Expression Profiles of Arabidopsis thaliana in Mineral Deficiencies Reveal Novel Transporters Involved in Metal Homeostasis

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Plants directly assimilate minerals from the environment and thus are key for acquisition of metals by all subsequent consumers. Limited bio-availability of copper, zinc and iron in soil decreases both the agronomic productivity and the nutrient quality of crops. Understanding the molecular mechanisms underlying metal homeostasis in plants is a prerequisite to optimizing plant yield and metal nutrient content. To absorb and maintain a balance of potentially toxic metal ions, plants utilize poorly understood mechanisms involving a large number of membrane transporters and metal-binding proteins with overlapping substrate specificities and complex regulation. To better understand the function and the integrated regulation, we analyzed in Arabidopsis the expression patterns in roots and in leaves of 53 genes coding for known or potential metal transporters, in response to copper, zinc, and iron deficiencies in Arabidopsis. Comparative analysis of gene expression profiles revealed specific transcriptional regulation by metals of the genes contrasting with the known wide substrate specificities of the encoded transporters. Our analysis suggested novel transport roles for several gene products and we used functional complementation of yeast mutants to correlate specific regulation by metals with transport activity. We demonstrate that two ZIP genes, ZIP2 and ZIP4, are involved in copper transport. We also present evidence that AtOPT3, a member of the oligopeptide transporter gene family with significant similarities to the maize iron-phytochelatin transporter YS1, is regulated by metals and heterologous expression AtOPT3 can rescue yeast mutants deficient in metal transport.

All organisms require metal prosthetic groups for their unique catalytic and structural properties. In proteins, copper and iron catalyze reduction-oxidation reactions, while zinc plays an essential structural or enzymatic role. Yet most metal ions are very reactive and can be toxic to cells when present in excess. Thus, it is important for organisms to maintain adequate levels of metals in tight homeostasis using complex, and often evolutionarily conserved mechanisms for the uptake and transport of low solubility metals and storage of metal ions in a non-toxic form. Despite rapid progress in recent years of our understanding of metal homeostasis in yeast, (1) our knowledge of metal metabolism in plants is still rudimentary (2). A large number of cation transporters potentially involved in metal ion homeostasis have been identified on the genome of the model plant Arabidopsis thaliana (3). Several members of the 15 ZIP gene family (4) and of the 6 NRAMP family of transporters (3) have been characterized and shown to be involved in metal uptake and transport in plants (5–10). ABC transporters (11) and P-type ATPase pumps (12, 13) are also known to be involved in metal ions trafficking metals into organelles. Since metals are cytotoxic as free ions, they are chelated for both intracellular storage and long-distance transport. These chelators are either enzymatically synthesized small molecular weight compounds, such as metallothionein (NA)3 and phytochelatin, or proteins, such as metallothionein (MT) and metal chaperones. Four NA synthase genes, NAS1–3 (14), two phytochelatin synthase (PCS) genes (15), and seven MT genes (16–18) have been identified. AtCCH, a homologue to the copper chaperone Atx1p of Saccharomyces cerevisiae, plays a role in intracellular copper transport (19, 20), while the function of 29 additional proteins containing an Atx1p-like heavy metal binding site (HMA) is unknown (21, 22). Furthermore, four genes encoding ferritin subunits have been identified (23), which form iron storage complexes within chloroplasts. The individual analysis of components of copper, zinc, and iron metabolism has provided important but limited insight into metal homeostasis in plants (for recent reviews, see Refs. 3 and 24). These findings do not provide a cohesive integrated model of the mechanisms used by organisms to regulate metal uptake. Moreover, large numbers of genes encoding proteins known or likely to play a role in metal transport. Second, the plant transporters that have been studied in heterologous systems exhibit low selectivity in the metal species transported.

Functional studies using yeast complementation have revealed wide substrate specificities but failed to identify the specific in planta function for most of the transporters. The transcriptional patterns in response to metal deficiencies could

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1 The abbreviations used are: NA, nicotianamine; MT, metallothionein; MES, 4-morpholineethanesulfonic acid; HEDTA, hydroxylated ethylenediaminetriacetate; ICP, inductively coupled plasma; AID, average difference intensity; RT, reverse transcription; SOD, superoxide dismutase; PC, phytochelatin; PS, phytosiderophore.

