The walnut *JrVHAG1* gene is involved in cadmium stress response through ABA-signal pathway and MYB transcription regulation

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**Abstract**

**Background:** Vacuolar H⁺-ATPase (V-ATPase) is a vital protein complex involved in abiotic stress response in plants. The G subunit of *Juglans regia* (*JrVHAG1*) was previously identified as a drought tolerance-related gene involved in the ABA (abscisic acid)-signal pathway. Heavy metal stress is becoming a major detriment for plant growth, development, and production. In order to understand the role of *JrVHAG1*, the potential function mechanism of *JrVHAG1* exposed to CdCl₂ stress was confirmed in this study.

**Results:** Transcription of *JrVHAG1* was induced by ABA and increased to 58.89-fold (roots) and 7.38-fold (leaves) and by CdCl₂ to 2.65- (roots) and 11.42-fold (leaves) relative to control, respectively. Moreover, when treated simultaneously with ABA and CdCl₂ (*ABA+CdCl₂*), *JrVHAG1* was up-regulated to 110.13- as well as 165.42-fold relative to control in the roots and leaves, accordingly. Compared to the wild type (WT) Arabidopsis plants, the transgenic plants with overexpression of *JrVHAG1* (G2, G6, and G9) exhibited increased seed germination rate, biomass accumulation, proline content, and activities of superoxide dismutase (SOD) and peroxidase (POD) under ABA, CdCl₂, and ABA+CdCl₂ treatments. In contrast, the reactive oxygen species (ROS) staining, malondialdehyde (MDA) content, hydrogen dioxide (H₂O₂) content, as well as electrolyte leakage (EL) rates of transgenic seedlings were all lower than those of WT exposed to ABA, CdCl₂ and ABA+CdCl₂ stresses. Furthermore, a 1200 bp promoter fragment of *JrVHAG1* was isolated by analyzing the genome of *J. regia*, in which the cis-elements were identified. This *JrVHAG1* promoter fragment showed expression activity that was enhanced significantly when subjected to the above treatments. Yeast one-hybrid assay and transient expression analysis demonstrated that *JrMYB2* specifically bound to the MYBCORE motif and shared similar expression patterns with *JrVHAG1* under ABA, CdCl₂ and ABA+CdCl₂ stress conditions.

**Conclusions:** Our results suggested that the *JrVHAG1* gene functions as a CdCl₂ stress response regulator by participating in ABA-signal pathway and MYB transcription regulation network. *JrVHAG1* gene is a useful candidate gene for heavy metal stress tolerance in plant molecular breeding.

**Keywords:** Heavy metal stress, *JrVHAG1* gene, ABA-signal pathway, Promoter, MYB transcription factor
Background

Heavy metals include micronutrients (such as Fe, Mn, and Mo), trace elements (such as Cu, Zn, Ni, and W), and stress factors (such as Cd, Pb, Hg, Ag, and U) that are toxic to plants [1]. Heavy metal stress not only leads to decrease in plant seed germination, plant growth, or increase of reactive oxygen species (ROS) accumulation and cell death but also induces chlorosis, necrosis, and turgor loss, even modifies the protein profile [2, 3]. More importantly, enrichment of heavy metals in plants and animals causes serious harm to the food chain and human health. Therefore, adequate understanding of the regulatory mechanism and screening the vital functions of genes from corresponding plants are vital to understand plant heavy metal stress responses.

Among the stress related heavy metals, Cu, Zn, Pb, Ni, Se, Cr, and Co were moderately enriched, whereas Sb and cadmium (Cd) were extremely highly enriched in the majority of soil samples, therefore, Cd is regarded as one of the most phytotoxic heavy metals [4, 5]. In soil, Cd contamination could severely affect the performance of agricultural fields [6]. Cd toxicity in agricultural soil has received significant attention because of its high penetration in the food chain and its toxicity to humans [7]. Thus, characterization the molecular mechanisms of potential Cd stress response genes is crucial [8, 9].

Several studies on Cd stress-related genes and mechanisms are available, for instance, the phytochelatin synthetic apparatus [11]. Heavy metal ATPase 3 (HMA3), a P1_1/2-ATPase, is a key tonoplast transporter involved in mediating the vacuolar sequestration of Cd to detoxify the intake of this element by plants. The HMA3 from Festuclus loliaceum (FIHMA3) plays an important role in Cd\(^{2+}\) sequestration in root cell vacuoles, thereby limiting the entry of Cd\(^{2+}\) into the cytoplasm and reducing Cd\(^{2+}\) toxicity [12]. Vacular H\(^{+}\)-ATPase (V-ATPase) and its subunits play important roles in heavy metal stress response, and the c subunit from Tamarix hispida (ThVHAc1) confers plants with enhanced CdCl\(_2\) stress tolerance through WRKY transcription factor and ROS scavenging [5].

