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Abstract: Cadmium (Cd) is a major heavy metal pollutant and a significant cause of abiotic stress in the environment. Organisms have developed various pathways to protect themselves against Cd stress. MYB protein is a large transcription factor family in plants. Many members of the family have been found to regulate developmental processes and stress responses. However, the function and mechanism of MYB116 are far from clear. In a recent study, we found that MYB116 of Chinese cabbage enhances Cd tolerance in yeast. BrMYB116 is transiently inducible by Cd treatment in Chinese cabbage. When expressed in yeast cells, it enhances tolerance against Cd stress and reduces Cd accumulation in the cells. Further analysis shows that BrMYB116 protein enhances Cd tolerance in yeast cells by activating one target gene that encodes the facilitator of iron transport 3 (FIT3). ScFIT3 is activated through specific binding with its promoter. More importantly, the expression of ScFIT3, leads to a substantial reduction of Cd accumulation in both yeast and Chinese cabbage. This finding implies that a similar network may exist in other biological systems, which presents a promising avenue for enhancing Cd tolerance in plants and developing Cd-resistant crops.

Keywords: MYB116; transcription factor; facilitator of iron transport 3 (FIT3); reduction of Cd accumulation; Chinese cabbage

1. Introduction

Over the last two decades, Cd has emerged as a severe environmental threat to both the plant kingdom and human health. Cd is a highly toxic heavy metal that is easily transferrable in soil and absorbed by various plants such as rice, Chinese cabbage and carrot [1–3]. Cd has been linked to a variety of human health issues including cancer and cardiovascular diseases [4–6]. Due to the rapid increase in industrialization and improper application of pesticides and chemical fertilizers, the soil is widely contaminated by Cd, affecting not only the plants but also humans through the food chain. The accumulation of Cd in cells or tissues can inhibit cell growth through a wide range of physiological, biochemical, morphological, cellular and ultrastructural changes [7–11]. Cd enhances the accumulation of ROS and MDA, which are also highly toxic and trigger various cellular alterations, such as DNA repair, DNA methylation, gene transcription and gene translation [12–15]. At the molecular level, extensive research has been conducted on Cd stress, revealing a multitude of candidate genes that encode metal transporters and transcription factors involved in Cd detoxification and tolerance in plants and other species [16–20].

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The MYB proteins belong to a superfamily of plant transcription factors, which play a central role in regulating plant biotic and abiotic stresses. The MYB transcription factor family is the largest family of transcriptional regulators, involved not only in plant growth and developmental processes, but also in abiotic stress tolerance [21,22]. The MYB family genes are classified into four subfamilies: 1R, 2R, 3R and 4R-MYB [23,24], each containing one or more conserved structural domains. 2R-MYB is the most abundant type in plants [24,25]. Thousands of MYB genes have been identified in various plant species that regulate physiological, biochemical and molecular processes under normal and abiotic stresses [26–28]. Several studies have reported on MYB genes conferring stress tolerance in plants. For example, *AtMYB20* and *MYBS1* induce drought stress tolerance in *Arabidopsis*, *PsnMYB108* enhances salinity stress in tobacco, and *RmMYB108* enhances cold stress in *Arabidopsis* [29–32]. MYB15 regulates cold stress tolerance by interacting with the inducer of C-repeat binding factor (CBF) Expression 1 (ICE1) and binding to the promoter of CBF [33]. The R2R3-MYB transcription factor AtMYB49 positively regulates Cd accumulation through associating with the basic helix-look-helix transcription factors bHLH38 and bHLH101, which activates iron-regulated transporter 1 (IRT1) and heavy metal-associated isoprenylated proteins (HIPP22 and HIPP44) [34]. In addition, overexpression of the MYB transcription factor BnMYB2 from ramie (*Boehmeria nivea*) regulates Cd tolerance and Cd accumulation in *Arabidopsis* [35]. OsMYB45 plays an important role in resistance to Cd stress in rice [36]. AtMYB4 regulates Cd tolerance by providing enhanced protection against oxidative damage and upregulating the expression of PCS1 and MT1C in *Arabidopsis* [37]. SbMYB15 from the succulent halophyte *Salicornia Brachiata Roxb* mitigates cadmium and nickel stress in transgenic tobacco by limiting uptake and modulating antioxidative defense systems [38]. However, most of these MYB genes identified in Cd tolerance do not influence Cd accumulation, with the exceptions of AtMYB49 and BnMYB2.

Chinese cabbage (*Brassica rapa*) is one of the most popular leafy vegetables in East Asia, and like many other leafy vegetables, it has a high capacity for accumulating Cadmium (Cd) in its leaves [39–41]. It is essential to specifically identify Cd tolerance candidate genes in Chinese cabbage. In this study, we investigated genes through a transcript profiling analysis conducted on Chinese cabbage "Guangdongzao" cultivar [42]; and BrMYB116, a MYB transcription factor in Chinese cabbage, has been discovered to enhance Cd tolerance using a yeast system. Previously, MYB116 from the sweetpotato was described to play a role in drought tolerance [43]. However, as a transcription factor, the downstream targets were not identified. To gain further insights, we generated the BrMYB116 overexpressing Chinese cabbage. However, the transgenic Chinese cabbage did not enhance the Cd tolerance comparing with the wild type control, which is different from the phenotype in yeast. Thus, we performed RNA-seq transcriptome analysis on Cd-treated yeast cells carrying either an empty vector or the BrMYB16 transgene vector, leading to the identification of eighteen differentially expressed genes (DEGs). Notably, overexpressing one of these DEGs, only DEG5 (FIT3, CENPK1137D_2397), in yeast greatly enhanced Cd tolerance while reducing Cd accumulation in the cells, which aligned with the Cd tolerance phenotype displayed by BrMYB116. More importantly, FIT3 can enhance Cd tolerance and reduce Cd accumulation in Chinese cabbage. Considering there are no homologs of FIT3 in Chinese cabbage, the loss function of MYB116 in the Chinese cabbage in Cd tolerance is probably due to the loss of FIT3 in the evolutionary process. Our results further demonstrated that the MYB116 protein is directly bound to the FIT3 promoter, thereby activating FIT3 in response to Cd stress. We hypothesize that *FIT3* acts as a downstream gene to mediate the iron transporter channel in Cd stress tolerance and Cd exclusion. This study has shed light on a novel molecular basis for Cd stress tolerance, paving the way for future investigations on genetically modified crops.