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yield useful information bearing on the function of the genes. We have therefore undertaken a large-scale analysis of gene expression profiles in Arabidopsis plants subjected to nutritional deficiencies in three essential metals using Affymetrix DNA microarrays containing 8,300 Arabidopsis genes. The response of plants to mineral deficiencies likely involves a complex regulatory cascade ultimately resulting in changes in expression of key transporters and metal homeostasis proteins as well as by inducing changes in their growth patterns. In this report, we focus on the changes in expression of genes coding for copper deficient metal homeostasis proteins that were germinated and grown on control growth medium (contain-

EXPERIMENTAL PROCEDURES

Plant Growth—Arabidopsis thaliana Columbia were aseptically grown on hydroponics at 20 °C under a 16-h light/8-h dark cycle. Control plants were grown on a modified Gamborg’s B-5 medium containing full-strength Gamborg’s B-5 salts, 1 × Gamborg’s vitamins, and 2 mM MES-KOH, pH 5.50. All chemical were purchased from Sigma. To induce copper deficiency, plants were first germinated on the same medium lacking copper. These plants remain healthy due to trace copper contamination, since the free copper activity must be kept below ~1 × 10⁻¹⁴ M to induce copper deficiency. After 5 weeks of growth, the nutrient solution was replaced by a chelate-buffered medium (25) to induce zinc deficiency, in which HEDTA (hydroxyethylethiolenediaminetriacetate) was added to copper-free medium at a level 25 μM in excess of the sum of the divalent metal concentrations (iron, zinc, manganese, and nickel). The speciation program Geochem-PC was employed to calculate free metal activities to design a hydroponics solution that was specific for copper deficiency while ensuring sufficient copper in other metals. Zinc was accordingly increased to 0.7 mM to keep its activity above ~1 × 10⁻₁⁹ M. The copper-deficient chelate buffer thus consisted of Gamborg’s B-5 salts lacking copper, 0.7 mM ZnSO₄, 0.85 mM HEDTA, 2 mM MES-KOH, pH 5.50, and Gamborg’s vitamins. Plants were grown for 5 days on HEDTA-buffered copper-deficient medium before the roots and the leaves were harvested for RNA extraction. A similar approach was used to induce zinc deficiency as in Grotz before the roots and the leaves were harvested for RNA extraction. A 

for transporters and metal homeostasis proteins that are present on the 8.3K Arabidopsis DNA chip and were shown previously or in this study to be regulated in response to copper, zinc, and iron deficiency. The very specific transcriptional regulation observed, in contrast to the wide substrate specificity of many metal transporters, suggests primarily transcriptional regulatory control of metal homeostasis in plants. In addition, our expression analysis and confirmatory functional complementation studies revealed previously unsuspected roles for several trans-membrane transporters in metal homeostasis.

Metal Homeostasis in Arabidopsis

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RNA Blots and Reverse Transcription (RT)-PCR—For Northern blots 10 μg of RNA were run in a 1.2% agarose-formaldehyde gel and transferred onto nylon membrane (Amersham Biosciences). Hybridization to (29)CTP-labeled DNA probes was carried out at 42 °C in 50% formamide, 6 × SSC, 0.1% SDS, 2 × Denharts solution. For RT, 5 μg of RNA were reverse-transcribed using Superscript reverse transcriptase (Inveritrogen) according to the manufacturer’s instructions. Quantitative RT-PCR was carried out using Taq DNA polymerase as directed by the supplier. (Takara Shuzo Co., Japan). Between 20 and 27 cycles (30 s at 94 °C, 60 s at 50 °C, and 60 s at 72 °C) were performed in a 50-μl volume. 5 μl of the reaction were analyzed on a 1% agarose + ethidium bromide gel that was photographed using a digital imager (Chemillmager 4400, Alpha Innotech Corp., CA) and analyzed using image analysis software (AlphaEase™, Alpha Innotech Corp.). Amplified β-tubulin gene was used to normalize the data. Oligonucleotides were designed to amplify 500–600 bp of the 5’ end of the genes (ZIP-2F, TAGGACCGCGTCTGATTGTCG; ZIP-2R, GAGATGGTTAACCGGCAACGTACA; ZIP-4F, GCTGCTGTTAGTTAAGGAGAT; ZIP-4R, ATACGCTGCGATTGCTCA; ZIP-5F, GATTTAACGGCGAGTGAACTAGC; ZIP-5R, AGATGGTTAACCGGCAACGTACA; ZIP-6F, GATTTAACGGCGAGTGAACTAGC; ZIP-6R, TAGCAGCCGCTGGATATTGC; ZIP-7F, CACCTCATCTTCTTGCTCAAG; ZIP-7R, GTTGGGTTGCACCACTCAC; B-TUBULIN-F, GAGTTTCCGTTCACAGGCTT; B-TUBULIN-R, GAGATGGTTAACCGCAACGTACA; B-TUBULIN-F, GCTGCTGTTAGTTAAGGAGAT; B-TUBULIN-R, ATACGCTGCGATTGCTCA).

Yeast Work—The S. cerevisiae KO strains used in this study are derived from BY4741 and were obtained from Research Genetics (www.resgen.com). The fett4Δmutant was in the DEY1453 background (S). Yeast functional complementation studies, cDNAs were amplified using ex Taq polymerase (Takara Bio Inc.), cloned into the yeast vector pFL61, sequence-verified, and transformed into yeast mutants using standard procedures. Yeast growth media were purchased from (OriGene, CA). Niotiamine was purchased from Hasegawa Co. (Tokyo, Japan).

