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Full-length transcriptome assembly and RNA-Seq integration of diploid and tetraploid ryegrass to investigate differences in cd uptake and accumulation among ryegrass with different ploidy levels

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Abstract

Background The accumulation of cadmium (Cd) in ryegrass (*Lolium multiflorum* Lamk.) as a widely used pasture plant poses a serious risk to food safety. This study aimed to investigate the differences in phenotypes, physiology, and expression of metal transporters between four ryegrass genotypes (diploid/tetraploid and Cd-tolerant/sensitive).

Results The diploid/Cd-sensitive genotypes were found to uptake, accumulate, and translocate more Cd compared to the tetraploid/Cd-tolerant genotypes. Cd with more soluble components facilitated the transfer of Cd from root to shoot in the sensitive genotypes. Tetraploid and Cd-tolerant Chuansi No.1 accumulated less Cd in shoots but higher ratio in root cell wall, making it a promising model for studying the mechanisms of plant resistance to Cd stress. The complex regulatory system and dilution effect contributed to the lower uptake and accumulation of Cd in tetraploid genotypes. Moreover, tetraploid genotypes exhibited higher expression of genes that promoted Cd efflux, which could contribute to their lower Cd accumulation.

Conclusions Overall, this study sheds light on the physiological and transcriptional mechanisms of Cd uptake and accumulation by different polyploids, providing guidance for ryegrass breeding and soil improvement.

Keywords *Lolium multiflorum* Lamk., Cd stress, Cd accumulation, Cd distribution, Diploid and tetraploid, Transcriptome

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Background

Cadmium (Cd) is a toxic heavy metal with serious implications for human food safety [1–3]. Humans primarily ingest Cd through food, and previous studies have shown that excessive intake of Cd can lead to "Itai-Itai disease", a condition characterized by severe bone deformities such as brittle and easily broken bones, first reported in Japan [4]. Therefore, it is essential to reduce the Cd content in drinking water and cultivated land and to strictly monitor the Cd content in food to minimize environmental pressure and maintain food safety [5, 6]. Moreover, the Cd content of forage plays a critical role in ensuring the safety of human meat and milk [7]. Thus, exploring the mechanism of Cd absorption and enrichment in forage is crucial for ensuring food safety.

Ryegrass (*Lolium multiflorum* Lamk.), also known as annual ryegrass, is a widely cultivated forage crop in Europe and Asia that originated in Italy. Due to its fast growth, large biomass, easy management, and high productivity, it is widely favored by farmers [8]. Additionally, it can be used in annual rotations with rice to make better use of winter fallow fields in rice planting areas. Ryegrass is tolerant to Cd and can even germinate under 1000 μmol·L^{-1} Cd treatment [9]. Therefore, it is considered an essential crop for exploring the mechanism of Cd absorption and accumulation due to its tolerance to Cd. Furthermore, a Cd stress-responsive gene, *LmAUX1*, has been identified in ryegrass [10].

Ryegrass is naturally present in the form of a diploid genotype. With the development of science and technology, tetraploid genotypes were discovered to have higher yield, faster growth, and stronger adaptability to the environment after autopolyploidization, and were widely promoted in the last century [11]. Polyploidy tends to make plants significantly more adaptive and tolerant to abiotic stresses than their diploid progenitors [12–14]. In our previous study, we found that tetraploid ryegrass genotypes were more tolerant to Cd than diploid genotypes at the germination stage [15]. The specific mechanism, however, remains to be further explored.

In this study, we intend to explore the relationship between Cd uptake and accumulation of diploid/Cd sensitive and tetraploid/Cd tolerance genotypes through four ryegrass genotypes (PI 577241, diploid Cd sensitive genotype, DS; PI 636508, diploid Cd tolerance genotype, DT; PI 611145, tetraploid Cd sensitive genotype, TS; Chuansi No.1, tetraploid Cd tolerance genotype, TT). We evaluated phenotypic parameters (biomass, root parameters, and photosynthetic pigment contents), physiological parameters (Cd and other metal content, Cd fluxes, Cd subcellular distributions, and Cd chemical forms), and metal transporter expression (ISO-seq and RNA-seq) in this study. Our ultimate aim was to improve the understanding of the adaptability of diploid and tetraploid ryegrass genotypes to Cd-polluted environments and provide new guidance for the molecular breeding of ryegrass.

Results

Plant growth and root morphological parameters

Plant growth and root morphological parameters were differentially affected by Cd treatment in the four genotypes (Fig. 1). The dry weight (DW) of both tolerant genotypes (DT and TT) did not change significantly under Cd stress. The root DW of DS decreased significantly, and the DW of roots and shoots of TS were significantly reduced (Fig. 1 A and B). Moreover, both total root length and root surface area of the two sensitive genotypes (DS and TS) were significantly reduced under Cd treatment. The total root length of the two tolerant genotypes was not affected by Cd stress, but their root surface area decreased significantly under Cd stress (Fig. 1 C and D).

Effects of Cd on chlorophyll content

Photosynthetic performance of four genotypes was affected under Cd stress. The Chl *a*, Chl *b* and Chl (*a* + *b*) of four genotypes all decreased significantly under Cd stress (Fig. 1 E to G). The ratio of Chl *a* and Chl *b* per plant did not differ among the genotypes (Fig. 1 H).

Cd content and flux

Following Cd treatment, the tissue Cd concentrations of both tolerant genotypes were significantly lower than the sensitive genotypes in the same ploidy. Besides, diploid genotypes accumulated more Cd than tetraploid genotypes when the genotype was the same (both sensitive and tolerant genotypes) (Fig. 2 A to C). However, there was no difference in Cd translocation from root to shoot between the two diploid genotypes, and both were significantly higher than both tetraploid genotypes. Moreover, Cd translocation from root to shoot of TS was significantly higher than that of TT (Fig. 2 D).

The non-invasive micro-test technology (NMT) probe was used to detect Cd flux in the meristem of the four ryegrass genotypes (Fig. 2 E and F). The level of Cd flux in the meristem was in the order of DS > TS > DT > TT. Moreover, Cd flux in the sensitive genotypes was significantly higher (22.72 ± 3.45 $cm^{-1}·s^{-1}$) compared to the tolerant genotypes (9.39 ± 2.59 $cm^{-1}·s^{-1}$).

Other metals concentrations

The concentrations of Ca, Fe, Mg, Mn, Zn, and Cu in the four genotypes were affected differently under Cd stress (Fig. 3). In DS, the concentrations of Mg, Mn, and Cu in the shoot were drastically decreased, and the concentrations of Fe, Mg, Mn, Zn, and Cu in the root

