrogen, and then stored at -85^{o}C prior to processing.

The plant tissue was ground to powder under liquid N, and then RNA was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Precipitated RNA was stored in 75% ethanol at -20^{o}C until being dissolved in DEPC-treated water prior to quantification and use in reverse-transcription reaction.

An aliquot of all RNA samples were treated with RNase-free DNase I (Fermentas) for 30 min at 37^{o}C, and then denatured at 70^{o}C for 10 min, following the addition of dilute EDTA to 4.5 mM. cDNA was generated from these treated RNA samples using oligo-dT primers and RevertAid^{TM} First Strand cDNA Synthesis Kit (M-MuLV RT enzyme; Fermentas), in half reactions of 10 µl. Finally, the first-strand cDNA was treated with RNase H (Fermentas) for 20 min at 37^{o}C. The resultant cDNA product was diluted to 1-4 ng/µl in TE for use in real-time PCR.

Quantitative PCR

Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System. Reactions of 20 µl were performed on 96-well plates, using hot-start SYBR-green master mixes, KAPA SYBR® FAST qPCR Master Mix, or TaKaRa SYBR® *Premix Ex Taq^{TM}* (Perfect Real Time), with ROX™ reference dye.

Gene expression was quantified based on a standard curve of a serial dilution of a pooled sample of WT shoot cDNA from both ± Fe treatments. In this way, expression was calculated relative to this pooled WT sample. The target gene expression in each sample was normalised, for variability in mRNA content, by dividing by the geometric mean of Os*e*EF-1α and OsUBC expression.

Table 6.3 contains a list of the primer pairs used in this study. In addition to the OsNRAMP1 primers successfully used in previous sections (designated here, "ORF"), another primer pair was used to check suppression of OsNRAMP1. This set was designed for the 3' UTR of this gene, closer to the 3' poly-A tail and overlapping with the location of the RNA- interfering fragment. This set of primers also allowed quantification of OsNRAMP1 expression in the OX-5 line independent of the over-expressed ORF transgene.

Table 6.3: Primers used to measure the expression of selected IDR genes and house-keeping genes. Except for the *e*EF-1α and UBC, which were taken from Jain *et al.* (2006), all primers were designed using the PerlPrimer program (Marshall 2004). Two different primer pairs were used for OsNRAMP1, one which anneals to a section of the gene ORF (also detecting the over-expressed cloned gene, of OX-5), and another located on the 3' UTR (overlapping with the region targeted by the interfering fragment of these RNAi lines).

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'	Annealing temp. (°C)
OsIRT1	GATGTGCTTCCACCAGATGTTC	GAAGAAGACGAGCACCGA	63
OsNRAMP1 (ORF)	AGGAATGAAGGATGTCTGTAG	AGCATCTTCTGGTGAAAGG	59
OsNRAMP1 (3' UTR)	CACCCGTGTAGTACCCTTCCA	TCAGCCGGTTATCACTGCTAACC	65
Os*e*EF-1α	TTTCACTCTTGGTGTGAAGCAGAT	GACTTCCTTCACGATTTCATCGTAA	64
OsUBC	CCGTTTGTAGAGCCATAATTGCA	AGGTTGCCTGAGTCACAGTTAAGTG	63

Plant tissue processing and analysis

All plant material was dried completely at 70°C. After being weighed, root and shoot material was ground to a fine powder under liquid N with a mortar and pestle; with exception of the 2 DAT root samples, which were too small, and so were cut to 1-2 mm

pieces with scissors. The plant material was re-dried in the oven before weighing out: for the 2 DAT and 10 DAT experiments, respectively, up to 15 mg and 45 mg of root, and 70 mg and 200 mg of shoot material, was weighed into acid-washed plastic tubes.

Concentrated HNO_3 was added to each tube (≥2 ml acid per 200 mg sample) and the samples were allowed to pre-digest overnight. The following morning, an equal volume of 30% H_2O_2 was added to each tube. After frothing had subsided, racks of tubes (with lids in place but not tight) were placed in a 100^{o}C water bath, for at least 3 h digestion. For shoot samples, a further half volume of H_2O_2 was added after boiling to ensure the breakdown of all organic matter.

All the plant digests were diluted 16.5x, to make a final acid concentration of 3% (assuming an approx. 50% loss through reaction during digestion). The final weight of the dilution was recorded and subsamples were taken and passed through a 0.22 µm filter. Root and shoot samples were diluted a further 20x and 3x, respectively, using 3% HNO_3, and then analysed by ICP-MS (Agilent) for Fe, Mn, Zn, Cu, As and Cd.

Results

OsNRAMP1 transgenics

The transgenic lines in this experiment were sourced from Takahashi *et al.* (2011), and the overexpression lines are the same as in that published paper. Figure 6.6 shows the expression analysis of these lines when tested during their initial selection. The seeds used here are also T1 seeds, and expression analysis was performed as a part of this experiment but with a different primer pair for OsNRAMP1. OX-6 was grown only in the potted soil experiment.

The OsNRAMP1 transgenic lines were tested in a similar way to the previous hydroponic experiment (Chapter 6.1). The gene expression and Cd uptake in the early stages of Fe deficiency was compared to that of sufficient plants to examine the roles of the genes OsIRT1 and OsNRAMP1. This experiment was carried out in a different lab to the first and unfortunately the Fe deficiency brought about in the 10 days of -Fe treatment was not consistently strong. This was likely because of small amounts of Fe contamination in single-deionised water. Figure 6.7 and Table 6.4 show the results for these two genes at 2 and 10 DAT. OsNRAMP1 was upregulated in all -Fe treated plants except for those of OX-5, which had greatly increased gene expression in both Fe treatments (note the log scale).

The increase in OsNRAMP1 in the OX varieties was much higher than that found initially for these varieties (Fig. 6.6) which was around 4-fold. In this experiment, there was a difference between the WT and OX varieties of 1-2 orders of magnitude. In the +Fe (control) treatment, OX-5 exhibited an average 100-200x overexpression above the WT. The 3'UTR primers measured the 'native' expression of OsNRAMP1 in OX-5, independent from the cloned gene (see Table 6.4 & Appendix 1). The expression pattern was not significantly different to the RNAi varieties, but still indicated native upregulation of OsNRAMP1 in response to Fe deficiency treatment.

The suppression of OsNRAMP1 in the RNAi varieties was not as large as initially found (Fig. 6.7). The difference between the WT and the RNAi varieties was significant in the smaller +Fe plants at the 2 DAT sampling, but was not significant at 10 DAT. These expression results were also confirmed with an additional, independent set of primers for OsNRAMP1 that was within the 3'-UTR region, the location of interfering fragment, and a similar result was found (Table 6.4 and Appendix 1).

The rate of Cd uptake (per 24 h) was measured for the plants at these time points (Table 5). The accumulation of other elements such as Fe and Mn from the base nutrient

solution was also quantified. There was increased accumulation of Cd in the WT compared with the RNAi lines. The result is also complicated by the results for OsIRT1, which also showed decreased expression in the RNAi lines at 2 DAT (Fig. 6.7). In addition there were significant differences in plant size between the WT and the transgenics (Table 6.5).

Minus Fe treatment had a significant effect on plant biomass at 10 DAT; this difference was seen in root dry weight for all varieties except RNAi-1 (Table 6.5). In terms of shoot weight, there was more variability, and the differences between plus and minus Fe treatments were not significant by L.S.D. (5% level). At 10 DAT there were also noticeable differences between the growth rates of the rice lines. The WT had the largest shoot and root weight under both ±Fe, and OX-5 had the slowest growth and had the smallest plant size under Fe deficiency.

There was a difference between the transgenics and the WT in their Fe deficiency response. At both 2 and 10 DAT, OsIRT1 expression in response to -Fe treatment was not significantly increased in the WT. In addition, at 10 DAT, the -Fe effect on upregulation of OsNRAMP1 was lower in the WT than in the other varieties. This was despite the fact the WT was the only line to have significantly reduced shoot Fe concentration.

