BrpNAC895 and BrpABI449 coregulate the transcription of the afflux-type cadmium transporter BrpHMA2 in Brassica parachinensis

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Abstract

Brassica parachinensis is a popular leafy vegetable. It is able to accumulate high concentrations of cadmium (Cd), but the molecular mechanism of Cd accumulation is unknown. This study investigated the function and regulatory mechanism of the Cd-responsive metal ion transporter gene BrpHMA2. BrpHMA2 was induced by Cd stress and specifically expressed in vascular tissues, and the protein was localized in the plasma membrane. Heterologous expression of BrpHMA2 enhanced Cd accumulation and Cd sensitivity in transgenic Arabidopsis and yeast. After Cd stress, the transcription factors BrpNAC895 and BrpABI449, which may recognize the abscisic acid-responsive elements in the BrpHMA2 promoter, were also differentially expressed. The transcriptional regulation of BrpHMA2 was further investigated using the chromatin immunoprecipitation–quantitative PCR (ChIP–qPCR) assay, the electrophoretic mobility shift assay (EMSA), and luciferase (LUC) reporter activity analysis employing the transient expression system of B. parachinensis protoplasts and tobacco leaves and the Escherichia coli expression system. By binding to the promoter, BrpNAC895 induced the transcription of BrpHMA2. BrpABI449 might bind to the BrpHMA2 promoter or interact with BrpNAC895 to interfere with the action of BrpNAC895. The findings suggest that BrpHMA2 is a membrane-based afflux-type Cd transporter involved in Cd $^{2+}$ uptake and long-distance transport in plants. BrpNAC895 and BrpABI449, which function as the transcription activator and repressor, respectively, coregulate BrpHMA2 expression.

Introduction

Cadmium (Cd) is one of the major environmental pollutants and a potential hazard to worldwide agriculture. Excess Cd uptake in plants normally induces the accumulation of reactive oxygen species (ROS) in plants and has severe consequences, such as chromosome aberrations, protein inactivation, membrane damage, and further leading to leaf chlorosis and root growth inhibition [1]. Furthermore, accumulation of Cd in crops enhances the risk of Cd poisoning in humans and animals [1]. Brassica species have been identified as Cd hyperaccumulators [2]. Brassica parachinensis L.H. Bailey (Chinese flowering cabbage) is a leafy vegetable widely consumed in China, Europe, and other regions of the world [3]. Thus, elucidating the molecular mechanisms of Cd accumulation in this plant is essential for developing effective strategies to control Cd accumulation in the plant’s edible parts.

Cd accumulation in plant tissues generally involves a three-step process: (i) absorption and accumulation of Cd in roots from the soil, (ii) translocation of Cd to the shoot via vascular tissue, and (iii) Cd storage in leaves [4]. Cd transporters are considered to play central roles in various physiological activities. The HMA (heavy metal ATPase), ZIP (zinc-regulated transporter protein), and Nramp (natural resistance-associated macrophage protein) families are among the transporter families that have been identified as being involved in these processes [5–7]. Our previous transcriptome analyses of B. parachinensis also showed that differentially expressed genes enriched in the gene ontology (GO)
terms ‘transmembrane transport’ and ‘metal ion transport’ may be involved in response to Cd, including genes encoding members of some transporter families, such as the subfamily C of ATP-binding cassette proteins (ABCCs) and HMAs [8].

HMAs, which belong to the P1B subfamily of the P-type ATPase superfamily, have been extensively investigated in the model plant Arabidopsis as well as in some crop plants, and the main focus of these studies has been on their functions [9]. For example, eight members of HMAs have been identified in Arabidopsis thaliana, and among these, AtHMA1–AtHMA4 are thought to specifically transport divalent cations, such as Zn²⁺, Cd²⁺, Co²⁺, and Pb²⁺ [10]. AtHMA2 is generally regarded as a Zn²⁺-ATPase [11–15]. It contains a conserved short metal-binding domain in the N-terminus and a long metal-binding domain in the C-terminal end; Zn²⁺-binding affinity was detected in both domains [13, 14], and Cd²⁺- and Cu²⁺-binding affinity was detected in the N-terminal domain [13]. Some studies showed that AtHMA2 functioned as an efflux to drive the outward transport of metals from the cell cytoplasm and responsible for cytoplasmic Zn²⁺ homeostasis and Cd detoxification [14, 15]. Some researchers proposed that AtHMA2 together with AtHMA4 played key roles in the long-distance root-to-shoot transport of Zn²⁺ and Cd²⁺ by loading these ions into the xylem [10, 13]. Similar results were also reported in wheat TaHMA2 [16, 17]. However, it seems that OsHMA2 in rice has a different role. The enhanced sensitivity to Cd and tolerance to zinc (Zn) deprivation afforded by heterologous expression of OsHMA2 in yeast cells suggest that OsHMA2 functions as a Cd influx transporter [18]. These studies showed that HMA2 and its subfamily members in different plants may function differently. There is a lack of thorough knowledge of the role of BrpHMA2 in Cd hyperaccumulation in the leafy vegetable B. parachinensis. The function of BrpHMA2 and the mechanisms that regulate its expression must be elucidated.

Previous studies have indicated that plants employ a universal and conserved approach to regulate the transcription of heavy metal uptake and tolerance genes. For example, in a bean (Phaseolus vulgaris), PvMTF-1 (metal response element-binding transcription factor 1), which could be induced by PvERF15 (ethylene response factor 15), may regulate the expression of the stress-related gene PvSR2 and confer Cd tolerance to the plant [19]. In Arabidopsis, two basic helix–loop–helix (bHLH) transcription factors (TFs), FIT (Fer-like iron deficiency-induced transcription factor) and PYE (POPEYE), modulate iron (Fe) deficiency responses by regulating the expression of IRT1 and FRO2 [20], whereas the bHLH TFs IAA-leucine resistant 3 (ILR3) and bHLH104 can form heterodimers and bind to specific elements in the promoter of PYE to regulate PYE [21].