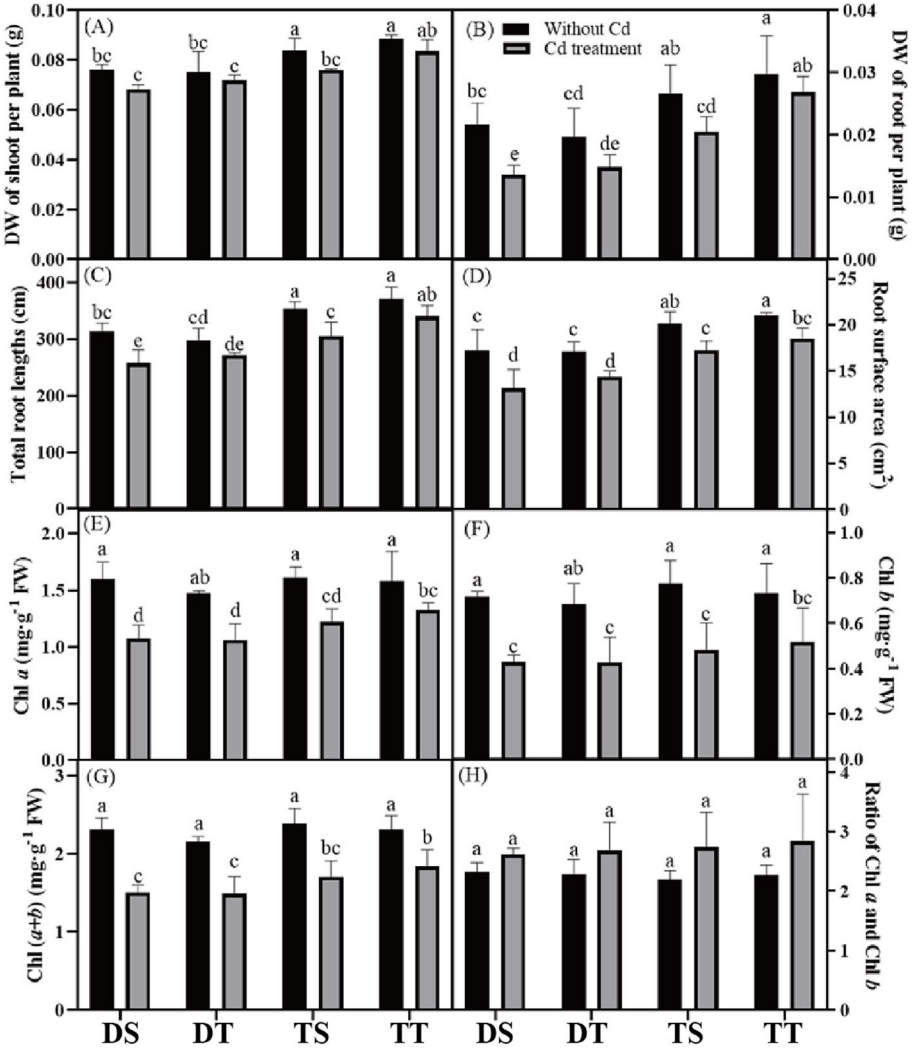

Fig. 1 Effects of Cd on the phenotypic parameters of ryegrass. Dry weight (DW) of shoots (**A**) and roots (**B**), total root length (**C**), root surface area (**D**), Chl *a* (**E**), Chl *b* (**F**), Chl (*a* + *b*) (**G**), and ratio of Chl *a* and Chl *b* (**H**). FW, fresh weight. Values were the mean of three biological replicates. Data presented as mean ± SD. Different letters indicate a significant difference at $p < 0.05$ by one-way ANOVA (Duncan test)

were significantly different after Cd treatment. In DT, the concentrations of Fe, Mn, Zn, and Cu in the shoot were significantly different under Cd stress, and the concentrations of Fe, Mg, and Cu in the root under Cd treatment were significantly higher than without Cd. In TS, there were no significant differences in the metal content of the shoot, but the concentrations of Ca and Cu in the root were significantly increased after Cd treatment. In TT, the concentrations of Ca, Fe, Mn, and Zn in the shoot were significantly different after Cd treatment. Moreover, the concentrations of Ca, Fe, Mn, Zn, and Cu in the shoot were significantly increased under Cd stress.

Cd chemical forms and subcellular distribution

We investigated the effect of Cd treatment on the accumulation and translocation of six Cd chemical forms in four ryegrass genotypes (Table S1). Due to differences in the uptake of Cd among the four genotypes, we analyzed the difference between the six Cd forms by ratio. In the roots of tolerant genotypes, the ratios of F_w (sensitive genotypes = 16.19% ± 1.93%, tolerant genotypes = 10.02% ± 0.81%) and F_{HAC} (sensitive genotypes = 11.78% ± 0.01%, tolerant genotypes = 8.77% ± 0.01%) decreased specifically, whereas the ratios of F_{NaCl} (sensitive genotypes = 4.93% ± 0.01%, tolerant genotypes = 6.65% ± 0.01%) and F_E (sensitive genotypes = 66.48% ± 2.62%, tolerant genotypes = 74.13% ± 1.82%)

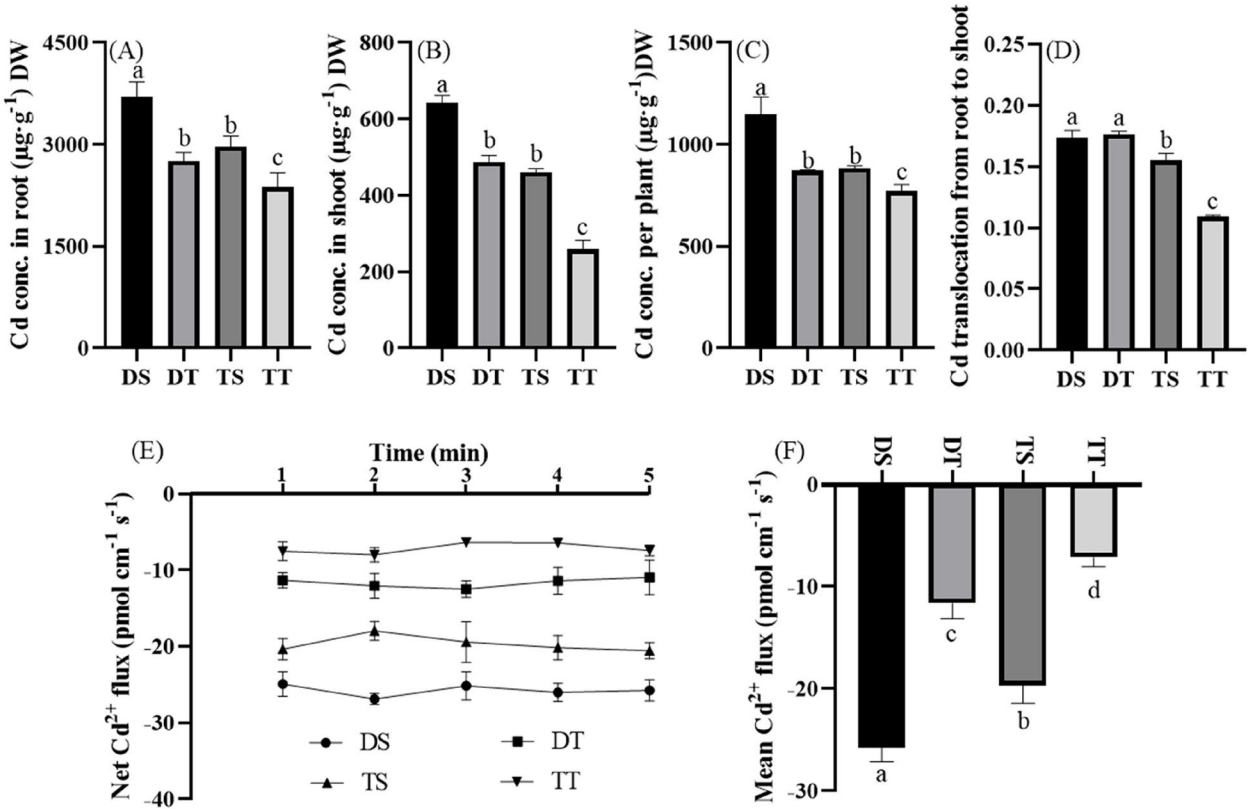

Fig. 2 Cd concentrations in root (**A**), shoot (**B**), total plant (**C**), Cd translocation from roots to shoots (**D**), and Cd flux in root (**E** and **F**). DW, dry weight. Values were the mean of three biological replicates. Data presented as mean ± SD. Different letters indicate a significant difference at $p < 0.05$ by one-way ANOVA (Duncan test)

were significantly increased compared to the sensitive genotypes (Fig. 4 A). In diploid genotypes, the ratio of F_{NaCl} in the root (diploid genotypes = 71.19% ± 0.13%, tetraploid genotypes = 68.64% ± 0.27%) was significantly increased in diploids compared to tetraploid genotypes (Fig. 4 B). Additionally, the F_E ratio of TS in the shoot was significantly higher than that of the other three genotypes.

