Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator Thlaspi caerulescens

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Abstract

Zinc (Zn) hyperaccumulation is a constitutive property of Thlaspi caerulescens, whereas cadmium (Cd) hyperaccumulation varies greatly among different ecotypes. The molecular basis of this variation is unknown. Ecotypic differences in the sequences and expression of four representative ZIP family transporter genes were investigated. Genome analysis indicated the presence of at least two closely related copies of the TcIRT1 gene in both Ganges (high Cd accumulating) and Prayon (low Cd accumulating) ecotypes, with different copies being expressed in each, and, furthermore, the two genes potentially encode different length transcripts. The predominant transcript in Prayon was truncated, missing sequence coding for the putative metal-binding site and the five C-terminal transmembrane helices. The two ecotypes were grown hydroponically and mRNA abundance determined for four ZIP genes. The four ZIP genes studied (TcIRT1, TcIRT2, TcZNT1, and TcZNT5) were expressed in roots only. TcIRT1 expression (full-length in Ganges, TcIRT1-1G; truncated in Prayon, TcIRT1-2P) was enhanced by Fe deficiency or by exposure to Cd. TcIRT2 expression was induced by Fe deficiency, but was unaffected by Cd exposure. TcZNT5-G showed greater expression in Prayon compared with Ganges. The functions of TcIRT1 from Ganges and Prayon and the Arabidopsis homologue were analysed by heterologous expression in yeast. All three IRT1 genes were able to facilitate growth on low Fe concentrations. Cd sensitivity of yeast was conferred in the order AtIRT1>TcIRT1-1G>TcIRT1-2P (truncated). Cd uptake after 4 h was only detectable following complementation by AtIRT1. The results suggest that although TcIRT1-G may be involved in Cd hyperaccumulation in the Ganges ecotype of T. caerulescens, the transporter expressed in yeast does not have an enhanced ability to transport Cd compared with AtIRT1. Therefore, the unique Cd-accumulating ability of the T. caerulescens Ganges ecotype must be due to the levels of expression of the protein or to other factors such as interacting proteins.

Key words: Cadmium, hyperaccumulation, IRT1, Thlaspi caerulescens, zinc, ZIP transporters.

Introduction

Metal hyperaccumulator is a term used to define plants that are able to accumulate metals (or metalloids) in their above-ground parts to extremely high concentrations, usually 100–1000-fold higher than those found in normal plant species (Baker et al., 2000; McGrath et al., 2002). Hyperaccumulators are highly tolerant to the metals they accumulate. There has been much interest over the last decade in the metal hyperaccumulation phenomenon, mainly because of the potential use of metal hyperaccumulators for phytoremediation or phytomining (Chaney et al., 1997; McGrath and Zhao, 2003). However, many hyperaccumulators are slow-growing, low-biomass plants and therefore not suitable for the purpose of
phytoremediation. One solution would be to transfer genes responsible for metal hyperaccumulation and detoxification to high-biomass plants. This approach requires an understanding of the molecular mechanisms of metal hyperaccumulation.

*Thlaspi caerulescens* J. & S. Presl (Brassicaceae) is a well-known zinc (Zn) hyperaccumulator, with the ability to accumulate and tolerate >25 000 mg Zn kg⁻¹ dry weight (DW) in the shoots (Baker et al., 1994; Shen et al., 1997). Zn hyperaccumulation is a constitutive and species-wide trait in *T. caerulescens*, although there is a considerable variation between different populations when grown under controlled conditions (Baker et al., 1994; Meerts and van Isacker, 1997; Escarré et al., 2000; Lombi et al., 2000; Assunção et al., 2003a; Roosens et al., 2003).

*Thlaspi caerulescens* is able to accumulate other metals, including Ni and Cd (Baker et al., 1994). When grown under the same conditions, different populations were found to vary widely in Cd accumulation (Lombi et al., 2000; Roosens et al., 2003). In particular, populations from southern France (Ganges ecotype) were far superior in Cd accumulation compared with those tested from other regions of Europe, including the Prayon population from Belgium that is widely used in physiological and molecular studies (Lombi et al., 2000; Roosens et al., 2003). Studies of metal influx showed the V_max for Cd uptake to be approximately five times higher in Ganges than Prayon (Lombi et al., 2001; Zhao et al., 2002). This suggested that either the transporters for Cd were more highly expressed in Ganges or that those expressed are more active. The Cd influx kinetics for Ganges also showed a saturable component at low Cd concentrations, which was not seen in Prayon, suggesting the existence of a specific transport system with high affinity for Cd, present predominantly in the Ganges ecotype. Cd uptake was significantly enhanced by Fe deficiency in the Ganges but not the Prayon ecotype (Lombi et al., 2002). In contrast, Zn uptake was not affected by the Fe status of the plants of either ecotype (Lombi et al., 2002). Recent studies using inter-ecotypic crosses of *T. caerulescens* suggest that multiple genes are involved in the accumulation of Zn (Assunção et al., 2003b) and Cd (Zha et al., 2004). The molecular mechanisms responsible for the extraordinary ability to hyperaccumulate Cd in the Southern French Ganges ecotype of *T. caerulescens* have not been elucidated.

