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ORIGINAL ARTICLE

Expression of TpNRAMP5, a metal transporter from Polish wheat (*Triticum polonicum* L.), enhances the accumulation of Cd, Co and Mn in transgenic *Arabidopsis* plants

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Abstract

Main conclusion **TpRNAMP5 is mainly expressed in the plasma membrane of roots and basal stems. It functions as a metal transporter for Cd, Mn and Co accumulation.**

Numerous natural resistance-associated macrophage proteins (NRAMPs) have been functionally identified in various plant species, including *Arabidopsis*, rice, soybean and tobacco, but no information is available on *NRAMP* genes in wheat. In this study, we isolated a *TpNRAMP5* from dwarf Polish wheat (DPW, *Triticum polonicum* L.), a species with high tolerance to Cd and Zn. Expression pattern analysis revealed that *TpNRAMP5* is mainly expressed in roots and basal stems of DPW. TpNRAMP5 was localized at the plasma membrane of *Arabidopsis* leaf protoplast. Expression of *TpNRAMP5* in yeast significantly increased yeast sensitivity to Cd and Co, but not Zn, and enhanced Cd and Co concentrations. Expression of *TpNRAMP5* in *Arabidopsis* significantly increased Cd, Co and Mn concentrations in roots, shoots and whole plants, but had no effect on Fe and Zn concentrations. These results indicate that TpNRAMP5 is a metal transporter enhancing the accumulation of Cd, Co and Mn, but not Zn and Fe. Genetic manipulation of TpNRAMP5 can be applied in the future to limit the transfer of Cd from soil to wheat grains, thereby protecting human health.

Keywords Expression pattern · Functional characterization · Metal transporter · Natural resistance-associated macrophage protein · Plasma membrane · Wheat

Abbreviations

DPW	Dwarf Polish wheat
IRT	Iron-regulated transporter
NRAMP	Natural resistance-associated macrophage protein

Introduction

Metal transporters play crucial roles in metal cation homeostasis, a process essential for maintaining plant nutrient balance and detoxifying nonessential heavy metals (Thomine et al. 2000). Natural resistance-associated macrophage proteins (NRAMPs), which constitute an integral-membrane metal transporter family, function as metal transporters for the uptake, translocation, intracellular transport, and detoxification of transition metals (Nevo and Nelson 2006; Sasaki et al. 2012). Since the first identification of an *NRAMP* gene (*NRAMP1*) from

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mouse (Vidal et al. 1993), increasing numbers of *NRAMP* genes have been cloned and functionally characterized in bacteria, fungi, plants and animals (Thomine et al. 2000; Nevo and Nelson 2006; Sasaki et al. 2012; Tejada-Jiménez et al. 2015; Qin et al. 2017; Gao et al. 2018). Numerous *NRAMP* genes have been discovered and characterized in plants. Six *NRAMP* genes have been discovered in *Arabidopsis*, five of which have been functionally identified (Curie et al. 2000; Thomine et al. 2000, 2003; Lanquar et al. 2005, 2010; Cailliatte et al. 2009, 2010; Gao et al. 2018). AtNRAMP1 is localized at the plasma membrane and intracellular vesicles of root cells (Agorio et al. 2017). This protein functions as a high-affinity manganese (Mn) transporter for Mn uptake, restores an *iron-regulated transporter1* (*IRT1*) mutant to take up iron (Fe) and cobalt (Co) (Cailliatte et al. 2010) and regulates Fe homoeostasis (Curie et al. 2000; Castaings et al. 2016). AtNRAMP2 functions as a trans-Golgi network-localized Mn transporter, participates in Mn remobilization in the Golgi apparatus of plants and transports Fe and zinc (Zn) in yeast (Gao et al. 2018). AtNRAMP3 and AtNRAMP4 are localized at the vacuolar membrane; they export Fe and Mn from vacuoles to individually maintain seed germination under low Fe conditions and photosynthesis in adult plants with low Mn concentrations (Lanquar et al. 2005, 2010). AtNRAMP4 also transports Zn and Cd in both yeast and plants (Pottier et al. 2015). AtNRAMP6, which is targeted to a vesicular-shaped endomembrane compartment, functions as an intracellular transporter for Cd, but not Fe, Mn and Zn (Cailliatte et al. 2009).

Seven *NRAMP* genes, namely, *OsNRAMP1–OsNRAMP7*, have been investigated in the rice genome, and five have been functionally characterized. OsNRAMP1 is localized at the plasma membrane and participates in Cd, Fe and arsenic (As) uptake and translocation, but not Mn (Curie et al. 2000; Takahashi et al. 2011; Tiwari et al. 2014). OsNRAMP2 is unable to transport Fe in yeast (Curie et al. 2000); its actual function needs to be discovered. OsNRAMP3 is also localized at the plasma membrane of phloem parenchyma cells; it participates in the distribution and remobilization of Mn, but not that of Fe, Cd or Zn (Yamaji et al. 2013; Yang et al. 2013). OsNRAMP4, also named Nrat1 and localized at the root plasma membrane, functions as a trivalent aluminum transporter, but does not transport Fe, Mn or Cd (Xia et al. 2010; Li et al. 2014). OsNRAMP5 is localized at the root plasma membrane and functions as a metal transporter for Mn and Cd uptake (Ishikawa et al. 2012; Ishimaru et al. 2012a, b; Sasaki et al. 2012; Yang et al. 2014). Knockdown of *OsNRAMP5* has been found to reduce shoot Fe concentration (Sasaki et al. 2012), which suggests that it is also a Fe transporter. OsNRAMP6 is localized at the root plasma membrane and transports Fe and Mn, but not Cd, in yeast (Peris-Peris et al. 2017).

Numerous *NRAMP* genes have also been identified from other plant species and investigated. For example, 13 *NRAMP* genes have been characterized from soybean (Qin et al. 2017). Of them, GmDMT1 transports Mn in yeast (Kaiser et al. 2003). MbNRAMP1 isolated from *Malus baccata* transports Fe, Mn and Cd in yeast (Xiao et al. 2008). NtNRAMP5 is localized at the plasma membrane in tobacco and functions as a Mn and Cd transporter, thereby possibly mediating Cd and Mn accumulations (Tang et al. 2017). HvNRAMP5 is localized at the plasma membrane and functions as a transporter for the uptake of Cd and Mn, but not Fe (Wu et al. 2016). TjNRAMP4 cloned from *Thlaspi japonicum* transports Ni, but not Zn, Cd and Mn, in yeast (Mizuno et al. 2005). No information is available, however, on *NRAMP* genes from either hexaploid or tetraploid wheat.

In the present study, we isolated a *NRAMP* gene from dwarf Polish wheat (DPW, *Triticum polonicum* L.; $2n=4x=28$, AABB) using our previously published DPW transcriptome data (Wang et al. 2016). DPW seedlings accumulate high concentrations of Cd and Zn and show high tolerances to Cd and Zn (Wang et al. 2017). With respect to this gene, NRAMPs exhibit functional divergence in different species. In rice, for example, only OsNRAMP1 and OsNRAMP5 function as metal transporters for Cd and Mn uptake (Takahashi et al. 2011; Ishimaru et al. 2012a, b; Sasaki et al. 2012; Yang et al. 2014). We therefore hypothesized that the product of the putative homologous gene, *TpNRAMP5*, would also transport Cd, Mn, Fe, Zn and/or Co. To test this hypothesis, we investigated the biological functions of this gene by analyzing the following: its expression pattern in different tissues at three growth stages, TpNRAMP5 subcellular localization in *Arabidopsis* leaf protoplast, and the effects of *TpNRAMP5* expression on metal tolerance and accumulation in yeast and on metal transport properties in *Arabidopsis*.