NAC (NAM, ATAF1, and CUC2) TFs are members of the most prominent TF families in plants. These TFs play essential roles in diverse biological processes, such as growth, development, senescence, and morphogenesis, and are widely involved in various signaling pathways in response to different phytohormones and multiple abiotic and biotic stresses [22, 23]. For example, NAC019, NAC055, and NAC072 negatively regulate drought stress-responsive signaling [24]. NAC096 is associated with drought stress. It could exert its function via a mechanism like that of basic leucine zipper protein (bZIP)-type TFs to bind specifically to abscisic acid (ABA)-responsive elements in the promoters of several drought stress-responsive genes [25]. This finding implies that NAC096 and bZIP-type TFs can sometimes regulate the same target genes [26]. Studies have also shown that the core DNA-binding sequences of NACRE (NAC-responsive element) and ABRE (ABA-responsive element) are PyCAG and PyACGTGG/TC (Py, pyrimidine), respectively [26].

In a previous study, we identified a few NAC and AREB (ABA-responsive element-binding protein) TFs triggered by Cd stress in B. parachinensis [8]. However, their functions remain unclear. To clarify the molecular mechanisms of Cd accumulation in B. parachinensis, the function of a Cd-responsive metal ion transporter gene BrpHMA2 and the coregulation of BrpHMA2 transcription by two TFs (BrpNAC895 and BrpABI449) were examined in this study. The findings reveal a precise regulatory mechanism in B. parachinensis in response to Cd stress.

**Results**

**Expression pattern of BrpHMA2**

We previously analyzed the Cd-induced mRNA transcriptome of B. parachinensis and found that several HMA homologs were substantially expressed under Cd stress [8]. We cloned one of the HMA2 homologs and constructed the phylogenetic tree of this HMA2 homolog with other HMAs in A. thaliana, Oryza sativa, Zea mays, and Alfred stonecrop by the neighbor-joining method using MEGA5. The results revealed that the sequence of this HMA2 homolog is closer to that of the AtHMA2 gene (Supporting Information Figure S1), and thus it was named BrpHMA2.

The transcript level of BrpHMA2 in seedlings grown hydroponically was examined using reverse transcription–quantitative PCR (RT–qPCR) to investigate the expression pattern of BrpHMA2 in B. parachinensis. According to the results, BrpHMA2 was expressed at higher levels in leaves than in roots. Cd stress may increase BrpHMA2 expression in leaves and roots, although BrpHMA2 expression in leaves fluctuates owing to developmental regulation (Fig. 1a). The GUS gene was transformed and expressed in Arabidopsis using the promoter of BrpHMA2 (pBrpHMA2::GUS) to corroborate the expression pattern, and histochemical assays were performed. Instant β-glucuronidase (GUS) staining for 0.5 hours showed that the GUS signal was visible in the vascular bundles of the leaves and roots of the plants treated with 50 μM Cd (NO₃)₂ for 2 days, but not in vascular bundles of...
seedlings that were not treated with Cd (Fig. 1c). Results from an examination of transcripts of the GUS gene in the reporter line were also consistent with these findings (Fig. 1c). This showed that BrpHMA2 could be induced by Cd stress.

However, when the pBrpHMA2::GUS transgenic seedlings were subjected to GUS staining for 3 hours, a strong GUS signal could be observed in the vascular bundles of the cotyledons, true leaves, stems, petals, filaments, and the carpodipodium of the seeds in young siliques. The blue GUS signal was particularly strong in the tissue junction regions where the vascular bundles were clustered (Figure S2). These results indicate that BrpHMA2 may function primarily in transport in vascular tissues. The fluorescent signal of BrpHMA-GFP (GFP, green fluorescent protein) was detected at the plasma membrane by transient expression analysis in protoplasts of B. parachinensis leaf cells (Fig. 1b), indicating that BrpHMA2 is localized at the plasma membrane.

Overexpression of BrpHMA2 enhances cadmium accumulation in Arabidopsis

To investigate the physiological role of BrpHMA2 in plants, transgenic A. thaliana lines expressing BrpHMA2 (OE-BrpHMA2) were generated. The Cd distribution and accumulation in seedlings of Col and BrpHMA2-overexpressing lines (OE-1, OE-2) were investigated. Dithizone staining showed that Cd was mainly located in the epidermal hairs of the leaves in both Col and OE-BrpHMA2 seedlings, but more Cd-dithizone precipitates were found in the OE-BrpHMA2 lines (Fig. 1d). Although Cd stress inhibited the growth of both Col and transgenic plants, the extent of growth inhibition in the OE-BrpHMA2 plants was stronger (based on leaf size and color) than in the Col plants after 6 days of Cd exposure (Fig. 1e). Moreover, Cd content assay revealed that the roots and shoots of the OE-BrpHMA2 plants had considerably more Cd than those of the Col plants (Fig. 1f).

Overexpression of BrpHMA2 enhances cadmium accumulation in yeast

To further analyze the function of BrpHMA2, BrpHMA2 was fused with the galactose-inducible promoter was transformed into a Cd-hypersensitive yeast mutant, Δycf [18]. In the presence of the transcriptional inducer galactose, Cd^{2+} considerably inhibited the growth of yeast cells with heterologous expression of BrpHMA2 compared with that of cells transformed with the empty vector (Fig. 1g). However, when gene expression was suppressed by the presence of glucose, no growth differences were detected between the cells transformed with BrpHMA2 and those transformed with the empty vector. The Cd content in the heterologous transgenic cells grown in liquid medium was higher than that in the control cells (Fig. 1h). These results indicate that BrpHMA2 functions as an afflux-type Cd transporter.

