

Ectopic expression of the *Sesuvium portulacastrum* genes SpHDZ39 and SpHDZ41 enhances cadmium tolerance in *Arabidopsis*

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Abstract

The *homeodomain-leucine zippers* (HDZs) function as key regulators to regulating plant growth, development, and environmental responses. Its functions have been extensively investigated in many plant species, while the HDZs family in *Sesuvium portulacastrum* has not been thoroughly explored. We carried out a genome wide analysis of *S. portulacastrum* to identify and characterize the *SpHDZs*. The functional of SpHDZ39 and SpHDZ41 were investigated through subcellular localization, transient expression, and yeast one hybrid assays. Their roles in the response to cadmium stress were further analyzed using quantitative real time polymerase chain reaction and transgenic approaches. We identified 46 *SpHDZs* in the *S. portulacastrum* genome and classified into four subgroups. The *SpHDZs* were unevenly distributed across 21 chromosomes. Their physicochemical features were examined, including isoelectric points, hydrophobicity, stability, and predicted secondary and tertiary structures. Gene duplication analysis revealed that all identified duplications resulted from whole-genome or segmental events, and most gene pairs exhibited signs of purifying selection. Promoter analysis indicated the presence of numerous *cis*-elements associated with stress signaling and development. Expression profiling of heavy metal and salt treatment identified a subset of *SpHDZs* that respond to these treatments, of which, *SpHDZ39* and *SpHDZ41* showed significant induction under cadmium treatment. Both SpHDZ39 and SpHDZ41 functioned as transcription factors, and transgenic experiments demonstrated that they enhanced cadmium tolerance by promoting root growth and increasing biomass. These results contribute to a deeper understanding of *SpHDZs* function in halophytic species and provide candidate genes for future applications in low cadmium crop breeding.

Introduction

Sesuvium portulacastrum, a salt-tolerant herb that grows as either annual or perennial is widely found in coastal zones across tropics and subtropics, and exhibits remarkable resistance to drought stress and salinity (Sukhorukov et al., 2021). *S. portulacastrum* is well-adapted to saline environments, characterized by succulent, prostrate stems, adventitious roots arising from nodes enabling clonal propagation, and habitats such as sandy shores, limestone coastlines, salt flats, and marshes (Zhang et al., 2024). Its leaves are opposite and linear-lanceolate, equipped with epidermal wax coatings and water-storage tissues that collectively maintain cellular water balance under salt stress (Sukhorukov et al., 2018).

S. portulacastrum can efficiently utilize both sodium and potassium ions. Seedling growth remains largely stable even when exposed to high concentration of sodium chloride (NaCl), with only slight curling observed in young leaves. The plant rapidly accumulates osmoprotective substances in salt stress, which helps sustain photosynthetic performance (Muchate et al., 2016; Kulkarni et al., 2024). NaCl also alleviates cadmium (Cd) toxicity by change water content, redox balance, while promoting the synthesis of chelating agents like glutathione and proline (Mariem et al., 2014; Wali et al., 2016; Peng et al., 2019). This species shows strong environmental adaptability, including tolerance to heavy metal stress (Ghnaya et al., 2005; Lokhande et al., 2020). After copper (Cu) is absorbed by the roots, the plant

