Short heading: Malate partitioning affects mineral nutrition in rice

3 Altered expression of a malate-permeable anion channel, OsALMT4, disrupts mineral nutrition

Jie Liu¹,², Meixue Zhou², Emmanuel Delhaize¹, Peter R Ryan¹

¹CSIRO Agriculture and Food, GPO Box 1700, Canberra, ACT 2601, Australia;
²School of Land and Food and Tasmanian Institute for Agriculture, University of Tasmania, Private Bag 1375, Prospect, TAS 7250, Australia.

One sentence summary: Increased expression of a malate-permeable anion channel induced malate release from cells which altered malate and Mn compartmentation in rice leaves leading to increased sensitivity to Mn

Authors' contributions: J.L. assisted in the design and performed most experiments, analysed the data and assisted with writing the article; E.D. assisted in experimental design and assisted with writing the article; M.Z. complemented the writing; P.R.R. conceived the project, assisted in experimental design and coordinated writing of the article; P.R.R., E.D. and M.Z. supervised J.L.

Funding: J.L. received funding from the China Scholarship Council.

Correspondence to: Peter.Ryan@csiro.au
Abstract

Aluminium-activated malate transporters (ALMT) form a family of anions channels in plants but little is known about most of its members. This study examined the function of OsALMT4 from rice (Oryza sativa L.). We show that OsALMT4 is expressed in roots and shoots and the OsALMT4 protein localizes to the plasma membrane. Transgenic rice lines over-expressing (OX) OsALMT4 released malate from the roots constitutively and had two-fold higher malate concentrations in the xylem sap than nulls indicating greater concentrations of malate in the apoplast. OX lines developed brown necrotic spots on the leaves which did not appear on nulls. These symptoms were not associated with altered concentrations of any mineral element in the leaves although the OX lines had higher concentrations of Mn and B in their grain compared with nulls. While total leaf Mn concentrations were not different between the OX and null lines, Mn concentrations in the apoplast were greater in the OX plants. The OX lines also displayed increased expression of Mn transporters and were more sensitive to Mn toxicity than null plants. We showed that growth of wild-type rice was unaffected by 100 µM Mn in hydroponics but, when combined with 1 mM malate, this concentration inhibited growth. We conclude that increasing OsALMT4 expression affected malate efflux and compartmentation within the tissues which increased Mn concentrations in the apoplast of leaves and induced the toxicity symptoms. This study reveals new links between malate transport and mineral nutrition.
Introduction

The aluminum-activated malate transporter (ALMT) family of genes encode anion channels in plants with 14 members in the Arabidopsis genome and nine in rice (Oryza sativa L.) (Delhaize et al., 2007b; Barbier-Brygoo et al., 2011; Dreyer et al., 2012; De Angeli et al., 2013). The family is named after the first member to be identified, TaALMT1, which controls the major mechanism for aluminium (Al\(^{3+}\)) resistance in wheat (Triticum aestivum L.) (Sasaki et al., 2004). TaALMT1 is expressed in the root apices of Al-resistant wheat germplasm and in acid soils the high concentration of soluble Al\(^{3+}\) activates the channel protein to release malate anions into the apoplast. Malate anions then bind with the toxic Al\(^{3+}\) to protect root cells and maintain root growth. Al\(^{3+}\) resistance in Arabidopsis also relies, in part, on the ALMT-dependent efflux of malate and a recent study proposed that its function relies on the presence of an aquaporin-type transporter (Wang et al., 2017). Malate efflux is not a major mechanism of Al resistance in rice. Instead, multiple mechanisms contribute to the very high resistance of this species some of which are regulated by the ART1 transcription factor (Yamaji et al., 2009). Among these the NRAT1 transporter transports Al into the root cells for final sequestration to the vacuole (Xia et al., 2010).

Although the ALMT family is widespread in plants only a small number have been studied in detail. Some contribute to Al resistance in a similar way to TaALMT1 but most perform different functions transporting organic and inorganic anions across the tonoplast and plasma membrane (Barbier-Brygoo et al., 2011; Sharma et al., 2016). For example, three members in Arabidopsis (AtALMT6, AtALMT9, AtALMT12) and one in barley (Hordeum vulgare; HvALMT1) contribute to guard cell function by transporting organic and inorganic anions across the plasma membrane or tonoplast. This is consistent with the involvement of anion transport in signal transduction and osmotic adjustment in modulating stomatal aperture (Kovermann et al., 2007; Gruber et al., 2010; Meyer et al., 2010; Sasaki et al., 2010; Dreyer et al., 2012; Xu et al., 2015). Electrophysiological studies in Xenopus oocytes revealed similarities between the currents generated by AtALMT12 and the previously characterised rapid or R-type/QUAC anion currents (Hedrich, 2012). A more recent study proposed that ALMT proteins function as GABA receptors in plants to transduce chemical signals into electrical signals by modulating anion fluxes across membranes (Ramesh et al., 2015).

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We examined the function of OsALMT4, the first member of the family to be investigated in rice and likely the only functioning member in clade 5. During this study we noticed that brown and necrotic spots developed on the leaves of independent lines over-expressing OsALMT4. The causes of this phenotype have wider implications for the influence of organic anions on the acquisition and distribution of mineral nutrients in plants.

Results

ALMT family in rice

A phylogenetic tree generated with the nine ALMT proteins from rice, 14 proteins in Arabidopsis and predicted proteins from other species formed five clades which is consistent with Dreyer et al. (2012) (Figure S1). In hydroponically grown plants OsALMT1, OsALMT2, OsALMT3 and OsALMT4 were expressed in leaves while OsALMT1, OsALMT2, OsALMT4, OsALMT7 and OsALMT9 were expressed in roots. No expression of OsALMT5, OsALMT6 or OsALMT8 was detected in roots or shoots under these growth conditions (data not shown).

Clade 5 was unusual by including two closely-related members from rice, OsALMT4 and OsALMT5, but no representatives from Arabidopsis. We sequenced the genomic DNA of these two rice genes and found that the OsALMT4 sequence is identical to the GenBank entry Os01g0221600 with five predicted introns and six exons. The putative 524-amino acid long protein has a hydrophobic domain in the N-terminal half with five to seven transmembrane regions (TMRs) depending on the algorithm applied (Table S1). Examination of OsALMT5 genomic DNA detected a premature stop codon that would truncate the C-terminal tail. A similar mutation in the TaALMT1 protein from wheat reduced transport activity (Furuichi et al., 2010; Ligaba et al., 2013). This study examined the function of OsALMT4 that is likely to be the only functional member in clade 5.

Subcellular localisation of OsALMT4

Subcellular localisation of OsALMT4 was examined by fusing the cDNA with the green fluorescent protein (GFP) gene and transiently expressing the constructs in leek (Allium...
porrum L.) and tobacco (Nicotiana benthamiana L.) cells. Fluorescence from N-terminal (GFP::OsALMT4) and C-terminal (OsALMT4::GFP) fusions was consistently observed at the periphery of tobacco (Figure 1a) and leek cells (Figure 1g). No fluorescent signals were detected in any internal organelles which suggests that OsALMT4 was confined to the plasma membrane. This was tested by co-expressing the GFP::OsALMT4 fusion protein in tobacco with a control protein, pm-rk, known to localise to the plasma membrane (Nelson, 2007). The pm-rk protein was tagged with the mCherry fluorophore which allows signals from both fluorophores to be detected in the same cell. Fluorescence from the pm-rk control was detected at the periphery of tobacco cells (Figure 1b) and overlapped with signals from GFP::OsALMT4 (Figure 1c). In a second test, T. benthamiana cells expressing GFP::OsALMT4 were plasmolysed by applying drops of 50% sucrose to the surface of the leaf. After several minutes, water movement out of the cytoplasm caused the plasma membrane to retract from the cell wall (Figure 1f). Fluorescence was detected in the Hechtian strands that are the residual connections between the plasma membrane and cell wall (Lang-Pauluzzi, 2000). These results are consistent with OsALMT4 being located on the plasma membrane. In the controls, which expressed GFP only, fluorescence was detected in the cytosol and nucleus of tobacco (Figure 1e) and leek cells (Figure 1i) which is typical of soluble GFP protein.

Characterising transgenic rice lines with altered OsALMT4 expression

To investigate the function of the OsALMT4 proteins we generated transgenic plants with altered OsALMT4 expression. Rice (cv. Nipponbare) was stably transformed with OsALMT4 cDNA using a constitutive promoter. More than 30 primary (T₀) transgenic lines were generated (Figure S2a) and three lines with high expression and single inserts (based on segregation ratios in the T₁ generation) were used to generate homozygous T₂ lines (see Materials and Methods). These homozygous lines, OX2, OX5 and OX8 had 15 to 40-fold higher OsALMT4 expression levels than their respective non-transgenic sister lines OX2_null, OX5_null, OX8_null (Figure S3a). Thirty five T₀ plants with RNAi constructs to reduce OsALMT4 expression were generated (Figure S2b). Three plants were chosen to generate homozygous T₂ lines (R24, R58 and R78) and the corresponding null sister lines (R24_null, R58_null and R78_null). OsALMT4 expression in the homozygous T₂ lines was reduced to 10 to 20% of null plants (Figure S3b).
The OX lines were fertile, showed similar root/shoot ratios, leaf chlorophyll content and tiller numbers as the nulls but produced slightly lighter grain (Table S2). The OX lines tended to accumulate less biomass than their null lines when grown in hydroponics or soil (Table S3). The RNAi plants were fertile and grew similarly to the nulls but produced ~6% heavier grain (Table S2).