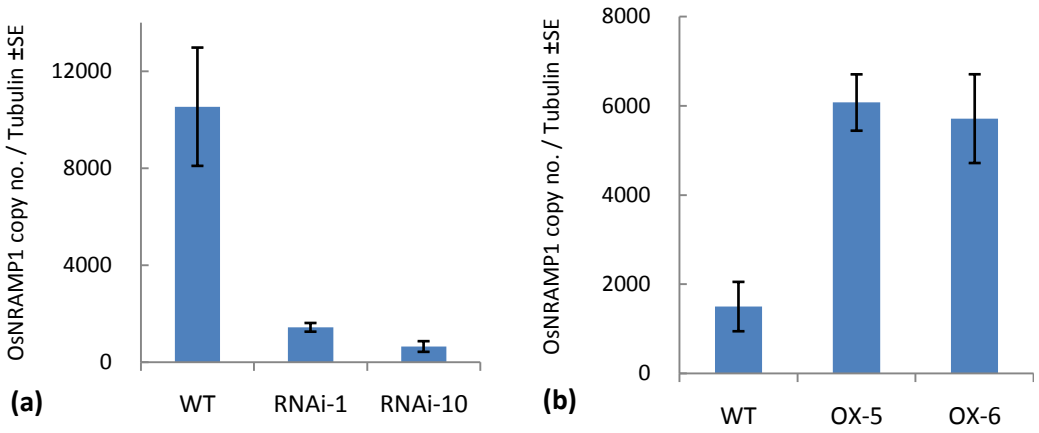
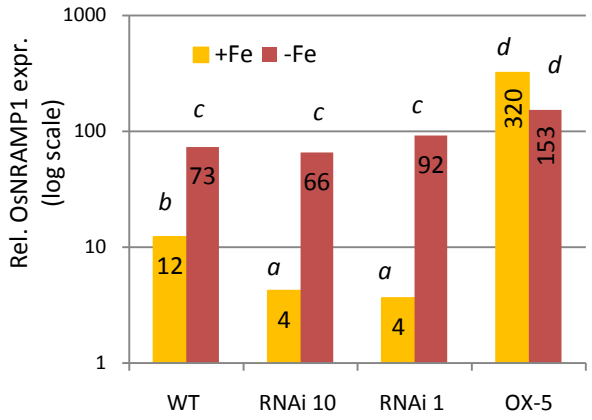
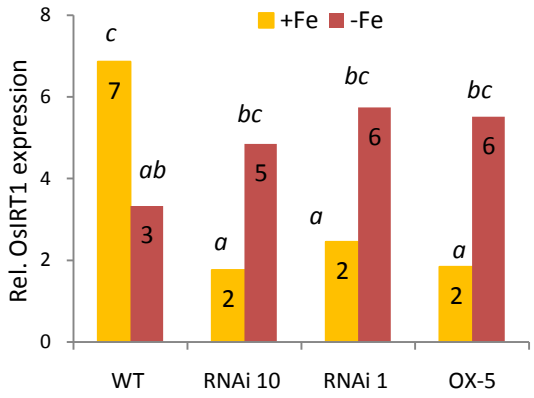


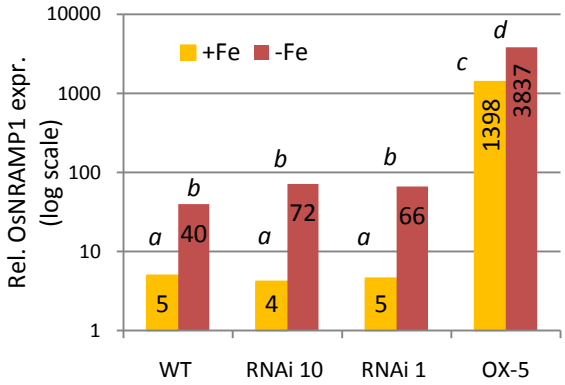
Fig. 6.6: Expression analysis performed in rice roots during the initial selection of these transgenic plants, compared with WT. Expression in (a) OsNRAMP1-RNAi plants, as well as (b) OsNRAMP1-OX plants, relative to α-tubulin expression. Data was provided by the Laboratory of Plant Biotechnology at the Graduate School of Agricultural and Life Sciences, the University of Tokyo, Japan.



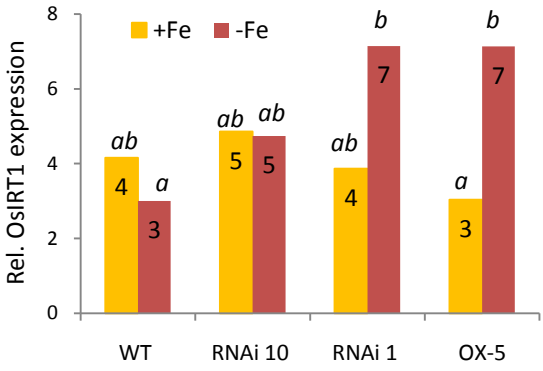
(a) 2 DAT OsNRAMP1 - Root



(b) 2 DAT OsIRT1 - Root



(c) 10 DAT OsNRAMP1 -Root



(d) 10 DAT OsIRT1 -Root

Fig. 6.7: Expression of OsNRAMP1 (a & c) and OsIRT1 (b & d) in roots of transgenic rice plants 2 d (a-b) and 10 d (c-d) after the beginning of treatments of plus or minus Fe treatment. Gene expression was quantified relative to a single cDNA pool and normalised against the (geometric) mean expression of two internal control genes, OsUBC and Os*e*EF1α. Different letters above columns represent means which are significantly different to each other by ANOVA-L.S.D. (5% level); for the OsNRAMP1 primers, this was of log-transformed data. Relative expression values are shown inside the columns. Full expression data (including shoot results) can be found in Appendix 1).

Table 6.4: Simplified representation of relative expression of the genes OsNRAMP1 and OsIRT1 (summary of expression data in Appendix 1 and Fig. 6.7). The symbols "**+**"/"**–**" represent significant increase/decrease in expression relative to the WT plants of the same treatment (ANOVA L.S.D. 5% level, of log-transformed data). Double symbols indicate a larger increase or decrease in expression. Blank space indicates there was no significant difference to the WT. Two different primer pairs were used for OsNRAMP1, one which anneals to a section of the gene ORF (also detecting the over-expressed cloned gene of OX-5), and another located on the 3' UTR (overlapping with the region targeted by the interfering fragment of these RNAi lines).

	2 DAT Experiment						**10 DAT Experiment**					
	Root			**Shoot**			**Root**			**Shoot**		
	NRAMP1-**ORF**	NRAMP1-**3'UTR**	**IRT1**	NRAMP1-**ORF**	NRAMP1-**3'UTR**	**IRT1**	NRAMP1-**ORF**	NRAMP1-**3'UTR**	**IRT1**	NRAMP1-**ORF**	NRAMP1-**3'UTR**	**IRT1**
+Fe												
RNAi 10	–	–	–					–				
RNAi 1	–	–	–									–
OX–5	++	–	–	+++			+++	–		+++		+
-Fe												
RNAi 10		–		– –	– –	–				++	++	
RNAi 1		–		– –	– –	–		+	+	++	++	
OX–5	+			+	– –	–	++	+	+	+++		+

Key:

"– –"	≤14% expression of WT
"–"	ca. 20-70% of the expression of WT (significant differences only)
"+"	> 1.5-fold expression of the WT (significant differences only)
"++"	> 10-fold expression of the WT
"+++"	> 100-fold expression of the WT

Elemental uptake

Analysis results for the two uptake experiments can be found in Table 6.5. The WT plants exhibited the highest Cd accumulation (or equal highest) in roots and shoots for both treatments in both uptake experiments (2 and 10 DAT). OX-5 did not display increased Cd accumulation, but rather in both uptake experiments, under ±Fe treatments, the OX-5 result was not significantly different to the lowest level of Cd accumulation (i.e. one of the RNAi plants).

At 2 DAT, there was a clear result of the effect of -Fe treatment on the uptake of Mn and Fe in the transgenic varieties. In the case of shoot Fe and Mn accumulation, this effect was not seen in the WT. In fact, the WT plants were least able to maintain shoot Fe concentration following removal of Fe supply. At 2 DAT, RNAi-10 and OX-5 had significantly increased shoot Fe in -Fe treatment, but the WT concentration was somewhat reduced and not significantly different to +Fe (Table 6.5). At 10DAT, the root Mn content of WT plants was not significantly increased by -Fe, but was increased 1.5-2.1–fold in the others.

By 10 DAT, all the RNAi plants no longer had a significant increase in shoot Fe content under -Fe. This indicated that the lack of Fe in roots was limiting the plants ability to transfer it to the shoots. The WT was the only genotype to show significantly reduced shoot Fe at this point.

At both time points RNAi-1 had the lowest, or equal lowest, shoot Cd concentration under plus and minus Fe treatment, and also the lowest increase in response to -Fe treatment.

Over-expression of OsNRAMP1 alone did not cause an increase in Fe, Cd or Mn, as seen in OX-5, however, the combination of Fe deficiency and OsNRAMP1 overexpression brought about increased shoot Fe and Mn uptake. Under -Fe treatment, OX-5 displayed an

approximate doubling in the amount of Fe and Mn translocation, per g $root_{DW}$, at both 2 and 10 DAT (Table 6.5). This effect was not matched by a similar increase in Cd uptake.

Contrast with gene expression

The major difference in Cd accumulation seen was higher Cd in WT compared with the transgenics. This difference aligned somewhat with OsNRAMP1 expression at 2 DAT, for the RNAi varieties, but not so for OX-5 which had greatly increased OsNRAMP1 expression and no observable link with Cd uptake.