regulates metal ion homeostasis through transport proteins belonging to families such as ZIP, which control Cu uptake and internal distribution (Colangelo & Guerinot, 2006; Alagarasan et al., 2017). Under different salt conditions, *S. portulacastrum* is capable of retaining high levels of heavy metals in its root tissues (Bonaventure et al., 2025). Plant reduces oxidative damage caused by Cu through enhanced antioxidant enzyme activity, stimulation of MAPK signaling and ascorbate-glutathione cycle. Genes relate to metal binding and transport also contribute to Cu detoxification by directing it into vacuoles, where it is safely compartmentalized (Xiang et al., 2025). In addition to its physiological resilience, this halophyte is valued for its nutritional and medicinal properties. It is considered an edible marine plant, rich in minerals, vitamin C, vitamin K, and antioxidant substances (Mathieu & Meissa, 2007; Lokhande et al., 2009; Boxman et al., 2018;). High levels of phenolics and other bioactive compounds are responsible for its traditional use in treating diseases and suppressing microbial growth (Magwa et al., 2006; Chandrasekaran et al., 2011). From an ecological standpoint, *S. portulacastrum* contributes to environmental restoration. It is used in floating bed systems to absorb excess nitrogen and phosphorus from nutrient-rich waters and can also serve as feed in aquaculture settings (Liu et al., 2019; Senff et al., 2020). Its strong capacity for sodium absorption supports soil desalination, making degraded lands more suitable for the cultivation of salt-sensitive crops (Rabhi et al., 2010).

The *Homeodomain-Leucine Zippers* (HDZs) encode transcription factors central to plant growth, development, and environmental responses (Henriksson et al., 2005). These proteins are defined by a conserved homeodomain (HD), responsible for sequence-specific DNA binding, and a leucine zipper (LZ) domain that mediates dimerization and DNA recognition (Moens & Selleri, 2006). HDZs are classified into four subfamilies based on their structural motifs and functional diversity (Ariel et al., 2007). HDZ I and HDZ II subfamilies contain only HD and LZ domains, while HDZ III and HDZ IV include additional motifs such as the START domain, and in HDZ III, the MEKHLA domain, which are associated with hormonal and developmental signaling pathways (Ariel et al., 2007; Zhu et al., 2018).

HDZ I plays a key role in mediating abiotic stress responses (Gu et al., 2019; Li et al., 2022). *HDZ II* is primarily involved in regulating light signaling and morphogenesis (Qi et al., 2013), while *HDZ III* regulates apical meristem development, vascular patterning, and organ polarity (Turchi et al., 2015). The *HDZ IV* subgroup is implicated in epidermal differentiation, root hair formation, and cuticle development (Chew et al., 2013; Schrick et al., 2023). In *Arabidopsis*, *AtHB7* and *AtHB12* function antagonistically for aluminum stress (Liu et al., 2020). Specifically, *AtHB7* promotes stomatal closure to reduce water loss, while *AtHB12* facilitates stomatal opening and is beneficial for seed production (Olsson et al., 2004; Ré et al., 2014). Moreover, *AtHB21*, *AtHB40*, and *AtHB53* show upregulated expression under abiotic stress conditions (Skinner & Gasser, 2009). The transcripts of *OsHOX22* and *OsHOX24* are significantly upregulated in both rice cultivars, whereas *OsHOX6* shows only a slight increase in the drought-sensitive rice under prolonged drought conditions (Agalou et al., 2008). *OsHOX22* fine-tunes drought and salinity adaptation by regulating the ABA-signaling cascade. (Zhang et al., 2012). The expression of *OsHOX19* is enhanced by drought stress application in both drought-sensitive and drought-resistant cultivars (Harris et al., 2011). Overexpression of *OsTF1L* has been shown to promote lignin biosynthesis and stomatal closure, improving drought tolerance (Bang et al., 2019).