A large number of transporter proteins are involved in the uptake and homeostasis of transition metals in plant cells, including members of the families ZIP (ZRT, IRT-like Proteins), NRAMP (Natural Resistance-Associated Macrophyge Proteins), P-type ATPases, and CDF (Cation Diffusion Facilitators) (Mäser et al., 2001). Notable examples are several ZIP transporters that mediate uptake of Fe(II) and Zn. Fifteen potential ZIP genes may be identified in the *Arabidopsis thaliana* genome. Of these, AtIRT1 has been demonstrated to be essential for Fe acquisition from the soil in *A. thaliana* (Connolly et al., 2002; Vert et al., 2002), whereas AtZIP1 and AtZIP3 may be responsible for uptake of Zn in the roots (Grotz et al., 1998). Expression of several ZIP genes is regulated by the status of Fe or Zn in plants (Guerinot, 2000). Some of the ZIP transporters are not metal-specific, but can transport different metals with varying affinities; for example, AtIRT1 is able to transport several divalent metal ions, including Cd, Co, Mn, and Zn (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002). In Fe-deficient *A. thaliana* there is evidence for a role for AtIRT1 in Cd uptake; *A. thaliana* plants engineered to overexpress AtIRT1 accumulated greater amounts of Cd than wild-type plants (Connolly et al., 2002).

*TcZNT1* was the first ZIP gene isolated from *T. caerulescens* Prayon, and mediates high-affinity Zn transport, as well as a low-affinity Cd uptake (Pence et al., 2000). *TcZNT1* has since been cloned from *T. caerulescens* Ganges (Lombi et al., 2002) and *TcZNT5* from Prayon (AF292029). *TcZNT1* is homologous to the *A. thaliana* gene AtZIP4, with 76% identity at the amino acid level. *TcZNT2* is a close homologue of *TcZNT1* (Assunção et al., 2001). *TcZNT1* and *TcZNT2* showed increased gene expression in *T. caerulescens* roots compared with the non-hyperaccumulator *Thlaspi arvense* (Pence et al., 2000; Assunção et al., 2001). Expression of both genes was hardly responsive to the Zn status of *T. caerulescens*, whereas they were expressed in *T. arvense* roots only when the plants were Zn-deficient. Constitutively high expression of *TcZNT1* and *TcZNT2* may explain the ability of *T. caerulescens* to hyperaccumulate Zn (Pence et al., 2000; Assunção et al., 2001). *TcIRT1-G*, cloned previously from the Ganges ecotype (Lombi et al., 2002), is a close homologue of *AtIRT1*, with 90.2% identity at the amino acid level. A previous study showed that the high expression of *TcIRT1* under iron deficiency in the roots of Ganges and no expression in Prayon corresponded to the ecotypic difference in the Fe deficiency-induced response in Cd uptake (Lombi et al., 2002). This, together with the fact that AtIRT1 can mediate Cd uptake in *A. thaliana*, implies a role for *TcIRT1* in Cd accumulation in *T. caerulescens*, particularly the Ganges ecotype. However, the functions of *TcIRT1* have not been analysed.

In the present study, four representative ZIP genes from both Ganges and Prayon ecotypes are cloned. Expression patterns in response to different Fe and Cd exposures are presented with the aim of identifying candidate genes that could explain the ecotypic difference in Cd accumulation. Furthermore, the functions of *TcIRT1* were analysed using heterologous expression in *Saccharomyces cerevisiae*.

**Materials and methods**

**Plant materials and culture conditions**

Seeds of two ecotypes of *T. caerulescens* J & C Presl were collected in Belgium (Prayon ecotype) and near St Laurent le
Minier, Southern France (Ganges ecotype). The seeds were germinated on a mixture of perlite and vermiculite moistened with deionized water and, after germination, with nutrient solution. After 21 d, seedlings were transferred to 1.0 l vessels (five seedlings per vessel) filled with a nutrient solution containing: 1000 μM Ca(NO₃)₂, 500 μM MgSO₄, 50 μM K₂HPO₄, 100 μM KCl, 10 μM H₂BO₃, 1.8 μM MnSO₄, 0.2 μM Na₂MoO₄, 0.31 μM CuSO₄, 0.5 μM NiSO₄, 100 μM Fe(III)-EDDHA [ethylene-diamine-di(o-hydroxy-phenylacetic acid)] and 5 μM ZnSO₄, buffered at pH 6.0 with 2 mM MES (2-morpholineoethanesulphonic acid; solution pH was adjusted with 1 M KOH so that the final concentration of KOH was about 1 mM). Nutrient solution was aerated continuously and renewed every 3 d. All plants were grown in a controlled environment with a 16 h day length, 20/16 °C day/night temperature and 60–70% relative humidity.

Iron treatments

Eight days after plants were transferred to the full nutrient solution, Fe treatments were imposed. Control plants (+Fe) were given full nutrient solution, and –Fe plants were given full nutrient solution without Fe(III)-EDDHA. Before applying the –Fe treatment, plant roots were washed in deionized water to remove any Fe remaining from the full nutrient solution. After 8 d of growth under these conditions plants were harvested, and roots and leaves were separated and frozen in liquid nitrogen. Tissue was stored at conditions plants were harvested, and roots and leaves were stored at –80 °C.

Cadmium treatments

Eight days after plants were transferred to full nutrient solution, 0 or 100 μM CdCl₂ was added to the nutrient solution. Each treatment was repeated three times. Plants were harvested on day 8 and stored at –80 °C.