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RESULTS

Regulation of ZIP Genes by Copper, Zinc, and Iron—Six members of the 15-member ZIP family of genes coding for zinc/iron peremeses are present on the DNA microarrays used in this work (Table I). The gene coding for IRT1, the major uptake transporter in Arabidopsis, is not present in the chips and thus not included in this analysis. Our results showed that ZIP genes can be regulated by zinc (ZIP4, ZIP5, ZIP9), iron (IRT2), and copper (ZIP2 and ZIP4) both in the roots and in the leaves (Table IIA and Figs. 1 and 2).
Microarray and RT-PCR results indicated that ZIP2 and ZIP4 are induced in copper deficiency and repressed in copper excess (Table IIA and Fig. 2). However, they were not responsive to the same levels of copper deficiency; we observed a sharp increase in expression of ZIP4 only in plants subjected to 3 weeks of growth on a copper-deficient HEDTA buffered medium (Fig. 2) but not after the 5-day treatment used in the microarray experiments. Copper deficiency was confirmed by measuring copper content in whole plants using ICP-EAS. Copper levels in deficient plants were 2.5 μg/g of dry weight compared with 7 μg/g of dry weight in control plants. The response of ZIP5 to copper deficiency observed in the microarray experiment could not be confirmed by RT-PCR, suggesting that the induction we observed in our microarray analysis may not be significant.

These results imply that ZIP2 and ZIP4 are involved in copper transport. To confirm this, we have cloned ZIP2 and ZIP4 cDNAs in the yeast expression vector pFL61 (30) and expressed it in yeast lacking high affinity copper uptake (A004 ctr1). Both ZIP2 and ZIP4 can restore growth of A004 ctr1 on a non-fermentable medium (1% yeast extract, 2% peptone, 2% glycerol (YPG)), indicating that both genes can function as copper transporter in yeast (Fig. 3A). Measurement of copper content of ZIP2 expressing A004 ctr1 cells harvested in log phase of growth

| Family                        | Gene Name | Potential or Known Function | Affy ID  | AGI  |
|-------------------------------|-----------|-------------------------------|----------|------|
| Aminoacid/auxin permeases     | AAP       | aminoacid/auxin transport     | 13426_at | A0g41190 |
| Cation diffusion facilitators | AMTP1 (ZAT1) | zinc efflux                 | 17046_s_at | A0g46800 |
|                               | AMTP6     | zinc efflux                 | 15813_at | A0g29410 |
|                               | unknown   | homolog to yeast MMT2 Fe transporter | 14509_at | A0g47830 |
|                               | unknown   | homolog to yeast MMT2 Fe transporter | 15018_at | A0g79520 |
|                               | unknown   | homolog to yeast MMT2 Fe transporter | 15274_at | A0g39450 |
| CTR2 copper transporters homologues | COPT1 | copper transport            | 15452_at | A0g59030 |
|                               | COPT2     | metal transport             | 15448_at | A0g46900 |
| Ferritins                     | AIFER1    | iron storage                | 16031_at | A0g01600 |
| Ferroportin-like              | Ferroportin-2 | iron transport            | 17631_at | A0g26820 |
|                               | Ferroportin-1 | iron transport            | 15067_at | A0g38460 |
| Glutamate-gated channels      | AGLR2.3   | Signal transduction        | 18836_at | A0g24710 |
| Heavy metal binding proteins | ACCS1     | Copper chaperone for SOD    | 15606_s_at | A1g12520 |
|                               | ATFP7     | metal binding               | 20720_at | A1g22990 |
|                               | ATFP6     | metal binding               | 18189_at | A0g38580 |
|                               | HMA-1     | metal binding               | 12004_at | A0g35060 |
|                               | HMA-2     | metal binding               | 14104_at | A0g53530 |
| Iron-sulfur cluster assembly genes | CTAU | Copper binding chloroplast | 19192_at | A0g33740 |
|                               | AtmIniJS  | NIFS-like amino transferase | 15941_at | A0g56720 |
|                               | AtmIniLU  | mitochondrial Fe-S cluster assembly | 16297_at | A0g22220 |
|                               | ArpCuNi   | plastid Fe-S cluster assembly  | 19146_at | A0g01940 |
|                               | AtCuSi    | plastid Fe-S cluster assembly  | 16296_at | A0g47770 |
| Major Intrinsic Proteins      | NIP9      | plasma membrane water/solute transport | 19481_s_at | A0g34390 |
|                               | PIP2d     | plasma membrane water/solute transport | 17322_at | A0g54820 |
|                               | MIPS epsilon | tonoplastic water/solute transport | 17488_at | A0g25810 |
| Metal ion transporters        | NRAMP1    | Fe/Cd transport            | 17993_at | A1g80830 |
|                               | NRAMP3    | Fe/Cd transport            | 18109_s_at | A0g23150 |
| Metallothioneins              | MT1A      | heavy metal (Cu, Zn, Cd) binding | 10011_at | A0g03930 |
|                               | MT2b      | heavy metal (Cu, Zn, Cd) binding | 10055_at | A0g02380 |
|                               | MT3       | heavy metal (Cu, Zn, Cd) binding | 10436_at | A0g67234 |
|                               | EC protein homologe 3 | heavy metal (Cu, Zn, Cd) binding | 18617_at | A0g42900 |
|                               | EC protein homologe 2 | heavy metal (Cu, Zn, Cd) binding | 18319_at | A0g23240 |
| Monovalent cation/proton antiporter 2 | CHX17 | Na/H exchange protein | 13627_at | A0g32700 |
|                               | AtTCR1    | metal/tetracycline exchange | 13523_s_at | A0g16080 |
|                               | AmMRP4    | glutathione S-conjugate transport | 20245_s_at | A0g74800 |
| NADPH oxidase-associated cytochrome b558 | FRO2 | ferric reductase | 19737_at | A0g19580 |
|                               | FRO3      | ferric reductase             | 19759_at | A0g23020 |
| Nicotianamine synthases       | NAS1      | nicotianamine synthesis     | 20047_at | A0g49950 |
|                               | NAS2      | nicotianamine synthesis     | 20044_at | BA474590 |
|                               | NAS3      | nicotianamine synthesis     | 13003_s_at | A0g05240 |
| Oligopeptide transporters     | AtOPT3    | oligopeptide transport      | 20117_at | A0g16370 |
|                               | AtOPT2    | oligopeptide transport      | 19477_at | A0g09930 |
|                               | YSL1      | iron chelate transport      | 17451_s_at | A0g24120 |
| P-type ATPases                | C/A/Zn pATPase | metal transporting pATPase | 20090_at | A0g30110 |
| Phytochelatin synthase        | RAN1      | copper transporting pATPase | 14641_s_at | A0g44790 |
| Zinc-Iron permeases           | PCS-1     | phytochelin synthesis       | 16077_s_at | A0g44070 |
|                               | ZIP1      | metal transport             | 15666_s_at | A0g55820 |
|                               | ZIP4      | metal transport             | 15884_at | A1g10970 |
|                               | ZIP5      | metal transport             | 19718_at | A0g05300 |
|                               | ZIP6      | metal transport             | 15807_s_at | A0g30800 |
|                               | ZIP9      | metal transport             | 14831_at | A0g33020 |
|                               | IRT2      | iron transport              | 14054_at | A0g19080 |

