Overexpression of *ThVHAc1* and its potential upstream regulator, *ThWRKY7*, improved plant tolerance of Cadmium stress

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As one of the most toxic heavy metals in the environment, cadmium (Cd) poses a severe threat to plant growth. We previously reported that overexpression of the *Tamarix hispida* V-ATPase c subunit (*ThVHAc1*) improved the Cd tolerance of *Saccharomyces cerevisiae*. In the current study, we further explored the Cd tolerance conferred by *ThVHAc1* in *Arabidopsis* and *T. hispida*. *ThVHAc1* transgenic *Arabidopsis* had higher seed germination, biomass, and chlorophyll content under CdCl₂ treatment. In Cd-stressed plants, overexpression of *ThVHAc1* significantly improved V-ATPase activity and affected the expression of other V-ATPase subunit-encoding genes. Intriguingly, the lower level of ROS accumulation in *ThVHAc1*-overexpressing lines under CdCl₂ treatment demonstrated that *ThVHAc1* may modulate Cd stress tolerance by regulating ROS homeostasis. Transient expression of *ThVHAc1* in *T. hispida* further confirmed these findings. Furthermore, promoter analysis and yeast one-hybrid assay revealed that the transcription factor *ThWRKY7* can specifically bind to the WRKY cis-element in the *ThVHAc1* promoter. *ThWRKY7* exhibited similar expression patterns as *ThVHAc1* under CdCl₂ treatment and improved Cd tolerance, suggesting that *ThWRKY7* may be an upstream regulatory gene of *ThVHAc1*. Therefore, our results show that the combination of *ThVHAc1* and its upstream regulator could be used to improve Cd stress tolerance in woody plants.

Unlike other heavy metals that function as micronutrients (such as Fe, Mn and Mo) or trace elements (such as Cu, Zn, Ni and W), Cd, Pb, Hg, Ag and U have no known nutritional function and are toxic to plants¹. These toxicities can cause many detrimental effects, including modification of protein profiles, reduced plant growth, decreased seed germination rates, the induction of reactive oxygen species (ROS) production, cell death, chlorosis/necrosis, and turgor loss¹⁻³. Furthermore, the high solubility of heavy metals in water results in uptake by plants, which can cause serious harm to the food chain and human health.

Cd is regarded as one of the most phytotoxic heavy metals. In a majority of soil samples, Ni, Cu, Co, Zn, Se, Pb, and Cr have been found to be moderately enriched, whereas Sb and Cd were extremely highly enriched⁴. Because soil Cd contamination affects the ceramic industry⁵, agricultural fields⁶, and the growth of sea rocket⁷, among others, understanding the molecular mechanisms of plants’ responses to Cd stress is crucial for improving plants’ adjustment and/or adaptation to Cd stress⁸⁻¹⁰. Functional genomics technologies and protein analysis methods have been used to broaden our knowledge of the pathways that respond to Cd stress in plants⁸. For example, overexpression of *BjCdR15/TGA3*, a bZIP transcription factor, effectively improved Cd uptake by roots and enhanced Cd tolerance in *Arabidopsis* and tobacco⁹. Moreover, *AtMYB4* in *Arabidopsis* and *WRKY53* in *Thlaspi caerulescens* have also been reported to play a potential role in Cd stress response¹¹,¹². In plants, the regulation of Cd tolerance involves several enzymes, including ATPase, HMA2, and HMA4, which are essential for Cd transport¹³. Cd is known to affect the activity of plasma membrane ATPase¹⁴. The V-ATPase motive force-dependent cation antiporter can significantly contribute to Cd detoxification by vacuolar compartmentalization in barley roots¹⁵. V-ATPase is a multi-subunit complex comprising domains *V₁* (600–650 kDa

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membrane-peripheral domain) and V₆ (260 kDa membrane-integral domain). The V₁ domain contains eight different subunits (A–H) and is responsible for ATP hydrolysis, while the V₆ domain includes six different subunits (a, d, c, c', c'' and e) and is responsible for proton translocation. There are few reports on the mechanism of V-ATPase regulation in response to various adverse conditions. However, some results have indicated that the expression levels of V-ATPase subunits are involved in various abiotic stresses. For example, overexpression of the wheat (RH8706-49) V-ATPase B subunit (TaVVB) in Arabidopsis conferred higher V-ATPase activity and overall salt tolerance than were observed in the wild type (WT)⁴⁶. Therefore, the cloning and characterization of the V-ATPase subunits may be an effective way to understand the regulation of this molecule and its response mechanism during abiotic stress.

The V-ATPase c subunit (VHAc) participates in the formation of a proton channel that is responsible for proton transport and is an essential factor in the assembly of V₁-V₆. Mutated yeast lacking VHAc fail to assemble V₁ into the membrane⁷. Some studies have demonstrated the salt regulation capacity of the VHAc gene. For example, a transcript analysis of Pennisetum glaucum PgVHA-c1 showed that the expression of PgVHA-c1 was increased in response to salinity stress⁸. Under salt stress conditions, overexpression of LbVHA-c1 (Limonium bicolor) in tobacco led to higher activity of superoxide dismutase (SOD) and peroxidase (POD) and lower levels of malondialdehyde (MDA) than in the WT⁹. Despite these findings, there are few reports on gene expression in response to heavy metals, especially related to the VHAc gene in a woody halophyte.

