agronomy

MDPI

Communication

Isolation of Three Metallothionein Genes and Their Roles in Mediating Cadmium Resistance

Pei-Hong Zhang [1,†], Xue-Jie Zhang [1,†], Ting-Wei Tang [2,†], Heng-Liang Hu [1], Ning-Ning Bai [1], Da-Wei Zhang [1,3,4], Shuan Meng [2,*] and Jia-Shi Peng [1,3,4,*]

1 School of Life and Health Sciences, Hunan University of Science and Technology, Xiangtan 411201, China
2 College of Agronomy, Hunan Agricultural University, Changsha 410128, China
3 Hunan Key Laboratory of Economic Crops Genetic Improvement and Integrated Utilization, Hunan University of Science and Technology, Xiangtan 411201, China
4 Key Laboratory of Ecological Remediation and Safe Utilization of Heavy Metal-Polluted Soils, Hunan University of Science and Technology, Xiangtan 411201, China
* Correspondence: mengs@hunau.edu.cn (S.M.); jspeng@hnust.edu.cn (J.-S.P.)
† These authors contributed equally to this work.

Abstract: Isolating the genes responsible for cadmium (Cd) accumulation and tolerance in oilseed rape and uncovering their functional mechanism is of great significance for guiding genetic improvement to cope with heavy metal pollution. In this study, we screened the cDNA library of *Brassica napus* cv. Westar using a yeast genetic complementation system and isolated *BnMT2-22a*, *BnMT2-22b* and *BnMT3b*, which can mediate Cd tolerance in yeast. They all have two cysteine-rich domains in their sequence. Ectopic expression of these MTs demonstrated that all of them enhanced Cd and Cu tolerance in yeast, but had no effect on Mn and Zn tolerance. The fusion of the red fluorescent protein mRFP did not affect their function in mediating Cd tolerance, and using these functional fusion proteins we observed that they were all localized in cytosol. Meanwhile, their expression in yeast did not affect the accumulation of Cd in the yeast transformants. Gene expression analyses found that *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* were all induced by Cd in roots, and *BnMT3b* was also significantly induced in shoots. These results indicate that the genes *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* isolated with cDNA library screening can mediate Cd tolerance, and they may detoxify Cd via cytosolic chelation.

Keywords: *Brassica napus*; cadmium; metallothionein; heavy metal

Citation: Zhang, P.-H.; Zhang, X.-J.; Tang, T.-W.; Hu, H.-L.; Bai, N.-N.; Zhang, D.-W.; Meng, S.; Peng, J.-S. Isolation of Three Metallothionein Genes and Their Roles in Mediating Cadmium Resistance. *Agronomy* **2022**, *12*, 2971. https://doi.org/10.3390/agronomy12122971

Academic Editors: Xin He and Yingpeng Hua

Received: 30 October 2022
Accepted: 24 November 2022
Published: 26 November 2022

1. Introduction

Brassica napus is tolerant to low Cd conditions; however, high Cd stress leads to great influence on various physiological processes, resulting in reduced biomass and yield in *Brassica napus* [1,2]. Therefore, the isolation of genes for Cd accumulation and tolerance in *Brassica napus* and uncovering their mechanisms are important for the safe production of *Brassica napus*, and will be helpful for the application of *Brassica napus* in phytoremediation of lightly polluted cropland.

Comparative studies on high and low Cd accumulation varieties of rapeseed found that *Brassica napus* can improve tolerance to Cd toxicity by regulating the antioxidant system, including enhancing the scavenging of reactive oxygen species, increasing antioxidant enzyme activity, and promoting the synthesis of antioxidants such as glutathione and ascorbic acid [3,4]. In addition, sequestration of Cd in the cell wall and vacuoles may play an important role in the process of detoxification of Cd in *Brassica napus* [5]. These processes were also found in other plants, including metal hyperaccumulators [6]; however, the molecular mechanisms behind these physiological processes are still poorly understood. Metallothioneins are a class of small proteins (4–8 kDa) widely found in eukaryotes that are rich in cysteine that can bind to heavy metal ions, and thus they play an important role in

the defense against heavy metal toxicity [7,8]. The metallothioneins in most angiosperms can be divided into four types according to their conservative cysteine order, namely MT1, MT2, MT3 andMT4 [9,10]. Unlike plant phytochelatins, another class of small polypeptides in plants that can bind and detoxify heavy metals, metallothioneins are encoded by genes. For example, there are seven functional metallothionein coding genes *AtMT1a*, *AtMT1c*, *AtMT2a*, *AtMT2b*, *AtMT3*, *AtMT4a* and *AtMT4b* in *Arabidopsis*, which play important roles in copper (Cu), zinc (Zn) and Cd tolerance and long-distance transport [11–14]. However, the role of metallothionein family members in *Brassica napus* upon resistance to heavy metal toxicity and the homeostasis of other elements such as Cu is still poorly studied.

We have an efficient transformation method and mature operating system for yeast, which is an excellent expression system for eukaryotic genes. Many key genes in plants that mediate Cd tolerance are obtained by screening their cDNA libraries using yeast complementary systems [15–22]. Thus, we took this advantage and screened a cDNA library of *Brassica napus* under Cd stress conditions and obtained gene resources mediating Cd transport and tolerance in *Brassica napus*. Three metallothionein family member genes, *BnMT2-22a*, *BnMT2-22b* and *BnMT3b*, were cloned from *Brassica napus* cv. Westar through library screening, and their characteristics and functional mechanisms in mediating Cd tolerance were investigated in detail. The current study provides a reference for exploring the role of metallothionein family members in accumulation and tolerance of heavy metal Cd in *Brassica napus*.

2. Materials and Methods

2.1. Biological Materials

Plant material *Brassica napus* cv. Westar was used in this study. Yeast strains: cadmium-sensitive yeast mutant Δ*yap1* [23] (*MAT α ura3 lys2 ade2 trp1 leu2 yap1::LEU2*) and its wild-type Y252 *(MAT α ura3 lys2 ade2 trp1 leu2*), zinc-sensitive yeast mutant Δ*zrc1* [24] [*MAT α can1-100 (oc) leu2trp1 ura3-52 zrc1:: HIS3*] and its wild-type CM100[*MATα can1-100 (oc) his3 leu2 trp1 ura3-52*], copper-sensitive yeast mutant Δ*cup2* [25] (*MATa his3 leu2 met15 ura YGL166w::kan MX4*) and its wild-type BY4741 (*MATa his3 leu2 met15 ura3D0*).