The difference between the WT and transgenics aligns with root OsIRT1 expression under +Fe treatment at 2 DAT. In Fig. 6.7, it can be seen that after 2 d of -Fe treatment, OsIRT1 was upregulated in all varieties except the WT. This link was not seen at 10 DAT, where RNAi-10 and the WT did not have significant increase in OsIRT1 expression, but still had an increase in Cd uptake. RNAi-10 also displayed a significant increase in distribution of Cd to the shoot under -Fe (as a proportion of plant Cd content), and this was not explained by OsIRT1 expression.

Table 6.5: Analysis of root and shoot material following 24 h Cd uptake in hydroponic culture at two days after the beginning of ±Fe treatments (**a,** 2 DAT and **b,** 10 DAT). Following Ca-citrate desorption of plant roots, oven-dried plant tissue was digested with concentrated HNO_3 and H_2O_2, and analysed by ICP-MS.

(a) 2 DAT	Root Dry Wt (mg plant^{-1})		Shoot Dry Wt (mg plant^{-1})		Root Cd conc. µg g^{-1}		Shoot Cd conc. µg g^{-1}		"-Fe" effect	Root Fe conc. mg g^{-1}		Shoot Fe conc. µg g^{-1}		"-Fe" effect	Root Mn conc. µg g^{-1}		Shoot Mn conc. µg g^{-1}		"-Fe" effect
-Fe		**		*		**		**	↑		**		*	↑		**		**	↑
WT	10	*b*	49	*bc*	56	*c*	3.3	*c*	1.5x	15	*a*	258	*ab*	0.8x	72	*c*	357	*bc*	1.0x
RNAi 10	7	*a*	39	*a*	54	*c*	2.6	*b*	1.6x	21	*b*	406	*cd*	1.5x	73	*c*	442	*de*	1.2x
RNAi 1	7	*a*	47	*bc*	54	*c*	2.4	*b*	1.5x	18	*ab*	316	*bc*	1.3x	99	*d*	404	*cd*	1.4x
OX-5	7	*a*	43	*ab*	55	*c*	2.7	*b*	1.9x	20	*ab*	540	*d*	2.7x	91	*d*	468	*e*	1.7x
+Fe																			
WT	10	*b*	54	*c*	36	*b*	2.2	*b*		29	*c*	315	*b*		42	*b*	356	*b*	
RNAi 10	10	*b*	47	*bc*	27	*a*	1.6	*a*		36	*d*	264	*ab*		38	*ab*	356	*b*	
RNAi 1	7	*a*	49	*bc*	31	*ab*	1.6	*a*		28	*c*	251	*ab*		45	*b*	281	*a*	
OX-5	7	*a*	43	*ab*	28	*a*	1.4	*a*		36	*d*	204	*a*		50	*b*	282	*a*	

Continued on following page

(b) **10 DAT**	Root Dry Wt (mg plant^{-1})		Shoot Dry Wt (mg plant^{-1})		Root Cd conc. µg g^{-1}		Shoot Cd conc. µg g^{-1}		"-Fe" effect	Root Fe conc. mg g^{-1}		Shoot Fe conc. µg g^{-1}		"-Fe" effect	Root Mn conc. µg g^{-1}		Shoot Mn conc. µg g^{-1}		"-Fe" effect
-Fe		*		*		***		**	↑		**		**	↑		**		**	↑
WT	31	*cd*	193	*c*	56	*d*	3.0	*e*	1.7x	4	*a*	104	*a*	0.4x	47	*b*	531	*e*	1.1x
RNAi 10	25	*b*	143	*b*	49	*c*	2.8	*e*	2.4x	6	*b*	131	*ab*	0.7x	61	*c*	499	*de*	1.6x
RNAi 1	24	*b*	138	*ab*	47	*c*	2.3	*c*	1.8x	5	*a*	180	*abc*	0.8x	65	*cd*	448	*c*	1.3x
OX-5	19	*a*	116	*a*	49	*c*	2.4	*cd*	2.0x	5	*a*	338	*d*	1.5x	73	*d*	543	*e*	1.8x
+Fe						*													
WT	40	*e*	195	*c*	29	*b*	1.8	*b*		27	*c*	239	*cd*		44	*b*	478	*cd*	
RNAi 10	34	*d*	153	*b*	26	*a*	1.2	*a*		28	*c*	187	*bcd*		30	*a*	321	*ab*	
RNAi 1	28	*bc*	151	*b*	26	*a*	1.3	*a*		31	*c*	219	*bcd*		39	*b*	348	*b*	
OX-5	25	*b*	132	*ab*	27	*a*	1.2	*a*		31	*c*	223	*cd*		47	*b*	297	*a*	

N.B. Different letters in the column adjacent the means indicate values that are significantly different (within a single column) according to ANOVA-L.S.D. (significance level is indicated by asterisks above: ***, F pr. < 0.001; **, F pr. < 0.01; *, F pr. < 0.05).

Columns labelled “-Fe effect” display the increase in uptake caused by -Fe treatment: mean value for -Fe treatment divided by the value for +Fe treatment, for each genotype.

Discussion

OsNRAMP1 transgenics

The suppression of the OsNRAMP1 transcript in the RNAi varieties of these experiments was not consistent. These lines have been previously tested for suppression of this gene, and in root cDNA under control conditions, RNAi-10 and RNAi-1 had 6% and 14% of WT transcript levels (R. Takahashi, pers. comm., Fig. 6.6.). In the +Fe conditions of this experiment, which included $Fe(II)SO_4$ for Fe nutrition supplied daily, there was suppression of OsNRAMP1 in the root cDNA of the RNAi varieties, to 10-38% of the WT levels (at 2 DAT). Consistent with the initial screening of these lines (Fig. 6.6), RNAi-10 was found here to have the most significant suppression in root and shoots. Unfortunately, the pattern of suppression for the other RNAi plants did not continue to the 10 DAT experiment, and so the result is suspect.

It is possible that the inconsistency of OsNRAMP1 suppression was related to the form of Fe nutrition used. When these RNAi lines were originally screened, it was under Fe-EDTA nutrition. In this study, at 10 DAT, the +Fe plants had been receiving EDTA-free Fe(II) daily for 14 d. In the same way that Fe deficiency caused perturbations of the RNAi lines' OsNRAMP1 expression, it is possible that the transient availability of Fe(II) in the +Fe solution elicited some amount of deficiency response.

Relative to the differences in OsNRAMP1 expression seen in these experiments, the degree of difference in Cd uptake between the genotypes was small. This was especially true for OX-5, which had greatly increased OsNRAMP1 expression but not increased Cd. Therefore, while differences occurred between the RNAi plants and WT in both OsNRAMP1 expression and Cd content, these correlations were eclipsed by the lack of relationship between OsNRAMP1 overexpression and Cd uptake. In terms of elemental content that

aligned with expression of OsNRAMP1, more significant results were seen for Fe translocation in OX-5 under conditions of Fe deficiency.

One significant effect seen in OX-5 was the increased shoot Fe content under conditions of Fe deficiency of even 10 d. The WT plants were the most rapidly affected by lack of Fe supply and their shoot Fe content was rapidly reduced. The RNAi lines also showed transiently increased shoot Fe content brought about by 2 d of -Fe treatment, associated with increased expression of IDR genes, but by 10 d the ability to scavenge root Fe was showing limitations. OX-5 rather, had the greatest increase in shoot Fe at 2 DAT, and still had significantly higher shoot Fe after 10 d without fresh Fe supply. Importantly, however, this result was not seen under +Fe conditions, which explains why Takahashi *et al.* (2011) did not find evidence of this increased Fe translocation. It seems that OsNRAMP1 can increase shoot Fe content when there is concomitant expression of other IDR genes.

The negative result for a relationship between OsNRAMP1 expression and Cd uptake was unequivocal in these experiments, however this contradicts the published results of a study using this same OX variety, where the OX line had increased shoot Cd (Takahashi *et al.* 2011). Instead of OsNRAMP1 activity, a possible explanation for the Cd accumulation pattern at 2 DAT is the expression of OsIRT1. At 2 DAT, the reduced expression of OsNRAMP1 coincided to a large degree with reduced OsIRT1 expression (Fig. 6.7b), indicating that decreased expression of OsNRAMP1 at that time (Fig. 6.7a) may have been the result of overall changes in plant Fe status, rather than actual RNAi suppression.

Timing of regulation of OsNRAMP1

This study confirmed the results of Chapter 6.1; that regulatory mechanisms involved in OsNRAMP1 expression respond to Fe deficiency more rapidly than OsIRT1. The OsNRAMP1 results, especially for root cDNA, displayed strong upregulation because of -Fe treatment.