Gene cloning

Cloning of T. caerulescens cDNAs, TcZNT1-G (G indicates Ganges) and TcIRT1-1G, from the Ganges ecotype was described previously (Lombi et al., 2002). The T. caerulescens cDNA fragments reported here were cloned using a 3′ RACE system for rapid amplification of cDNA ends (Invitrogen, Paisley, UK) or RT-PCR from total RNA extracted from Ganges and Prayon leaf or root tissue. For the first strand cDNA synthesis, TcIRT1-2P (P indicates Prayon) cDNA was amplified by PCR using the AUAP (5′-GGCCACGCGTCGAC-TAGTAC-3′) RACE primer (Invitrogen, Paisley, UK) and the same primer as used for TcIRT1-1G, which included the start codon (PCR conditions: initial denaturing for 3 min at 95 °C, 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 90 s at 72 °C with a final 5 min at 72 °C for elongation). TcZNT5 was amplified by RT-PCR using oligonucleotide primer sequences designed to the original accession (AF292029) (PCR conditions: initial denaturing of 3 min 95 °C, followed by 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 80 s at 72 °C, with a final 5 min at 72 °C for elongation). The TcZNT5 was amplified by RT-PCR using oligonucleotide primer sequences designed to the original accession (AF292029) (PCR conditions: initial denaturing for 3 min at 95 °C, 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 80 s at 72 °C, with a final 5 min at 72 °C for elongation). The TcZNT5 gene fragment, TcIRT2, was amplified via RT-PCR from the Prayon and Ganges ecotypes using oligonucleotide primers designed to the AtIRT2 sequence (Atg419680) (Vert et al., 2001) (PCR conditions: initial denaturing: 3 min 95 °C, followed by 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 80 s at 72 °C, with a final 5 min at 72 °C for elongation). TcZNT5 was amplified by RT-PCR using oligonucleotide primer sequences designed to the original accession (AF292029) (PCR conditions: initial denaturing for 3 min at 95 °C, 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 80 s at 72 °C, with a final 5 min at 72 °C for elongation). The primers used for RT-PCR were as follows: TcZNT5 primer 1, 5′-CCCTTAATTICCGAGGATTT-3′; TcZNT5 primer 2, 5′-ATTGAGCTCTTGAAATTTGA-3′; TcIRT2 primer 1, 5′-GACGCTTCCCTGTTTGCACG-3′; TcIRT2 primer 2, 5′-GAAACATACACCACAGGGT-3′.

After gel purification, the cDNA products from PCR were cloned into the pGEM-T Easy vector (Promega, Madison, WI) for transformation into Escherichia coli. Sequencing of the plasmid insert was performed on an ABI Prism 310 genetic analyser using Big Dye™ sequencing reagents (PE-Applied Biosystems, Foster City, CA).

Expression analysis

RNA was extracted from Prayon and Ganges roots or leaves by the method of Verwoerd et al. (1989). Ten micrograms of total RNA from each treatment was separated on a 1% (w/v) agarose gel containing 3% (v/v) formaldehyde and blotted onto a nylon membrane as described in Sambrook et al. (1989). Membranes were hybridized overnight at 65 °C using TcZNT1-G (AJ313521, 1078 bp; Lombi et al., 2002), TcIRT1-G (AJ320253, 1321 bp; Lombi et al., 2002), TcIRT2-G (AJ53844, 645 bp; this manuscript), and TcZNT5-G (1203 bp) cDNA fragments radiolabelled with [α-32P]dCTP using the Prime-A-Gene oligonucleotide labelling system (Promega). Membranes were washed to high stringency in 1× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and 0.1% (w/v) SDS at 65 °C before visualizing on Biomax MS autoradiography film (Eastman-Kodak, Rochester, NY). Between successive hybridizations, probes were stripped from nylon membranes with boiling 0.1% (w/v) SDS. Equal loading of RNA in each lane was verified by ethidium bromide staining.

Genomic DNA extraction

To obtain genomic DNA, 1.0–1.5 g of frozen T. caerulescens shoot tissue was ground to a fine powder and extracted in 0.2 M TRIS-HCl pH 7.5, 0.25 M NaCl, 25 mM EDTA, and 0.5% (w/v) SDS for 20 min at 60 °C with frequent mixing. After centrifugation at 14 000 g for 5 min, phenol/chloroform/isooamyl alcohol (IAA) (25:24:1 by vol.) was added to the supernatant and the aqueous phase separated by centrifugation at 14 000 g for 10 min. A second extraction with chloroform/IAA was performed. An equal volume of isopropanol was mixed with the upper aqueous phase and incubated at 4 °C for 20 min. The genomic DNA was pelleted by centrifugation at 14 000 g for 10 min and washed once with 70% (v/v) ethanol, spun at 14 000 g for 10 min, air-dried for 10–15 min, and resuspended in 50 μl of sterile water. The samples were afterwards treated with RNase A (10 mg ml⁻¹) (2.5 μl per 100 ml) for 1.5 h at 37 °C, extracted with phenol/chloroform/IAA and then with chloroform/IAA and precipitated with 1/10 vols of 3 M Na-acetate and 2.6 vols of absolute ethanol at –20 °C for 1 h. The genomic DNA pellets were collected by centrifugation at 14 000 g for 10 min and washed with 70% (v/v) ethanol, spun at 14 000 g for 10 min, air-dried for 10–15 min, and resuspended in 50 μl of sterile water.

Southern blotting and hybridization

For genomic Southern analysis, 25 μg of genomic DNA was digested with 60 U of BamHI, EcoRI, XhoI, or PstI, for 15 h at 37 °C. The resulting fragments were separated by electrophoresis on a 1.5% (w/v) agarose TRIS-acetate-EDTA gel, which was then depurinated (0.25 M HCl), denatured (1.5 M NaCl, 0.5 M NaOH) and neutralized (1.5 M NaCl, 0.5 M TRIS-HCl pH 7.5, and 0.001 M EDTA) prior to capillary blotting onto positively charged nylon membrane (Hybond N®, Amersham Biosciences, Buckinghamshire, UK) overnight using 20× SSC. The genomic DNA was UV-crosslinked (UV-Stratilinker 2400, Amsterdam, The Netherlands).

