Journal of Genetics (2021)100:89
https://doi.org/10.1007/s12041-021-01339-6

RESEARCH NOTE

Identification of *NRAMP4* from *Arabis paniculata* enhance cadmium tolerance in transgenic *Arabidopsis*

BIN ZHU[1,2], CHENCHEN GAN[1], LEI GU[1], XUYE DU[1]* and HONGCHENG WANG[1]*

[1]*School of Life Sciences, Guizhou Province, Guizhou Normal University, Guiyang, People's Republic of China*
[2]*Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture and Rural Affairs, Wuhan, People's Republic of China*
*For correspondence. E-mail: Xuye Du, duxuye@gznu.edu.cn; Hongcheng Wang, besthongcheng@163.com.

Received 26 April 2021; revised 23 July 2021; accepted 3 August 2021

Abstract. *Arabis paniculata* has been reported as a hyperaccumulator and functions in cadmium (Cd) tolerance and accumulation. However, the genes involved in Cd stress resistance in *A. paniculata* are still unknown. In this work, genes of the natural resistance-associated macrophage proteins (*NRAMPs*) were characterized in *A. paniculata*, and their evolutionary relationship and expression patterns were analysed. Expression profiles indicated that *ApNRAMPs* showed large differences in response to Cd stress. It was highly induced by Cd in root and shoot tissues. To investigate the function of *ApNRAMP4* under Cd stress, *ApNRAMP4* was cloned and expressed in yeast and *Arabidopsis*. The results indicated that yeast and *Arabidopsis* expressing *ApNRAMP4* showed normal growth under Cd stress. In addition, transgenic yeast and *Arabidopsis* showed the ability to concentrate Cd. Under 20 μM $CdCl_2$, Cd concentrations in wild type (WT) and transgenic yeast were 3.11 and 5.92 mg/kg, respectively. Cd concentrations in root tissues of WT and transgenic *Arabidopsis* were 0.18 and 0.54 mg/kg, respectively. In shoot tissues of WT and transgenic *Arabidopsis*, Cd concentrations were 0.13 and 0.49 mg/kg, respectively. This report provides genomic information on hyperaccumulator *A. paniculata*. In addition, the present work identified key *NRAMP* genes that may serve as resources for heavy metal phytoremediation.

Keywords. full-length transcriptome; *NRAMP* gene; gene expression; cadmium tolerance; *Arabis paniculata*.

Introduction

Cd is a common toxic heavy metal in polluted soil and has been regarded as highly toxic to plants, animals, and humans (Meena *et al.* 2018). In plants, roots take up Cd from the soil, and then, it is transported into cells through epidermal cell gaps or by metal transporters (Oono *et al.* 2016). Natural resistance-associated macrophage protein (*NRAMP*) mainly function as divalent metal transporters to a range of divalent metal cations, including Fe^{2+}, Mn^{2+}, Cd^{2+}, and Zn^{2+} (Wang *et al.* 2019). *NRAMPs* have been reported in several plants, and some of the functions of heavy metal tolerance have been characterized. In *Arabidopsis*, AtNRAMP1 was identified as a transporter of Fe^{2+}, Mn^{2+}, and Cd^{2+} (Cailliatte *et al.* 2010). *AtNRAMP3* and *AtNRAMP4* were found to be involved in Fe^{2+} and Mn^{2+} transport (Lanquar *et al.* 2010). In *Arabidopsis*, *AtNRAMP2* and *AtNRAMP6* were involved in the distribution of Mn^{2+} and Cd^{2+}, respectively (Alejandro *et al.* 2017). In rice, seven *NRAMPs* have been characterized (Mani *et al.* 2018). Unlike *NRAMPs* in *Arabidopsis*, *OsNRAMP4* demonstrated the ability to act as a trivalent Al^{3+} transporter (Xia *et al.* 2010).

A. paniculata is widely distributed in southwest China. It is regarded as a hyperaccumulator that showed the ability to accumulate Cd, Pb, and Zn (Tang *et al.* 2009; 2016). Until now, information on *NRAMPs* in *A. paniculata* is limited. In this work, the full-length transcriptome of *A. paniculata* was sequenced, and *ApNRAMPs* were characterized. Subsequently, the response of *ApNRAMPs* to Cd stress was

Bin Zhu and Chenchen Gan contributed equally to this work.

Supplementary Information: The online version contains supplementary material available at https://doi.org/10.1007/s12041-021-01339-6.