Similarly, the subcellular distribution of Cd (F_{cw}, F_s, F_{co}) is also displayed by converting the original data (Table S2) into ratios (Fig. 4 C and D). In DS, DT, and TS, the level of Cd subcellular distribution in roots and shoots was $F_s \geq F_{cw} > F_{co}$. However, in TT, it was $F_{cw} > F_s > F_{co}$ in roots and $F_s \geq F_{cw} > F_{co}$ in shoots. In the roots of Cd diploid genotypes, the ratio of F_{co} in DT was significantly higher than in PI577241, and diploid genotypes were also higher than tetraploid genotypes. However, the ratio of F_{cw} in tetraploid genotypes was specifically decreased in shoots. In addition, the ratio of F_s in tolerant genotypes was significantly reduced when compared with the sensitive genotypes.

Full-length reference transcriptome

The differences in physiological parameters of roots were more significant after Cd stress, and roots acted as the first barrier for plants to come into contact with Cd. Therefore, we investigated the transcriptomics of roots to further explore the differences in Cd uptake and accumulation between diploid and tetraploid ryegrass genotypes.

We obtained 440,626 and 617,904 polymerase reads in DT and TT, respectively (Table S3). The full-length non-chimeric (FLNC) reads were 333,697 and 492,615 for the DT library and TT library, respectively (Table S4). We used the hierarchical n*log(n) algorithm and arrow software to correct the consensus sequence and finally obtained the polished consensus sequence (Table S5). We corrected the transcripts on the LoRDEC platform (http://atgc.lirmm.fr/lordec) to improve data accuracy (Table S6), and we used CD-HIT software (http://www.bioinformatics.org/cd-hit) to remove redundant isomers (Table S7). Finally, we obtained two high-quality transcripts by correction and de-redundancy.

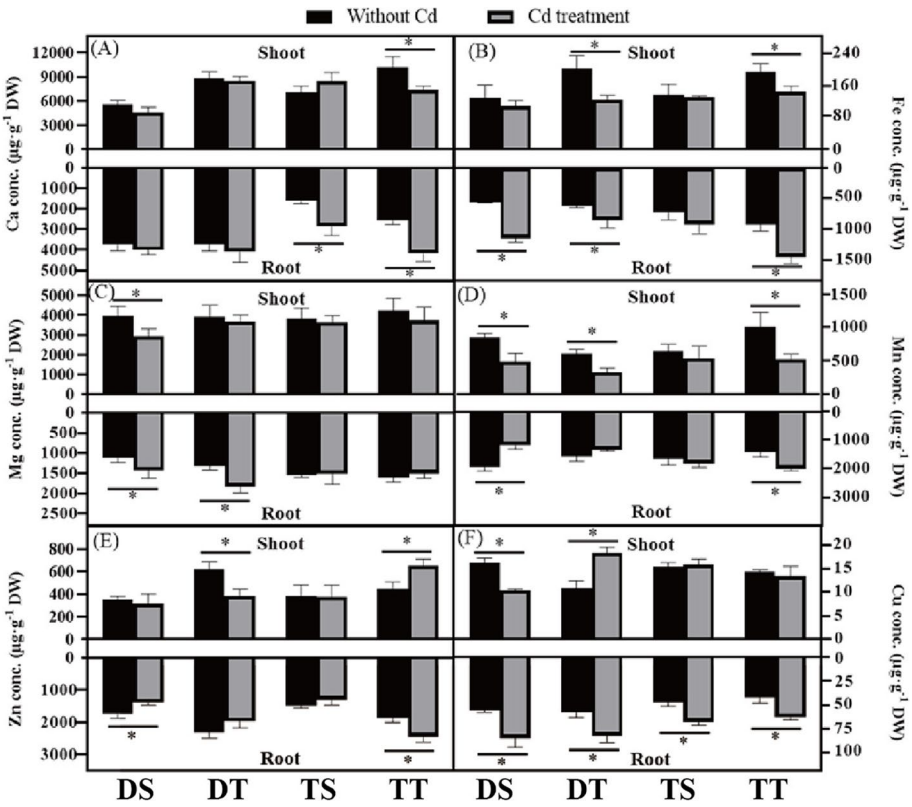

Fig. 3 Concentrations of micronutrients in root and shoot: Ca (**A**), Fe (**B**), Mg (**C**), Mn (**D**), Zn (**E**) and Cu (**F**). DW, dry weight. Values were the mean of three biological replicates. Data presented as mean ± SD. * indicates a significant difference compared to without Cd treatment at $p < 0.05$ with Student's t-test

The DT library had a total of 33,941 transcripts and 19,345 annotated genes, and the TT library had a total of 48,757 transcripts and 25,963 annotated genes. We considered the DT library as the reference transcriptome of the diploid genotypes, and the TT library as the reference transcriptome of the tetraploid genotypes.

Venn diagrams were created based on the results of transcriptional analysis (Fig. 5 A). Among annotated genes, 8,568 transcripts were commonly annotated in both DT and TT. All transcripts from ISO-seq were aligned to seven databases, including NCBI non-redundant proteins (NR), NCBI non-redundant nucleotides (NT), Protein family (Pfam), euKaryotic Ortholog Groups (KOG) / Clusters of Orthologous Groups of proteins (COG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Ontology (GO) (Figure S1 A and B). Venn diagrams based on the annotation results of five common databases were selected from the seven database annotations (Figure S1 C and D).

Regarding the GO analysis, the transcripts were mainly associated with metabolic process (GO:0008152) and cellular process (GO:0009987) in the biological process category (DT = 46.30%, TT = 46.32%). In cellular component category (DT = 22.77%, TT = 22.68%), the transcripts were mainly associated with cell (GO:0005623) and cell part (GO:0044464). In molecular function category (DT = 30.93%, TT = 31.00%), the transcripts were mainly associated with binding (GO:0005488) and catalytic activity (GO:0003824) (Fig. 5 B and C). As for the KEGG analysis, transport and catabolism were the prominent pathways in cellular processes, signal transduction was the prominent pathway in environmental information processing, translation and folding, sorting and degradation were the prominent pathways in genetic information processing, and carbohydrate metabolism and amino acid metabolism were the prominent pathways in metabolism (Fig. 5 D and E).

DEGs response to Cd stress

A total of 3363, 2953, 6548, and 5491 differentially expressed genes (DEGs) were obtained under Cd stress in DS, DT, TS, and TT, respectively (Figure S2 A to D). Venn diagrams in Figure S2 E illustrate the DEG response in all genotypes. Approximately 833 transcripts were collectively annotated in all four genotypes, while 624, 1015, 2465, and 1587 transcripts were independently annotated in DS, DT, TS, and TT, respectively.

