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Response to zinc deficiency of two rice lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates, and not by zinc-transporter activity

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Summary

• Zinc (Zn)-deficient soils constrain rice (*Oryza sativa*) production and cause Zn malnutrition. The identification of Zn-deficiency-tolerant rice lines indicates that breeding might overcome these constraints. Here, we seek to identify processes underlying Zn-deficiency tolerance in rice at the physiological and transcriptional levels.

• A Zn-deficiency-tolerant line RIL46 acquires Zn more efficiently and produces more biomass than its nontolerant maternal line (IR74) at low $[Zn]_{ext}$ under field conditions. We tested if this was the result of increased expression of Zn^{2+} transporters; increased root exudation of deoxymugineic acid (DMA) or low-molecular-weight organic acids (LMWOAs); and/or increased root production. Experiments were performed in field and controlled environment conditions.

• There was little genotypic variation in transcript abundance of Zn-responsive root Zn^{2+}-transporters between the RIL46 and IR74. However, root exudation of DMA and LMWOA was greater in RIL46, coinciding with increased root expression of putative ligand-efflux genes. Adventitious root production was maintained in RIL46 at low $[Zn]_{ext}$, correlating with altered expression of root-specific auxin-responsive genes.

• Zinc-deficiency tolerance in RIL46 is most likely the result of maintenance of root growth, increased efflux of Zn ligands, and increased uptake of Zn-ligand complexes at low $[Zn]_{ext}$; these traits are potential breeding targets.

Introduction

Zinc (Zn) deficiency is the most common micronutrient disorder in rice, affecting up to 50% of lowland rice soils (White & Zasoski, 1999; Dobermann & Fairhurst, 2000). Zinc deficiency may result when soils are formed from parent materials containing little Zn, but more frequently immobilization of soil Zn in forms of low plant availability is the main cause of Zn deficiency. Such immobilization can result from high soil pH and excess bicarbonate, factors that typically occur in calcareous soils of the Indo-Gangetic plains of India and Pakistan (Qadar, 2002), whereas perennial wetness and low redox potential induce Zn deficiency in peat and coastal saline soils (Neue & Lantin, 1994; Quijano-Guerta *et al.*, 2002). Zinc is an essential component of thousands of proteins in plants (Broadley *et al.*, 2007); Zn

deficiency therefore causes a variety of disorders in rice, such as seedling mortality, stunting, leaf bronzing, and delayed flowering.

Quantitative trait loci (QTLs) affecting leaf bronzing, seedling mortality and relative dry weight have been identified in a recombinant inbred line (RIL) population derived from a cross between the Zn-deficiency-intolerant parent 'IR74' and the tolerant North Indian landrace 'Jalmagna' (Wissuwa *et al.*, 2006). A line (RIL46) from this QTL mapping population surpassed Jalmagna in tolerance, acquired more Zn and maintained higher relative root growth rates (Ismail *et al.*, 2007). Increased Zn uptake by RIL46 could be the result of constitutively greater expression of Zn-deficiency-responsive Zn^{2+} transporters and/or enhanced exudation of organic acids (Hoffland *et al.*, 2006) or other substances capable of releasing soil-bound Zn into the rhizosphere. Global expression profiling techniques now allow these rice traits to be dissected at the physiological and transcriptional levels (Walia *et al.*, 2005). Here, we test three hypotheses explaining increased tolerance to Zn-deficient soils in RIL46 using physiological and transcriptomics analyses of field-grown rice.

Hypothesis 1: increased Zn uptake by RIL46 from low $[Zn]_{ext}$ is the result of more efficient regulation of root- or shoot-expressed Zn^{2+} transporters Influx of Zn^{2+} from extracellular compartments to the cytoplasm is likely mediated by Zn/Fe permease (ZIP) family genes (Colangelo & Guerinot, 2006). A total of 14 ZIP family genes have been identified in rice (Narayanan *et al.*, 2007; Yang *et al.*, 2009) and a possible role in Zn uptake was deduced from semiquantitative analyses of the *OsZIP1*, *OsZIP3* and *OsZIP4* transporter, indicating that these are up-regulated in roots of Zn-deficient rice plants (Chen *et al.*, 2008). Several other gene families likely have Zn^{2+} transport functions in rice. Among these are Zn^{2+}-efflux proteins such as HMAs (heavy metal-associated genes of the P_{1B}-ATPase family), Zn^{2+}/H^{+} antiporters of the cation diffusion facilitator (CDF) family, Mg^{2+}/H^{+} exchanger (MHX) transporters, and Zn-induced facilitator-1 (ZIF1) and natural resistance-associated macrophage protein (NRAMPs) type genes (Colangelo & Guerinot, 2006; Narayanan *et al.*, 2007).

Hypothesis 2: increased Zn uptake by RIL46 from low $[Zn]_{ext}$ is the result of enhanced root exudation of low-molecular-weight organic acids (LMWOAs) and/or deoxymugineic acid (DMA) Because soil Zn is chemically bound in forms with a low plant availability (Neue & Lantin, 1994), the release of compounds capable of chelating soil-bound Zn, and the subsequent uptake of either Zn–chelate complexes or dissociated Zn^{2+} could overcome Zn deficiency. Hoffland *et al.* (2006) observed that Zn deficiency triggered the release of LMWOAs from rice roots and that intolerant IR74 had lower exudation rates than more tolerant lines. An alternative could be exudation of phytosiderophores. Whilst typically associated with Fe deficiency (Ishimaru *et al.*, 2006), some evidence also links phytosiderophores with Zn deficiency (Suzuki *et al.*, 2006).

Hypothesis 3: increased Zn uptake by RIL46 from low $[Zn]_{ext}$ is the result of maintenance of adventitious root development at low $[Zn]_{ext}$ Unlike most upland crops, transplanted rice requires rapid development of new adventitious or crown roots because seminal roots are damaged during transplanting. This could be a key adaptive factor in nutrient-limited environments, especially for Zn and nutrients with low soil mobility which must be 'intercepted' by an expanding root system. Despite the need to rapidly establish new adventitious roots, we observed a reduction in total root biomass under Zn-deficient conditions in field experiments (Ismail *et al.*, 2007). It is possible that reductions in root growth are not solely the result of assimilate availability, but also of the negative effects of high soil solution bicarbonate concentrations on root growth, which become more pronounced under Zn deficiency (Hajiboland *et al.*, 2005; Wang *et al.*, 2008).

These three hypotheses are based on evidence collected from plants grown in artificial media. The main aim of this study was to test them in a field environment to ensure downstream relevance for breeding. We used transcriptome data obtained from roots and shoots of the two contrasting genotypes, RIL46 and IR74, grown in the field at low or high external Zn concentrations ($[Zn]_{ext}$). Additional experiments were carried out in nutrient solutions or soil under controlled-environment (CE) conditions for phenotypic validation.

Materials and Methods

Transcriptomic analyses of two varieties of rice (*Oryza sativa* L.) with contrasting Zn-deficiency tolerance, grown under field conditions, were conducted in addition to detailed physiological studies in the laboratory. Previous experiments had identified a highly Zn-deficiency-tolerant recombinant inbred line (RIL46) in a QTL mapping population that had been used to identify QTLs associated with tolerance to Zn deficiency, with 'Jalmagna' being the tolerant donor parent and 'IR74' the intolerant parent (Wissuwa *et al.*, 2006).

Field experiments

Field experiments were conducted at the International Rice Research Institute (IRRI), Los Baños, Philippines, in the dry seasons (February–May) of 2006 and 2007. The 2006 trial was used for the microarray experiment and first phenotypic characterization. In 2007, an identical experiment was conducted and plant samples were used for detailed

analysis of adventitious root growth and for confirmation of transcriptome levels by quantitative RT-PCR (qPCR). The soil is a Typic Hydraquent (silty loam) with a low concentration of plant available Zn (*c.* 0.8 mg DTPA extractable Zn kg^{-1} soil; Wissuwa *et al.*, 2008). Zinc deficiency is induced by a combination of high pH (7.8; 1 : 1 w/v H_2O), high organic matter (2.8%) and low redox potential as a result of continued flooding. Recommended doses of NPK were applied to all plots at rates of 80 : 37.5 : 47 kg ha^{-1} (N : P : K). At the booting stage, a second dose of N fertilizer was applied as 68 kg ha^{-1}. The +Zn plot received 15 kg ha^{-1} (Zn) as $ZnSO_4{\cdot}7H_2O$, whereas the −Zn plot received no Zn and had no recent history of Zn fertilization. IR74 and RIL46 were seeded in shallow (2 cm depth) seedling trays filled with regular unfertilized soil from the IRRI farm. Three-week-old seedlings were transplanted as single-plant hills in 2.4 m^2 microplots (three rows of 4 m length, 0.2 m spacing between and within rows). Within Zn treatments, the experimental design was a randomized complete block design with three replications.