NACs and abscisic acid-responsive element-binding proteins are differentially expressed in B. parachinensis after cadmium stress

To determine the TFs responsible for BrpHMA2 expression in B. parachinensis, a cis-element analysis (Plant-CARE) of 2000 bp of the BrpHMA2 promoter was performed. In the promoter region, three ABRE cis elements (PyACGTGG/TC) were identified, all of which contain the G-box family core sequence ACCT (Fig. 2a). The NAC recognition site (NACRES) CGTG is likewise present in these ABREs. In the promoter of BrpHMA2, two additional NAC recognition motifs, CDS5 (core DNA-binding sequence) and CACG, were found. Three ABREs (containing three NACRESs), four NACRESs, and four CDS cis elements were found in the promoter of BrpHMA2 (Fig. 2a). These findings suggest that certain transcription factors, such as NACs or AAREBs, may control BrpHMA2 in B. parachinensis via these cis elements. To confirm this deduction and identify the regulatory pathways involved in the response to Cd stress, the transcriptome of B. parachinensis as mentioned above was used to collect data for the NAC and AREB genes that showed differential expression following Cd stress [8]. Eighteen NAC genes and 11 AREB genes were selected to create a heat map, and three NAC TFs and three AREB TFs were identified as Cd-induced TFs (Figure S3). Their transcription levels were further analyzed by RT-qPCR. The results showed that the NAC TF genes BraA03000895, BraA010004584, and BraA10002796 were upregulated in the roots of the plants exposed to Cd for 1 day (Figure S4a–c). After 4 days of Cd exposure, the AREB TF gene BraA01000449 was induced in roots, and BraA05001227, BraA01000449, and BraA01003678 were induced in leaves (Figure S4d–f). Similar to the findings for BrpHMA2, our results suggest that these TF genes may respond to Cd.

The coding sequences (CDSs) of the three NAC TFs and three AREB TFs listed above were cloned and submitted to the NCBI database. The last three or four numbers of each gene’s full name was used as the gene name. MEGA5 was used to create a phylogenetic tree of these NAC TF or AREB TF genes and Arabidopsis NAC or AREB genes using the neighbor-joining method. The results revealed that the BrpNAC4584 and BrpNAC895 sequences were closer to those of Arabidopsis ANAC046 and ANAC087, respectively (Figure S5); in addition, the BrpABI227 and BrpABI449 sequences were closer to that of AtABF4, and the BrpABI449 sequence was more comparable to that of AtABF3 (Figure S6).

BrpNAC895 binds to NAC-responsive element motifs in the promoter of BrpHMA2 and upregulates BrpHMA2 transcription

To determine whether the NAC TFs can regulate BrpHMA2 expression, each BrpNAC driven by 3SS (p3SS::BrpNACs) and the promoter of BrpHMA2 fused to the firefly LUC (luciferase) gene (pBrpHMA2::LUC) were employed as effector and reporter, respectively, and cotransfected
Figure 1. Expression pattern of BrpHMA2 in B. parachinensis and the impact of BrpHMA2 on Cd tolerance and accumulation in transgenic Arabidopsis and yeast cells. 

a) Relative expression of BrpHMA2 in B. parachinensis. Fourteen-day-old hydroponic seedlings of B. parachinensis were treated with 50 μM Cd(NO₃)₂ for 1, 2 and 4 days; expression of BrpHMA2 was then analyzed by RT–qPCR. DAC, days after Cd stress.

b) Subcellular localization of BrpHMA2. Protoplasts of B. parachinensis leaves with transient expression of BrpHMA2-GFP or GFP were observed under a confocal microscope. GFP fluorescence (left), bright-field (middle), and merged images (right) are shown. Scale bars: 20 μm.

c) Histochemical assays (left) and RT–qPCR analysis (right) of pBrpHMA2::GUS transgenic Arabidopsis. Analysis was performed on 9-day-old transgenic Arabidopsis seedlings treated for 2 days with or without Cd. Scale bar = 1 mm.

d) Cd localization in leaves of Col and p35S::BrpHMA2 transgenic Arabidopsis (OE-BrpHMA2) seedlings. Arrows point to precipitates of Cd-dithizone.

e) Phenotype of transgenic plants and f) Cd contents in Arabidopsis seedlings. Three-week-old hydroponic seedlings of Arabidopsis were exposed to 0 or 20 μM Cd(NO₃)₂ for 4 days for Cd precipitate observation and for 6 days for the phenotype observation and the Cd content analysis.

g) Growth of yeast cells transformed with empty vector or BrpHMA2. Cells were inoculated on SD plates containing different concentrations of Cd(NO₃)₂ in the presence of 2% glucose or 2% galactose and cultured for 3 days.

h) Cd content in yeast cells grown in liquid medium containing 2% galactose and 60 μM Cd(NO₃)₂ for 24 hours. EV, empty vector. Each experiment (biological repeat) used pooled RNA extracted from tissues collected from three independent plants in (a) and 25 seedlings in (c). Error bars represent the SD of three biological replicates. Asterisks indicate significant differences with respect to means of the control plants (Student’s t-test): *P < .05; **P < .01.
Figure 2. BrpNCA895 directly binds to the promoter of BrpHMA2 and promotes BrpHMA2 transcription. a Schematic diagram of the cis elements (ABRE, NACRES, CDBS) and the fragments used for ChIP-qPCR in the promoter of BrpHMA2 (pF1–pF3). b, c Regulation of the expression of pBrpHMA2::LUC by BrpNAC895, BrpNAC4584 and BrpNAC796 analyzed in the transient expression system of (b) tobacco leaves and (c) B. parachinensis protoplasts. Different letters next to each bar indicate statistically significant differences as determined by one-way ANOVA followed by Tukey’s multiple comparison test ($p < .05$). d Upregulated transcription of BrpHMA2 detected in B. parachinensis protoplasts with transient expression of BrpNAC895. e Schematic diagram of probe positions in the BrpHMA2 promoter and the probe sequences. f Binding of BrpNAC895-MBP to the promoter regions of BrpHMA2 detected by EMSA. g Binding of BrpNAC895-GFP to BrpHMA2 promoter regions detected by ChIP assay with an anti-GFP trap. Actin was used as a negative control. Asterisks indicate significant differences with respect to means of the control (Student’s t-test): **$p < .01$. 