In this work, we identified that 46 *SpHDZs* in the *S. portulacastrum* genome. These genes were classified into four subgroups, comprising 25 members in subgroup I, 6 members in subgroup II, 6 members in subgroup III, and 9 members in subgroup IV. The identified *SpHDZs* were unevenly distributed across 21 chromosomes, and both intra- and inter-species collinearity relationships were observed. Protein sequence analysis revealed the presence of six conserved motifs and structural domains, reflecting evolutionary conservation and gene duplication events. The exons number was ranged from 2 to 19, while introns varied between 1 and 5. The encoded proteins had lengths between 215 and 865 amino acids, with predicted molecular weights ranging from 24,651.80 to 94,974.01 Da. The theoretical isoelectric points (pI) were found to vary from 4.56 to 9.07. In addition, physicochemical analyses demonstrated wide variation in instability indices, aliphatic indices, and GRAVY values, which ranged from 43.50 to 75.89, 54.65 to 86.69, and - 1.174 to -0.062, respectively. *Cis*-element on the promoters associated with light responsiveness, hormone signaling, stress responses, and developmental processes. Transcriptome profiling under Cd, Cu, and NaCl treatment indicated that the expression of *SpHDZs* were differentially regulated in response to these abiotic stress. The expression pattern of *SpHDZ39* and *SpHDZ41* were further validated by quantitative real-time PCR. In addition, subcellular localization and yeast one-hybrid assays confirmed that *SpHDZ39* and *SpHDZ41* function as transcription factors. Their overexpression in *A. thaliana* enhanced Cd tolerance. Overall, our results suggest that *SpHDZs* play important roles in Cd stress and may provide genetic resources for improving Cd tolerance in crops through molecular breeding strategies.

Materials And Methods

Identification of *SpHDZs* in *S. portulacastrum* genome

The reference genome of *S. portulacastrum* was obtained from the laboratory at Guizhou University. *AtHDZs* sequences from *Arabidopsis thaliana*, available in the TAIR database, were used as queries to blast in the *S. portulacastrum* proteome using TBtools software. Candidate gene sequences were validated through the identification of conserved Pfam domains, specifically PF00046 and PF02183.

Phylogenetic and sequence analysis

Protein sequences of the identified *SpHDZs*, together with *AtHDZs* from *A. thaliana* and *OsHDZs* from *O. sativa*, were aligned and constructed a phylogenetic tree in MEGA11 as described previously (Wu et al., 2025). The tree was edited and graphically refined using Adobe Illustrator. Protein subcellular localization was predicted using the WoLF PSORT online tool. Physicochemical properties such as molecular weight and isoelectric point were analyzed through the ProtParam server. Motif structures were identified using the MEME suite under default settings.

Promoter analysis of *cis*-acting elements

Promoter sequences extending 2,000 bps upstream of the start codon of each *SpHDZ* were extracted and analyzed for *cis*-acting elements using the PLANTCARE database. The results were visualized using

the Simple BioSequence Viewer module in TBtools. To assess the distribution of regulatory elements, a statistical summary was generated using PivotCharts in Microsoft Excel, and a heatmap representing the abundance and classification of elements was produced using the HeatMap module in TBtools.

Synteny analysis of the SpHDZs

Syntenic relationships among *SpHDZs* within the *S. portulacastrum* genome and between *S. portulacastrum* and *A. thaliana* were investigated using TBtools. Data required for MCScanX-based analysis were processed using the File Merger module. The synteny maps were visualized with the Circle Gene Viewer function.

Chromosomal localization of SpHDZs

Chromosomal distribution of *SpHDZs* was analyzed using TBtools. The genomic annotation file and filtered gene list were imported, and genes were positioned based on their physical coordinates along the chromosomes. The resulting map was adjusted for clarity and visualization quality.

Structural and evolutionary characterization

The secondary structure of each SpHDZ protein was predicted using SOPMA, and the tertiary structure was modeled with SWISS-MODEL. Gene duplication patterns were determined based on synteny analysis, and types of duplication events were identified accordingly. Non-synonymous (Ka) and synonymous (Ks) substitution rates were calculated in TBtools to evaluate evolutionary pressure among gene pairs.