As hybridization probes, a TcIRT1 Ganges full-length DNA (TcIRT1-1G) and a partial 645 bp TcIRT2 Ganges cDNA probe covering nucleotides 1719–840 of the corresponding Arabidopsis AtIRT2 were used. Cross-hybridization could not be excluded because of the 72–73% nucleotide identity between the IRT1 and IRT2 probe. For probe preparation, 50 ng of DNA (probe) was denatured at 95 °C for 5 min and labelled with 5 μl of [α-32P]dATP (10 μCi ml⁻¹) as detailed by the manufacturer (RadPrime DNA Labeling System, Invitrogen, Paisley, UK) and purified by using the NucTrap probe purification column (Stratagene, Orange, CA).
The yeast strains were sequenced (four colonies of each in full agreement with the cDNA sequences. The cDNAs were sequenced by the lithium acetate method (Gietz et al., 2002) and the PCR conditions were initial denaturing for 3 min 95 °C, followed by 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 90 s at 72 °C, with a final elongation step for 5 min at 72 °C. The splicing sites were obtained by the alignment of the cDNA sequences. The cDNAs were sequenced by the lithium acetate method (Gietz et al., 2002) and the PCR conditions were initial denaturing for 3 min 95 °C, followed by 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 90 s at 72 °C, with a final elongation step for 5 min at 72 °C. The splicing sites were obtained by the alignment of the cDNA and the present genes using CLUSTAL W and confirmed with NetGene2 (http://www.cds.dtu.dk/services/NetGene2).

Genomic sequences

A 1 μg aliquot of genomic DNA of Ganges and Prayon ecotypes was used for PCR amplification of the respective genomic TcIRT1 sequences. The primers were the same as used in Lombi et al. (2002) and the PCR conditions were initial denaturing for 3 min 95 °C, followed by 40 cycles of 30 s at 95 °C, 20 s at 55 °C, and 90 s at 72 °C, with a final elongation step for 5 min at 72 °C. The splicing sites were obtained by the alignment of the cDNA and the present genes using CLUSTAL W and confirmed with NetGene2 (http://www.cds.dtu.dk/services/NetGene2).

Functional analysis

Constructs and transformation: RT-PCR was used to produce full-length TcIRT1-1G and TcIRT1-2P cDNA sequences. The cDNAs were sequenced (four colonies of each in full agreement with the corresponding genomic DNAs) before the cloning to ensure fidelity of clones used in the constructs for yeast transformation. TcIRT1-1G was amplified using the same primers as indicated above, whereas for the TcIRT1-2P sequence, a specific primer for the end of the insert was used (IRT1-PR-noncod, 5′-GGACACGGGATTGATATGAGATATTGATGTT-3′). The resulting PCR products were subcloned into the pGEWT-easy vector (Promega UK, Southampton, UK). Sequencing of the plasmid insert was performed on an ABI Prism 310 genetic analyzer using Big Dye sequencing (PE-Applied Biosystems, Foster City, CA) to ensure that no PCR errors had been introduced. The NotI-TcIRT1-1G or NotI-TcIRT1-2P fragments were ligated into the yeast expression vector pFL61 (Minet et al., 1992). The orientation of the insert was established by restriction digestion. pFL61 containing AdIR1 (pFL61/AdIRT1) was obtained from Professor M-L Guerinot (Dartmouth College, Hanover, USA) and Professor D Eide (University of Wisconsin-Madison, USA).

The yeast strains fet3fet4 DEY1453 (MATa/MATa ade2/can11 his3/his3 leu2/trp11 ura3/ura3 fet3-2/his3/fet3-2/His3 fet4-1::LEU2/fet4-1::LEU2) and parent strain (wild type) DEY1457 (MATa/MATa ade2/can11 his3/his3 leu2/trp11 ura3) were grown in standard defined media (1.7 g l−1 yeast nitrogen base, 5 g l−1 ammonium sulphate, 5 g l−1 D-glucose, 0.83 g l−1 amino acid supplements), supplemented with 0.2 mM FeCl3 in the case of the fet3fet4 mutant. The fet3fet4 strain was obtained from Professor S Thomine (Gif-sur-Yvette, France). These strains were transformed with pFL61, pFL61/TcIRT1-1G, pFL61/TcIRT1-2P, or pFL61/AdIRT1 constructs by the lithium acetate method (Gietz et al., 1992).

Yeast functional complementation: Minimal medium (SD) lacking uracil (-ura) was supplemented to generate the desired conditions.

To assess Cd sensitivity, 2.5, 5, 7.5, or 10 μM CdSO4 was used. For the iron complementation of the fet3fet4 mutant, plates without a supplement of 0.2 mM FeCl3 were tested. Three dilutions of the culture (of optical density at 600 nm of 0.2, 0.02, and 0.002) were spotted onto the plates from the left to the right. Growth was carried out for 4 d at 30 °C.

Cd-uptake in wild type yeast: Overnight cultures of single wild-type (WT) yeast colonies transformed with pFL61/TcIRT1-1G, pFL61/TcIRT1-2P, pFL61/AdIRT1, or the empty pFL61 vector were added to 200 ml volumes of SD-ura to obtain an OD600nm of 0.2 and incubated until an OD600nm of approximately 0.4 was reached (time 0). CdSO4(50 μM) was then added to each culture. Twenty ml volumes were removed from each culture, washed with 20 ml of 1 mM EDTA followed by sterilized deionized water (SDW) and the cells harvested by vacuum filtration 4 h after the initial addition of CdCl2. A sample was analysed in order to obtain the OD600nm. The yeast samples were digested with HNO3 at 160 °C, and Cd concentrations determined by inductively-coupled plasma mass spectrometry (ICP-MS, Agilent 7500ce, Agilent Technologies, Palo Alto, CA, USA).