**OsALMT4 facilitates malate efflux from cells**

ALMT proteins characterised in other species function as anion channels that facilitate organic or inorganic anion movement across membranes. We tested the hypothesis that OsALMT4 transports organic anions by measuring organic anion release from roots using two methods. One method analysed the nutrient solution from flasks in which several seedlings were grown and the other measured efflux from excised root apices. After 16 h malate was detected in the flasks containing OX2, OX5 and OX8 seedlings but not in flasks with null or wild-type plants (Figure 2a). Citrate efflux was also detected and the fluxes from OX lines tended to be smaller than for nulls and wild-type plants. Malate release was also measured from excised root apices of OX5 and the result of 0.5 nmol apex\(^{-1}\) h\(^{-1}\) was similar to rates from aluminium-resistant wheat roots using a similar technique (Ryan et al., 1995). To examine whether other organic anions were being released the exudates from OX5 and OX5_null plants were also analysed with HPLC. This method detected fumarate efflux in addition to malate but the rates were much slower (0.07 nmol seedling\(^{-1}\) h\(^{-1}\)) (Table S4).

We tested whether the malate efflux from roots of OX lines protected plants from Al stress. Al resistance of the OX5 and OX2 lines was estimated by relative root length (RRL) after growing plants for seven days in nutrient solutions containing 0, 200 and 400 μM Al concentrations. RRL in the two transgenic lines (OX5 and OX2) was approximately 32% in 200 μM Al and 25% in 400 μM Al which were two-fold greater than the nulls and wild-type plants (Figure 2b). Therefore both OX lines had enhanced Al resistance compared to the null controls.

Metabolomic analyses of organic anions in the root and shoot tissues of the transgenic lines revealed very few differences between OX5 and OX5_null lines (Table S5). OX5 had a 43% lower malate concentration in the roots compared to OX5_null (7.4 ± 0.4 and 13.0 ± 2.0 nmol mg\(^{-1}\) tissue respectively) and a lower citrate concentration in roots (2.9...
± 0.2 and 5.0 ± 0.6 nmol mg⁻¹ tissue, respectively.). No concentration differences were detected between the OX and null lines for any organic anion in the shoots.

**Leaf symptoms associated with increased OsALMT4 expression**

All three OX lines developed brown spots on their leaves after four to six weeks (Figure 3) which were not observed on the leaves of nulls, wild-type or RNAi plants. Leaf tip necrosis was observed on some plants but this was not a consistent phenotype. The brown spots tended to appear on older leaves first but developed on most leaves with time. A general correlation was apparent between the severity of the phenotypes and the level of OsALMT4 expression in the transgenic lines since the symptoms appeared earliest, and were most severe on OX5, and appeared last, and were least severe, on OX8. This leaf phenotypes occurred in all growth conditions including hydroponics, flooded soil and unflooded soil but the symptoms developed more rapidly under high light intensity and high temperatures (Figure S4). This was demonstrated by scoring the leaf symptoms regularly on two OX lines and nulls grown either in a glasshouse, which had the highest daily light intensity (up to 1500 µmol m⁻² s⁻¹) and highest temperatures (32/26°C for day/night), or in growth cabinets with different combinations of maximum light intensity (300 or 600 µmol m⁻² s⁻¹) and temperatures (22/20°C or 29/24°C for day/night). Plants grown in the glasshouse developed the leaf symptoms earliest. Plants grown in chambers with the high light intensity and temperatures developed the symptoms next while plants grown with the lowest light intensity developed the spots last. No leaf symptoms developed on the null lines in any treatment (Figure S4).

**Elemental analysis of grain and leaves**

Elemental concentrations were measured in grain and leaves collected from transgenic and null plants grown in flooded soil. Figure 4 summarises the raw data (Table S6) by showing the ratio of concentrations in each transgenic line relative to null plants grown along side. Mn and B were the only elements that differed between the transgenic and null lines (Figure 4a). OX5 had two-fold higher B and Mn concentrations compared to nulls. OX2 also showed a higher B concentration than null plants but the difference was not quite significant at P<0.05. Concentrations of all mineral elements in grain of RNAi line R24 were similar to its null sister line (Figure 4b).
Brown and necrotic spots on leaves are typical symptoms of Mn toxicity while chlorotic and necrotic leaf margins can be associated with B stress (Marschner, 1995). The finding that high light intensity exacerbates the symptoms is also consistent with Mn stress (Fernando and Lynch, 2015; Nable et al., 1988; Gonzalez et al., 1998; Fernando et al., 2016). Elemental concentrations in the leaves of the OX and RNAi plants were measured in young and old plants grown in various conditions and showing varying severity of leaf symptoms. No consistent differences were detected in the concentration of any element between the transgenic and null lines over these different conditions (data not shown). In some experiments the Mn concentrations in OX lines were different from their nulls but this was not always apparent (Table S7). Furthermore, no correlation was detected between leaf concentrations of Mn or B and the appearance of the brown spot phenotype in the OX lines. Indeed, symptoms developed on leaves of OX plants that had very different Mn concentrations. Even when plants were sampled through time no pattern emerged despite the appearance of brown spots on the OX lines (Table S7). Null plants and RNAi plants never developed the brown spots despite having similar or higher Mn concentrations than the OX lines.

Increased OsALMT4 expression alters the compartmentation of Mn in leaves

Since the symptoms were not correlated with total Mn or B concentrations we measured the compartmentation of elements between the symplast and apoplast in plants grown in flooded soil for eight weeks. Mean Mn concentrations in the leaf apoplast of the two OX lines were five-fold greater than the null lines (Figure 5). These changes were significant for OX2 (P=0.007) and almost significant for OX5 (P=0.055). Mn concentrations in the symplast also tended to be higher in the OX lines than nulls but the differences were not significant. Importantly, the ratio of Mn concentrations in the apoplast and symplast was three-fold greater in OX5 and OX2 compared to their nulls. While the trend was clear for both lines the difference was statistically significant for OX2 (P<0.05) but not for OX5 (P<0.065) (Figure 5). To determine whether this pattern occurred for other elements we also measured the partitioning of B and Ca. The results showed that the distribution of these elements within the leaves were not affected in the same way and the ratio of apoplastic to symplastic concentrations for these elements was similar in the OX and null lines. These results indicate that the distribution of Mn within leaf tissues was altered by increasing OsALMT4 expression such that OX lines have a greater proportion of leaf Mn residing in the apoplast than the null lines.
Overexpression of *OsALMT4* increases malate concentration in the xylem sap

We investigated whether the compartmentation of malate within tissue was altered by measuring malate concentrations in the xylem sap collected from the cut stems of plants grown in flooded soil. Malate concentrations in sap of wild-type plants, RNAi lines and all null plants were similar at 1.0 mM which is consistent with previous reports (Yokosho et al., 2009) (Figure 6). By contrast, malate concentrations in the sap of the OX5 and OX2 lines were significantly greater than all control lines at approximately 2.0 mM. This result demonstrates that increasing *OsALMT4* expression increased malate concentrations in the leaf xylem sap, an apoplastic compartment.

Expression of Mn transporters is altered in the OX lines

To further examine how Mn nutrition was perturbed in the OX lines we measured the expression of genes encoding Mn transporters. *OsNramp5* and *OsMTP9* were mainly expressed in the roots, *OsMTP8.1* was mainly expressed in the shoots and *OsYSL2* and *OsNramp3* were expressed in both tissues (Figure 7). Expression of *OsYSL2* in roots of OX5 was 60-fold greater than in null plants and six-fold greater in the shoots. Similarly, the expression of *OsNramp5* was 30-fold greater in the roots of OX5 compared to OX5_null. Expression of *OsNramp3* was unchanged. *OsMTP8.1* and *OsMTP9* expression tended to be higher in the shoots of OX5 plants than OX5_nulls but the differences were not significant.