Published online: 24 November 2021

analysed, and the function of *ApNRAMP4* in transgenic *Arabidopsis* was further investigated.

Materials and methods

Plant materials

For this study, *A. paniculata* was collected from Huaxi, Guiyang, Guizhou province. *Arabidopsis* (Col) was used as a recipient material for gene transformation.

Plant growth conditions

A. paniculata seed was germinated in double distilled H_2O and grown in 1/2 Murashige and Skoog (MS) solution until the stage of six leaves. Then, the plants were harvested and washed thrice using double distilled H_2O and frozen in liquid nitrogen.

RNA preparation and PacBio sequencing

The plants were collected and stored at −80°C. For further sequencing, the roots and shoots of *A. paniculata* were collected from five independent plants. Samples were immediately frozen in liquid nitrogen for RNA sequencing. Total RNA from root and shoot tissues was extracted using TRIzol reagent (CwBio, Beijing, China). To ensure the accuracy of sequencing data, a Nanodrop 2000 was used to detect RNA purity, and an Agilent 2100 was used to detect RNA integrity. The same amount of RNA from the root and shoot tissues was mixed for sequencing analysis. Sequencing was carried out by BerryGenomics (Beijing, China) using the Pacific Bioscience RS II platform.

Analysis of ApNRAMP genes in response to Cd stress

Primers for qRT-PCR were designed according to the coding sequences of *ApNRAMP* genes (table 1 in electronic supplementary materials at http://www.ias.ac.in/jgenet/). Total RNA was extracted from root or shoot tissue of *A. paniculata* using an RNApure plant kit (CwBio, Beijing, China). qRT-PCR was further carried out using an UltraSYBR One Step RT-qPCR Kit (CwBio, Beijing, China). *β-actin* was used as an internal control gene. The $2^{-\Delta\Delta CT}$ method was carried out to normalize gene expression (Livak and Schmitten 2001).

Yeast expression

The coding sequence of *ApNRAMP4* was sub-cloned into *Hin*dIII and *Eco*RI sites of the yeast expression vector pYES2. The recombinant plasmid and the empty vector were individually transformed into *Saccharomyces cerevisiae* strain BY4743. Cd concentrations and growth curves of yeast were calculated according to Peng *et al.* (2018).

Generation of transgenic Arabidopsis expressing ApNRAMP4

The coding sequence of *ApNRAMP4* was inserted into pCAMBIA3201, and transgenic *Arabidopsis* was generated according to Peng *et al.* (2018). GUS histochemical assays and GUS activity detection were carried out according to Zhao *et al.* (2019).

Measurement of electrolytic leakage and antioxidant enzyme activity

The WT and transgenic lines were grown in 1/2 MS medium for three weeks and transferred to the 1/2 MS medium with 20 μM $CdCl_2$ for three days. Electrolyte leakage was analysed as described by Lutts *et al.* (1996). H_2O_2 and MDA content were measured according to Zhang *et al.* (2020). SOD, CAT, and POD activity were measured using the testing kit (Solarbio, Beijing, China).

Fluorescence detection of Cd in plants

Fluorescence detection of Cd was carried out using Leadmium Green AM Dye (Invitrogen, Shanghai, China). Leadmium Green AM Dye was prepared according to the operating instructions of the kit.

Primers and statistical analyses

All the primers used in this study are listed in table 1 in electronic supplementary material. Data were statistically analysed using Tukey's test on the basis of three biological replicates. Bars in histograms are presented as the mean ± standard deviation (SD).

Results

Characterization of NRAMPs in A. paniculata

A total of 9.4 Gb clean data and 26,770 sequences were annotated in the transcriptome. The clean data were uploaded to BIG Sub (https://bigd.big.ac.cn/gsub/) with accession CRA002602. Five *NRAMP* genes were characterized and named *ApNRAMP1–ApNRAMP5*. The sequences of these genes are shown in the electronic supplementary material. As indicated in table 2 in electronic supplementary material, wide variations were present in *ApNRAMPs*. The length of