Plants were sampled 3, 4 and 7 wk after transplanting (WAT) but only the sample from the fourth week was used in this study. Three subsamples, each of four plants, were taken per microplot. By gently agitating the root zone by hand, the structure of the puddled soil became loose enough to pull plants out of the soil without causing visible damage to roots. Roots and shoots were repeatedly washed under running water to remove any adhering soil. For two subsamples destined for RNA extraction and enzyme activity analysis, roots and shoots were separated and quickly frozen in liquid nitrogen. The third subsample was brought to the laboratory and numbers of new and old roots per plant were counted, new roots being white without laterals. Leaf bronzing was scored on a scale of 0–9 (9 having most symptoms) according to Wissuwa *et al.* (2006). In 2007 additional earlier samples were taken at 0, 3, 7 and 14 d after transplanting for the determination of root number. Root and shoot dry weights were determined after oven drying samples at 70°C for 4 d. Plant samples were then ground to a fine powder and 0.5 g tissue was digested in 5 ml of concentrated H_2SO_4/H_2O_2 and analyzed by atomic absorption spectrophotometry (AAS).

Data from the +Zn control were used to calculate relative values. The main data analysis then focused on the Zn-deficient plot. For analysis of variance within Zn treatments, the procedure PROC MIXED of SAS (SAS Institute, Cary, NC, USA) was used which allows for analysis of variance of models composed of 'fixed' effects (here: genotype) and 'random' effects (blocks). For separation of genotypic means within each Zn treatment, we used Tukey's HSD test with a significance threshold of $P < 0.05$.

Microarray experiment

RNA preparation and GeneChip hybridization RNA extraction, clean-up, labeling and hybridization to GeneChips for rice shoot and root samples were done as described in Walia *et al.* (2005). Briefly, total RNA was isolated using the TRIzol reagent. The RNA was cleaned by passing through an RNAeasy spin column (Qiagen, Chatsworth, CA, USA) and on-column DNase1 treated according to the manufacturer's protocol. The RNA quality was assessed using the RNA Lab-On-A-Chip (Caliper Technologies Corp., Mountain View, CA, USA) on a Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Further labeling and hybridization steps were performed as recommended by Affymetrix, Inc. (Affymetrix Genechip Expression Analysis Technical Manual, Affymetrix, Inc., Santa Clara, CA, USA). Each biological replicate was hybridized to an array to obtain a total of three replicates for each genotype and treatment. Raw data files have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and will be publicly available.

Statistical analysis of rice transcriptome data RNA CEL files (roots and shoots) were globally normalized before further analysis using the RMA pre-processor in GeneSpring GX Version 7.3 (Agilent). Subsequently, per-gene normalizations were applied whereby the signal intensity of each probe set was normalized to the median value of the probe set across all samples. Data were analyzed initially using the 'Condition Tree' clustering program in GeneSpring. Shoot and root data separated at the first division, then genotypes at the second division and then high or low Zn. Probe sets with differential hybridization intensities between growth environments (E; high and low Zn) and genotype (G; IR74 and 46), were identified subsequently using a two-way ANOVA, with a model of E (high vs low Zn, averaged over both genotypes), G (IR74 vs RIL46, averaged over Zn treatments) and an interaction term (G × E), with the Benjamini-Hochberg false discovery rate (FDR) multiple testing correction at $P = 0.05$, in GeneSpring.

Organic acid and DMA exudation experiment

Organic acid exudation experiment After germination, seedlings were grown for 10 d in 0.5 mM $CaCl_2$ with 10 μM Fe-EDTA; seedlings were then transferred to 45 l plastic boxes and raised for 11 d on half-strength Yoshida *et al.* (1976) solution containing only 5 nM Zn. Three-week-old plants were transferred in 45 l boxes containing full-strength Yoshida solution with either zero Zn (Zn-deficient treatment) or 1 μM Zn as $ZnSO_4.7H_2O$ (Zn-replete treatment) and grown for a further 11 d to establish Zn-deficient vs Zn-replete plants. All solutions also contained 1 mM bicarbonate (as $NaHCO_3$) with pH adjusted to 8.0

with NaOH to simulate paddy conditions. Preliminary studies showed no reduction in shoot weight, root weight or root length of Zn-efficient or Zn-inefficient rice genotypes grown with 1 mM bicarbonate compared with nil bicarbonate (T. Rose and M. Wissuwa, unpublished data). Seedlings were grown in pairs in 20-mm-diameter holes in the container lids, supported by foam material. Each box contained four pairs of each of the two genotypes randomly positioned within the box and each treatment was replicated four times.

For exudate collection, two pairs of plants of each genotype from each replicate were placed separately in 75 ml glass test tubes filled with deionized water, and covered with aluminum foil to prevent light degradation of exudates. After 3 h collection, exudates were filtered through a 0.20 μm syringe filter into 200 ml plastic bottles and a 20 ml subsample was immediately frozen at −20°C. Remaining exudates were refrigerated at 4°C. After exudate collection, shoots were severed *c.* 1 mm above the highest adventitious root, dried in an air-forced oven at 70°C for 72 h and weighed. Roots of the two pairs of the same plants used for exudate collection were severed at the crown and dried in an air-forced oven at 70°C for 48 h and weighed. Roots from the remaining two pairs of each genotype per box were flash-frozen in liquid nitrogen. They were then ground in liquid nitrogen and a sample of *c.* 0.5 g fresh weight was digested in 5 ml of 5% H_3PO_3 (Hajiboland *et al.*, 2005) and filtered though Advantec filter paper into sterilized storage tubes.

Determination of organic acid concentration in exudates Frozen 20 ml aliquots of exudate solution were freeze-dried at −80°C for 5 d and salts were re-dissolved in 1 ml sterilized milliQ water. Exudates and root extracts were then analyzed for malate and citrate by enzyme assay using kits following the manufacturer's instructions (Boehringer-Mannheim, Germany). Organic acid efflux (μmol g^{-1} h^{-1}) was calculated by taking root DW and duration of collection into account (Ryan *et al.*, 1995).

DMA exudation experiment Seedlings of RIL46 and IR74 were grown in nutrient solution for 4 wk: for the first week, in 0.5 mM $CaCl_2$ with 10 μM Fe-EDTA; for the second and third weeks, in half-strength Yoshida's solution (Yoshida *et al.*, 1976) with 75 nM Zn; and for the fourth week, in full-strength Yoshida's solution with either 1 nM Zn (–Zn treatment) or 1 μM Zn (+Zn). After washing the roots three times in deionized water, individual plants were transferred to flasks containing 200 cm^3 of deionized water and root exudates were collected over 4 h starting at 06:30 h. At the end of the collection period, an antimicrobial agent (Micropur, Katadyn Products, Wallisellen, Switzerland) was added and the solutions were filtered and passed through an amberlite cation exchange column to obtain the cationic fraction. This fraction was eluted with NH_4OH, and, after evaporating to dryness, dissolved in high-performance liquid chromatography (HPLC) buffer solution and analyzed by HPLC (Mori *et al.*, 1987).

Results

Growth and mineral uptake

Biomass was restricted in low-$[Zn]_{ext}$ plots relative to high-$[Zn]_{ext}$ plots (Table 1). Genotypic differences in growth between RIL46 and IR74 were not apparent at high $[Zn]_{ext}$ but at low $[Zn]_{ext}$, RIL46 had greater shoot and root DW, developed more roots (old and new) and showed fewer leaf-bronzing symptoms compared with IR74. That these differences were caused by genotype-specific responses to Zn-limited growth conditions was evident by the severe reduction in plant Zn content at low $[Zn]_{ext}$ of 84% in RIL46 and 92% in IR74. Total plant Zn content was 74% higher in RIL46 (30.9 μg Zn; Table 1) than in IR74 (17.8 μg Zn). Both genotypes reacted to Zn deficiency by lowering their shoot Zn concentrations by *c.* 50% to a similarly low concentration of *c.* 14 μg Zn g^{-1}, which is below the 15–20 μg Zn g^{-1} critical Zn-deficiency threshold (Dobermann & Fairhurst, 2000). As a result of more efficient internal Zn use, shoot DW was reduced less than Zn content, averaging 56% reduction in RIL46 but 78% in IR74.