Expression pattern of SpHDZ39 and SpHDZ41

To examine the expression of *SpHDZ39* and *SpHDZ41* under Cd treatment, *S. portulacastrum* seedlings were treated with 200 μ M Cd for 0, 7, 14, and 21 days. Total RNA was extracted from root tissues using a commercial RNA extraction kit. First-strand cDNA was synthesized using a reverse transcription kit. The *SpGAPDH* was used as an internal control. Quantitative real-time PCR was performed with three biological and three technical replicates per treatment group with gene specific primers (Table S5). Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Subcellular localization of SpHDZ39 and SpHDZ41

The coding sequences of *SpHDZ39* and *SpHDZ41* without stop codons were cloned into vectors, generating 35S::SpHDZ39-GFP and 35S::SpHDZ41-GFP constructs with gene specific primers (Tab S5). These constructs were transformed into *Agrobacterium tumefaciens* and infiltrated into tobacco leaves. After dark incubation, fluorescence signals were observed at 72 hours post-inoculation using a Leica TCS SP8 confocal microscope (Leica, Wetzlar, Germany). The 35S::TksARF3-mCherry construct served as a nuclear marker for co-localization assessment (Wu et al., 2025).

DNA-binding activity of SpHDZ39 and SpHDZ41

To assess DNA-binding activity, the coding regions of *SpHDZ39* and *SpHDZ41* were cloned into the pGADT7-rec2 vector with gene special primers (Table S5). Synthetic DNA sequences containing triple tandem repeat binding motifs, including MA0573, MA0573_mutant, MA0008, and MA0008_mutant, were inserted individually into the pHIS2 vector with gene special primers (Table S5). These constructs were co-transformed into *Saccharomyces cerevisiae* strain Y187 using a polyethylene glycol and lithium acetate protocol. Protein-DNA interaction was assessed by growing transformants on SD/-Leu/-Trp (DDO) and SD/-His/-Leu/-Trp (TDO) media. Plates supplemented with 50 mM 3-aminotriazole were used for further validation of DNA-binding specificity.

Plant transformation

The constructs 35S::SpHDZ39-GFP and 35S::SpHDZ41-GFP were introduced into *Agrobacterium tumefaciens* GV3101. Transformation of *A. thaliana* was performed using the floral dip method, and the resulting seeds were collected for subsequent selection and analysis.

Results

Genome-wide identification and classification of SpHDZs

A comprehensive genome-wide analysis uncovered 46 HDZs in *S. portulacastrum*. Based on phylogenetic relationships with *A. thaliana* and *O. sativa*, these genes were categorized into HDZ I, HDZ II, HDZ III, and HDZ IV subgroups (Elhiti & Stasolla, 2009). Among them, 25 *SpHDZs* belonged to subgroup I, 6 to subgroup II, another 6 to subgroup III, and 9 to subgroup IV (Fig. 1).

Based on chromosomal locations and gene annotations, the 46 *SpHDZs* were renamed as *SpHDZ1* to *SpHDZ46*, sequentially (Table S1). Among them, *SpHDZ16* encodes the longest protein, consisting of 865 amino acids and exhibiting the highest molecular weight, whereas *SpHDZ34* encodes the shortest protein, with 215 amino acids and the lowest molecular weight. The predicted isoelectric points (pI) of the *SpHDZs* ranged from 4.56 (*SpHDZ14*) to 9.07 (*SpHDZ19*). All *SpHDZs* displayed negative grand average of hydropathicity (GRAVY) values, ranging from -1.174 (*SpHDZ41*) to -0.062 (*SpHDZ4*), suggesting that these proteins are generally hydrophilic. The aliphatic index values varied between 54.65 (*SpHDZ39*) and 86.69 (*SpHDZ17*), while all proteins had instability index values above 43.50 (*SpHDZ26*) and below 75.89 (*SpHDZ7*), indicating potential instability *in vitro*. Subcellular localization predictions indicated that 41 *SpHDZs* are primarily localized in the nucleus. Notable exceptions include *SpHDZ17*, *SpHDZ35*, and *SpHDZ43*, which are predicted to be localized in the cytoplasm, as well as *SpHDZ22* and *SpHDZ33*, which are likely localized in the chloroplast.