Materials and methods

Cloning of the full-length cDNA of *TpNRAMP5*

Total RNA was extracted from *Triticum polonicum* L., dwarf Polish wheat (DPW, collected from Xinjiang province, China) seedlings using a Total RNA Kit II (Omega Bio-Tek, Norcross, GA, USA). cDNA was synthesized from 2 μg total RNA using an M-MLV First Strand cDNA Synthesis kit (Omega).

PCR primers (forward: 5′-GCAATGGAGATCGAGAGGGA-3′; reverse: 5′-TTCACAAGACGAGGCGAGAT-3′) based on reference sequences (Wang et al. 2016) were designed using Beacon Designer 7.0. The amplified fragment was introduced into a pMD19-T vector (TaKaRa, Dalian, China) for sequencing.

Bioinformatics and phylogenetic analyses

The amino acid sequence encoded by *TpNRAMP5* was deduced using ExPASy software (http://web.expasy.org/translate/). The gene structure and chromosomal location of *TpNRAMP5* were predicted by blasting against the genome of *Triticum aestivum* (wheat)'Chinese Spring' (The International Wheat Genome Sequencing Consortium 2014) on the Ensemblplants website (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast?db=core). Putative subcellular localization was determined using ProtComp 9.0 (http://linux1.softberry.com/berry.phtml?group=programs&subgroup=proloc&topic=protcomppl), and transmembrane domains were predicted using SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html).

Amino acid sequences of 14 NRAMPs, including TpNRAMP5, six *Arabidopsis* NRAMPs (AtNRAMP1–AtNRAMP6) and seven rice NRAMPs (OsNRAMP1–OsNRAMP7), were aligned using Vector NTI 11.5.1 (Invitrogen, Carlsbad, CA, USA). A phylogenetic tree based on these sequences was constructed using the neighbor-joining algorithm in MEGA5.

Expression analysis of *TpNRAMP5*

DPW was grown in a field during the regular wheat growing season (October 2016 to May 2017). Tissues at various growth stages were collected, including roots, basal stems, leaf sheaths, leaf blades, young leaves, lower leaf sheaths, lower leaf blades, first, second and third nodes, second internodes, flag leaf sheaths, flag leaf blades, peduncles, lemmas and grains. The collected tissues were snap-frozen in liquid nitrogen and stored at − 80 °C for RNA extraction. Total RNA isolation and cDNA synthesis were performed as described above.

To investigate response to metal stresses, DPW seeds were sterilized with 5% NaClO for 5 min and then germinated in the dark for 5 days. The germinated seedlings were cultured in Hoagland's nutrient solution in a growth chamber at 25 °C under a 16-h/8-h light/dark photoperiod. After 2 weeks, the seedlings were treated with 8 mM $MgCl_2$ (Mg), 8 mM $ZnSO_4$ (Zn), 8 mM $FeCl_3$ (Fe), 8 mM $CuCl_2$ (Cu), 40 μM $CdSO_4$ (Cd), 40 μM $PbCl_2$ (Pb) or 40 μM $NiCl_2$ (Ni). After treatment for 24 h, roots and leaves were individually collected, snap-frozen in liquid nitrogen and then stored at − 80 °C for RNA extraction.

Quantitative real-time PCR was performed on a CFX-96 system (Bio-Rad, Hercules, CA, USA) as described by Wang et al. (2015) using *TpNRAMP5*-specific primers (forward: 5′-TGGCTGAGGAACCTGATGA-3′; reverse: 5′-TGCTGCTGCTGAACTTGAG-3′). To normalize gene expression levels, the *Actin* gene was used as a reference as described in Wang et al. (2015). Relative expression levels were calculated according to the $2^{\Delta\Delta Ct}$ method using CFX Manager 3.1 software (Bio-Rad).

Yeast expression

The open reading frame of *TpNRAMP5* was sub-cloned into the BamHI and EcoRI sites of the yeast expression vector pYES2. The recombinant plasmid and the empty vector were individually transformed with an S. C. EasyComp Transformation kit (Invitrogen) into the following yeast strains: normal *Saccharomyces cerevisiae* strain BY4743 (wild type, WT; Mat α; his3Δ1; leu2Δ0; met15Δ0; lys2Δ0; ura3Δ0), Cd-sensitive strain Δ*ycf1* (Mat α; his3Δ1; leu2Δ0; lys2Δ0; met15Δ0; ura3Δ0; YDR135c::kanMX4), Zn-sensitive strain Δ*zrc1* (Mat α; his3Δ1; leu2Δ0; lys2Δ0; met15Δ0; ura3Δ0; YMR243c::kanMX4) and Co-sensitive strain *YK40* (ura3-52 his3-200, Δcot1, mat α).

Metal tolerance of the transformed yeast strains on synthetic defined (SD) plate medium was analyzed as described by Wang et al. (2012) with minor modifications of metal stresses. Briefly, the medium was supplemented with $CdCl_2$ (0 or 80 μM), $ZnSO_4$ (0 or 4 mM) or $CoCl_2$ (0 or 200 μM).

To confirm metal tolerance using yeast growth curves, 50 μl of pre-cultured transformed cells (OD_{600} = 0.8) were added to 10 ml SD liquid medium containing 2% galactose and $CdCl_2$ (0, 20 or 40 μM) or $CoCl_2$ (0, 200 or 400 μM). All cells were cultured at 30 °C with shaking at 250 rpm. For Cd tolerance, OD_{600} values were measured at 0, 6, 12, 24 and 36 h using a microplate spectrophotometer (Fisher Scientific, MA, USA); for Co tolerance, OD_{600} values were recorded at 0, 12, 24, 36 and 48 h.

To measure yeast metal concentrations, transformed yeast cells were grown in SD liquid medium with 2% galactose and $CdCl_2$ (20 μM) for 48 h or $CoCl_2$ (50 μM) for 60 h and then collected by centrifugation. The collected cells were washed with 100 μM EDTA for 5 min, rinsed three times with deionized water, and dried at 80 °C for 24 h. All samples were digested in 80% nitric acid at 220–280 °C for 30 min and then diluted in deionized water. The metal concentration of each sample was determined by inductively coupled plasma mass spectrometry (ICP-MS, Fisher Scientific).

Subcellular localization of TpNRAMP5 in *Arabidopsis* leaf protoplast

The open reading frame of *TpNRAMP5* was inserted into the BamHI and KpnI sites of the *Arabidopsis* protoplast expression vector HBT95-GFP under the control of the 35S promoter. The preparation and transformation of *Arabidopsis* leaf mesophyll protoplast was performed as described by Yoo et al. (2007). A plasmid construct encoding a plasma membrane marker (RFP-SCAMP1; Cai et al. 2011) was

co-transformed to confirm the subcellular localization. GFP and RFP signals were detected on a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Expression of *TpNRAMP5* in *Arabidopsis thaliana*

The open reading frame of *TpNRAMP5* was inserted into the BamHI and SalI sites of the *Arabidopsis* expression vector pCAMBIA1305.1 driven by the 35S promoter. The transformation and selection of homozygous lines was performed as described by Clough and Bent (1998).