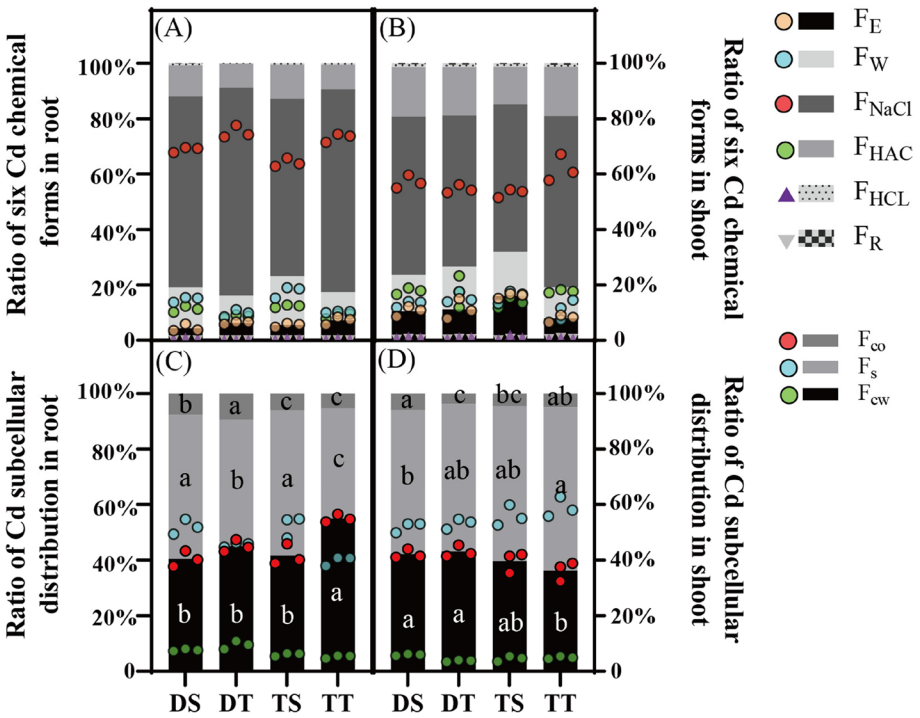

Fig. 4 The ratio of six Cd chemical forms (**A** and **B**) and Cd subcellular distribution (**C** and **D**) in root and shoot. Values were the mean of three biological replicates. Different letters indicate a significant difference at $p < 0.05$ by one-way ANOVA (Duncan test)

Since the genes responsible for metal transporters are usually associated with Cd uptake and accumulation, we focused on screening metal transporters. A total of 103, 92, 182, and 127 transcripts of metal transporters were annotated in DS, DT, TS, and TT, respectively. To better understand the differences between different genotypes, gene differential expression of diploid and tetraploid genotypes was analyzed separately (Fig. 6). In diploid genotypes, a total of 20 transcripts/11 metal transporters were found to have differences between DS and DT (Fig. 6 A). Among them, NRAMP1, ABCC2, and ABCG28 were specifically up-regulated, while ACA13, ABCG14, ABCG25, YSL16, and YSL6 were significantly down-regulated in DS. ATX1, CCH, ZIP2, and ABCG23 were up-regulated in DT. In TS, ABCG23, ABCG28, ABCG53, NRAMP4, and YSL16 were specifically up-regulated, while ABCB8, ABCG5, HMA7, and ACA2 were specifically down-regulated. In TT, ABCG33, IRT1, NRAMP2, and ZIP10 were specifically up-regulated, and ACA13 was significantly down-regulated. NRAMP5 was specifically decreased in TS but significantly increased in TT. Among all transcripts, ABCB9 and COPT3 were only differentially expressed in diploid genotypes, while ABCG31, ACA9, HMA1, NRAMP5, YSL12, and ABCG37 were only differentially expressed in tetraploid genotypes (Fig. 6 C). Compared to in TS, NRAMP5 in TT was found to be an early termination mutation (Fig. 7). Moreover, ZIP1 was specifically decreased in diploid genotypes but significantly increased in tetraploid genotypes.

Eight metal transporters were randomly selected to verify the accuracy of RNA-seq by qRT-PCR (Fig. 8). Among them, ZIP1, COPT3, ACA9, and NRAMP5 were differentially expressed genes in diploid or tetraploid genotypes, and ABCG14, CCH, HMA7, and NRAMP2 were independently differentially expressed genes in four genotypes, respectively. All qRT-PCR results were consistent with RNA-seq results.

Discussion

Tetraploid/tolerant genotypes accumulate less Cd than diploid/ sensitive genotypes

Cd treatment significantly affects the biomass of ryegrass (Fig. 1), and similar results have been previously reported in maize [3]. Besides, as the same species, there might be differences in Cd uptake and accumulation among different genotypes [16, 17]. Our results revealed that tetraploid/tolerant genotypes had fewer changes in root morphology parameters and pigment content (Fig. 1) as well as lower Cd concentrations and Cd^{2+} flux under Cd stress (Fig. 2), suggesting that these genotypes had superior potential in maintaining homeostasis and reducing the accumulation of Cd. In terms of Cd^{2+} flux parameters, TS showed higher values than DT, but there was no difference between them in Cd concentration. The two parameters may seem