We found that V-ATPase activity and ThVHAc1 protein expression in T. hispida under CdCl₂ treatment were much higher than those under NaCl, PEG, or CaSO₄ treatments (data not shown), indicating that ThVHAc1 may play a key role in Cd stress tolerance in T. hispida. Consistently, we confirmed that the expression of ThVHAc1 was induced by CdCl₂ treatment in T. hispida roots, stems, and leaves and that the overexpression of ThVHAc1 in yeast improved Cd tolerance²⁰. However, the role of ThVHAc1 in response to Cd stress and the mechanisms of ThVHAc1 regulation under Cd stress remain far from being fully elucidated. In this study, we identified a potential upstream regulator of ThVHAc1, ThWRKY7, which showed expression patterns similar to those of ThVHAc1 and which improved Cd stress tolerance. In addition, the regulation of plant Cd tolerance by the ThVHAc1 gene and the relationship between ThVHAc1 and V-ATPase activity under CdCl₂ treatment were further analyzed. Our results demonstrate that ThVHAc1 may participate in Cd tolerance through the reactive oxygen species (ROS) scavenging system to alleviate cell damage. The heterologous expression of ThVHAc1 effectively improved V-ATPase activity and affected the expression of other subunits and related genes. This study expands our knowledge of the response of T. hispida to Cd stress and the relationship of the c subunit with the entire V-ATPase enzyme. Further, our findings provide new insights into the role and regulatory mechanism of ThVHAc1 upon exposure to Cd stress, which will be beneficial for providing candidate genes to genetically improve tolerance of Cd stress in woody plants.

Materials and Methods
Plant materials and treatments. Two-month-old T. hispida seedlings were grown in a greenhouse on a 14 h light/10 h dark cycle, with 70–75% relative humidity and an average temperature of 24 °C. The seedlings were well watered at the roots with 150 μM CdCl₂ for 6, 12, 24, 48, and 72 h, as indicated. Well-watered seedlings were used as the control. The roots, stems and leaves of every treated seedling (sample size of 20 seedlings) were harvested for quantitative real-time PCR (qRT-PCR) analyses. All treatments were applied at least three times (as biological replicates).

Cloning and expression analysis of the ThVHAc1 promoter. The ThVHAc1 promoter was PCR-amplified from T. hispida genomic DNA using a genome walking kit (Takara, Japan). The cis-elements in the ThVHAc1 promoter were analyzed using the PLACE database (http://www.dna.affrc.go.jp/PLACE)²¹. The 35S promoter in pCAMBIA1301 was replaced with the ThVHAc1 promoter to drive the expression of β-glucuronidase (GUS) (Fig. S1a), and this construct (named pThVHAc::GUS) was transferred into Arabidopsis²². The T₃ generation seedlings were used to study the ThVHAc1 temporal and spatial expression patterns through GUS staining. The 30 d transgenic Arabidopsis and T. hispida transiently expressing pThVHAc::GUS were independently treated with 100 μM CdCl₂ or H₂O (as a control) for 0 h, 12 h, or 24 h. Samples were then collected and labeled, and GUS activities were used to analyze the expression of the ThVHAc1 promoter. The GUS activity was measured according to Hunter and Watson (2008)²³. When the protein concentrations were high, the samples were diluted with sterile water to maintain A₅₉₅ at less than 2.0. Thus, the determination of GUS activity is precise and avoids the errors generated by saturated staining.

RNA isolation and qRT-PCR. Total RNA was isolated from each sample using the CTAB method. qRT-PCR was carried out using an MJ OpticonTM machine (Bio-Rad, Hercules, CA, USA) with the reaction system and procedures from Gao et al. (2011)²⁰, and α-Tubulin (FJ618518), β-actin (FJ618517), and β-tubulin (FJ618519) were used as internal controls. The primer sequences are listed in Table S1. Relative expression levels were calculated using the ∆∆Ct method²⁴.

Identification of the upstream regulator of ThVHAc1. The WRKY motif (“TGACA”) was identified in the ThVHAc1 promoter (Fig. S1b, S2). A yeast one-hybrid assay was used to find the transcription factors that recognize the WRKY motif. Three tandem copies of the WRKY motif were cloned into a pHis2 vector (pHis2-WRKY) (Fig. S1c). WRKY TFs were identified from seven T. hispida libraries and cloned into pGADT7-Rec2 (Clontech, Palo Alto, CA, USA) to produce a cDNA library for use in the one-hybrid assay²⁵.

To confirm the interactions between the motif and positive clones, the WRKY core “TGAC” was mutated to “GTCAC” and cloned into pHis2 (pHis2-WRKY-M). Fragments of the ThVHAc1 promoter, including the WRKY motif (pHis2-WRKY-Seg), excluding the WRKY motif (pHis2-WRKY-Seg-M1), or including the mutated WRKY motif (pHis2-WRKY-Seg-M2) were separately cloned into pHis2 (Fig. S1c). The pHis2 plasmid containing three
The reaction buffer included 10 mM Mes (adjusted to pH 7.5 with Tris), 250 mM sorbitol, 3 mM MgSO4, 5 mM CdCl2 for another 12 d to compare the fresh weight and root length between lines. All experiments were performed three times.

ThVHAc1 transgenic Arabidopsis. The ORF of ThVHAc1 was amplified and cloned into a prokII vector (35S::ThVHAc1). The primers are shown in Table S1. 35S::ThVHAc1 was transferred into Arabidopsis using the Agrobacterium-mediated transformation method35. An empty prokII plasmid was also transferred into Arabidopsis and used as a control (ck). Kanamycin-resistant lines were detected by PCR using vector-specific primers. The expression level of ThVHAc1 was confirmed by qRT-PCR, and two transgenic lines with intermediate expression levels (c1#10 and c1#17) were selected for further analysis.

Stress tolerance analysis. Seeds from the control and T4 transgenic Arabidopsis were sown on 1/2 Murashige & Skoog (MS) agar medium with 100 μM CdCl2. The germination and fresh weight were recorded after 8 d. Six-day-old seedlings sown on 1/2 MS were transferred to 1/2 MS agar plates with an additional 100 μM CdCl2 for another 12 d to compare the fresh weight and root length between lines. All experiments were performed three times.