V-ATPase is a multi-subunit complex comprising domains \(V_1\) (600–650 kDa membrane-peripheral domain) and \(V_0\) (260 kDa membrane-integral domain). The \(V_1\) domain contains eight different subunits (A–H) and is responsible for ATP hydrolysis, while the \(V_0\) domain includes six different subunits (a, d, c, c', c'\(^+\), and e) and is responsible for proton translocation [13]. Some subunits of the V-ATPase were previously characterized, such as Malus domestica A subunit (MdVHA-A) [14], T. aestivum (RH8706–49) B subunit (TaVb) [15], Pennisetum glaucum c subunit (PgVHA-c1) [16], and Arabidopsis thaliana VHA-B, –E, –G, and -a subunits [17]. Therefore, understanding the abiotic stress response function and the mechanism of V-ATPase as well as its subunits sounds important.

Juglans regia is a nut tree cultivated worldwide for its nutritious fruits [18], whose production is limited by various environmental stimuli. Studies on the stress response mechanism of walnut trees are currently lacking; therefore, achieving a better understanding of the mechanisms involved in abiotic stress response of J. regia is timely and essential [19]. In previous studies, we identified a few candidate genes in walnut involved in stress response, including the G subunit (JrVHA1G), which is a known drought-inducible osmotic stress gene and correlated with the ABA (abscisic acid)-signal pathway [19]. In this study, we found that the expression of JrVHA1G was induced by CdCl\(_2\) stress in J. regia leaves and roots, and the function of JrVHA1G exposed to CdCl\(_2\) treatment was further verified. Meanwhile, the expression activities of JrVHA1G promoter segment and upstream regulatory genes were analyzed, which suggested a potential CdCl\(_2\) response mechanism of JrVHA1G involving in ABA-signal pathway and MYB transcription regulation.

Results

Expression of J. regia V-ATPase subunits and response to CdCl\(_2\) and ABA treatments

To better understand the role of V-ATPase subunits in abiotic stress response in walnut tree, 15 V-ATPase subunits were identified from the J. regia transcriptome in tissues using functional annotation of non-redundant unigenes (NRUs), which were analyzed by blast and denoted as VHA-A, B, C, D, E, F, G (JrVHA1G), H, a1, a3, c1, c4, d1, d2, and e1, respectively. Transcript level of these subunits under CdCl\(_2\), ABA, and ABA+CdCl\(_2\) (CdCl\(_2\) plus ABA) were analyzed by quantitative real time PCR (qRT-PCR) in the roots and leaves of J. regia. The results showed that most of these subunits were induced by CdCl\(_2\) and ABA treatments (Fig. 1). In leaves, the expression patterns upon exposure to ABA, CdCl\(_2\), ABA+CdCl\(_2\) could be classified into four groups. VHA-A and e1 had the maximum transcription under CdCl\(_2\), but had the minimum under ABA stress; While VHA-D, E, F, H, a1, a3, d1 subunits were grouped together based
on their expression patterns that were contrary to VHA-A and e1; they hit a peak upon exposure to ABA, but declined to bottom upon exposure to CdCl₂ stress. The expression levels of VHA-B, C, JrVHAG1, c1 and c4 subunits were increased for ABA and ABA+CdCl₂ stress, whose transcription was up-regulated upon addition of ABA to CdCl₂ treatments, while the VHA-d2 subunit gene was down-regulated by all the stresses, and the tendency was reverse to the levels of VHA-B, C and G (Fig. 1). Under ABA treatment, the VHA-D, E, G, H, a1, a3, c1, c4 were induced to 7.38~30.20-fold of the control; VHA-B, d1, d2, e1 were suppressed. Under CdCl₂ stress, VHA-D, E, F, d1, d2 and e1 subunits were down-regulated, among which the F subunit was the least, whereas VHA-G, c1 and c4 were the top induced three genes. When treated with ABA+CdCl₂, VHA-G, c1 and c4 were induced to 165.42-, 105.42-, 119.43-folds of the control, respectively. Moreover, the transcription of JrVHAG1 was increased much more obviously by ABA+CdCl₂ than all other subunits (Fig. 1).