Figure 2. BrpNCA895 directly binds to the promoter of BrpHMA2 and promotes BrpHMA2 transcription. The empty vector (pGreenII 0062SK) was used as a negative control of the effector. The cotransformation of p35S:BrpNAC895 with pBrpHMA2::LUC resulted in stronger LUC fluorescence (Fig. 2b). The cotransformation of p35S::BrpNAC895 and pBrpHMA2::LUC into protoplasts of B. parachinensis leaves could also result in a significant increase in the activity of the LUC reporter (Fig. 2c). Similarly, the transformation of BrpNAC895 in B. parachinensis leaf protoplasts yielded a significant increase in the BrpHMA2 transcription level (Fig. 2d). However, no positive regulation effects of the other two NAC TFs (BrpNAC796, BrpNAC4584) on the expression of LUC were detected (Fig. 2b and c). The results show that BrpNAC895 could promote the transcriptional activity of the BrpHMA2 promoter.
Several AREB genes could reduce the BrpNAC895-activated transcription of BrpHMA2. Effect of BrpABI449, BrpABI227, and BrpABI678 on the expression of pBrpHMA2::LUC when transiently expressed in (a) tobacco leaves and (b) protoplasts of B. parachinensis. c, d Coregulation of expression of pBrpHMA2::LUC by BrpNAC895 and several AREBs when transiently expressed in (c) tobacco leaves and (d) protoplasts of B. parachinensis. Different letters next to each bar in (b) and (d) indicate statistically significant differences as determined by one-way ANOVA followed by Tukey’s multiple comparison test (P < .05).

Electrophoretic mobility shift assays (EMSAs) were conducted to investigate whether the BrpNAC895 protein directly binds to the promoter of BrpHMA2. Three probes containing NACRES and CBDS motifs on the BrpHMA2 promoter were designed and used for the EMSA. The results revealed that the BrpNAC895-MBP (MBP, maltose binding protein) fusion protein could bind to the three probes in vitro (Fig. 2e and f). A chromatin immunoprecipitation (ChIP) assay was performed using an anti-GFP antibody to precipitate BrpNAC895-GFP fusion proteins expressed in B. parachinensis protoplasts, and three fragments (pF1, pF2, and pF3) covering the NACRES and CBDS motifs on the BrpHMA2 promoter were designed and used for PCR. Moreover, there is only one base interval between the last two NACRES cis elements, so they were considered as one fragment (pF3) (Fig. 2a). Approximately 1.5- to 2-fold enrichment of fragments pF1, pF2, and pF3 was detected compared with those found in the control (Fig. 2e and g). The results demonstrate that BrpNAC895 can promote the expression of BrpHMA2 by binding directly to the NACRES and CBDS motifs of its promoter.

**BrpNAC895 and BrpABI449 coregulate the expression of BrpHMA2**

Transient expression assays were performed to determine the function of the selected AREB genes in regulating BrpHMA2 transcription. The visualization...
of LUC activity revealed that these AREB genes could slightly downregulate the LUC activity of the reporter pBrpHMA2::LUC, even though the quantitative analysis indicated no significant difference between the transformations with each AREB gene or the empty vector (Fig. 3a and b). The complex relationship between NAC and AREB TFs has been confirmed mostly in Arabidopsis [25]. Three AREB TFs were cotransformed with p35S::BrpNAC895 as the effector and pBrpHMA2::LUC as the reporter to assess the influence of AREB TFs on BrpNAC895 function. The results showed that all three AREB TFs could reduce the BrpNAC895-activated transcription of BrpHMA2, and BrpABI449 exerted a stronger influence than the other two AREBs (Fig. 3c and d).

**BrpABI449 binds to the promoter of BrpHMA2 directly**

To investigate the mechanism of BrpHMA2 coregulation by BrpNAC895 and BrpABI449, a ChIP assay was performed by expressing BrpABI449-GFP in B. parachinensis protoplasts to analyze the binding affinity of BrpABI449 with the promoter of BrpHMA2. A qPCR analysis revealed that the BrpABI449 protein was enriched with fragments containing pF2 and pF3 of the BrpHMA2 promoter (Fig. 4a). We further performed an EMSA to confirm the binding of BrpABI449 to ABRE motifs in the promoter of BrpHMA2. The results proved that BrpABI449 could bind directly to the probes containing ABRE cis elements in the pF2 and pF3 regions of the BrpHMA2 promoter (Fig. 4b).

Two NAC-responsive element motifs in the promoter of BrpHMA2 are required for BrpNAC895 transcriptional regulation

The roles of BrpNAC895-binding loci in BrpHAM2 transcriptional regulation were investigated by constructing a series of BrpHMA2 promoter mutants (MUT1–MUT7) by changing CACG/CGTG in the NACRES or CBDS to AAAA (Fig. 5a). A dual LUC assay was performed using the effector p35S::BrpNAC895 vector and the reporter vector was cotransformed into B. parachinensis protoplasts. Compared with pBrpHMA2::LUC, the cotransformation of p35S::BrpNAC895 with pMUT1::LUC or pMUT3::LUC resulted in much reduced LUC activity, but the cotransformation of the p35S::BrpNAC895 effector with pMUT2::LUC resulted in considerably higher LUC activity (Fig. 5b). Among the cotransformations of the promoter of BrpHMA2 with two or more mutations, substantially weaker LUC activity could only be seen in the transformations with promoters mutated at both locus 1 and locus 3 (Fig. 5c). These findings indicate that the mutation in the first and third NACRES motifs reduced the BrpNAC895-activated transcription of BrpHMA2, and these two binding loci may play central roles in the BrpNAC895-activated transcription of BrpHMA2.