Results

Gene expression in response to Fe treatments

Expression of four members of the ZIP gene family (TcIRT1, TcIRT2, TcZNT1, TcZNT5) was investigated in root and leaf tissues of the two ecotypes of T. caerulescens under Fe-sufficient or -deficient conditions (Fig. 1). TcZNT1 was expressed in the roots strongly and to a much lesser extent in the leaves of both T. caerulescens ecotypes (Fig. 1A). Little difference in the abundance of TcZNT1 mRNA was observed between the two ecotypes or between the +Fe and –Fe treatments. In contrast, the transcript abundance of TcZNT5 mRNA was higher in the Prayon roots than in the Ganges roots (Fig. 1B). There were no differences in the abundance of the other genes (Fig. 1C–D).

Northern blot analysis of total RNA extracted from root and leaf tissues. mRNA was detected by 18S rRNA was used as a loading control. The approximate sizes of the transcripts were TcIRT1-1G: 0.6 kbp, TcIRT1-2P: 0.6 kbp, TcZNT1: 1.3 kbp and TcZNT5: 1.3 kbp.

Fig. 1. Effect of Fe status on ZIP gene expression in the T. caerulescens Prayon and Ganges ecotypes. mRNA was detected by northern blot analysis of total RNA extracted from root and leaf tissues. Each lane contained 10 μg of total RNA. Expression of (A) TcZNT1-G (AJ313521), (B) TcZNT5-G (AJ937739), (C) TcIRT1-G (AJ320253), and (D) TcZRT2-G (AJ538344) in the roots and leaves of Ganges and Prayon. (E) 18S rRNA was used as a loading control. The approximate sizes of the transcripts are TcIRT1-1G: 1.4 kbp, TcIRT1-2P: 0.5 kbp, TcZNT2: 0.6 kbp, TcZNT1: 1.3 kbp and TcZNT5: 1.3 kbp.
was little change in the mRNA abundance between the Fe treatments for Prayon; however, for Ganges there was an apparent increase in \( TcZNT5 \) expression under Fe deficiency. No expression of \( TcZNT5 \) was observed in the leaves of either ecotype.

As previously reported (Lombi et al., 2002), \( TcIRT1 \) (\( TcIRT1-1G \)) was predominantly expressed in the roots of the Ganges ecotype. In addition, as shown here, a smaller mRNA (c. 0.5 kbp) from Prayon roots cross-hybridizes with the Ganges \( TcIRT1 \) probe (\( TcIRT1-2P \)) (Fig. 1C). The abundances of mRNAs hybridizing with \( TcIRT1 \) in both Ganges and Prayon were greatly increased in root tissue under Fe-deficient conditions when compared with Fe-sufficient conditions.

In contrast to \( TcIRT1 \), \( TcIRT2 \) was expressed in the roots of both ecotypes (Fig. 1D). The expression was enhanced greatly by Fe deficiency. No difference in the \( TcIRT2 \) transcript level was observed between the two ecotypes under either Fe-sufficient or Fe-deficient conditions. There was no expression of either \( TcIRT1 \) or \( TcIRT2 \) in the leaves.

**Analysis of \( TcIRT1 \) genes**

As indicated by the northerns (Fig. 1), the \( TcIRT1 \) transcripts for each ecotype differed in their size. The cDNAs for both of these transcripts were isolated and sequenced. Whereas Ganges expressed the full-size gene (\( TcIRT1-1G \)), the transcript of Prayon (\( TcIRT1-2P \)) was truncated, resulting in a polypeptide lacking the putative metal-binding site of the ZIP family and the five C-terminal transmembrane helices (Eng et al., 1998) (Fig. 2). The 3’-end non-coding region of Prayon [around 150 bp until the poly (A) tail] showed no similarity to any known genes. To investigate this further, the genomic structure was analysed (Southernns, Fig. 3). Probing genomic DNA with the full-length size \( TcIRT1-1G \), multiple \( TcIRT1 \)-hybridizing bands were apparent in each ecotype (Fig. 3A). To examine cross-hybridization with the closely homologous \( TcIRT2 \), the same membrane was re-hybridized with a \( TcIRT2 \)-specific probe, and a single band corresponding to the single copy of this gene in \( T. caerulescens \) was observed (Fig. 3B). Superimposing the two images indicates possible cross-hybridization of \( TcIRT2 \) and \( TcIRT1 \) (marked by an asterisk in Fig. 3C). At least two bands remain which are specific for \( TcIRT1 \), suggesting at least two copies of the \( IRT1 \) gene in each ecotype, given that none of the sequences contains \( BamHI \) or \( EcoRI \) sites. The multiple band pattern of the Ganges \( EcoRI \) hybridization may be a sign of other unidentified isoforms which are not apparent in the \( BamHI \) hybridization or may be because the genomic DNA was isolated from a population of non-isogenic seedlings. Using specific primers for the full and truncated length sequences (\( TcIRT1-1G \) and \( TcIRT1-2P \), respectively), two isoforms of \( TcIRT1 \) were amplified in

![Fig. 2. Alignment of Arabidopsis thaliana IRT1 (accession number NM_118089) and TcIRT1 putative amino acid sequences predicted from the genomic sequences, AM293353 and AM293350 from Prayon and Ganges ecotypes, respectively. Amino acids are shaded grey or black when conserved in two or three sequences, respectively. Transmembrane (TM) domains (Eide et al., 1996) are underlined with a broken line. The putative heavy metal binding site is underlined with a continuous line.](https://academic.oup.com/jxb/article-abstract/58/7/1717/515150)
the genomic DNA for each of the two ecotypes, corresponding to the genes for the full (TcIRT1-1G: AM293350 and TcIRT1-1P: AM293351, for Ganges and Prayon, respectively) and truncated forms (TcIRT1-2G: AM293352 and TcIRT1-2P: AM293353, for Ganges and Prayon, respectively) (Fig. 3D). A Vista alignment (http://genome.lbl.gov/vista/index.shtml) indicating homology of the genomic sequence of A. thaliana IRT1 with the four genomic sequences of TcIRT1 is shown in (E). Percentage similarities were calculated using a 100 bp moving average.