**A Role for ZIP2 and ZIP4 in Copper Transport**—Microarray and RT-PCR results indicated that ZIP2 and ZIP4 are induced in copper deficiency and repressed in copper excess (Table IIA and Fig. 2). However, they were not responsive to the same levels of copper deficiency; we observed a sharp increase in expression of ZIP4 only in plants subjected to 3 weeks of growth on a copper-deficient HEDTA buffered medium (Fig. 2) but not after the 5-day treatment used in the microarray experiments. Copper deficiency was confirmed by measuring copper content in whole plants using ICP-EAS. Copper levels in deficient plants were 2.5 μg/g of dry weight compared with 7 μg/g of dry weight in control plants. The response of ZIP5 to copper deficiency observed in the microarray experiment could not be confirmed by RT-PCR, suggesting that the induction we observed in our microarray analysis may not be significant. These results imply that ZIP2 and ZIP4 are involved in copper transport. To confirm this, we have cloned ZIP2 and ZIP4 cDNAs in the yeast expression vector pFL61 (30) and expressed it in yeast lacking high affinity copper uptake (Δctr1). Both ZIP2 and ZIP4 can restore growth of Δctr1 on a non-fermentable medium (1% yeast extract, 2% peptone, 2% glycerol (YPG)), indicating that both genes can function as copper transporter in yeast (Fig. 3A). Measurement of copper content of ZIP2 expressing Δctr1 cells harvested in log phase of growth
on fermentable SD medium (Fig. 3B) indicate that ZIP2-expressing cells have a higher content of copper compared with the 3xcr1 mutant transformed with the vector alone (Fig. 3B). Our results and previous work (26) suggest and are consistent with a role for ZIP2 in root copper and zinc homeostasis and a specific role in root copper and zinc transport in roots and in shoots. Most Transporters Regulated by Copper Are Also Regulated by Other Metals—Although we found copper deficiency-regulated transporters, we did not identify metal transporters specifically induced by copper deficiency in the 8,300 genes tested. In addition to ZIP2, ZIP4, and possibly ZIP5, two other potential copper transporter genes also appeared to be regulated by zinc, namely COPT2, a homologue of a yeast copper transporter (31) in leaves, and Zn/Cd pATPase3, a putative Zn/Cd-transporting P-type ATPase in roots (Table IIA and Fig. 1). Both COPT2 and the Zn/Cd p-ATPase3 showed up-regulation in zinc deficiency. COPT1, a known copper transporter (31), appeared not to be regulated by copper deficiency, and its expression was higher in leaves than in roots (Fig. 1). CHX17, coding for a potential Na+/H+ exchange protein, is the only transporter gene that appears to be specifically regulated by copper (Table IIB, Fig. 1). Six other members of the cation/proton antiporter family present on the array (CHX13, CHX15, CHX21, KEA3, NHX1, and NHX17) do not appear to be regulated in response to any of the metal deficiencies tested. Our analysis indicates that RAN1, a known copper-transporting P-ATPase that plays a role in copper assembly into the ethylene receptor ETR1 (12, 13), is expressed constitutively at comparatively high levels, and its expression is higher in the roots than in the leaves (Fig. 1).