WT plants, 2 d after the beginning of -Fe treatment, had 15-fold increase in the expression of OsNRAMP1 in both roots and shoots. RNAi-10 and RNAi-1 also displayed an approximate 15-fold increase in OsNRAMP1 in root cDNA, at both 2 and 10 DAT. RNAi-6 results were more variable, but the level of upregulation under -Fe was the same or greater than the other plants.

The expression of OsIRT1 under the -Fe conditions of this study were more ambiguous than in the experiments of Chapter 6.1. For some cultivars the upregulation of OsIRT1 under -Fe treatment was not significant, and the upregulation in the WT plants was never significant in the samples taken here.

As expected, OX-5 had strong overexpression of OsNRAMP1 in roots and shoots. In addition to this, these plants also had significant gene response to Fe-deficiency. When native OsNRAMP1 expression was measured (using qPCR primers for the 3'UTR), OX-5 had 40-50x increase in root expression of OsNRAMP1. OsIRT1 expression also showed a 2-3 fold increase under -Fe treatment.

Induced Fe deficiency

There were conflicting results between OsNRAMP1 and OsIRT1 for the level of Fe deficiency induced in the WT plants. The WT plants, under -Fe, always showed some degree of increased OsNRAMP1 in roots and shoots at both time points, but for OsIRT1, there was not a significant increase in expression in any tissue. This difference could be the result of a reduced level of Fe deficiency induced in the WT plants, compared with that of the transgenics (both RNAi and OX). This was evident in the root OsIRT1 expression of 2 DAT and 10 DAT, and also the shoot OsNRAMP1 expression at 10 DAT. This observable difference in expression of IDR genes correlates with the '-Fe effect' on shoot

concentration of Cd, Fe and Mn. For all three elements, WT plants showed the smallest increase in translocation because of -Fe treatment, at both 2 and 10 DAT (Table 6.5).

In the WT -Fe treatment, there was a greater upregulation of OsNRAMP1 at 2 DAT than at 10 DAT (Fig. 6.7). Conversely, for RNAi-10 and RNAi-1, OsNRAMP1 was not as greatly increased at 2 DAT, but spiked relative to the WT at 10 DAT. It seems, in the OsNRAMP1 results, that the Fe deficiency response was slightly delayed in the RNAi plants. The faster growing WT plants experienced Fe deficiency earlier than the smaller RNAi.

It is likely that the overall stronger Fe deficiency in the transgenics was related to the difference in plant size. It is not clear whether the growth differences were related to the hygromycin treatment of the transgenic seedlings, but the WT plants grew consistently quicker and larger than the transgenics. The larger root systems of the WT may have allowed it to scavenge minute quantities of Fe present in the -Fe solution. Alternatively, it could have been because of the greater amount of Fe retained in the root-cell apoplasm.

In Chapter 3.3, the shoot Cd ratio (proportional to total plant Cd) was discussed in relation to the effect of Fe deficiency on partitioning of Cd in the plant. Fe deficiency was seen to cause an increase in the proportion of plant Cd found in the shoot. This is caused by the transport activity of membrane transporters which, responding to Fe deficiency, increase the transport of substrate metals to the shoot. Of note in this study was that, in the 2 DAT experiment, -Fe treatment did not cause an increase in the proportion of plant Cd found in the shoot for any of the lines tested. This contrasts with the 10 DAT experiment, in which Fe deficiency resulted an increase in the shoot:total Cd ratio for RNAi 10, RNAi 1 and OX-5 (data not shown, but calculated from results in Table 6.5). Although not conclusive, this difference provides an indication that the induced gene response influenced Cd translocation more at 10 DAT than at 2 DAT. At 2 DAT, it is more likely that

the competitive interaction between Fe and Cd, or lack thereof, may have been the main cause of the increased Cd uptake (the evidence for which was presented in Chapter 6.1).

Manganese

In view of the evidence (Takahashi *et al.* 2011), it is probable that OsNRAMP1 is not a Mn transporter. The increase in Mn uptake seen could have been an indirect effect of the Fe transport ability of OsNRAMP1. The increase in shoot Mn content that was seen in OX-5 (during Fe deficiency, at 2 DAT) was not as strong as the concomitant increase in Fe translocation (Table 6.5). As these plants were in an Fe-limited situation, it is possible that an increased amount of Mn found its way to the shoot because of the depletion of (cytoplasmic) root Fe reserves, and hence competition for another transport pathway that Fe and Mn share.

Mn has different plant distribution patterns to both Fe and Cd. Shoot Mn concentrations were significantly higher in plant shoots than roots, vastly different to Fe and Cd, for which the majority of the element accumulates in the roots. This Mn partitioning is evidence of the lack of homeostatic mechanisms for regulation of uptake and efflux of Mn in plants (Cailliatte et al 2010). Plants have not developed effective ways of limiting shoot Mn accumulation.

Conclusion

None of the results of this study provided evidence for an important role for OsNRAMP1 in rice Cd accumulation. Over-expression of OsNRAMP1 in combination with Fe deficiency resulted in greatly increased root to shoot Fe translocation, more than the WT plants under the same treatments. This confirms the hypothesised role for OsNRAMP1 as a transporter

that works cooperatively with root epidermal membrane transporters like OsIRT1 to increase Fe translocation to shoot during Fe deficiency stress (Takahashi *et al.* 2011).

The RNAi varieties did not show consistent suppression of OsNRAMP1 transcripts under these conditions. At the 2 d sampling, under +Fe (control) conditions, there was some suppression of OsNRAMP1. It is possible that the increased expression of OsNRAMP1 at later time points, especially under -Fe, is because of reduced plant Fe content from the early suppression of OsNRAMP1. Taken with the original results for these transgenic lines (Fig. 6.6), it seems that under normal Fe conditions, there is reduced expression of OsNRAMP1 in these varieties. In Chapter 6.3 these transgenic lines were used again in a potted soil experiment.

6.3: The performance of transgenic rice lines with modified expression of OsNRAMP1 in soil culture

Introduction

If Fe deficiency response in rice plants is related to changes in Cd uptake, and OsNRAMP1 takes part in that uptake, the transgenic varieties tested under short-term hydroponic Cd uptake (Chapter 6.2) may perform differently under the variable redox conditions that can exist in paddy soil. This section presents a greenhouse experiment with potted paddy soil, testing the shoot and grain Cd accumulation of two OsNRAMP1 over-expressing (OX) rice lines and two OsNRAMP1–RNAi rice lines. These four transgenics were grown alongside the WT cultivar Tsukinohikari (*O. sativa,* subsp. *japonica*) under two different irrigation treatments.

The preceding pot experiment in Chapter 5 demonstrated that intermittent flooding treatment can result in the same or higher grain Cd uptake as aerobic, non-flooded soil; it also demonstrated that the Fe availability to plants in intermittently flooded soil is much closer to that of aerobic conditions than it is to anoxic conditions. For this reason, in this experiment, these seven varieties were compared in (1) intermittent and (2) continuous flooded paddy soil with Cd spiked at a concentration of 0.4 mg kg^{-1}.

Materials and Methods

Plant material and experimental setup

The paddy soil used for this experiment, the same as Chapter 5.3.2, was sourced from a rice growing area near Xiamen, Fujian province, China. It was a sandy loam soil, with pH 6.09 (2.5:1 water:soil ratio). The background Cd concentration was measured to be 0.02 $mg.kg^{-1}$.

Prior to use in the pot experiment, all the soil was passed through a 2mm sieve, with large clods broken up.

Germinated seedlings were selected from hygromycin plates (except for WT; as per Chapter 6.2) and at 12 DAS, transferred to tubs of perlite and a basic nutrient solution. These tubs were placed in a greenhouse with natural light conditions. The seedlings were grown until 32 DAS, with nutrient solution topped up once per week.

Paddy soil was weighed into PVC pots (3.5 kg each) and spiked with $Cd(NO_3)_2$ at a rate of 0.4 mg kg^{-1} Cd. This was applied as a diluted solution used to saturate the soil. Flooded conditions were maintained in the prepared pots for 38 days, at which time the soil of every pot was hand-mixed and uniform seedlings of the seven varieties were planted. Shallow flooded conditions (<5 cm) were maintained for the first three weeks after transplant, after which the continuous flooding treatment was switched to deep flooding (ca. 10 cm standing water) and the intermittent flooding treatment alternated between flooding and drying, watering every 4-5 days, and then every 3-4 days as temperatures increased in the summer. During the growth of these plants, daily temperatures were in the range 27-35^{o}C.

Soil solution sampling and soil redox

The soil and watering conditions of this experiment were essentially a repeat of the previous section's (Chapter 5) setup. In this pot trial, Rhizon soil moisture samplers (Rhizon Flex 10 cm; Rhizosphere Research Products, Wageningen, Netherlands) were used to compare the Cd and Fe availability under these treatments. Ten samplers (in the two treatments; n=5) were placed in pots of the OX-5 variety three weeks after planting and were used at three times over the growing season to take soil moisture samples: once three days after insertion; once at pre-heading stage; and finally, once at early flowering.