To test metal sensitivity of the *Arabidopsis* seedlings, the WT, two independent homozygous lines and an empty vector line were germinated on half-strength MS solid plates containing $CdCl_2$ (0 or 25 μM), $CoCl_2$ (0 or 80 μM) or $MnCl_2$ (0 or 500 μM). All plates were grown in a growth chamber at 22 °C under an illumination of 120 μmol photons m^{-2} s^{-1}, a 16-h/8-h light/dark photoperiod and 50% humidity. After 10 days, the root lengths of different treated plants were measured.

To test metal transport properties of *TpNRAMP5*-expressing lines, the WT, two independent homozygous lines and an empty vector line were germinated in half-strength MS medium. Seedlings with four leaves were then cultured in soil. After 3 weeks, the soil was watered once with 40 mg kg^{-1} $CdCl_2$ or $CoCl_2$ dissolved in water. After a month, the dry weight of each plant was measured, and the roots and aerial parts were individually collected. All dried samples were digested in 80% nitric acid at 220–280 °C for 45 min and diluted in deionized water. Metal concentrations in each sample were determined by ICP-MS.

Data analysis

All data (three biological replicates per sample) were statistically analyzed using Tukey's test at $P \leq 0.05$ in SPSS 20.0. All figures were drawn in SigmaPlot 12.0.

Results

Cloning and phylogenetic analysis of *TpNRAMP5*

The full-length amplified cDNA of *TpNRAMP5*, consisting of 1647 bp, included a 1617-bp open reading frame encoding a polypeptide of 539 amino acids. Blasting of the open reading frame of *TpNRAMP5* against the wheat genome (The International Wheat Genome Sequencing Consortium 2014) revealed that *TpNRAMP5* is located on chromosome 4AS (gene: TRIAE_CS42_4AS_TGACv1_306761_AA1013050) and comprises 11 introns and 12 exons. Phylogenetic analysis closely grouped the deduced amino acid sequence of TpNRAMP5 with OsNRAMP5, with which it shared 83.1% identity, and then with OsNRAMP1 (70.4% identity). In contrast, the TpNRAMP5 sequence was only 33.9% identical to AtNRAMP5 (Fig. S1). These results suggest that the function of TpNRAMP5 is similar to that of OsNRAMP5 and OsNRAMP1, but different from AtNRAMP5.

Expression patterns of *TpNRAMP5*

TpNRAMP5 expression levels were investigated in different wheat tissues at jointing, booting and grain-filling stages. At all stages, *TpNRAMP5* was mainly expressed in roots, followed by basal stems (Fig. 1a).

Previous studies have indicated that different *NRAMP* genes have different responses to metal supplementation. We therefore investigated the expression of *TpNRAMP5* in seedlings treated with Mg, Zn, Fe, Cu, Cd, Pb or Ni. *TpNRAMP5* expression was significantly down-regulated in roots by Mg, Zn, Fe, Pb and Ni (Fig. 1b) and in leaves by Cu and Ni (Fig. 1c). Interestingly, no change was induced by Cd in either roots or leaves (Fig. 1b, c).

Subcellular localization of *TpNRAMP5*

TpNRAMP5 was predicted to be a plasma membrane protein with ten transmembrane domains (Fig. S2). To confirm the subcellular localization of TpNRAMP5, a TpNRAMP5-GFP fusion and RFP-SCAMP1 were transiently co-transformed into *Arabidopsis* leaf protoplast. Green fluorescence of the empty vector (HBT95) was confined to the cytosol, the nucleus and the plasma membrane. In contrast, the green fluorescence of the fusion protein (HBT95-TpRNAMP5-GFP) was completely merged with the red fluorescence of the plasma membrane marker, which indicates that TpNRAMP5 is localized at the plasma membrane (Fig. 2).

Functional expression of *TpNRAMP5* in yeast

To investigate whether TpNRAMP5 is a functional metal transporter and to examine its transport properties, we expressed *TpNRAMP5* or the empty vector pYES2 in different yeast strains. In the presence of galactose, Cd seriously inhibited the growth of Δ*ycf1* transformed with pYES2 relative to that of BY4743 harboring the empty vector. In Δ*ycf1*, sensitivity to Cd was strongly increased by expression of *TpNRAMP5* compared with pYES2 (Fig. 3a). This enhanced sensitivity to Cd was further confirmed by examination of growth curves under 20 and 40 μM $CdCl_2$ stresses (Fig. 3b). The highest Cd accumulation was detected in *TpNRAMP5*-expressing Δ*ycf1* (Fig. 3c), and the lowest accumulation was in BY4743 with pYES2 (Fig. 3c).

We also investigated Co tolerance and accumulation (Fig. 4). Co strongly inhibited the growth of *YK40*