Coordinated Transcriptional Regulation of Copper-binding Proteins—AtCCS, a homologue to the yeast copper chaperone CCS, and both cysteolic and chloroplast Cu,Zn-SODs transcript levels fall in copper and in zinc deficiency (Table IIC, Fig. 4). Two other SOD coding genes, the iron-SOD3 gene (accession number AAC24834) and a SOD-like gene (accession number AAC2483), do not respond to metal deficiencies (Fig. 4). Three genes coding for AtCCS-related metal-binding proteins (21, 22) are expressed only at very low levels and do not appear to be regulated by any of the metal deficiencies tested (see Supplementary Table S1). In contrast, steady-state RNA levels of MT2a, MT2b, and MT3 in normal growth conditions are very high and remained unchanged in all deficiencies, except for a slight decrease in expression of MT2a in copper deficiency (Fig. 5). Finally AtCuA4, a homologue to a bacterial copper-binding protein (32), is not regulated by copper and does not show tissue specific expression (Table S1).

Inter-relationship of Zinc and Iron Transport—Zinc and Iron deficiency led to specific changes in ZnP transcript expression. Transcription profiles showed that ZIP5 and ZIP9 are expressed at very low levels in normal growth conditions, suggesting that they play a specific role in root and shoot zinc transport in response to zinc deficiency. Only IRT2 of the ZIP family, which was previously shown to be involved in iron uptake (9), was specifically increased in iron deficient roots. It is not induced in iron-deficient shoots or in zinc and copper deficiency. None of the ZIP genes studied appears to be co-regulated by zinc and iron. However, iron deficiency led to down-regulation of zinc-regulated ZIP4 and ZIP5 (Table IIA and Fig. 2). This down-regulation could reflect an increase in cellular zinc levels probably via IRT1, an iron-regulated transporter that is known to also transport zinc (8). Similarly, we have observed a sharp increase in expression of iron-regulated ZIP4 in zinc excess (Fig. 2), indicating that plants exposed to excess zinc become iron-deficient, probably because zinc and iron are competing for the same transporters. The expression of ZIP6 was not affected by any of the metal deficiencies and excess tested (Figs. 1 and 2), suggesting either that this permease is constitutively expressed or is regulated by other metals.

Up-regulation of Iron Reductases and IRT2 and Down-regulation of Ferritins in Iron Deficiency—The FRO2 ferric reductase gene, controlling the rate-limiting step of root iron uptake (33), is strongly induced by iron deficiency. Fig. 1 shows that the induction was specific to iron deficiency, and the absolute level of FRO2 transcripts in iron-deficient roots was higher than all other metal-regulated genes assayed. While FRO2 was not expressed in leaves, FRO3 was increased under iron de-
efficiency both in the roots and in the leaves. The increase in leaf-localized FRO3 shows that reduction of ferric iron to ferrous is also a component of metal transport in the leaves (Table IIA) (34). Iron deficiency also led to decreased expression of ferritin genes in roots (AtFer1) and leaves (AtFer1 and AtFer4) (Table IIC). Decreased expression in iron deficiency is consist-
Gene, and a FeSOD gene in control plants and plants grown under Actin is used as a control. ADIs of individual replicates (shown in Table S1) were averaged, and error bars represent S.D. The scale of the y-axis is different in the two graphs.

FIG. 4. Co-regulation of AtCCS and Cu,Zn-SOD genes. ADIs of AtCCS, Cu,Zn-SOD, chloroplast Cu,Zn-SOD (cpSOD), an SOD-like gene, and a FeSOD gene in control plants and plants grown under copper, zinc, or iron deficiency. R and L denote roots and leaves, respectively. ADIs of individual replicates (shown in Table S1) were averaged, and error bars represent S.D.