Five-week-old WT and transgenic plants were used to determine the stress tolerance of the transgenic lines. SOD activity, POD activity, glutathione transferase (GST) activity, total chlorophyll content (Tc), H2O2 content, proline content, MDA content and electrolyte leakage (EL) were measured after treatment with 100 μM CdCl2 for 6 d27,28. The fresh weights of the aerial parts of seedlings placed on clean filter paper were measured to compare their water loss. The Cd content was determined using atomic fluorescence spectrometry29. Seedlings watered with 1 μM CdCl2 for 6 d were used as the control. Leaves sampled from the above lines and treated with 100 μM CdCl2 for 0 (control), 1, and 2 h were stained with nitroblue tetrazolium (NBT), 3,3′-diaminobenzidine (DAB), and Evans blue to analyze the in vivo accumulation of O2−, H2O2, and cell death in leaves, respectively. ROS produced by intact guard cells and roots were stained with 3 μM 2,7-dichlorofluorescein diacetate (H2DCF-DA, Fluka)30, and dead cells in the main roots were stained by propidium iodide31. ROS and cell death were visualized using a confocal laser-scanning microscope (CLSM) featuring an LSM410 microscope (Zeiss, Jena, Germany) with excitation at 488 nm and emission at 525 nm. Images were acquired using the ZEN 2009 “lite” edition32.

Five-week-old transgenic and control Arabidopsis treated with 100 μM CdCl2 for 0, 3, 6, 9, 12, and 24 h were collected for isolation of total RNA. The expression of 28 V-ATPase subunits, other V-ATPase-related genes, and stress-related genes was examined by RT-PCR or qRT-PCR. The gene names and primer sequences are listed in Table S3. Stress tolerance analysis. Seeds from the control and T4 transgenic Arabidopsis were sown on 1/2 MS agar plates with an additional 100 μM CdCl2. The germination and fresh weight were recorded after 8 d. Six-day-old seedlings sown on 1/2 MS were transferred to 1/2 MS agar plates with an additional 100μM CdCl2 for another 12 d to compare the fresh weight and root length between lines. All experiments were performed three times.

Transient expression of ThVHAc1 in T. hispida. 35S::ThVHAc1, RNAi::ThVHAc1 and empty Agrobacterium (used as control, labeled as T-ck) were transiently transformed into the aerial parts of five-week-old T. hispida seedlings33. The transforming seedlings were stained with DAB and Evans blue to visualize the ROS and cell death after treatment with 100 μM CdCl2 for 0 (control), 1, or 2 h. ThVHAc1 expression was analyzed using qRT-PCR. Meanwhile, the SOD, POD, GST, and glutathione peroxidase (GPX) activities, as well as MDA and EL, were assayed. Furthermore, the expression levels of ThSOD, ThPOD, ThGSTZ1, and ThGPX, as well as 15 subunits of V-ATPase were examined using qRT-PCR. Two other V-ATPase-related genes and five stress response genes were also examined using RT-PCR. The primers are shown in Table S1. Meanwhile, 35S::ThWTKY7 was transiently transformed into T. hispida for analysis of Cd tolerance.

Tonoplast isolation, SDS-PAGE and western blotting. Using a modification of the method of Ma et al. (2002)34, tonoplasts were isolated from 200 g of aerial tissue from five-week-old transgenic and WT Arabidopsis either treated with 100 μM CdCl2 or well watered (as a control) for 6 d on a 0–25% sucrose solution plate. Similarly, tonoplasts from T. hispida seedlings with transient expression of 35S::ThVHAc1 were isolated using 15% polyacrylamide gels according to a previously published procedure34,35. The blots were performed based on the recommendations of Ma et al. (2002)34 and Burnette et al. (1981)36. Meanwhile, the relative activity levels of P-ATPase and F-ATPase from the 25%–50% sucrose solution plate were also tested34,35.

Proton pumping assays, ATPase hydrolysis activity, ATPase activity, and protein concentration. A total of 60 μg of the membrane protein from each sample was used to monitor proton pumping activity. The reaction buffer included 10 mM Mes (adjusted to pH 7.5 with Tris), 250 mM sorbitol, 3 mM MgSO4, 5 mM ascorbic acid, and 100 mM KCl, and the reaction was initiated using 2.5 mM MgSO4. The ATPase hydrolysis activity was measured by the P1 released from ATP34,37. The reaction buffer contained 40 μg protein, 25 mM Mes (adjusted to pH 7.5 with Tris), 50 mM KCl, 3 mM MgSO4, 3 mM ATP, inhibitor, and 0.0125 (V/V) Triton X-100. ATPase activity was measured, the reaction buffer included 40 μg protein, 30 mM Tris (adjusted to pH 7.8 with Mes), 50 mM KCl, 0.3 mM MgSO4, 0.3 mM PEPi-Tris (pH = 7.8), and inhibitor. Protein concentrations were estimated as described by Lowry et al. (1951)38. The inhibitors used for determining V-ATPase-, P-ATPase-, and F-ATPase-related activities were NaN3 (0.6) + Na3VO4 (0.6), NaNO3 (50) + Na3 (0.6), and NaNO3 (50) + Na2VO3 (0.6) [mM], respectively. All experiments were performed at least three times.
Results and Discussion

Cloning and analysis of ThVHAc1 promoter. A 1,164 bp promoter fragment (from −1 to −1164) was amplified by TAIL-PCR, and pThVHAc::GUS transgenic Arabidopsis was generated. GUS staining in Arabidopsis revealed GUS activity in mature seeds, cotyledons, leaves, stems, roots, petals, stamen, stigma, pistils, and anthers but not in fresh pods or immature seeds (Fig. 1a–q), indicating that ThVHAc1 expression is tissue-specific. Consistent with this finding, Padmanaban et al.39 (2004) detected strong GUS activity in the root cap in AtVHA-c3 promoter::GUS transgenic plants, and AtVHA-c3 dsRNA-mediated mutant lines exhibited decreased root length and diminished salt tolerance. These results indicated that the roots play a role in the regulation of abiotic stresses, especially Cd tolerance, by ThVHAc1. Furthermore, GUS activity was more obvious in roots and leaves than in stems (Fig. 1a–l). Upon exposure to CdCl2 for 12 h, the GUS activity increased 2.04-fold in aerial parts and 1.77-fold in roots compared to control conditions. When treated for 24 h, the increase was 1.87-fold in aerial parts and 1.22-fold in roots, indicating that the expression pattern in plants exposed to Cd stress. Furthermore, GUS staining also revealed the transient expression of pThVHAc::GUS transgenic leaves under the same treatments, indicating a correlation between the promoter activity and ThVHAc1 gene expression pattern in plants exposed to Cd stress.