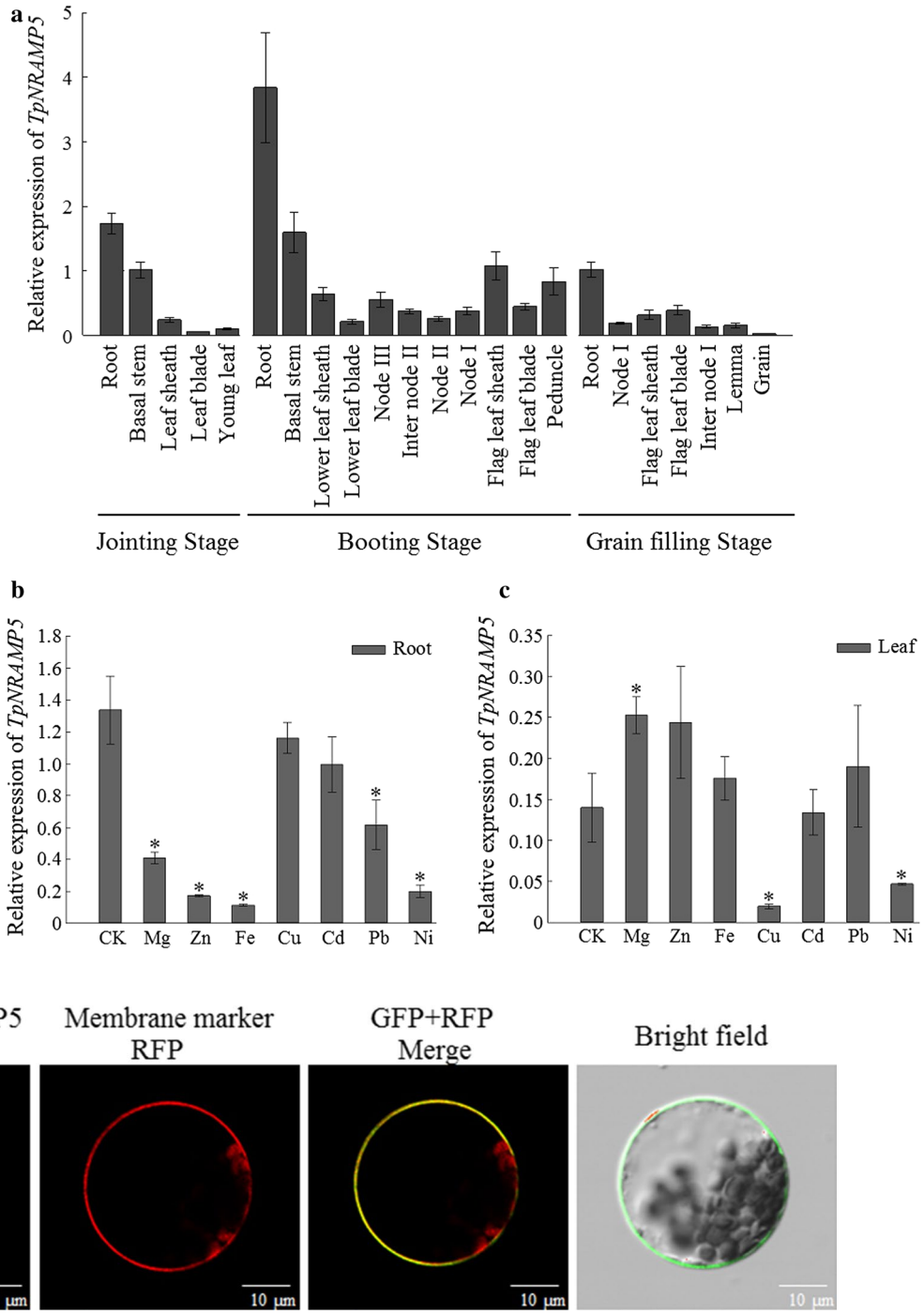

Fig. 1 Expression pattern of *TpNRAMP5*. **a** Relative expression of *TpNRAMP5* in various wheat tissues at jointing, booting and grain-filling stages. Relative expression of *TpNRAMP5* in roots (**b**) and leaves (**c**) of seedlings under Mg, Zn, Fe, Cu, Cd, Pb or Ni stress for 24 h. Asterisks indicate significant differences from the control (CK) at $P < 0.05$ according to Tukey's test based on three independent biological replicates

Fig. 2 Subcellular localization of TpNRAMP5 in *Arabidopsis* leaf protoplast. A HBT95-TpNRAMP5-GFP vector and a plasma membrane marker (RFP-SCAMP1) were co-transformed into *Arabidopsis* leaf mesophyll protoplast. GFP and RFP signals were detected using a confocal laser scanning microscope

carrying pYES2 compared with that of pYES2-transformed BY4743. The expression of *TpNRAMP5* strongly increased the Co sensitivity of *YK40* relative to that of *YK40* with pYES2 (Fig. 4a). This increased sensitivity was also apparent in growth curves under 200 and 400 μM $CoCl_2$ stresses (Fig. 4b). The expression of *TpNRAMP5* in *YK40* resulted in the highest accumulation of Co, i.e., to levels significantly higher than those of BY4743 and *YK40* transformed with pYES2 (Fig. 4c). In contrast, *TpNRAMP5* expression did not promote Zn transport (Fig. S3).

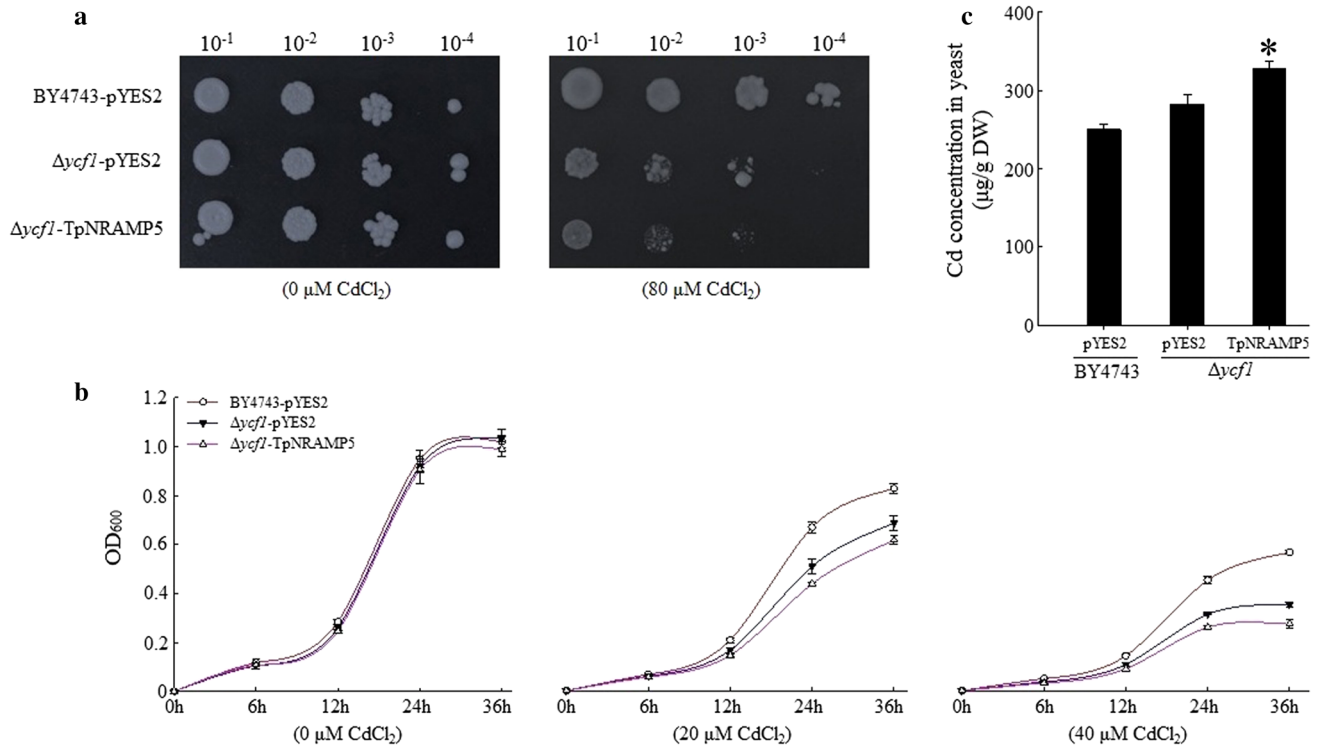

Fig. 3 Sensitivity to Cd and concentrations of Cd in yeast. **a** Cd sensitivity of yeast grown on synthetic defined (SD) plates with 80 μM $CdCl_2$. **b** Cd sensitivity of yeast grown in liquid SD medium with 20 and 40 μM $CdCl_2$. OD_{600} values were measured at 0, 6, 12, 24 and 36 h. **c** Cd concentrations in yeast grown in liquid SD medium with 20 μM $CdCl_2$ for 48 h. Asterisks indicate significant differences from Δ*ycf1* harboring pYES2 at $P < 0.05$ according to Tukey's test based on three independent biological replicates