In the roots, the expression profiles of most subunits exposed to ABA, CdCl₂, ABA+CdCl₂ were different from those seen in the leaves, excluding VHA-F, d1 and e1 (Fig. 1). According to their transcription patterns, these subunits could also be divided into four groups. Group 1 contained VHA-A, B, F, d1, and d2, whose expression reached a peak when subjected to ABA treatment, while reached the least when subjected to CdCl₂ stress. The VHA-e1 subunit formed a separate group for its specific expression pattern. VHA-a1 was also by itself in a class as the expression was highest when exposed to ABA+CdCl₂, while it became the lowest when subjected to ABA. The other subunits were grouped as one family, whose maximum transcription was noticed when treated with ABA+CdCl₂, while the minimum occurred when subjected to CdCl₂ stress (Fig. 1). Under ABA treatment, the expression of VHA-C, G, c1, c4 were induced much more obviously than others, and the expression values were 18.55~58.89-fold of the control. The VHA-D, E, a1, a3 were all down-regulated to lower levels compared to the other subunits. Under CdCl₂ stress, the VHA-F subunit was strongly suppressed. The VHA-C, G, c1, c4 were up-regulated to 2.65~6.65-fold of the control. When treated with ABA+CdCl₂, VHA-C, G, c1, c4 were strongly induced to a much higher level than other subunits, and the highest transcription level was JrVHAG1 (110.13-fold of those of the control) (Fig. 1).

**Overexpression of JrVHAG1 improves plant CdCl₂ stress tolerance**

For a complete characterization of the CdCl₂ stress tolerance of JrVHAG1 gene, whether it involves the ABA-
signal pathway, the germination, biomass, ROS metabolism, and physiological performance of three transgenic lines that overexpression of JrVHAG1 (G2, G6, and G9) [19] and wild type (WT) Arabidopsis were analyzed when exposed to CdCl2, ABA and ABA+CdCl2. The results showed that the germination rates of G2, G6, and G9 were significantly higher \( (p < 0.05) \) than that of WT under the three treatments, which averaged 1.10-, 1.35-, and 1.51-fold increase compared to that of the WT under CdCl2, ABA, and ABA+CdCl2, respectively (Fig. 2a-e). The average fresh weight of the germinated seedlings of G2, G6, and G9 were 1.48-, 1.19-, 1.55-folds higher than those of WT when subjected to the above treatments, accordingly (Fig. 2f). When 8-day-old seedlings of WT, G2, G6, and G9 grown on 1/2 MS were transferred to 1/2 MS with or without ABA, CdCl2, ABA+CdCl2 and cultured for additional 8 d, the fresh weight and primary root length were similar among the four lines under control conditions. However, the biomass accumulation (fresh weight and primary root length) of the transgenic lines were significantly higher \( (p < 0.05) \) than that of WT (Fig. 3a-d). The average fresh weight of G2, G6, and G9 lines were 1.26- (CdCl2) and 1.34-folds (ABA+CdCl2) of that of WT (Fig. 3e). Growth of the primary roots of the WT were severely affected by the three treatments, among which the most serious inhibition was happened on ABA+CdCl2 stress (Fig. 3f). These results suggest that overexpression of JrVHAG1 plays a positive role in the response to CdCl2 stress in plants.

To further verify the CdCl2 tolerance role of JrVHAG1, 47-d-old WT and transgenic plants were treated with ABA, or CdCl2, or ABA+CdCl2. 3, 3′-Diaminobenzidine (DAB) and nitrogen blue tetrazolium (NBT) staining clarified that the WT seedlings were stained deeper than those of G2, G6, and G9 when exposed to all above three treatments (Fig. 4a, b). The hydrogen dioxide \( (\text{H}_2\text{O}_2) \) accumulation in the WT plants was also significantly higher than those seen in the transgenic plants (Fig. 4c). Furthermore, the electrolyte leakage (EL) rates of these lines had a trend similar to that as \( \text{H}_2\text{O}_2 \). Under ABA treatment, the EL rates of G2, G6, G9 were 69.28%~73.57% of that of WT; but when exposed to CdCl2, the differences were much more obvious compared to those under ABA stress and they were 45.14%~63.98% of that of WT; When treated with ABA+CdCl2, the corresponding rates were 49.57%~69.13% of that of WT, respectively (Fig. 4d). The superoxide dismutase (SOD) and peroxidase (POD) activities, proline and malondialdehyde (MDA) contents were also supportive of the role of JrVHAG1 that it could effectively improve plant tolerance to CdCl2 (Fig. 5). Upon exposure to ABA, CdCl2, ABA+CdCl2 stresses, the MDA contents of the tested lines were similar to that of the EL rates and \( \text{H}_2\text{O}_2 \) contents, WT had higher values, 1.24~1.75-fold, 1.19~1.67-fold and 1.16~1.64-fold of transgenic seedlings, respectively. However, the SOD, POD and proline levels were contrasting with MDA levels that the SOD, POD and proline levels of three transgenic

![Fig. 2](image-url)
lines were 2.08-~ 2.75-fold, 1.32-~ 1.60-fold and 1.31-~ 1.45-fold of that of WT, accordingly (Figs. 4, 5).

