TpIRT1 is a transition metal transporter in Polish Wheat (*Triticum polonicum* L.) with a broad substrate specificity

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Abstract

Aims (1) Explore the metal substrate specificity of homologous TplIRT1A and TplIRT1B transporters from dwarf Polish wheat by expressing them in protoplast, yeast, and transgenic *Arabidopsis*; (2) screen polymorphic residues of IRT1 homologs from tetraploid and diploid ancestral wheat species that change the substrate specificity.

Methods Two *IRT1* homoeologs were isolated from A (*TpIRT1A*) and B (*TpIRT1B*) genomes of a tetraploid crop, polish wheat (*Triticum polonicum*). Both of them were analysed by expressing them in yeast and *Arabidopsis* protoplast, respectively. Then we constructed over-expressing transgenic plants of *TpIRT1B* for metals property analysis in *Arabidopsis*. We also isolated 22 *IRT1* homoeologs from tetraploid and diploid ancestral wheat species and expressed them in yeast for function analysis.

Results Our data highlighted the importance of TplIRT1 in the uptake and translocation of Fe, Mn, Co, and Cd with direct implications for wheat yield potential. Both TplIRT1A and TplIRT1B were located at the plasma membrane and internal vesicles in *Arabidopsis* protoplasts, and responsible for Cd and Co sensitivity in yeast. The over-expression of *TpIRT1B* in *A. thaliana* increased Fe, Mn, Co, and Cd concentration in its tissues and improved plant growth under Fe, Mn, and Co deficiencies, while causing more sensitivity to Cd than wild-type plant. Functional analysis of *IRT1* homoeologs from tetraploid and diploid ancestral wheat species in yeast disclosed four distinct amino acid residues in TdiIRT1B (*T. dicoccum*) and TtuIRT1B (*T. turgidum*). Altogether, these results increase the knowledge of IRT1 function in a global crop, wheat.

Introduction

Adequate uptake of micronutrients including iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) from the soil into plant roots, root-to-shoot delivery and lateral distribution is critical for the growth and development of plants (Kumar et al. 2009; Kobayashi and Nishizawa 2012; Yamaji et al. 2013). Thus, micronutrient deficiency in soils adversely influences crop growth and yield and results in a poor quality of plant-based food, which, in turn, negatively influences the health and well-being of the world's population (Grotz and Guerinot 2006). However, the over-accumulation of Fe, Zn, and Mn in plant cells causes toxicity with the detrimental consequences for crop growth and yield as well (Uriu-Adams and Keen 2005; Valko et al. 2005). Additionally, highly toxic and non-essential metals, such as cadmium (Cd) and lead (Pb), compete with micronutrients for uptake and internal transport, posing a threat to plant growth and human health (Benavides et al. 2005; Wierzbicka et al. 2007; Zhai et al. 2014).

A number of mineral nutrient transport proteins facilitate a controlled absorption of minerals from the soil into root epidermal cells, internal mineral elements transport and loading to edible plant tissues, including grains (Gayomba et al. 2015). These transporters include heavy metal ATPases (HMAs) (Sasaki et al. 2014; Wang et al. 2018), natural resistance-associated macrophage proteins (NRAMPs) (Pottier et al. 2015), cation diffusion facilitators (CDFs) (Williams et al. 2000), ZRT/IRT-related protein family (ZIPs)

(Guerinot 2000), ABC transporters (Cheng et al. 2011; Song et al. 2014), Yellow Stripe-Like (YSL) proteins (Curie et al. 2001), Cu Transporters (COPTs) (Yuan et al. 2011), and oligopeptide transporters (OPTs) (Zhai et al. 2014). Among these transporters, Iron Regulated Transporters (IRTs) that belong to the ZIP family have been functionally characterized in many plant species (Korshunova et al. 1999; Ivanov et al. 2012; Urzica et al. 2012). It is well-established now that IRT1-like proteins are the main contributors to Fe^{2+} transport in non-grass-species that primarily rely on the reduction-based strategy for Fe uptake (Korshunova et al. 1999; Vert et al. 2001, 2002; Cohen et al. 2004; Schikora et al. 2006; Barberona et al. 2014). In contrast, grasses, including major cereal crops, rice, maize and wheat, mainly use the chelation-based strategy and rely on members of the YSL transporter family mediating transport of Fe-phytosiderophore chelates (Inoue et al. 2009; Lee et al. 2009c; Araki et al. 2011; Kakei et al. 2012; Senoura et al. 2017; Zhang et al. 2018). In addition, rice, maize, and barley also possess functional IRT1 homologs (Ishimaru et al. 2006; Li et al. 2015; Long et al. 2017).

Concerning IRT1-like proteins in non-grass species, the *A. thaliana* genome possesses three *IRTs*: *AtIRT1*, *AtIRT2*, and *AtIRT3* (Vert et al. 2001, 2002; Lin et al. 2009). *AtIRT1* localizes to the plasma membrane and participates in the absorption of essential metals including Fe, Zn, Mn, cobalt (Co), and also toxic metals such as Cd and Ni from the soil to root epidermal cells (Korshunova et al. 1999; Rogers et al. 2000; Nishida et al. 2011; Barberona et al. 2014). *AtIRT2* localizes to intracellular vesicles and plays a role mainly in Fe and Zn compartmentalization into internal storage vesicles to alleviate metal toxicity (Vert et al. 2001, 2009). *AtIRT3*, localized to the plasma membrane and facilitates the uptake of Fe and Zn but not of Cd and Mn (Lin et al. 2009). IRT1 homolog from pea (*Pisum sativum*), *PsIRT1* mediates Fe and Zn uptake as evidenced by its ability to complement growth of *Saccharomyces cerevisiae* Fe and Zn uptake mutants (Cohen et al. 1998, 2004); *PsIRT2* localizes to the mitochondria and controls Fe transport in the vasculature (Alagarasan 2016). In tomato, both *LeIRT1* and *LeIRT2* transport Fe, Zn, Mn, and Cu (Schikora et al. 2006).