Functional analysis of TcIRT1-G in S. cerevisiae

The A. thaliana IRT1 has been shown to complement the yeast double mutant fet3fet4 (strain DEY1453), lacking the high- and low-affinity iron-uptake systems (Eide et al., 1996). Therefore, in order to investigate the role of TcIRT1 isoforms in Fe assimilation in yeast, fet3fet4 was complemented with the full-length TcIRT1-1G from Ganges and the truncated transcript from Prayon (TcIRT1-2P). Since the fet3fet4 mutant cannot grow under Fe-limited conditions, the ability of the transformants to grow on minimal medium without or with a supplement of Fe (200 μM) was tested. Transformants with the pFL61 control vector were only able to grow in the media supplemented with 200 μM Fe, whereas complementation with IRT1 from A. thaliana and T. caerulescens (including the truncated TcIRT1-2P) allowed growth in the normal minimum medium without addition of 200 μM Fe (Fig. 4A).
Expression of IRT1 from A. thaliana in wild-type yeast increases the sensitivity to the presence of Cd

To investigate this potential role of IRT1, wild-type yeast was complemented with IRT1 cDNAs of T. caerulescens and A. thaliana. In the presence of 10 μM CdSO₄ there was an increase of sensitivity to Cd in yeast transformed with AtIRT1 or TcIRT1-1G but not with TcIRT1-2P. At the lower Cd concentrations used, the presence of the A. thaliana IRT1 increased the sensitivity of the yeast the most (Fig. 4B). These yeast functional assays indicated that TcIRT1 is involved in the transport of Cd as well as Fe, but that in the case of Cd, the full-length transcript was required, suggesting a requirement for the full-length sequence, which includes the histidine-rich putative metal-binding site (see Fig. 2), in order to be able to transport Cd.

The uptake of Cd into yeast was investigated by measuring the amount of Cd taken up by yeast cultures using ICP-MS. Yeast complemented with IRT1 from Arabidopsis showed the greatest uptake after 4 h, whilst little or no uptake above that of the wild type was observed with TcIRT1-1G or TcIRT1-2P (Fig. 4C). These results indicated that TcIRT1-1G had no detectable Cd transport activity under the conditions used and at this time scale, a phenomenon observed by Hassinen et al. (2007) with TcMRP10. Initial Cd uptake rates were assayed using radiolabelled Cd; however, uptake was too low to determine the kinetics reproducibly.

Gene expression in response to Cd treatments

Expression of the ZIP genes was analysed in roots of T. caerulescens ecotypes in the absence or presence of Cd, when plants were supplied with sufficient Fe. Representative blots are shown in Fig. 5. TcZNT1 expression was not affected by Cd and expression levels were similar for both ecotypes. Similar to the Fe treatment experiment, the expression level of TcZNT5 in Ganges was below that seen for Prayon. In both ecotypes, there was no clear effect of Cd treatments on the expression of TcZNT5-G in the roots. Expression of TcIRT1-1G was observed in Ganges roots in the presence of Cd, even though, in this experiment, plants were grown with a sufficient level of Fe.
Expression of the truncated TcIRT1-2P was observed in Prayon roots in response to Cd. TcIRT2 transcript levels were not detectable in either ecotype, in the presence or absence of Cd.

Phylogenetic relationship of ZIP genes

It is probable that T. caerulescens homologues exist for most, if not all of the A. thaliana ZIP genes, and T. caerulescens homologues have been cloned from three out of four of the main clusters (shaded, Fig. 6).

The two TcIRT1 transporters align together along with the Arabidopsis homologue. Of the other T. caerulescens ZIP genes, TcIRT2 was cloned for the first time in the present study: for both Prayon and Ganges, the TcIRT2 cDNA fragment was 645 bp long, spanning nucleotides 189–840 of AtIRT2. The TcIRT2-G and TcIRT2-P fragments showed 99% identity at the nucleotide level and 98% identity at the protein level. They are close homologues of AtIRT2, with 91% identity at the amino acid level. When compared with AtIRT2, TcIRT2 from both T. caerulescens ecotypes has an extra histidine and glycine residue pair at amino acid 114 of accession AJ538344/AJ53844, an area thought to be involved in heavy metal binding (Eide et al., 1996). There is also a four amino acid insert in the Prayon and Ganges TcIRT2 sequences at amino acid 127, when compared with the equivalent position in the A. thaliana homologues.

The phylogenetic analysis indicates that TcZNT1 is a close homologue of AtZIP4 from A. thaliana (previously reported by Assunção et al., 2001) and TcZNT5 is a close homologue of ZIP5. For TcZNT5-P, the resulting cDNA fragment was 1023 bp long, spanning nucleotides 52–1073 of the ZNT5 sequence reported in the database (GenBank AF292029). The Ganges ZNT5 cDNA fragment (TcZNT5-G) was 1014 bp long, spanning the same region of ZNT5 as the Prayon fragment. However, a three amino acid segment was absent from TcZNT5-G at amino acid 20. TcZNT5-P and TcZNT5-G have 98% nucleotide identity. There are some discrepancies between the Prayon cDNA cloned here and that already reported in the database although the nucleotide identity is still over 99%. These small differences may be due to variation within the Prayon ecotype.