2.2. cDNA Library Construction and Cd Tolerance Gene Screening

Brassica napus cv. Westar was cultured for 6 weeks in Hogland solution, RNAs were extracted from the roots and shoots, then were mixed with an equal amount of each other. A complete yeast-expressed cDNA library was constructed according to the kit instructions (Invitrogen Corporation), and the library was constructed into the yeast expression vector pYES2-DEST52 under the control of the *GAL1* promoter. Then the library was transformed into *E.coli* for amplification, and clones were pooled. Plasmids were extracted and transformed into the yeast Y252 using the lithium acetate method [26]. Yeast transformants were grown on more than 200 large plates (150 mm × 150 mm) using glucose as the carbon source. Yeast cells were collected and pooled in sterile water, and then were plated to SD medium supplemented with 50 μM $CdCl_2$ using galactose as the carbon source for 7–10 d. Tolerant colonies were further analyzed via restriction digestion and sequencing.

2.3. Bioinformatics Analysis

Westar genome sequence files (Westar.genome.fa) and gff3 data format files (westar.v0.gff3) were downloaded from BnPIR (http://cbi.hzau.edu.cn/bnapus/ (accessed on 23 May 2021)). MT sequences from the *Brassica napus* cv. Westar genome sequence file were extracted using Tbtools (v1.089) and subjected to BLASTP search using *Arabidopsis* MT protein as the reference sequence with default E-value. Conserved motifs of MT proteins were obtained using MEME (https://meme-suite.org/meme/tools/meme (accessed on 1 October 2022)) with the given motif number as 4 and E-value $<10^{-10}$ [27]. The neighbor-joining trees were constructed using MEGA-X with default parameters.

2.4. Yeast Experiments

The primers L and R1 (primers are listed in Table S1) were used to amplify the CDS sequence of each target gene. The amplified fragments were cloned into the yeast expression vector pYES2 with *BamH1* and *EcoR1* restriction sites, and then transformed into the corresponding yeast strain as described by Elble [28]. The positive single clones were cultured with fresh liquid SD medium to the logarithmic growth phase, then the yeast cells were collected by centrifugation, suspended with sterile water, and diluted to OD600 = 1, 0.1, 0.01 and 0.001. After that, 15 µL suspensions were dropped onto medium plates containing different concentrations of heavy metals as indicated, were cultured at 30 °C for about 7 d, and then were photographed.

2.5. Construction of Vector for Subcellular Localization Analysis and Fluorescence Observation

The mRFP was amplified using primers mRFP-L and mRFP-R listed in Table S1, and the pYES2-mRFP vector was obtained by introducing the above amplified products into the yeast expression vector pYES2 with the *BamH1* and *EcoR1* restriction sites. The CDSs of BnMTs were amplified using the corresponding L and R2 primers listed in Table S1, and then the vector pYES2-BnMTs-mRFP was obtained by introducing the amplified products into the yeast expression vector pYES2-mRFP with *HindIII* and *BamHI* restriction sites, then transformed into yeast. Subcellular localizations for BnMTs were analyzed using laser confocal microscopy.

2.6. Determination of Cd Content

The yeast were cultured to the logarithmic growth phase, then was treated with 20 µM Cd for 6 h, then washed twice with ultrapure (>18 MΩ cm^{-1}) water, 5 mmol·L^{-1} EDTA and ultrapure water again in succession, and dried at 80 °C to constant weight. The samples were digested in 70% nitric acid at 100 °C for 2 h and diluted with ultrapure water, after which the Cd content was determined via inductively coupled plasma mass spectrometry (ICP-MS) [29].

2.7. Gene Expression Analysis

Brassica napus cv. Westar plants were grown hydroponically for three weeks using Hogland and then were treated with 20 µM Cd for 7 days. Roots and shoots were sampled and subjected to RNA extraction followed by first-strand cDNA synthesis and quantitative RT–PCR as described [30]. The primers used in the assays are listed in Table S1, and *ACTIN* was selected as internal reference gene.

2.8. Sequence Information

The sequences of *Arabidopsis* MT proteins, rice MT proteins and *Brassica rapa* MT proteins were downloaded from the UniPro database (https://www.uniprot.org/ (accessed on 1 October 2022)) or GenBank/EMBL databases. The serial numbers are as follows: AtMT1a (P43392), AtMT1b (Q38803), AtMT1c (Q38804), AtMT2a (P25860), AtMT2b (Q38805), AtMT3 (O22433), AtMT4a (P93746), AtMT4b (Q42377), OsMT1a-1 (A2ZH20), OsMT1a-2 (P0C5B3), OsMT1b (Q10N03), OsMT2a (P94029), OsMT2b (Q5JM82), OsMT2c-1 (A3AZ88), OsMT2c-2 (A2XZL0), OsMT3a-1 (A1YTM8), OsMT3a-2 (A2WLS0), OsMT3b-1 (A2Y1D7), OsMT3b-2 (A3B0Y1), OsMT4a (Q0IMG5), OsMT4b (Q2QNE8), OsMT4c (Q2QNC3), BrMT1a (MT361644), BrMT1b (MT361645), BrMT1c (MT361646), BrMT2a (MT361647), BrMT2b (MT361648), BrMT3 (MT361649).

3. Results

3.1. Cloning and Bioinformatics Analysis of BnMT2-22a, BnMT2-22b and BnMT3b

In order to isolate the key genes mediating the accumulation and tolerance of heavy metal Cd in rape, a yeast-expressed cDNA library of Westar rape was constructed. After the library was transformed into the yeast Cd-sensitive mutant Δ*yap1*, the yeast transformants were placed on a plate containing 50 µmol·L^{-1} $CdCl_2$, and more than 1000 single clones with resistance

phenotypes were obtained. After re-verification, 13 Cd tolerant genes were identified via yeast plasmid extraction and sequencing, including 3 members from the metallothionein family. After alignment in the BnPIR database (http://cbi.hzau.edu.cn/bnapus/ (accessed on 1 October 2022)), one of the genes was named *BnMT3b (BnaC05G0424300WE)*, and the other two were named *BnMT2-22a (Bnascaffold3320T0000200WE/BnaC09T0587800WE/BnaA10T0290800WE)* and *BnMT2-22b (BnaC02T0000200WE/BnaA02T0003200WE/BnaA02T0007100WE)*.