Concerning IRT1 homologs in grasses, rice (*Oryza sativa*), possesses two IRT1-like proteins, *OsiIRT1* and *OsiIRT2* that contribute to the uptake of Fe, Zn and Cd (Bughio et al. 2002; Ishimaru et al. 2006) but not Mn, Co, and Cu (Nakanishi et al. 2006; Lee et al. 2009a, b). Maize IRT1 homolog, *ZmIRT1*, localizes to the plasma membrane and endoplasmic reticulum of silk and embryos, and is involved in Fe and Zn transport (Li et al. 2015). IRT1 from barley (*Hordeum vulgare*), *HvIRT1*, plays an essential role in Mn uptake, translocation and grain accumulation (Long et al. 2017). These results indicate that although IRTs have broad transport substrate specificities and transport both essential and non-essential metals, their transport specificities and cellular localization differ among different species. Here, we thought to characterize IRT1 homoeologous from wheat. We specifically focused on Dwarf Polish Wheat (DPW) because it can accumulate high concentrations of Cd, Zn, and Fe in its seedlings without showing toxicity symptoms (Wang et al. 2016). DPW (*Triticum polonicum* L., $2n = 4x = 28$, AABB) was originally collected from Tulufan, Xinjiang province, China, by Prof. Chi Yen of the Sichuan Agricultural University, China. In the present study, two *IRT1* homoeologous, *TpIRT1A* and *TpIRT1B* have been isolated from DPW and analyzed for the tissue specificity of their expression, the subcellular localization and metal transport

capabilities by using functional complementation studies in yeast and over-expressing *TpIRT1B* in *A. thaliana*. We have also initiated functional analysis of *TpIRT1* homologs from tetraploid (*T. dicoccum*) and diploid ancestral (*T. turgidum*) wheat species.

Materials And Methods

Plant materials and growth conditions

Dwarf Polish wheat (DPW, *Triticum polonicum* L., $2n = 4x = 28$, AABB) were grown either hydroponically in the greenhouse or in the Wenjiang experimental field of Triticeae Research Institute (30.6822° N, 103.8566° E), Sichuan Agricultural University, Sichuan, China as detailed below. For analyses of the expression pattern of *TpIRT1* homoeologs in different tissues, seeds of DPW were sowed on October 29th, 2015 and October 30th, 2016. Field trials were performed in a randomized complete block design with three replications, and each plot included one row with 20 plants. Tissues were collected at the three growth stages including the jointing stage (root, basal stem, leaf sheath, leaf blade, and young leaf), flowering stage (root, stem, leaf, leaf I, flag leaf, sheath, node, rachis, rachilla, lemma, palea, awn, ovary, and anther), and filling stage (root, stem, leaf, leaf I, flag leaf, sheath, node, rachis, rachilla, lemma, palea, awn, and immature grain). All tissues were frozen in liquid nitrogen, then stored at -80 °C for RNA isolation.

To impose Fe, Zn, Mn or Co deficiency, or Cd toxicity, the uniform-size seeds were sterilized for 15 min in 5% (m/v) sodium hypochlorite (NaClO), rinsed five times with double-distilled water (ddH₂O) and germinated on two layers of filter paper in glass Petri dishes. After five days of growth, uniform-size seedlings were transplanted into lucifugal plastic containers with a full Hoagland nutrient solution that was changed once per week. The full Hoagland nutrient solution contained 5 μM ZnSO₄, 250 μM FeCl₃, 350 μM MnCl₂, 8 μM CoCl₂. After two weeks of growth, a subset of seedlings was transferred to the same medium or to the medium lacking either ZnSO₄, or FeCl₃, or MnCl₂, or CoCl₂, or containing 25 μM CdSO₄. After additional one week of growth, roots of plants were collected, frozen in liquid nitrogen, and stored at -80° C for RNA isolation. In these experiments, plants were grown in the growth chamber under a 16 h light/8 h dark photoperiod, an irradiance of 150 μmol mEm⁻² s⁻¹, a constant temperature of 23° C, and relative humidity of 75%. Each sample contains tissues that were pooled from five plants and experiments were repeated three times. Transgenic *Arabidopsis* lines generated as described below were grown in the growth chamber under 120 mEm⁻² s⁻¹ illumination intensity, a 16 h light/8 h dark period, a constant temperature of 22° C, and 50% humidity.

RNA extraction, cDNA synthesis, and quantitative Real-Time PCR

Total RNA was extracted using the Total RNA Kit II (Omega Bio-tek, Georgia, USA). The cDNA was synthesized from 2 μg of total RNA using the M-MLV First Strand cDNA Synthesis Kit (Omega Bio-tek, Georgia, USA). qRT-PCR was performed in 96-well plates with CFX-96™ system (Bio-Rad Laboratories,

California, USA) according to the user manual. The *Actin* gene (Forward-CCGATTGCTTGTTATCTGTT and Reverse-GAGGATGAAGACGAGAGTTT) was used to normalize the relative expression level of *TpIRT1* (Wang et al. 2015). The relative expression was calculated using the software of *Bio-Rad CFX manager* v3.1 with the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Statistical analysis was performed using Student's *t*-test ($p < 0.05$) in the software of *IBM SPSS Statistics* v22 (International Business Machines Corporation, New York, USA).

Gene cloning, bioinformatics, and phylogenetic analysis

The full-length cDNAs of *TpIRT1A* and *TpIRT1B* were amplified from leaves. PCR primers were selected based on the reference sequence of the wheat genome (Wang et al. 2016) were designed using *Beacon Designer* v7.0 (PREMIER Biosoft International, California, USA) (Table S1). Ten tetraploid wheat and two diploid ancestral species were used to investigate *IRT1* homoeologs with the same primers (Table S2). Deduced amino acid sequences, gene structures, chromosome localization and phylogenetic analysis were predicated according to Jiang et al. (2017). Putative transmembrane (TM) domains were identified using *TMHMM Server* v2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>).