Identification and expression activity of JrVHAG1 promoter
A 1200 bp promoter segment of JrVHAG1 was identified from the J. regia genome that was located in the 538,241–534,657 region of the walnut genome (NW017389860.1). The promoter sequence and the relevant elements such as, ARE, LTR, MYBCORE, W-box are shown in Additional file 1: Figure S2. This promoter fragment was inserted into a β-glucuronidase (GUS) expression vector pCAMBIA1301, and Arabidopsis plants were transformed using this vector. GUS staining revealed that the promoter caused GUS expression in the leaves and roots (Fig. 6). When treated with ABA, CdCl₂, and ABA+CdCl₂, the transgenic plants containing this promoter showed increased GUS activity overall in the aerial parts and roots compared to control. The total GUS activities exposed to ABA, CdCl₂, and ABA+CdCl₂ stresses were 1.22-, 1.30-, and 1.49-fold higher of that under normal condition, correspondingly, and the differences were significant (Fig. 6).

Up-stream regulation of JrVHAG1 involves MYB transcriptional activation
To verify the up-stream regulation of JrVHAG1, yeast one-hybrid assay was employed to study the interactions
between transcription factors (TFs) and MYBCORE in the promoter. It was found that JrMYB2 binds to the MYBCORE motif, which was confirmed by the interaction between pHis2-MYB-M (mutated MYBCORE), pHis2-MYB-S (the JrVHAG1 promoter including the MYBCORE motif), pHis2-MYB-M1 (the JrVHAG1 promoter excluding the MYBCORE motif), or pHis2-MYB-M2 (the JrVHAG1 promoter including the mutated MYBCORE motif) and JrMYB2 on the SD (synthetic drop-out medium)/−Trp-Leu-His/50 mM 3-AT (3-amino-1, 2, 4-triazole) solid medium (Fig. 7a). Moreover, the co-transformation of the reporter and effector demonstrated that the GUS activities of the leaves that were transformed by the MYBCORE motif or the promoter fragments containing the MYBCORE motif were similar to that of the positive control, and significantly higher than those of the negative control and mutated reporters. The GUS activities of leaves transformed by effector and the mutated reporter was similar to that of the negative control, further suggesting that JrMYB2 binds specifically to the MYBCORE motif in the JrVHAG1 promoter (Fig. 7b).

The results of qRT-PCR analysis of JrMYB2 clearly showed that JrMYB2 was induced by ABA, CdCl₂, and ABA+CdCl₂ in the leaves and roots (Fig. 7c). In leaves, the transcription of JrMYB2 was induced by 3.97-, 7.01-, and 12.04-fold of control when subjected to ABA, CdCl₂, and ABA+CdCl₂ stresses, and the corresponding gene expression in the roots were 11.96-, 3.34-, and 15.14-fold compared to control, respectively (Fig. 7c). It appears that the expression profiles of JrMYB2 under these stresses in both tissues are similar to those of JrVHAG1, especially when subjected to the same conditions (Fig. 1), indicating that JrMYB2 may act as an upstream regulator of JrVHAG1 to either control JrVHAG1 or act along with JrVHAG1 to improve CdCl₂ stress tolerance of plants.

**Discussion**

The V-ATPase regulates and is also self-regulated by various signaling cascades controlling nutrient supply and metabolism [20]. The up-regulation of the V-ATPase subunits is beneficial to plants as part of the heavy metal stress response. For instance, Cucumis sativus CsVHA-c1, CsVHA-c2, and CsVHP1;1 are essential elements of the mechanisms involved in the adaptation of cucumber plants to copper toxicity [21]. T. hispida c subunit (ThVHAc1) was up-regulated by CdCl₂ stress,
and the overexpression could effectively improve the CdCl₂ tolerance of plants [5]. In a previous study, we demonstrated that the involvement of JrVHAG1 in drought-inducible osmotic stress correlated with ABA-signal pathway [19], implying the potential response mechanism of J. regia V-ATPase and its subunits involve in ABA-signal regulation. To well understand the adaptation mechanism of J. regia to heavy metal stress, in this study, we firstly identified 15 V-ATPase subunits and their expression were analyzed by qRT-PCR under CdCl₂ and ABA treatments. The results showed that the expression of VHA-B, C, JrVHAG1, c1 and c4 subunits were similar and induced much more obviously than others exposed to the three treatments, whose transcription was up-regulated more obviously by ABA+CdCl₂ than single stress of ABA or CdCl₂ (Fig. 1), suggesting the possibly positive role of VHA-B, C, JrVHAG1, c1 and c4 to CdCl₂ stress and may involve in ABA-signal pathway. To understand rapidly of the prediction, the open reading frames (ORFs) of VHA-B, C, JrVHAG1, c1 and c4 were inserted into the pYES2 and transformed into INVSC1, respectively [22]. Then the transgenic yeasts were treated with ABA, CdCl₂, ABA+CdCl₂ and compared to the control yeast. The growth abilities of VHA-B, C, JrVHAG1, c1 and c4 transformed yeasts were higher than that of the control yeast. The most important is that the JrVHAG1 transformed yeasts showed the best growth activities under the three treatments, and the differences between JrVHAG1 transgenic yeast and others were significant under ABA+CdCl₂ stress (Additional file 1: Figure S1), indicating that the ABA truly promoted the function of JrVHAG1 in CdCl₂ tolerance. Considering that the expression of JrVHAG1 was highly induced by CdCl₂ stress and was enhanced to a very high level by ABA+CdCl₂ (Fig. 1), we think JrVHAG1 was a potential candidate gene for CdCl₂ stress tolerance and was likely to connect with ABA signal.