A PLACE database (http://www.dna.affrc.go.jp/PLACE) comparison of the ThVHAc1 promoter revealed many abiotic stress-related cis-elements, such as ARR1AT, CAATBOX, DOFCOREZM, EBOXBNNAPA, GT1CONSSENSUS, MYCCONSSENSUSAT, NODCON1GM, WBOX, and WRKY710S (Fig. S2), indicating that ThVHAc1 may be regulated by different types of transcription factors (TFs). In particular, the promoter contains eighteen WBOX and WRKY motifs that are found in many promoters of stress tolerance genes, such as TaElf5440, Propep2 and Propep341, and GbDXS and GbgGpPs42. The promoter of pathogenesis-related protein (PR) in parsley also contains WRKY motifs to which PR proteins43 specifically bind, and these motifs were further used to identify PR proteins43. In bananas, ethylene-induced ripening induces the expression of both the PR gene and the V-ATPase c′ subunit in fruit tissue, suggesting a probable interaction between the PR and V-ATPase c′ genes44. Thus, WRKY genes may also regulate V-ATPase c′ gene expression. Moreover, the V-ATPase c′ subunit shares high homology with VHA-c, and their expression patterns under different stresses were highly similar in T. hispida roots, stems, and leaves (data not shown), suggesting a possible synergistic function in stress tolerance. These results led us to investigate whether WRKY motif-binding TFs bind specifically to the ThVHAc1 promoter and function as its upstream regulator to control ThVHAc1 expression during stress tolerance.

ThWRKY7 is an upstream regulator of ThVHAc1. A yeast one-hybrid assay was used to verify the interaction between TFs and the WRKY motif in the promoter. ThWRKY7 was found to bind to the WRKY motif, as shown by the interaction between pHis2-WRKY-Seq and ThWRKY7 and the absence of an interaction between pHis2-WRKY-M/M1/M2 and ThWRKY7 on the SD-/Trp-Leu-His/50 mM 3-AT (3-amino-1, 2, 4-triazole) solid medium (Fig. 3a). Furthermore, the effector construct prokII-ThWRKY7 was transferred into Arabidopsis, and T1 seedlings were used to detect transient expression of reporter plasmids. The reporter plasmids were constructed in pCAMBIA1301 harboring the intact or mutated WRKY motif, harboring the ThVHAc1 promoter fragment with its intact or mutated WRKY motif, or without the WRKY motif, followed by a 46bp minimal promoter. The leaves of prokII-ThWRKY7 transgenic T1 seedlings were then transiently transformed with one of the above-mentioned reporter plasmids. GUS activity was clearly detected in prokII-ThWRKY7 transgenic leaves with the reporter plasmid containing the ThVHAc1 promoter fragment with its intact WRKY motif, whereas
Figure 2. The GUS staining expression analysis in pThVHAc::GUS-transformed Arabidopsis after CdCl₂ treatment and transient expression in T. hispida. (a) 30-d-old transformed Arabidopsis under normal conditions and CdCl₂ treatment. (b) The GUS activity according to a. (c) GUS staining of T. hispida transiently transformed with pThVHAc::GUS. (d) The GUS activity according to (c) Data are shown as the mean ± SD. The asterisks in (b,d) indicate significant differences between treatment and control (P < 0.05).

Figure 3. Yeast one-hybrid analyses of the upstream regulators of ThVHAc1. (a) The clones on SD/-Leu-Trp were used as positive controls, further confirmed by spotting serial dilutions (1/1, 1/10, 1/100, 1/1000) onto SD/-His/-Leu/-Trp plates with 50 mM 3-AT, and the triangle indicates the dilutions from 1/10 to 1/1000. (b) The effect of the overexpression of ThWRKY7 in Arabidopsis on the transiently expressed reporter. M, mutated WRKY motif. M1, ThVHAc1 promoter fragment without the WRKY motif. M2, ThVHAc1 promoter fragment containing the mutated WRKY motif. (c) GUS activity according to (b).
The expression patterns of ThVHAc1 and ThWRKY7 under CdCl2 treatment. (a) Roots. (b) Stems. (c) Leaves. The relative expression levels were all log2 transformed. All experiments were repeated three times. The data are shown as the means ± SD of three independent experiments.

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Heterologous expression of ThVHAc1 improves Cd tolerance in Arabidopsis. To study the function of ThVHAc1 in plants, ThVHAc1 was overexpressed in Arabidopsis, and two transgenic lines (c1#10 and c1#17) were subjected to CdCl2 treatment. The results showed that the germination, fresh weight, and main root length did not differ among c1#10, c1#17, WT, and ck under normal conditions (Fig. 5). However, when exposed to the CdCl2 treatment c1#10 and c1#17 showed better biomass accumulation than WT and ck. The average germination length did not differ among c1#10, c1#17, WT, and ck under normal conditions (Fig. 5). However, when exposed to CdCl2 treatment, all lines displayed similar hydro- lytic activity, ATPase activity, and proton transport activity under normal conditions. After CdCl2 treatment, all lines, but the activities in the transgenic ThVHAc1 lines were increased more than those in the control WT and ck lines. The average V-ATPase activities, hydrolytic activities, V-ATPase-related activities were increased in all lines, but the activities in the transgenic ThVHAc1 lines were markedly overexpressed in T. hispida. The leaves transformed with 35S::ThWRKY7 showed lower levels of DAB and Evans blue staining as well as slower accumulation of MDA and EL than did leaves transformed with T-ck. Moreover, the SOD, POD, GST, and GPX activities of leaves transformed with 35S::ThWRKY7 were significantly higher than those of leaves transformed with T-ck (Fig. S3), suggesting that overexpression of ThWRKY7 also markedly improves Cd tolerance, further demonstrating that ThWRKY7 may fine-tune the ThVHAc1-mediated Cd stress response.