Functional complementation assays in *Saccharomyces cerevisiae*

The open reading frames (ORFs) of *TpIRT1A* and *TpIRT1B* were sub-cloned into the yeast expression vector pYES2 (Invitrogen, Carlsbad, USA) using primers with the *Hind*III and *Xba*I sites (Table S1). These constructs and the empty vector (EV) were transformed into different yeast mutants as well as the isogenic wild-type BY4743 (*MATa; his3Δ1; leu2Δ0; met15Δ0; lys2Δ0; ura3Δ0*), Cd-sensitive mutant $\Delta ycf1$ (*MATa; his3Δ1; leu2Δ0; lys2Δ0; met15Δ0; ura3Δ0; YDR135c::kanMX4*), Zn-sensitive mutant $\Delta zrc1$ (*MATa; his3Δ1; leu2Δ0; lys2Δ0; met15Δ0; ura3Δ0; YMR243c::kanMX4*), and Co-sensitive mutant $\Delta cot1$ (*YK40*) (*MATa; ura3-52 his3-200, Δcot1*) (Euroscarf Lab, Germany). Transformants were selected for uracil prototrophy on yeast nitrogen base drop-out medium lacking uracil (SD-Ura) (Sigma, Missouri, USA) and by colony PCR using primer pairs specific to each *TpIRT1* homoeolog. Functional complementation assays were performed on a solid or liquid SD-Ura media as described by Peng et al. (2018a) with minor modification for the concentration of mineral elements Cd (0 μ M and 40 μ M), Zn (0 μ M and 4 mM), and Co (0 μ M and 250 μ M). For the functional complementation assays in a liquid medium, the single yeast colony was selected and grown overnight in SD-Ura liquid medium. The overnight culture was then diluted to an $OD_{600} = 0.8$ and one mL of the diluted yeast culture was added to a 50 mL of the SD liquid media with 1 mM $ZnCl_2$, 20 μ M $CdCl_2$, or 100 μ M $CoCl_2$, respectively. OD_{600} was measured every 12 hours using a microplate spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) with three replicates per each measurement. Each experiment was repeated three times.

The subcellular localization of *TpIRT1A* and *TpIRT1B* in the leaf protoplast of *A. thaliana*

The ORFs of *TpIRT1A* and *TpIRT1B* without stop codon were individually fused at the C terminus with the modified green fluorescent protein (EGFP) and placed under the control of the cauliflower mosaic virus

35S promoter (*CaMV35s*) in the *pSAT6-N1-EGFP-Gate* vector (Jung et al. 2012). *TpIRT1A-EGFP* and *TpIRT1B-EGFP* constructs and the vector lacking cDNA inserts were transfected into *A. thaliana* protoplasts prepared as described by Jung et al. (2012). EGFP-mediated fluorescence and chlorophyll auto-fluorescence were visualized and collected using FITC (for EGFP) or rhodamine (for chlorophyll) filter sets of the Axio Imager M2 microscope equipped with the motorized *Z-drive* and the high-resolution 25 Axio Cam MR Camera (Zeiss, Oberkochen, Germany).

The generation and characterization of transgenic *A. thaliana* ectopically expressing *TpIRT1B*

The ORF of *TpIRT1B* with the stop codon was inserted between *HindIII* and *XbaI* sites of the pCambia1305.1 vector (Jefferson et al. 1987). The recombined constructs and the vector lacking the cDNA insert were individually transformed into *A. thaliana* wild-type (*cv. Col-0*) by floral infiltration (Bent et al. 1994). Transgenic plants were selected on ½ MS medium for the resistance to 25 mg/L hygromycin. The presence of *TpIRT1B* was also verified by PCR and sequencing using primer pairs *TpIRT1B-HindIII-F* and *TpIRT1A/B-XbaI-R* (Table S1). The T4 generation of homozygous lines was used for analyses. The expression level of *TpIRT1* in transgenic lines was analyzed by qRT-PCR as described above.

To test the role of *TpIRT1B* in mineral element deficiency and Cd toxicity, transgenic plants and *A. thaliana* wild-type were grown hydroponically. To do so, seeds were sterilized and treated with 4°C for two days, then sowed onto ½ MS media. After one week, the uniform-size seedlings were transplanted into the fresh hydroponic medium containing 1.25 mM KNO₃, 0.5 mM MgSO₄, 0.625 mM KH₂PO₄, 0.5 mM Ca (NO₃)₂, 2.5 µM NaCl, 10 µM Fe (III)-HBED, 3.5 µM MnCl₂, 0.25 µM ZnSO₄, 0.125 µM CuSO₄, 17.5 µM H₃BO₃, 0.05 µM Na₂MoO₄, and 0.025 µM CoCl₂. After two weeks of growth, a subset of plants was transferred either to the same medium or a medium with the reduced concentration of ZnSO₄ (50 nM) or Fe (III)-HBED (2.5 µM), or MnCl₂ (3.5 µM), or CoCl₂ (2.5 nM). A subset of plants was also transferred to a hydroponic medium with 20 µM CdCl₂. Root lengths were measured after five days of growth for a subset (eight plants/line) of plants. In addition, roots and leaves were collected after two weeks of growth, dried at 80°C for two days, and dry weight was analysed.

To investigate tolerance to excess metals, the sterilized seeds of control and *TpIRT1B*-overexpression lines were sowed on ½ MS medium plates with normal or elevated concentrations of one of the following mineral elements: Fe(III)-HBED (250 µM), MnCl₂ (0.8 mM), CoCl₂ (25 µM), CdCl₂ (20 µM), or ZnCl₂ (15 µM). After one week of growth, plants were imaged and root lengths were measured from eight plants per line, per experiment (n = 3).

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Different plant lines were grown hydroponically for four weeks as described above. Plants were collected and mineral elements were desorbed from the root surface as described by Zhai et al. (2014). Briefly, plant roots were washed for 10 min in a medium containing 10 µM EDTA (ethylenediaminetetraacetic acid), then transferred for 5 min to a medium containing 0.3 µM BPS (4,7-diphenyl-1,10-

phenanthrolinedisulfonic acid) and 5.7 mM Na₂S₂O₃, followed by five sequential washes in ddH₂O. Roots were then dried in 80° C, ground, digested in 80% nitric acid at 220–280° C, and plant residue was dissolved in 25 mL of ddH₂O. Mineral concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Fisher Scientific, Massachusetts, USA).