Shoot concentrations of other nutrients did not change in one specific pattern. For both genotypes, shoot Mg concentration increased, whereas shoot Mn, P and K concentrations decreased at low $[Zn]_{ext}$ while remaining clearly above deficiency thresholds (Table 1). Owing to the strong reduction in total DW, overall nutrient content decreased at low $[Zn]_{ext}$, but less so in RIL46 because it maintained a greater total shoot and root biomass. Since grain Zn content is too low (*c.* 0.5 μg per seed) to have lasting effects on rice growth, and since seedling DW or Zn content at transplanting did not differ between genotypes (data not shown), we conclude that the higher biomass in RIL46 is the result of genotypic differences in the ability to maintain relatively high uptake of Zn and other mineral elements.

Global transcript analysis

Since genome annotation of rice is not as detailed as Arabidopsis, we combined HarvEST:Rice (Close *et al.*, 2006) and a local Basic Local Alignment Search Tool (BLAST) to determine relevant categories of genes, based on similarity to Arabidopsis gene categories likely to be involved in Zn acquisition and transport. A detailed description of the alternative gene annotation procedures and gene categories used is given in Supporting Information, Table S1.

There are 57 381 probe sets on the Affymetrix rice array. Probes whose normalized $\log_e$ expression did not exhibit < 0.9 or > 1.1-fold change in any of the four conditions (two Zn concentrations × two genotypes) were excluded

New Phytologist

Table 1 Growth and mineral composition of rice (*Oryza sativa*) IR74 and RIL46 grown in field conditions under Zn-deficient (−Zn) or -fertilized (+Zn) conditions

	−Zn			+Zn		
	RIL46	IR74	HSD	RIL46	IR74	HSD
Shoot DW (g per plant)	1.31	0.71	*	3.0	3.3	ns
Root DW (g per plant)	0.34	0.17	**	0.83	0.71	ns
Total root number	169.0	83.8	**	190.2	194.6	ns
New root number	55.4	25.2	*	104.2	123.0	ns
Leaf bronzing	0.8	4.2	**	0.3	0	ns
Plant mortality (%)	2.7	11.4	ns	5.1	10.6	ns
Shoot Zn content (μg per plant)	17.9	10.1	*	73.8	103.9	ns
Zn content (μg per plant)	30.9	17.8	*	193.3	211.7	ns
Shoot nutrient concentrations						
Zn (μg g^{-1})	13.7	14.3	ns	27.0	30.4	ns
Fe	1005.0	1206.2	ns	1126.4	1177.0	ns
Mn	185.8	198.4	ns	244.7	226.1	ns
Cu	4.7	6.0	ns	6.1	6.9	ns
Mg (mg g^{-1})	4.08	3.28	ns	2.51	2.28	ns
Ca	3.57	3.76	ns	3.17	4.30	ns
P	1.83	2.03	ns	3.32	4.06	ns
K	34.9	29.7	*	40.1	38.2	ns
S	2.61	2.53	ns	2.54	2.41	ns

*, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant. Plants were sampled 4 wk after transplanting. The HSD compares means within Zn treatments. DW, dry weight.

from the analysis, that is, 22 453 and 24 463 genes from the root and shoot sets, respectively. The root expression of 3214 genes was affected by $[Zn]_{ext}$ ($P < 0.05$), and 3118 genes were differentially expressed between RIL46 and IR74 ($P < 0.05$). The major gene categories of 'Zn-related', 'CA (carboxylic acid) transport', 'OA (organic acid) metabolism' and 'TCA (tricarboxylic acid cycle) cycle' were not significantly overrepresented among $[Zn]_{ext}$-regulated groups or among those expressed differentially between genotypes. MATE effluxer genes were overrepresented among genes that were differentially expressed between genotypes (Table 2; values for total genes within categories shown may differ from the total number of genes given here as a result of exclusion of probes with fold changes between 0.9 and 1.1 in statistical analyses). Shoot expression of 7308 genes was significantly affected by $[Zn]_{ext}$ ($P < 0.05$). In contrast to roots, all selected categories of genes were overrepresented. Approximately 5364 genes were differentially expressed between genotypes, and 1265 showed a $[Zn]_{ext} \times$ genotype interaction. 'CA transport, 'OA function', and 'TCA cycle' genes were overrepresented among these groups.

A set of significantly expressed genes was selected to validate the microarray data through qPCR, using RNA from an independent experiment conducted in 2007 under identical conditions. A general description of phenotypic data from that experiment is given in Arnold *et al.* (2010). Results of both experiments were very similar but plants in 2007 were a little smaller and genotypic differences were a little less pronounced compared with 2006. Microarray and qPCR data were in general agreement ($r = 0.63$, Fig. S1; including methods of qPCR validation). The correlation was tighter for the comparison of factor G ($r = 0.72$) than for factor E ($r = 0.51$). Excessively high fold differences detected by qPCR were typically the result of the absence of detectable gene expression in one genotype. Specific cases of agreement or mismatches between the array and qPCR data will be discussed in subsequent paragraphs dealing with genes related to our hypotheses.

Hypothesis 1: increased Zn uptake by RIL46 from low $[Zn]_{ext}$ is the result of more efficient regulation of root- or shoot-expressed Zn^{2+} transporters

Root expression of five ZIP family genes increased at low $[Zn]_{ext}$, with *OsZIP8* showing the highest (4.1-fold) and *OsZIP2* the lowest (1.4-fold) response (Table 3), but expression did not differ between genotypes. A similar picture emerged for other putative members of the zinc/iron transport gene family (IRT/ZIP; Table 3) with Os06g37010 being responsive to Zn deficiency but equally so in both genotypes (Table 3). By contrast, root expression of the rice gene family of P-type ATPases (Baxter *et al.*, 2003) showed minimal response to low $[Zn]_{ext}$, and, again, differed little between genotypes. Thus, among HMAs with known functions in Zn transport, there were no significant differences in root expression at low $[Zn]_{ext}$ and only one between genotypes, with RIL46 being 2.1-fold higher for Os09g09930.

Among other P-type ATPases at low $[Zn]_{ext}$, *OsALA1*, *OsAHA7*, *OsECA1*, *OsACA8*, and *OsACA9* showed between 1.4- and 2.7-fold greater expression at low $[Zn]_{ext}$

Table 2 Global analysis of transcript differences between $[Zn]_{ext}$ and/or Zn-deficiency-tolerant and -intolerant rice (*Oryza sativa*), based on gene categories likely to be Zn-regulated

	Factor	No. of differentially expressed genes/total	*Arabidopsis* gene categories	No. of genes/total	
Root	E (Zn response)	3214/34 928	Zn-related genes	444/4741	ns
			GO:46942 carboxylic acid transport	15/154	ns
			GO:6082, organic acid metabolism	110/1137	ns
			KEGG TCA cycle	6/59	ns
			ABC/MATE/Multidrug	70/613	ns
			Phytosiderophore-related	4/34	ns
			Auxin-related	19/263	ns
	G (genetic response)	3118/34 928	Zn-related genes	421/4741	ns
			GO:46942 carboxylic acid transport	11/154	ns
			GO:6082, organic acid metabolism	112/1137	ns
			KEGG TCA cycle	7/59	ns
			ABC/MATE/Multidrug	70/613	*
			Phytosiderophore-related	4/34	ns
			Auxin-related	15/263	ns
	Interaction	2/34 928	–	–	–
Shoot	E (Zn response)	7308/34 243	Zn-related genes	1031/4564	*
			GO:46942 carboxylic acid transport	46/151	**
			GO:6082, organic acid metabolism	295/1115	***
			KEGG TCA cycle	27/69	***
			ABC/MATE/Multidrug	159/630	*
			Phytosiderophore-related	13/34	**
			Auxin-related	57/263	**
	G (genetic response)	5364/34 243	Zn-related genes	709/4564	ns
			GO:46942 carboxylic acid transport	33/151	*
			GO:6082, organic acid metabolism	232/1115	***
			KEGG TCA Cycle	21/69	**
			ABC/MATE/Multidrug	111/630	ns
			Phytosiderophore-related	7/34	ns
			Auxin-related	28/263	
	Interaction	1265/34 243	Zn-related genes	167/4564	ns
			GO:46942 carboxylic acid transport	7/151	ns
			GO:6082, organic acid metabolism	69/1115	***
			KEGG TCA cycle	9/69	***
			ABC/MATE/Multidrug	22/630	ns
			Phytosiderophore-related	–	ns
			Auxin-related	2/263	

***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.
TCA, tricarboxylic acid cycle.

(Table S1), whereas root expression of *OsP5* (0.7-fold) and *OsAHA8* (0.5-fold) was less at low $[Zn]_{ext}$. Only *OsALA8* (Os05g04150; Table 3) had greater root expression in RIL46 than in IR74, others having 0.5- to 0.8-fold lower expression in RIL46 than in IR74 (*(Os05g49770*, *Os12g39660*, *Os09g04670*). One root-expressed putative *OsNRAMP* (Os07g06130) decreased 0.7-fold in abundance at low $[Zn]_{ext}$ and in RIL46 (0.7-fold; Table 3).