Increasing *OsALMT4* expression decreased tolerance to Mn toxicity

We investigated whether changing *OsALMT4* expression affected the sensitivity of rice to Mn. In the first set of experiments, OX, RNAi and null lines were grown in hydroponics with 1 µM Mn (control), 250 µM or 500 µM MnCl₂. Plants were harvested after 28 d of treatment which was before the OX lines in control treatments had developed strong leaf symptoms. This avoided the confounding factor of leaf symptoms having secondary effects on growth. Root and shoot growth of all lines was inhibited by high Mn concentrations (Figure S5) but the decreases in OX5 and OX2 were significantly larger than in their nulls, OX5_null and OX2_null. Figure 8 summarises these findings by showing the relative biomass for each line (biomass in high Mn relative to biomass in control). The relative root and shoot biomass for OX5 and OX2 at 500 µM Mn was significantly smaller than the null lines. Similarly, the relative shoot biomass for OX2 in
269 250 µM Mn was significantly smaller than OX2_null. OX5 and OX2 plants in control
treatment showed no leaf symptom during this experiment but severe symptoms did
develop on these lines at 500 µM MnCl₂ (Figure S6). The leaf symptoms and greater
sensitivity to Mn could not be explained by total accumulation of Mn, or any other
mineral element, as leaves of the OX and null lines had similar Mn concentrations of
~7000 mg kg⁻¹ (Table S8). These results indicate that overexpression of OsALMT4
increased the sensitivity of OX plants to Mn but this was not caused by greater Mn
accumulation in the leaves.

Relative biomass of the RNAi lines were not significantly different between R24 and
R24_null or between R58 and R58_null lines (Figure 8). Mild leaf symptoms did appear
on leaves but only at the highest Mn treatment as described for the null lines above (data
not shown).

**External malate increases sensitivity to Mn toxicity**

It is possible that malate efflux from roots contributes to the leaf symptoms by facilitating
excessive nutrient uptake either from the soil or hydroponics. We tested this by adding
external malate to the standard hydroponics (1 µM Mn) and examined whether brown
spots appeared on null plants or whether the severity of this phenotype increased on OX
plants. Plants were grown in hydroponics with and without 2 mM malate for 35 d.
Solutions were replaced regularly. External malate did not affect final biomass of any line
(data not shown) and no brown spots were detected on the null lines whether malate was
present or absent from the solution. Brown spots did appear on OX5 and OX2 after
approximately 30 d as expected but neither the timing of their appearance nor the severity
of the final symptoms was affected by the presence or absence of malate in the solution
(data not shown).

We then tested whether adding malate altered the sensitivity of rice to higher Mn
concentrations. Wild-type plants were grown in a standard control hydroponics (1 µM
Mn) or in 10 or 100 µM Mn with and without 1 mM malate for 28 d. The 10 or 100 µM
Mn treatments alone had no effect on shoot biomass which is consistent with the
relatively small effect of 250 µM Mn on growth of null plants observed previously
(Figure 8; Figure S5). However the combination of higher Mn and 1 mM malate
significantly inhibited biomass (Figure 9, Figure S7). In the absence of malate the leaf
symptoms on the wild-type plants were mild and did not appear regardless of the Mn
concentration. By contrast, brown spots did develop on leaves when malate was added to the 10 or 100 µM Mn treatments. The appearance of these leaf symptoms was not correlated with Mn accumulation because the total concentration in leaves were similar at ~15,000 mg kg\(^{-1}\) in the presence and absence of malate. The combination of Mn and malate in the hydroponics decreased Fe concentrations in leaves by 40% and increased Cu and Zn concentrations but not to toxic levels (Reuter and Robinson, 1986) (Figure S8).

These results demonstrate that in standard growth conditions, with 1 µM Mn, external malate alone did not induce the brown spots that consistently developed on transgenic plants with increased \(OsALMT4\) expression. However when malate was added with Mn concentrations that are higher, but normally non-toxic when applied alone, the combination was inhibitory to growth.

**OX lines have increased expression of B transporters and show greater tolerance to B toxicity**

B concentrations were higher in grain of the OX5 line compared to nulls. We examined how expression of \(OsALMT4\) affected B nutrition by measuring the expression of B transporters and comparing the tolerance of the OX and null lines to B toxicity.

Expression of genes encoding B transporters (\(OsBOR1\) and \(OsNIP3.1\)) was measured in OX5 and null plants grown in hydroponics with 5.5 µM \(H_3BO_3\) (control) or with 5 mM \(H_3BO_3\) (Figure 10). \(OsBOR1\) was mainly expressed in the roots while \(OsNIP3.1\) was expressed in roots and shoots which is consistent with previous reports (Nakagawa et al., 2007; Hanaoka et al., 2014). In the control treatment \(OsBOR1\) expression in roots was five-fold greater in OX5 than nulls and \(OsNIP3.1\) expression was two-fold greater in OX5 than OX5_null. The high B treatment decreased \(OsBOR1\) expression to 5% in OX5_null and to ~1% in OX5 plants. High B also reduced \(OsNIP3.1\) expression in shoots and roots of null plants and OX5 plants. \(OsALMT4\) expression remained more than 50-fold greater in the roots and shoots of OX5 compared to nulls regardless of B treatment. Interestingly, the high B treatment caused a large decrease of \(OsALMT4\) expression in roots of null plants but no changes occurred in the shoots. These changes were too small to be seen in Figure 10 due to the much higher expression in OX5 but in control treatment \(OsALMT4\) expression in null plants (0.013±0.006) was reduced to
0.00014±0.00006 in high B. These results show that increased OsALMT4 expression was associated with large increases in the expression of B transporters in roots and shoots.

Growth of the OX lines, RNAi lines and nulls were then compared in hydroponics with 5.5 µM (control) or 5 mM H$_3$BO$_3$. After 28 d the high B treatment reduced shoot growth of all lines by 20 to 40% and reduced root growth by 50 to 70% (Figure S9). Relative root biomass for OX5 and OX2 remained significantly greater than OX5_null and OX2_null (Figure 11). Similar trends occurred with relative shoot biomass however the difference was significant for OX5 only in this case (Figure 11). By contrast, relative root and shoot biomass for the RNAi lines, R24 and R58, were not significantly different from their null lines (Figure 11). These results indicate that increasing OsALMT4 expression increased the tolerance of rice to B toxicity.

Discussion

Malate efflux from cells disrupts Mn nutrition in rice

By investigating lesions that developed on the leaves of transgenic rice plants over-expressing OsALMT4 this study has revealed novel links between organic anions and mineral nutrition. Three independent transgenic lines (OX5, OX2 and OX8) over-expressing OsALMT4 developed brown spots and lesions on their leaves that were not observed in null, wild-type or RNAi plants (Figure 3). These general symptoms have been associated with many disorders including oxidative stress, pathogens, and nutrient deficiencies and nutrient toxicities - including Mn and B (Marschner, 1995; Fuhrs et al., 2010). Pathogen infection was discounted here since symptoms occurred in all growth environments (hydroponics, soil, glasshouses and cabinets) and only on the OX lines. Furthermore similar symptoms could be induced in the null and wild-type plants with certain growth conditions. We propose that the leaf symptoms are most likely related to mineral imbalances and especially to Mn toxicity.

We first established that OsALMT4 is expressed in the roots and shoots and likely encodes an anion channel that localises to the plasma membrane (Figure 1) and facilitates malate efflux from cells (Figure 2). The OX lines had lower concentrations of malate and citrate in the roots than null plants but there were no differences in the shoots.
The results that support our conclusion that the leaf symptoms on the OX lines are linked to altered Mn nutrition include the following: (1) the symptoms appeared more rapidly and were more severe at higher Mn concentrations (Figure S6), (2) the expression of several Mn transporters was altered in OX lines compared to the nulls (Figure 7), (3) OX lines were significantly more sensitive to higher Mn concentrations than nulls (Figure 8), (4) partitioning of Mn between the symplast and apoplast was altered in the OX plants such that a greater proportion of total leaf Mn was retained in the apoplast compared to nulls (Figure 5), and (5) the symptoms appeared more rapidly and were more severe when plants were grown in higher light intensity (Fernando and Lynch, 2015; Nable et al., 1988; Gonzalez et al., 1998; Fernando et al., 2016) (Figure S4).

Plants absorb Mn from the soil in its reduced form, Mn(II), so excess uptake commonly occurs in waterlogged conditions where the low redox potential reduces oxidised Mn(III) ions to Mn(II). Many different transport proteins are involved in taking up Mn from the soil and transferring it to the shoots and grain (see later). Rice is relatively tolerant of Mn compared to other cereals and grows well on the highly-reduced conditions in paddy fields. Indeed, rice can accumulate 1% Mn w/w with minimal signs of stress (Vlamis and Williams, 1964). Mn toxicity can likely be triggered at different cellular sites but the apoplast of leaves appears to be important (Fuehrs et al., 2009; Fuhrs et al., 2010). Mn toxicity induces brown spots and necrotic lesions on the leaves which is caused, in part, by the oxidisation of Mn and phenolic compounds in the apoplast which damage proteins and lipids (Fecht-Christoffers et al., 2003; Fecht-Christoffers et al., 2006). The greater tolerance to Mn stress exhibited by some species has been attributed to the binding of Mn in the apoplast or sequestration of Mn into subcellular compartments such as the vacuole, Golgi and endoplasmic reticulum and the induction of protective proteins in the chloroplast (Lidon and Teixeira, 2000; Delhaize et al., 2007a; Fuhrs et al., 2010; Fernando and Lynch, 2015).