WRKY transcription factors are a complex family with previously reported relationships to the plant immune response, in which they function as either positive or negative regulators65,66. Various functions in the protection process are a basic feature of WRKY genes, and the redundant elements in the promoters of their target genes imply a regulatory capacity of WRKY47. WRKY TFs regulate plant tolerance to abiotic stress by binding to the WRKY cis-element present in many stress-related co-regulated gene promoters in Arabidopsis46. Regarding our results, the binding of ThWRKY7 to the WRKY element in the ThVHAc1 promoter, as well as the similar expression profiles of these elements over time when exposed to Cd stress, suggest that ThVHAc1 and ThWRKY7 may co-regulate Cd tolerance and that ThWRKY7 may control ThVHAc1 in the improvement of abiotic stress tolerance.

Figure 4. The expression patterns of ThVHAc1 and ThWRKY7 under CdCl2 treatment. (a) Roots. (b) Stems. (c) Leaves. The relative expression levels were all log2 transformed. All experiments were repeated three times. The data are shown as the means ± SD of three independent experiments.

other mutated reporters did not activate GUS expression (Fig. 3b,c). These results further demonstrate that ThWRKY7 may bind specifically to the WRKY motif in the ThVHAc1 promoter.

qRT-PCR analysis of ThVHAc1 and ThWRKY7 showed that the two genes displayed similar expression patterns under CdCl2 treatment. Specifically, the relative expression levels of both genes in roots increased before 12 h after CdCl2 treatment and then decreased, reaching their lowest levels at 24 or 48 h (Fig. 4a). In stems, the expression of ThWRKY7 was slightly higher than that of ThVHAc1 at every time point. However, the two genes exhibited the same expression pattern, with the expression levels being highest at 24 h and lowest at 72 h (Fig. 4b). In leaves, the two genes were downregulated at 6 h and exhibited peak expression at 48 h (Fig. 4c). This synchronized expression patterns indicate that ThWRKY7 may play a critical role in either regulating the ThVHAc1 expression or cooperating with ThVHAc1 to improve plant Cd stress tolerance. To further confirm this conclusion, ThWRKY7 was transiently overexpressed in T. hispida. The leaves transformed with 35S::ThWRKY7 showed lower levels of DAB and Evans blue staining as well as slower accumulation of MDA and EL than did leaves transformed with T-ck. Moreover, the SOD, POD, GST, and GPX activities of leaves transformed with 35S::ThWRKY7 were significantly higher than those of leaves transformed with T-ck (Fig. S3), suggesting that overexpression of ThWRKY7 also markedly improves Cd tolerance, further demonstrating that ThWRKY7 may fine-tune the ThVHAc1-mediated Cd stress response.

ThVHAc1, as a regulator of Cd stress, may fine-tune the ThVHAc1-mediated Cd stress response. This fine-tuning effect may be achieved by increasing the production of a series of enzymes such as V-ATPase, SOD, POD, GPX, ascorbate peroxidase (APX), and GST48,49. Therefore, we compared the V-ATPase activity between the control and transgenic lines. Assays for V-ATPase-related activities were performed, and all four Arabidopsis lines displayed similar hydro-lytic activity, ATPase activity, and proton transport activity under normal conditions. After CdCl2 treatment, all V-ATPase-related activities were increased in all lines, but the activities in the transgenic ThVHAc1 lines were increased more than those in the control WT and ck lines. The average V-ATPase activities, hydrolytic activities, and proton transport activities of c1#10 and c1#17 were 1.8-, 1.2-, and 1.4-fold greater than those of the control lines, respectively (Fig. 7). These results indicate that the heterologous expression of ThVHAc1 leads to an increase in V-ATPase-related activities in response to Cd stress.

Similarly to V-ATPase, F-ATPase and P-ATPase may also be involved in stress tolerance. Lemos et al. (2005) showed that F-ATPase functions in maintaining cytoplasmic pH, determining the acid tolerance of cariogenic Streptococci mutans50. P-ATPase was previously shown to be an important factor in salt tolerance48-52. In this
Figure 5. Cd tolerance assay of wild type (WT), empty prokII-transformed line (control, marked as ck), and transgenic Arabidopsis c1#10 and c1#17. (a) Germination under 100μM CdCl₂ treatment for 8 days. (b) Germination percentage according to (a). (c) 6-day-old seedlings of WT, ck, c1#10, and c1#17 grown under normal conditions (1/2 MS medium) were transferred to medium supplemented with 100μM CdCl₂ for another 12 d. (d,e) Fresh weight and root length of the transgenic Arabidopsis under Cd stress. The all experiments were repeated three times, and thirty Arabidopsis seedlings were used for each treatment. All data are shown as the mean ± SD. The asterisks in (b,d,e) indicate significant differences between transgenic lines and WT (P < 0.05).

Figure 6. Cd stress tolerance analysis in soil growth conditions. (a) Growth states of the four lines before and after treatment. (b) Cd content comparison between control lines and ThVHAc1 transgenic seedlings after CdCl₂ treatment. (c) Western blotting analysis of tonoplast V-ATPase, using antibodies against the ORF full-length protein synthesized from ThVHAc1 (Abmart, Inc., Shanghai, China) and AHA3 (bs-2247R).
study, the activity of F-ATPase and P-ATPase in isolated tonoplast samples was measured after the addition of the corresponding inhibitors, and the results were very similar to those for V-ATPase activity (Fig. 7). Compared with WT and ck, the activity of F-ATPase and P-ATPase were increased 1.2- and 1.3-fold, respectively, in Arabidopsis lines overexpressing ThVHAc1. Taken together, this finding demonstrates that the regulation of Cd tolerance is complex, and the possible roles of F-ATPase and P-ATPase in Cd tolerance merit further study.