Perl's staining with DAB/H₂O₂ Intensification

The Perl's staining with DAB/H₂O₂ (3,3'-diaminobenzidine tetrahydrochloride/Hydrogen peroxide) was performed according to Brumbarova and Ivanov (2014). Briefly, the five-day-old seedlings grown on ½ MS media were collected and washed with ddH₂O. Samples were vacuum-infiltrated (500 mbar, 30 min) with the fixation solution containing (methanol: chloroform: glacial acetic acid in 6:3:1 ratio) and washed with ddH₂O for 1 min three times. Fixed samples were incubated with the pre-warmed staining solution (4% K₄Fe(Cn)₆ : 4% HCl in 1:1 ratio) for 15 min under vacuum (500 mbar) and washed with 0.1 M phosphate buffer (pH 7.4) three times. For the intensification reaction, samples were applied with intensification solution (0.1 M Phosphate buffer (pH 7.0) containing 0.025% DAB, 0.005% H₂O₂, and 0.005% CoCl₂) for 10 min, then the reaction was terminated by rinsing with ddH₂O. Samples were stored in ddH₂O one week prior to analyses. Images were collected using the Axio Imager M2 microscope equipped with the motorized *Z-drive* (Zeiss, Oberkochen, Germany).

Results

Cloning and characterization of *TpIRT1A* and *TpIRT1B*

TpIRT1A and *TpIRT1B* cDNAs were cloned from A and B genomes of DPW, respectively. The full-length cDNA of *TpIRT1A* included a 127 bp 5'-UTR, 1110 bp ORF, and 383 bp 3'-UTR; the full-length cDNA of *TpIRT1B* included a 124 bp 5'-UTR, 1119 bp ORF, and 104 bp 3'-UTR (Table S3). Alignment of the sequenced *TpIRT1A* and *TpIRT1B* to the reference sequence of the hexaploid wheat genome (The International Wheat Genome Sequencing Consortium 2018; Elizabeth 2018) revealed that *TpIRT1A* and *TpIRT1B* are localized on the chromosome 4AL (TRIAE_CS42_4AL_TGACv1_290140_AA0982100) and 4BS (TRIAE_CS42_4BS_TGACv1_328611_AA1090980), respectively, and each gene included an intron and two exons. Analysis of the deduced 370 and 373 amino acid sequences of *TpIRT1A* and *TpIRT1B* polypeptides, respectively, revealed that *TpIRT1A* shares 95.44% amino acid identity to *TpIRT1B* and each polypeptide contained eight predicted transmembrane domains (Table S3).

The expression pattern of *TpIRT1A/B* in DPW

To analyse the tissue-specificity and dynamics of the expression pattern of *TpIRT1A* and *TpIRT1B* we used plants at different developmental stages. Due to the high sequence identity of *TpIRT1A* and *TpIRT1B* genes, we were not able to identify specific regions for distinguishing the expression pattern of *TpIRT1A* from *TpIRT1B*. Thus, the results below show the expression pattern for both *TpIRT1A* and *TpIRT1B*, and indicated from here on as *TpIRT1A/B*. We found that *TpIRT1A/B* was highly expressed in

flag leaves, followed by roots and lemma at the flowering stage (Fig. 1A). The high expression of *TpIRT1A/B* in flag leaves and roots was maintained throughout the grain filling stage (Fig. 1B). In this developmental stage, the expression of *TpIRT1A/B* was also evident in reproductive tissues including lemma, palea, awn, rachilla, and grain (Fig. 1B). At the jointing stage, *TpIRT1A/B* was highly expressed in old leaves and was also evident in roots with the lowest expression in young leaves (Fig. 1C). The expression of *TpIRT1A/B* was up-regulated only in response to Mn, but not to Fe, Zn or Co deficiencies (Fig. 1D). In contrast, the expression of *TpIRT1A/B* was considerably up-regulated by Fe, Mn, Co, and Cd, but not Zn excess (Fig. 1E).

Subcellular localization of *TpIRT1A* and *TpIRT1B*

To establish the cellular localization of *TpIRT1A* and *TpIRT1B*, *TpIRT1A-EGFP*, *TpIRT1B-EGFP* and the empty *pSAT6-N1-EGFP-Gate* vector were transiently expressed in protoplasts isolated from *A. thaliana* mesophyll cells. The EGFP signal from the *pSAT6-N1-EGFP-Gate* vector was found in the entire cell (Fig. 2A-C). The fluorescence of *TpIRT1A-EGFP* and *TpIRT1B-EGFP*-transfected protoplasts were detected at the cell periphery suggesting that both proteins are associated with the plasma membrane (Fig. 2E-L). The *TpIRT1A-EGFP* and *TpIRT1B-EGFP*-mediated fluorescence was also found to be associated with internal vesicles. While the nature of these vesicles is unknown, we speculate that they might be related to the endocytic pathway and participate in the recycling of *TpIRT1A-EGFP* and *TpIRT1B-EGFP*.

TpIRT1A and *TpIRT1B* increase the sensitivity of *S. cerevisiae* mutants lacking Cd and Co-transporters to metals

We next tested the transport capabilities of *TpIRT1A* and *TpIRT1B* using different *S. cerevisiae* mutants including: 1) a Cd-hypersensitive the *Dycf1* mutant lacking the vacuolar ATP-binding cassette transporter responsible for the sequestration of Cd, chelated with a ubiquitous cellular tripeptide, glutathione (Cd-GS₂) (Li et al. 1996); 2) a Co-sensitive mutant (*Dcot1*), lacking a Co transporter COT1 (Conklin et al. 1992; Bloß et al. 2002; Lang et al. 2011); 3) and Zn-hypersensitive mutant (*Δzrc1*) lacking a vacuolar-membrane-localized transporter, ZRC1 (Miyabe et al. 2001).