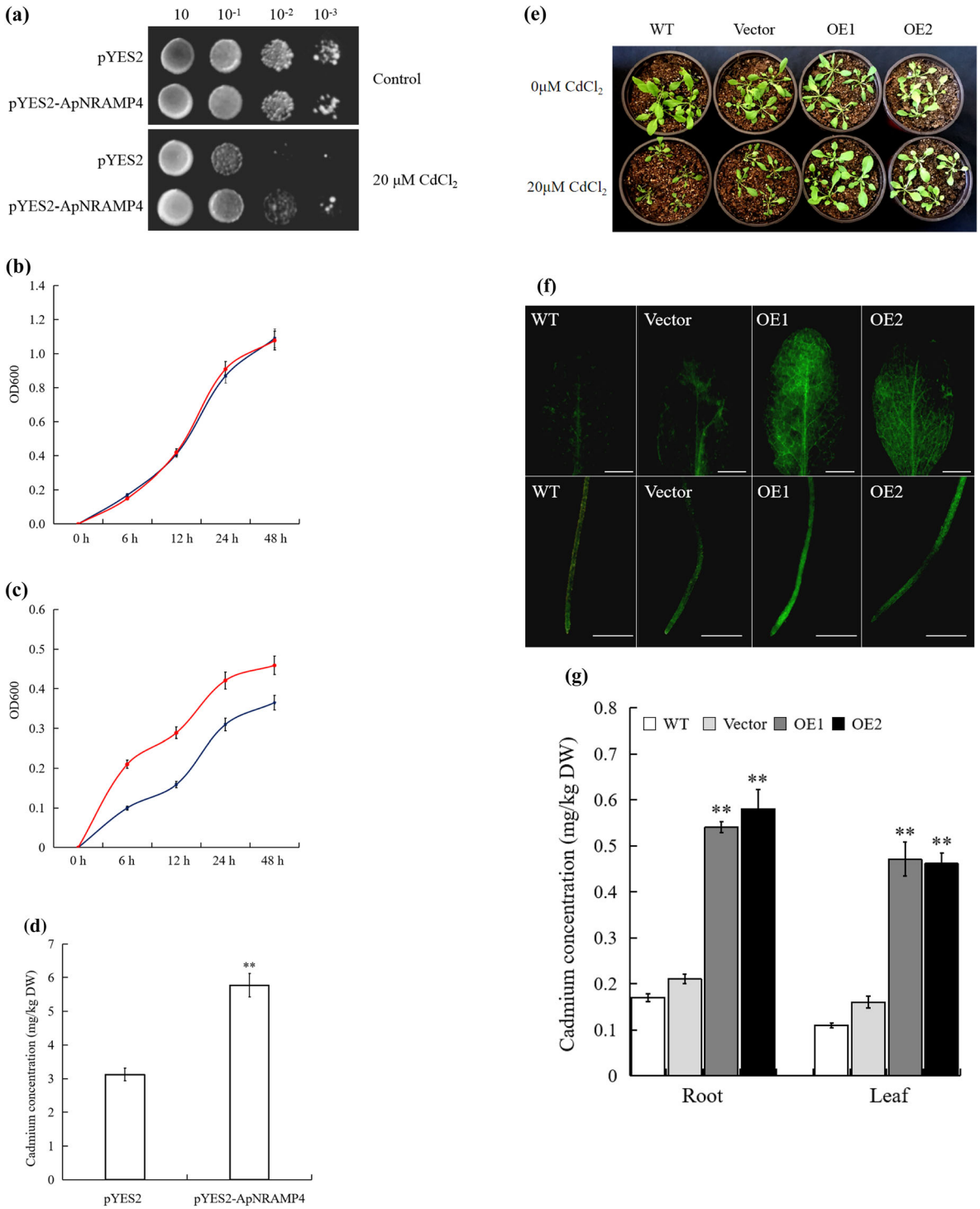

Figure 1. (a) Cd sensitivity of yeast grown on synthetic defined (SD) plates with 20 μM $CdCl_2$. (b) Cd concentrations in yeast grown in liquid SD medium with 20 μM $CdCl_2$ for 48 h. (c) OD600 values of empty yeast grown in liquid SD medium without $CdCl_2$. The values were measured at 0, 6, 12, 24 and 48 h. (d) OD600 values of yeast grown in liquid SD medium with 20 μM $CdCl_2$. The values were measured at 0, 6, 12, 24 and 48 h. Red and blue curve indicate pYES2-*ApNRAMP4* and pYES2, respectively. (e) Phenotype of WT and transgenic *Arabidopsis* under Cd tolerance; (f) Cd concentration of transgenic *Arabidopsis* under Cd tolerance, bar means 0.5 cm; (g) Cd fluorescence of transgenic *Arabidopsis* under Cd tolerance. **Significant differences at $P = 0.05$ according to Tukey's test based on three independent biological replicates.