Functional expression of *TpNRAMP5* in *Arabidopsis*

To confirm whether TpNRAMP5 transports Cd, Co, Zn, Fe or Mn in *Arabidopsis*, we expressed *TpNRAMP5* or an empty vector (pCAMBIA1305.1) in *Arabidopsis* under the 35S promoter. Two independent homozygous *TpNRAMP5*-expressing lines were analyzed (Fig. S4a). The growth of *TpNRAMP5*-expressing lines was enhanced compared with the WT in the absence of metal stress conditions (Fig. 5a). At the seedling stage, 500 μM $MnCl_2$ significantly increased root growth (Fig. 5b), whereas 25 μM $CdCl_2$ and 80 μM $CoCl_2$ had no effect (Fig. S4b, c). Addition of 40 mg kg^{-1} $CoCl_2$ did not affect dry weights of the WT, empty vector line or *TpNRAMP5*-expressing lines (Fig. 5c). *TpNRAMP5*-expressing lines with 40 mg kg^{-1} $CdCl_2$ displayed symptoms of Cd toxicity in seedling leaves (red speckles and eventual chlorosis; Fig. 5d) and significantly reduced the dry weights of adult-stage soil-grown plants (Fig. 5e).

We also analyzed metal concentrations. Mn concentrations of roots (Fig. 6a), shoots (Fig. 6b) and whole plants (Fig. 6c) of *TpNRAMP5*-expressing lines were significantly increased when compared with WT, which indicates that expression of *TpNRAMP5* enhances the root accumulation of Mn absorbed from soil. However, the shoot-to-root concentration ratio [translocation factor (TF)] was significantly reduced in *TpNRAMP5*-expressing lines (Fig. 6d), thus leading to greater Mn retention in roots (Fig. 6a). Under 40 mg kg^{-1} $CoCl_2$ stress, Co concentrations of roots (Fig. 7a), shoots (Fig. 7b) and whole plants (Fig. 7c) of *TpNRAMP5*-expressing lines were significantly enhanced when compared with WT; whilst, the translocation of Co from roots to shoots was not affected (Fig. 7d). Under 40 mg kg^{-1} $CdCl_2$ stress, Cd concentrations in roots (Fig. 8a), shoots (Fig. 8b) and whole plants (Fig. 8c) of *TpNRAMP5*-expressing lines were significantly increased, but Cd translocation from roots to shoots was unaffected (Fig. 8d). This indicates that TpNRAMP5 is also involved in Cd accumulation. Finally, concentrations of Fe and Zn in roots and shoots of *TpNRAMP5*-expressing lines were unaltered (Fig. S5), thus implying that TpNRAMP5 is not a metal transporter for Fe and Zn accumulation.

Discussion

The uptake, translocation and sequestration of Mn, an essential metal nutrient in plants, is mediated by various transporters (Socha and Guerinot 2014). IRT, a high-affinity Fe

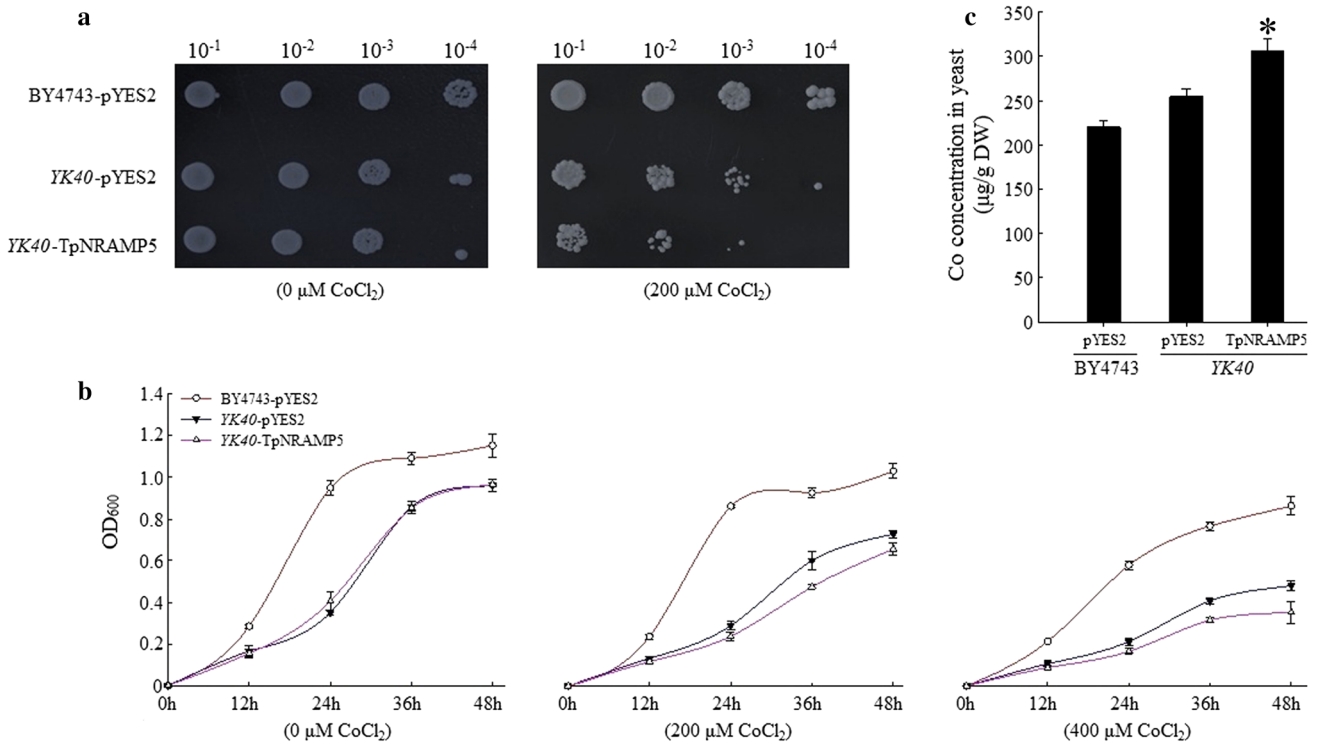

Fig. 4 Sensitivity to Co and concentrations of Co in yeast. **a** Co sensitivity in yeast grown on synthetic defined (SD) plates with 200 μM $CoCl_2$. **b** Co sensitivity in yeast grown in liquid SD medium with 200 and 400 μM $CoCl_2$. OD_{600} values were measured at 0, 12, 24, 36 and 48 h. **c** Co concentrations in yeast grown in liquid SD medium with 50 μM $CoCl_2$ for 60 h. Asterisks indicate significant differences from *YK40* harboring pYES2 at $P < 0.05$ according to Tukey's test based on three independent biological replicates