As would be expected for plasma membrane-localized Cd uptake transporters, the expression of both *TpIRT1A* and *TpIRT1B* increased the sensitivity of the *Δycf1* mutant to 40 μM Cd in a solid medium compared to the *Δycf1* mutant expressing the vector without cDNA inserts (EV) (Fig. 3A). The increased sensitivity of *Δycf1-TpIRT1A* and *Δycf1-TpIRT1B* strains to Cd compared to the EV-transformed mutant was also observed in a liquid medium (Fig. 3B-C). Under Co stress, the expression of both *TpIRT1A* and *TpIRT1B* also enhanced the sensitivity of the *COT1* mutant to 250 μM Co on plates and 100 μM Co in liquid media compared to EV (Fig. 3D-F). However, the growth of *Δzrc1-TpIRT1A* and *Δzrc1-TpIRT1B* was similar to *Δzrc1-EV* cell lines on solid or in liquid medium with high Zn (Fig. 3G-I).

Transgenic *A. thaliana* expressing *TpIRT1B* accumulates more Fe, Mn, and Co

To determine the metal transport properties of *TpIRT1* in plants, we over-expressed *TpIRT1B* in *Arabidopsis* and generated two independent *TpIRT1B*-overexpressing lines (*TpIRT1B-OE-L1* and *TpIRT1B-OE-L2*). The expression level of *TpIRT1B* in *TpIRT1B*-overexpressing lines was much higher than that in control lines (Fig. S1). Although *TpIRT1B*-overexpressing lines and control lines were indistinguishable when they were grown under control conditions, the over-expression of *TpIRT1B* significantly increased Fe and Mn concentrations in leaves and roots of plants (Fig. 4A-F). The concentration of Co was elevated only in leaves in both *TpIRT1B-OE* lines compared to wild-type, correspondingly the translocation factor was also improved (Fig. 4G-I). Furthermore, Perls-DAB staining showed that plants ectopically expressing *TpIRT1B* accumulated more Fe at the root tip, the zone of maturation, the root-to-shoot junction, leaf vasculature, as well as stomatal area including stomatal cavity and guard cells of the leaf (Fig. 5).

***A. thaliana* transgenic lines expressing *TpIRT1B* are more tolerant to Fe and Mn deficiencies**

We then exposed different plant lines to mineral deficiencies and, as expected, root and leaf biomass of control lines was significantly lower when they were grown under Fe or Mn deficiency and leaf biomass was lower when plants were grown under Co deficiency compared to plants grown under control conditions. Concerning *TpIRT1B* expressing plants, while Mn deficiency decreased root biomass, Fe and Co deficiencies decreased shoot biomass, overall, plant growth was less affected compared to wild-type or empty vector expressing plants (Fig. 6). Specifically, the over-expression of *TpIRT1B* partly rescued the plant root growth (Fig. 6E) and even increased the root length (Fig. 6D) and the leaf biomass (Fig. 6F) of Mn-deficient plants compared to control lines. Although no significant differences were found in root length or biomass between the transgenic and control lines grown under Co deficiency (Fig. 6G-H), leaf biomass was higher in *TpIRT1B*-overexpressing lines than in the wild-type or vector expressing plants (Fig. 6I). The increased tolerance of *TpIRT1B*-overexpressing plants to Fe, Mn, and Co deficiencies may result from their increased ability to accumulate these minerals when they are in abundance (Fig. 4) and use them under mineral deficiency. Consistently, as evidenced by the decreased root length of *TpIRT1B*-overexpressing lines, these plant lines were more sensitive than controls to elevated concentrations of Fe, Mn, and Co (Fig. S2). Interestingly, over-expression of *TpIRT1B* had no influence on plant growth under both either low or excess Zn and did not change internal Zn concentration when compared to control lines (Fig. S3).

***A. thaliana* transgenic lines expressing *TpIRT1B* are more sensitive to Cd toxicity**

We then examined the sensitivity of *TpIRT1B*-overexpressing lines to excess Cd. To do that different plant lines were grown either hydroponically (Fig. 7A-G) or on solid ½ MS medium (Fig. 7H). The over-expression of *TpIRT1B* caused more serious toxicity symptoms including lesion and chlorosis of leaves, inhibited root lengths and decreased biomass when compared to control lines (Fig. 7A-D, H). Consistently, *TpIRT1B*-overexpressing plants accumulated more Cd in roots and leaves (Fig. 7E-G).

The identification of IRT1 homoeologs from different wheat subgenomes

We then used an *in silico* analysis to identify *IRT1* homoeologs in different wheat genomes. Twenty *IRT1* homoeologs that were isolated from ten tetraploid wheat genomes included ten homoeologs from the genome A and ten homoeologs from the genome B (Table S3). In addition, two *IRT1* homoeologs, *TuIRT1* and *AsIRT1*, were also identified from two diploid wheat species *T. urartu* and *Aegilops speltoides*, and classified as *TuIRT1A* and *AsIRT1B* because *T. urartu* and *A. speltoides* are considered as the A and B genome donors, respectively (Haider 2013). The ORF lengths of a total of 22 *IRT1* homoeologs ranged from 1094 bp to 1121 bp (Fig. S4), which encoded 363 to 372 amino acids (Fig. S5). According to amino acid alignment, two amino acid substitutions were identified in 12 *IRT1A* members while the other ten proteins shared the same sequence; five polymorphisms were found in *IRT1B* members (Fig. S5). Most *IRT1* proteins were predicted to possess eight TMs, except for *TdiIRT1B* that was predicted to have nine TMs (Fig. S6).

In total, 32 *IRT1* proteins were grouped into two clades with 100% bootstrap values. All *IRT1*s from wheat were clustered into the Poaceae subclade (red arrow) with 96% bootstrap values (Fig. 8). All *IRT1* homoeologs from A genome were grouped with 99% bootstrap values (Fig. 8). Interestingly, *TaIRT1D* from the D genome of hexaploidy wheat was clustered with *TpIRT1B* and *TdiIRT1B* with 98% bootstrap values (Fig. 8).