Root expression of *MTP1*, *MTP3*, or *AtMHX* orthologs did not differ at low $[Zn]_{ext}$ or between genotypes. Among other transporter genes potentially linked to altered Zn nutrition, nine putative phosphate transporters (category PT; Table S1) showed increased root expression under Zn deficiency (from 1.5- to 7.2-fold). For two root-specific P transporters (Os08g45000 and Os03g05640) the expression level was significantly higher in RIL46.

Overall there was little evidence that efficient Zn uptake in RIL46 is caused by altered shoot expression of ZIP family genes, although *OsZIP11* (Os03g29850) and Os02g10230 expression differed 0.8- and 1.2-fold, respectively, in shoots of RIL46 compared with IR74. Genotypic differences existed for a few other genes but fold changes were small (Table 3).

Hypothesis 2: increased Zn uptake by RIL46 is to the result of root exudation of LMWOAs and/or DMA

Gene expression analysis: LMWOA-related genes Transcriptome analyses indicate that orthologs of Arabidopsis genes

Table 3 Zinc (Zn) and cation transport-related genes in rice (*Oryza sativa*) significantly affected by factors environment (E) or genotype (G)

Putative gene	Locus	Root			Shoot	
		Fold_E	Fold_G	R/Sh	Fold_E	Fold_G
OsZIP1	Os01g74110	1.8	1.5	74.6	1.1	1.2
OsZIP2(11)	Os03g29850	**1.4**	1.0	1.8	0.9	**0.8**
OsZIP3	Os04g52310	0.9	1.0	1.1	1.1	1.0
OsZIP4	Os08g10630	**3.4**	0.9	0.2	**8.4**	0.8
OsZIP5/9	Os05g39560	**2.6**	1.1	0.4	**45.5**	0.5
OsZIP6	Os05g07210	1.1	1.2	2.9	1.1	0.8
OsZIP7	Os05g10940	**2.5**	0.9	1.0	**2.4**	0.9
OsZIP8	Os07g12890	**4.1**	0.8	0.0	**2.0**	1.1
OsIRT1	Os03g46470	1.1	1.4	15.0	0.3	1.6
IRT/ZIP	Os02g10230	0.8	0.9	1.2	1.0	**1.2**
IRT/ZIP	Os05g39540	**4.5**	1.5	1.3	**50.1**	0.7
IRT/ZIP	Os06g37010	**7.9**	1.0	0.4	**5.6**	0.9
IRT/ZIP	Os09g31442	**3.6**	1.4	1.7	**45.0**	0.7
HMA	Os06g47550	1.0	0.7	0.1	**5.2**	1.2
HMA	Os09g09930	0.7	**2.1**		1.2	**1.3**
AHA	Os05g49770	**0.5**	**0.5**	3.2	1	**1.8**
AHA	Os09g04670	**0.8**	**1.1**	0.4	**1.4**	**0.8**
ALA	Os05g04150	1	**1.6**	1.8	**1.2**	**1.3**
NRAMP	Os01g13430	0.8	0.9	0.2	1.1	**1.6**
NRAMP	Os03g11010	0.8	0.7	1.1	**1.4**	0.9
NRAMP	Os06g46310	0.9	0.7	0.8	**1.5**	1
NRAMP	Os07g06130	**0.7**	**0.7**	1.1.	0.9	1
NRAMP	Os07g15460	0.8	1.1	1	**2.1**	0.6
CDF	Os02g28980	1.2	1	0.9	**1.7**	**0.8**
CDF	Os03g61990	1.2	0.8	1.5	**0.6**	1
CDF	Os04g23180	1.1	0.7	0.5	1.1	**0.6**
CDF	Os07g08330	1.1	1	2.2	**0.8**	0.9
CDF	Os11g25800	1.3	1.1	0.1	**0.7**	1.1

Folds E, G: fold changes in the low-Zn treatment relative to +Zn (E) or of RIL46 relative to IR74 (G); bold font represents significance for respective factor. Shading represents the overall expression level: dark, highly expressed genes (1SD above mean); none, very low expression (below 100). Values for R/Sh describe average root expression level relative to shoot expression.

categorized as OA metabolism-related (GO:6082), TCA (KEGG pathway) and CA transport (GO:46942) are highly overrepresented among shoot but not root genes differentially regulated by factors E and G (Tables 2, Table S1).

Organic acid exudation-mediated solubilization of soil-bound Zn would be caused by either increased OA synthesis, regulated by genes involved in central carbon metabolism/TCA cycle and/or with enhanced root OA efflux. The two key enzymes directing carbon flow to the TCA cycle are phosphoenolpyruvate carboxylase (PEPCase) and pyruvate dehydrogenase (PDH). PEPCase was up-regulated by Zn deficiency in roots but equally in both genotypes (Table 4). The regulation pattern was similar in shoots, with the exception that one additional PEPCase (Os12g36590) was only detected in RIL46. This gene was not expressed in roots. PDH was not regulated in roots but significant G, E and G × E effects existed in shoots. In all cases, expression was either constitutively higher in IR74 (Os03g44300, Os08g42410) or IR74 showed some induction by Zn deficiency (Os02g01500, Os06g01630, Os07g22720). Other genes involved in central carbon metabolism, such as phosphofructokinase (PFK) and pyruvate kinase, were not affected by factor E in roots, and when genotype effects were significant, expression levels were higher in IR74 (Table S1).

Among TCA cycle-related genes, up-regulation of citrate synthase (CS) and malate dehydrogenase (MDH) and down-regulation of aconitase would increase cellular concentrations of citrate and malate that are known to effectively solubilize soil Zn. Nine putative MDHs were expressed in roots but only two differed between genotypes, with Os04g46560 being 1.4-fold higher in RIL46 and root-specific Os05g02220 being only detectable in IR74 (Table 4). The only MDH affected by low $[Zn]_{ext}$ was Os08g33720 (1.6-fold). Five CS genes were expressed in roots but none were affected by $[Zn]_{ext}$ and two (Os01g19450, Os04g14450) had lower expression in RIL46. Aconitases were not regulated by factors E or G in roots. By contrast, six (of nine) MDHs in shoots were affected by E and five by G. All were more highly expressed in IR74 or under low $[Zn]_{ext}$. This pattern also applied for CS genes, with Os01g19450 and Os02g10070 induced by Zn deficiency in IR74 but not in RIL46.

Gene expression analysis: DMA biosynthesis-related genes

Mugineic acid (MA) is synthesized by rice and exuded by roots in a variety of structural forms. The predominant form (DMA) is biosynthesized from L-methionine and nicotianamine, via S-adenosyl-L-methionine (SAM), and catalyzed by SAM synthetase (SAMS), nicotianamine synthase (NAS), nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS).

Putative *OsSAMS* genes were highly expressed in roots but were not affected by factor E (Table 4). *OsSAMS2* showed constitutively lower expression in RIL46 (0.6-fold). In shoots, *OsSAMS2* was up-regulated by low $[Zn]_{ext}$ and more so in IR74, while *OsSAMS1* was down-regulated by low $[Zn]_{ext}$. Genotypic effects were significant but fold changes were small, with Os01g18860 having higher, and Os05g04510 lower, expression in RIL46. Of the three NAS genes identified in rice, root-specific *OsNAS1* and *OsNAS2* had high constitutive expression. *OsNAS3* (Os07g48980) was expressed equally in roots and shoots and showed four- to fivefold increased expression at low $[Zn]_{ext}$ (Table 4). *OsNAAT1* showed the highest expression level of all six *OsNAAT* genes in roots and shoots. Zn deficiency led to an up-regulation in shoots as seen by Suzuki *et al.* (2008). A 1.6- to twofold up-regulation in shoots was also observed for *OsNAAT4*, *5* and *6*. *OsNAAT6* was not detected in roots and *OsNAAT3* was not detected at all. Significant genotype effects were seen

Table 4 Phytosiderophore biosynthesis and transport-related genes in rice (*Oryza sativa*) significantly affected by factors environment (E) or genotype (G)