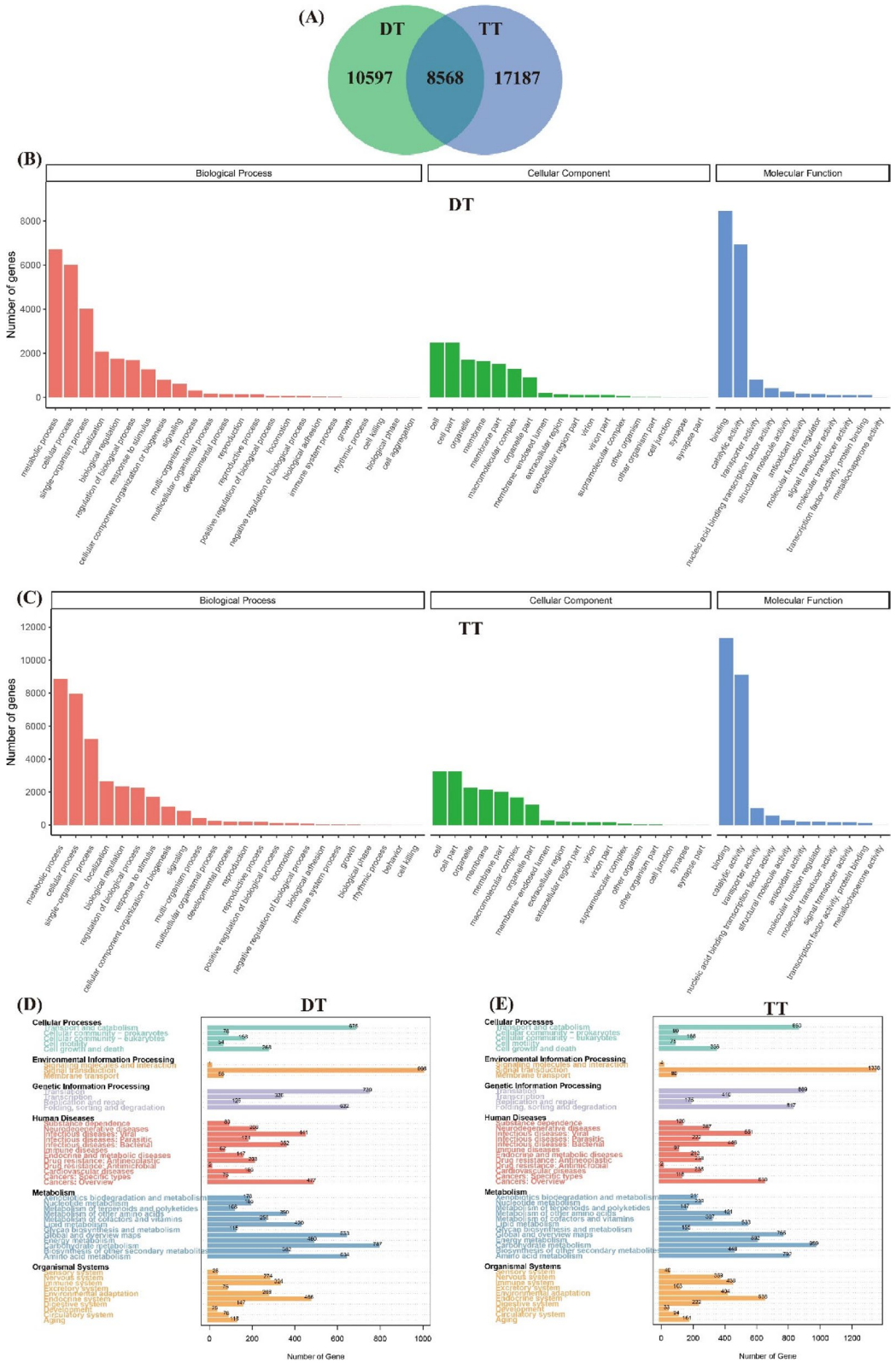

Fig. 5 Full-length reference transcriptome annotation for DT and TT. **A** Venn diagram of all transcripts from DT and TT. **B** and **C** Gene function classification for GO. **D** and **E** Gene function classification for KEGG

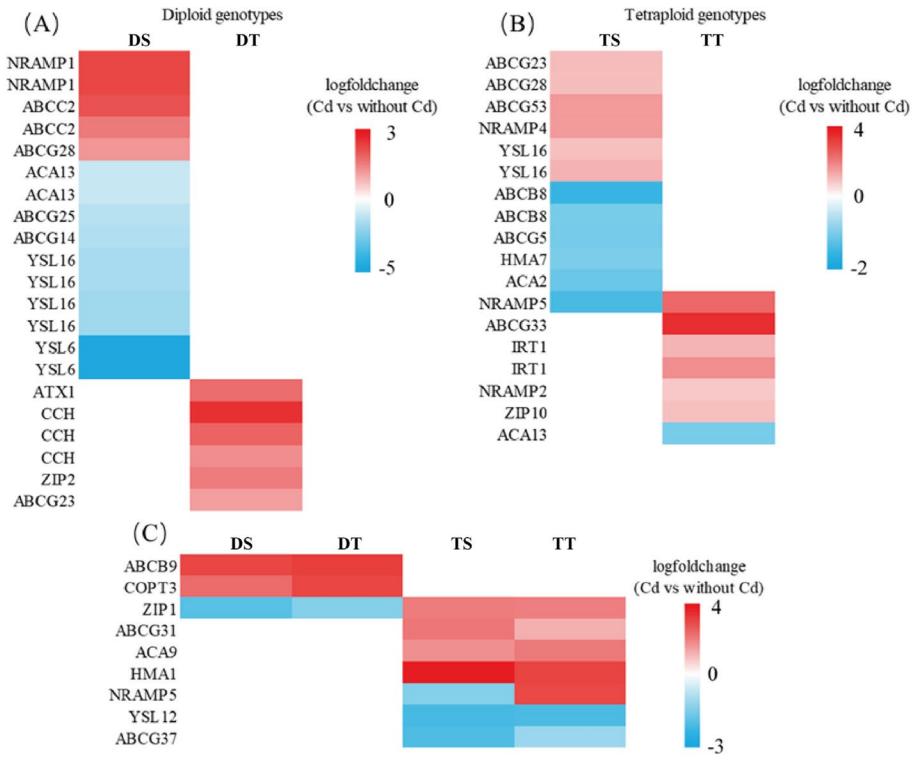

Fig. 6 Differential expression of metal transporters in diploid genotypes (**A**), tetraploid genotypes (**B**), and between diploid and tetraploid genotypes (**C**)

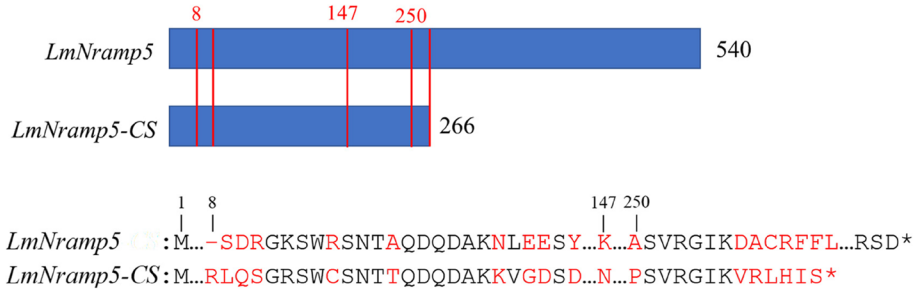

Fig. 7 Alignment of the amino acid sequence of *LmNramp5* and *LmNramp5 -CS*

contradictory, but the dry weight of roots in TS was significantly higher than that of DT. In addition, the dry weight of tetraploid genotypes was significantly higher than that of diploid genotypes, and its Cd content was significantly lower (in the same Cd tolerance/ sensitive genotype), indicating that the difference in growth speed between tetraploid genotypes and diploid genotypes also led to a certain degree of dilution effect. Tetraploid/tolerant genotypes exhibited superior adaptation to Cd stress compared to diploid/sensitive genotypes, which is consistent with previous findings on autopolyploidy [18, 19]. However, there was no significant difference between the Cd concentrations of DT and TS in this study (Fig. 2 A and B). This result indicates that not all diploids take up less Cd than tetraploids. Moreover, PI 611146, the parent of TT, was also recognized as a Cd-tolerant genotype in previous studies [15], but was not as tolerant as TT. Therefore, we believe that the Cd tolerance in TT was inherited from the parent.

Differences in Cd uptake and transport were caused by competitive/synergistic absorption of metal ions, Cd forms, and subcellular distribution

Essential metal transport pathways often mediate the uptake and accumulation of Cd in plants [9, 20,