Functional characterization of wheat *IRT1* homoeologs

To reveal the function of different *IRT1* homoeologs, we individually introduced them into Zn, Cd and Co sensitive yeast strains, $\Delta zrc1$, $\Delta ycf1$, and $\Delta cot1$ respectively. We found that two homoeologs were distinct from others in their ability to complement yeast mutants. First, the expression of *TdiIRT1B* increased the sensitivity of the $\Delta zrc1$ mutant to Zn toxicity when compared with other wheat *IRT1*s and empty pYES2 vector (Fig. 9C). Second, while all *IRT1*s tested increased the sensitivity of $\Delta ycf1$ to Cd, the expression of *TtuIRT1B* dramatically increased Cd tolerance of this mutant strain (Fig. 9D-F). Meanwhile, *TtuIRT1B*-expression did not affect the growth of $\Delta cot1$ mutant under Co stress in comparison to other *YK40-IRT1*s that increased the Co sensitivity of this mutant (Fig. 9G-I). The comparison of *IRT1* sequences disclosed that *TdiIRT1B* has two SNPs (571 and 773 sites) distinguishing it from other *IRT1* homoeologs that converted the Histidine (His/H) to Tyrosine (Tyr/Y) at the positions of 193 (H193Y) and 260 (H260Y) (Fig. 9J). In addition, two SNPs were found in *TtuIRT1B* at positions 253 and 657 that converted Arginine⁸⁹ to His⁸⁹ (R89H) and Isoleucine²³⁰ to Tyr²³⁰ (I230T), respectively (Fig. 9J). The nucleotide variations in *TdiIRT1B* and *TtuIRT1B* may have altered the function of these genes, causing the functional differentiation of metal transport.

Discussion

***TpIRT1B* transports Fe, Mn, Co, and Cd transport, but not Zn unlike its homologs from other species**

In this manuscript, we show that *TpIRT1B* mediates uptake of Fe, Mn, Co, and Cd, but not Zn. This conclusion was made based on the following findings: first, the expression of *TpIRT1B* increased the

sensitivity of *Δcot1* and *Δycf1* mutant yeast cells to Co and Cd, respectively. This suggests that *TpIRT1B* promoted the accumulation of Co and Cd in cells and caused toxicity. In contrast, Zn sensitivity of the *Δzrc1* did not change (Fig. 3). Second, the over-expression of *TpIRT1B* enhanced Fe, Mn, and Co concentrations in roots and shoots in *A. thaliana* and improved the tolerance of plants to these mineral deficiencies (Fig. 4–6). These data imply that Fe, Mn, and Co accumulation in plant tissues during growth under control conditions have helped to sustain normal growth under mineral deficiencies (Fig. 6) and increased the sensitivity to excess Fe, Mn, and Co stresses (Fig. S2). Third, the over-expression of *TpIRT1B* enhanced Cd concentration in roots and shoots, increasing the sensitivity to Cd stress (Fig. 7). Fourth, the over-expression of *TpIRT1B* in *A. thaliana* did not change Zn concentration and the growth of transgenic plants (Fig. S3).

Previous studies indicated that all IRTs from plants studied to date including AtIRT1, AtIRT2, AtIRT3, OsIRT1, OsIRT2, ZmIRT1, HvIRT1, LeIRT1, and LeIRT2 transport Fe and Zn; AtIRT1, HvIRT1, LeIRT1, and LeIRT2 transport Mn; AtIRT1, OsIRT1, OsIRT2, and HvIRT1 transport Cd; additionally, AtIRT2, AtIRT3, OsIRT1, and OsIRT2 do not transport Mn, Cd, and/or Co (Korshunova et al. 1999; Rogers et al. 2000; Vert et al. 2001, 2002; Bughio et al. 2002; Ishimaru et al. 2006; Nakanishi et al. 2006; Schikora et al. 2006; Pedas et al. 2008; Vert et al. 2009; Lin et al. 2009; Lee et al. 2009a, b; Nishida et al. 2011; Barberona et al. 2014; Li et al. 2015; Ismael et al. 2018). Since *TpIRT1B* from Polish wheat transports Fe, Mn, Co, and Cd, but not Zn, its transport function differs from its homologs in other species.

The *TpIRT1* is preferentially expressed in roots, leaves, lemma, and palea (Fig. 1A-C). Previous studies demonstrated that *OsIRT1* is mainly expressed in roots and leaves; *AtIRT1*, *ZmIRT1*, and *HvIRT1* are mainly expressed in roots but also found in anthers (Bughio et al. 2002; Vert et al. 2002; Ishimaru et al. 2006; Li et al. 2015; Long et al. 2017). In this study, the *TpIRT1* expression in roots was up-regulated by Mn deficiency (Fig. 1D). Surprisingly, *TpIRT1* transcript abundance did not change in response to Fe, Zn or Co deficiency; on the contrary, its expression was up-regulated by excess Fe, Mn, and Co (Fig. 1D-E). Our results differ from reports on *IRT1s* from other species. Specifically, *AtIRT1*, *OsIRT1*, *AhIRT1*, *LeIRT1*, and *ZmIRT1* are up-regulated by Fe and Zn deficiencies; *OsIRT1*, *AtIRT1*, and *HvIRT1* are up-regulated by Mn deficiency; *AtIRT1* and *ZmIRT1* are up-regulated and down-regulated by Fe and Zn deficiency and sufficiency, respectively (Vert et al. 2002, 2003; Ishimaru et al. 2006; Schikora et al. 2006; Lee and An 2009a; Ding et al. 2010; Shanmugam et al. 2010; Nakanishi et al. 2010; Li et al. 2013; Long et al. 2017; Zheng et al. 2018). Similar to *AtIRT1* and *OsIRT1*, *TpIRT1* was up-regulated by Cd toxicity (Fig. 1E). We also found that *TpIRT1s* were potentially located at plasma membrane and internal vesicles (Fig. 2). This result is in agreement with data showing that *AtIRT1* resides at the plasma membrane and *AtIRT2* on a periphery of small vesicles (Vert et al. 2001, 2002); *ZmIRT1*, in addition to plasma membrane is also associated with endoplasmic reticulum (ER) (Li et al. 2013). Together, our results suggest that *TpIRT1* is involved in Fe, Mn, Co, and Cd uptake and internal distribution in the plant.