transporter, plays crucial roles in Mn uptake from soil to roots (Vert et al. 2002; Pedas et al. 2008). Most members of the NRAMP family function as Mn transporters to facilitate Mn distribution, sequestration and translocation (Lanquar et al. 2005, 2010; Yamaji et al. 2013; Yang et al. 2013; Gao et al. 2018), but only AtNRAMP1 and OsNRAMP5 promote Mn uptake (Cailliatte et al. 2010; Ishimaru et al. 2012a, b; Sasaki et al. 2012; Yang et al. 2014). In this study, Mn accumulation in tissues and whole plants of *TpNRAMP5*-expressing *Arabidopsis* lines were enhanced (Fig. 6a–c). Consequently, TpNRAMP5 is a Mn transporter for Mn accumulation. This information may explain why expression of *TpNRAMP5* promoted growth in either normal soil (Fig. 5a) or on medium with 500 μM $MnCl_2$ (Fig. 5b). In *TpNRAMP5*-expressing lines, the Mn TF was decreased (Fig. 6d). In *Arabidopsis*, AtZIP1 and AtZIP2 are responsible for Mn translocation from roots to shoots (Milner et al. 2013). The expressions of *AtZIP1* and *AtZIP2* were probably not elevated by *TpNRAMP5* in *TpNRAMP5*-expressing *Arabidopsis* lines, which ultimately led to greater TpNRAMP5-induced Mn retention in roots (Fig. 6a). Mn accumulation in the roots of *TpNRAMP5*-expressing lines was enhanced (Fig. 6a); as a result, the translocation efficiency was insufficient to translocate the large amounts of Mn, which finally led to a decrease in the TF.

Co, a metal that is not essential for plant growth, disrupts Fe homeostasis and competes with Fe for access to transporters in many organisms (Morrissey et al. 2009; Barras and Fontecave 2011). Several Co transporters, such as AtIRT1, IRON REGULATED1 (IREG1/FPN1), IREG2/FPN2 and AtHMA3, have been identified to date (Korshunova et al. 1999; Morrissey et al. 2009; Morel et al. 2009; Barberon et al. 2014). Only AtIRT1, however, is responsible for Co uptake, a process that is Fe regulated (Korshunova et al. 1999; Morrissey et al. 2009; Barberon et al. 2014). TpNRAMP5, identified in this study, is a novel Co transporter for Co accumulation, a conclusion supported by the following evidence: (1) expression of *TpNRAMP5* in yeast increased sensitivity to Co (Fig. 4a, b) and enhanced Co accumulation (Fig. 4c); (2) *TpNRAMP5* was mainly expressed in roots and basal stems (Fig. 1a) and was localized at the plasma membrane (Fig. 2); (3) expression of *TpNRAMP5* in *Arabidopsis* significantly enhanced Co concentrations in roots (Fig. 7a), shoots (Fig. 7b) and whole plants (Fig. 7c). However, Co translocation from roots to shoots in *TpNRAMP5*-expressing lines was not changed (Fig. 7d). Furthermore, Fe and Zn

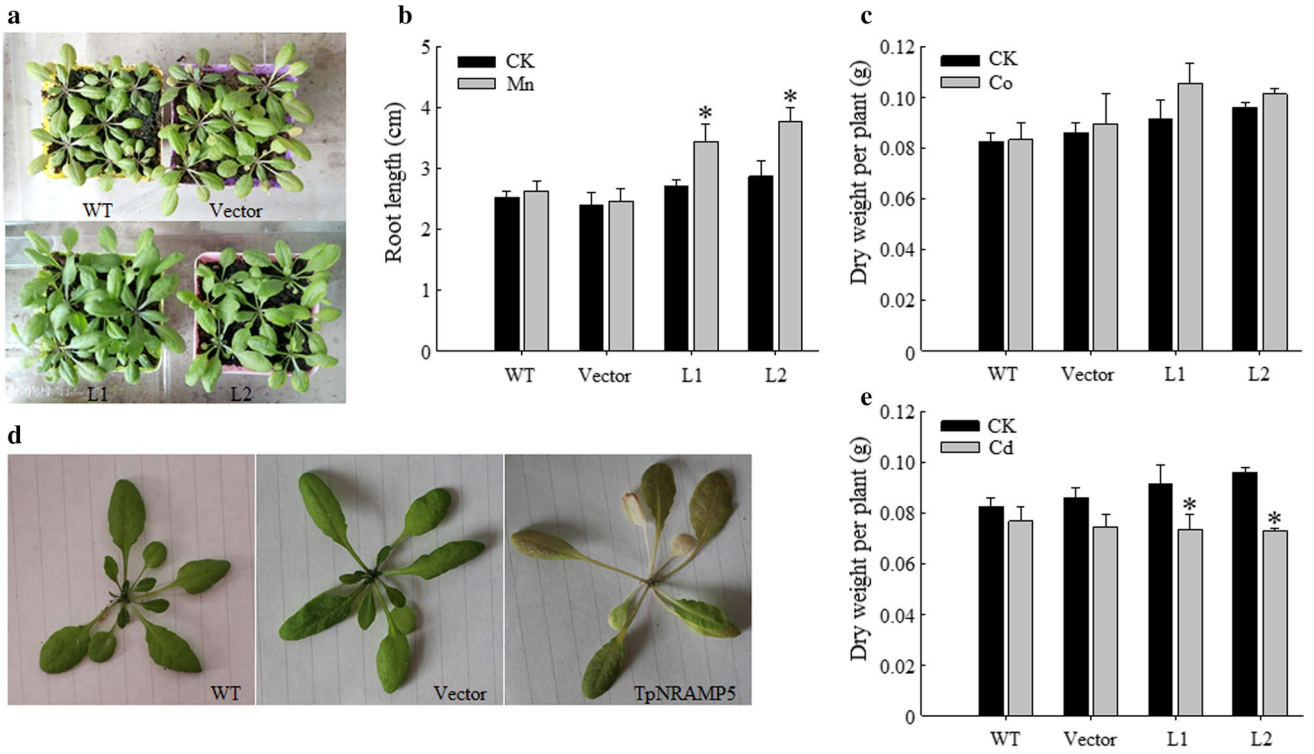

Fig. 5 Phenotypic assay of *TpNRAMP5*-expressing *Arabidopsis* under Mn, Co and Cd stresses**. a** Growth of the WT, vector line and two *TpNRAMP5*-expressing lines cultivated in soil for 4 weeks. **b** Root lengths of the WT, vector line and two *TpNRAMP5*-expressing lines grown on half-strength MS medium with 500 μM $MnCl_2$ for 10 days. **c** Dry weight of the WT, vector line and two *TpNRAMP5*-expressing lines grown in soil with 40 mg kg^{-1} Co stress. **d, e** Cd toxicity symptoms (**d**) and dry weights (**e**) of *TpNRAMP5*-expressing lines grown in soil with 40 mg kg^{-1} Cd stress. Asterisks indicate significant differences from individual controls (CK) at $P < 0.05$ according to Tukey's test based on three independent biological replicates