ApNRAMPs ranged from 453 to 1575 bp, and the molecular weight of the deduced protein ranged from 16.83 to 57.64 kDa.

Phylogenetic analysis of ApNRAMPs with published NRAMPs

A phylogenetic tree was constructed with *NRAMP* genes from *A. paniculata* and published *NRAMP* genes from *Brassica napus*, *Arabidopsis*, and *Oryza sativa* (figure 1a in electronic supplementary material). *NRAMPs* were divided into two clades. *ApNRAMP4* and *ApNRAMP5* clustered in one group and showed a close relationship with *AtNRAMP4* from *Arabidopsis*. *ApNRAMP1*, *ApNRAMP2* and *ApNRAMP3* clustered in another group; *ApNRAMP2* and *ApNRAMP3* showed close relationships with *BnNRAMP5* and *AtNRAMP2*, respectively (figure 1a in electronic supplementary material).

NRAMP genes of A. paniculata in response to Cd stress

qRT-PCR was carried out to investigate *ApNRAMPs* in response to Cd stress. *ApNRAMP4* was highly induced by Cd stress in root and shoot tissues. In addition, *ApNRAMP5* was highly induced by Cd stress but only in shoot tissue (figure 1, b&c in electronic supplementary material).

ApNRAMP4 increased Cd tolerance in transgenic yeast

The status and growth curve of yeast were highly inhibited by Cd stress (figure 1, a–c). Compared with the WT yeast strain, transgenic yeast showed higher OD values (figure 1, b&c), demonstrating that the expression of *ApNRAMP4* increased Cd tolerance in yeast. As an important index reflecting the response to Cd stress in yeast, Cd concentrations in transgenic yeast were significantly higher than those of WT yeast (figure 1d). The results indicated that *ApNRAMP4* increased Cd tolerance in transgenic yeast.

Detection of transgenic events in Arabidopsis

Double digestion by *Hin*dIII and *Eco*RI verified that the vector was successfully constructed (figure 1 in electronic supplementary material). PCR amplification indicated that the PCR products were only present in *A. paniculata* and transgenic *Arabidopsis* (figure 2a in electronic supplementary material). To further explore the transcriptional activation of *ApNRAMP4* in transgenic *Arabidopsis*, GUS staining and activity were analysed (figure 2, b–d in electronic supplementary material). The results suggested that Cd stress increased GUS dye and activity in transgenic *Arabidopsis*. Two transgenic *Arabidopsis* lines expressing *ApNRAMP4* and one transgenic line expressing the empty vector (EV) were verified.

ApNRAMP4 increased Cd tolerance and accumulation in transgenic Arabidopsis

Arabidopsis expressing *ApNRAMP4* showed better growth than that of WT or EV lines (figure 1e). The fluorescence intensity of Cd in root and leaf tissues indicated that Cd accumulation was higher in *Arabidopsis* expressing *ApNRAMP4* than in WT or EV lines (figure 1f). Cd concentrations in the plants further confirmed these results (figure 1g).

Expression of ApNRAMP4 alleviated Cd-induced damage to Arabidopsis

As indicated in figure 2, electrolyte leakage, H_2O_2 content, and MDA content were increased by Cd stress in *Arabidopsis*. Compared with the WT and *Arabidopsis* expressing the empty vector, the transgenic lines exhibited lower electrolyte leakage, H_2O_2 content, and MDA content. SOD and POD activity were also increased by the expression of *ApNRAMP4*.

Discussion

The *NRAMP* gene family in hyperaccumulator *A. paniculata* was identified and characterized in the present work. The expression of *ApNRAMPs* under Cd stress was analysed, and *ApNRAMP4* was identified as an important gene in response to Cd stress. The results were further verified in transgenic *Arabidopsis*.

Several *NRAMPs* have been identified in plants. In *Arabidopsis*, six *NRAMPs* were identified and further divided into two groups according to phylogenetic analysis (Maser *et al.* 2001). Hyperaccumulator *Sedum alfredii* possessed six *NTAMPs* and showed high homology to NRAMPs in *Arabidopsis* (Zhang *et al.* 2020). *ApNRAMPs* in *A. paniculata* were identified and further divided into two groups. *ApNRAMP1*, *ApNRAMP2*, and *ApNRAMP3* belonged to one group, while *ApNRAMP4* and *ApNRAMP5* belonged to another group (figure 1a in electronic supplementary material). In addition, *ApNRAMP4* and *ApNRAMP5* showed a close relationship with *AtNRAMP4* of *Arabidopsis*, suggesting that they share a similar function in the response to heavy metal stress.