The sequence data indicate that ecotypic differences in amino acid sequence exist between the Prayon and Ganges gene homologues. The significance of these differences in terms of metal ion uptake requires investigation through functional analysis.

Discussion

Expression analysis

Physiological differences between the Ganges and Prayon ecotypes of T. caerulescens, in terms of Cd and Zn hyperaccumulation, have been well characterized (Lombi et al., 2001, 2002; Zhao et al., 2002; Roosens et al., 2003; Zha et al., 2004). These studies have provided strong...
physiological evidence that multiple uptake systems are involved in the root uptake of Zn and Cd in T. caerulescens. A number of ZIP genes have been found to be constitutively highly expressed in T. caerulescens (Pence et al., 2000; Assunção et al., 2001; Hammond et al., 2006) and in another Zn/Cd hyperaccumulator Arabidopsis halleri (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006), when compared with non-hyperaccumulator species. These genes may play a role in the hyper-accumulation of Zn and Cd. In the present study, ecotypic variations in the amino acid sequences and expression patterns of four ZIP genes representing three separate clusters of the ZIP family were investigated (Fig. 6).

The cloning of the respective ZIP gene homologues from both Prayon and Ganges led to the identification of amino acid sequence differences that may contribute to the differing abilities of the two ecotypes to accumulate Cd. The most significant difference was the truncated TcIRT1 transcript in Prayon, TcIRT1-2P. The resulting predicted polypeptide would be less than one-half in length, and lack the putative metal-binding site (HGHGHGHG) and five of the eight transmembrane domains (Fig. 2). It would be surprising if such a truncated protein could catalyse any transport function.

Other point differences between amino acid sequences were noted. Additional histidine and glycine residues (G116 and H117) were present in TcIRT2 (AJ538344 and AJ538345) but not AtIRT2 (NM_001036593 or NM_118088), and may be of particular interest due to the location in the potential metal-binding site (data not shown). Other amino acid substitutions identified may also be of importance as such substitutions have previously been shown to affect transport by ZIP proteins. For example, a single amino acid substitution in the yeast ZIP gene ZRT1 blocked endocytosis of the ZRT1 protein from the plasma membrane (Gitan and Eide, 2000). In the most comprehensive analysis to date (Rogers et al., 2000), 14 amino acid substitutions in the AtIRT1 sequence were investigated for effects on transport properties including ion selectivity. Six of these substitutions which were in the region deleted in TcIRT1-2P were essential for transport activity, suggesting that the truncated TcIRT1-2P would not have transport function. Altered transport properties were observed for single amino acid substitutions in the N-terminal region of AtIRT1; however, all of these are absolutely conserved in TcIRT1-1G (except an E103D conservative substitution) and TcIRT1-2P.

All four ZIP genes (TcZNT1, TcZNT5, TcIRT1, and TcIRT2) were expressed mainly in the root tissues, with little or no expression in leaves (Fig. 1). This is consistent with previous studies with A. thaliana (Grotz et al., 1998; Vert et al., 2001, 2002) and with T. caerulescens for TcZNT1 and TcZNT2 (Pence et al., 2000; Assunção et al., 2001), suggesting that the transporters are probably involved in root uptake or xylem loading of metals.

TcZNT1 expression was equally high in the roots of both ecotypes, despite the plants being grown with 5 μM Zn. Furthermore, expression of this gene was not responsive to different Fe or Cd treatments (Figs 1, 5). Pence et al. (2000) showed that TcZNT1 can mediate high-affinity Zn uptake, as well as low-affinity Cd uptake, when the gene was expressed in yeast. The constitutively high expression of TcZNT1 may be an important reason for the hyperaccumulation of Zn by this plant (Pence et al., 2000; Assunção et al., 2001). However, the superior Cd-accumulating ability exhibited by the Ganges ecotype is unlikely to be due to TcZNT1, because of its low affinity for Cd and the similar levels of expression in the two contrasting ecotypes. Although the TcZNT5 sequence has been submitted in the database (AF292029), there is little knowledge regarding the expression of this gene and its function. The expression of TcZNT5 was much higher in Prayon than in Ganges (Figs 1, 5). This ecotypic difference is notable, but is opposite to the pattern of Cd accumulation. This would rule out TcZNT5 as a major Cd transporter involved in the influx of Cd in Ganges roots. However, if TcZNT5 were to mediate Cd efflux from cytoplasm to the apoplast, the observed expression pattern could explain the ecotypic difference in Cd accumulation. TcZNT5 is most closely related to AtZIP5, and to a slightly lesser extent also to AtZIP3 (Fig. 6). No expression or functional data currently exist for AtZIP5, but AtZIP3 is expressed in Zn-deficient A. thaliana roots and can rescue a Zn-uptake defective mutant of yeast (Grotz et al., 1998).