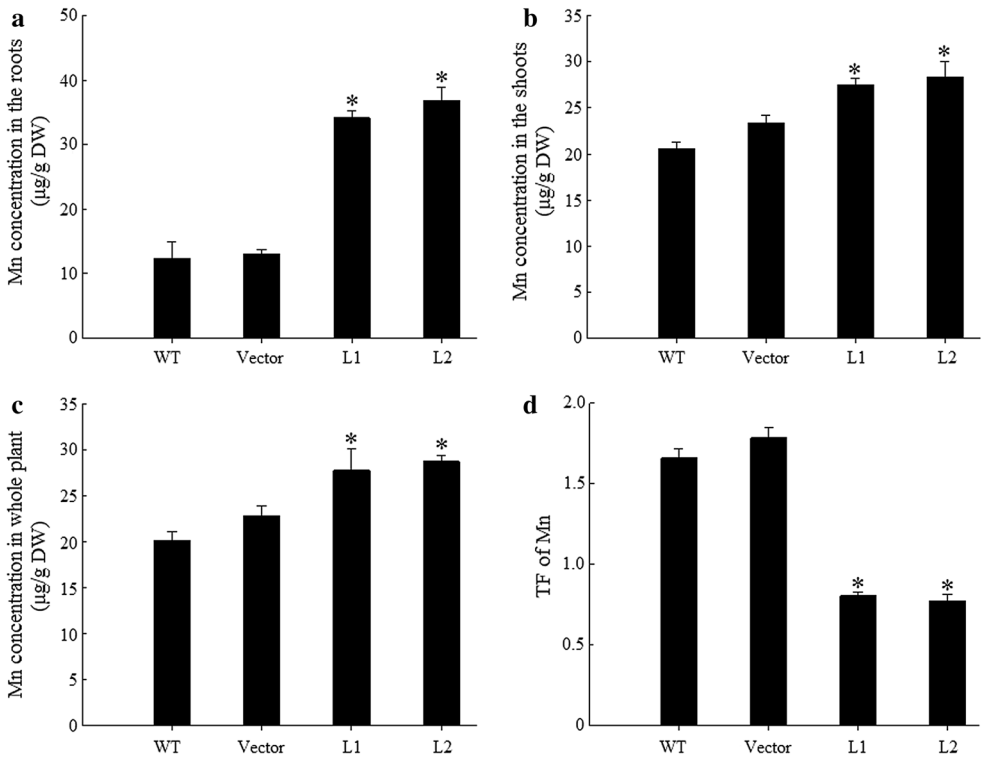

Fig. 6 Concentrations and translocation factors (TFs) of Mn in *Arabidopsis*. Concentrations of Mn in roots (**a**), shoots (**b**) and whole plants (**c**). **d** Mn TFs. All plants were grown in soil. Asterisks indicate significant differences from the WT at $P < 0.05$ according to Tukey's test based on three independent biological replicates

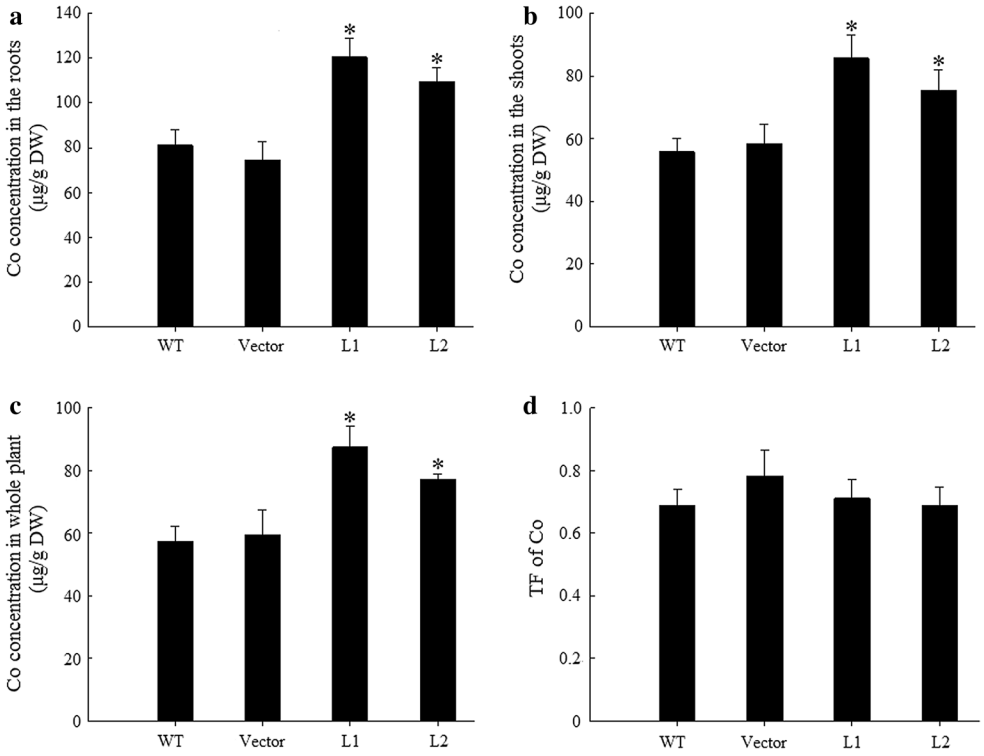

Fig. 7 Concentrations and translocation factors (TFs) of Co in *Arabidopsis*. Concentrations of Co in roots (**a**), shoots (**b**) and whole plants (**c**). **d** Co TFs. All plants were grown in soil with 40 mg kg^{-1} Co. Asterisks indicate significant differences from the WT at $P < 0.05$ according to Tukey's test based on three independent biological replicates

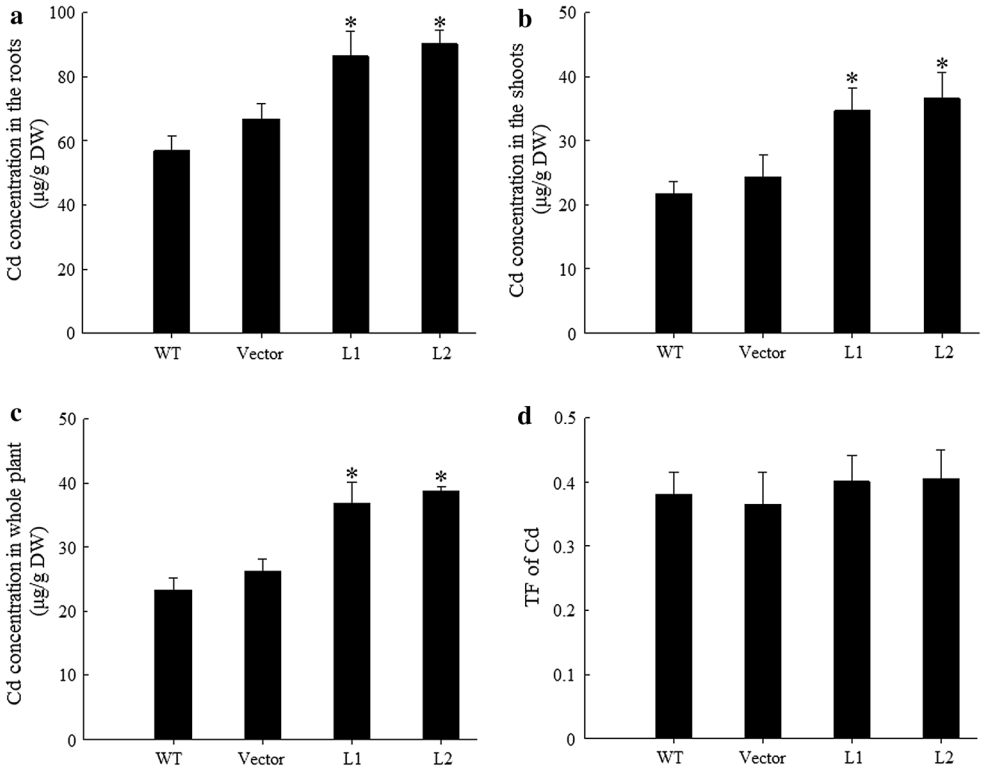

Fig. 8 Concentrations and translocation factors (TFs) of Cd in *Arabidopsis*. Concentrations of Cd in roots (**a**), shoots (**b**) and whole plants **(c)**. **d** Cd TFs. All plants were grown in soil with 40 mg kg^{-1} Cd. Asterisks indicate significant differences from the WT at $P < 0.05$ according to Tukey's test based on three independent biological replicates

concentrations in roots and shoots of *TpNRAMP5*-expressing lines were unchanged (Fig. S5).