FIG. 5. Expression levels of genes involved in metal binding and chelation. ADIs were used to assess and compare expression levels of genes involved in metal binding and chelation. ADI for genes encoding ferritin (AtFer1 and AtFer4), nicotianamine synthase (NAS1–3), phytochelatin synthase (PCS-1), and metallothioneins (Mt2a, MT2, MT3) from control plants, and plants grown under copper, zinc, or iron deficiencies were plotted. R and L denote roots and leaves, respectively. Actin is used as a control. ADI of individual replicates (shown in Table S1) were averaged, and error bars represent S.D. The scale of the y axis is different in the two graphs.

consistent with previous observations of increased ferritin transcription in iron overload (23).

Increased Nicotianamine Synthase Gene Expression in Response to Iron and Zinc Deficiency—Enzymatically synthesized small molecular weight compounds such as NA and phytochelatins (PCs) bind metals in cells. NA plays an unidentified role in long distance metal transport, possibly related to entry of iron into the phloem and/or xylem (35) and in cellular transport of iron (36). Three nicotianamine synthase genes (AtNAS1–3) of the four known Arabidopsis nicotianamine synthase genes are present on the DNA chips. AtNAS1 and AtNAS3 transcripts were increased by both zinc and iron deficiencies in the roots. AtNAS2 transcript levels appeared to be increased by zinc deficiency rather than iron deficiency in both roots and leaves. In leaves, copper deficiency may also increase AtNAS2 transcript levels slightly (Table IIC, Fig. 5). In contrast to the NAS genes, the PC synthase gene (PCS-1) was not affected by any of the deficiencies studied (Fig. 5), which is

Members of Oligopeptide Transporters Family OPT Are Regulated by Metals—Our results indicate that AtOPT2 and AtOPT3, two members of an oligopeptide transporter family (38), are regulated in response to metal deficiencies. AtOPT2 and AtOPT3 are highly induced in iron deficiency (Table IIB), and in fact, transcript levels of AtOPT3 in roots are nearly as high as the ferric reductase transcript FRO2 (Fig. 1). Northern blot hybridizations confirmed increased expression of AtOPT3 in iron deficiency and also suggested increased expression in copper and revealed a dramatic expression increase in manganese deficiency (Fig. 6A).

Functional Complementation Demonstrates That AtOPT3 Is a Metal Transporter—To test the ability of OPT3 to transport metals we expressed the AtOPT3 cDNA in three different yeast mutants fet3fet4, Δctr1, and Δsmt1 (39), which are deficient in iron, copper, and manganese transport, respectively. Expression of AtOPT3 could restore growth of Δctr1 on a non-fermentable medium (Fig. 6B). ICP measurements of copper content in Δctr1 expressing AtOPT3 revealed a higher copper content than the cells transformed with the vector alone supporting a role of AtOPT3 in copper transport (Fig. 3B). Fig. 6C shows that AtOPT3 can restore growth of Δsmt1 on manganese-limited medium and that this growth can be reduced by addition of EGTA to the medium. We noted that AtOPT3 shares sequence similarities with YSL1, a gene involved in transport of iron-phytosiderophore complexes in maize roots, and with the Arabidopsis genes of the YSL (YSL1-like) family (59). In our experiments, AtYSL1 is not detectably expressed in roots and has very low levels of expression in leaves. AtYSL1, if regulated, appears to be down-regulated, not up-regulated, in iron and copper deficiency and unaffected by zinc deficiency (Table S1). Monocotyledonous plants such as Arabidopsis do not synthesize phytosiderophores; however, they synthesize NA an immediate precursor of the phytosiderophore mugineic acid. To test whether AtOPT3 can transport iron or NA/iron chelates, we
have expressed the gene in the yeast mutant fet3fet4 deficient in iron uptake. We observed improved growth in iron-limited medium of the mutant expressing AtOPT3 (Fig. 7) in liquid cultures; however, the addition of NA did not affect the growth significantly. Similarly we did not observe a significant difference in the growth of the yeast mutant Δctr1 expressing AtOPT3 in the presence or in the absence of NA (Fig. 7).

Iron Regulation of a Chloroplast ABC Transporter—We found nine membrane transporters in addition to the ones discussed above that showed regulation by at least one metal (Table IIB, Fig. 1). One gene, similar to a large number of bacterial and cyanobacterial ABC transporters was down-regulated by iron deficiency in leaves and is a particularly interesting candidate for iron transport into chloroplasts. This gene, named AtSBL (SufB-like), is similar to SufB, an Escherichia coli iron-regulated gene that encodes a NiFS-like protein required for the assembly of iron sulfur clusters (40, 41). AtSBL is predicted to reside in chloroplasts and thus could play a parallel role in iron transport into plastids. Another gene, AtTCR1, was down-regulated by iron deficiency in leaves and is highly induced by iron deficiency (45).