Putative gene	Locus	Root			Shoot	
		Fold_E	Fold_G	R/Sh	Fold_E	Fold_G
Carbon flow to TCA						
PEPCase	Os02g41580	**2.8**	0.8	4.2	1.2	1.1
PEPCase	Os02g41580	1.7	1.1	1.3	**2.5**	1.7
PEPCase	Os08g27840	3.2	0.9	0.0	**1.8**	1.0
PEPCase	Os08g27840	1.5	1.1	0.0	**3.4**	1.0
PEPCase	Os09g14670	**1.9**	1.3	0.6	**2.2**	1.0
PEPCase	Os09g14670	**2.4**	1.2	0.5	**2.7**	1.0
PEPCase	Os12g36590	0.6	3.8	0.1	1.0	**15.5**
PDH	Os02g01500	1.0	0.6	2.1	1.2	**0.7**
PDH	Os08g42410	1.3	1.0	1.6	1.1	**0.9**
PDH	Os07g22720	1.1	1.0	1.9	**1.1**	1.0
PDH	Os06g01630	1.2	1.0	1.2	**1.3**	0.9
PDH	Os02g50620	1.1	1.0	1.0	**1.3**	1.0
PDH	Os03g44300	1.0	1.1	1.3	1.0	**0.8**
TCA cycle						
CS	Os01g19450	1.1	**0.8**	1.4	**1.1**	**0.8**
CS	Os02g13840	0.8	0.9	1.5	1.0	0.9
CS	Os02g10070	1.1	0.9	1.2	**1.4**	0.9
CS	Os04g14450	0.7	**0.5**	1.1	**0.8**	0.9
CS	Os11g33240	1.3	1.1	2.8	1.1	0.8
MDH	Os05g49880	1.3	1.2	1.0	**1.5**	1.0
MDH	Os10g33800	1.1	0.9	1.2	1.0	**0.9**
MDH	Os01g46070	1.2	1.2	4.1	**1.5**	**0.8**
MDH	Os08g33720	**1.6**	1.2	0.6	**1.7**	**0.7**
MDH	Os07g43700	0.9	0.9	0.0	**1.3**	1.0
MDH	Os04g46560	1.0	**1.4**	1.6	0.7	**0.5**
MDH	Os03g56280	0.6	0.8	0.0	1.0	1.1
MDH	Os05g02220	0.8	**0.1**	7.8	1.0	1.0
MDH	Os12g43630	0.9	1.0	4.2	**1.5**	0.8
MDH	Os01g61380	1.1	1.0	12.0	**1.7**	0.7
Phytosiderophore biosynthesis						
OsSAMS1	Os05g04510	1.0	1.0	2.3	0.8	**0.8**
OsSAMS1	Os01g18860	1.3	1.2	0.5	**0.8**	**1.2**
OsSAMS2	Os01g22010	0.9	**0.6**	1.8	**1.4**	**0.6**
OsNAS1	Os03g19427	1.1	1.0	409	0.5	2.3
OsNAS2	Os03g19420	1.1	1.1	126	0.7	0.9
OsNAS3	Os07g48980	**5.7**	0.9	0.8	**4.2**	1.1
OsNAAT1	Os02g20360	1.2	1.2	0.3	**1.5**	1.1
OsNAAT2	Os02g19970	0.9	0.9	2.3	0.9	1.4
OsNAAT3	Os02g19924	1.1	0.9	1.1	1.0	1.1
OsNAAT4	Os06g23684	0.8	**0.6**	1.7	**2.1**	**1.9**
OsNAAT5	Os11g35040	0.9	1.2	2.5	**1.6**	**1.4**
OsNAAT6	Os12g39840	1.1	1.0	0.0	**1.6**	0.9
OsDMAS1	Os03g13390	0.8	0.9	3.8	1.0	0.8
Phytosiderophore transport						
OsYSL2	Os02g43370	0.9	**0.4**	1.0	**1.9**	**0.3**
OsYSL5	Os04g32060	0.8	0.8	1.0	0.8	0.9
OsYSL6	Os04g32050	**0.8**	1.2	0.7	**0.9**	**1.2**
OsYSL9	Os04g45860	0.9	1.4	2.6	**0.7**	1.2
OsYSL12	Os04g44320	**0.6**	1.1	2.0	**0.5**	1.2
OsYSL13	Os04g44300	0.8	**0.5**	2.0	0.9	0.9
OsYSL14	Os02g42220	0.6	0.9	2.7	**0.6**	1.1
OsYSL15	Os02g43410	1.2	1.0	11.3	1.0	1.2
OsYSL16	Os04g45900	**2.0**	1.0	0.1	1.1	1.1
ABC transporters (efflux)						
ABC	Os08g07590	**2.8**	**2.5**	25.8	0.8	0.8
ABC	Os08g30740	**2.1**	**1.8**	5.6	**1.3**	0.9
ABC	Os06g38950	**2.0**	**1.6**	26.0	1.3	0.9
ABC	Os02g11760	1.7	**2.3**	3.3	1.2	0.9
MDR	Os01g50160	**1.6**	**1.5**	7.9	1.1	1.2
ABC	Os11g05700	1.5	**1.7**	0.3	**2.4**	0.9
ABC	Os05g02750	**1.4**	**2.2**	3.2	0.8	0.9
ABC	Os01g42380	1.3	**1.7**	3.6	**1.4**	0.7
MATE	Os03g48626	1.2	**1.7**	1.8	0.9	**1.6**
ABC	Os04g08060	1.2	**1.7**	11.5	0.7	1.0
ABC	Os06g41770	1.1	**1.9**	5.2	1.0	1.0
ABC	Os07g45194	**0.8**	**3.6**	0.8	0.8	**0.4**
ABC	Os02g32690	0.8	**3.9**	0.2	1.2	**15.1**
ABC	Os09g16330	0.8	**2.7**	0.4	0.9	0.8
ABC	Os12g22284	**0.4**	**3.3**	2.5	0.8	**3.6**

Annotations are based on Kobayashi *et al.* (2005) for OsSAMS, on Suzuki *et al.* (2008) for OsNAS and OsDMAS, Inoue *et al.* (2008) for OsNAAT, and on Narayanan *et al.* (2007) and Nozoye *et al.* (2007) for OsYSLs.
Folds E, G: fold changes in the low-Zn treatment relative to +Zn (E) or of RIL46 relative to IR74 (G); bold font represents significance for respective factor. Shading represents the overall expression level: dark, highly expressed genes (1SD above mean); none, very low expression (below 100). Values for R/Sh describe average root expression level relative to shoot expression.
TCA, tricarboxylic acid cycle.

in shoots for *OsNAAT4* and *5*, RIL46 being 1.9- and 1.4-fold higher but with lower expression in roots of RIL46. *OsDMAS1* was expressed at a low level in both tissues, and neither E nor G effects were significant.

Gene expression analysis: OA/DMA efflux transporters

Rhizosphere activity of AO and DMA is typically limited by their exudation rate. As yet, DMA efflux transporters have not been identified but candidates for OA/DMA efflux transporters are likely to be found among ABC/MATE/Multidrug-related genes which were overrepresented in genes whose expression differs between RIL46 and IR74 roots (Table 2). Higher expression in RIL46 was detected for 33, with some degree of root-specificity and induction by $[Zn]_{ext}$ seen in 15 (Table 4). These can be considered interesting candidates for OA/DMA efflux transporters. Homologs of malate/citrate efflux transporters identified in other plant species were not among those significant for factor G. However, the rice homolog (Os03g11734) of the barley aluminum activated citrate transporter (*HvAACT1*) and a gene (Os02g45160) similar to *AtAlMT1* were both root-specific and induced by Zn deficiency (1.8-fold) but not different between genotypes (Table S1). No expression was detected for Os01g69010 and Os04g34010, the homologs of the sorghum Alt_{SB} and wheat *AlMT* genes.

Gene expression analysis: YSL transporters Transport of metal–MA complexes into roots (and within rice plants) is mediated by the family of YSL transporters and 18 members have been identified in rice (Koike *et al.*, 2004). *OsYSL6* has a reportedly higher constitutive expression in roots and shoots and we can basically confirm this except that expression levels decreased slightly at low $[Zn]_{ext}$, with RIL46 maintaining slightly higher expression in both tissues (Table 4, S1). *OsYSL9, OsYSL12, OsYSL13* and *OsYSL14* were expressed at higher levels in roots than in shoots. Only *OsYSL15* was truly root-specific, confirming observations by Koike *et al.* (2004) that expression was highly inducible in roots by Fe deficiency, making *OsYSL15* the main candidate for uptake of metal–MA complexes into roots (Lee *et al.*, 2009). No expression at any factor combination was detectable for YSL 1, 3, 4, 7, 8, 10, 17, 18 (Table S1). Significant genotype effects indicative of more efficient uptake of the complexes were not detected in the array data.