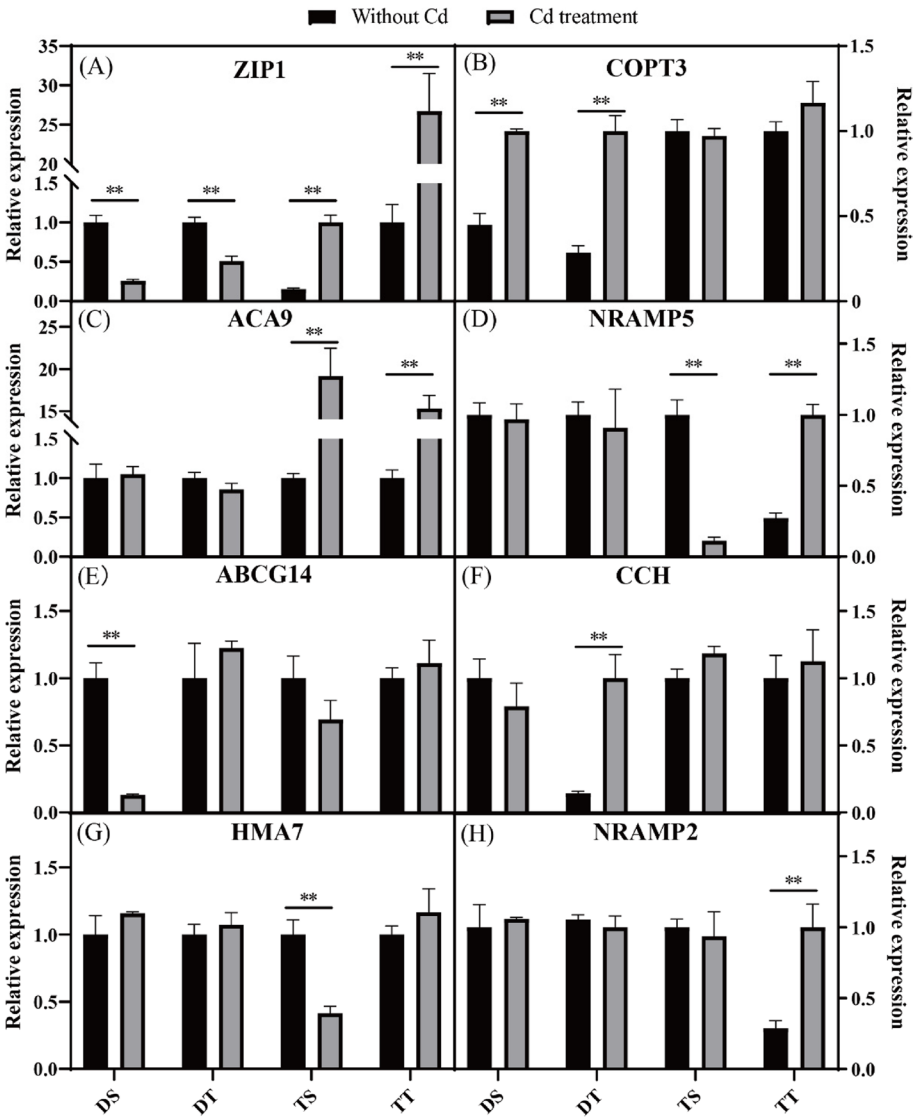

Fig. 8 Identification of 8 Cd transport-related DEGs based on a qRT-PCR assay. Values were the mean of three biological replicates. Data presented as mean ± SD. ** indicates a significant difference compared to without Cd treatment at $p < 0.01$ with Student's t-test

21]. Our study showed that Cd treatments altered the uptake and accumulation of other metals in ryegrass (Fig. 3), suggesting that there may be competition or cooperation between metal transporters for translocation of metals in ryegrass. Exposure to Cd also resulted in differences in the concentration of essential metals in the four genotypes, indicating differences in metal transport systems between diploid and tetraploid genotypes, particularly in TT. Studies on potato (*Solanum tuberosum*) and ryegrass have demonstrated that the uptake of micronutrients, such as essential metals, is affected by the genotype and tissue-specific Cd levels [9, 20].

The migration and binding of Cd are also affected by different forms of Cd [22, 23]. F_W has greater mobility than other forms [22, 24]. However, other forms of Cd are mainly bound in cell walls or other organelles [25]. In sensitive genotypes, the ratio of F_W was significantly higher than that in tolerant genotypes (Fig. 4 A). The results showed that the Cd translocation in sensitive genotypes was significantly higher than in tolerant genotypes, probably due to the greater mobility of Cd in sensitive genotypes. These results were also supported by the Cd subcellular concentration (Fig. 4 C and D).

Our study revealed that Cd subcellular distribution also varied significantly among the four genotypes, which

could have influenced Cd migration [22, 25, 26]. The root cell wall (F_{cw}) could limit Cd translocation, but F_s was easily translocated to other organs due to its high mobility [22]. In this study, the ratio of F_s in sensitive genotypes was higher than in tolerant genotypes, indicating that higher ratio of Fs in sensitive genotypes promoted the transport of Cd from the root to the shoot. In tetraploid genotypes, the ratio of F_{cw} in TT was significantly higher than in TS, while the ratio of F_s in TT was significantly lower, which indicates that Cd was mainly stored in the cell wall in TT and restricted Cd transport (Fig. 4 C). On the other hand, there were more proportions of F_{co} in the roots of DT, but the specific reason was not clear.

Differential expression of metal transporters in tetraploid and diploid genotypes

As the first barrier for plants to encounter Cd, roots can provide timely feedback on the impact of Cd stress on ryegrass. Therefore, our transcriptomic exploration and discussion mainly focus on the changes of Cd in roots. Differences in metal concentrations, such as Fe and Mg, chemical forms, subcellular distribution of Cd, and differentially expressed genes (DEGs) encoding metal transporters, could possibly explain the variations in Cd uptake and accumulation among the four genotypes [27]. At the transcriptome level, tetraploid genotypes exhibit more shared or unique DEGs than diploids (Fig. 6). This may be attributed to the fact that tetraploids have more annotated transcripts and a more complex regulatory system. For instance, ZIP1 is a metal efflux transporter that helps in the removal of excess Zn, Cd, or Cu. In rice, overexpression of *OsZIP1* allowed it to become more adaptive and accumulate fewer metals under excessive metal stress [28]. In our study, the expression levels of ZIP1 were significantly lower in diploid genotypes but higher in tetraploid genotypes (Fig. 6 C), which might explain the lower Cd accumulation in tetraploid genotypes (Fig. 2). The difference in the differential expression of ZIP1 between diploid and tetraploid genotypes may be attributed to the more complex regulatory network in tetraploid genotypes, but the specific mechanism requires further exploration. In addition, the impact of the copy number of a gene on function cannot be ignored. Unlike the stoichiometric effects that commonly result from multicopying genes[29, 30], multicopying of the Cd master transporter NRAMP5 further reduces Cd accumulation shoot and grains in plants [31]. This might also be one of the reasons for the reduced Cd accumulation in tetraploid genotypes.

We propose a model (Fig. 9) to illustrate the functions of metal transporters in diploid and tetraploid genotypes based on the differential expression of metal transporters and differences in metal accumulation among the four genotypes.

NRAMP1 is one of the metal transporters responsible for Cd entry into root cells and has similar functions with NRAMP5. Knocking out its homologous gene *OSNRAMP1* in rice significantly decreased Cd content [32]. Previous studies have shown that up-regulation of ACA13 can down-regulate the expression of Cd transporters such as NRAMP5 and HMA2, thereby reducing Cd concentration in rice [33]. In DS, NRAMP1 was specifically up-regulated and ACA13 was significantly down-regulated, which could explain why more Cd is accumulated in DS. Moreover, ACA13 may regulate Cd in plants by mediating NRAMP1 expression due to the negative correlation between them and the similar functions of NRAMP1 and NRAMP5, but this still needs further verification. YSL6 is a Mn-nicotianamine transporter that plays a crucial role in Mn detoxification [34, 35], and was significantly down-regulated in DS. This result is consistent with the observed decrease in root Mn content in DS (Fig. 3 D). YSL16 is important in maintaining plant iron homeostasis [36], and its significant down-regulation under Cd stress leads to the destruction of iron homeostasis, thus increasing Fe uptake in DS.

CCH and ATX1 are involved in the maintenance of intercellular Cu homeostasis and long-distance transport of Cu in plants [37–39]. Their significant up-regulation in DT suggests that the transport coefficient of Cu was specifically higher in this genotype than in DS ($DS = 0.12 \pm 0.01$; $DT = 0.22 \pm 0.02$) under Cd stress. We speculate that CCH or ATX1 might also participate in the long-distance transport of Cd, as there was no difference in the Cd transport coefficient of DT, a Cd-tolerant genotype, compared to DS (Fig. 2 D).

ABC transporter C and B family members have been widely reported to be involved in vacuolar Cd storage [40–42]. Although there is no accurate report on the function of ABCC2, previous studies suggested that ABCC2 and ABCC9 have similar functions and can fix Cd in vacuoles [27, 43].