No Evidence of Metal-specific Regulation of Several Metal Transporters—We found multiple metal transporters that were not regulated by the metal deficiencies tested though transcript levels appear to be tissue specific. Two zinc efflux genes (ZAT1 and AtMTPb), COPT1, RAN1 (a copper transporting P-type ATPase), AtNRAMP1, and AtNRAMP3 all showed tissue-specific, but not metal-regulated, expression. COPT1 is expressed at higher levels in the leaves, while ZAT1, RAN1, and AtNRAMP1 are more highly expressed in the roots. AtNRAMP3 expression was very low in both roots and leaves. Finally, AtMTPb, a potential zinc efflux gene, and two Ferroportin1/IREG1 homologues were not expressed at detectable levels in our experiments (Fig. 5, Table S1), contrasting with results in animal cells in which IREG1/Ferroportin1 is highly induced by iron deficiency (45).

Comparison with Previously Reported Regulation Patterns of Transporter Genes—The expression profiles for 9 genes out of the 53 analyzed are available in the literature for comparison. Our array results were consistent with published data (Table III) with the exception of two NRAMP genes. The expression of NRAMP1 and NRAMP3 results contrasted with previous reports showing that members of this family are induced by iron deficiency in roots (10, 46). Differences in the growth conditions, treatment, and developmental stage could be reasons for the difference in expression patterns observed with NRAMP3. These factors may also account for the differences in the published expression patterns for NRAMP1. While expression of NRAMP1 in the roots under normal growth conditions is observed in one report (10), no expression of NRAMP1 is seen in the roots in normal growth conditions in an independent report (46). In addition, the results for NRAMP1 may be affected by the fact that NRAMP1 and NRAMP6 mRNAs have 85% sequence similarity over their entire length which could be sufficient for cross-hybridization on northern blots. For the metallothionein gene MT2a we observed the same tissue specificity as published. Based on our results, MT2a is among the most highly transcribed gene in Arabidopsis. Because of this, we were likely beyond the dynamic range of the DNA chips (47) and thus could not see a robust regulation by copper for this gene despite its known regulation by copper (48).

**DISCUSSION**

Genome-wide Analysis Provides Insight into Metal Transport—We have used Affymetrix Arabidopsis DNA chips containing ~8,300 genes (which cover about one-third of the ge-
ZIP proteins. This wide substrate specificity appears to be supported by work in Arabidopsis proteins in yeast mutants (5, 6, 26), suggesting wide substrate specificities for the ZIP family of proteins. Transcription of the genes is regulated in a way to prevent excessive uptake of the most toxic metal (copper) while ensuring proper zinc (the least toxic metal) uptake and transport. This is particularly striking for ZIP4, which is highly induced in zinc deficiency but is completely turned off in copper excess. Our results confirm that transcriptional regulation plays an important role in regulating the expression of the metal transporters and we have shown that knowledge of the transcriptional regulation patterns can yield information on the function of the protein encoded. However, post-transcriptional regulation should be taken into consideration when analyzing regulation of metal homeostasis, as shown for IRT1 an iron transporter of the ZIP genes family (7).

### Metal Homeostasis in Arabidopsis

**TABLE III**

| Gene      | Regulation by metals | Tissue-specific expression |
|-----------|----------------------|---------------------------|
|           | This work            | Published                  |
|           | Root-specific        | Root-specific (Northern)   |
|           | Leaves and roots     | Leaves and roots (Northern) |
|           | IRT1                 | Root-specific (Northern)   |
|           | ZIP4                 | Root-specific (Northern)   |
|           | NRAMP3               | Low expression in roots    |
|           | NRAMP1               | Roots > leaves (Northern)  |

| Gene      | Regulation by metals | Tissue-specific expression |
|-----------|----------------------|---------------------------|
| FRO2      | Iron-regulated       | Root-specific (Northern)   |
| ZIP4      | Zinc-regulated       | Leaves and roots (Northern) |
| AtFer1    | Iron-regulated       | Leaves and roots (Northern) |
| AtFer4    | Iron-regulated       | Leaves > roots (Northern)  |
| IRT2      | Iron-regulated       | Root-specific (Northern)   |
| ZAT1      | No regulation by metals deficiencies | Leaves > roots |
| MT2a      | Possible copper regulation in cotyledon (RT-PCR) | High expression in leaves (RT-PCR Northern) |
| NRAMP3    | No metal regulation  | Very low expression in roots and in leaves |
| NRAMP1    | No metal regulation  | Roots > leaves (Northern)  |

* N/A, not available.