The full-length coding regions (CDS) of *BnMT3b*, *BnMT2-22a* and *BnMT2-22b* genes were 204 bp, 243 bp and 246 bp, respectively. The proteins they encode are rich in cysteines (Cys) at both ends. Phylogenetic analysis found that BnMT2-22a, BnMT2-22b and BnMT3b were closely related to the MT2 subfamily and MT3 subfamily of *Arabidopsis*, rice and *Brassica rapa*, respectively (Figure 1a). Further analysis found that the Cys arrangement positions of BnMT2-22a, BnMT2-22b and BnMT3b were completely conserved compared with MT2 and MT3 from *Arabidopsis thaliana* and *Brassica rapa*, which was also consistent with the characteristics of the metallothionein family (Figure 1b,c).

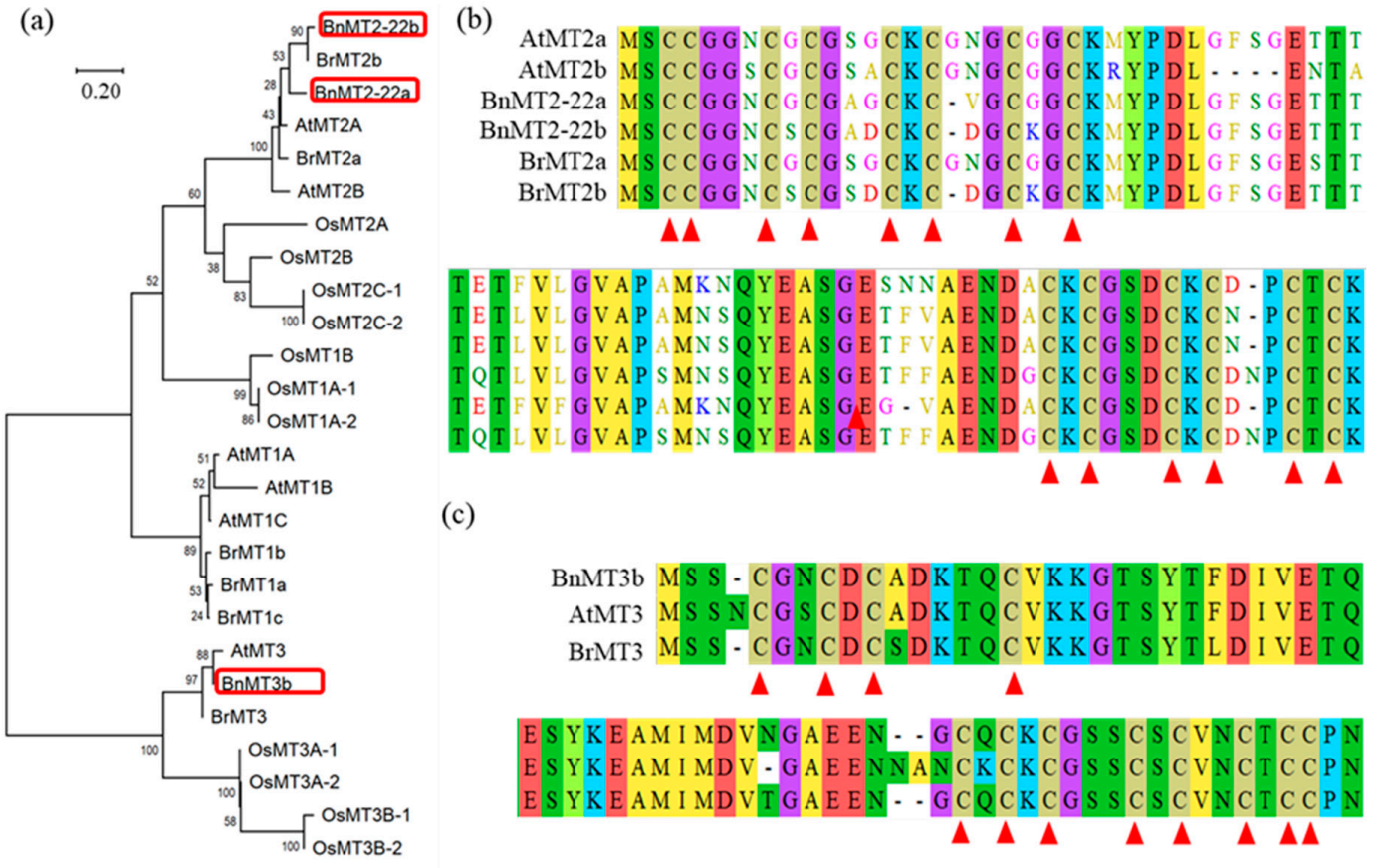

Figure 1. Sequence analysis of BnMTs. (**a**) Phylogenetic tree analysis of BnMTs with metallothionein family from *Arabidopsis thaliana* (At), Rice (Os) and *Brassica rapa* (Br). (**b**,**c**) Sequence alignment of BnMT2-22a, BnMT2-22b (B)and BnMT3b (C)with their homologues of *Arabidopsis thaliana* and *Brassica rapa*.

We then identified 13 other members of the metallothionein family from the Westar rapeseed genome. Among the 16 metallothionein members, 1 member belonged to the MT1 subfamily, 7 members to the MT2 subfamily, 3 members to the MT3 subfamily, and 2 to MT4 subfamily members. There were three members that are still not named in the BnPIR database. Phylogenetic tree analysis indicated that BnaC01T04104400WE and BnaA01T0299100WE are members of the MT2 subfamily, while BnaA08T0282500WE is a member of the MT1 subfamily (Figure 2a). Conserved motif prediction revealed that these members contained four conserved structural domains. The number and types of

motifs are differentially among these members, but all members contained motif 2, and most members contained motif 3 (Figure 2a). Interestingly, motif 2 and motif 3 are enriched in conserved Cys sequences (Figure 2b), which are key domains for the functioning of the metallothionein family [19].