2. Materials and methods

2.1. Plant treatments

Chinese cabbage (Cv. Guangdongzao) seeds were subjected to a pretreatment by soaking them with 8% sodium hypochlorite for 3 min, followed by thorough washing with ddH_2O at least five

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times to remove any excessive sodium hypochlorite. The retreated seeds were then germinated in half MS media in a growth chamber. The uniform seedlings were transferred to a hydroponic culture and grew for five days before being exposed to a treatment of 75 μM Cd. At different time points (0, 2, 4, 8, 12, and 24 hours) after the Cd treatment, samples were collected, and the plant tissues were rapidly ground in liquid nitrogen to extract total RNA for further analysis. For the generation of transformed Chinese cabbage plants, the floral dip method and vernalization infiltration technique were employed [44].

2.2. Gene clone and plasmid construction

To construct the overexpression vectors for yeast (*Saccharomyces cerevisiae*), the coding sequences of Chinese cabbage gene BrMYB116 and ScFIT3 were cloned into vector pRS416-GFP, and the gene NSR1 and VLD1 were cloned and into the vector pRS423 in fusion with the RFP sequence. The coding sequence of BrMYB116 was amplified from the cDNA of Chinese cabbage (Cv. Guangdongzao), while the coding sequence of ScFIT3, and NSR1 and VLD1 were amplified from the cDNA of yeast (JRY472) with specific primers. Subsequently, the amplified sequences were inserted into pRS416-GFP and pRS423 in fusion with the RFP sequence using the infusion cloning kit (Catalog no. 011614; Clontech). For expression constructs BrMYB116 and ScFIT3, DNA was PCR amplified from Chinese cabbage (Cv. Guangdongzao) and yeast (JRY472) genomic DNA with specific primers. And then, the amplified sequences were inserted into the XbaI site of binary vector pCambia3300 to yield pBrMYB116::BrMYB116 and pScFIT3::ZmO2L1 using the infusion cloning kit (Catalog no. 011614; Clontech). The sequence insertions were confirmed through SANGER sequencing. All primers are listed in Supplementary Table S1.

2.3. Total RNA extraction, RT-qPCR and quantitative real-time PCR analysis

The total RNA was extracted from Chinese cabbage tissues using TRIzol, while yeast RNA was extracted using a M5 EASYspin yeast RNA rapid extraction kit and MF158-01 (Mei5 Biotechnology, Co., Ltd). For yeast RNA extraction, the cells were grown to OD_{600}~ 0.3 at 30°C, and then treated with 75 μM Cd for 12 h before the total RNA was harvested. The double-stand cDNA was synthesized using a PrimeScript and RT reagent kit with gDNA Eraser (TAKARA). A SYBR Premix Ex-Taq Kit (TAKARA) was used for quantitative real time PCR. All experiments were performed with three independent biological replications. The transcript levels were calculated using the $2^{\Delta\Delta}$-CT method. The primers used for RT-qPCR are presented in Supplementary Table S1.

2.4. Tolerance assay and growth curve

The yeast cells transformed with BrMYB116 and ScFIT3 expressing pRS416-GFP vectors, along with those transformed with empty pRS416-GFP (WT) vector, were cultured overnight in SC (URA^-) fluid medium at 30°C. The cell solutions were then diluted to reach an OD_{600} of approximately 0.1, and allowed to grow to OD_{600}~0.3. After precisely adjusting the OD_{600} to 0.3, the cell solutions were five-fold diluted and spotted onto plates without or with 75 μM Cd. The cells were incubated at 30°C for two days. The experiments were repeated three times. The growth curve of BrMYB116 and WT was conducted after being grown in liquid SC (URA^-) medium at 30°C. The solutions were diluted to an OD600 of approximately 0.1, and then further incubated to reach an OD_{600} of approximately 0.3. After precisely adjusting the OD600 to 0.3, the solution was treated with 75 μM Cd for 12 h. Subsequently, the OD600 was measured every 2 hours to monitor the growth pattern.

2.5. Protein subcellular localization assay

The yeast cells co-transformed with pRS416-GFP vectors expressing or BrMYB116 ScFIT3 and pRS423 vector expressing NSR1 and VLD1 in fusion with RFP were cultured overnight in SC (URA- and HIS-) fluid medium at 30°C. The yeast cells transformed with an empty pRS416-GFP vector were also cultured overnight in SC (URA-) fluid medium at 30°C. Then, the solutions were diluted to an OD600 of approximately 0.1 and allowed to grow until reaching an OD600 of approximately 0.3. After

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adjusting the OD600 to 0.3 exactly, the solutions were treated without or with 75 μM Cd. After incubating for about 12 h at 30°C, the yeast cells were observed under a Zeiss 300 confocal microscope.