To verify the function of JrVHAG1 in Cd stress, transgenic Arabidopsis lines with high expression of this gene were evaluated. The results showed that the germination rates, biomass accumulations, and protective enzyme activity of the transgenic lines were significantly higher than those of the WT plants; while the ROS generation of G2, G6, and G9 were significantly lower than that of WT (Figs. 2, 3, 4, 5) and perhaps indicating the JrVHAG1 gene functions in ROS scavenging, suggesting that the expression of JrVHAG1 could improve plant Cd stress tolerance. These physiological index endowing by JrVHAG1 under ABA, CdCl₂ and ABA+CdCl₂ treatments were similar to other subunits those were verified to be abiotic stress tolerance genes. Such as M. domestica MdVHA-A and Puccinellia tenuiflora PtVHAc. Overexpression of MdVHA-A conferred transgenic tobacco seedlings with enhanced drought tolerance by improving important attributes such as dry weight, fresh weight, MDA, and relative water content [14]. Transgenic A. thaliana lines expressing PtVHAc exhibited improved tolerance to salt-induced osmotic stress as
revealed from the analysis of the fresh weight, root length, siliques number, and other parameters [23]. Moreover, ABA is known as a stress hormone that takes part in the integration of signals [24]. Germination and plant growth under heavy metal stress is related to ABA [25, 26]. The current results also revealed that the difference of germination rates and average fresh weight between the transgenic lines and WT under ABA+CdCl₂ stress was significant than that exposed to CdCl₂ (Fig. 2), implying that the ABA promotes the function of JrVHAG1 gene in response to CdCl₂ stress, which were just similar to other previous reports. For example, after exposure to CdCl₂ stress, the S. lycopersicum Micro-Tom (MT) siliques ABA-deficient mutant (sit) and its WT counterpart displayed differences in lipid peroxidation, hydrogen peroxide content, and activities of some key antioxidant enzymes such as catalase, glutathione reductase, and ascorbate peroxidase, highlighting the relative importance of ABA in Cd stress response of plants [24]. The basic helix-loop-helix (bHLH) in tartary buckwheat (Fagopyrum tataricum) (FtbHLH3) was induced by polyethylene glycol 6000 (PEG₆₀₀₀) and ABA treatment. Overexpression of FtbHLH3 in Arabidopsis resulted in increased drought/oxidative tolerance and indicates that FtbHLH3 may function as a positive regulator of drought/oxidative stress tolerance through an ABA-dependent pathway [27]. These results told us that JrVHAG1 function as a positive CdCl₂ response gene involved in ABA-signaling pathway.