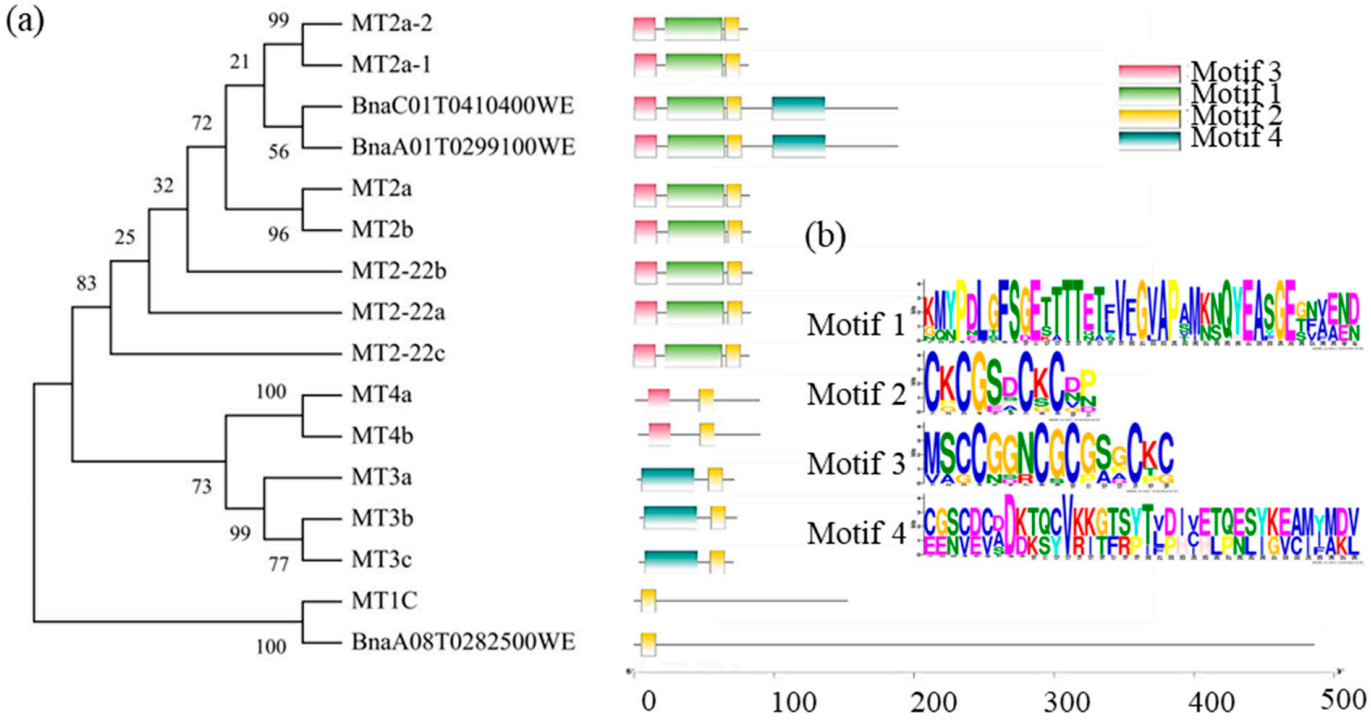

Figure 2. Identification and motif analysis of metallothionein proteins in *Brassica napus* cv. Westar. (**a**) Phylogenetic tree and motifs analysis of metallothionein family from *Brassica napus* cv. Westar. (**b**) Details of the motifs of metallothioneins from *Brassica napus* cv. Westar.

3.2. BnMT2-22a, BnMT2-22b and BnMT3b Mediate the Yeast Tolerance to Cd and Cu

To further verify the functions of BnMT2-22a, BnMT2-22b, and BnMT3b, we used the yeast Cd-sensitive mutant Δ*yap1*, Zn-sensitive mutant Δ*zrc1* and Cu-sensitive mutant Δ*cup2* to verify that they function in mediating tolerance to different metal stresses. The results demonstrated that the yeast expressing *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* under control of *GAL1* promoter exhibited greatly enhanced tolerance to 40 μmol·L^{-1} $CdCl_2$ and 30 μmol·L^{-1} $CuSO_4$ stresses compared with the control yeast transformed with empty vector (Figure 3a,c). However, the growth of the three metallothionein-transferred yeast transformants under Mn stress or Zn stress was similar to that of control yeast (Figure 3a,b). In conclusion, the expression of BnMT2-22a, BnMT2-22b and BnMT3b could enhance the tolerance of yeast to Cd and Cu, but did not affect the resistance to Mn and Zn.