**Strict and Multilevel Transcriptional Regulation by Metals**

The robust regulation we identified suggests an important role for transcription in metal homeostasis. Previous functional studies indicate that plant metal transporters have wide substrate specificities with limited ion selectivity. Overlapping specificities do not provide an obvious mechanism for plants to differentially regulate uptake of specific metals. One possibility is that specificity observed in heterologous systems utilized do not accurately reflect the in planta situation and additional proteins such as reductases may provide specificity. Alternatively, we suggest that the unique transcriptional response to each individual metal deficiency results in the expression of a specific mix of transporters with limited selectivity, which together provide effective combinatorial control of metal transport. Our results confirm that transporters of the ZIP family of genes play a fundamental role in regulating metal uptake as they appear to be the most highly regulated genes in response to metals deficiencies. Heterologous expression studies of ZIP proteins in yeast mutants (5, 6, 26), supported by work in Arabidopsis (7, 8), suggest wide substrate specificities for the ZIP proteins. This wide substrate specificity appears to be compensated for by a strict transcriptional regulation of the genes. Transcription of the genes is regulated in a way to prevent excessive uptake of the most toxic metal (copper) while ensuring proper zinc (the least toxic metal) uptake and transport. This is particularly striking for ZIP4, which is highly induced in zinc deficiency but is completely turned off in copper excess. Our results confirm that transcriptional regulation plays an important role in regulating the expression of the metal transporters and we have shown that knowledge of the transcriptional regulation patterns can yield information on the function of the protein encoded. However, post-transcriptional regulation should be taken into consideration when analyzing regulation of metal homeostasis, as shown for IRT1 an iron transporter of the ZIP genes family (7).
Atx1p and Csep in yeast is to overcome the high thermodynamic capacity for nonspecific binding of copper in the cell to ensure proper delivery of copper to metalloproteins and organelles while minimizing toxic free copper levels (53, 54). Interestingly, we have observed a decrease in the expression of the AtCCS in copper deficiency, which raises the question about whether AtCCS is down-regulated to shunt copper into other pathways.

Independent Systems of Copper and Iron Homeostasis in Arabidopsis—Copper plays a pivotal role in mammalian and yeast iron transport. These organisms rely on the activity of multicopper oxidases, such as ceruloplasmin (55, 56) and heme-oxin (57) in mammals and Fet3p (58) in yeast, for iron transport. Copper-dependant iron assimilation is also found in the green algae Chlamydomonas reinhardtii (59). In contrast, copper deficiency did not have a major effect on the expression of any iron regulated transporter or metal homeostasis gene in Arabidopsis (Table II) or on any other genes tested that are affected by iron deficiency (data not shown). Of the 8,300 genes tested, only 5 genes were co-regulated in response to both iron and copper deficiencies. More overlap was found between copper and zinc deficiencies (29 genes co-regulated), and zinc and iron deficiencies (32 genes co-regulated). This suggests that iron transport in plants may not depend on the activity of multi-copper oxidases like in yeast and in mammals.

Role of Nicotianamine Synthase and Oligopeptide Transporters in the Metal Deficiency Response—Arabidopsis, and dicotyledonous plants in general, differs from grasses in iron uptake strategy. Grasses such as corn, “Strategy II” plants convert NA into phytosiderophores (PSs) such as mugineic acid that are excreted into the soil to bind ferric iron. In maize, Fe(III)-PS complex are transported the into the roots by YS1 (60). All non-grass plants (“Strategy I” plants like Arabidopsis and tomato) lack the ability to synthesize and secrete PS and furthermore do not take up iron as the Fe(III)-PS complex. Instead, trans-membrane ferric reductases reduce and presumably release iron from Fe(III)-chelates (including phytosiderophores) for subsequent uptake by Fe(II) ion transporters. Despite this, the phenotype of the NA-less tomato chloronerva demonstrates that NA plays an important role in iron/metal homeostasis in Strategy I plants (61). Our results suggest that NAS genes are activated in metal deficiencies, and thus, NA participates in the response to metal deficiency (our data) as well as to iron toxicity (36). Induction of NAS in iron deficiency is also observed in monocots (62), suggesting that the regulation pattern of NAS genes has been conserved in monocots and dicots. It has been postulated that Arabidopsis homologues of YS1, the 8-member YSL (YS-like) family, may be involved in transporting Na-metal chelates within the plant (60). OPT and YSL are two divergent families of proteins (63) with homology to ISP4, a fungal oligopeptide transporter (64). Our yeast complementation experiments have shown that AtOPT3 can transport copper, manganese, and possibly iron as it was suggested by the transcriptional regulation pattern. However, we have no evidence that these metals are transported into yeast via AtOPT3 as a complex with nicotianamine. The potential role of NA in metal transport via AtOPT3 will have to be further addressed in planta. Interestingly, recent work by Koh et al. (38) has demonstrated that all members of the Arabidopsis OPT family can transport leu tetra- and pentapeptides with the exception of OPT2 and OPT3. It was also shown that OPT3 is required for embryo development and that it is expressed in vascular tissues consistent with a role in long distance transport of metals (49). Our results indicate that YSL1, the only YSL gene present on the chip, is not involved in the response to metal deficiency. Thus, both YLS and OPT could be involved in different aspects of metal transport.

Conclusion—Transcriptional regulation of genes plays an important role in metal homeostasis. By using transcription profiling we have been able to identify novel components of the metal transport machinery and an intricate pattern of regulation.

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