In plants, transcription factors are important components of stress response pathways involving in many stress-response genes. There are several studies on the function of Cd-responsive genes; however, the upstream transcriptional regulatory pathways that modulate their responses to Cd are less clear [28]. To better understand the Cd stress response mechanism of JrVHAG1, the promoter of JrVHAG1 was identified and the expression activity was tested. The promoter displayed different expression activities in the roots and leaves under normal and CdCl₂ treated conditions (Fig. 6), which was similar to the expression of JrVHAG1 upon exposure to CdCl₂ stress (Fig. 1), indicating the effectiveness of the selected promoter fragment. Further, the up-stream regulator of JrVHAG1 was identified through yeast one-hybrid assay and co-transient expression experiments. JrMYB2 was found to specially bind to the MYBCORE motif present in the JrVHAG1 promoter, and the expression of JrMYB2 was similar to that of JrVHAG1 under ABA, CdCl₂, and ABA+CdCl₂ treatments (Fig. 7), indicating that JrMYB2 acts as an up-stream regulator of JrVHAG1 and could regulate or combine functionality with JrVHAG1 in CdCl₂ stress response. This prediction is similar to that in other studies. For instance, T. hispida ThWRKY7, the up-stream regulator of ThVHAc1, regulates plant CdCl₂ tolerance and controls the involvement of ThVHAc1 in plant CdCl₂ stress response by binding to the WRKY motif present in the ThVHAc1
promoter [5]. Lin et al. demonstrated that the Phaseolus vulgaris ethylene response factor 15 (PvERF15), an upstream transcriptional regulator of the Cd metal response element-binding transcription factor 1 (PvMTF1) was identified using a yeast one-hybrid system. It was strongly induced by CdCl2 stress and activated the PvERF15/PvMTF1 transcriptional interaction [28]. These results suggested that the Cd stress response was controlled by upstream regulators and yeast one-hybrid assay is an effective way to screen the potential regulatory factors. Meanwhile, MYB TFs are members of a large family that have multiple functions in plant growth and development, flavonoid metabolism, and biotic/abiotic stress responses [29]. In A. thaliana, the root-specific transcription factor MYB72 is required for the onset of induced systemic resistance (ISR) and is also associated with plant survival under conditions of iron deficiency [30]. Ectopic expression of orchid (Dendrobium sp. XMW-2002-2) R2R3-MYB gene DwMYB2 in Arabidopsis confers hypersensitivity to iron deficiency in the transgenic plants. The ferric-chelate reductase gene, AtFRO2, and the iron transporter genes, AtIRT1 and AtIRT2, are consistently up-regulated by the expression of DwMYB2, while other potential iron transporters such as AtIREG1, AtFRD3, and NRAMP1 are down-regulated, indicating the role of DwMYB2 in iron transportation impairment [31]. SbMYB44, a R2R3-MYB cloned from Salicornia brachiata Roxb., was up-regulated in response to salinity, desiccation, high temperature, and treatments with ABA and salicylic acid (SA). Overexpression of SbMYB44 enhanced the growth of yeast cells under both ionic and osmotic stresses [32]. Considering all these studies and our results, we consider that the JrVHAG1 gene is an important CdCl2 response gene and is involved in MYB transcription pathway.

**Conclusion**

The transcription of JrVHAG1 was highly up-regulated by CdCl2 and ABA stress in J. regia roots and leaf tissues. Heterologous overexpression of JrVHAG1 in A. thaliana conferred the transgenic seedlings with increased fresh weight and primary root length, higher SOD and POD activities, as well as lower levels of ROS, H2O2, MDA, and EL rates than those of WT plants.
exposed to ABA, CdCl₂, ABA+CdCl₂ treatments. A 1200 bp promoter fragment containing many cis-elements of JrVHAG1 was isolated and demonstrated to show high expression activity, which was induced by CdCl₂ and ABA treatments. Yeast one-hybrid and cotransient transformation assays in tobacco showed that JrMYB2 could specifically bind to the MYBCORE motif in the JrVHAG1 promoter. Similar to JrVHAG1, JrMYB2 could also be induced by CdCl₂ and ABA. Considering that the clear responses of JrVHAG1 and JrMYB2 to CdCl₂ and ABA treatments in walnut root and leaf tissues and the CdCl₂ tolerance conferred by overexpression of JrVHAG1 in transgenic Arabidopsis, it can be concluded that JrVHAG1 plays a positive role in Cd tolerance through ABA-signal pathway and involves in MYB transcription regulation.

Methods
Plant materials and growth conditions
New branches from 6-year-old grafted ‘Xiangling’ seedlings (a genotype of J. regia widely planted in China) were obtained and grafted to 2-year-old stocks of the same ‘Xiangling’. The grafted seedlings were cultivated for 2-years in a greenhouse (22 ± 2 °C, relative humidity 70 ± 5%, illumination cycle 14/10 h) [33], and treated with 50 μM ABA, or 150 μM CdCl₂, or 150 μM CdCl₂ plus 50 μM ABA (ABA+CdCl₂) by watering the roots for 60 h. The roots and leaves were harvested independently and stored at –80 °C for qRT-PCR analysis. The seedlings watered only with fresh water for 60 h served as control. Every treatment was applied three times and each treatment contained at least 9 seedlings.