2.6. RNA-Seq library construction and sequencing

The transgenic yeast cells expressing *BrMYB116* were treated with 75 μM Cd for 14 h, and then the cells were harvested and stored at -80°C before being sent to Noveogen for RNA-seq analysis. Total RNA isolation, library construction, sequencing, and basic data analysis were carried out by Novogene. Three independent biological replicates were performed. Briefly, the RNA integrity was evaluated using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Total RNA was used as input material for RNA sample preparation. The mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was conducted using divalent cations under elevated temperatures in First Strand Synthesis Reaction Buffer. The first strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second strand cDNA synthesis was subsequently carried out using DNA Polymerase I and RNase H. Remaining overhangs were transferred into blunt ends via exonuclease/polymerase activities. After adenylation of the 3′ ends of the DNA fragments, adaptors with hairpin loop structures were ligated to prepare for hybridization. To select cDNA fragments within the preferred length range of 370~420 bp, the library fragments were purified with an AMPure XP system (Beckman Coulter, Beverly, USA). Subsequently, PCR was carried out with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. The resulting PCR products were purified using the AMPure XP system, and the library quality was evaluated on an Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was carried out on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumia). After cluster generation, the library was sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

2.7. Promoter activity assay

Nicotiana benthamiana seedlings were grown in a controlled environment at 22°C, with a 16-h-light/8-h-dark photoperiod. The infiltration process was conducted on 4-week-old plants. Single clones of GV3101, each carrying different vectors, were inoculated to YEP medium containing rifampicin and kanamycin and then grown for more than 20 h at 28°C. Next, approximately one microliter Agrobacterium was inoculated to 20 mL fresh YEP medium containing 10 mM MES (pH 5.6), and 20 mM acetosyringone and grown for about 4 h at 28°C. The cells were collected and resuspended in the infiltration medium (10 mM MgSO4, 200 mM acetosyringone, and 10 mM MES) to reach an OD_{600} of 1. The cells were incubated at room temperature for 2 h. The infiltration medium contains three *A. tumefaciens* strains: 1 mL of transcription activator strain (p35S::BrMYB116 or empty vector), 100 mL promoter strain (ScFIT3 promoter-*fluc*), and 5 mL reference strain (35S-*rLUC*). Infiltration was carried out with *N. benthamiana* leaves using a syringe. After infiltration, tobaccos were kept in a dark chamber with high humidity overnight and then transferred back to a normal growth room for 2 d. The luciferase values were measured using a Dual-Luciferase Reporter Assay System (catalog no. E1910; Promega).

2.8. Chromatin Immunoprecipitation

One hundred microliters of cells (OD_{600}~1.0) were subjected to cross-linking by 1% formaldehyde for 20 min at 30°C, which was stopped by adding 125 mM glycine and incubating for 5 min. Cell pellet was collected, washed twice with PBS (137 mM sodium chloride, 2.7 mM potassium chloride, and 11.9 mM phosphate buffer, pH 7.4), and then frozen immediately in liquid nitrogen. The cell pellets were resuspended in sorbitol buffer (1.2 M sorbitol in PBS buffer) containing 1.5 mg lyticase (Cat# L4025, Sigma) and incubated at 30°C for 30 min. Then, the pellets were washed twice with PBS buffer and resuspended in 0.2 ml lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1%Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF) containing 1% SDS. The lysates

were sonicated using a M220 sonicator (Covaris) to yield chromatin fragments with an average size of 500 bp. To reduce the SDS concentration to 0.1%, the lysis buffer was added to the solution to achieve a final volume of 2 ml. The samples were centrifuged at 4°C. One hundred microliter of the soluble chromatin was saved as the input, while the rest was immunoprecipitated with specific antibodies [2 μg monoclonal anti-GFP antibody (Cat# ab16918, Abcam) overnight at 4°C, and then 25 μl mixture (1:1) of Dynabeads® Protein A (Cat# 10002D, Life Technologies AS) and protein G (Cat#10004D, Life Technologies AS) was added and incubated at 4°C for over 6 h. The beads were washed twice in low salt buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1%Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), twice in high salt buffer (50 mM HEPES-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1%Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), twice in the washing buffer (10 mM Tris pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA and 1 mM PMSF) and twice in TE (10 mM Tris pH 8.0, 1 mM EDTA). DNA was eluted by incubating the beads overnight at 65°C with 0.3 ml elution buffer (50 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 1% SDS), and cross-linking was reversed. The proteins were digested by incubation for 2 h at 50°C with 0.3 mg/ml proteinase K (Cat# P6556, Sigma). Then, 1 μg RNase A (Cat# A1101A, TaKaRa) was added, and the samples were incubated for 2 h at 37°C. The ChIPed DNA was purified by phenol/chloroform extraction and precipitated with ethanol/NaOAC overnight at -20°C. DNA was resuspended in 0.2 ml ddH_2O. Actin 1 of *Saccharomyces cerevisiae was* used as the international control. Recovered DNA fragments were quantified by qPCR with a SYBR® *Premix Ex* TaqTM Mix (Cat# DRR820A, TaKaRa). The CP (crossing point) values of the immunoprecipitated DNA fractions with GFP or no antibody control (NoAb) were normalized to the CP value of the input DNA fractions for the same qPCR assay. The ChIP signals were calculated as the relative enrichment in signals relative to the NoAb control. All experiments were repeated three times. All primers are listed in Supplemental Table S1.

2.9. Cd accumulation measurement

For the measurement of the Cd concentration in the seedlings, the Chinese cabbage seedlings grown on half-strength MS solid media with Cd treatment were collected and washed as previously described [45] with minor modifications. Three biological replicates of each Cd-treated group were collected.

2.10. Statistical analysis

All experiments were conducted in triplicates. Data were analyzed using variance (ANOVA) analysis with Duncan's multiple range test (DMRT). Standard errors were calculated for all mean values, and differences were considered significant at the $P \leqslant 0.05$ level.

2.11. Accession numbers

Sequence data for the RNA-seq samples can be found in the NCBI's Sequence Read Archive (SRA) database under the following accession number SUB12330385.

3. Result and Discussion

3.1. Screening Cd tolerance genes from Chinese cabbage by a yeast system

In our previous report (He et al., 2022), a number of Cd response genes (CRGs) from the Chinese cabbage cultivar "Guangdongzao" have been identified by transcriptome profile analysis. Yeast is a common eukaryotic system for the study of Cd resistant mechanism. Hence, in this study, yeast was employed to identify the CRGs of Chinese cabbage that participates in Cd tolerance (He et al., 2022). The genes were fused after the glycerol phosphate dehydrogenase (GPD) promoter in a yeast expressing recombination vector pRS416 to yield pGPD::CRGs for transformation into budding yeast (cv JRY472). Serial dilution assays were conducted to test Cd tolerance. As shown in Figure 1, the strains expressing *BrMYB116* grew stronger than the wild-type JRY472 with the empty vector. We also conducted the growth curve of the yeast expressing *BrMYB116* without or with Cd stress.