Samples were extracted using vacuum tubes and immediately acidified with HNO_3 and stored at 4°C, before dilution for ICP-MS analysis at a later date.

Soil redox conditions were checked 24 d after transplanting (more than three weeks pre-heading) and also at full flower to confirm reduced conditions of flooded soil. See Chapter 5 for details of soil redox monitoring.

Plant harvest and analysis

Once the rice panicles had reached maturity they were harvested. The greenhouse had experienced a pest mouse problem, so a significant number of plants had lost grain to mice. For this reason, grain weight totals are not available for this experiment. Shoots were cut 6 cm from the base of the plant to avoid soil contamination from the section that was submerged when flooded.

All plant material was dried completely at 70°C. Rice husks were removed using a bench-top dehulling machine. The brown rice samples were ground to a powder in an electric grinder. Plant shoots were also ground in an electric grinder and a subsample was passed through a 2 mm sieve.

The plant material was re-dried in the oven before weighing 0.2 g grain and 0.2 g shoots into acid-washed plastic tubes. Concentrated HNO_3 was added to each tube (2 ml each) and samples were left overnight to pre-digest. The following morning an equal volume of 30% H_2O_2 was added to each tube. After frothing had subsided, racks of tubes (with lids on but not tight) were placed in a 100°C water bath for digestion for at least 3 h. After complete digestion, all samples were then diluted to 50 ml. The final weight of the dilution was recorded and aliquots were taken and passed through a 0.22 µm filter. The digests were analysed by ICP-MS for Fe, Mn, Zn, As and Cd.

Results

The two irrigation treatments of this pot trial resulted in noticeably different soil chemistry conditions and resultant plant elemental accumulation. Under continuously flooded soil conditions, Fe and Mn availability was significantly increased above intermittent flooding treatment and Cd was undetectable in soil pore water (Table 6.6). During this experiment, both RNAi and OX plants showed symptoms of interference to the homeostatic Fe balance leading to toxicity. This was visible in the plant shoots from tillering-stage, shown as leaf bronzing in plants of these lines, while the WT remained green and unaffected. The final harvest (Fig. 6.8b) confirmed that the transgenic varieties had increased shoot Fe content, under the continuous flooding treatment, where Fe was most available (Table 6.6).

A different pattern of results was seen under these conditions than in hydroponically grown seedlings. Grain Cd accumulation from plants in the continuous flooding treatment was very low, and variability between the varieties was also very low. Under intermittent flooding, Cd availability was higher and varieties of both RNAi and OX type exhibited increased grain Cd accumulation compared with WT control (Fig. 6.9a). In terms of shoot Cd accumulation, under both treatments only RNAi-10 had significantly higher Cd content (Fig 6.8).

Both RNAi 10 and the two OX lines had higher Fe content in brown rice under intermittent flooding, compared to continuous flooding and also the WT plants under intermittent flooding treatment (Fig 6.9b). This was despite the reduced Fe availability in the soil solution of this treatment and the fact that the WT plants had somewhat increased grain Fe under continuous flooding.

The results for plant and rice grain Zn and Cu content are not presented here. There were no significant differences in Zn content between the two treatments or any of the rice

varieties tested. There was a large variability in Cu content, and no significant trends were seen between the RNAi or OX varieties.

Soil pore water sampling

The soil pore water was sampled using a different method than that of Chapter 5 and the the elemental concentrations in soil solution were found to be different. The first of note was that the Fe concentration was an order of magnitude higher in the continuously flooded treatment, for what was the same soil under nearly identical conditions (Table 6.6). The difference must have come about because of differences in filtering. The centrifuged soil solution samples were filtered through 0.22 µm units. The Rhizon samplers are semi-permeable membranes; it is possible there was a larger pore size that allowed uptake of Fe-containing particles greater than 0.22 µm.

Table 6.6: Soil pore water concentrations of Fe, Cd and Mn in potted soil during the growth of rice plants. Sampled using Rhizon samplers (10 cm long) inserted and buried in the soil 3 d before the first sampling date. Zn content of pore water was below detection limit (because of high Zn in blanks).

	Sampling period	Fe (mg kg^{-1})	SE	Cd (µg kg^{-1})	SE	Mn (µg kg^{-1})	SE
Continuously flooded	Tillering	38.1	1.0	*n.d.*		3295	56
	Pre-heading	37.9	1.9	*n.d.*		3279	96
	Early flowering	36.6	0.8	*n.d.*		3375	99
Intermittent flooding	Tillering *	4.2	1.0	0.14	0.01	870	164
	Pre-heading	0.33	0.01	0.24	0.06	88	40
	Early flowering	0.35	0.04	0.31	0.07	40	6

N.B. * Tillering (Intermittent treatment) = 6 d after drainage of the initial flooding for transplanted seedlings, therefore conditions were more anaerobic than later sampling dates. *n.d.* = undetectable by ICP-MS analysis.

Table 6.7: Simplified summary of the Cd, Fe, Mn and Cu accumulation characteristics of the four transgenic rice lines tested here. Grain and shoot content which was significantly higher or lower than the WT of the same treatment is indicated, with its corresponding significance level (ANOVA-L.S.D.; ***, **, * = 0.1%, 1% & 5% level, respectively). Blank space indicates a mean not significantly different to the WT. ↑↓

	Brown Rice Cd	Shoot Cd	Brown Rice Fe	Shoot Fe	Brown Rice Mn	Shoot Mn	Shoot Cu
Continuous flooding							
WT							
RNAi 10		↑ **	↓ **			↑ *	
RNAi 1						↑ **	↑ *
OX-5							
OX-6		↓ **			↓ ***		
Intermittent flooding							
WT							
RNAi 10	↑ ***	↑ **	↑ *	↑ ***	↑ *		
RNAi 1	↑ *			↑ **	↑ *	↑ *	
OX-5	↑ **			↑ *		↓ *	
OX-6					↓ *		↓ *

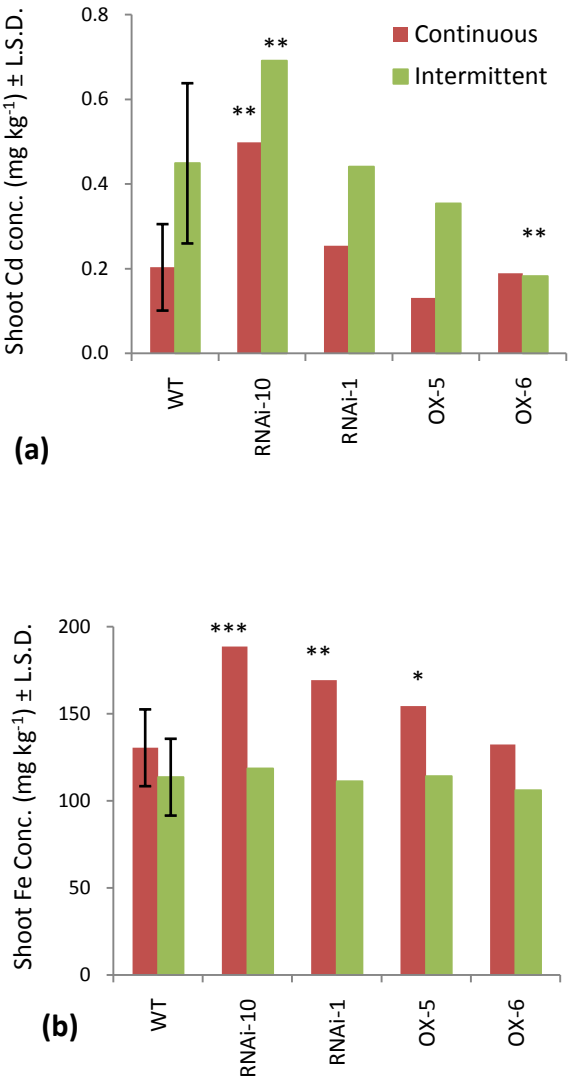


Figure 6.8: Cd concentration in brown rice (a) and shoots (b) of OsNRAMP1 transgenic plants grown in soil spiked with 0.4 mg.kg^{-1} Cd, under two different irrigation treatments: Darker (red) bars, continuous flooding (soil pore water Cd undetectable by ICP-MS); Lighter (blue) bars, intermittent flooding (avg. soil PW Cd conc. 0.27 µg.kg^{-1}). Asterisks above columns designate means that significantly differ from the WT, within a single treatment, by ANOVA-L.S.D. (***, **, * = 0.1%, 1% & 5% level, respectively).