**BrpABI449 interacts with BrpNAC895 and decreases BrpNAC895 DNA-binding activity**

To elucidate the relationship between NAC and AREB TFs, a bimolecular fluorescence complementation (BiFC) approach was used. Full-length BrpNAC895 and BrpABI449 were fused to the C-terminal and N-terminal halves of enhanced yellow fluorescent protein (eYFP), respectively. The different fusion combinations were as follows: (i) eYFPN and eYPFC, (ii) eYFPN and eYPFC-BrpNAC895, (iii) eYFPN-BrpNAC895 and eYPFC, (iv) eYPFN and eYPFC-BrpABI449, (v) eYPFN-ABI449 and eYPFC, (vi) eYFPN-BrpNAC895 and eYPFC-BrpABI449, and (vii) eYFPN-BrpABI449 and eYPFC-BrpNAC895. No signal was detected in the transformations of combinations (i)–(v). When BrpNAC895 and BrpABI449 fused respectively with the C-terminal or N-terminal halves of eYFP were coexpressed in tobacco leaves, the BiFC signal could be detected in the nucleus. This indicates that BrpNAC895 could interact with BrpABI449 in vivo (Fig. 6a). To confirm this interaction in vitro, MBP-BrpNAC895 and MBP-BrpABI449-GST (GST, glutathione-S-transferase) fusion proteins were expressed in Escherichia coli, and protein pull-down was then performed by incubating dextrin beads with the incubated mixtures of MBP-BrpNAC895 or MBP (negative control) and BrpABI449-GST. The results demonstrated that BrpABI449 could be detected in the precipitates of MBP-BrpNAC895 (Fig. 6b) and indicate that BrpNAC895 can interact with BrpABI449.

The cotransfection of BrpABI449 with BrpNAC895 reduced the BrpNAC895-activated transcription of BrpHMA2 (Fig. 3c and d); moreover, BrpABI449 could interact with BrpNAC895, which suggests that the interaction of BrpABI449 and BrpNAC895 inhibits the ability of BrpNAC895 to bind with the promoter of BrpHMA2. To confirm this speculation, we used an EMSA to analyze the binding ability of BrpNAC895 with the pF1 locus of the BrpHMA2 promoter in the presence of the BrpABI449 protein. The results demonstrated that the binding of BrpNAC895 on the promoter fragment pF1 of BrpHMA2 was reduced by the BrpABI449 protein (Fig. 6c). However, BrpABI449 could not bind to pF1 directly, as shown in Fig. 4c. These results demonstrate that the interaction between BrpABI449 and BrpNAC895 interferes with the binding of BrpNAC895 to the BrpHMA2 promoter.

**Discussion**

B. parachinensis is a popular leafy crop; however, it may collect significant levels of heavy metals, particularly Cd, when grown on Cd-polluted substrate soil [2, 8, 27]. Transcriptomes of B. parachinensis under Cd stress were previously generated to elucidate the mechanisms underlying Cd accumulation [8]. We reveal that BrpHMA2, which is differently expressed in plants, is involved in Cd uptake and accumulation (Fig. 1). Furthermore, BrpHMA2 expression is controlled by BrpNAC895 and BrpABI449, which operate as activators and inhibitors, respectively (Fig. 7).
BrpHMA2 functions in cadmium transport and is responsible for cadmium accumulation in plants

Our results reveal that BrpHMA2 could be activated by Cd$^{2+}$ (Fig. 1a), which is similar to the results found for HMA2 in Arabidopsis [14]. Results suggest that BrpHMA2 is involved in the Cd response of plants. BrpHMA2 was also found to be expressed explicitly in the vascular tissues of roots, stems, leaves, flowers, siliques, and carpopodia, and its protein was localized in the plasma membrane (Fig. 1; Figure S2). These results are consistent with previous findings for HMA2 in Arabidopsis [15], OsHMA2 in rice, and TaHMA2 in wheat [16,18]. The protein plasma membrane localization and the vascular-specific expression pattern of the genes (Fig. 1; Figure S2) revealed that HMA2 might function as a membrane transporter in long-distance transport in plants.

In recent years, some studies have investigated the function of HMA2. Most of these studies demonstrated that HMA2 is involved in Zn$^{2+}$ and Cd$^{2+}$ transmembrane transport and influences root-to-shoot Zn/Cd translocation. For example, HMA2 in Arabidopsis is thought to be involved in the outward transport of Zn$^{2+}$ and Cd$^{2+}$ from the cell cytoplasm, and HMA2 mutants are more sensitive to Cd stress and exhibit higher Zn or Cd accumulation than wild-type plants in the presence of high levels of Zn$^{2+}$ or Cd$^{2+}$ [14,15]. The overexpression of OsHMA2 in wheat, rice, and Arabidopsis improves root-to-shoot Zn/Cd translocation [16,17]. In addition, the transformation of TaHMA2 in yeast enhances the resistance of cells to Zn/Cd [16]. In rice, the suppression of OsHMA2 decreases the Zn and Cd concentrations in leaves, increases the retention of Zn in roots and reduces the translocation of Cd and Zn from roots to shoots compared with the results obtained with wild-type plants [28]. According to the literature, HMA2 is responsible for Zn$^{2+}$/Cd$^{2+}$ efflux from cells, plays roles in Zn and Cd loading to the xylem, and participates in the root-to-shoot translocation of Zn/Cd.