Symptoms of Mn toxicity are not correlated with total Mn concentrations

No consistent differences in leaf Mn concentrations were detected between the OX and null lines and total Mn concentrations were not correlated with the appearance of the brown spot phenotype (Table S7). Other reports have reported similar lack of correlations between tissue Mn concentration and stress symptoms (Fuhrs et al., 2010; Sasaki et al., 2014; Fuhrs et al., 2012) and several explanations could account for this.
For instance, mineral concentrations in leaves can vary due to plant age, nutrition supply and soil chemistry. Translocation of Mn in the phloem is lower than many other minerals but it does occur (Rengel, 2001) and some of the transporters have been identified (Ishimaru et al., 2010). It might be possible that tissue concentrations of Mn change even after the symptoms develop which reduces the association. Loading and unloading of Mn from the xylem and phloem relies on specific proteins that transport the ions in source and sink tissues. Some of these transport steps require Mn to be complexed with nicotianamine and perhaps other compounds and so the availability and concentration of these ligands will affect Mn distribution (Koike et al., 2004; Ishimaru et al., 2010).

However, we propose that the main reason that the leaf symptoms are poorly correlated with Mn concentrations is that the compartmentation of Mn within the leaves is altered as discussed below.

Malate release from all cells of the OX lines is greater than the nulls which would significantly increase the concentration of malate in the apoplast of all tissues. This is supported by the release of malate from the roots and the higher concentration of malate in the xylem sap (Figure 6), an apoplastic compartment. Increased malate concentrations in the xylem and leaf apoplast would affect the chemistry of these spaces. The formation of Mn:malate complexes in the xylem and apoplastic compartments could compete with nicotianamine and other ligand compounds that normally chelate Mn prior to transport across membranes. These changes would inhibit loading and unloading processes. This inhibition is possible for two reasons: the Mn:malate complex has a relatively high stability constant of \(~10^{2.24}\) (Furia, 1972) and the concentration of malate in the apoplast is much higher than nicotianamine. For instance, the concentration of nicotianamine (and similar ligands such as 2-deoxymuginenic acid) in the xylem is 10 to 50 µM (Kakei et al., 2009) whereas the measured malate concentration in the OX lines is 2 mM. If the OsYSL2 and OsYSL6 transporters are unable to transport Mn:complexes to the leaf symplast then the accumulation of Mn in the apoplast could trigger stress symptoms, even when the total concentrations of Mn in the leaves are not normally considered harmful to rice. This hypothesis is consistent with the role of the leaf apoplast in Mn toxicity convincingly proposed by others (Fuhrs et al., 2010) and with phenotypes described in ysl6 mutants in rice (see later). The model presented in Figure S10 attempts to summarise the proposed changes in the OX lines.
Support for the model that altered compartmentation of Mn in the leaf tissues can cause toxicity without changing total Mn concentrations comes from rice lines with knock-out mutations in the *OsYSL6* (Sasaki et al., 2011). These *osysl6* mutants also accumulated higher concentrations of Mn in the apoplast and experienced Mn toxicity at much lower tissue concentrations than wild-type plants. This mutation resulted in similar changes to Mn distribution in the leaves and generated a similar phenotypes to those described here for the OX lines.

**Attempts to mimick the OX phenotype in wild-type plants with external malate**

We tested whether adding 2 mM malate to a standard hydroponics (~1 µM Mn) could induce the same symptoms in non-transgenic plants but found that it had no effect on growth and did not induce leaf symptoms on null plants. Furthermore, external malate did not change the timing or severity of leaf symptoms on the OX plants. We conclude that external malate alone, at least at this concentration, cannot mimic the phenotype on the OX lines when external Mn concentrations are low. When malate was added with higher Mn concentrations the combination was more toxic than Mn alone. We found that the combination of 1 mM malate with 10 or 100 µM Mn was very inhibitory to growth of wild-type plants and induced similar symptoms in the leaves. These responses were unrelated to total Mn accumulation because the concentration in leaves was similar regardless of the malate treatment. Once again, we propose that malate induces stress by disrupting the distribution of Mn within the tissues. This model is consistent with the symptoms displayed by OX plants, their greater sensitivity to Mn ([Figure 8](#)).

Another example of malate efflux from roots interacting with metal ions was reported recently. *AtALMT1* is a member of the *ALMT* family in Arabidopsis which encodes a malate-permeable anion channel. *AtALMT1* is induced under conditions of low phosphorus and Mora-Macías et al. (2017) found that *atalmt1* mutants did not show the usual inhibition of primary root growth displayed by wild-type plants when they grew into media with low-P levels (Sánchez-Calderón et al., 2006). The authors proposed that, in wild-type plants, malate release via AtALMT1 interacts with iron in the media and causes Fe$^{3+}$ to aggregate in the apoplast of the root apices. This aggregation triggers oxidative stress and callose production in that tissue which inhibits root growth. Under the same conditions, roots of *atalmt1* mutants continue to grow normally because the absence of malate efflux prevents Fe$^{3+}$ from accumulating in the apoplast.
Some findings in the present study appear to contradict Chen et al. (2015) who reported that the addition of external malate increased Mn tolerance in yeast (Saccharomyces cerevisiae) and Stylosanthes guianensis, a forage legume species. That study concluded that part of the genotypic variation for Mn tolerance in Stylosanthes guianensis was attributable to greater malate efflux from roots. However important differences exist between the two experimental systems which could explain our contrasting conclusions. Chen et al. (2015) conducted much shorter growth assays on solid agar media and used very different ratios of malate:Mn. They found that 10 µM malate ameliorated the inhibition of Stylosanthes guianensis growth in 20 mM Mn (a 2000-fold concentration difference) and as little as 0.1 µM malate improved Saccharomyces cerevisiae growth on media containing 15 mM Mn (a 150,000-fold concentration difference). Interestingly, the authors mentioned that the ameliorative effects of malate became smaller or even disappeared when malate concentrations were raised (Chen et al., 2015). Therefore it is possible that even higher concentrations of malate would have increased the toxicity of Mn as we described here.

**OX lines showed increased expression of Mn transporters**

The expression of two Mn transporters was significantly enhanced in OX5 compared to OX5_null. One of these was OsNramp5 from the Natural Resistance-Associated Macrophage Protein family. OsNRAMP5 is a major transporter of Mn and Cd from the external solution to root cells. It is constitutively expressed in the roots and localizes to the distal side of the plasma membrane in both exodermal and endodermal cells and in the xylem parenchyma. It is important for Mn uptake from soil and loading in the xylem for distribution to the shoots (Sasaki et al., 2012; Yang et al., 2014). Another gene showing increased expression in the OX lines is OsYSL2, which encodes a transporter of Fe(II)-nicotianamine and Mn(II)-nicotianamine complexes (Koike et al., 2004) that facilitates Mn translocation in the phloem and loading to the grain (Ishimaru et al., 2010). The induction of these genes in OX plants could be a response to Mn stress and function to redistribute Mn around the plant via the xylem and phloem. Enhanced OsYSL2 expression provides an explanation for the tendency of OX grain to have higher Mn (and Fe) concentrations. Expression of OsMTP8.1, a member of the cation diffusion facilitator family, tended to be greater in OX5 than null plants as well. OsMTP8.1 localizes to the tonoplast of leaf cells and appears to be important for loading Mn into the vacuole since it
is induced by Mn toxicity (Chen et al., 2013). Other transporters encoded by OsYSL6 and OsMTP9 have previously been shown not to change expression with Mn supply (Sasaki et al., 2011; Ueno et al., 2015). OsYSL6 encodes a Mn-nicotianamine transporter that is critical for Mn movement from the apoplast to the symplast in leaves although its tissue localisation remains unclear (Sasaki et al., 2011). OsMTP9 is a transporter in roots that localises to the proximal side of the plasma membrane in the exodermis and endodermis (opposite side to OsNRAMP5). The current model proposes that OsNRAMP5 facilitates Mn uptake by roots at the exodermis and endodermis while OsMTP9 facilitates Mn efflux from those cell layers and that both transporters are required for efficient Mn movement to the stele (Sasaki et al., 2016).