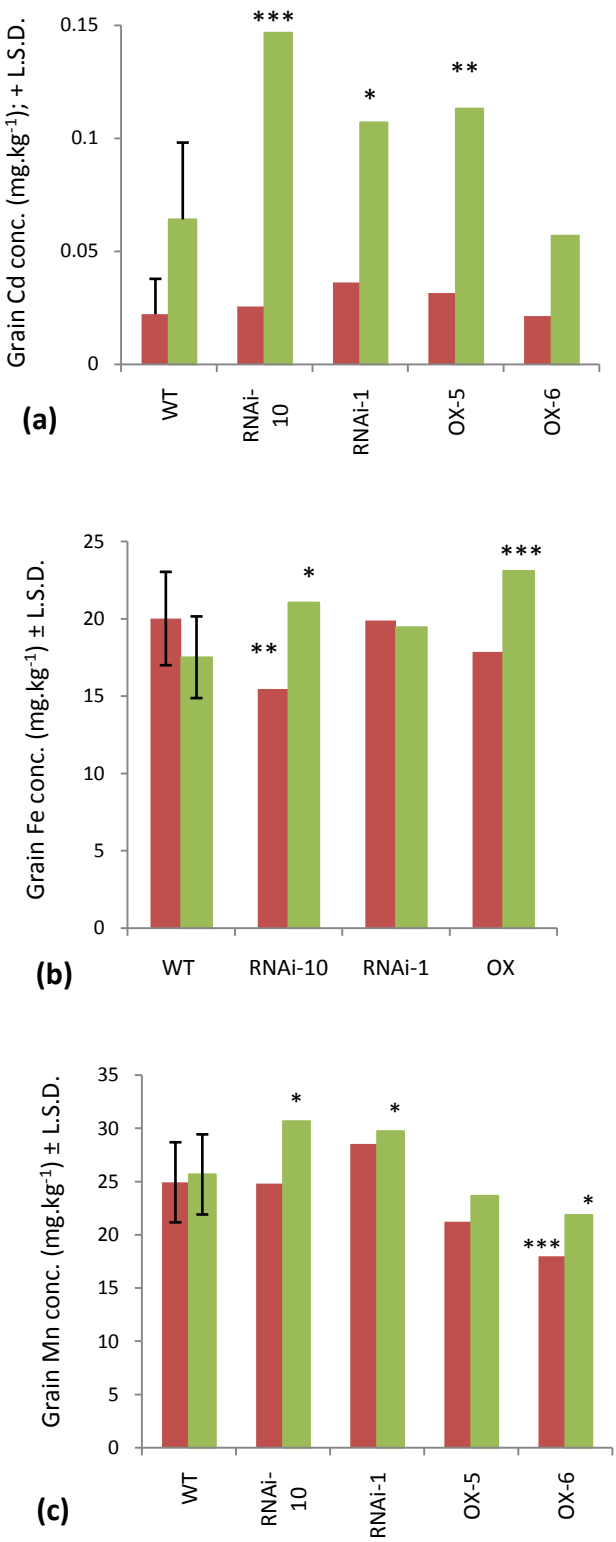


Figure 6.9: Iron concentration in brown rice (a) and shoots (b) of OsNRAMP1 transgenic plants grown under two different irrigation treatments. Darker (red) bars are of rice in continuously flooded soil; avg. soil pore water Fe conc. = 38 mg.kg^{-1}. Lighter (blue) bars are plants under intermittent flooding; avg. SPW Fe conc. = 0.3 mg.kg^{-1}. Asterisks above columns designate means that significantly differ from the WT, within a single treatment, by ANOVA-L.S.D. (***, **, * = 0.1%, 1% & 5% level, respectively). The value labelled as "OX" (a) represents an overall mean for OX-4, OX-5 and OX-6.

Discussion

Significant differences in rice Cd content were found between the germplasm tested in this experiment. These differences were only seen under the intermittent flooding treatment, in which a number of the transgenic varieties accumulated increased amounts of Cd. RNAi-10, RNAi-1, OX-4, and OX-5 had significantly higher brown rice Cd content. The Cd accumulation seen in the rice was similar to that of shoots, except the WT plants had higher accumulation in shoots, and there were larger differences between means under continuous flooding treatment. RNAi-10 was the only transgenic that had significantly higher shoot Cd content than the WT, but unlike grain Cd content, this difference occurred in both of the treatments.

In the initial screening of these RNAi varieties, and in Chapter 6.2, RNAi-10 had the most repeatable suppression of OsNRAMP1. In soil culture, with intermittent flooding treatment, this line showed the greatest Cd accumulation in both shoots and brown rice (Figs 6.8 & 6.9); it also had significantly increased grain Fe content. Under continuous flooding, RNAi-10 also accumulated the highest shoot concentration of Fe. Interpretation of these results in respect to OsNRAMP1 is difficult because, except for shoot Cd, the significant increase in element concentration seen in RNAi-10, relative to the WT, was in the same direction as the OX plants. Logically, the RNAi and OX lines should have had opposite results. Given that the short-term uptake experiments, in Chapter 6.2, showed no evidence of increased Cd uptake from OsNRAMP1 expression, the most likely explanation for increased Cd uptake in this experiment is an indirect response to Fe content.

The increase in shoot Fe content in a number of the transgenic varieties under continuous flooding (Fig. 6.8b) supports the visual observations of a disturbance in the Fe homeostatic mechanisms in these plants. Especially during tillering phase (when all the plants had initial flooding), leaf bronzing was seen on the leaves of the OX and RNAi rice

plants, evidence of probable Fe toxicity. Surprisingly, this occurred in both types of transgenics.

The OX varieties tested in this section showed an increase in grain Fe content in the intermittent flooding treatment, despite a higher shoot Fe concentration under continuous flooding (Fig 6.8b). There was a fairly high amount of variability in the OX grain Fe, so for statistical significance the replicates for all the OX lines were combined, as they had similar mean values, all higher than the WT. The grain Fe results of the continuously flooded plants were not significantly different to the WT plants. This characteristic of the OX varieties supports the hydroponic results, where OsNRAMP1 overexpression and low Fe supply caused an increase in Fe translocation in OX-5. The OX plants showed significantly increased grain Fe content under intermittent conditions, but not continuous flooding. The intermittent flooding conditions, which would have included times of drier, aerobic soil, would possibly have lead to transient expression of IDR genes.

It is unusual to see a large increase in the Fe content of rice grains. The results of the pot trial of Chapter 5 showed the small effect that soil Fe availability had on Fe content of brown rice. Although the DGT deployment in that study had demonstrated the marked difference in Fe concentration in the soil pore water between continuous flooding and intermittent flooding, for many of the varieties tested there was not an increase in grain Fe content, or they even showed a decrease. This characteristic of paddy grown rice is the result of the strong homeostatic control mechanisms that plants have for Fe. If the ability of the plant to maintain correct Fe distribution was compromised by OsNRAMP1 overexpression, it is feasible that higher amounts of Fe would reach the grain. The reason that this increase in Fe content only occurred in the intermittently-flooded pots of treatment B indicates that an additional factor besides the expression of OsNRAMP1 was at play.

In Chapter 6.2, only the minus Fe treatment caused shoot Fe content to be increased in the OX varieties. That experiment led to the conclusion that other IDR genes are also necessary to enable OsNRAMP1 overexpression to increase shoot Fe content. This is likely indicative of the function of OsNRAMP1 in normal situations. The current evidence suggests that OsNRAMP1 is primarily located near the vascular tissue and that other transporters like OsIRT1 drive increases in plant Fe uptake, after which OsNRAMP1 facilitates movement to the shoot (Takahashi *et al.* 2011). AtNRAMP1 was also found to be in the inner layer of cells in the root, rather than the epidermal cells (Cailliatte et al 2010).

The qRT-PCR results of Chapter 5 demonstrated that in rice plants grown in unflooded soil, OsNRAMP1 can be upregulated, and in this pot trial, soil porewater sampling showed the reduced availability of Fe that occurred in the intermittent flooding treatment. This reduced availability could have led to an increase in the expression of IDR genes that can also transport Fe and cooperate with OsNRAMP1.

Mn content was the only variate that displayed a trend of a difference between the RNAi plants and the OX plants. Under intermittent flooding treatment, RNAi-10 and RNAi-1 had significantly higher Mn concentration than the OX plants for both grain and shoot Mn. The explanation for the reduced Mn accumulation in the OX plants, and the increase in RNAi plants is uncertain. Takahashi *et al*. (2011) and Narayanan *et al.* (2006) could not find evidence for Mn transport by OsNRAMP1. Like the result for Cd in this experiment, Mn transport could have been affected by Fe homeostasis. If the OX plants accumulated increased amounts of root Fe, this could have led to more competition for Mn translocation. Conversely, in the RNAi plants, if suppression of OsNRAMP1 led to a greater Fe- deficiency response, OsIRT1 may have been upregulated.

Chapter 7

Final Discussion

This body of work examined Cd accumulation in rice in combination with genotypic and environmental effects that influence the availability and uptake of Cd. Of particular interest, the root membrane transporters that are responsible for movement of Cd were investigated.