However, Yamaji et al. [18] found that OsHMA2 is localized at the pericycle of the roots and in the phloem of enlarged and diffuse vascular bundles in the nodes. Their insertion lines of rice showed decreased concentrations of Zn and Cd in the upper nodes and reproductive organs. The study revealed that the heterologous expression of OsHMA2 in yeast is associated with the influx transport of Zn and Cd. These researchers suggested that OsHMA2

Figure 4. BrpABI449 directly binds to the BrpHMA2 promoter. a Binding of BrpABI449-GFP to BrpHMA2 promoter regions detected by ChIP assay with an anti-GFP trap. Actin was used as an unenriched control. b Binding of BrpABI449-MBP to the promoter regions of BrpHMA2 detected by EMSA. Probes 1–3 and pF1–3 are the same as those shown in Fig. 2. Asterisks indicate significant differences with respect to means of the control (Student’s t-test): **P < .01.
in the nodes plays an important role in the preferential distribution of Zn and Cd through the phloem to the developing tissues [28]. Our results also revealed that, in the presence of Cd\(^{2+}\), transgenic Arabidopsis seedlings and yeast overexpressing BrpHMA2 showed higher concentrations of Cd and enhanced Cd\(^{2+}\) sensitivity compared with the controls (Fig. 1). Thus, we propose that BrpHMA2 functions in Cd\(^{2+}\) transport in the phloem tissue of vascular systems through influx into cells, and the efflux from phloem cells during long-distance transport may be performed by other transporters. The differential function of HMA2 from various plants might come from the tiny difference in amino acids in their function domains; this puzzle requires further investigation.

**Expression of BrpHMA2 can be coregulated by BrpNAC895 and BrpABI449**

In this study, we identified the NAC TF gene BrpNAC895, a homolog of Arabidopsis ANAC087 (Figure S5), which could be induced by Cd\(^{2+}\) stress (Figure S4). We confirmed that BrpNAC895 has a role in the response of B. parachinensis to Cd\(^{2+}\) stress by upregulating BrpHMA2 expression through direct binding to the BrpHMA2 promoter using EMSA, ChIP–qPCR, and the transient transformation method with B. parachinensis protoplasts (Fig. 2). Previous studies have demonstrated that Arabidopsis ANAC087 is associated with plant programmed cell death (PCD). It functions along with the TF ANAC046 to show partial redundancy in coregulating the expression of some PCD genes in the root columella, including ZEN1, BFN1, and RNS3 [29]. Whether ANAC087 could participate in regulating Cd transporters in plants has not been reported. Our findings on BrpNAC895 show that this NAC TF has a novel role in upregulating BrpHMA2 expression in response to Cd\(^{2+}\) stress.

We also identified the Cd-responsive AREB TF BrpABI449 (Figure S4), which is a homolog of Arabidopsis ABF3 (Figure S6) and can bind to the promoter of BrpHMA2 (Fig. 4). ABF3 modulates the response to drought, salt, and other osmotic stresses as a master component in ABA signaling [30, 31]. This TF can also regulate the expression of multiple genes, such as the AGAMOUS-like MADS-box TF family gene SOC1, which is a floral...
integrator regulating flowering in response to drought [29], and the AREB TF ABI5, which is a core component in the ABA signaling pathway in the regulation of seed germination and early seedling growth during exposure to ABA and abiotic stresses [31, 32]. In general, ABF3 can form protein complexes with other TFs. For example, ABF3 forms homodimers or heterodimers with AREB1/AREB2 and acts cooperatively to regulate ABRE-dependent gene expression [30]. ABF3 forms a complex with NF-YC3 to promote the expression of the SOC1 gene and thus accelerate flowering and drought-escape responses [29]; ABF3 interacts with NAC072 to regulate RD29A and RD29B expression in response to ABA [33]. Thus, complex formation might be the important functional mechanism by which ABF3 regulates gene transcription.

Using EMSAs and ChIP–qPCR assays, we found that BrpABI449 could directly bind to regions of the BrpHMA2 promoter (Fig. 4). The interaction of BrpABI449 and BrpNAC895 was further confirmed by pull-down and BiFC assays (Fig. 6). The inhibition of BrpABI449 on the transcriptional regulatory role of BrpNAC895 was detected in the B. parachinensis protoplast transient system (Fig. 3). The inhibition by BrpABI449 of the transcriptional regulatory role of BrpNAC895 complex, likely interferes with BrpNAC895’s activity in the transcriptional activation of BrpHMA2 in response to Cd stress. It has also been reported that Cd stress can induce a stress response via ABA signaling [34]. Our results showing that BrpNAC895 and BrpABI449 are upregulated by Cd stress also support this point.

The uptake or homeostatic regulation of heavy metals needs proper modulation to ensure plant health. Previous studies have shown that Cd stress induces the MYB TF gene MYB49 in Arabidopsis. This TF may further positively regulate the downstream TF gene bHLH38 and bHLH101 by directly binding to their promoters, and activate iron-regulated transporter 1 (IRT1) to enhance Cd
Protoplasts have been widely used for subcellular protein localization and gene regulation analyses. In this study, the transient transformation of B. parachinensis protoplasts was demonstrated to be a powerful system for ChIP–qPCR analysis. Previous studies have applied a similar approach to Populus trichocarpa and Brassica napus [35–37]. Although the transient transformation system of B. parachinensis protoplasts was successfully used in this study of molecular mechanisms, the system cannot be easily used for phenotype and physiological analyses. The lack of BrpNAC895 and BrpABI449 transgenic B. parachinensis is a problem that severely limits research on this plant. New techniques, such as the transient reprogramming of plant traits via the transfection of RNA-based viral vectors using Agrobacterium and gene editing combined with fast-treated Agrobacterium coculture, may be useful approaches for comprehending gene function concerning physiology and for the further application of modifications of gene function to effectively control the accumulation of Cd in B. parachinensis [38, 39].