B nutrition is disrupted in the OX lines

The conclusion that B nutrition was also perturbed in the OX lines is supported by the higher B concentrations in the grain of OX5 compared to the null (Figure 4), altered expression of genes encoding B transporters (Figure 10) and the enhanced tolerance of OX plants to B toxicity (Figure 11). B uptake by plants occurs as the undissociated boric acid via members of the aquaporin-type family of transporters (Sutton et al., 2007; Takano et al., 2008; Miwa and Fujiwara, 2010). OsNIP3;1 is a candidate for B uptake in rice because it is mainly expressed in the exodermis and vascular tissues of roots and leaves and transcript levels increase during B starvation (Hanaoka et al., 2014). Furthermore reducing OsNIP3;1 expression impairs growth in B-limited conditions. In the current study we found that OsNIP3;1 expression was significantly greater in the OX line than null plants. Another important B transporter in rice is OsBOR1, an anion channel that facilitates the efflux of [B(OH)₄]⁻ or borate anions from cells. OsBOR1 is required for B uptake and accumulation, especially when B is limited, because reducing OsBOR1 expression reduces B uptake and B loading into the xylem (Nakagawa et al., 2007). In B-deficient Arabidopsis plants AtBOR1 transporters are expressed in the pericycle and endodermal cells of roots, where they also transport B to the xylem (Yoshinari et al., 2016). Interestingly, members of this family of transporters also contribute to B tolerance in plants as illustrated by HvBOT1 in barley (Hordeum vulgare L.). HvBOT1 is more highly expressed in the roots of B-tolerant genotypes than sensitive in genotypes and when B concentrations are high HvBOT1 facilitates borate efflux from root cells which reduces B accumulation in the shoots (Hayes and Reid, 2004; Reid et al., 2006).
In the present study the OX lines showed 30-fold greater expression of \textit{OsBOR1} than the null plants. While it remains unclear why over-expression of \textit{OsALMT4} alters the expression of \textit{OsNIP3;1} and \textit{OsBOR1} it does indicate B nutrition is disrupted. More intriguing was the finding that the OX lines are more tolerant to B toxicity. Since \textit{OsALMT4} is an anion channel one explanation is that \textit{OsALMT4} is permeable to borate anions and facilitates borate efflux from cells in a similar way as \textit{OsBOR1}. This hypothesis can be tested with electrophysiological and chemical analyses using the \textit{Xenopus} oocyte expression system.

**Mn accumulation in leaves as an indicator of organic anion release from roots**

The release of organic and inorganic compounds from roots, especially carboxylates and protons, is an effective strategy by which some species increase phosphorus (P) acquisition from soil (Ryan et al., 2001; Neumann and Martinoia, 2002; Vance et al., 2003; Lambers et al., 2015b; Ramirez-Flores et al., 2017). Plants that use these strategies also tend to have higher tissue concentrations of Mn because the carboxylates released, especially citrate and malate, help mobilise Mn in the soil for uptake by roots (Lambers et al., 2013). Indeed, the lower Mn concentration in leaves of maize and other plants infected with mycorrhizal fungi was attributed to their reduced exudation of carboxylates and other compounds that mobilise P (Kothari et al., 1991; Posta et al., 1994). Recognising this linkage, Lambers et al. (2015a) proposed that measurements of leaf Mn concentrations could form the basis of a convenient screen for identifying species that use these rhizosphere-based strategies for efficiently acquiring P. The current study supports that concept in principle since the OX lines with malate efflux from roots also showed altered Mn nutrition. However, in the present study, the changes were not correlated with higher Mn concentrations in the leaves but instead with signs of Mn toxicity. The proposal by Lambers et al. (2015a) should accommodate this scenario which might occur in species that release carboxylates in soils with higher Mn concentrations.

**Conclusions**

Increasing expression of \textit{OsALMT4} increased malate efflux from cells which increased malate concentrations in apoplast including the xylem. We conclude that these changes will likely disrupt transport processes in the vasculature and affect the distribution and...
compartmentation of nutrients, especially Mn, within the tissues. In addition, we speculate that OsALMT4, and perhaps other members of the ALMT family, may be permeable to borate anions. Future experiments should investigate this with electrophysiological approaches. This study reveals new links between organic anions and mineral nutrition in rice.

Materials and Methods

Plant Material and Growth Conditions

Rice (Oryza sativa L.; cv Nipponbare) seed were obtained from the collection at CSIRO. Grain was surface sterilized by 20% bleach for 15 min, rinsed thoroughly in water and pre-germinated under dark on ½ strength Murashige and Skoog (MS) media for two days in the 28 °C growth room.

Hydroponics: Germinated seedlings were then grown under continuous light for five days and moved into hydroponic tanks or soil. Hydroponic experiments were performed in 10 L tanks with ½ strength Kimura B nutrient solution (pH 5.6). The standard nutrient solution contained a final concentration of 250 µM KNO₃, 250 µM CaCl₂, 250 µM NH₄NO₃, 75 µM MgSO₄, 10 µM KH₂PO₄, 0.4 µM EDTA:Fe, 5.5 µM H₃BO₃, 1 µM MnCl₂, 0.175 µM ZnCl₂ and 0.1 µM CuCl₂. The pH was adjusted to 5.6-5.7 and the solution was changed every three days.

Aluminum resistance: Six day old seedlings were transferred to a series of hydroponic tanks (pH 5.6) and grown for a further two days after which the length of the longest root on each plant was measured. The solution were replaced with a new solution with reduced KH₂PO₄ concentration (1 µM) and pH (4.5) and with addition of 0, 200 or 400 µM AlCl₃. The solutions were aerated to mix the solution and replaced every three days. After seven days, the longest root length was measured again and the relative root length (RRL) calculated as root growth in Al³⁺ treatment compared to the root growth in...
control solution. Standard errors of the ratio or relative root growth (SERRG) were calculated as follows: $SERRG = \frac{RRG \times [(SEx/x)^2 + (SEy/y)^2]^{1/2}}{}$, where $x$ and $y$ represent the mean of root length under control and $\text{Al}^{3+}$ treatments, and $SEx$ and $SEy$ represent the standard errors of those means. To statistically compare relative data from one line with another we adapted an approach described previously (Zhou et al., 2013).

Soil experiments: Growth in flooded and unflooded soil used pots (8.5 cm diameter and 20 cm high) and “rice soil” prepared at CSIRO. Plants were maintained in a glasshouses or growth cabinets as detailed below. Growth experiments in various light and temperature regimes was conducted in soil over eight weeks in one of four conditions designated A, B, C, D: A – a glasshouse with light intensity up to 1500 µmol m$^{-2}$ s$^{-1}$ and maximum temperature of 30°C/24 °C (natural summer day/night cycle); B – a growth cabinet with 600 µmol m$^{-2}$ s$^{-1}$ light and 29/24°C temperature, C – a growth cabinet with 600 µmol m$^{-2}$ s$^{-1}$ light and 22/20°C temperature, and D – a growth cabinet with 300 µmol m$^{-2}$ s$^{-1}$ light and 29/24°C temperature. All cabinets had a diurnal cycle of 16 h/8 h (day/night).

Growth during Mn and B stress: Homozygous transgenic lines with altered OsALMT4 expression levels and associated null lines were germinated as described. Seven day old seedlings were transferred from MS media to nutrient solution (pH 5.6) and acclimated for another seven days. The solutions were then changed to control solution or control solution with the addition of 250/500 µM MnCl$_2$ or 5 mM H$_3$BO$_3$. All the solutions were renewed every three to four days and the plants were harvested after four weeks. Root and shoot tissues were harvested separately and dried in a 65 °C oven for 72 hours. Root and shoot dry weight were then measured and the total dry weight was calculated.

Relative dry weight was calculated as the biomass for each line in each treatment relative to that in the control treatment. Relative biomass and cumulative SE values were calculated (see below).

Plant growth with external malate: For growth experiments in which malate was added to the nutrient solution with standard Mn concentrations, OX5, OX2, and null lines were grown in standard nutrient solution with or without 2 mM sodium malate (pH 5.6). Root and shoot growth as well as leaf phenotype were assessed after five weeks. For growth experiments with added malate and high external Mn, wild-type rice plants were
germinated and grown in standard hydroponic solution for one week before being transferred to either standard control solution (1 µM Mn) or other treatments with 10 or 100 µM Mn with or without 1 mM malate. The plants were grown in these solutions for four weeks before the roots and shoots were harvested and the leaf phenotypes examined. Solutions were changed regularly for both series of experiments.

**Online sequence resources**

The ALMT gene sequences were obtained from The National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml) (Table S1). Online software placed OsALMT4 into a gene family is Pfam 27.0 (http://pfam.xfam.org/). The secondary structure of the protein was analysed by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui//sosui_submit.html), Kyte-Doolittle (http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm) and Protter (http://wlab.ethz.ch/protter/start.

**Expression analysis of rice ALMTs and cloning the OsALMT4 coding region**

Wild-type plants were grown by hydroponics as described above. cDNA was prepared from the leaves and roots of four-week old plants. Small diagnostic regions of each of the nine ALMT genes were amplified by gene specific primers (Table S9): OsALMT1 (Primers 1 and 2), OsALMT2 (Primers 3 and 4), OsALMT3 (Primers 5 and 6), OsALMT4 (Primers 7 and 8), OsALMT5 (Primers 9 and 10), OsALMT6 (Primers 11 and 12), OsALMT7 (Primers 13 and 14), OsALMT8 (Primers 15 and 16) and OsALMT9 (Primers 17 and 18). Full-length OsALMT4 cDNA was amplified from leaves with primers 19 and 20. OsALMT5 genomic DNA sequence was amplified from leaves with primers 21 and 22.