Fe was found to have a large effect on the uptake of Cd by rice plants, but only a small proportion of this effect could be attributed to the Fe deficiency response. In hydroponic experiments, despite the strong Cd uptake response shown to Fe deficiency in the literature, the increase in plant Cd uptake brought about by the upregulation of rice IDR genes was significant but small. It was rather competition with Fe^{2+} that had a large effect on the uptake of Cd supplied at low concentrations (Chapter 6.1). For rice plants grown in potted soil, Fe-deficiency-responsive (IDR) gene upregulation was detected in plants grown in unflooded paddy soil relative to those in flooded soil (Chapter 5). However, these transcriptional responses were not found to be linked to an increase in Cd uptake. Under environmentally-relevant conditions for rice, i.e. low redox, high Fe(II) availability, and relatively low Cd availability, the uptake of Cd by is likely severely inhibited by the high concentration of Fe^{2+} ions.

The competition between Fe^{2+} and Cd most likely occurs at the root Fe(II) transporter OsIRT1. Another possible candidate, OsNRAMP1, was not shown to be responsible for significant Cd transport. Plants were tested with manipulated expression of OsNRAMP1 by RNAi and overexpression (Chapters 6.2 & 6.3). Although overexpression of OsNRAMP1 led

to an increase in plant Fe content in Fe deficient plants, overexpression of OsNRAMP1 was not found to increase the uptake of Cd in rice.

Unexpectedly, Fe deficiency was not found to cause an overall increase in plant Cd accumulation, even after ten days of minus Fe hydroponic treatment, with strong upregulation of IDR genes. In fact, root Cd concentration was consistently higher in plants from Fe sufficient conditions than those of Fe deficient conditions. Fe deficiency was found repeatedly to result in a 20% increase in Cd concentration in shoots (at 2 and 10 DAT, in two different test solutions). However, at 5 d after beginning of Fe deficiency treatment, there was no increase in Cd translocation. This was thought to be associated with mobilisation of stored Fe at this time, facilitated through an increase in root nicotianamine (as a result of increased expression of NA-synthase genes). The lack of strong evidence of Fe-deficiency stimulated Cd uptake in rice is actually consistent with the results of another research paper on this topic (Nakanishi *et al.* 2006).

One section of this work sought to test the hypothesis that Fe deficiency response played a role in Cd accumulation in field-grown rice plants. Although there was evidence that rice plants grown under aerobic soil conditions experience some degree of Fe deficiency, this did not lead to an increase in Cd translocation other than what was associated with increases in Cd availability from redox changes.

In hydroponic experiments, the presence of Fe^{2+} ions in solution had a large effect on Cd uptake and translocation, whereas Fe in the form of Fe(III)EDTA did not. This showed that there was a direct effect of the divalent Fe ion on the uptake of Cd, rather than on overall plant Fe status.

OsNRAMP1 was confirmed to be an important transporter of Fe in rice, but its role in Cd transport was not clear. It was found that overexpression of OsNRAMP1 together with

Fe deficiency treatment (i.e. concomitant upregulation of other IDR genes such as OsIRT1) led to a significant increase in shoot Fe content (relative to WT plants). This is consistent with the hypothesised role for OsNRAMP1 as a transporter that works cooperatively with root epidermal membrane transporters like OsIRT1 to increase Fe translocation to the shoot during Fe deficiency stress (Takahashi *et al.* 2011). It is likely that OsNRAMP1 enables xylem loading of Fe.

This research has contributed to this field of study through clarification of the importance of Fe deficiency in Cd accumulation in rice. Results presented in the literature from dicot plants have shown a large role for Fe deficiency response in Cd uptake. In rice, at low concentrations of Cd, upregulation of IDR genes was only associated with small increases in root to shoot translocation rather than overall plant Cd uptake. This is possibly because of different affinities for Fe and Cd that the Fe transporters have in different plant species, as has been demonstrated previously (Zaharieva and Römheld 2000; Cohen *et al.* 2004).

This work has covered other areas of study relevant to our understanding of Cd accumulation in rice plants. This included experiments on the timing of Cd accumulation in rice, the effect of Si nutrition and the physiological basis of genotypic differences in Cd accumulation.

Manipulation of the supply of Cd, before and after flowering, revealed that grain Cd is not just the product of remobilised Cd accumulated in shoot, but that root Cd uptake after flowering contributes a significant (40%) amount of grain Cd. It is now evident that grain Cd content is not just a product of the vegetative accumulation of Cd in shoots which is remobilised to the maturing rice.

Under hydroponic conditions, Si supply was found to reduce Cd uptake and translocation by approximately two-fold. However, although the presence of silicic acid in

the nutrient solution was found to reduce root hydraulic conductivity, extended pre-treatment with Si (which would lead to the root apoplastic deposition of silica gel) did not have a significant effect on root hydraulic conductivity. The findings of this study were contrary to the idea that the action of Si is by blocking apoplastic bypass-flow around the Casparian strip. As it was found that Si pre-treatment has a significant effect on plant uptake of Cd (when tested in -Si solution), and it has previously been seen that Si nutrition reduces plant evapotranspiration, it can be inferred that it is within the shoot that Si supply reduces Cd translocation. As well as reducing transpirational flow, it is possible that the ionic affinity of deposited apoplastic silica for cations such as Cd, and the likelihood of Cd-Si complexes forming, is a possible reason for the reduced plant accumulation of Cd under Si supply (Wang et al. 2000).

In this study, genotypic variation in plant Cd accumulation was studied in an effort to understand the basis of differences between cultivars. Genotypic characteristics of Cd accumulation were not consistent across different growing conditions and different plant tissue types. Four Chinese rice cultivars, previously characterised for Cd uptake in potted paddy soil, did not show consistent rankings for Cd accumulation when tested in hydroponic culture or when grown in potting mix. In addition, in the same manner seen in other studies of rice, shoot Cd accumulation rankings were not found to match grain Cd accumulation. In a pot trial comparison of four rice varieties, those with higher harvest indices (i.e. ratio of grain yield to shoot biomass) had higher Cd concentrations than those that put on more shoot biomass during their reproductive phase.

When germplasm with a wider range of Cd accumulation was compared in flooded and unflooded soil, the ranking of the cultivars was essentially consistent. The large difference in redox, between the two flooding conditions, was associated with sizeable differences in Fe, Mn and Cd concentration but did not cause significant changes in the relative differences between

the cultivars. Genotype by environment interaction was present, but did not radically change the cultivar rankings.

A number of experiments in this study were used to try to relate documented grain Cd accumulation characteristics with root gene expression of membrane transporters putatively involved in Cd transport. General trends were noticed between the cultivars which exhibited greater variation in Cd accumulation. *Indica* rice varieties generally had higher expression of IDR genes than *japonica* varieties when tested at seedling stage, and also responded more strongly (and/or rapidly) to Fe deficiency treatment; i.e. expression of OsIRT1 and OsNRAMP1 was more quickly upregulated in *indica* seedlings subjected to minus Fe supply.

There was not a consistent pattern between expression of these candidate genes and Cd accumulation characteristics. It is likely that the molecular basis for differences in Cd accumulation characteristics is not the same in all cultivars. The importance of the functionality of tonoplast transporters that enable significant Cd sequestration has been shown recently through work on OsHMA3 (Ueno *et al.* 2010), but this gene is not responsible for differences in Cd accumulation between all rice cultivars (Takahashi *et al.* 2011). On the basis of the results of this work, OsNRAMP1 does not appear to be a likely candidate for causing large scale changes in Cd accumulation.

Building on this work, more research is needed in the molecular basis of genotypic variation between specific rice genotypes. Environmental and nutritional factors play a large role in determining plant uptake of Cd, especially through competition with other cations. There are possibilities to utilise field-applied Si to reduce Cd accumulation in rice paddies affected by Cd. However, an increased understanding of genotypic variation in rice will lead to new possibilities in the breeding of rice varieties with lower levels of Cd accumulation.

8. Appendix

The following two pages display the full gene expression analysis results by RT-qPCR for the transgenic varieties in Chapter 6.2. Both root and shoot expression in the two different treatments is shown, at 2 DAT (Fig. A1) and 10 DAT (Fig. A2).

Figure A1 (next page)**:** Expression of OsNRAMP1 (a-d) and OsIRT1 (e-f) in roots (a, c, e) and shoots (b, d, f) of transgenic rice plants 2 d after the beginning of treatments of plus or minus Fe treatment. Gene expression was quantified relative to a single cDNA pool and normalised against the (geometric) mean expression of two internal control genes, OsUBC and Os*e*EF1α. Different letters above columns represent means which are significantly different to each other by ANOVA-L.S.D. (5% level); for the NRAMP1 primers, this was of log-transformed data. Two different primer pairs were used for OsNRAMP1, one which anneals to a section of the gene ORF (also detecting the over-expressed cloned ORF in OX-5), shown in (a) and (b), and another located on the 3' UTR (overlapping with the region targeted by the interfering fragment of these RNAi lines), shown in (c) and (d).