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Compared with the wild-type control, the transgenic cells exhibited a Cd tolerance phenotype between 8 to 16 h after the Cd treatment. It is clear that BrMYB116 plays a role in Cd tolerance in yeast.

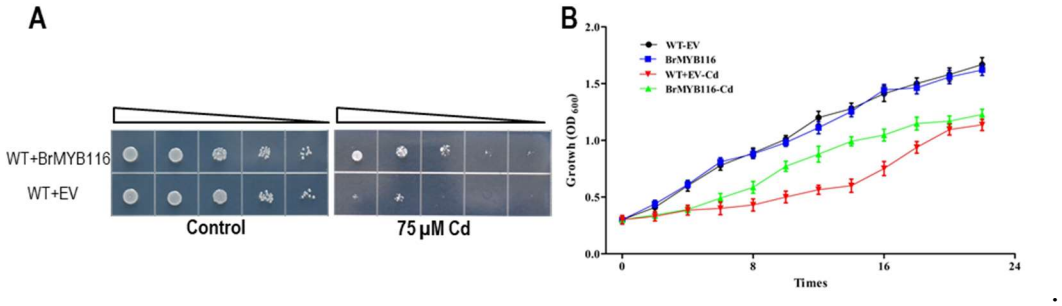

Figure 1. Cd tolerance tests in yeast. A. Yeast dilution bioassay with the wild-type strain and the wild-type strain transformed with a *ScMPC1* expressing pRS416 vector in SD medium. Triangles represent serial 10-fold dilutions (starting concentration of 0.3 OD_{600}). B. The wild-type yeast strain and transgenic cells were grown at 30°C in SD liquid media and exposed to 75 μM $CdCl_2$ at the concentration of 0.3 OD_{600}. Cell density was monitored with the absorbance at 600 nm at 12, 14, 16, 18, 20, and 22 h after the Cd treatment. Error Bars indicate ±SD from three independent experiments.

3.2. BrMYB116 was induced by Cd in Chinese cabbage

To investigate the expression of *BrMYB116* in response to Cd stress, Chinese cabbage seedlings were grown in MS nutrient solution without or with 200 μM $CdCl_2$. The whole seedlings were collected at different time points after the Cd treatment. Quantitative real-time PCR (RT-qPCR) data showed that the mRNA abundance increased steadily until the second hour after the Cd treatment, reaching its peak at the eighteenth hour (Figure 2). These results indicate that the BrMYB116 transcript is activated in response to Cd stress and may be involved in Cd responses in Chinese cabbage. As a transcription factor, BrMYB116 could potentially bind to specific downstream cis-elements associated with genes that confer resistance to Cd stress.

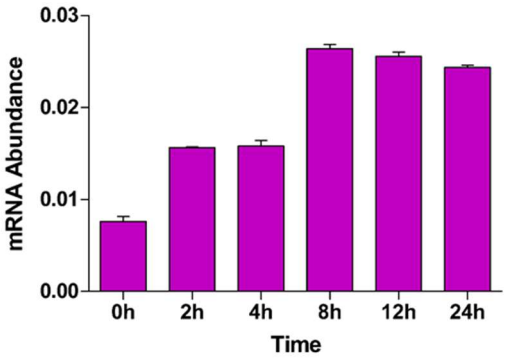

Figure 2. *BrMYB116* transcript abundance in Chinese cabbage without or with 75 μM $CdCl_2$. *BrMYB116* transcript abundance in Chinese cabbage seedlings (relative to *BrACT2* control) was determined by RT-qPCR. Six-day-old seedlings were exposed to 75 μM $CdCl_2$. Error bars indicate ± SD from three independent experiments.

3.3. BrMYB116 specifically accumulated in the nuclear under Cd stress

There is no evidence supporting the translocation of BrMYB116 into the nuclear under heavy metal stresses. To test whether BrMYB116 plays a specific role as a transcription factor under Cd stress, the coding region was fused to GFP and expressed in yeast cells. The vector pRS423 expressing yeast nuclear localized gene NSR1 (YGR159C) in fusion with RFP was used as a positive control [46], and the empty vector (pRS416-GFP) was used as a negative control. In the absence of Cd, the

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BrMYB116 fusion was localized in the cytoplasm. However, upon treatment with 75 μM $CdCl_2$, BrMYB116 was translocated into the nucleus, while the GFP signal remained unaffected by Cd stress (Figure 3), indicating that BrMYB116 specifically entered into the nuclear under Cd stress. The specific nuclear localization of BrMYB116 suggests that it may play a pivotal role in Cd tolerance at the molecular level. These results also implied that BrMYB116 might bind to cis-elements of its target genes within the nucleus, which are crucial for Cd stress responses in yeast.