Physiological evidence Previous experiments identified citrate and malate as the dominant LMWOAs exuded from roots of both genotypes, with oxalate present only in very low concentrations (M. Frei, unpublished). Therefore, in the present nutrient solution experiment, only citrate and malate were investigated. At the time of exudate collection, plants in the −Zn treatments showed a reduction in DW relative to the +Zn control of 33% for RIL46 and 40% for IR74. Root malate concentration increased threefold at low $[Zn]_{ext}$ in RIL46 and IR74, whereas increases in citrate were insignificant (Fig. 1). Malate exudation did not differ constitutively between RIL46 and IR74, but increased twofold in RIL46 at low $[Zn]_{ext}$, and was constant in IR74 (Fig. 2). By contrast, citrate exudation was neither significantly different between genotypes nor affected by Zn supply. DMA exudation in 28-d-old seedlings was constitutively higher in RIL46 (Fig. 3). No effect of Zn supply was detected, but at the time of exudate collection, visible symptoms of Zn deficiency were absent despite plants being grown without Zn for 7 d.

Hypothesis 3: root growth

Root development decreased under Zn deficiency, mainly because of a decline in newly developing adventitious roots

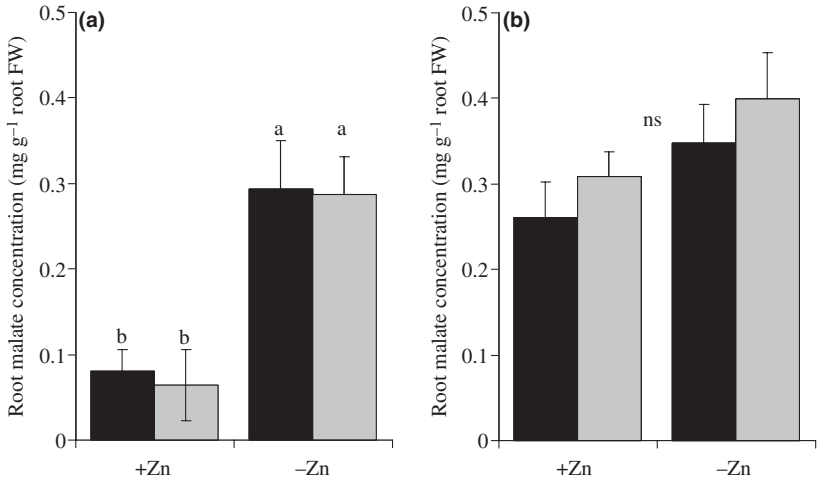

Fig. 1 Concentration of malate (a) and citrate (b) in roots of rice (*Oryza sativa*) RIL46 (black bars) and IR74 (gray bars) under zinc (Zn)-replete and Zn-deficient conditions in nutrient solution. Values shown are means ± SE (n = 4), and different letters above columns indicate significant difference between means, based on Tukey's HSD at $P < 0.05$.

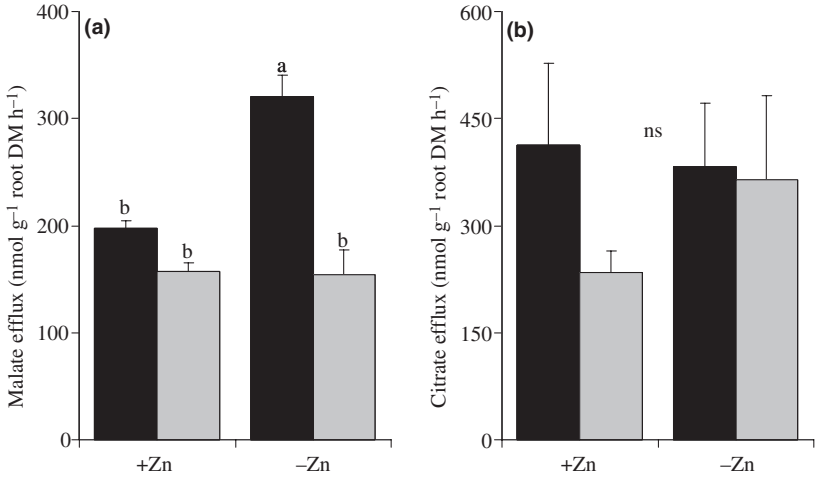

Fig. 2 Exudation of malate (a) and citrate (b) by roots of rice (*Oryza sativa*) RIL46 (black bars) and IR74 (gray bars) under zinc (Zn)-replete and Zn-deficient conditions in nutrient solution. Values shown are means ± SE (n = 8), and different letters above columns indicate significant difference between means, based on Tukey's HSD at $P < 0.05$.

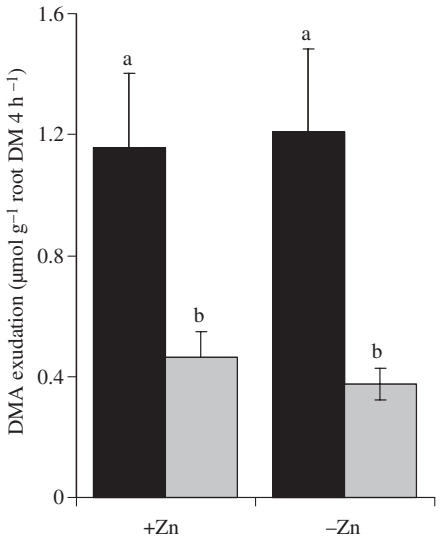

Fig. 3 Deoxymugineic acid (DMA) exudation in 21-d-old seedlings of rice (*Oryza sativa*) RIL46 (black bars) and IR74 (gray bars). Zinc (Zn) had been omitted from the nutrient solution 7 d before exudate collection in the –Zn treatment. In the control treatment, Zn was supplied at a concentration of 0.1 μM. Values shown are means ± SE (n = 4), and different letters above columns indicate significant difference between means, based on Tukey's HSD at $P < 0.05$.

Table 5 Root number and dry weight development in rice (*Oryza sativa*) following transplanting

	–Zn			+Zn		
DAT	RIL46	IR74	HSD[a]	RIL46	IR74	HSD
Root number per plant						
0	5.3	4.5	***	5.3	4.8	**
3	7.0	4.2	***	7.3	6.1	**
7	14.3	9.7	***	21.1	18.3	**
14	19.9	14.6	**	51.0	48.7	ns
21	52.7	33.3	***	148.6	115.9	***
Root DW (mg per plant)						
0	7.8	7.9	ns	7.0	7.4	ns
3	14.7	12.3	ns	15.9	14.1	ns
7	38.1	23.9	***	39.0	34.4	ns
14	52.4	30.5	**	117.8	87.7	*
21	65.4	29.7	**	322.6	237.6	ns

[a]Tukey's HSD was used for the comparison of genotypic means within Zn treatments: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.
At day 0 all roots were seminal roots. Adventitious roots developed after transplanting.
DW, dry weight.

that led to reduced total root number (Table 1), rather than inhibition of longitudinal root growth. This decline in new adventitious root formation 3–4 wk after transplanting was significantly higher in IR74 (−74%) compared with RIL46 (−49%). At that time, genotypes differed in Zn uptake; hence, better adventitious root formation in RIL46 may be a consequence of its higher Zn uptake rather than a direct tolerance mechanism. A subsequent experiment was conducted in 2007 to monitor adventitious root development before genotypic differences in Zn uptake could have confounded effects. At day 0, both genotypes only had seminal roots. Genotypic differences in the development of new adventitious roots following transplanting were detectable as early as 3 d after transplanting when RIL46 had produced two new adventitious roots, but IR74 had yet failed to produce any (Table 5). One week after transplanting, RIL46 had produced nine adventitious roots compared with five in IR74, suggesting that maintaining adventitious root formation may be a causal tolerance mechanism in RIL46. However, smaller differences between genotypes were maintained even in the +Zn treatment, which may indicate that IR74 is sensitive to additional stress factors, such as low redox potential and high bicarbonate, that are present in our soil irrespective of Zn treatment.

Several genes that alter adventitious root phenotype have been identified, such as the *OsPIN1* (Xu *et al.*, 2005) and crown rootless (crl) genes (Kitomi *et al.*, 2008). *OsPIN1*, *OsCrl1* and *OsCrl4* were expressed in roots and shoots of both genotypes, but the only significant effect was a 30% down-regulation of *OsCrl1* (Os03g049100) in Zn-deficient shoots of both genotypes. Adventitious root formation is generally thought to be controlled via auxin-mediated processes, and 21 putative auxin-induced/responsive genes showed a significant regulation pattern in roots (Table 6). Four genes with a significant genotype effect were root-specific and RIL46 had a higher expression level in all cases. Among these were the auxin-responsive protein *OsIAA7* (Os02g13520), and two putative auxin-induced protein *pCNT115* (Os04g26870 and Os04g27060).