ZIP2 is known to be an efflux transporter of Cd and its up-regulation has been shown to reduce Cd accumulation in *Arabidopsis* [44]. Therefore, the significant up-regulation of ZIP2 in DT may contribute to its lower Cd accumulation when compared with DS. ABC transporter G family members have also been reported to play a role in Cd efflux [45, 46]. For instance, the overexpression of *OsABCG36* in rice roots has been shown to promote Cd efflux [45]. In DS, ABCG28 was specifically up-regulated while ABCG14 and ABCG25 were significantly down-regulated. The opposing effects of the differential expression of these genes resulted in the accumulation of more Cd in the vacuoles, but not in its elimination through

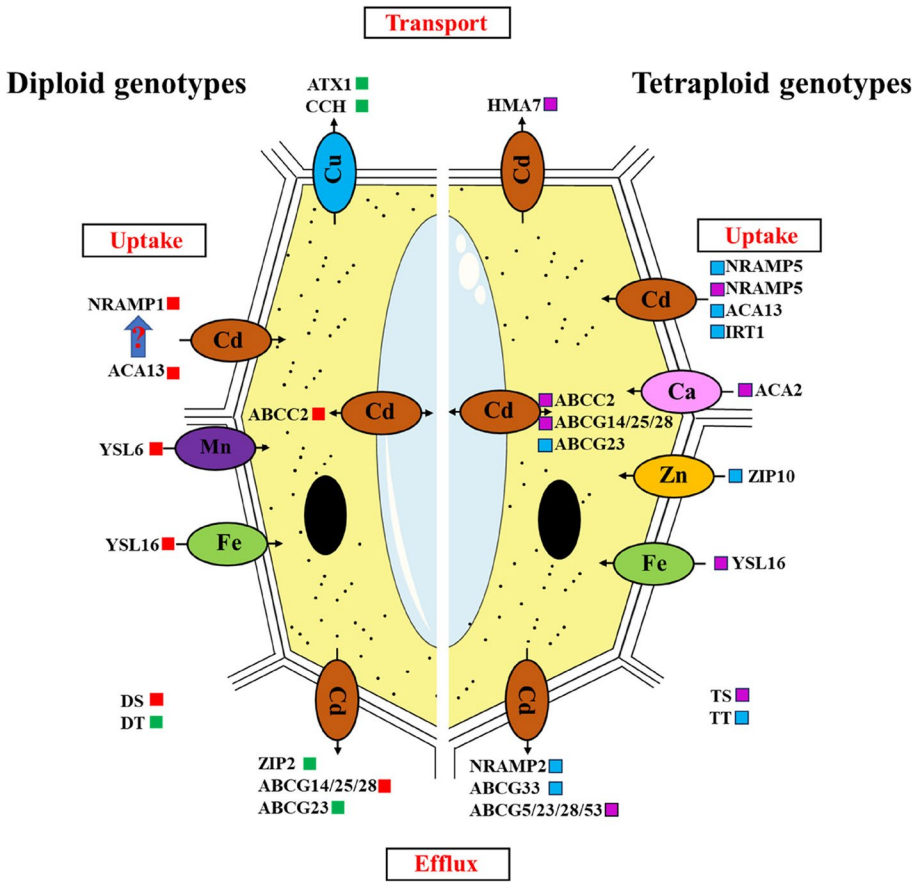

Fig. 9 The potential model for Cd distribution and differential expression of metal transporters in diploid and tetraploid genotypes

efflux transporters. This may also explain the higher Cd concentration in F_s (Fig. 4 C).

NRAMP5 is a key transporter for Cd uptake into root cells in plants [47, 48]. However, in TT, NRAMP5 was found to be spliced for unknown reasons (Fig. 7), which may explain the inconsistent expression of NRAMP5 and Cd concentration in comparison to previous studies. This phenomenon is referred to as subfunctionalization, and it has also been reported in Arabidopsis and cotton [49, 50]. The splicing of NRAMP5 in TT (*LmNRAMP5-CS*) may have altered its function and led to differential expression. In contrast, the un-spliced NRAMP5 (*LmNRAMP5*) was also annotated in the full-length transcriptome library of TT, but there was no differential expression observed in TT. This difference may be due to genotype variations or the binding of *LmNRAMP5-CS* to upstream products of *LmNRAMP5*, preventing the differential expression of *LmNRAMP5*. This genetic mutation might result in the loss of Cd transport function in TT. In addition, negative feedback regulation of ACA13 on NRAMP5 was observed in TT, but there was no differential expression of ACA13 or ACA3 in TS, indicating that NRAMP5 may also be regulated by other unknown genes.

Furthermore, IRT1 was considered as the Cd absorption transporter in TT [44, 51], while ZIP10 was found to help alleviate Cd toxicity and increase Zn absorption in rice [52], The significant up-regulation of ZIP10 in TT may explain the reduced Cd accumulation and increased Zn accumulation under Cd stress. Moreover, ACA2, a calmodulin-regulated pump [53], has been reported to respond to Ca [54] and salt stress [55]. The significant down-regulation of ACA2 may have contributed to the significant increase in Ca concentration observed in TS.

Cd can be distributed to organelles and whole plants through HMAs [38, 56]. In *Arabidopsis thaliana*, HMA2 and HMA4 were involved in long-distance transport of Cd in plants [56] and similar expression differences occurred in HMA4, HMA5 and HMA7 under Cd stress [38]. Although no report describes the roles related to Cd uptake, accumulation or translocation of single HMA7, the function of HMA7 may be similarly to HMA2 and HMA5, which might be involved in the long-distance transport of Cd by loading Cd into the xylem to facilitate

Cd transfer from roots to shoots [38, 57]. These results also explain why the Cd transport coefficient of TS was lower than that of DS with the same Cd sensitive genotype.

NRAMP4 was considered to be one of the genes that fix divalent cations in vacuoles [58, 59] and its significant up-regulation results in more Cd fixed in vacuoles of TS. Otherwise, the down-regulation of ABCB8 results in more Cd that cannot be transported into vacuoles and exist in cytoplasm. This was also verified in the results of Cd form and Cd distribution due to higher ratio of F_w (Fig. 4 A) and F_s (Fig. 4 C).

The expression of more efflux transporters (i.e., ABCG23, ABCG28, ABCG53 and ABCG33) makes the tetraploid genotype accumulate less Cd (Figs. 2 A and 6 B). Besides, as a gene that is located on the vacuolar membrane and participates in the metal ion efflux [60, 61], NRAMP2 also participates in the Cd efflux of TT.

Conclusion

This study has shown that diploid/Cd sensitive genotypes accumulate more Cd than tetraploid/Cd tolerant genotypes due to the differential expression of metal transporters. The Cd sensitive genotypes had more soluble Cd components, which facilitated the migration of Cd from roots to shoots. Additionally, TT accumulated higher ratio Cd in root cell wall, which could be a useful model for studying mechanisms of plant resistance to Cd stress. Tetraploid genotypes expressed more Cd-efflux promoting genes resulting in lower Cd accumulation compared to diploid genotypes. Moreover, the complex regulatory system and dilution effect in tetraploid genotypes contributed to lower Cd uptake and accumulation. These findings provide insights into the physiological and transcriptional mechanisms of Cd uptake and accumulation in different polyploid ryegrass genotypes, which could be useful for ryegrass breeding and soil Cd amelioration.

Materials and methods

Plant materials and growth

Four ryegrass genotypes, PI 577241 (diploid, Cd sensitive, DS), PI 636508 (diploid, Cd tolerant, DT), PI 611145 (tetraploid, Cd sensitive, TS) and Chuansi No.1 (tetraploid, Cd tolerant, TT), were used in this study following a previous study on the screening of Cd tolerance of 41 ryegrass during germination [15]. The genotypes with PI number were provided by American National Plant Germplasm System (Pullman, Washington, America) and the new cv. Chuansi No.1 was obtained from Sichuan Agricultural University (SICAU, Chengdu, China).