**Subcellular localization of OsALMT4 protein**

Both the N and C-terminal fusions of GFP to OsALMT4 were constructed. For the N-terminal fusion, GFP::OsALMT4, the OsALMT4 was amplified with primers 23 and 24 and the GFP fragment with primers 25 and 26 (Table S9). The PCR products were purified and fused by a fusion PCR reaction. For the C-terminal fusion OsALMT4::GFP, the OsALMT4 and GFP fragments were amplified with primers 27 and 28 and primers 29 and 30 respectively (Table S9). Once confirmed the constructs were prepared for bombardment into leek tissues and transformation of Nicotiana benthamiana leaf tissue by infiltration.
For leek transformation, the fusion constructs were inserted into the pWUbi plasmid vector under the control of the ubiquitin promoter. 1 mg plasmid was precipitated on gold particles (0.6 mm diameter; Bio-Rad) and bombarded into leek tissue as described previously (Delhaize et al., 2007a; Tovkach et al., 2013). The procedure used the Bio-Rad Biolistic Particle Delivery System model PDS-1000/He (600 mm Hg vacuum; 6,000 kP pressure). The tissues kept at room temperature in the dark. Images were captured after 6 to 28 hours using a Leica TCS SP2 confocal laser scanning microscope. For *N. benthamiana* transformation, the OsALMT4::GFP and GFP::OsALMT4 fusions were inserted into the pART plasmid vector under the control of the CaMV 35S promoter. The vector was transformed into *Agrobacterium tumefaciens* (strain AGL-1) by electroporation and transformed into *N. benthamiana* leaves infiltration method (Wood et al., 2009). Two days after inoculation, epidermal strips were peeled from the underside of the leaves and checked with the Leica TCS SP2 confocal microscope. Co-localization experiments also introduced into both systems a known plasma membrane protein fused with mCherry report gene to confirm the final result (Nelson, 2007).

Co-infiltration of tobacco leaves with Agrobacterium containing different constructs allowed two proteins tagged with different fluorophores. The plasma membrane localized control aquaporin protein, pm-rk, contains the mCherry fluorophore (Stock 4011591933, http://www.bio.utk.edu/cellbiol/markers/). The pm-rk plasmid was isolated and transformed into AGL-1 agrobacterium competent cells. These cultures were prepared as above and then mixed with the GFP constructs prior to infiltration. The same pm-rk contract was also co-bombarded in leek after the plasmid was mixed (1 mg/µl) with either the pART7-OsALMT4::GFP or pART7-GFP::OsALMT4 plasmid. Images from these two fluorescent probes were overlaid to assess their co-localisation within cells.

*Generating transgenic rice plants with increased and reduced OsALMT4 expression*

To over-express OsALMT4 in rice, *OsALMT4* cDNA was ligated into pWUbi vector which contains the ubiquitin (Ubi) promoter and transformed into electro-competent DH5α *E. coli* cells. A correct clone was digested with the *Not*I restriction enzyme and the excised fragment ligated into pWBVec8 (Tovkach et al., 2013). Positive clones were transformed into AGL-1 *Agrobacterium* competent cells for rice transformation. Knockout mutations in the *OsALMT4* gene of rice were not available from the rice genomic resource centre when this project started (http://signal.salk.edu/RiceGE/RiceGE_Data_Source.html) so instead RNAi
technology was used to generate transgenic rice lines with reduced OsALMT4 expression. An RNAi construct was prepared by amplifying the 1108-1335bp region of OsALMT4 cDNA using primers 31 and 32 (Table S9). This fragment was unique to OsALMT4 and so this construct would not be expected to reduce expression of any other member of the family. The rice transformation method used was as described previously (Toki et al., 2006).

The primary transgenic plants from tissue culture were called T0 plants. The seed harvested from T0 plants were called T1 seed. From a total of 31 T0 overexpression plants generated three pairs of homozygous T2 lines (OX2, OX5 and OX8) were developed along with their corresponding null sister lines (OX2_null, OX5_null and OX8_null). Since the segregation ratio of transgenic plants to non-transgenic plants in the T1 generation was 3:1 (see below) each of these homozygous lines are likely to possess a single transgene insertion. Since knockout mutant rice lines were not available in OsALMT4 when this project started RNAi lines were generated instead. Thirty five T0 RNAi plants showing a range of expression levels were initially selected. T1 lines showing low expression and a segregation ratio of 3:1 (transgenic to non-transgenic) were selected and three were chosen to generate homozygous T2 lines (R24, R58 and R78) as well as their corresponding null sister lines (R24_null, R58_null and R78_null). Transgenic and null lines were tested in the T2 generation by PCR using primers 33 and 34 (Table S9).

Mineral analysis of grain and leaves

Plant roots and shoots were dried at 70 °C for 24 hours. Between 50 to 250 mg dry biomass was digested in 8 mL concentrated nitric acid and 2 mL hydrochloric acid at 170 °C in a microwave oven for 1.5 h. The samples were diluted in 40 mL milliQ water and elements analysed with inductively coupled plasma mass spectrometry (ICP) analysis.

Expression of Mn and B transporter genes

Plants were grown for five weeks in hydroponics in a growth cabinet and cDNA prepared from roots and the most recently fully expanded leaves. For the expression of Mn transporters, the OsALMT4 over-expressing line OX5 and its null line were germinated and grown in standard nutrient solution (1 µM Mn) for five weeks. The root and shoot tissues were collected for RNA extraction to measure the expression of Mn transporters. The primers used were 35/36 (fwd/Rev) for OsNramp3, 37/38 for OsNramp5, 39/40 for OsYSL2, 41/42 for OsMPT8.1 and 43/44 for OsMPT9 (Table S9). For the expression of
B transporters, the OsALMT4 over-expressing line OX5 and its relative null line were
germinated and grown in standard nutrient solution (5.5 µM B) and high B (5 mM B) for
five weeks. Root and shoot material were collected for RNA extraction to measure the
expression of B transporters using the following primers: 45/46 (fwd/Rev) for OsBOR1- /
and 47/48 for OsNIP3.1. Quantitative RT-PCR was performed with gene-specific primers
by using SYBR Green (BIO-RAD) and the CFX96 Real-Time System (BIO-RAD)
following the manufacturer’s instructions. Reactions were performed with three
biological replicates and three technical replicates for each sample. Transcript levels were
normalized with the reference gene glyceraldehyde-3-phosphate dehydrogenase
(GAPDH, GenBank: GQ848049.1).

Xylem-sap assays

Plants were grown in hydroponics and solutions changed every five days, including 16 h
before the sap collection. Shoots were cut 3 cm above the root crown and xylem sap was
collected with micropipettes from the cut end as described by previously (Yokosho et al.,
2009). Leaf symptoms had appeared on the OX2 and OX5 lines when samples were
collected. The samples were diluted 1/100 and analysed for malate using an enzyme
assay (Ryan et al., 2009).

Partitioning of elements within leaves

Concentrations of Mn, B and Ca in leaf apoplast and symplast were measured following
the method of Nouchi et al. (2012). Plants were grown in flooded soil for eight weeks and
the most recently expanded leaves selected from four separate plants for each genotype.
Samples where the volume of apoplastic fluid extracted was less than 1.2 µL were
ignored.

Statistical analysis

Statistical analysis used SigmaPlot version 13 (Systat Software Inc) for t tests and one
way ANOVA analyses. Since growth and vigour of the transgenic and null lines often
varied in control conditions, relative measurements of a parameter were usually made and
compared (i.e. relative root growth or relative shoot growth). For example, relative root
growth is defined as growth in a treatment compared to the growth in control condition.
There is also a need to account for the accumulation of errors associated with deriving
these ratios. Following the example of relative root growth (RRG), the standard error for this ratio would be calculated as follows: $SE_{RRG} = RRG \left[ \left( SE_x/x \right)^2 + \left( SE_y/y \right)^2 \right]^{1/2}$ where $x$ and $y$ represent the mean growth in the control and treatment conditions and $SE_x$ and $SE_y$ are the standard errors for root growth in control and treatment conditions, respectively.

To statistically compare relative data from one line with another we adapted an approach based on overlapping confidence limits (CL) described previously (Zhou et al., 2013).

Supplemental Material

Table S1 Information on the OsALMT4 and OsALMT5 genes in clade 5.

Table S2 Phenotypic comparison of homozygous transgenic lines with increased.

Table S3 Comparing the biomass of transgenic and null lines in various growth conditions.

Table S4 Organic anion efflux measured from transgenic rice by HPLC.

Table S5 Metabolomic analysis of transgenic rice lines with increased and reduced expression of OsALMT4.

Table S6 Elemental analysis of transgenic rice grain.

Table S7 Linking Mn concentrations in the leaves with leaf phenotypes in OX and null lines.