Previous works indicated that *NRAMPs* increased heavy metal accumulation in bacteria and plants (Wang *et al.* 2019). In this work, expression of *ApNRAMP4* increased Cd tolerance and concentration in transgenic yeast (figure 1a). The results were further revealed by transgenic *Arabidopsis* (figure 1e). Several works have demonstrated that many *NRAMPs* are involved in Cd absorption and transport. *AtNRAMP4* and *AtNRAMP6* in *Arabidopsis* are involved in Cd transport of the intercellular (Lanquar *et al.* 2010); *OsNRAMP5* participates in Cd uptake in roots (Sasaki *et al.* 2012; Ishimaru *et al.* 2012), and *OsNRAMP1* is responsible for cellular Cd uptake and transport within plants (Takahashi

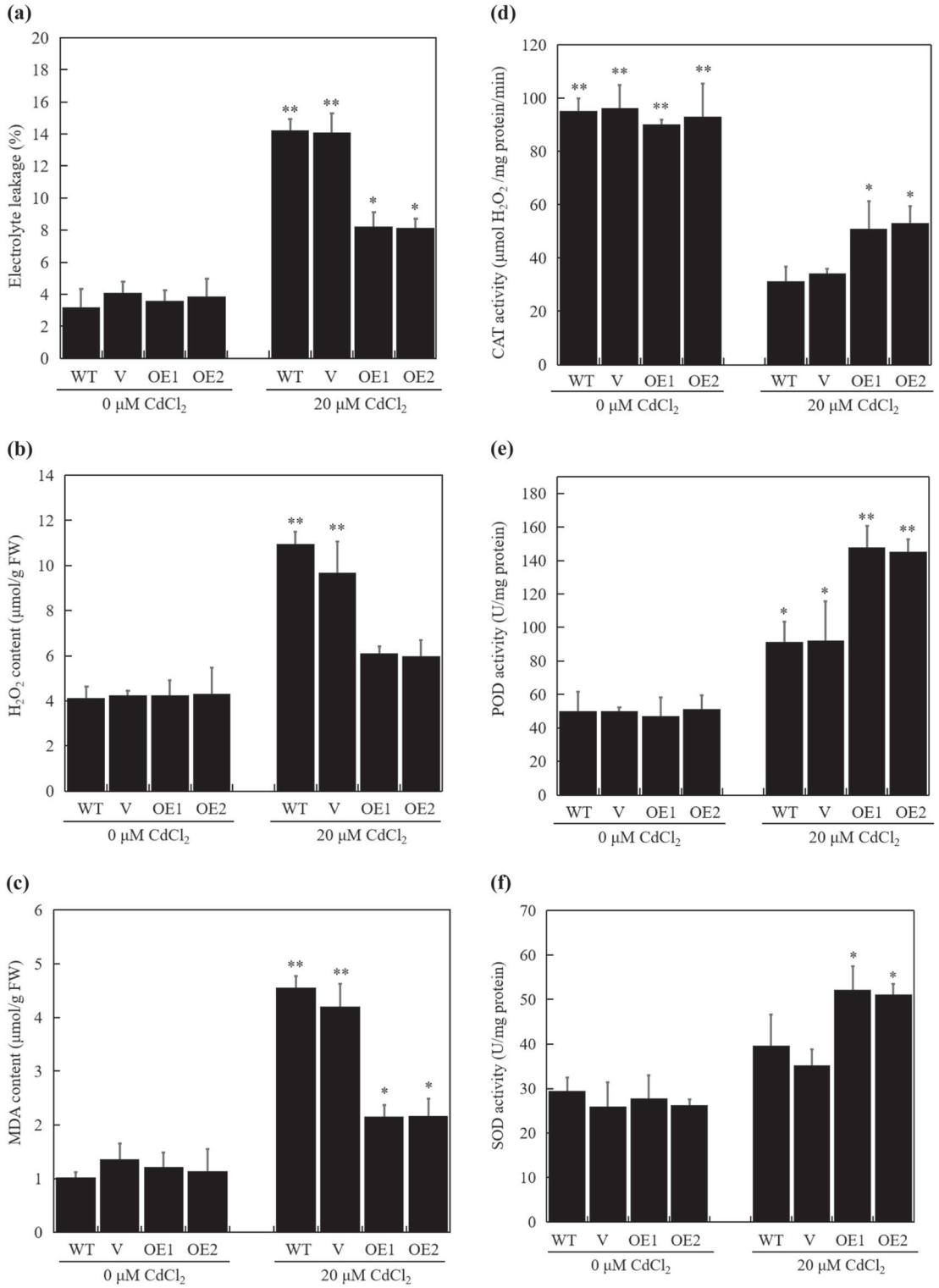

Figure 2. *ApNRAMP4* regulated the physiological and biochemical indexes of transgenic *Arabidopsis*. (a) Electrolyte leakage of WT and transgenic *A. thaliana* under 0 μM or 20 μM $CdCl_2$ stress. (b) SDS activity of WT and transgenic *A. thaliana* under 0 μM or 20 μM $CdCl_2$ stress. (c) MDA content of WT and transgenic *A. thaliana* under 0 μM or 20 μM $CdCl_2$ stress. (d) H_2O_2 content of WT and transgenic *A. thaliana* under 0 μM or 20 μM $CdCl_2$ stress. (f) POD activity of WT and transgenic *A. thaliana* under 0 μM or 20 μM $CdCl_2$ stress. (g) CAT activity of WT and transgenic *A. thaliana* under 0 μM or 20 μM $CdCl_2$ stress. The data are the means±SD of three independent measurements from three individual plants. * And **significantly different at P=0.05 and 0.01, respectively. The results were calculated according to Tukey's test based on three independent biological replicates.

et al. 2011). In the present work, expression of *ApNRAMP4* enhanced Cd accumulation in the root and shoot tissues of *Arabidopsis*, suggesting that *ApNRAMP4* is involved in the process of Cd uptake and transport.