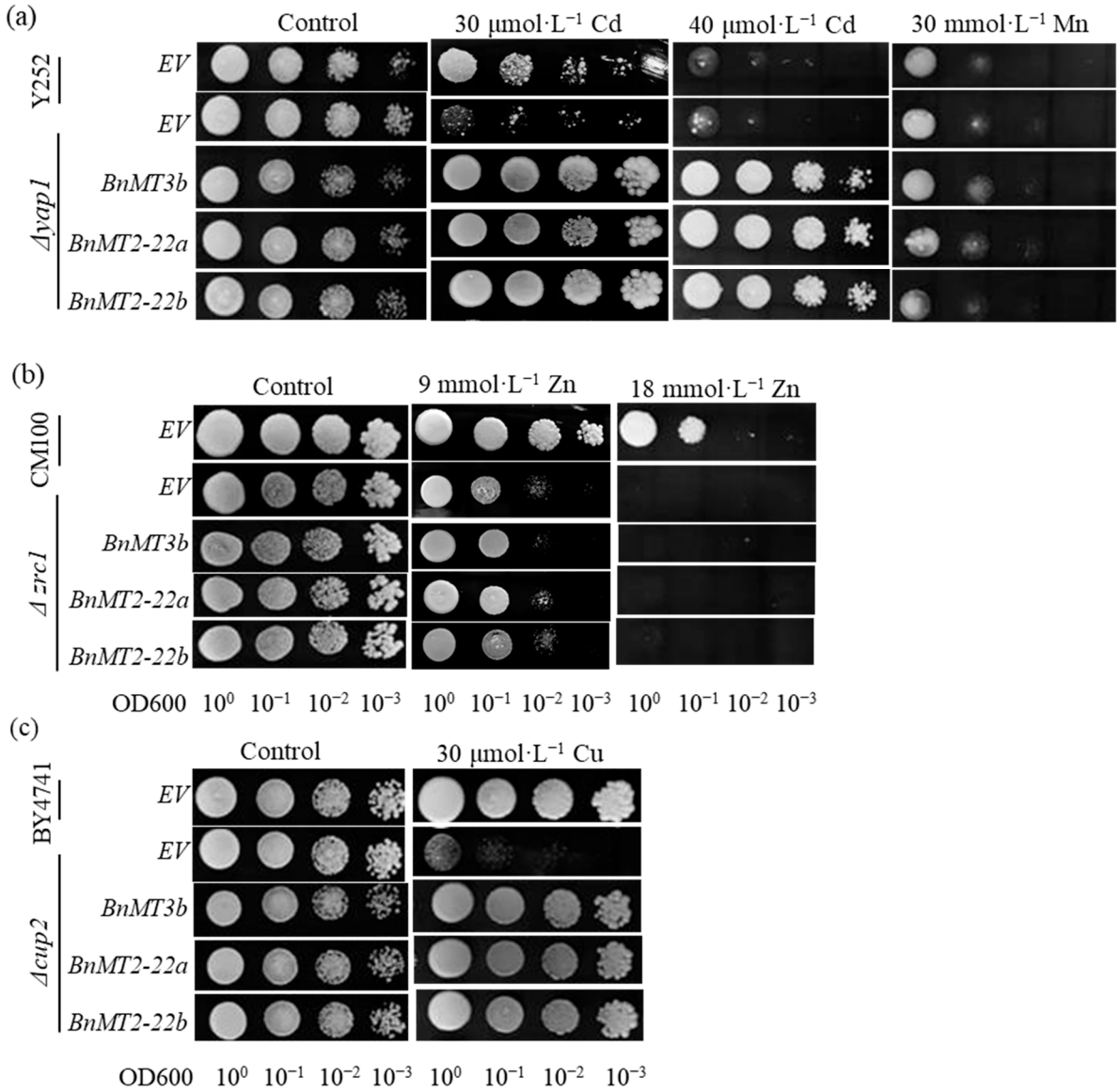

Figure 3. Expression of *BnMT*s enhanced Cd and Cu tolerance in yeast. (**a**–**c**) *BnMT3b*, *BnMT2-22a*, *BnMT2-22b* were transformed into Cd-sensitive yeast mutant Δ*yap1*, Zn-sensitive yeast mutant Δ*zrc1* and Cu-sensitive yeast mutant Δ*cup1*, and the yeast transformants were subjected to Cd, Mn (**a**), Zn (**b**) and Cu (**c**) tolerance assays. EV: empty vector.

3.3. BnMT2-22a, BnMT2-22b and BnMT3b Localized in the Cytosol

The subcellular localization of a protein in cells is critical for understanding the protein's function. To explore the subcellular localization of BnMT2-22a, BnMT2-22b and BnMT3b, we fused those members with the red fluorescent protein mRFP. Since these three metallothioneins as one protein are relatively small compared with mRFP, and fusion with fluorescent proteins is also very likely to affect the function of the target protein, we first verified whether the fusion with mRFP would affect their function. As shown in Figure 4a, fusion proteins BnMT2-22a-mRFP, BnMT2-22b-mRFP and BnMT3b-mRFP enhanced Cd tolerance in yeast. Then we carried out the subcellular localization analysis. The subcellular fluorescence of yeast transformants expressing fusion constructs was observed in the cytosol (Figure 4b), suggesting that they may function mainly in the cytoplasm and thus reducing the toxic effect of Cd.

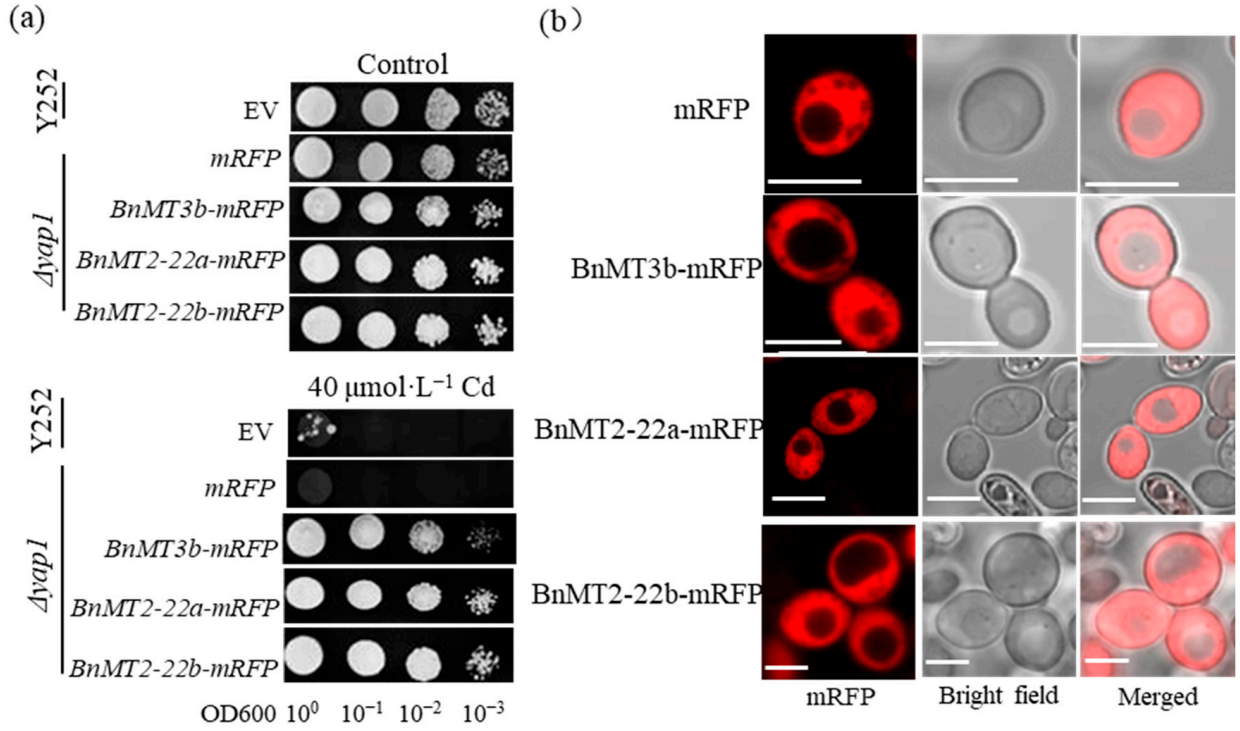

Figure 4. BnMTs were localized to the cytosol. (**a**) The BnMTs-mRFP fusion protein is functional in yeast. (**b**) Subcellular location of BnMTs-mRFP in yeast culture in liquid SD medium without Cd. Bar = 5 μm. EV: empty vector.