Chromosomal distribution and synteny

All 46 *SpHDZs* were successfully mapped across 21 chromosomes in *S. portulacastrum* (Fig. 2). The highest gene density was found on Chr7, Chr10, and Chr11, each containing four *SpHDZs*. Chr2, Chr3, and Chr5 each harbored three genes. Chr1, Chr8, Chr12, Chr13, Chr14, Chr18, Chr20, Chr21, Chr23, and Chr24 contained two *SpHDZs*, while Chr4, Chr15, Chr16, Chr17, and Chr19 were each associated with only one *SpHDZ* (Fig. 2).

To explore the structural diversity and potential functional roles of *SpHDZs*, conserved domain analysis was conducted. Members within the same subgroup generally exhibited similar domain architectures, whereas marked variation was observed among different subfamilies. The HD and LZ domains were consistently present in most *SpHDZ* I proteins, aligning with findings in other species. An exception was *SpHDZ34*, which lacked the LZ domain despite being classified in the *SpHDZ* I group. All six *HDZ* II proteins retained the LZ domain; however, only *SpHDZ1* possessed the HD domain, while the remaining members, *SpHDZ13*, *SpHDZ19*, *SpHDZ25*, *SpHDZ30*, and *SpHDZ38*, lacked it. Members of the *HDZ* III and IV subfamilies all contained both HD and START domains. Notably, *HDZ* III members also harbored a C-terminal MEKHLA domain, indicating a higher degree of structural complexity and possible functional divergence (Fig. 3B).

Motif analysis revealed that Motif1, Motif2, and Motif3 were highly conserved across all *SpHDZs*. Functional annotation showed that Motif1 and Motif2 together formed the canonical HD domain (Fig. 3A).

Gene structure analysis demonstrated that varying exon-intron organizations across subfamilies. Most *SpHDZs* contained 1 to 5 introns, but exon numbers ranged from 2 to 19. The *HDZ* III subgroup showed the most complex gene structure, with 18 to 19 exons. This was followed by *HDZ* IV, whose members had 8 to 11 exons. In contrast, *HDZ* I and II members generally exhibited simpler gene structures, typically containing only 2 to 4 exons (Fig. 3C).

Cis-acting regulatory element analysis of the *SpHDZs*

Cis-acting regulatory elements are crucial for transcriptional regulation and directly impact gene function. To investigate potential regulatory mechanisms, the 2,000 base pairs upstream promoter regions of *SpHDZs* were analyzed for the presence of *cis*-acting regulatory elements (Fig. 4A). Identified elements were grouped into light-responsive, hormone-responsive, biotic/abiotic stress-responsive, and plant growth and development-related elements (Fig. 4B).

Among these, hormone-responsive elements were the most prevalent, totaling 248, with jasmonate-responsive elements (JAREs) being the most dominant, present in over 59% of the genes. Light-responsive elements were the second most common, followed by stress-related elements, which included MYB-DRE (41.17%), ARE (35.88%), LTRE (11.76%), DSRE (7.06%), and ANX-Enh (4.12%). The plant development-related elements group was the least represented, with meristem-related motifs being the most enriched within this category.

Collinearity analysis of the SpHDZs

Collinearity analysis using MCScanX identified 39 collinear gene pairs within the *SpHDZs*, spanning all four subfamilies (Fig. 5B). These duplication events, primarily resulting from whole-genome duplication (WGD) or segmental duplication (Table S2), likely contributed to genome expansion and evolutionary diversification. The polyploidization or local genomic rearrangements driven events may enhance genetic variability, promoting functional innovation and adaptive responses.