2 DAT Experiment

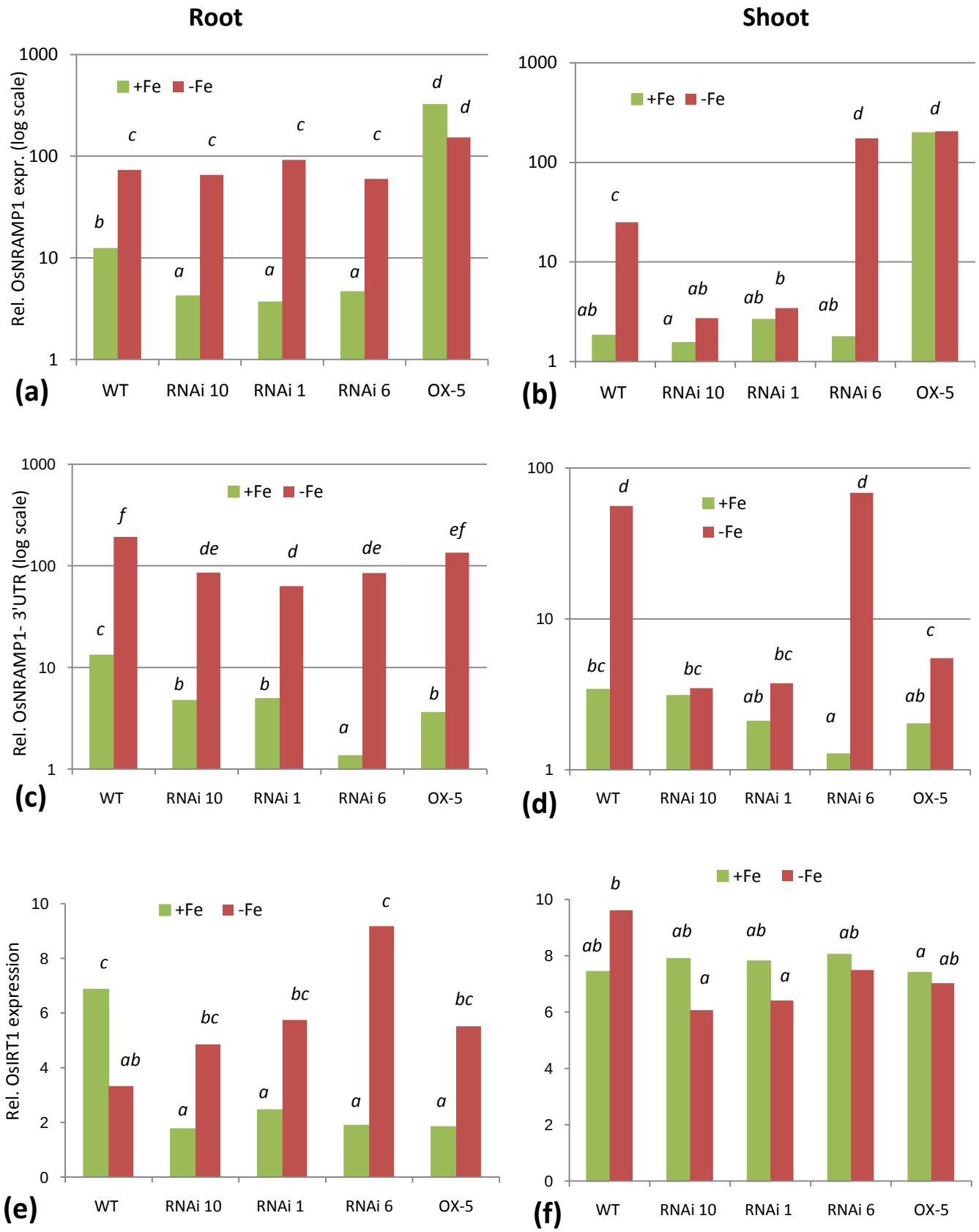
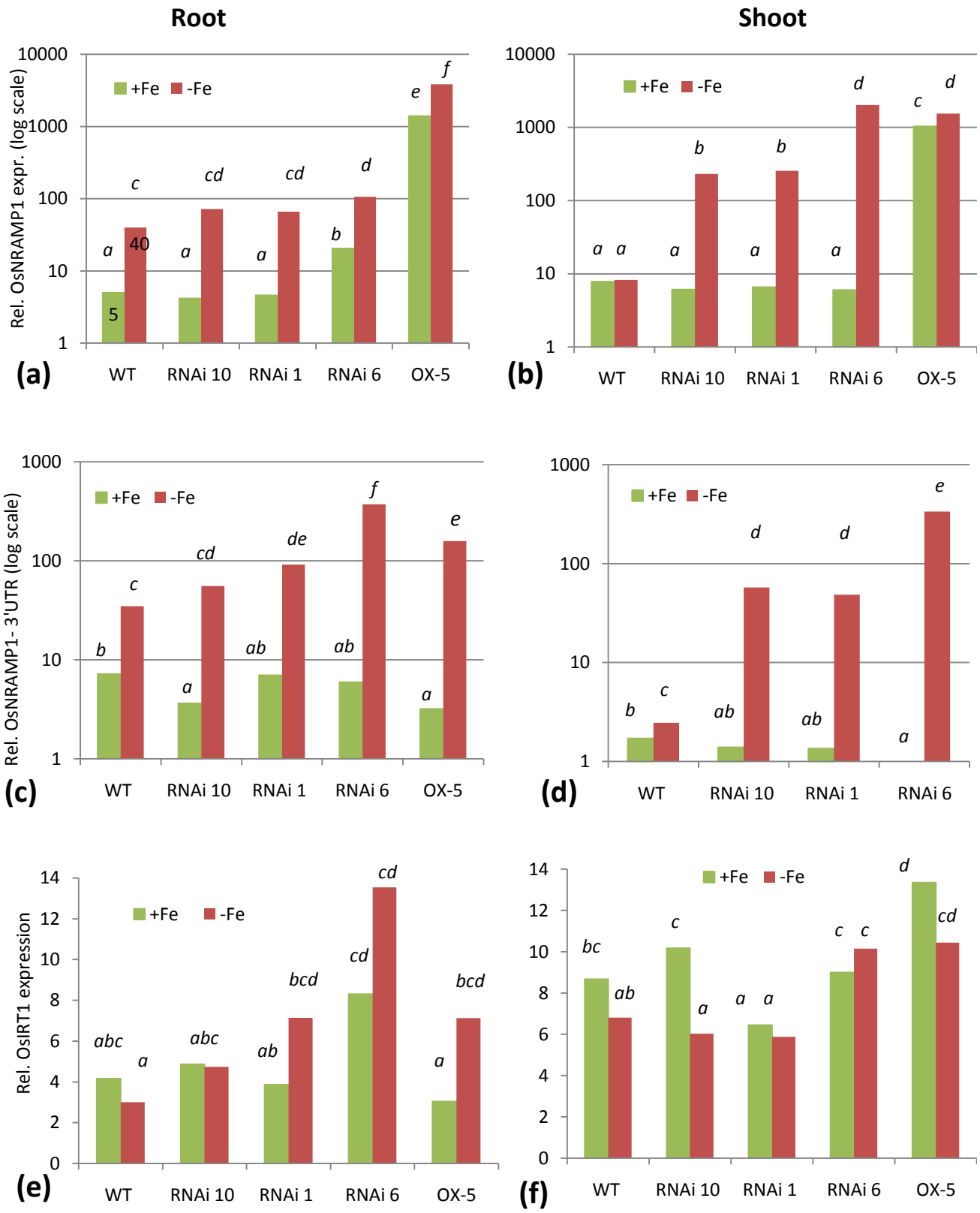


Figure A2 (next page)**:** Expression of OsNRAMP1 (a-d) and OsIRT1 (e-f) in roots (a, c, e) and shoots (b, d, f) of transgenic rice plants 10 d after the beginning of treatments of plus or minus Fe treatment. Gene expression was quantified relative to a single cDNA pool and normalised against the (geometric) mean expression of two internal control genes, OsUBC and Os*e*EF1α. Different letters above columns represent means which are significantly different to each other by ANOVA-L.S.D. (5% level); for the NRAMP1 primers, this was of log-transformed data. Two different primer pairs were used for OsNRAMP1, one which anneals to a section of the gene ORF (also detecting the over-expressed cloned ORF in OX-5), shown in (a) and (b), and another located on the 3' UTR (overlapping with the region targeted by the interfering fragment of these RNAi lines), shown in (c) and (d).

10 DAT Experiment



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