3.4. BnMT2-22a, BnMT2-22b, BnMT3b Hardly Affect the Accumulation of Cd in Cells

To further verify the above speculation that these three MTs function mainly in the cytoplasm, we then determined the content of Cd in yeast transformants. Compared with yeast transformants with empty vector, the Cd content was very close in *BnMT2-22a* or *BnMT3b* expressed transformants (Figure 5), which indicated that *BnMT2-22a* or *BnMT3b* did not affect the accumulation of Cd in yeast cells. Notably, expressing *BnMT2-22b* in yeast increased Cd content slightly, from 60.1 $\mu g \cdot g^{-1}$ to 63.2 $\mu g \cdot g^{-1}$, which was not too much difference (Figure 5). Therefore, the expression of BnMT2-22a, BnMT2-22b or BnMT3b hardly affects the accumulation of Cd in yeast.

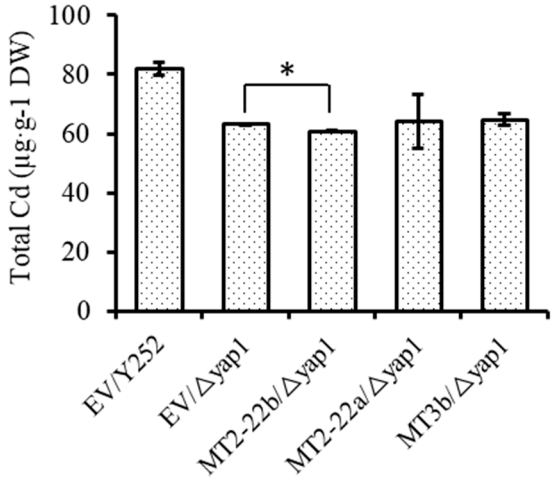

Figure 5. Total Cd accumulation in yeast transformants. Data are mean ± SD, n = 3. Statistical significance was indicated by * (Student's *t* test, $p < 0.05$).

3.5. Expression of BnMT3b, BnMT2-22a and BnMT2-22b Response to Cd Stress

In order to further elucidate the functional characteristics of *BnMT3b*, *BnMT2-22a* and *BnMT2-22b*, we carried out quantitative RT-PCR using rapeseed seedlings combined with Cd treatment. The results demonstrated that the expression level of *BnMT3* in roots and shoots was similar under normal conditions (Figure 6a). Upon Cd treatment, the expression of *BnMT3* in roots and shoots increased by 8.7 times and 2.9 times respectively, making its expression in roots higher than that in shoots (Figure 6a). The expression of *BnMT2-22a* and *BnMT2-22b* in roots and shoots was also induced by Cd (Figure 6b,c). Expression of *BnMT2-22a* and *BnMT2-22b* was up-regulated by 14.2 times and 8.6 times in roots, and up-regulated by 1.9 times and 1.6 times in shoots, respectively (Figure 6b,c). Notably, the expression of *BnMT2-22a* and *BnMT2-22b* in roots and shoots was much higher than that of *BnMT3b* (Figure 6a–c). The above results indicate that the expression of *BnMT3b*, *BnMT2-22a* and *BnMT2-22b* response to Cd stress occurs both in roots and shoots, and the response in roots is stronger than that in shoots.

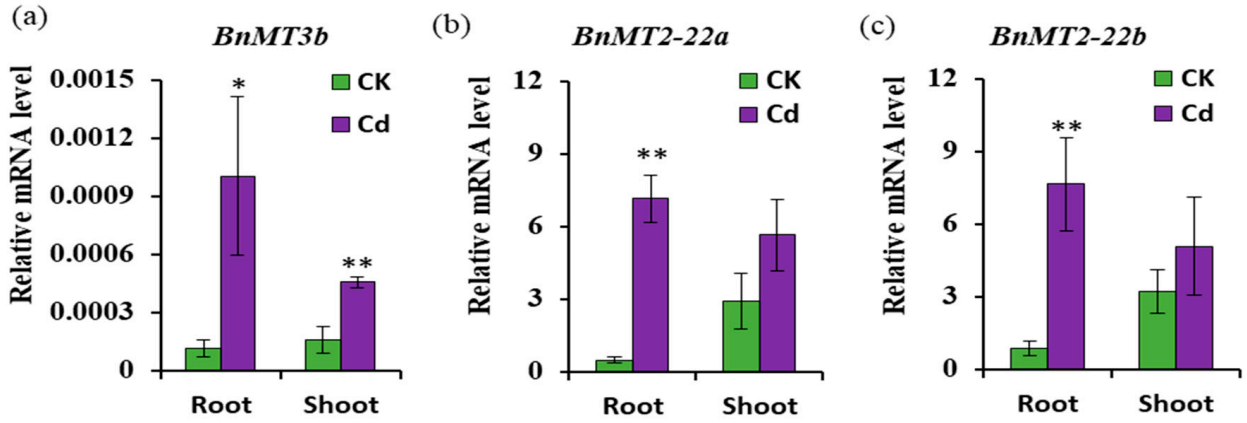

Figure 6. Expression levels of *BnMT3b*, *BnMT2-22a* and *BnMT2-22b* in response to Cd treatment. (**a**–**c**) Relative expression levels of *BnMT3b* (**a**), *BnMT2-22a* (**b**) and *BnMT2-22b* (**c**) under normal (CK) or Cd treatment (Cd) for 7 days. Data are mean ± SD, n = 3. Statistical significance was analyzed using Student's *t* test, and was indicated by * ($p < 0.05$) or ** ($p < 0.01$).

4. Discussion

Metallothionein family members generally chelate metal ions through their rich Cys to reduce the toxicity of these metal ions [18]. By measuring the accumulation of Cd in the yeast transformants, we found that expressing *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* could hardly affect the accumulation of Cd in yeast cells, though they greatly enhanced the tolerance of yeast to Cd stress (Figures 3a and 5), which indicated that the tolerance of yeast cells to Cd stress mediated by *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* was not achieved by reducing the accumulation of Cd. Combined with their subcellular localization in the cytoplasm, we speculated that *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* might chelate Cd in the cytoplasm to reduce the toxicity of free Cd to cells, which was similar to the results observed in other metallothionein studies [19,31].