A previous study showed that the expression of TcIRT1-IG was highly inducible under Fe deficiency in the Ganges ecotype, but little or no equivalent transcript was detectable in the Prayon ecotype (Lombi et al., 2002). In addition, a TcIRT1-IG homologue transcript could not be isolated from the Prayon ecotype by RT-PCR. This ecotypic difference was further confirmed in the present study (Fig. 1). The greatly increased transcript abundance of TcIRT1-IG in the Ganges ecotype in response to Fe deficiency corresponded to the greatly increased $V_{\text{max}}$ for Cd influx and Cd accumulation in the shoots of Ganges under Fe deficiency (Lombi et al., 2002). The expression was enhanced by Cd exposure in an experiment with sufficient Fe supply (Fig. 5). This could be due either to a direct effect of Cd or an indirect effect through Cd-induced Fe deficiency. However, the lack of expression of TcIRT2-G in the roots of both ecotypes in this experiment (see below) suggests that there was no Fe deficiency. The low or zero expression of a full-length TcIRT1 in the Prayon ecotype, even under Fe deficiency, implies that this ecotype must rely upon the truncated TcIRT1 (which appears to be able to transport Fe, see Fig. 4A) or other Fe transporters, such as other ZIP transporters or Nramp transporters, to acquire Fe. For example, it is possible that Prayon relies on TcIRT2 for the acquisition of Fe. Expression of TcIRT2-G was highly inducible under Fe
deficiency in both ecotypes, and there was little difference between the ecotypes in TcIRT2-G transcript level (Fig. 1). Unlike TcIRT1-G, expression of TcIRT2-G was not detectable in Fe-replete plants of either ecotype in the Cd exposure experiment (Fig. 5). The expression data suggest that TcIRT2-G is unlikely to play a significant role in the root uptake of Cd. Nor does it explain the ecotypic difference in Cd accumulation.

**Gene structure**

Quite surprisingly, a truncated transcript lacking the putative metal-binding site and the five C-terminal transmembrane helices has been identified in the low Cd-accumulating ecotype, which is unable to transport Cd as shown by the yeast functional assays (Fig. 4B). Truncated but not full-length transcripts were found in another low Cd-accumulating population (Bizkaia, Spain) of T. caerulescens (data not shown), suggesting that this mechanism is not restricted to Prayon but may be responsible for the high variability of this species for Cd accumulation.

The two ecotypes are similar at the genomic level, with each possessing two copies of TcIRT1, one corresponding to the full-length and one to the truncated genes. Differential expression suggests that the promoter regions or transcript stability must be different in the two ecotypes. Examination of promoters of each transcript for each ecotype is necessary to understand fully the mechanisms behind this differential transcription.

In A. halleri, several candidate genes encoding metal transporters have been found to have multiple gene copies, including MTP1, HMA4, ZIP3, ZIP6, and ZIP9, whose expression levels were also much elevated compared with A. thaliana (Dräger et al., 2004; Talke et al., 2006).

**Functional analysis**

AtIRT1 has been shown to mediate uptake of Fe(II) and Cd across plasma membranes of root cells in A. thaliana (Connolly et al., 2002; Vert et al., 2002). As the expression patterns of TcIRT1-1G were suggestive of a role in Cd uptake, this gene was subjected to functional characterization by heterologous expression in yeast. In agreement with the results reported for AtIRT1 (Eide et al., 1996; Korshunova et al., 1999; Rogers et al., 2000; Connolly et al., 2002), TcIRT1-1G as well as the truncated version were able to complement the Fe-uptake-deficient yeast mutant fet3fet4. The surprising complementation of the fet3fet4 mutant by the truncated version may indicate that the five C-terminal transmembrane helices and the putative metal-binding site are not critical for Fe transport. As kinetics of Fe transport were not examined, it is impossible to say to what extent Fe transport is modified; it may be speculated that the truncated version may allow some permeability of Fe, perhaps in a multimeric con-formation. The presence of TcIRT1-1G, at the level of expression achieved, did not apparently facilitate significant Cd uptake at the concentration and at the time scale (4 h) used (Fig 4C); however, an increase in the sensitivity of wild-type yeast strains to Cd was observed (after 3 d), whilst the truncated version did not modify the sensitivity of the wild-type strain to Cd. At low concentrations, the sensitivity of the yeast Wt-pFL61/AtIRT1 was higher than for Wt-pFL61/TcIRT1-1G, suggesting a superior transport function of the Arabidopsis transporter compared with Thlaspi at this low concentration (2.5–7.5 μM Cd in the agar medium), or possibly a higher level of expression (abundance). The yeast functional assays suggest that the putative metal-binding site is involved in Cd transport but not that of Fe in yeast. The medium used (SD) in all the experiments contains 1.23 μM Fe and 2.48 μM Zn, which is unlikely to have any direct competitive effects on Cd uptake (50 μM; Fig. 4C), although post-transcriptional regulation by the presence of these ions has been suggested (Connolly et al., 2002).

**Summary**

The results from the present study are inconclusive, but not inconsistent with the suggestion that TcIRT1-1G is involved in Cd uptake by the Ganges ecotype, as shown by an increase of Cd sensitivity of the yeast wild type (normally naturally resistant), due to the presence of a full-length cDNA, particularly under Fe deficiency. The fact that TcIRT1-1G was highly expressed in Ganges under Fe-sufficient conditions in the presence of Cd (Fig. 5) may explain the superior Cd-accumulating ability of this plant when compared with Prayon. Conversely, the suppressed expression of a full-length IRT1 transcript in Prayon under any of the observed conditions may be an adaptive strategy to limit metal uptake in this accession. Alternatively, it is possible that an as yet uncloned homologue (as indicated by the Southern data) may be responsible for the observed uptake. In all cases it is likely that patterns or levels of expression or post-transcriptional control rather than a specific characteristic of this transporter contribute to the observed Cd accumulation abilities of the different accessions. Other gene family members still require functional analysis to determine their role in metal ion uptake and hyperaccumulation. In addition, this study demonstrates the need for ecotype-specific studies.

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Metal transporter genes in Thlaspi caerulescens

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