It is well-recognized that non-gramineous plants use IRT1 to take up Fe(II) from the soil into root epidermal cells (Eckhardt et al. 2001; Varotto et al. 2002; Vert et al. 2002; Schikora et al. 2006; Hodoshima et al. 2007; Ding et al. 2009; Tan et al. 2015; Shanmugam et al. 2011). The non-gramineous plants absorb

Fe-phytosiderophores using transporters from the YSL family, but not IRT1 (Lee et al. 2009c; Inoue et al. 2009). Previous studies have shown that rice and maize use both strategies and rely on IRT1 and YSL transporters to absorb Fe from the soil (Ishimaru et al. 2006; Li et al. 2013, 2015, 2018). In this study, we show that *TpIRT1B* is expressed mainly in roots and transports Fe. These results imply that wheat, similar to rice and maize, uses both strategies for Fe absorption.

Among mineral elements studied here, Co, is beneficial but can be toxic if in excess, while Cd is highly toxic for plant growth and development (Battersby 1993; Palit et al. 1994; Arfin et al. 1995; Komeda et al. 1997; Bakkaus et al. 2005; Järup and Åkesson 2009; Ismael et al. 2018). Thus, uptake and internal transport of these minerals in the plant relies on transporters for essential metals (Thomine et al. 2000; Morel et al. 2009; Cheng et al. 2011; Takahashi et al. 2011, 2014; Wang et al. 2017, 2019). It is noteworthy that global crop wheat, when grown on Cd-polluted soils, is an entry point of Cd into a daily human diet (Greger and Löfstedt 2004). To date, several essential metal transporters from wheat including TaHMA2, TaVP1, TPNRAMP3, TPNRAMP5 and TPNRAMP6 have been discovered to transport Co and/or Cd (Peng et al. 2008a, b; Khoudi et al. 2012; Tan et al. 2013; Wang et al. 2019). Our results show that TPIRT1B also transports Co and Cd (Fig. 4, 6, 7). Thus, these transporters may contribute to Cd accumulation in wheat, which therefore are available genes for genetic manipulation to reduce the Cd accumulation in the food chain.

Amino acid residues involved in Zn, Cd, and Co transport in IRT1 homoeologs

It has been shown that point substitutions of several pivotal amino acid residues in mineral ion transporters can change their substrate specificity (Zhao and Eide 1996a, b; Rogers et al. 2000; Grosseohme et al. 2006). For example, the replacement of the key lysine (Lys) residue in the intracellular loop of ZRT1 from yeast and AtIRT1 alters their transport specificity and the ability to transport Cd and Co (Gitan et al. 2000; Dubeaux et al. 2018). In this study, two His substitutions (H193Y and H260Y) of TdiIRT1B increased the sensitivity of $\Delta zrc1$ -*TdiIRT1B* strain to Zn stress when compared to other wheat IRT1s and the empty pYES2 vector (Fig. 9A-C, J). This result suggested that this polymorphism increased TdiIRT1B specificity to Zn. Meanwhile, the unique substitution H260Y in TdiIRT1B potentially caused an additional TM (TM9) between TM5 and TM6 as predicated by TMHMM v2.0 (Fig. S6). TMs form a potential ion channel and allow metal ions to pass through the cell membrane (Kadir et al. 2018). In the P1B-type ATPase family, the difference in TMs number appears to confer their capability to transport metals (Argüello et al. 2007). Thus, it is tempting to speculate that an additional TM9 in TdiIRT1B might be a central component of Zn transport.

In addition, the expression of TtuIRT1B with two polymorphic residues (R89H and I230T) dramatically increased the tolerance of $\Delta ycf1$ strain to Cd; while, the expression of other IRT1s in $\Delta ycf1$ increased the sensitivity to Cd (Fig. 9D-F, J). Meanwhile, *TtuIRT1B*-expression did not change the growth of the $\Delta cot1$ strain under Co stress when compared to other *YK40-IRT1s* that were sensitive to Co (Fig. 9G-I). These results implied that the two polymorphic residues might change the transport properties of TtuIRT1B or its subcellular localization, or the ability to bind Cd or Co. For example, Cd normally binds to various

amino acid residues including His, Glu, Cys, Asp and Tyr (Friedman 2014). Thus, His⁸⁹ may be a potential Cd-binding site for intercellular mobilization at the IRT1. Consequently, the Ile²³⁰ seemed to be the Co-binding site, and its substitution may result in failing to transport Co in yeast.

Conclusion

Our results highlight the importance of *TpIRT1* in the uptake and translocation of Fe, Mn, Co, and Cd. *TpIRT1* is preferentially expressed in roots and leaves, and is significantly induced by Fe, Mn, Cd and Co excess, and Mn deficiency. *TpIRT1A* and *TpIRT1B* localize at the plasma membrane and small internal vesicles in *Arabidopsis* protoplasts. The expression of *TpIRT1A* and *TpIRT1B* increased yeast sensitivity to Cd and Co. The overexpression of *TpIRT1B* in *Arabidopsis* has led to Fe, Mn, and Co accumulation in plant tissues and partially rescued the plant defects under these metals deficiencies when compared to control lines. Meanwhile, the over-expression of *TpIRT1B* enhanced Cd concentration in *Arabidopsis*, resulting in the increased sensitivity of plants to Cd toxicity. Most of the *IRT1* homoeologs that exist in abundant natural variations of wheat species have shown a similar function in Zn, Co, and Cd transport in yeast. However, four SNPs were detected that, we speculate, might change the metal transport specificity. Our future studies will focus on the discovery of amino acid residues that contribute to metal selectivity of IRTs in wheat.

Declarations

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Author contributions

Y.J. and Y. W. designed the experiments. Y. J. performed most of experiments and analysed the data. Other authors assisted in experiments and discussed the results. Y. J., Y. W. and O. V. wrote the manuscript.

