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

Matthew Scott Rodda

School of Earth and Environmental Sciences

Submitted for fulfilment of the degree of

Doctor of Philosophy

University of Adelaide

August 2012

Table of Contents

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

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

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

The author acknowledges that copyright of published work contained within this thesis (as listed below*) resides with the copyright holder of that work.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines.

Matthew Scott Rodda

…………………………………………………

Signed

*__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

Acknowledgements

- My principal supervisor, Assoc. Prof. Rob Reid, who was always generous with his time and was greatly supportive while I carried out my studies
- Prof. Mike McLaughlin, for his advice and input during the initial stages of this project
- Prof. Yongguan Zhu, Chinese Academy of Sciences (CAS), for his supervision and provision of resources to carry out experimental work in Xiamen over a ten month period
- Prof. Nishizawa's research group at the University of Tokyo (particularly Dr. Ryuichi Takahashi and Dr. Yasuhiro Ishimaru) for helpful advice and supplying the transgenic rice plants that were used in experiments in Chapter 6
- The staff and graduate students at the Institute of Urban Environment, CAS, for their friendship and general assistance during my ten months in Xiamen
- Mr. Li Gang, PhD student (CAS) for going out of his way to assist with experimental work during my time in Xiamen
- Mr. Gaetan Dales, who provided technical assistance with experiments on hydraulic conductivity in Chapter 3.6
- My wife, Rebekah, for her continual support and encouragement during the completion of my research and thesis

- This research was supported under Australian Research Council's *Discovery Projects* funding scheme (project number DP0773638). Some experimental work in this project was also financially supported by the Chinese Academy of Sciences, International Collaboration Fund (GJHZ200828).

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

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

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

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

Signed ……………………………………………………………………………. *Date* …………………………………..