RNA isolation and expression analysis of V-ATPase subunits
V-ATPase subunits were identified from the J. regia transcriptome in tissues using functional NRUs, which were analyzed using BLAST tools. The ORFs were screened using the ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/), and the primers used for qRT-PCR analysis were designed from the 5′-end of the ORF (Additional file 1: Table S1). Total RNA from each sample was isolated using the cetyltrimethylammonium ammonium bromide (CTAB) method and reverse-transcribed into cDNA [34], which was diluted to 1/10 of the original concentration with sterile water for use as template of qRT-PCR. The 18S rRNA was used as an internal control gene [35]. The 20 μL reaction mixture contained 2 μL cDNA template (equivalent to 100 ng of total RNA), 0.5 μM of each forward and reverse primer (Additional file 1: Table S1), 10 μL of SYBR Green Real-time PCR Master Mix (CWBIO). The qRT-PCR was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Redmond, WA) [33]. The following cycling parameters were applied for amplification: 94 °C for 30 s followed by 44 cycles at 94 °C for 12 s, 60 °C for 30 s, 72 °C for 40 s, and 1 s at 81 °C for plate reading. To ensure the reproducibility of qRT-PCR results, three independent experiments were carried out. The relative expression levels were calculated based on the threshold cycle according to the \(2^{-\Delta\Delta CT}\) method [36].

Analysis of CdCl₂ stress tolerance in JrVHAG1 transgenic Arabidopsis plants
The JrVHAG1 gene was inserted into Arabidopsis plants and three transgenic lines (G2, G6, and G9) with the highest expression level were analyzed [19]. For the seed germination assay, the seeds of WT, G2, G6, and G9 lines were placed on 1/2 Murashige and Skoog (MS) agar medium with 0 (control), or 50 μM CdCl₂, 3 μM ABA, 50 μM CdCl₂ plus 3 μM ABA (ABA+CdCl₂) and grown for another 10 d after which fresh weight and primary root length were recorded. For analysis of ROS accumulation and variations in physiological performance under above-described treatments, 12-day-old seedlings of WT, G2, G6, and G9 were grown on 1/2 MS agar medium and were transferred to 1/2 MS agar medium with 0 (control), 50 μM CdCl₂, 3 μM ABA, 50 μM CdCl₂ plus 3 μM ABA (ABA+CdCl₂) and grown in a greenhouse for additional 5-weeks. The seedlings were then treated with 0 (normal watered, control), or 50 μM CdCl₂, 3 μM ABA, 50 μM CdCl₂ plus 3 μM ABA (ABA+CdCl₂) for 6 d, then the aerial parts were collected to determine ROS generation. Histochemical staining for ROS generation in the four lines was performed using DAB and NBT methods [5, 34, 37]. Determination of physiological indices including H₂O₂ content, MDA, and proline levels, activity levels of SOD and POD, as well as EL rate was according to previous studies [19, 38]. Every assay was applied at least three times and each replicate contained at least 30 seedlings.

Identification and expression analysis of the JrVHAG1 promoter
The JrVHAG1 promoter was identified from the walnut genome [39], and amplified by PCR reaction from the J. regia DNA. The cis-elements in the JrVHAG1 promoter were analyzed using the PLANTCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [40]. To understand the expression activity of JrVHAG1 promoter, this promoter was used to replace the 35S promoter.
and cloned into a pCAMBIA1301 vector to drive the expression of GUS gene (Additional file 1: Figure S2). The recombinant construct was transferred into Arabidopsis through Agrobacterium-mediated floral dip method [41]. Four-week-old transgenic seedlings were used to study the expression activity and level through GUS activity determination and staining [5, 42] under normal, CdCl₂ and ABA stress [50]. Four-week-old transgenic seedlings were used to study the expression activity and level through GUS activity determination and staining [5, 42] under normal, CdCl₂ and ABA stress [50]. Every treatment was replicated three times and each replicate contained at least 30 seedlings.

Identification of the upstream regulator of JrVHAG1

The core sequence of MYBCORE motif is “CNGTTR”, and three MYBCORE elements (“CAGTTG” and “CAGTTA”) were found in the JrVHAG1 promoter (Additional file 1: Figure S3). Yeast one-hybrid assay was employed to identify the up-stream transcription factors (TFs) capable of recognizing the MYBCORE motif (“CAGTTG” was used in this study). Three tandem copies of “CAGTTG” were cloned into pHis2 vector (pHis2-MYB) (Additional file 1: Figure S4A). MYB TFs were identified from the J. regia transcriptome and cloned into pGADT7-Rec2 vector to generate a cDNA library for use in one-hybrid assays [42].

To confirm the interactions between the motif and positive clones, the MYBCORE “CAGTTG” was mutated to “CCAGGG” and inserted into pHis2 (pHis2-MYB-M). Fragments of the JrVHAG1 promoter including the MYBCORE motif (pHis2-MYB-S), excluding the MYBCORE motif (pHis2-MYB-M1), and including the mutated MYBCORE motif (pHis2-MYB-M2) were all cloned into pHis2, respectively (Additional file 1: Figure S4A). The p53His2 construct was used as a control in the yeast one-hybrid assays [5, 42]. All the primers used are listed in Additional file 1: Table S2.