Declaration

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

RT-qPCR analysis of the tissue expression pattern of TpIRT1A/B The expression patterns of TpIRT1A/B in different tissues of DPW were analysed at the flowering stage (A), the filling stage (B) and the jointing stage (C). The relative expression levels of TpIRT1 in roots was also analysed in plants subjected for one week to Fe, Mn, Zn, Co deficiency (D) or metal excess (E). Asterisks indicate significant differences from control, grown under Fe, Mn, Zn, Co stresses ($p < 0.05$, Tukey's t-test, $n = 3$ independent experiments).

Figure 2

The subcellular localization of TpIRT1A-EGFP and TpIRT1B-EGFP in *A. thaliana* protoplasts pSAT6-N1-EGFP-Gate empty vector (EV, A-C), or the vector containing TpIRT1A (D-F) and TpIRT1B (G-I) cDNA inserts were transiently expressed in *A. thaliana* protoplasts. EGFP fluorescence (green), chlorophyll autofluorescence (red), and overlay(overlay) images are shown. Scale bar = 10 μ M.

Figure 3

TpIRT1A and TpIRT1B increase Cd and Co sensitivity of yeast cells. Cadmium sensitive $\Delta ycf1$ yeast cells (A to C), Co sensitive $\Delta cot1$ (D to F) or Zn sensitive $\Delta zrc1$ yeast cells (G) were transformed with the empty vector (EV) or a vector containing either TpIRT1A or TpIRT1B inserts. Cells were then spotted either on solid SD medium (A, D, G) or inoculated into liquid SD media with the indicated concentration of metals (B, C, E, F, H, I). A, D, and G show representative images from three independent experiments. In B, C, E, F, H, I, the presented values are mean $\pm$ standard deviation (n = 3 independent experiments).

Figure 4

Tissues of *A. thaliana* ectopically expressing TplIRT1B accumulate more Fe, Mn, and Co. The *A. thaliana* wild-type (WT), empty vector (EV) transformed plants and two TplIRT1B-overexpressing lines (Line 1 and Line 2) were grown hydroponically with the standard concentrations of minerals. Tissues of four-week-old plants were collected for ICP-MS analysis. (A to C) shows Fe concentration in roots (A), shoots (B) and Fe translocation from roots to shoots (C). (D to F) shows Mn concentration in roots (D), shoots (E) and Mn translocation from roots to shoots (F). (G to I) shows Co concentration in roots (G), shoots (H) and Co translocation from roots to shoots (I). Asterisks indicate significant differences between WT and the overexpressing lines.

and Mn translocation from roots to shoots (F). (G to I) shows Co concentration in roots (G), shoots (H) and Co translocation from roots to shoots (I). Asterisks indicate significant differences between WT and two *TpIRT1B*-overexpressing lines ($p < 0.05$, Student's t-test; $n = 3$ independent experiments with tissues pooled from eight plants in each independent experiment).

Figure 5

Perls-DAB staining to track Fe distribution in seedlings of WT and the *TpIRT1B*-overexpressing line. The WT and the *TpIRT1B*-overexpressing line (*TpIRT1B*-OE) were grown on $\frac{1}{2}$ MS medium for 5 days prior to staining with Perls-DAB solution. Bright-field images of the entire leaf of WT (A), *TpIRT1B*-OE (B), and a close-up of *TpIRT1B*-OE leaf (C). (D to E) shows root tips of WT (D) and *TpIRT1B*-OE (E). (F to G) shows root-to-shoot junction in WT (F) and *TpIRT1B*-OE (G). (H to I) shows the root zone of maturation WT (H) and *TpIRT1B*-OE (I). White arrows indicate Fe-rich sites; red arrows mark Fe accumulation in stomatal areas in leaves; guard cells of stoma are marked by red lines in (C). Photos were taken using the Axio Imager M2 microscope.

Figure 6

The phenotype and biomass of WT, EV- and *TpIRT1B*-overexpressing lines under low content of Fe, Mn, and Co stresses in hydroponics. The WT, EV, and two *TpIRT1B*-overexpressing lines (Line 1 and Line 2) were grown hydroponically with the standard condition of minerals for two weeks, then treated with low content of Fe, Mn, and Co for one week, respectively. The root length, dry weight of roots and shoots were averaged with eight plants per genotypes from low content of Fe (A to C), Mn (D to F), and Co (G to I).

treatment, respectively. Asterisks indicate significant differences from control vs. metal stresses and WT vs. overexpressing lines by Student's t-test ($p < 0.05$, $n = 3$ independent experiments).

Figure 7

The TplIRT1B-overexpressing lines are more sensitive to Cd and accumulate more Cd than control lines under Cd stress. The WT, EV, and two TplIRT1B-overexpressing lines (Line 1 and Line 2) were grown either hydroponically (A to G) or on solid $\frac{1}{2}$ MS medium (H). After two weeks of growth in hydroponics, plants were transferred to a fresh medium with or without 20 μM CdCl₂. A subset of plants was imaged (A) and root lengths (B) were measured after five days of treatment. Another subset of plants was grown for two more weeks prior to tissue collection, the analysis of root and shoot biomass (C, D, respectively) and ICP-MS analysis (E to G). Asterisks indicate significant differences from control vs. Cd stress and WT vs. overexpressing lines ($p < 0.05$, Student's t-test, $n = 3$ independently experiments with tissues pooled from eight plants per genotype). (H) shows a representative image of 7-day-old plants of indicated lines grown on $\frac{1}{2}$ MS soil medium with or without 20 μM CdCl₂.

Figure 8

Phylogenetic tree of IRT1 proteins from Arabidopsis, rice, maize, Brachypodium, barley, and wheat genomes. The neighbor-joining (NJ) tree of IRT1 proteins was performed by MEGA 6.0. AtZIP1 (AT3G12750) was used as an outgroup. Numbers at nodes indicate bootstrap values. Bar at the left bottom indicates the scale value. The red arrow indicates the Clade of Triticeae.

marked in red. (J) shows all mutant sites and marked the pivotal sites that changed the sensitivities of yeast mutants in red.

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