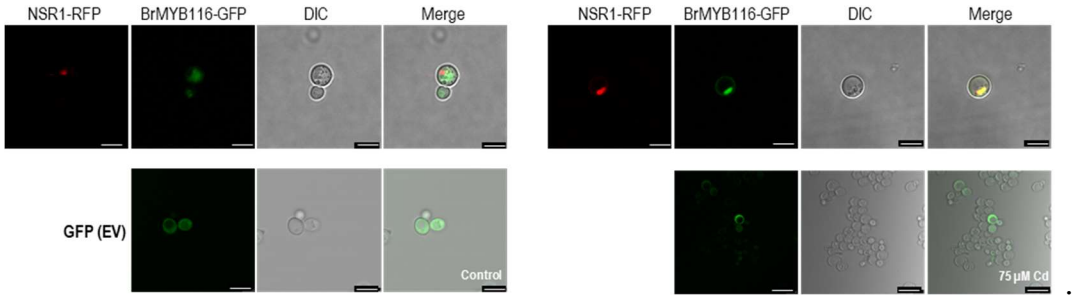
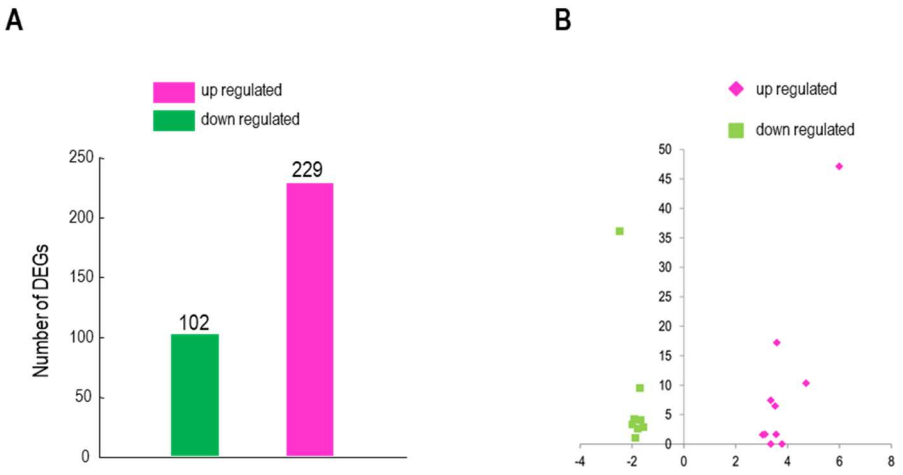


Figure 3. Subcellular localization of BrMYB116 and empty vector tagged with GFP and expressed in yeast cells without or with 75 μM $CdCl_2$. The nuclear localized gene NSR1 (YGR159C) was used as positive control. The images were obtained from the RFP channel, GFP channel, DIC channel, or a merged image of the two channels. Scale bar: 10 μm.

3.4. Differentially expressed genes identified from RNA-Seq analysis

The Cd tolerance phenotype associated with the expression of BrMYB116 is likely due to the expression change of genes regulated by BrMYB116. To examine if the gene expression pattern has changed, an RNA-seq analysis was conducted on the Cd-treated yeast cells transformed with BrMYB116-expressing vectors and empty vectors. A total of 331 DEGs with statistically significant changes (log2(FoldChange) ≤ 1 or ≥ 1, and the adjusted P value ≤ 0.05) were selected (Supplementary Table S2, Figure 4A). Gene Ontology analysis shows that many of these DEGs are involved in transporter activity and transmembrane transporter activity (Supplementary Table S3), indicating that BrMYB116 could enhance Cd tolerance through regulating Cd ion transport. Given that BrMYB116 could regulate several or one target gene to confer Cd tolerance, we narrowed our focus to a smaller group of DEGs with log2(FoldChange) ≤ -1.5 or ≥ 3, which is composed of 18 DEGs. These 18 DEGs were named DEG1 to DEG18 from top to bottom in the clustering analysis map (Fig. 4B).



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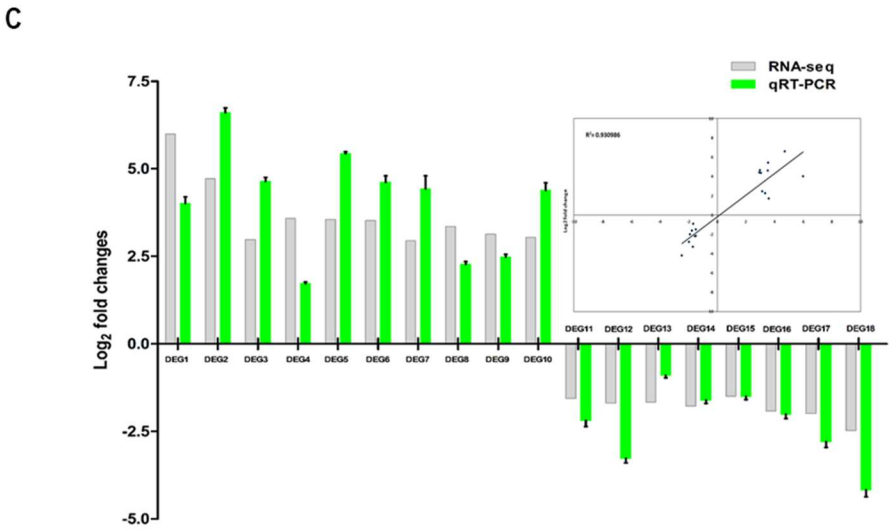

Figure 4. RNA-seq analysis. A. Numbers of DEGs in transgenic cells expressing BrMYB116 VS WT group. B. Volcano plot of the selected DEGs (log2 values >3 or < -1.5) from (A). X axis represents fold change shown as log2 values; Y axis represents adjust P value shown as -log10. Magenta represents increased expression; green represents decreased expression compared to WT. C. qPCR identification of the selected DEGs (relative to ACT2) in the same batch Cd-treated yeast cells of RNA-seq. Error bars indicate ±SD from three independent experiments.

3.5. Overexpression of DEG5 enhanced Cd tolerance in yeast

To validate the expression pattern obtained from RNA-seq analysis, RT-qPCR was conducted on these 18 DEGs, which showed a similar expression pattern compared with that of the RNA-seq data for most of the genes (Figure. 4C). Each of the eighteen DEG candidates was fused to the GPD promoter for expression in yeast cells. When tested under Cd stress (75 μM), only yeast cells overexpressing DEG5 (CENPK1137D_2397), which encodes a putative member of the facilitator of iron transport 3 (FIT3), exhibited stronger growth than the wild-type control (Figure. 5A). This finding indicates that overexpression of DEG5 alone is sufficient to enhance Cd tolerance, highlighting its crucial role in ameliorating the toxic effects of Cd. Furthermore, the Cd content in the transgenic yeast cells was assessed, and reductions in Cd accumulation were observed among JRY472 (EV), JRY472 (BrMYB16) and JRY472 (ScFIT3) (Figure 5B). These results suggest that the Cd tolerance phenotype mediated by BrMYB116 and ScFIT3 is mainly attributed to reductions in Cd accumulation.