The activity of the antioxidants SOD, POD and GST also did not differ among the four lines under normal conditions. After exposure to CdCl2 treatment, the SOD, POD, and GST activities in c1#10 and c1#17 were significantly higher than those in WT and ck. The SOD activity of c1#17 was 1.92-fold greater than that of WT, the POD activity of c1#17 was 1.77-fold greater than that of the control lines, and the average GST of the transgenic lines was 1.46-fold greater than that of the control lines (Fig. 7f–h). These results suggested that the heterologous expression of ThVHAc1 correlates with increased activities of protective regulatory enzymes under Cd stress. A previous study suggested that antioxidant activity, such as that of POD in Kandelia candel and lipid peroxidation in Bruguiera gymnorrhiza, can be used as a biomarker for heavy metal stress conditions53. In the current study, the activities of V-ATPase, SOD, POD, and GST were all increased and higher than the corresponding activities in the control lines exposed to Cd stress, indicating that the heterologous expression of ThVHAc1 increased the activities of the above enzymes, keeping the ROS level low in transgenic Arabidopsis.

Figure 7. V-ATPase activity and other related enzymes activities analysis of five-week-old Arabidopsis with heterologous expression of ThVHAc1 and treated with CdCl2 for 6 d. V-ATPase-related activities were measured under the addition of the inhibitor 0.6 mM NaN3 + Na3VO3. P-ATPase-related activity was measured under the addition of the inhibitor 0.6 mM NaN3 + 50 mM NaNO3. F-ATPase-related activity was measured under the addition of the inhibitor 50 mM NaNO3 + 0.6 mM Na3VO3. (a) Hydrolytic activity. (b) ATPase activity. (c) Proton transport activity under control. Gramicidin D was used to collapse the residual pH gradient. (d) Proton transport activity under Cd stress. (e) The proton transport activity according to (c,d). Proton transport activity was evaluated by fluorescence quenching as measured by a Hitachi 4010 fluorescence spectrophotometer with 495 nm excitation and 525 nm emission. The reaction was started with MgSO4 after equilibrium, with 4 mM gramicidin D to collapse the pH gradient. The equilibrium fluorescence quenching after treatment with 4 mM gramicidin D. (f) SOD activity. (g) POD activity. (h) GST activity. Data are shown as the mean ± SD. The asterisks indicate significant differences between transgenic lines and WT (P < 0.05).
Consistent with the above results, the histochemical staining of the ROS level showed that under normal conditions, levels of DAB staining for H$_2$O$_2$ and NBT staining for O$_2$$^-$$^-$ in leaves were similar among WT, ck, c1#10, and c1#17. After CdCl$_2$ treatment, c1#10 and c1#17 accumulated less H$_2$O$_2$ and O$_2$$^-$$^-$ than did WT and ck (Fig. 8a,b). The H$_2$DCF staining of ROS in intact guard cells and main roots also showed that ROS accumulation in WT and ck was higher than that in c1#10 and c1#17 under CdCl$_2$ treatment (Fig. 8d,e), suggesting a positive role for ThVHAc1 in regulating the ROS level in plants under Cd stress. In addition, Evans blue and propidium iodide staining for cell damage in leaves and main roots also revealed less cell damage in transgenic lines than in WT and ck (Fig. 8c,f). Meanwhile, the EL rate and MDA, H$_2$O$_2$ and proline contents of WT and ck were also significantly higher than those of c1#10 and c1#17 (p < 0.05) (Fig. 9a–d), confirming that the Cd stress response involves ROS metabolism and that ThVHAc1 may play a positive role in Cd stress tolerance by controlling ROS homeostasis.

Plants exposed to various stresses need to maintain normal metabolic functions, such as growth\textsuperscript{54}, water-holding capacity\textsuperscript{55} and chlorophyll content\textsuperscript{56}. Water and chlorophyll are requirements for photosynthesis. Our results showed that the transgenic lines had higher water-holding capacity than the control lines (Fig. 9e) and that after CdCl$_2$ treatment, the chlorophyll content of c1#10 and c1#17 was higher than that of WT and ck (Fig. 9f). Figure 6a also shows that growth for all four lines is similar under normal conditions; however, after exposure to CdCl$_2$ treatment for 6 d, c1#10 and c1#17 displayed greener leaves than WT and ck. All these results suggest that the heterologous expression of ThVHAc1 in Arabidopsis increased the activity of both V-ATPase and antioxidants, which may regulate ROS homeostasis, cell damage, and photosynthesis for better Cd stress tolerance.

Cd tolerance analysis in T. hispida transiently expressing ThVHAc1. To further confirm the results of the heterologous expression of ThVHAc1 in Arabidopsis, the overexpression vector 35S::ThVHAc1, the suppression expression vector RNAi::ThVHAc1 and the empty vector T-ck were transiently expressed in T. hispida. qRT-PCR results revealed an expression level of ThVHAc1 in 35S::ThVHAc1 that was 47.17-fold greater than that
of T-ck, and the expression of RNAi::ThVHAc1 was only 6.79% that of T-ck (Fig. 10a), indicating that the transient expression in these lines was successful.

Figure 9. Physiological index analyses of five-week-old seedlings of WT, ck, c1#10, and c1#17 under 100 μM CdCl2 for 6 d. Well-watered seedlings were used as controls. (a) Electrolyte leakage. (b) MDA content. (c) H2O2 content. (d) Proline content. (e) Weight of water loss. (f) Total chlorophyll. All experiments were repeated three times. The data are the means ± SD of three independent experiments. All data are shown as the mean ± SD. The asterisks indicate significant differences between transgenic lines and WT (P < 0.05).