Metallothionein family members were reported to specifically bind to Cd without affecting cell resistance to other metal ions in some specialized plants adapted to habitats rich in Cd and other heavy metals [19]. Meanwhile, the three metallothionein family members identified in this study not only mediate Cd tolerance, but also significantly increase tolerance to Cu (Figure 3). Notably, *BjMT2* also demonstrated enhanced tolerance to Cu and Cd when expressed in *Escherichia coli*, and yeast cells expressing *BrMT1*, *BrMT2a*, *BrMT2b* and *BrMT3* were more tolerant to Cd and Zn [32,33]. As *Brassica napus* is not a plant that adapts to a Cd habitat, we speculate that *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* may be more important for dynamic balance of Cu in *Brassica napus*, and the role of these three metallothionein family members with Cd may be due to the similar chemical structure

of Cd and Cu. This is similar to the results of some metallothionein family members in *Arabidopsis thaliana* [11].

5. Conclusions

Using a yeast genetic complementation system, we isolated three metallothionein family member genes *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* via screening the cDNA library of rapeseed. *BnMT2-22a*, *BnMT2-22b* and *BnMT3b* response to Cd stress, localized in cytoplasm, could greatly improve yeast tolerance to Cd and Cu without obvious change in Cd accumulation in cells. Thus, BnMT2-22a, BnMT2-22b and BnMT3b might chelate Cd in the cytoplasm to increase the tolerance of cells to Cd stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12122971/s1, Table S1: Primer sequences used in this study.

Author Contributions: Conceptualization, S.M. and J.-S.P.; investigation, P.-H.Z., X.-J.Z., T.-W.T. and H.-L.H.; project administration, N.-N.B. and D.-W.Z.; supervision, J.-S.P.; visualization, S.M. and J.-S.P.; writing–original draft, S.M. and J.-S.P.; writing–review and editing, S.M. and J.-S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the National Natural Science Foundation of China 31900219 and U20A2024, the Scientific Research Fund of Hunan Provincial Education Department 19B273 and 20K055, and the Hunan Provincial Natural Science Foundation of China 2022JJ20022 and 2022JJ30284.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Ji-Ming Gong (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences) for kindly providing yeast strains Δ*yap1*, Y252, Δ*zrc1*, CM100, Δ*cup2* and BY4741.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ali, B.; Tao, Q.; Zhou, Y.; Gill, R.; Ali, S.; Rafiq, M.T.; Xu, L.; Zhou, W. 5-Aminolevolinic acid mitigates the cadmium-induced changes in *Brassica napus* as revealed by the biochemical and ultra-structural evaluation of roots. *Ecotoxicol. Environ. Saf.* **2013**, *92*, 271–280. [CrossRef]
2. Ali, B.; Gill, R.A.; Yang, S.; Gill, M.B.; Ali, S.; Rafiq, M.T.; Zhou, W. Hydrogen sulfide alleviates cadmium-induced morpho-physiological and ultrastructural changes in *Brassica napus*. *Ecotoxicol. Environ. Saf.* **2014**, *110*, 197–207. [CrossRef] [PubMed]
3. Lv, W.; Yang, L.; Xu, C.; Shi, Z.; Shao, J.; Xian, M.; Chen, J. Cadmium disrupts the balance between hydrogen peroxide and superoxide radical by regulating endogenous hydrogen sulfide in the root tip of *Brassica rapa*. *Front. Plant Sci.* **2017**, *8*, 232. [CrossRef] [PubMed]
4. Wu, Z.; Zhao, X.; Sum, X.; Tan, Q.; Tang, Y.; Nie, Z.; Qu, C.; Chen, Z.; Hu, C. Antioxidant enzyme systems and the ascorbate-glutathione cycle as contributing factors to cadmium accumulation and tolerance in two oilseed rape cultivars *Brassica napus* L. under moderate cadmium stress. *Chemosphere* **2015**, *138*, 526–536. [CrossRef]
5. Zhang, Z.; Zhou, T.; Tang, T.; Song, H.; Guan, C.; Huang, J.; Hua, Y. A multiomics approach reveals the pivotal role of subcellular reallocation in determining rapeseed resistance to cadmium toxicity. *J. Exp. Bot.* **2019**, *70*, 5437–5455. [CrossRef]
6. Peng, J.S.; Guan, Y.H.; Lin, X.J.; Xu, X.J.; Xiao, L.; Wang, H.H.; Meng, S. Comparative understanding of metal hyperaccumulation in plants: A mini-review. *Environ. Geochem. Health* **2021**, *43*, 1599–1607. [CrossRef]
7. Cobbett, C.; Goldsbrough, P. Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annu Rev. Plant Biol.* **2002**, *53*, 159–182. [CrossRef] [PubMed]
8. Leszczyszyn, O.I.; Imam, H.T.; Blindauer, C.A. Diversity and distribution of plant metallothioneins: A review of structure properties and functions. *Metallomics* **2013**, *5*, 1146–1169. [CrossRef]
9. Freisinger, E. Structural features specific to plant metallothioneins. *J. Bio. Inorg. Chem.* **2011**, *16*, 1035–1045. [CrossRef]
10. Zimeri, A.M.; Dhankher, O.P.; Mccaig, B.; Meagher, R.B. The plant MT1 metallothioneins are stabilized by binding cadmiums and are required for cadmium tolerance and accumulation. *Plant Mol. Bio.* **2005**, *58*, 839–855. [CrossRef]
11. Benatti, M.R.; Yookongkaew, N.; Meetam, M.; Guo, W.J.; Punyasuk, N.; Abuqamar, S.; Goldsbrough, P. Metallothionein deficiency impacts copper accumulation and redistribution in leaves and seeds of Arabidopsis. *New Phytol.* **2014**, *202*, 940–951. [CrossRef]
12. Guo, W.J.; Meetam, M.; Goldsbrough, P.B. Examining the specific contributions of individual *Arabidopsis* metallothioneins to copper distribution and metal tolerance. *Plant Physiol.* **2008**, *146*, 1697–1706. [CrossRef]