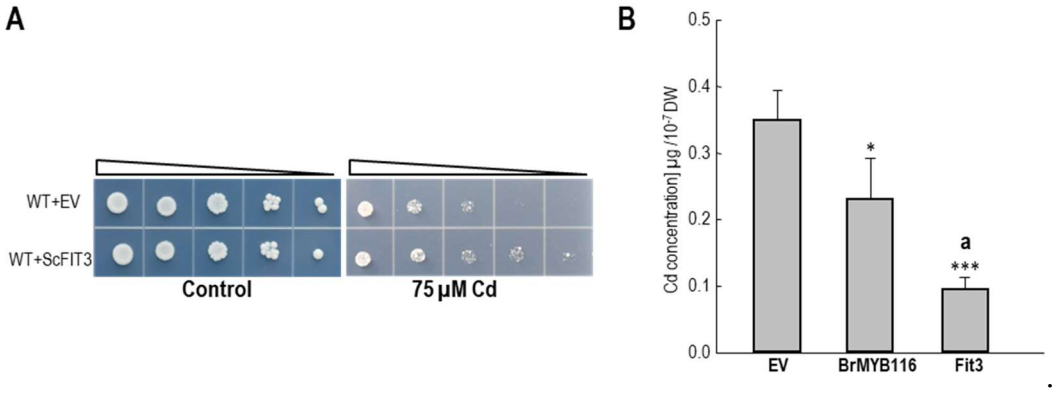

Figure 5. Cd tolerance phenotype and accumulation. A. Dilution bioassay for the wild-type yeast strain and the wild-type strain overexpressing *ScFIT3* in SC medium. Triangles represent serial 10-fold dilutions (starting concentration of 0.3 OD_{600}). Representative test from three reproducible experiments was shown. B. Cd concentration in yeast cells. Yeast strains grown on SC solid plates without or with 75 μM $CdCl_2$ at 30℃ for 2 d. Cells were collected in liquid SC with 75 μM Cd and

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the OD_{600} was recorded before atomic absorption spectrometer measuring. Error bars indicate ±SD of three independent experiments. P value of student's t test: BrMYB116 compared with empty vector. *P<0.05; **P<0.01; ***P<0.001. . P value of student's t test: BrMYB116 compared with ScFIT3. [a]P<0.05.

3.6. ScFIT3 was ubiquitously present in yeast cells from vacuole under Cd stress

The Cd tolerance candidate ScFIT3 is a facilitator of iron transport 3, and studies have shown that some iron transporters can transfer Cd ions in addition to iron ions. To investigate the subcellular localization of ScFIT3, the coding region of *ScFIT3* was fused to GFP and expressed in yeast cells. The vector pRS423 expressing vacuole localized gene VLD1 (YIR014W) in fusion with RFP was used as a positive control [47], and the empty vector (pRS416-GFP) was used as a negative control. As shown in Figure 6, in the absence of Cd, ScFIT3 was localized in the vacuole. However, upon Cd treatment, ScFIT3 was distributed throughout the entire cell. The absence of VLD1 fused with RFP in yeast cells under Cd stress suggests that Cd might have disrupted its expression. It is clear that ScFIT3 changes its subcellular localization when treated with Cd stress. Under normal conditions without Cd stress, ScFIT3 may play a role in facilitating the transport of iron ions in the vacuole. However, in the presence of Cd, ScFIT3 appears to facilitate the transport of the Cd ions from the vacuole, and throughout the whole cell. The Cd content measurement (Figure. 5B) showed that it is plausible that the change in ScFIT3 localization is associated with the exclusion of Cd ions from the cell membrane.

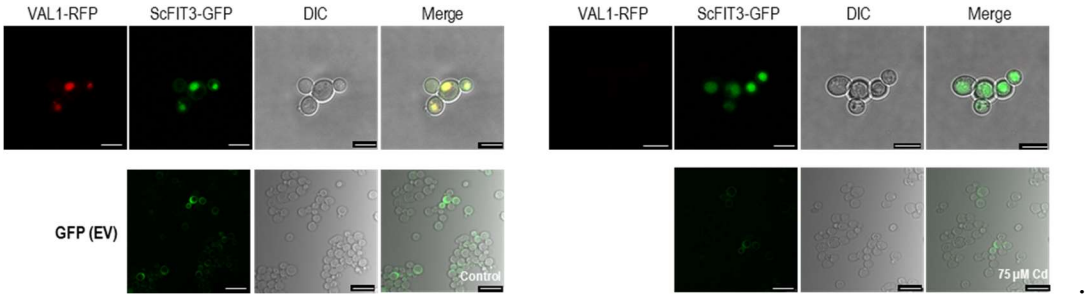

Figure 6. Subcellular localization of ScFIT3 and empty vector tagged with GFP and expressed in yeast cells without or with 75 μM $CdCl_2$. The vacuole localized gene VLD1 (YIR014W) was used as positive control. The images were obtained from the RFP channel, GFP channel, DIC channel, or a merged image of the two channels. Scale bar: 10 μm.