Figure 10. Analysis of transient expression of ThVHAc1 in T. hispida under 100 μM CdCl2 treatment for 1 h and 2 h. (a) qRT-PCR analysis of ThVHAc1 in T. hispida seedlings transiently transformed with 35S::ThVHAc1, RNAi::ThVHAc1 compared with T-ck. The relative expression levels were all log2 transformed. (b) DAB staining. (c) Evans blue staining. (d) MDA content. (e) Electrolyte leakage. All data are displayed as the mean ± SD of three independent experiments, and significant differences between transgenic lines and WT (P < 0.05) are indicated by asterisks.
DAB and Evans blue staining of these transient expression lines showed that the ROS accumulation in RNAi::ThVHAc1 was higher than that in T-ck, while the lowest ROS accumulation was observed in 35S::ThVHAc1 under CdCl2 treatment (Fig. 10b,c). The EL and MDA levels in 35S::ThVHAc1 were also significantly lower than those in T-ck and RNAi::ThVHAc1. Specifically, the EL in 35S::ThVHAc1 was 73.6% of that in T-ck and 58.9% of that in RNAi::ThVHAc1, and the MDA content in 35S::ThVHAc1 was 78.2% of that in T-ck and 69.0% of that in RNAi::ThVHAc1 (Fig. 10d,e). The tonoplasts of these lines were isolated, as confirmed by western blotting (Fig. 11a), and all control, RNAi::ThVHAc1, and 35S::ThVHAc1 lines showed similar V-ATPase activities before Cd stress. However, after treatment with CdCl2, 35S::ThVHAc1 displayed the highest V-ATPase activity and RNAi::ThVHAc1 the lowest. The V-ATPase activity, hydrolytic activity and proton transport activity of 35S::ThVHAc1 were 1.3-, 1.4-, and 1.6-fold greater than those in the RNAi::ThVHAc1 line, respectively (Fig. 11). The F-ATPase and P-ATPase activities showed tendencies similar to that of V-ATPase activity. In 35S::ThVHAc1, the F-ATPase and P-ATPase activities were 1.5- and 1.4-fold greater than those in RNAi::ThVHAc1, respectively, while the corresponding hydrolytic activities were 1.4- and 1.2-fold greater than those in RNAi::ThVHAc1 (Fig. 11).

Furthermore, the activities of protective enzymes, including SOD, POD, GST, and GPX, were significantly higher in 35S::ThVHAc1 than in RNAi::ThVHAc1 and T-ck after CdCl2 treatment (Fig. 12). These results further suggest that ThVHAc1 participated in the regulation of Cd tolerance by increasing the activity of protective enzymes to maintain ROS homeostasis in cells. Taken together, these results indicate that ThVHAc1 may be an effective gene for improving plants’ Cd tolerance.

Expression of ThVHAc1 influenced other related genes and V-ATPase subunits. To investigate whether other genes were affected by the expression of ThVHAc1, the expression levels of five AHA (H+-ATPase), five ACA (auto-inhibited Ca2+-ATPase), and eight stress-related genes were analyzed by RT-PCR. AHA genes primarily participate in ATP binding, the biosynthetic process, protein phosphorylation-dependent regulation, and

Figure 11. V-ATPase activity analysis of transient expression of ThVHAc1 in T. hispida under CdCl2 treatment for 2h. V-ATPase related activities were measured as those in Arabidopsis. (a) Western blotting analysis of tonoplast V-ATPase. (b) Hydrolytic activity. (c) ATPase activity. (d) Proton transport activity under control. (e) Proton transport activity under Cd stress. (f) Proton transport activity according to (d,e). All data are displayed as the mean ± SD, and significant differences (P < 0.05) are indicated by asterisks.
coupling with transmembrane ion movement\textsuperscript{57}. One of the AHA genes, At3g42640, was reported to be induced during Arabidopsis pollen development and during fertilization in B. campestris subsp. Chinensis\textsuperscript{58}. ACA genes function in ATP activity, calcium channel activity, catalytic activity, hydrolase activity, and carbonate dehydratase activity\textsuperscript{59}. When exposed to a boron deficiency for 24 h, the transcriptional level of the ACA gene At1g27770 increased by 1.43-fold\textsuperscript{60}. CSD (cytosolic copper/zinc superoxide dismutase, At1g08830) is involved in ROS accumulation\textsuperscript{61}, and APX (ascorbate peroxidase, At1g07890) is an ascorbate peroxidase with increased activity under oxidative stress in DET2-mutant Arabidopsis\textsuperscript{62}. The transcription levels of RBOHC (respiratory burst oxidase homolog c, At5g51060) and LOX1 (lipoxygenase, At3g45140) were markedly upregulated when Arabidopsis was exposed to Cd stress\textsuperscript{63}. The results of the present study revealed that three AHA genes (At2g07560, At1g80660, and At3g42640), three ACA genes (At1g27770, At1g08065, and At1g08080) and four stress-related genes (LOX1 (At3g45140), CSD (At1g08830), APX (At1g07890), and RBOHC (At5g51060)) were expressed at higher levels in the transgenic Arabidopsis c1#10 and c1#17 lines than in the WT and ck lines. For example, the ThVHAc1 transgenic lines

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Figure 12. The SOD, POD, GST, GPX activities, and the expression of ThSOD, ThPOD, ThGSTZ1, ThGPX analysis of T-ck, 35S::ThVHAc1, RNAi::ThVHAc1 seedlings. All data are displayed as the mean ± SD of three independent experiments, and significant differences between transgenic lines and WT (P < 0.05) are indicated by asterisks.
expressed the AHA genes at levels more than 3-fold those of the WT. The highest expression level of the AHA gene was approximately 4.9-fold higher (relative to ck) in c1#10, while that of the CSD gene was 5.3-fold higher (relative to ck) (Fig. 13a–d).

In three transiently transformed T. hispida lines, four antioxidant genes were analyzed using qRT-PCR. ThSOD, ThPOD, ThGSTZ1 and ThGPX showed similar expression profiles, all of which were upregulated after CdCl2 treatment. The expression levels of these genes were highest in 35S::ThVHAc1 and lowest in RNAi::ThVHAc1 (Fig. 12). We also characterized the expression of several genes associated with stress-related functions and V-ATPase activity in T. hispida. The upregulated genes are shown in Fig. 13e,f, including one vacuolar cation/proton exchanger isoform CAX2 gene, three chloroplast protease genes (CSB), one ATP-dependent protease proteolytic subunit (ADP), one glycoside hydrolase protein (GLH) and one NADPH gene. These results indicate that the expression of ThVHAc1 changed the expression of other stress-related and V-ATPase-related genes, suggesting a complex network of Cd tolerance regulation.