Cd, a nonessential heavy metal that is toxic to all living organisms, disrupts nutrient homeostasis in plants (Verbruggen et al. 2009), thereby ultimately inhibiting plant growth and development. At the same time, Cd possesses physical and chemical characteristics similar to those of some essential metals, such as Zn and Fe (Chesworth 1991);

its transport thus usually relies on Zn and Fe transporters, including IRTs, NRAMPs and HMAs (Thomine et al. 2000; Nakanishi et al. 2006; Morel et al. 2009; Cailliatte et al. 2010; Takahashi et al. 2011; Ishimaru et al. 2012a; Sasaki et al. 2012; Tang et al. 2017). Plant NRAMPs currently identified as Cd transporters include AtNRAMP1, AtNRAMP3, AtNRAMP4, AtNRAMP6, OsNRAMP1, OsNRAMP5, HvNRAMP5, TcNRAMP3, TcNRAMP4 and MbNRAMP1 (Thomine et al. 2000; Xiao et al. 2008; Oomen et al. 2008; Cailliatte et al. 2009; Takahashi et al. 2011; Ishimaru et al. 2012a, b; Sasaki et al. 2012; Yang et al. 2014; Wu et al. 2016). Other NRAMPs, no evidence for Cd transport by TjNRAMP4, OsNRAMP3, OsNRAMP4 and OsNRAMP6 could be obtained (Mizuno et al. 2005; Xia et al. 2010; Yamaji et al. 2013; Peris-Peris et al. 2017). Among uncovered Cd transporters, only AtNRAMP1, OsNRAMP1, OsNRAMP5, HvNRAMP5 and NtNRAMP5 are involved in Cd uptake and accumulation (Thomine et al. 2000; Takahashi et al. 2011; Ishimaru et al. 2012a, b; Sasaki et al. 2012; Yang et al. 2014; Wu et al. 2016; Tang et al. 2017). In the present study, expression of *TpNRAMP5* in *Arabidopsis* significantly increased Cd concentrations in roots (Fig. 8a), shoots (Fig. 8b) and whole plants (Fig. 8c), but had no effect on Cd translocation from roots to shoots (Fig. 8d). At the same time, expression of *TpNRAMP5* in yeast significantly increased Cd sensitivity (Fig. 3a, b) and enhanced Cd concentrations (Fig. 3c). We therefore conclude that TpNRAMP5 is a Cd transporter for Cd accumulation. Under Cd stress, expression of *TpNRAMP5* in *Arabidopsis* thus increased Cd concentration, which caused seedling leaves to suffer from Cd toxicity (Fig. 5d) and decreased dry weights at the adult stage (Fig. 5e). Direct or indirect consumption of Cd-contaminated wheat by humans is a potential cause of disorders (Grant et al. 2008) such as renal proximal tubular dysfunction (Stinson et al. 2003) and itai-itai bone disease (Nogawa et al. 1987). Future genetic manipulation of TpNRAMP5 could limit Cd transfer from soil to wheat grains and therefore protect human health.

In our study, TpNRAMP5 shared 83.1 and 70.4% identities with its putative homologous gene OsNRAMP5 and OsNRAMP1, respectively (Fig. S1). However, OsNRAMP5 absorbs Cd, Mn and Fe (Ishimaru et al. 2012a, b; Sasaki et al. 2012; Yang et al. 2014); OsNRAMP1 takes up Cd, As and Fe, but not Mn (Curie et al. 2000; Takahashi et al. 2011; Tiwari et al. 2014). We actually found evidence that expression of *TpNRAMP5* promotes the accumulation of Cd, Mn and Co (Figs. 3, 4, 6, 7, 8), but not that of Fe and Zn (Fig. S5). Differences in a few residues of their primary sequences discriminate the substrate range of AtNRAMP3 and AtNRAMP4 (Lanquar et al. 2005). In addition, mutations in AtNRAMP4 affect Zn, Mn and Cd transport (Pottier et al. 2015), and allelic variation in the C-terminal of NtNRAMP5 impairs Mn and Cd transport (Tang et al. 2017). Differences in the functions of TpNRAMP5, OsNRAMP5 and OsNRAMP1 may thus be due to differences in their amino acid sequences (Figs. S1, S2).

The expression of *OsNRAMP5* is significantly regulated by Zn and Fe deficiency and unaffected by insufficient Cu and Mn (Sasaki et al. 2012), while the expression of *OsNRAMP1* is regulated by Fe deficiency and Cd supplementation (Takahashi et al. 2011). In our study, the expression of *TpNRAMP5* was significantly induced by Mg, Zn, Fe, Pb, Cu and Ni supplementation (Fig. 1b, c) but was not induced by treatment with Cd (Fig. 1b, c). In addition, the expression pattern of *TpNRAMP5* also differed from that of *AtNRAMP6* (Cailliatte et al. 2009), *OsNRAMP1* (Takahashi et al. 2011), *OsNRAMP3* (Yamaji et al. 2013; Yang et al. 2013), *GmNRAMP1–6* (Qin et al. 2017) and *AtNRAMP2* (Gao et al. 2018) and where expression was respectively observed mainly in first nodes and culms, roots and leaves, flowers and young leaves, dry seed embryos, and multiple tissues except roots.

Conclusion

TpNRAMP5, located on chromosome 4AS, is mainly expressed in roots and basal stems and localized at the plasma membrane. The expression of *TpNRAMP5* in seedling roots is significantly regulated by Mg, Zn, Fe, Pb and Ni. Expression of *TpNRAMP5* in yeast revealed that TpNRAMP5 functions as a metal transporter for Cd and Co accumulation, but not Zn. Expression analysis of *TpNRAMP5* in *Arabidopsis* illustrated that TpNRAMP5 is mainly involved in Cd, Co and Mn accumulation, but not that of Fe and Zn. The function of TpNRAMP5 is thus different from OsNRAMP5 transport of Cd, Mn and Fe (Sasaki et al. 2012; Ishimaru et al. 2012a) and the involvement of OsNRAMP1 in uptake and translocation of Cd and Fe, but not Mn (Curie et al. 2000; Takahashi et al. 2011; Tiwari et al. 2014).

Author contribution statement FP, CW, JZ and YW designed the experiments; FP, CW and JZ performed the experiments and drafted the manuscript; FP, JZ, HK, XF, LS and HZ analyzed the data; FP, YZ and YW revised the manuscript.

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