For additional root growth-related genes we detected 17 cellulose synthase genes with at least fivefold higher expression in roots. Five of these differed between genotypes, but in four cases, IR74 had higher expression (factor E being not significant; Table S1), indicating that cellulose synthesis was not linked to Zn deficiency and tolerance thereof. Similar results were obtained for other cell wall-related genes (glucan- and xyloglucan-related). Twelve putative xylanase inhibitors were only expressed in roots, of which four differed between genotypes, and in all four cases (Os08g40680, Os11g47560, Os11g47580, Os11g47590) expression was only detected in RIL46.

Candidate genes

Physiological evidence suggested OA/DMA efflux as a key tolerance mechanism in RIL46; expression of several OA/DMA-related genes were therefore evaluated by qPCR in the 2007 experiment. *OsNAS3* and *OsNAAT4* showed twofold higher expression in RIL46 (Table 7), whereas there was no genotypic effect in the microarray. However,

Table 6 Auxin-related genes significantly affected by factors environment (E) or genotype (G) in roots of rice (*Oryza sativa*)

Putative gene	Locus	E	G	R/Sh
Auxin-induced or -responsive proteins				
OsIAA10	Os01g43090	**0.5**	0.6	0.3
OsSAUR26	Os02g13520	1.1	**1.6**	8.8
OsIAA7	Os02g30810	**1.5**	1.4	400
OsIAA10	Os02g57250	**0.7**	1.0	0.4
OsSAUR53	Os03g53140	0.8	**1.8**	0.8
pCNT115	Os04g26870	0.8	**1.8**	8.2
pCNT115	Os04g27060	**0.4**	**3.0**	9.2
	Os05g05180	**1.9**	1.2	4.8
IAA7	Os05g08570	**1.3**	**1.3**	1.7
OsSAUR26	Os06g45970	**2.1**	0.6	0.5
	Os08g41280	**0.6**	0.9	1.9
	Os08g41290	**0.8**	0.9	118
IAA18	Os09g26550	1.1	**0.5**	0.9
OsSAUR53	Os09g37480	**0.5**	1.1	0.1
ARP1	Os11g44810	**0.8**	**0.7**	3.3
Auxin response factors				
ARF3	Os01g54990	**0.6**	0.7	0.7
	AK064860	0.6	**2.3**	0.9
ARF16	Os10g33940	**0.5**	0.8	1.7
	Os11g10530	0.6	**2.3**	1.2
Auxin efflux carrier				
	Os08g09190	**0.8**	0.9	1.1
	Os09g31478	1.0	**1.2**	0.9
	Os11g44680	0.4	**7.7**	4.6

Numbers in columns E, G: fold changes in the low-Zn treatment relative to +Zn (E) or of RIL46 relative to IR74 (G); bold font represents significance for respective factor. Shading represents the overall expression level: dark, highly expressed genes (1SD above mean); none, very low expression (below 100). Values for R/Sh describe average root expression level relative to shoot expression.

this may only affect within-plant transport of phytosiderophore-bound Zn rather than imply higher exudation of DMA since no genotypic effect was seen for *OsDMAS1*. One potentially important difference in the 2007 field trial was seen for *YSL15*, which was clearly inducible by Zn deficiency (3.3-fold; Table 7), and was constitutively higher in RIL46 (2.9-fold). This higher expression of *YSL15* suggests that uptake of Fe/Zn–DMA chelates would be higher in RIL46, presumably because RIL46 exudes more DMA. The key candidate gene may therefore be found among putative efflux transporters, and several ABC transporters were confirmed as being more highly expressed in RIL46, namely Os02g11760, Os02g32690, and Os08g07590. Of the three candidates genes putatively involved in auxin-mediated adventitious root formation, only one (Os04g27060) could be confirmed through qPCR.

Table 7 Comparison of microarray (MA) and quantitative RT-PCR (qPCR) results in selected groups of significant genes in rice (*Oryza sativa*)

TIGR6 locus			Root		Root/ shoot	Shoot	
			E	G		E	G
Os01g22010	OsSAMS2	MA	0.9	**0.6**	1.8	**1.4**	**0.6**
		qPCR	**1.3**	**0.7**		**6.1**	1.4
Os03g19420	OsNAS2	MA	1.1	1.1		0.7	0.9
		qPCR	1.4	1.3		**2.5**	1.0
Os07g48980	OsNAS3	MA	**5.7**	0.9	0.8	**4.2**	1.1
		qPCR	**10.9**	**2.0**		**2.8**	0.9
Os06g23684	OsNAAT4	MA	0.8	**0.6**	1.7	**2.1**	**1.9**
		qPCR	0.8	**1.9**		0.8	**1.9**
Os03g13390	OsDMAS1	MA	0.8	0.9		1.0	0.8
		qPCR	**1.8**	1.1		1.5	**1.6**
Os02g43370	YSL2	MA	0.9	**0.4**		**1.9**	**0.3**
		qPCR	**0.4**	**0.0**		**3.1**	**0.2**
Os04g44300	YSL13	MA	0.8	**0.5**	2.0	0.9	0.9
		qPCR	0.9	0.9		0.8	1.6
Os02g43410	YSL15	MA	1.2	1.0	11.3		
		qPCR	**3.3**	**2.9**			
Os06g38950	ABC	MA	**2.0**	**1.6**	26.0		
		qPCR	1.2	1.0			
Os08g07590	ABC	MA	**2.8**	**2.5**	25.8		
		qPCR	**1.8**	**1.5**			
Os04g08060	ABC	MA	1.2	**1.7**	11.5		
		qPCR	**0.4**	1.1			
Os01g50160	ABC	MA	**1.6**	**1.5**	7.9		
		qPCR	1.0	1.5			
Os02g11760	ABC	MA	1.7	**2.3**	3.3		
		qPCR	19.7	**49.4**			
Os02g32690	ABC	MA	0.8	**3.9**	0.2	1.2	**15.1**
		qPCR	0.8	**70.9**		1.0	**2162**
Os06g41770	ABC	MA	1.1	**1.9**	5.2		
		qPCR	1.3	1.3			
Os08g30740	ABC	MA	**2.1**	**1.8**	5.6		
		qPCR	1.1	0.8			
Os08g45000	P transporter	MA	**7.2**	**4.6**	6.6		
		qPCR	**0.6**	**2.3**			
Os02g47090	OPT	MA	1.3	**3.5**	79		
		qPCR	1.3	**2.3**			
Os09g14670	PEPCase	MA	**2.4**	1.2	0.5	2.7	1.0
		qPCR	1.0	1.5		**5.8**	**1.5**
Os02g13520	IAA7	MA	1.1	**1.6**	8.8		
		qPCR	0.9	0.8			
Os04g26870	pCNT115	MA	0.8	**1.8**	8.2		
		qPCR	**4.3**	0.6			
Os04g27060	pCNT115	MA	**0.4**	**3.0**	9.2		
		qPCR	0.9	**1.6**			

Data are presented as relative comparison (–Zn/+Zn) for environment (E) or (RIL46/IR74) for genotype (G), and are the means of three independent replications. Effects highlighted in bold are significant based on two-factorial ANOVAs conducted separately for array and qPCR data.

Discussion

Genotypic variation for tolerance to Zn deficiency exists in rice (Quijano-Guerta *et al.*, 2002; Wissuwa *et al.*, 2006) and could be exploited in plant breeding; however, our present knowledge regarding effective tolerance mechanisms and underlying genetic factors is very limited. This knowledge gap is addressed in the present study. In contrast to previously published transcriptome analyses in other plant

species under Zn deficiency (to our knowledge no data have yet been published for rice), we used two genotypes with contrasting tolerance to Zn deficiency and obtained samples from field-grown rather than from hydroponically grown plants. A key difference between tolerant RIL46 and intolerant IR74 was a more than 50% higher Zn uptake when grown in a Zn-deficient field. The discussion will therefore focus on mechanisms and underlying genes that could contribute to higher Zn uptake. Other genotypic differences, such as the high amount of visible leaf symptoms (bronzing) in Zn-deficient IR74, possibly caused by differences in the detoxification of oxygen radicals, are discussed in a separate paper (Frei *et al.*, 2010).