After sterilization and germination [62], seedlings in two-leaf stage were transplanted to the modified 1/3 Hoagland's nutrient solution [1.5 mmol·L $^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 1.25 mmol·L $^{-1}$ KNO_3, 0.5 mmol·L $^{-1}$ KH_2PO_4, 0.5 mmol·L $^{-1}$ $MgSO_4 \cdot 7H_2O$, 2.5 μmol·L $^{-1}$ H_3BO_3, 1.5 μmol·L $^{-1}$ $MnSO_4$, 0.5 μmol·L $^{-1}$ Na_2MoO_4, 0.5 μmol·L $^{-1}$ $CuSO_4$, 1.5 μmol·L $^{-1}$ $ZnSO_4$ and 25 μmol·L $^{-1}$ Fe-EDDHA, PH = 6.8] in a 25 ℃ greenhouse with a photoperiod of 16 h-light and 8 h-dark, light intensity of 180 μmol m^{-2} S^{-1} and relative humidity of 70%. After preculture for 14 days, plants were treated with 80 μmol L^{-1} $CdCl_2 \cdot 2.5H_2O$ (Cd treatment) or without Cd. After 7 days treatment, seedlings were collected for further analyses. All data in this study were three biological replicates and every six plants were combined as one biological replicate. In this study, all data were collected from 7 days after treatment.

Dry weight determination

The harvested above-ground and below-ground parts were dried in an oven at 75℃ for 10 days. On the 11th day they were taken out to normal environment for 1 day and then dried in the oven for 1 day and their dry weight was measured. This was repeated until the dry weight was constant and recorded.

Determination of root parameters

Root scanner (Epson Seiko Corporation, Nagano, Japan) was used to scan fresh roots. The total root length and surface area were measured by WinRHIZO software (Regent Instruments Canada Inc., Quebec, Canada) and subjected to statistical analysis.

Estimation of photosynthetic pigment contents

Chl *a*, Chl *b*, Chl (*a* + *b*), and ratio of Chl *a* and Chl *b* were estimated according to the method of Fang et al. (2017). The fresh leaf surface pigment was extracted with 10 ml 95% ethanol. The pigment extract was analyzed by enzyme-labeled instrument (Varioskan LUX; Thermo Scientific, Massachusetts, America) at wavelengths of 665 and 649 nm.

Quantification of Cd and other metals

Concentrations of Cd, calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), zinc (Zn), and copper (Cu) were determined as described by Cheng et al. (2020). Approximately, 0.2 g of plant material were ground to a fine powder after quick freezing in liquid nitrogen. The dried samples were digested with 5 ml HNO_3 and $HClO_4$ mix solution (v/v = 4/1) at 240 ℃ until the digest was clear and less than 0.5 ml. The digested solution was diluted to 25 ml and filtered by filter paper. The concentrations of the above 7 metal elements were measured by ICP-MS (7900, Agilent, America).

Measurement of Cd fluxes

After Cd treatment, the meristem area of root tip was immobilized by fixative and the net Cd flux from roots was analyzed for 5 min by the Noninvasive Microtest System (NMT100 Series; Amherst, Massachusetts, America) previously described [63, 64].

Determination of Cd subcellular distributions

The subcellular distribution of Cd was determined using the following protocol [21]: Fresh root and shoot tissues were homogenized in 50 mM Trimethylolamine buffer. The homogenate was then centrifuged at 4000 rpm for 15 min and at 12,000 rpm for 45 min to obtain the fractions of cell wall (F_{cw}), cell organelles (F_{co}), and soluble fraction (F_s). The subcellular distribution of Cd was measured as described in the previous section after drying.

Measurement of Cd chemical forms

Six Cd chemical forms were extracted from roots and shoots by ethanol (Aminophenol, nitrate/nitrite, and chloride forms of Cd, F_E), water (Organic acids and water-soluble forms of Cd, F_W), NaCl (Protein, pectin, and cellulose forms of Cd, F_{NaCl}), Hac (Binding forms of Cd with $HPO_4^{2-}$and PO_4^{3-}, F_{HAC}), HCl (Binding form of Cd with oxalic, F_{HCl}) and the remainder (F_R), respectively, as described by previous studies [23, 65]. After drying the extracted components, measure the Cd content as described in the above section.

Assembly and annotation of a full-length reference transcriptome database (ISO-seq)

As there is no publicly available genome-wide data for ryegrass, the limitations of RNA-seq, such as short read lengths and amplification biases, can be overcome by using single-molecule real-time (SMRT) sequencing, which generates long reads without requiring further assembly [10, 66]. Isoform sequencing (ISO-seq) has been successfully applied to ryegrass in previous studies [10]. In this study, two ISO-seq libraries were prepared, one for DT with and without Cd stress treatments and the other for TT with and without Cd treatments in the roots of ryegrass by PacBio RSII Sequencing Platform. ISO-seq was performed at the Novogene Bioinformatics Institute (Beijing, China). See Supplementary File 1 for detailed methodology. The DT library was used as the reference transcriptome for diploid genotypes, and the TT library was used as the reference transcriptome for tetraploid genotypes.

DEGs analysis of metal transporter genes

Eight RNA-Seq libraries were created using the roots of the samples through the Illumina platform, including eight treatments for four genotypes. RNA-seq was performed at the Novogene Bioinformatics Institute (Beijing, China). See Supplementary File 2 for detailed methodology. Genes with log2 (fold change) > 1 or < -1 and Padj < 0.005 (*P*-value adjusted for multiple testing with the Benjamini–Hochberg program) were considered differentially expressed. Since Cd uptake and accumulation are often associated with metal transporter genes, changes in metal transporter gene expression were primarily investigated. Metal transporters were annotated using information from the reference ISO-seq libraries and the Swiss-Prot database, and transcript-level expression differences of metal transporters were analyzed using the eight RNA-Seq libraries. Differential expression of genes (DEG) was calculated using the DEGseq method [27].

Validation of DEGs with qRT-PCR

The qRT-PCR was used to verify the relative expression levels of eight corrected DEGs [10, 63]. Briefly, the cDNA synthesis from re-extracted new total RNA and used for qRT-PCR. Validation of RNA-seq library reliability by qRT-PCR with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, America).

Statistical analysis

SPSS 27.0 (IBM Corporation, Armonk, New York, America) was used for statistical analysis. Figures were produced with the Prism program (Graphpad Software, San Diego, Canada) and Photoshop CS2 programs.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11325-2.

Supplementary Material 1

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Not applicable.

Authors' contributions

X. Y.: Conceptualization, Data curation, Investigation Formal analysis, Investigation, Visualization, Writing – original draft. X. W.: Formal analysis, Investigation, Visualization. X. Z.: Formal analysis, Investigation, Validation. D. W.: Writing – review & editing. Y. C.: Writing – review & editing. Y. W.: Writing – review & editing. L. S.: Writing – review & editing. J. Z.: Methodology, Writing – review & editing. H. K.: Writing – review & editing. X. F.: Writing – review & editing. L. H.: Writing – review & editing. Y. C.: Writing – review & editing. Y. Z.: Supervision, Project administration, Funding acquisition, Writing – review & editing. H. Z.: Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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Data availability
ISO-seq and RNA-seq data have been uploaded to the NCBI database (accession number: PRJNA779241).

Declarations

Ethics approval and consent to participate
We obtained permission to use the seeds of ryegrass from the American National Plant Germplasm System and Sichuan Agricultural University. This study did not involve any human or animal research participant data. The plant sample tested in this study was not an endangered species, and the collection of samples did not cause any environmental problems. All experimental procedures were approved by the Academic Committee of Sichuan Agricultural University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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