13. Lee, J.; Shim, D.; Song, W.; Hwang, I.; Lee, Y. *Arabidopsis* metallothioneins 2a and 3 enhance resistance to cadmium when expressed in *Vicia faba* guard cells. *Plant Mol. Bio.* **2004**, *54*, 805–815. [CrossRef] [PubMed]
14. Ren, Y.; Liu, Y.; Chen, H.; Li, G.; Zhang, X.; Zhao, J. Type 4 metallothionein genes are involved in regulating Zn ion accumulation in late embryo and in controlling early seedling growth in *Arabidopsis*. *Plant Cell Environ.* **2012**, *35*, 770–789. [CrossRef]
15. Blanvillain, R.; Kim, J.H.; Wu, S.; Lima, A.; Ow, D.W. OXIDATIVE STRESS 3 is a chromatin-associated factor involved in tolerance to heavy metals and oxidative stress. *Plant J.* **2009**, *57*, 654–665. [CrossRef]
16. Clemens, S.; Kim, E.J.; Neumann, D.; Schroeder, J.I. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO J.* **1999**, *18*, 3325–3333. [CrossRef]
17. Kim, Y.Y.; Kim, D.Y.; Shim, D.; Song, W.Y.; Lee, J.; Schroeder, J.I.; Kim, S.; Moran, N.; Lee, Y. Expression of the novel wheat gene TM20 confers enhanced cadmium tolerance to bakers' yeast. *J. Bio. Chem.* **2008**, *283*, 15893–15902. [CrossRef]
18. Papoyan, A.; Kochian, L.V. Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiol.* **2004**, *136*, 3814–3823. [CrossRef]
19. Peng, J.S.; Ding, G.; Meng, S.; Yi, H.Y.; Gong, J.M. Enhanced metal tolerance correlates with heterotypic variation in SpMTL, a metallothionein-like protein from the hyperaccumulator *Sedum plumbizincicola*. *Plant Cell Environ.* **2017**, *40*, 1368–1378. [CrossRef] [PubMed]
20. Song, W.Y.; Martinoia, E.; Lee, J.; Kim, D.; Kim, D.Y.; Vogt, E.; Shim, D.; Choi, K.S.; Hwang, I.; Lee, Y. A novel family of cys-rich membrane proteins mediates cadmium resistance in *Arabidopsis*. *Plant Physiol.* **2004**, *135*, 1027–1039. [CrossRef] [PubMed]
21. Shim, D.; Hwang, J.U.; Lee, J.; Lee, S.; Choi, Y.; An, G.; Martinoia, A.E.; Lee, Y. Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice. *Plant Cell* **2009**, *21*, 4031–4043. [CrossRef]
22. Vatamaniuk, O.K.; Mari, S.; Lu, Y.P.; Rea, P.A. AtPCS1, a phytochelatin synthase from *Arabidopsis*: Isolation and in vitro reconstitution. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 7110–7115. [CrossRef]
23. Wemmie, J.A.; Wu, A.L.; Harshman, K.D.; Parker, C.S.; Moye-Rowley, W.S. Transcriptional activation mediated by the yeast AP-1 protein is required for normal cadmium tolerance. *J. Biol. Chem.* **1994**, *269*, 14690–14697. [CrossRef]
24. MacDiarmid, C.W.; Gaither, L.A.; Eide, D. Zinc transporters that regulate vacuolar zinc storage in Saccharomyces cerevisiae. *EMBO J.* **2000**, *19*, 2845–2855. [CrossRef]
25. Welch, J.; Fogel, S.; Buchman, C.; Karin, M. The *CUP2* gene product regulates the expression of the *CUP1* gene, coding for yeast metallothionein. *EMBO J.* **1989**, *8*, 255–260. [CrossRef]
26. Gietz, R.D.; Schiestl, R.H. Large-scale high-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2007**, *2*, 38–41. [CrossRef]
27. Pan, Y.; Zhu, M.; Wang, S.; Ma, G.; Huang, X.; Qiao, C.; Wang, R.; Xu, X.; Liang, Y.; Lu, K.; et al. Genome-Wide characterization and analysis of metallothionein family genes that function in metal stress tolerance in *Brassica napus* L. *Int. J. Mol. Sci.* **2018**, *19*, 2181. [CrossRef]
28. Elble, R. A simple and efficient procedure for transformation of yeasts. *Biotechniques* **1992**, *13*, 18–20.
29. Peng, J.S.; Wang, Y.J.; Ding, G.; Ma, H.L.; Zhang, Y.J.; Gong, J.M. A pivotal role of cell wall in cadmium accumulation in the crassulaceae hyperaccumulator *Sedum plumbizincicola*. *Mol. Plant.* **2017**, *10*, 771–774. [CrossRef] [PubMed]
30. Lin, J.H.; Xu, Z.J.; Peng, J.S.; Zhao, J.; Zhang, G.B.; Xie, J.; Yi, Z.X.; Zhang, J.H.; Gong, J.M.; Ye, N.H.; et al. *OsProT1* and *OsProT3* function to mediate proline- and γ-aminobutyric acid-specific transport in yeast and are differentially expressed in rice (*Oryza sativa* L.). *Rice* **2019**, *12*, 79. [CrossRef]
31. Peng, J.S.; Yi, H.Y.; Gong, J.M. Isolation and characterization of cadmium tolerant gene *SpMT2* in the hyperaccumulator *Sedum plumbizincicola*. *Sheng Wu Gong Cheng Xue Bao* **2020**, *36*, 541–548. [PubMed]
32. An, Z.; Li, C.; Zu, Y.; Du, Y.; Wachter, A.; Gromes, R.; Rausch, T. Expression of *BjMT2*, a metallothionein 2 from *Brassica juncea*, increases copper and cadmium tolerance in *Escherichia coli* and *Arabidopsis thaliana*, but inhibits root elongation in *Arabidopsis thaliana* seedlings. *J. Exp. Bot.* **2006**, *57*, 3575–3582.
33. Liu, J.; Zhang, J.; Kim, S.H.; Lee, H.S.; Marinoia, E.; Song, W.Y. Characterization of *Brassica rapa* metallothionein and phytochelatin synthase genes potentially involved in heavy metal detoxification. *PLoS ONE* **2021**, *16*, e0252899. [CrossRef] [PubMed]