Table S8 Elemental concentration in the leaves of OX5 plants grown in 1 and 500 µM Mn.

Table S9 Primers used in this study.

Figure S1 Phylogenetic tree of some members of the ALMT family.

Figure S2 OsALMT4 expression in primary (T0).

Figure S3 OsALMT4 expression in homozygous transgenic rice lines.

Figure S4 Severity of the leaf symptoms in various light and temperature treatments.

Figure S5 Shoot and root biomass of transgenic and null lines grown in different Mn treatments.

Figure S6 Leaf symptoms on OX5 plants grown in different Mn concentrations.

Figure S7 Photograph of representative plants after 28 d in hydroponic solution with different concentrations of Mn (µM) with or without 1 mM malate.
**Figure S8** Mineral concentration in leaves of wild-type plants grown in hydroponics with various concentrations of Mn and malate.

**Figure S9** Shoot and root biomass of transgenic and null lines grown in 5.5 μM or 5 mM B concentrations.

**Figure S10** Cartoon showing the possible transport pathways and distribution of malate and Mn in tissues of the OX lines.

**Acknowledgements**

The authors acknowledge support from the China Scholarship Council for J.L., helpful comments from Jian Feng Ma and technical assistance from Kumara Weligama, Muyun Xu, Chunyan Zhang and Rosemary White.

**Figure legends**

**Figure 1 OsALMT4 localises to the plasma membrane.**

GFP was fused to the N and C-terminal ends of the OsALMT1 protein and transiently expressed in tobacco (*Nicotiana benthamiana*) leaves (a-f) and leek tissue (*Allium ampeloprasum*) (g-i). (a) GFP fluorescence of a tobacco leaf cell co-infiltrated with GFP::OsALMT1 and a construct containing a plasma membrane-localising control protein pm-rk fused to the mCherry fluorescent protein; (b) mCherry fluorescence of the cell shown in a; (c) Overlapping signals (yellow) after merging the GFP and mCherry images in a and b. (d) Bright-field image of the cell in a-c; (e) Tobacco cell expressing a control construct with soluble GFP protein showing the typical high expression in the cytosol and nucleus; (f) GFP fluorescence image of a tobacco cell expressing the GFP::OsALMT1 construct after plasmolysis with 50% sucrose showing the cytoplasm receding from the cell wall (CW; arrow) and the Hechtian strands (HS) stretching between the cell wall and plasma membrane (arrow); (g) GFP fluorescence of a leek cell expressing a GFP::OsALMT1 construct; (h) Brightfield image of the cell in g; (i) Leek cell expressing a control construct with soluble GFP protein. Bars = 20 μm in a-f; 10 μm in g,h; 40 μm in i.

**Figure 2 Organic anion efflux from the roots of OX lines**

Organic anion efflux from the roots and Al tolerance were compared in the OX lines, RNAi lines and nulls. (a) Malate and citrate efflux were measured from seedlings grown in flasks with sterile nutrient solution (five plants per flask). The bathing solution was measured after 16 h (mean and SE, n=4) and organic anions measured with enzyme assays. (b) Al resistance of OX lines was estimated by measuring relative root length (RRL) in six day old seedlings of OX and null lines after seven days in 0,
200 and 400 µM AlCl₃. RRL was calculated as the ratio of root length in Al to root length in the control solution (no Al) (mean and SE, n=5-9). An asterisk in part (a) indicates a significant difference (t test; P<0.05) between the OX line and its respective null line. An asterisk in part (b) indicates a significant difference (t test; P<0.05) between the RRL of the OX line and its respective null line within each Al concentration. The statistical test used to compare these ratios is described in Materials and Methods.

Figure 3 Leaf symptoms appearing on the leaves of OX lines
Brown and necrotic spots appeared on rice plants over-expressing OsALMT4 but not on the null lines, RNAi lines or wild-type plants. (a) Leaves from OX5 (homozygous line with enhanced OsALMT4 expression) and OX5_null plants grown in flooded soil. (b), (c) and (d) Leaves from OX5, OX2 and OX8 plants (left side) and their respective nulls (right side). (e) and (f) Brown spots on OX5 leaves under higher magnification (bar is 0.5 mm). (g) Leaves from left to right are R58 (homozygous RNAi line with reduced OsALMT4 expression), R58_null, wildtype, OX2_null and OX2. Scale bar in (e) and (f) is 1 mm.

Figure 4 Concentration of elements in grain of OX and RNAi lines relative to their nulls.
The figure shows a summary of the data in Table S6 and provides the ratio of concentrations in the OX lines relative to their respective null lines. Grain were collected from OX and null plants grown at the same time in flooded soil in a glasshouse. The asterisks indicate significant differences (P<0.05) in the ratio of concentrations in each OX line relative to its respective null line. The statistical test used to compare these ratios is described in Materials and Methods.

Figure 5 Partitioning of Mn in the leaves of OX and null lines.
The concentrations of Mn, B and Ca in the apoplastic and symplastic compartments of rice leaves was estimated after growing plants in the glasshouse in flooded soil for eight weeks. Data are means and SE (n=4). The asterisk in the apoplastic data indicates a significant difference (P<0.05) between the OX and its null using a t test. The asterisks for the ratio data indicates a significant difference (P<0.05) between the OX and its null lines using the statistical method described in Materials and Methods.

Figure 6 Malate concentrations in the xylem sap of OX lines and nulls
Xylem sap was collected from two OX lines (OX2 and OX5) and their nulls and two RNAi lines (R24 and R58) and their nulls using the method described previously.
Plants were grown in non-flooded soil for six weeks and malate concentrations were measured by enzyme analysis. Wild-type plants are also included. Data show the means and SE (n=4 replicate plants). Asterisk indicates significant differences (P<0.01) using t tests to compare each pair of OX and null lines.

**Figure 7** Expression of Mn transporters is altered in the OX lines

RNA was extracted from roots and the most recent fully-expanded leaf of OX5 and OX5_null plants grown in standard hydroponics in a growth chamber for 28 d. Expression of genes encoding Mn transporters was estimated with qRT-PCR in shoots (a) and roots (b) using GAPDH as the reference. Data show means and SE (n = 3 biological replicates from different plants). Asterisk indicates significant differences (P<0.05) using t tests to compare each pair of OX and null lines.

**Figure 8** OX lines are more sensitive to Mn toxicity

Growth of OX and RNAi lines was compared with their respective nulls after 28 d growth in hydroponics with 1 μM (control), 250 μM or 500 μM Mn. Shown is the relative shoot (a) or root (b) biomass for each genotype which is defined as biomass in the high Mn treatments compared to biomass in the control treatment (see Figure S5). Data show the mean and SE (n=5). An asterisk indicates a significant difference (P<0.05) between the relative biomass of a transgenic line and its corresponding null line. The statistical test used to compare these ratios is described in Materials and Methods.

**Figure 9** Effect of external malate on plant growth at different Mn concentrations

Wild-type rice seedlings were transferred to standard hydroponic solutions amended with various concentrations of Mn and malate. These treatments included: 1 μM (control), 10 μM Mn, 100 μM Mn, 10 μM Mn + 1 mM malate and 100 μM Mn + 1 mM malate. Solutions were changed every three to seven days depending on the size of the plants. Shoot biomass and mineral content were measured after eight weeks. (a) Final shoot biomass and (b) Mn concentration in the leaves. Data show means and SE (n = 4 replicate plants). Different letters indicate significant differences (P<0.05) using a one factor ANOVA. Photographs of representative plants at harvest are shown in Figure S7 and mineral analysis of leaf material from the control and 500 μM Mn treatments is shown in Figure S8.

**Figure 10** Expression of B transporters is altered in the OX lines
RNA was extracted from roots and the most recent fully-expanded leaf of OX5 and OX5_null plants grown in hydroponics with 5.5 µM (control) or 5 mM H$_3$BO$_3$ for 28 d. Expression of genes encoding B transporters was estimated with qRT-PCR in shoots (a) and roots (b) using GAPDH as the reference. Also shown is relative expression of the OsALMT4 gene. Data show means and SE (n = 3 biological replicates from different plants). An asterisk indicates significant differences (P<0.05) between the OX and its null line within each treatment using a t test.

**Figure 11** OX lines are more tolerant to B toxicity

Growth of OX and RNAi lines was compared with their nulls after 28 d growth in hydroponics with 5.5 µM (control) or 5 mM H$_3$BO$_3$. Shown is the relative shoot (a) or root (b) biomass for each genotype which is defined as biomass in the high B treatment compared to biomass in the control treatment (see Figure S9). Data show the mean and SE (n=5). An asterisk indicates a significant difference (P<0.05) between the relative biomass of a transgenic line and its corresponding null line. The statistical test used to compare these ratios is described in Materials and Methods.