3.7. BrMYB116 is associated with the promoter of ScFIT3

To investigate the interaction between BrMYB116 and ScFIT3, we fused the firefly luciferase gene (*luc*) to a 2-kb promoter including its untranslated region (UTR) fragment from *ScFIT3* to generate the promoter-*luc* fusion. The construct was transiently introduced into tobacco leaf tissue by agroinfiltration, along with p35S::*BrMYB116*, or an empty vector control. In the transactivation assay, enhanced expression of pScFIT3-*luc* by p35S::*BrMYB116* was observed under the Cd treatment (Figure 7A), whereas the promoter activity was not affected in the absence of Cd. These results indicated that the activity of pScFIT3 was specifically induced by BrMYB116 under Cd stress. A chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) analysis was also performed to test the *in vivo* interaction of the promoter in transgenic yeast cells producing GFP-tagged BrMYB16. Following immunoprecipitation with anti-GFP antibody, four pairs of primers were used for each part (500 bp) of the promoter corresponding to fragments F1 - F4, respectively (Figure 7B). Positive interaction for BrMYB116 was found for F1 (Figure 7B), but not for the other fragments, including the ACT1 (XM_009117825, NCBI) promoter used as the negative control. These results suggested that BrMYB116 binds to a specific region of the promoter of *ScFIT3* to regulate its expression.

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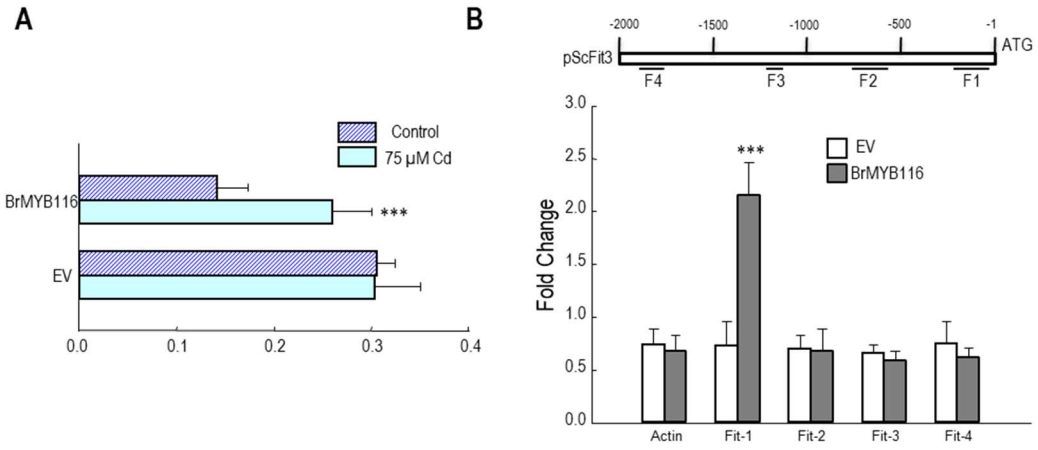

Figure 7. BrMYB116 associates the fragment of the ScFIT3 promoter under Cd stress. A. BrMYB116 activation of the ScFIT3 promoter (include 5′ UTR) determined by infiltration mediated transient expression assay. The X axis is the ratio of LUC to rLUC activity two days after infiltration. The numbers indicate the position of the starting nucleotide of each BOXS2 relative to the translation start. Error bars indicate ± SD of three biological repeats. P value of student's t test: BrMYB116 compared with empty vector. *P<0.05; **P<0.01; ***P<0.001. B. BrMYB116 binds the promoter of ScFit3. ChIP-qPCR was performed to test the *in vivo* interaction of promoters (including 5′UTR) with BrMYB116 in cells from WT, and WT (*BrMYB116-GFP*) treated with 75 μM Cd. DNA fragments from different parts of the promoter were tested (labeled with F1 - F4). The CP (crossing point) value of immuno-precipitated DNA fractions with GFP or the no-antibody control (NoAb) normalized to the CP value of the input DNA fractions for the same qPCR assay. The Y axis is the ChIP signals calculated as the enrichment relative to the no-antibody control (NoAb). Error bars indicate ± SD from three independent experiments.

3.8. FIT3 reduced the Cd content in the Chinese cabbage.

To investigate the function of FIT3 in preventing Cd accumulation in plants, we transferred ScFIT3 to the Chinese cabbage cultivar "Guangdongzao". After confirming the successful integration of ScFIT3 through RT-PCR, two independent lines were selected for further analysis of Cd tolerance phenotype and Cd accumulation. As shown in Figure 8A and B, under the Cd stress, the transgenic seedlings exhibited better growth conditions and longer root lengths compared to the wild-type control. Moreover, the Cd content was significantly reduced in these two transgenic lines compared to the wild type control (Figure 8C), indicating that the improved growth in the transgenic lines was attributed to reduced Cd accumulation and toxicity. Since Chinese cabbage is a vital vegetable, we also examined the content of several classical nutritional ions in the transgenic seedlings. As shown in Figures 8D-F, the contents of K, Cd and P ions were indistinguishable between the transgenic seedlings and the wild type control, suggesting that the expression of FIT3 did not affect the uptake of main nutritional ions.

To elucidate the function of BrMYB116 in Cd tolerance, we had transferred the BrMYB116 gene to Chinese cabbage before. After the quantitative PCR identification, two independent lines were selected for the further Cd tolerance phenotype and Cd accumulation tests. As shown in Figure 8A, under Cd stress, there was no obvious difference between the BrMYB116 overexpressing lines and the wild type control. Considering the absence of a homologous gene to FIT3 in Chinese cabbage, the association of BrMYB116 with the promoter of FIT3, and the ability of FIT3 to alleviate Cd toxicity in Chinese cabbage, it is plausible that FIT3 is the real target candidate gene in the Cd tolerance pathway, which was regulated by the transcription factor BrMYB116 and lost in the evolutionary process.