Cd-induced toxicity in plants mainly causes growth inhibition and leaf chlorosis and damages the redox system (Dias *et al.* 2013; Ehsan *et al.* 2014). Here, we investigated the growth conditions of *Arabidopsis* expressing *ApNRAMP4* compared to the WT. The reduced biomass of the WT lines under Cd stress was obvious compared with transgenic lines (figure 1e). Additionally, Cd-induced toxicity could be correlated with ROS accumulation, lipid peroxidation, and cell damage (Meena *et al.* 2018). In this work, electrolyte leakage and H_2O_2 content in transgenic lines were significantly lower than that in WT lines, suggesting that expression of *ApNRAMP4* inhibits cell damage in *Arabidopsis*. ROS accumulation induced oxidative deterioration when the plants were under Cd stress (Schutzendubel *et al.* 2001). Moreover, the levels of antioxidative enzymatic activity in the present work indicated that *ApNRAMP4* increases tolerance to Cd stress in transgenic *Arabidopsis* (figure 2, d–f).

In conclusion, the full-length transcriptome of hyperaccumulator *A. paniculata* was obtained, and *NRAMP* genes were further identified. Further study indicated that *ApNRAMP4* promoted Cd accumulation in transgenic *Arabidopsis*. This work provides important information on genes in *A. paniculate*, and the gene families involved in heavy metal absorption and transport will be further analysed.

Acknowledgements

This work was supported by Guizhou Normal University (2018-5769-16) and Open Project of Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture and Rural Affairs, China (KF2019005). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

References

Alejandro S., Cailliatte R., Alcon C., Dirick L., Domergue F., Correia D. *et al*. 2017 Intracellular distribution of manganese by the trans-Golgi network transporter *NRAMP2* is critical for photosynthesis and cellular redox homeostasis. *Plant Cell* **29**, 3068–3084.

Cailliatte R., Schikora A., Briat J. F., Mari S. and Curie C. 2010 High-affinity manganese uptake by the metal transporter *NRAMP1* is essential for *Arabidopsis* growth in low manganese conditions. *Plant Cell* **22**, 904–917.

Dias M. C., Monteiro C., Moutinho-Pereira J., Correia C., Gonçalves B. and Santos C. 2013 Cadmium toxicity affects photosynthesis and plant growth at different levels. *Acta Physiol. Plant.* **35**, 1281–1289.