Hypothesis 1: Zn transporters

Since RIL46 takes up more Zn compared with IR74 at low $[Zn]_{ext}$, we tested if this correlated with increased expression of Zn^{2+} transporters in roots and shoots of RIL46. Chen *et al.* (2008) detected higher gene expression of *OsZIP3* and *OsZIP4* in roots of Zn-efficient IR8192 compared with inefficient Erjiufeng and proposed that the higher expression of these two zinc transporters is related to Zn efficiency. Expression of *OsZIP3* was not detected in our study on field-grown plants, whereas expression of *OsZIP4* was highly induced by Zn deficiency but equally so in our contrasting pair of lines. The induction of Zn^{2+} transporter in response to low $[Zn]_{ext}$ was also seen for many other putative Zn^{2+} transporters; however, there was no compelling evidence indicating that intolerance of IR74 was caused by insufficient up-regulation of these transporters, assuming that post-transcriptional regulation did not reverse the up-regulation seen in IR74. This conclusion is supported by Zn uptake data from low-Zn nutrient solutions that showed Zn uptake in IR74 to be not different from several RILs (sister lines of RIL46) with higher field tolerance than IR74 (Wissuwa *et al.*, 2006). In fact, several genotypes showing superior Zn uptake from low-Zn nutrient solutions did not show a similarly high uptake of Zn in field studies. Low availability of free Zn^{2+} in Zn-deficient soils likely means that up-regulation of transporters alone does not confer tolerance because substrate (Zn^{2+}) availability is the limiting factor that would best be overcome by mechanisms that increase Zn availability at the root surface or that increase relative root growth and thereby total root surface area.

Hypothesis 2: root exudation of substances increasing Zn availability

One potential mechanism increasing Zn availability in the rhizosphere is root exudation of citrate (Hoffland *et al.*, 2006) and malate (Gao, 2007) for which respective studies found higher exudation rates in genotypes with higher tolerance to Zn deficiency. Induction of malate efflux detected in our study for RIL46 under Zn deficiency may thus contribute to its high tolerance. However, high malate efflux does not appear to be linked to higher malate biosynthesis since root-internal malate concentrations were similar between genotypes, as were expression levels of genes favoring citrate or malate biosynthesis. Our observation is consistent with the conclusion of Delhaize *et al.* (2001) that the limiting step is not the plant-internal concentration of OAs (synthesis) but their rate of release. This release is mediated by specific efflux transporters and their level of activity was shown to be directly linked to malate-mediated tolerance to excess aluminum in barley and sorghum (Delhaize *et al.*, 2004; Magalhaes *et al.*, 2007).

Transcript abundances of the rice homologs of known OA efflux transporters *HvAACT1* and *AtAlMT1* were both root-specific and induced by Zn deficiency (1.8-fold) but did not differ between genotypes, whereas no expression was detected for the homologs of the sorghum Alt_{SB} and wheat *AlMT* genes. Several candidates were identified among genes annotated as putative ABC/MATEs, using root specificity, response to Zn deficiency, and higher expression in RIL46 than in IR74 as selection criteria. Based on the independent confirmation via qPCR, three candidates (Os02g11760, Os02g32690, and Os08g07590) appear particularly promising.

A possible role of Zn-phytosiderophore uptake in the tolerance of RIL46 has recently been suggested based on the analysis of Zn isotope ratios in leaves of RIL46 and IR74 obtained from the field experiment conducted at IRRI in 2007. A shift toward the heavy ^{66}Zn in RIL46 but not in IR74 suggested that Zn uptake pathways/mechanisms differed between both lines (Arnold *et al.*, 2010). The relative enrichment with ^{66}Zn in RIL46 implied the involvement of a Zn-binding process. A similar shift had been observed for Fe in strategy II plants (Guelke & von Blanckenburg, 2007) that rely on the Fe-phytosiderophore uptake pathway. This observation, in combination with higher DMA exudation rates detected for RIL46 in the present study, suggests that tolerance of RIL46 is in part the result of enhanced DMA exudation and subsequent uptake of DMA-bound Zn. That we detected 2.9-fold higher transcript abundance for *OsYSL15* in RIL46 in the experiment that showed the heavy Zn enrichment in RIL46 further corroborates this hypothesis.

OsYSL15 has recently been confirmed as the dominant transporter involved in uptake of the Fe(III)–DMA complex (Inoue *et al.*, 2009; Lee *et al.*, 2009), but little is known about its ability to transport Zn–DMA as well. The threefold increase in *OsYSL15* expression under Zn deficiency is, to our knowledge, the first report linking it with response to Zn deficiency. That it is up-regulated under Zn deficiency under lowland conditions, where rice plants typically experience excess Fe in the rhizosphere (both genotypes had shoot Fe concentrations > 1000 μg Fe g^{-1}, bordering Fe toxicity), would suggest that the up-regulation

is a specific response to Zn deficiency. It is not known whether the uptake of Zn–DMA could contribute substantially to tolerance of Zn deficiency, given that concentrations of Fe will be several orders of magnitude higher than Zn in the rhizosphere of paddy rice. However, in highly reduced paddy soil, reduced Fe(II) is expected to predominate in the soil solution. Furthermore, evidence reviewed by Hiradate *et al.* (2007) indicates that the affinity of DMA for Zn would be higher than for Fe(II), thus allowing Zn–DMA chelate formation, even under condition of excess Fe(II) availability. *OsYSL15* is not necessarily the key candidate gene since the most limiting factor in rice is expected to be DMA exudation (Suzuki *et al.*, 2008). Since transcriptome evidence did not suggest that DMA biosynthesis differed markedly in RIL46, we conclude that higher efflux transporter activity is the most plausible tolerance mechanism. The same putative candidates discussed as potentially involved in malate efflux (Os02g11760, Os02g32690, and Os08g07590) would therefore also be DMA efflux transporter candidates.

Hypothesis 3: root growth

Phenotypic evidence clearly showed that a key difference between RIL46 and IR74 is a severe reduction in adventitious root development in IR74. This effect occurred 3 d after transplanting, suggesting that root growth is an independent tolerance mechanism rather than a subsequent effect of higher Zn uptake in RIL46. Differences in gene expression between RIL46 and IR74 for root-specific auxin-responsive proteins (Table 6) could suggest their involvement in maintaining adventitious root formation in tolerant RIL46. However, the reduction in adventitious root development seen in the field experiment may not be solely to the result of Zn deficiency. High bicarbonate concentrations in soil solutions, as present at our field site, reportedly reduce root growth in Zn-inefficient but not in Zn-efficient rice genotypes (Hajiboland *et al.*, 2005). First tests with our genotypes have indeed found a negative effect of bicarbonate on root growth that was more pronounced in IR74 compared with RIL46 (Widodo *et al.*, unpublished). We hypothesize that roots of RIL46 may be tolerant to the radical oxygen stress caused by high HCO_3^-, as a result of superior ROS scavenging mechanisms which are apparent in shoots of RIL46 (Frei *et al.*, 2010). Regardless, it is clear that knowledge of adventitious development (and interactions with bicarbonate) remains very limited and the three root-specific auxin-responsive proteins identified here may just serve as candidates for further studies on this topic rather than as proof of its involvement in the tolerance response of RIL46. In further studies, it would be crucial to evaluate tissue-specific gene expression levels, particularly for the crown region where adventitious roots originate, rather than relying on whole root/shoot expression profiles.

Conclusions

Zinc deficiency in rice is induced by several factors, including high pH, excess bicarbonate and low redox potential. It is therefore likely that several tolerance mechanisms need to be combined if a genotype is to fully overcome the effects of Zn deficiency. This complexity had already been indicated in the QTL analysis conducted with the RIL population from which RIL46 is derived: several putative QTLs were detected, with epistatic interactions between QTLs playing an unusually important role. Since RIL46 contains donor parent introgressions spanning several of these QTLs, it is expected that no single mechanism would explain the high tolerance of RIL46.

Here we identified three possible tolerance mechanisms based on phenotypic evidence: DMA and/or OA exudation, and adventitious root growth. Based on the transcriptome data, several putative OA/DMA efflux transporters were identified and these need to be studied in more detail. Overall this holds great promise for breeding, because the efficacy of selecting for high efflux activity has repeatedly been shown for tolerance of other stresses such as excess aluminum. Improving adventitious root formation under stress has equally high potential; however, our understanding of the processes involved is too incomplete at the moment to identify clear candidate genes. The present transcriptome dataset of genotypes with contrasting adventitious root formation may therefore serve as a base for data mining to shed further light on this process.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Comparison of gene expression patterns between microarray data and subsequent quantitative RT-PCR (qPCR) data.

Table S1 Main data file with accession numbers, *P*-values, normalized expression levels, fold changes, annotations and chromosomal locations of the 57 381 probes on the Affymetrix GeneChip array

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