Consistent with this result, other researchers have also shown that the overexpression or suppression of some genes always affects other genes. For example, overexpression of a DREB gene affected the expression of SOD, GST, and other stress-related genes27. R740S mutation in the a3 subunit of V-ATPase decreased the expression of key osteoclast markers (TRAP, cathepsin K, OSCAR, DC-STAMP, and NFATc1)64. The overexpression of SaVHAc1 in rice upregulated many stress-related genes, such as cysteine synthase, the pathogenesis-related protein Bet vI family protein, and glutamine synthetase under salt stress65.

V-ATPase is a multi-subunit enzyme. Overexpression of the ThVHAc1 gene affected many aspects of V-ATPase activity under CdCl2 treatment (Figs 7 and 11). To better understand whether other subunits of V-ATPase were also affected by the expression of ThVHAc1 under CdCl2 treatment, the expression profiles of 28 subunits in Arabidopsis were analyzed by qRT-PCR in c1#10 and c1#17 at different times. Clustering analysis of the expression patterns of all 28 subunits in c1#10 showed that they were primarily clustered into three groups. All subunits in group 1, including AtVHA-E2, F, G1, G2 and E1, were upregulated. Meanwhile, most subunits in group 2, including AtVHA-B1, C, B3, a2, H, c2, A, E3, d1 and d2, were induced after 12h of treatment. The remaining subunits, except a1 and c1, belong to group 3 and were primarily suppressed, especially after 24h of treatment (Fig. 14).
expression of the five AtVHA-c subunits was unchanged except for AtVHA-c3, which was induced at 6 h (Fig. 15). At the same time, ThVHAc1 showed much higher expression under the same conditions, especially at 12 h (Fig. 15), suggesting that expression of the exogenous VHA-c subunit may suppress the expression of intrinsic VHA-c genes. The expression patterns of all subunits were also similar in c1#17 (Fig. S4), indicating that expression of ThVHAc1 may cause other subunits to participate in V-ATPase regulation under Cd stress and that V-ATPase activity is controlled by a complex network.

Interestingly, transient overexpression of ThVHAc1 in T. hispida had a different effect. Fifteen subunits were amplified from the T. hispida cDNA library. All subunits except ThVHA-H in 35S::ThVHAc1 and ThVHAc1 in RNAi::ThVHAc1 showed positive expression levels under the control conditions, and most subunits showed greater expression in RNAi::ThVHAc1 than in 35S::ThVHAc1 (Fig. 16). However, when treated with CdCl2, although all subunits except ThVHA-e in 35S::ThVHAc1 were induced, their expression was higher in 35S::ThVHAc1 than in RNAi::ThVHAc1 (Fig. 16). These results suggest that ThVHAc1 responds to Cd stress and that all subunits may

Figure 14. Cluster analysis of the expression levels of all subunits in c1#10 based on WT. The subunits were classified into three groups. The log2-transformed expression levels were calculated according to the three groups. The x-axis shows the stress time point. The data are shown as the means ± SD of three independent experiments.

Figure 15. qRT-PCR analysis and comparison of five AtVHA-c subunits and ThVHAc1 in c1#10. The relative expression levels were all log2 transformed. The x-axis shows the stress time point. The data are shown as the means ± SD of three independent experiments.
participate in the regulation of V-ATPase activity. However, the mechanisms by which all subunits act in such a complex network of V-ATPase regulation require further study.

A previous study indicated that the expression patterns of different subunits of V-ATPase may differ under the same stress. The *Mesembryanthemum crystallinum* V-ATPase subunits A, B and c were all upregulated approximately 2-fold relative to the control plant in roots and young leaves when exposed to salt stress. However, when the leaves fully expanded, only the c subunit was induced in reaction to salt66. Sugar beet VHA-A and VHA-c were coordinately expressed during plant development and are induced in response to high salinity67. The subunit E was also induced after treatment with salt for 3 d in mature common ice plant leaves, but it was not induced in juvenile leaves under the same conditions68.

**Conclusion**

In the plant kingdom, the V-ATPase c subunit (VHAc) is an important component of V-ATPase, which mediates abiotic stress responses. Some studies have demonstrated the salt regulation capacity of the VHAc gene. However, there are few reports on VHAc gene function in response to heavy metal stresses in a woody halophyte. Because *ThVHAc1* rapidly responded to Cd stress in *T. hispida*, in this study, we further investigated the role of *ThVHAc1* in Cd tolerance regulation. Our results showed that overexpression of *ThVHAc1* effectively enhanced the tolerance of the transgenic *Arabidopsis* and *T. hispida* plants to Cd stress and that *ThVHAc1* may modulate Cd stress tolerance by improving protective enzyme activities and strengthening the reactive oxygen species (ROS) scavenging system to decrease the cell damage when exposed to CdCl₂ treatment. Moreover, we identified a potential upstream regulator of *ThVHAc1*, *ThWRKY7*, which also responded to Cd stress, showed expression patterns similar to those of *ThVHAc1*, and improved the Cd stress tolerance of transgenic *T. hispida*. Although it remains unclear whether *ThWRKY7* and *ThVHAc1* cooperate to participate in the regulation of tolerance to other stresses, the current study provides new insights into the role and regulatory mechanism of *ThVHAc1* in the regulation of tolerance to Cd stress in *T. hispida*.

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Author Contributions
All authors reviewed the manuscript. G.Y. wrote the paper, analyzed the data and did/participated most of the assays (main on yeast one-hybrid relative experiments), meanwhile the figures and tables were done by G.Y. and C.W. cloned the promoter and analyzed the expression in Arabidopsis. Y.W. and C.Y. design mainly on the current study, afford some guidance on some assays, examined the data analysis, meanwhile afford some funds on the assays. Y.G. and Y.Z. did the staining assay (DAB, evans bule and so on) and tested the physiological index. C.G. revised the paper and did the expression assay (qRT-PCR and RT-PCR), and afford main fund on the study.

Additional Information
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