**Literature cited**


Furia TE (1972) Stability Constants (log K1) of Various Metal Chelates, In CRC Handbook of Food Additives, 2nd Ed. In. CRC Press, p 1016


Hanaoka H, Uraguchi S, Takano J, Tanaka M, Fujiwara T (2014) OsNIP3;1, a rice boric acid channel, regulates boron distribution and is essential for growth under boron-deficient conditions. Plant Journal 78: 890-902

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Sasaki A, Yamaji N, Xia J, Ma JF (2011) OsYSL6 is involved in the detoxification of excess manganese in rice. Plant Physiology 157: 1832-1840


Vlamis J, Williams DE (1964) Iron and manganese relations in rice and barley. Plant and Soil 20: 221-231


**Figure 1** OsALMT4 localises to the plasma membrane.

GFP was fused to the N and C-terminal ends of the OsALMT1 protein and transiently expressed in tobacco (Nicotiana benthamiana) leaves (a-f) and leek tissue (Allium ampeloprasum) (g-i). (a) GFP fluorescence of a tobacco leaf cell co-infiltrated with GFP::OsALMT1 and a construct containing a plasma membrane-localising control protein pm-rk fused to the mCherry fluorescent protein; (b) mCherry fluorescence of the cell shown in a; (c) Overlapping signals (yellow) after merging the GFP and mCherry images in a and b. (d) Bright-field image of the cell in a-c; (e) Tobacco cell expressing a control construct with soluble GFP protein showing the typical high expression in the cytosol and nucleus; (f) GFP fluorescence image of a tobacco cell expressing the GFP::OsALMT1 construct after plasmolysis with 50% sucrose showing the cytoplasm receding from the cell wall (CW; arrow) and the Hechtian strands (HS) stretching between the cell wall and plasma membrane (arrow); (g) GFP fluorescence of a leek cell expressing a GFP::OsALMT1 construct; (h) Brightfield image of the cell in g; (i) Leek cell expressing a control construct with soluble GFP protein. Bars = 20 µm in a-f; 10 µm in g,h; 40 µm in i.
Figure 2  Organic anion efflux from the roots of OX lines

Organic anion efflux from the roots and Al tolerance were compared in the OX lines, RNAi lines and nulls. (a) Malate and citrate efflux were measured from seedlings grown in flasks with sterile nutrient solution (five plants per flask). The bathing solution was measured after 16 h (mean and SE, n=4) and organic anions measured with enzyme assays. (b) Al resistance of OX lines was estimated by measuring relative root length (RRL) in six day old seedlings of OX and null lines after seven days in 0, 200 and 400 µM AlCl₃. RRL was calculated as the ratio of root length in Al to root length in the control solution (no Al) (mean and SE, n=5-9). An asterisk in part (a) indicates a significant difference (t test; P<0.05) between the OX line and its respective null line. An asterisk in part (b) indicates a significant difference (t test; P<0.05) between the RRL of the OX line and its respective null line within each Al concentration. The statistical test used to compare these ratios is described in Materials and Methods.
Brown and necrotic spots appeared on rice plants over-expressing *OsALMT4* but not on the null lines, RNAi lines or wild-type plants. (a) Leaves from OX5 (homozygous line with enhanced *OsALMT4* expression) and OX5_null plants grown in flooded soil. (b), (c) and (d) Leaves from OX5, OX2 and OX8 plants (left side) and their respective nulls (right side). (e) and (f) Brown spots on OX5 leaves under higher magnification (bar is 0.5 mm). (g) Leaves from left to right are R58 (homozygous RNAi line with reduced *OsALMT4* expression), R58_null, wildtype, OX2_null and OX2. Scale bar in (e) and (f) is 1 mm.
Figure 4  Concentration of elements in grain of OX and RNAi lines relative to their nulls.

The figure shows a summary of the data in Table S6 and provides the ratio of concentrations in the OX lines relative to their respective null lines. Grain were collected from OX and null plants grown at the same time in flooded soil in a glasshouse. The asterisks indicate significant differences (P<0.05) in the ratio of concentrations in each OX line relative to its respective null line. The statistical test used to compare these ratios is described in Materials and Methods.
The concentrations of Mn, B and Ca in the apoplastic and symplastic compartments of rice leaves was estimated after growing plants in the glasshouse in flooded soil for eight weeks. Data are means and SE (n=4). The asterisk in the apoplastic data indicates a significant difference (P<0.05) between the OX and its null using a t test. The asterisks for the ratio data indicates a significant difference (P<0.05) between the OX and its null lines using the statistical method described in Materials and Methods.

Figure 5  Partitioning of Mn in the leaves of OX and null lines.

The concentrations of Mn, B and Ca in the apoplastic and symplastic compartments of rice leaves was estimated after growing plants in the glasshouse in flooded soil for eight weeks. Data are means and SE (n=4). The asterisk in the apoplastic data indicates a significant difference (P<0.05) between the OX and its null using a t test. The asterisks for the ratio data indicates a significant difference (P<0.05) between the OX and its null lines using the statistical method described in Materials and Methods.
Figure 6 Malate concentrations in the xylem sap of OX lines and nulls

Xylem sap was collected from two OX lines (OX2 and OX5) and their nulls and two RNAi lines (R24 and R58) and their nulls using the method described previously (Yokosho et al., 2009). Plants were grown in non-flooded soil for six weeks and malate concentrations were measured by enzyme analysis. Wild-type plants are also included. Data show the means and SE (n=4 replicate plants). Asterisk indicates significant differences (P<0.01) using t tests to compare each pair of OX and null lines.
Figure 7 Expression of Mn transporters is altered in the OX lines

RNA was extracted from roots and the most recent fully-expanded leaf of OX5 and OX5_null plants grown in standard hydroponics in a growth chamber for 28 d. Expression of genes encoding Mn transporters was estimated with qRT-PCR in shoots (a) and roots (b) using GAPDH as the reference. Data show means and SE (n = 3 biological replicates from different plants). Asterisk indicates significant differences (P<0.05) using t tests to compare each pair of OX and null lines.
Figure 8  OX lines are more sensitive to Mn toxicity

Growth of OX and RNAi lines was compared with their respective nulls after 28 d growth in hydroponics with 1 µM (control), 250 µM or 500 µM Mn. Shown is the relative shoot (a) or root (b) biomass for each genotype which is defined as biomass in the high Mn treatments compared to biomass in the control treatment (see Figure S5). Data show the mean and SE (n=5). An asterisk indicates a significant difference (P<0.05) between the relative biomass of a transgenic line and its corresponding null line. The statistical test used to compare these ratios is described in Materials and Methods.
Wild-type rice seedlings were transferred to standard hydroponic solutions amended with various concentrations of Mn and malate. These treatments included: 1 µM (control), 10 µM Mn, 100 µM Mn, 10 µM Mn + 1 mM malate and 100 µM Mn + 1 mM malate. Solutions were changed every three to seven days depending on the size of the plants. Shoot biomass and mineral content were measured after eight weeks. (a) Final shoot biomass and (b) Mn concentration in the leaves. Data show means and SE (n = 4 replicate plants). Different letters indicate significant differences (P<0.05) using a one factor ANOVA. Photographs of representative plants at harvest are shown in Figure S7 and mineral analysis of leaf material from the control and 500 µM Mn treatments is shown in Figure S8.
Figure 10  Expression of B transporters is altered in the OX lines

RNA was extracted from roots and the most recent fully-expanded leaf of OX5 and OX5_null plants grown in hydroponics with 5.5 µM (control) or 5 mM H_3BO_3 for 28 d. Expression of genes encoding B transporters was estimated with qRT-PCR in shoots (a) and roots (b) using GAPDH as the reference. Also shown is relative expression of the OsALMT4 gene. Data show means and SE (n = 3 biological replicates from different plants). An asterisk indicates significant differences (P<0.05) between the OX and its null line within each treatment using a t test.
Figure 11  OX lines are more tolerant to B toxicity

Growth of OX and RNAi lines was compared with their nulls after 28 d growth in hydroponics with 5.5 µM (control) or 5 mM H$_3$BO$_3$. Shown is the relative shoot (a) or root (b) biomass for each genotype which is defined as biomass in the high B treatment compared to biomass in the control treatment (see Figure S9). Data show the mean and SE (n=5). An asterisk indicates a significant difference (P<0.05) between the relative biomass of a transgenic line and its corresponding null line. The statistical test used to compare these ratios is described in Materials and Methods.


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Google Scholar: Author Only Title Only Author and Title

Furia TE (1972) Stability Constants (log K1) of Various Metal Chelates, In CRC Handbook of Food Additives, 2nd Ed. In CRC Press, p 1016

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Hanaoka H, Uraguchi S, Takano J, Tanaka M, Fujiwara T (2014) OsNIP3;1, a rice boric acid channel, regulates boron distribution and is essential for growth under boron-deficient conditions. Plant Journal 78: 890-902

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Sasaki A, Yamaji N, Xia J, Ma JF (2011) OsYSL6 is involved in the detoxification of excess manganese in rice. Plant Physiology 157: 1832-1840