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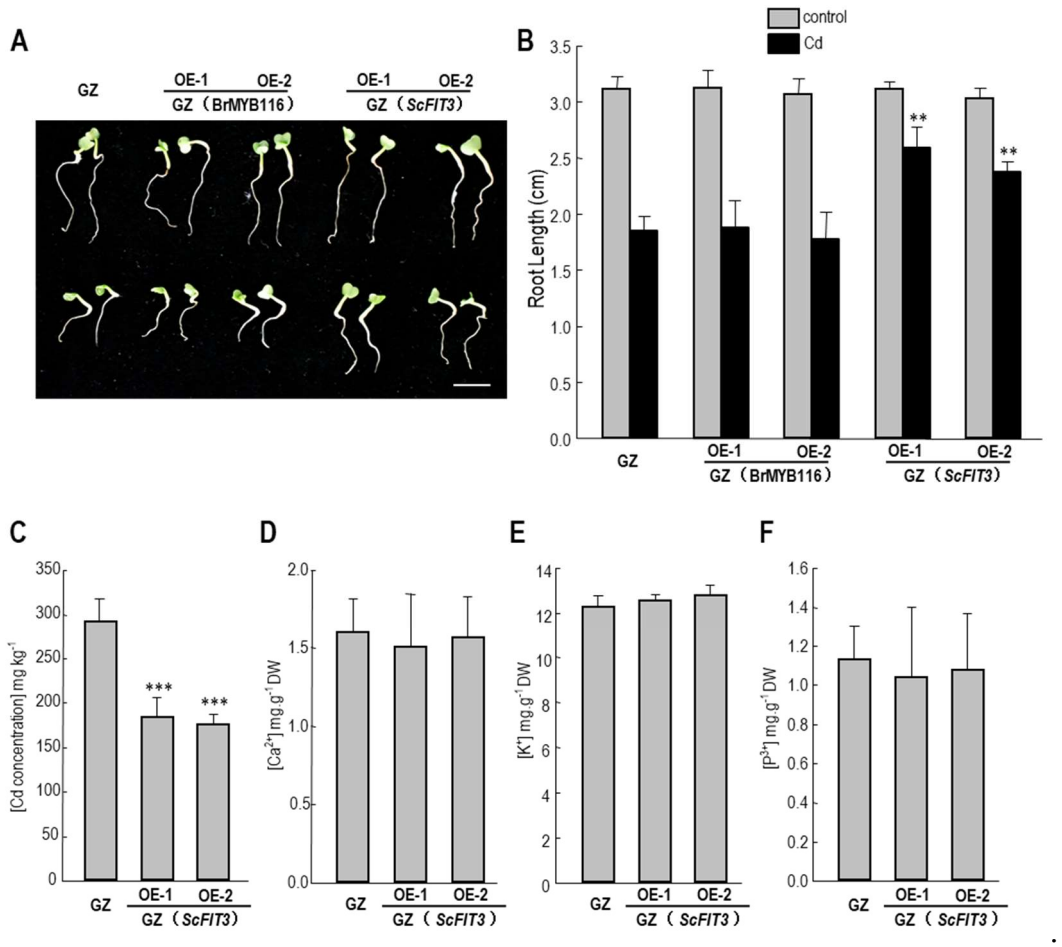

Figure 8. Cd tolerance and content, and some nutrition ion contents in the Chinese cabbage. A. Chinese cabbage plants germinated for 2 d were transferred to hydroponic MS media without or with 50 μM $CdCl_2$ for another 3 d. Representative results from three reproducible experiments are shown. OE-1 and OE-2 are two independent transgenic lines. B. Average root length of seedlings cultured under the same growth condition as shown in A. The root length of three seedlings of each class was measured as the mean value. The content of Cd (C), Ca^{2+} (D), K^{+} (E), p^{3+} (F) in the wild-type and ScFIT3 transgenic Chinese cabbage. Error bars indicate ± SD of three biological repeats. P value of student's t test: BrMYB116 or ScFIT3 transgenic plants compared with the wild-type control. **P; ***P<0.001.

4. Conclusions

Cd toxicity poses a threat to human health through food contamination. Therefore, compared with other methods for the remediation of Cd contamination, a novel Cd transporter which can exclude Cd ions from the cells or the plants is imperative and can be easily used for genetic engineering of vegetables and crops. In this study, we identified a classical MYB transcriptional factor from Chinese cabbage, BrMYB116, which associated the iron ion transporter gene *ScFIT3* of yeast and facilitates the exclusion of Cd in both yeast and Chinese cabbage. Although homologous genes of FIT3 have not been found in other plant species, it is possible that FIT3 was lost during the evolutionary process. The ancient heavy metal resistance mechanism regulated by FIT3 and MYB116 might be elucidated and utilized by plants to remediate Cd toxicity and exclude Cd from plant tissues (Figure 9).

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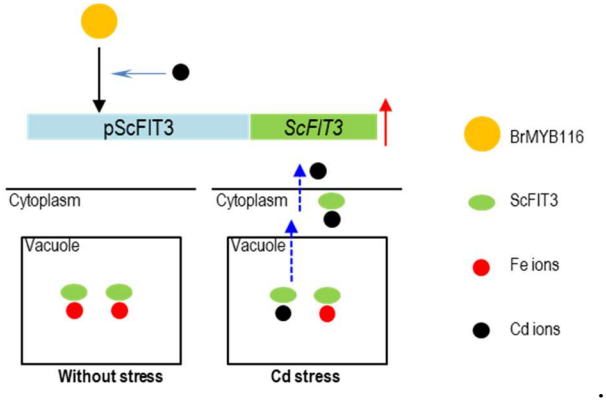

Figure 9. The hypothesis model of BrMYB116 regulating Cd tolerance in yeast cells. BrMYB116 is shown in yellow roundness, ScFIT3 is shown in green oval, Fe ions is shown in red roundness, and Cd ions is shown in black roundness.

Contributions: Lilong He and Anwar Ali performed the experiments with the assistance from Chao Yuan, Bing Cui, Lixia Wang, Shu Zhang, Cheng Li and Jingjuan Li; Lilong He, Anwar Ali, and Chao Yuan carried out most of the analyses; Lilong He and Jianwei Gao designed the project and experiments; Lilong He and Anwar Ali wrote the manuscript, and Jianwei Gao reviewed the draft. All authors read and approved the final manuscript.

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Conflicts of Interest: The authors declare that there are no conflicts of interest.

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