Furthermore, pHis2-MYB/M/S/M1/M2 were independently fused with a CaMV35S-46 minimal promoter and cloned into pCAMBIA1301 to drive the GUS gene (reportors) expression (Additional file 1: Figure S4C) to confirm the above-described interactions. The ORF of JrMYB2 (screened TF) was cloned into prokII vector such that it is placed under the control of a 35S promoter (prokII-JrMYB2) (Additional file 1: Figure S4B), and can act as an effector. Every reporter was transiently co-transformed with the effector in tobacco leaves using Agrobacterium-mediated transformation method, and all co-transformed tobacco leaves were used to measure the GUS activity [42, 43]. Every co-transformation was replicated three times and every replicate contained at least 15 leaves.

**Statistical analysis**

All of the data were analyzed using the Statistical Package for Social Science (SPSS) (SPSS, Chicago, Illinois, USA). The differences between the transgenic and WT lines were evaluated using Tukey's multiple comparison test with the significance level set at p < 0.05, and sample variability is reported as standard deviation (S.D.).

**Additional file**

**Additional file 1: Table S1.** The primers used in qRT-PCR analysis. **Table S2.** The primers used in yeast one-hybrid assay and pCAMBIA1301 recombinant vector construction. **Figure S1.** The abiotic stress tolerance analysis of VHA-B, C, JrVHAG1, c1 and c4 in yeast expression system compared with empty pYES2 (CK) yeast. The six yeast cultures were independently grown in SC-Ura liquid medium containing 2% (w/v) galactose for 20 h at 30 °C to OD₆₀ₐ₅ = 0.4, then collecting and adjusting the yeast with SC-Ura including 2% galactose cultivated to OD₆₀₅ = 1.6 for stress analysis. Yeast cell densities (OD₆₀₅) of VHA-B, C, JrVHAG1, c1 and c4 transgenic yeasts and CK were treated with 50 μM ABA, or 150 μM CdCl₂, or 150 μM CdCl₂ plus 50 μM ABA (ABA+CdCl₂) for 0 or 24 h (0 h was set as control) were tested. All data are displayed as the mean ± S.D. of three independent experiments, the significant differences among the six yeasts were compared under the same treatment and indicated by a, b, c (p < 0.05), respectively. **Figure S2.** Mapping the reconstruct of JrVHAG1' promoter that inserted into pCAMBIA1301 vector. **Figure S3.** The promoter and elements of JrVHAG1 promoter. The MBS is the ‘MYBCORE’ element. **Figure S4.** The binding sites analysis of JrMYB2 to the MYBCORE motif. (A) Diagram of the reporter and effector vectors. Three tandem copies of the MYBCORE were inserted into the pHIS2 vector as the reportor vector. (B) The CDS of JrMYB2 was cloned into pGADT7-Rec2 as the effector construct. The effector and reporter constructs were co-transformed into the yeast strain Y187. (C) Diagram of the reportors and effectors. Triple tandem copies of the W-box were fused with the 35S CaMV46 minimal promoter and cloned into pCAMBIA1301 for driving the GUS gene as the reportor construct. The CDS of JrMYB2 was cloned into prokII under the control of the 35S promoter as the effector constructs. (DOC 1310 kb)

**Abbreviations**

3-AT: 3-amino-1, 2, 4-triazole; ABA: Abscisic acid; bHLH: Basic helix-loop-helix; CAGTTG: Promoter of JrVHAG1; CaMV35S: Cauliflower mosaic virus 35S promoter; CNTTTR: Core sequence of MYBCORE motif; DAB: 3,3′-Diaminobenzidine; DMSO: Dimethyl sulfoxide; DPA: 3-amino-1, 2, 4-triazole; DAB: 3,3′-Diaminobenzidine; DMSO: Dimethyl sulfoxide; DPA: 3-amino-1, 2, 4-triazole; 3-AT: 3-amino-1, 2, 4-triazole; ABA: Abscisic acid; bHLH: Basic helix-loop-helix; CTAB: Cetyltrimethylammonium ammonium bromide; DAB: 3,3′-Diaminobenzidine; EL: Electrolyte leakage; H₂O₂: Hydrogen dioxide; MDA: Malondialdehyde; MS: Murashige and Skoog; NBT: Nitrogen blue tetrazolium; POD: Peroxidase; qRT-PCR: Quantitative Real-Time PCR; ROS: Reactive oxygen species; S.D.: Standard deviation; SD: Synthetic drop-out medium; SOD: Superoxide dismutase

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article [and its supplementary information files].

**Authors’ contributions**

ZX wrote the paper and analyzed all the data; YG did the yeast one-hybrid assay and tested the physiological index; YG and WZ did the staining assays...
and all the expression analysis, YZ and GY designed and fund for the current study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
All the authors declare that they have no conflict of interest.

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