Ehsan S., Ali S., Noureen S., Mahmood K., Farid M., Ishaque W. *et al*. 2014 Citric acid assisted phytoremediation of cadmium by *Brassica napus* L. *Ecotoxcol. Environ. Safe.* **106**, 164–172.

Hou W. Q., Feng W., Yu G. H., Du X. Y. and Ren M. J. 2017 Cloning and functional analysis of a novel x-type high-molecular-weight glutenin subunit with altered cysteine residues from *Aegilops umbellulata*. *Crop Pasture Sci.* **68**, 409–414.

Khan A., Khan S., Alam M., Khan M. A., Aamir M., Qamar Z. *et al*. 2016 Toxic metal interactions affect the bioaccumulation and dietary intake of macro- and micro-nutrients. *Chemosphere* **146**, 121–128.

Livak J. and Schmitten T. D. 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Lanquar V., Ramos M. S., Lelievre F., Barbier-Brygoo H., Krieger-Liszkay A., Kramer U. *et al*. 2010 Export of vacuolar manganese by *AtNRAMP3* and *AtNRAMP4* is required for optimal photosynthesis and growth under manganese deficiency. *Plant Physiol.* **152**, 1986–1999.

Maser P., Thomine S., Schroeder J. I., Ward J. M., Hirschi K., Sze H., Talke I. N. *et al*. 2001 Phylogenetic relationships within cation transporter families of *Arabidopsis*. *Plant Physiol.* **126**, 1646–1667.

Meena M., Aamir M., Kumar V., Swapnil P. and Upadhyay R. S. 2018 Evaluation of morpho-physiological growth parameters of tomato in response to Cd induced toxicity and characterization of metal sensitive *NRAMP3* transporter protein. *Environ. Exp. Bot.* **148**, 144–167.

Oono Y., Yazawa T., Kanamori H., Sasaki H., Mori S., Handa H. *et al.* 2016 Genome-wide transcriptome analysis of cadmium stress in rice. *Biomed. Res. Int.* 9739505.

Peng F., Wang C., Zhu J. S., Zeng J., Kang H. Y., Fan X. *et al*. 2018 Expression of *TpNRAMP5*, a metal transporter from Polish wheat (*Triticum polonicum* L.), enhances the accumulation of Cd, Co and Mn in transgenic *Arabidopsis* plants. *Planta* **247**, 1395–1406.

Schutzendubel A., Schwanz P., Teichmann T., Gross K., Langenfeld-Heyser R., Godbold D. L. *et al*. 2001 Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. *Plant Physiol.* **127**, 887–898.

Takahashi R., Ishimaru Y., Senoura T., Shimo H., Ishikawa S., Arao T. *et al*. 2011 The *OsNRAMP1* iron transporter is involved in Cd accumulation in rice. *J. Exp. Bot.* **62**, 4843–4850.

Tang T., Liu P. L., Zheng G. W. and Li W. Q. 2016 Two phases of response to long-term moderate heat: variation in thermotolerance between *Arabidopsis thaliana* and its relative *Arabis paniculata*. *Phytochem.* **122**, 81–90.

Tang Y. T., Qiu R. L., Zeng X. W., Yi R. R., Yu F. M. and Zhou X. Y. 2009 Lead, zinc, cadmium hyperaccumulation and growth stimulation in *Arabis paniculata* Franch. *Environ. Exp. Bot.* **66**, 126–134.

Wang C., Chen X., Yao Q., Long D., Fan X., Kang H. Y. *et al*. 2019 Overexpression of *TtNRAMP6* enhances the accumulation of Cd in *Arabidopsis*. *Gene* **696**, 225–232.

Xia J., Yamaji N., Kasai T. and Ma J. F. 2010 Plasma membrane localized transporter for aluminum in rice. *Proc. Natl. Acad. Sci. USA* **107**, 18381–18385.

Zhao X. Y., Qi C. H., Jiang H., Zhong M. S., You C. X., Li Y. Y. *et al*. 2019 *MdHIR4* transcription and translation levels associated with disease in apple are regulated by *MdWRKY31*. *Plant Mol. Biol.* **101**, 149–162.

Zhang J., Zhang M., Song H. Y., Zhao J. Q., Shabala S., Tian S. K. *et al.* 2020 A novel plasma membrane-based *NRAMP* transporter contributes to Cd an Zn hyperaccumulation in *Sedum alfredii* Hance. *Environ. Exp. Bot*. **176**, 104121.

Corresponding editor: Manoj Prasad