Materials and methods

Plant material and growth conditions

A. thaliana (wild-type Columbia-0), tobacco (Nicotiana benthamiana), and Chinese flowering cabbage (B. parachinensis L.H. Bailey, cultivar ‘YQ’) were used in this study. The B. parachinensis cultivar ‘YQ’ was obtained from the Vegetable Research Institute of Guangdong Academy of Agricultural Sciences. Seeds were surface-sterilized, grown on 1/2 Murashige and Skoog (MS) medium for 7 days and then transferred to a simple hydroponic culture device with 1/2 Hoagland nutrient solution (pH 5.8–6.0) [8]. For Cd treatment, a final concentration of 50 μM Cd(NO₃)₂ was applied to the nutrient solution 7 days after transplantation. Arabidopsis seedlings were cultured in 1/2 Hoagland nutrient solution (pH 5.8–6.0) as described by Conn et al. [40]. The Cd treatments were conducted with 21-day-old Arabidopsis seedlings by adding Cd(NO₃)₂ to the nutrient solution at a final Cd²⁺ concentration of 20 μM. Tobacco plants were grown in a mix of soil, perlite, and vermiculite at a ratio of 1:1:1. All seedlings were grown at 22°C and 60% relative humidity with a 16-hour photoperiod (~100 mmol m⁻² s⁻¹).

Analysis of the cadmium tolerance of yeast

The Saccharomyces cerevisiae Δycf mutant strain was used to investigate the function of BrpHMA2 [16]. BrpHMA2 was included in the pAG413 vector, and the resulting vector was transformed into Δycf mediated by polyethylene glycol as previously described by Ito et al. [41]. For the evaluation of Cd tolerance, cultures of the transformants were sequentially 10x diluted and 4 μl of each diluted culture was spotted on solidified SD medium containing 0, 15, 30 and 45 μM Cd(NO₃)₂ in the presence of 2% glucose, an expression suppressor, or 2% galactose, an expression inducer. The plates were incubated at 30°C for 3 days. For measurement of the Cd contents, yeast cells uptake [34]. In contrast, Cd stress upregulates the expression of ABI5. ABI5 interacts with MYB49, prevents its binding to the promoters of downstream genes, and functions as a negative regulator to control Cd uptake and accumulation [34]. Our present results also demonstrate a mechanism for controlling the expression of the heavy metal transporter gene BrpHMA2 under Cd stress. We propose that Cd²⁺ induces the expression of BrpNAC895 and BrpABI449, which might be mediated by ABA signaling. BrpNAC895 then promotes the transcription of BrpHMA2 by binding directly to its promoter (Fig. 7). The activation of BrpHMA2 enhances Cd²⁺ uptake and may induce cell damage. Negative regulation of BrpHMA2 is then achieved by the upregulation of another AREB TF, BrpABI449, which interacts with BrpNAC895 and forms BrpNAC895-BrpABI449 protein complexes to inhibit the BrpHMA2 transcription activated by BrpNAC895 (Fig. 7). BrpABI449 could also bind to the promoter of BrpHMA2 directly to compete with BrpNAC895 in binding to the BrpHMA2 promoter. This negative regulation may play a supplementary role in the uptake and transport of Cd.

Application of B. parachinensis protoplasts

Many plant species of Brassicaceae, including Arabidopsis, turnip, and oilseed rape, can be genetically modified, but the creation of transgenic B. parachinensis remains difficult. Therefore, we overexpressed BrpHMA2 in Arabidopsis to investigate the function of BrpHMA2 and established a transient transformation system in B. parachinensis protoplasts to perform gene regulatory network analysis.

Figure 7. A proposed model for the transcriptional regulation of BrpHMA2 by BrpNAC895 and BrpABI449 in B. parachinensis. Cd induces the expression of BrpNAC895 and BrpABI449. BrpNAC895 functions as an activator to promote the transcription of BrpHMA2 by binding to the loci pF1 and pF3 in its promoter. BrpABI449 could also bind to the promoter of BrpHMA2 in the loci pF2 and pF3 or form a complex with BrpNAC895, and interfere with the binding action of BrpNAC895. BrpABI449 serves as a transcriptional repressor and coregulates the transcription of BrpHMA2 with BrpNAC895.

A. thaliana (wild-type Columbia-0), tobacco (Nicotiana benthamiana), and Chinese flowering cabbage (B. parachinensis L.H. Bailey, cultivar ‘YQ’) were used in this study. The B. parachinensis cultivar ‘YQ’ was obtained from the Vegetable Research Institute of Guangdong Academy of Agricultural Sciences. Seeds were surface-sterilized, grown on 1/2 Murashige and Skoog (MS) medium for 7 days and then transferred to a simple hydroponic culture device with 1/2 Hoagland nutrient solution (pH 5.8–6.0) [8]. For Cd treatment, a final concentration of 50 μM Cd(NO₃)₂ was applied to the nutrient solution 7 days after transplantation. Arabidopsis seedlings were cultured in 1/2 Hoagland nutrient solution (pH 5.8–6.0) as described by Conn et al. [40]. The Cd treatments were conducted with 21-day-old Arabidopsis seedlings by adding Cd(NO₃)₂ to the nutrient solution at a final Cd²⁺ concentration of 20 μM. Tobacco plants were grown in a mix of soil, perlite, and vermiculite at a ratio of 1:1:1. All seedlings were grown at 22°C and 60% relative humidity with a 16-hour photoperiod (~100 mmol m⁻² s⁻¹).

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were grown in liquid medium containing 2% galactose with shaking at 200 rpm to induce heterologous gene expression. After 24 hours of culture, 60 μM Cd(NO₃)₂ was added to the medium, and the cells were incubated for an additional 24 hours and harvested by centrifugation.

**Analyses of metal element contents and cadmium localization**

*Arabidopsis* seedlings treated with Cd for 6 days and yeast cells treated with Cd for 24 hours were harvested. The samples were washed thoroughly with deionized water, 10 mM EDTA, 10 mM CaCl₂, and deionized water successively. The samples were then dried and digested with 65% HNO₃ using a microwave digestion system (Ethos ONE, Milestone, Italy). The Cd contents were analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 7000DV, Perkin Elmer, USA). Three biological replicates were examined.

To analyze the distribution of Cd at the tissue level, intact fresh leaves of *Arabidopsis* were rinsed in deionized H₂O and then infiltrated in a staining solution (15 mg of diphenylthiocarbazole in 30 ml of acetone, 10 ml of H₂O₂ and 50 ml of glacial acetic acid) for 1 hour. After a brief rinse in deionized H₂O, well-stained leaf samples were photographed under a light microscope (Eclipse E200, Nikon) to show the red-black Cd-dithizone precipitates [42].

**Gene cloning**

The CDSs of *BrpHMA2*, *BrpNAC895*, *BrpNAC796*, *BrpNA C4578*, *BrpABI449*, *BrpABI227*, and *BrpABI678* were amplified from the cDNA of ‘YQ’ by PCR using PrimerStar (Takara). The PCR amplicons were cloned into the empty pEASY®-Blunt Zero Cloning vector (Transgen).

**β-Glucuronidase staining assay**

The 2.0-kb promoter of *BrpHMA2* was amplified by PCR from the genomic DNA of *YQ* and cloned into the pLp100 vector carrying the GUS reporter gene to generate the *BrpHMA2::GUS* structure. GUS staining of the transgenic *BrpHMA2::GUS* precipitates was performed as described by Ivanov et al. [43].

**Reverse transcription–quantitative PCR**

Total RNA was extracted from the samples using the TRIzol reagent according to the manufacturer’s protocol (Takara). RT–qPCR was performed using TransStart® Tip Green qPCR SuperMix (Transgen) with a Roche LightCycler 480 real-time PCR instrument. Ubiquitin and Actin7 were used as the internal control genes for *Arabidopsis* and *B. parachinensis*, respectively.

**Recombinant protein purification, electrophoretic mobility shift assays, and pull-down assays**

The full-length coding sequence of *BrpNAC895* or *BrpABI449* was cloned into the pMAL c5x vector with an MBP tag. The constructs were transferred into E. coli BL21(DE3) for recombinant protein production, and the recombinant protein was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside at 16°C for 12 hours in a shaking incubator at 100 rpm and purified using the pMAL Protein Fusion and Purification System (NEB). EMSA was performed with the EMSA/Gel-Shift kit (Beyotime, China) according to the users’ guide. For pull-down assays, *BrpABI449* was fused to the tag of GST. The protein interactions were detected by western blot analysis using anti-MBP and anti-GST antibodies (Transgen).

**Gene expression analysis, dual-luciferase reporter assay and chromatin immunoprecipitation–quantitative PCR of *B. parachinensis* protoplasts**

The CDS of NACs and bZIPs without a stop codon was cloned into the pGreenII 0062SK vector [44]. The promoter of *BrpHMA2* was fused upstream of the firefly luciferase gene (LUC) in the pGreenII0800-LUC vector, which contains 35S::RLuc (Renilla luciferase, REN) [44]. Protoplasts of *B. parachinensis* were prepared as described previously [36].

RNA extraction and gene expression analysis by RT–qPCR was performed as described above.

Dual LUC assays were performed using a Dual-Luciferase Reporter Assay Kit (Promega) as previously described [44]. The ability of TFs to bind to the promoter regions of *BrpHMA2* was indicated by the ratio of LUC to REN activity.

The CDS of *BrpNAC895* or *BrpABI449* without a stop codon was cloned into the pGreenII background for expression of the fusion protein with a GFP tag. As previously described, ChIP assays of *B. parachinensis* protoplasts were performed with slight modifications [42]. Briefly, ~50 μg of plasmid DNA of pGreenII-*BrpNAC895* or pGreenII-*BrpABI449* and 1 × 10⁷ protoplasts were used for each transfection. Chromatin was fragmented by sonication with a Covaris S220 (Thermo) to obtain fragments of ~500 bp. Actin7 was used as the internal control. Transfection with the pGreenII 62-SK vector for the overexpression of GFP was used as a negative control. The primers used for ChIP–qPCR can be found in Supplemental Table S1. Three biological replicates of each transfection and three technical repeats of each biological replicate were included in the experiment.

**Transient expression assays in tobacco leaves**

*BrpHMA2* fused to the firefly LUC gene (*BrpHMA2::LUC*) was constructed in pGreenII 0800-LUC as a reporter, and *BrpNACs* or other AREBs were constructed in the pGreenII 62-SK vector as effectors. pGreenII 62-SK and pGreenII 0800-LUC vectors were used for transient expression assays in tobacco leaves. The reporter and effector were transiently expressed in tobacco leaves mediated by *Agrobacterium GV3101* (pSoup). *Agrobacterium*-infected plants were cultivated under low irradiance for 24 hours and then transferred to light for an additional 2 days [45].
The LUC signal in the transfected leaves was detected with a CCD camera (Vilber Newton 7.0).

**Subcellular localization and bimolecular fluorescence complementation assays**

BrpHMA2 was inserted into the 0062SK vector for the localization analysis of BrpHMA2-GFP. BrpNAC895 and BrpABI449 were fused to the N-terminus or C-terminus of YFP in the pGreen background, respectively. The constructs were then transformed or cotransformed into tobacco leaves by Agrobacterium strain GV3101 through infection-mediated transfection. Three days later, the fluorescence of GFP or YFP was imaged using a confocal laser-scanning microscope (Zeiss).

**Statistical analysis**

All data comprised at least three biological replicates. Statistical analysis between the control and test groups was performed using Tukey’s test program. Significant differences were evaluated using one-way ANOVA. All analyses were performed using SPSS for Windows.

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**Author contributions**

X.C. and Y.T. designed the research; S.L. performed the research; L.L. performed the LUC analyses; Y.D. conducted gene expression analyses; Y.B. contributed to the EMSA assays; C.S. contributed to the pull-down assays; S.H. and J.Z. contributed to protoplast isolation, L.S. and X.Y. performed the yeast cell analysis, and L.L. provided technical support. All authors read and approved the final manuscript.

**Data availability**

All data supporting the study findings are available from the corresponding author upon reasonable request.

**Conflict of interest**

The authors declare no conflicts of interest.

**Supplementary data**

Supplementary data is available at Horticulture Research online.

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