To evaluate evolutionary pressures acting on these duplicated genes, nonsynonymous/synonymous substitution rate (K_a/K_s) ratios were calculated (Table S3). It showed that most gene pairs showed K_a/K_s values between 0.03 and 0.88 ($K_a/K_s < 1$), indicating that purifying selection has predominantly preserved protein function by eliminating deleterious mutations. Notably, only *SpHDZ7/SpHDZ8* pair exhibited a K_a/K_s ratio of 1.09, suggesting positive selection, which may have driven functional divergence through advantageous amino acid changes. Structural modeling of these collinear gene products revealed substantial tertiary structural conservation, reinforcing the presence of functional constraints. Furthermore, interspecies synteny analysis between *S. portulacastrum* and *A. thaliana* uncovered 57 orthologous gene pairs, while *SpHDZ24*, *SpHDZ30*, and *SpHDZ45* were lacked identifiable syntenic counterparts (Fig. 5A). This high level of conservation reflects shared evolutionary ancestry and underscores the structural and functional preservation of the *SpHDZ* family across plant species.

Expression profiling of SpHDZs under different Abiotic stress conditions

To assess the transcriptional responses of *SpHDZs* to salt stress, RNA-seq data from salt-treated *S. portulacastrum* leaves were analyzed under varying NaCl concentrations (Fig. 6C). The results revealed diverse expression trends, with both upregulation and downregulation observed across different genes as salt levels increased. The *SpHDZ1*, *SpHDZ6*, *SpHDZ7*, *SpHDZ8*, *SpHDZ10*, *SpHDZ24*, *SpHDZ26*, *SpHDZ29*, *SpHDZ30*, *SpHDZ34*, *SpHDZ40*, and *SpHDZ41* were exhibited marked upregulation under salt concentrations. Notably, 10 of these genes belonged to the HDZ I subgroup, while *SpHDZ1* and *SpHDZ30* were classified under HDZ II. Conversely, *SpHDZ5*, *SpHDZ16*, *SpHDZ20*, *SpHDZ22*, *SpHDZ23*, and *SpHDZ32* were displayed consistent downregulation. Several other genes showed peak expression at intermediate salt concentrations.

To explore the involvement of *SpHDZs* in heavy metal stress responses, transcriptomic data were analyzed from roots and leaves subjected to Cd and Cu treatment. The *SpHDZ6*, *SpHDZ13*, *SpHDZ14*, *SpHDZ29*, and *SpHDZ38* were upregulated with prolonged Cd exposure compared with control conditions in roots (Fig. 6A). However, most genes were also upregulated at 7 days of Cd exposure compared with control, while *SpHDZ4*, *SpHDZ5*, *SpHDZ6*, *SpHDZ14*, *SpHDZ17*, and *SpHDZ33* maintained elevated expression at either 7 days, 14 days, or 21 days, compared with control in leaves (Fig. 6A). In contrast, *SpHDZ10*, *SpHDZ23*, *SpHDZ25*, *SpHDZ31*, *SpHDZ32*, and *SpHDZ45* showed downregulated with Cd treatment compared with control. Furthermore, the expression of *SpHDZ39* and *SpHDZ41* under Cd treatment was validated by qRT-PCR (Fig. 6D). Data analysis revealed that the expression level of *SpHDZ39* showed a gradual downregulation trend with the extension of Cd stress duration. In contrast,

SpHDZ41 exhibited an upregulation at 7 days of treatment, followed by a downregulation relative to the control group as the stress duration prolonged. Similarly, *SpHDZ5*, *SpHDZ15*, *SpHDZ16*, and *SpHDZ17* showed upregulation at 50 μ M Cu treatment compared with control, *SpHDZ7*, *SpHDZ8*, *SpHDZ26*, *SpHDZ29*, and *SpHDZ41* showed upregulation at 100 μ M Cu treatment, *SpHDZ4*, *SpHDZ6*, *SpHDZ18*, *SpHDZ24*, and *SpHDZ46* were induced at 200 μ M Cu treatment, compared with control in roots, respectively (Fig. 6B). In leaves, exposure to 50 μ M Cu elicited significant upregulation of *SpHDZ12*, *SpHDZ23*, *SpHDZ32*, and *SpHDZ34* compared with untreated controls. Treatment with 100 μ M Cu induced the expression of *SpHDZ10*, *SpHDZ14*, and *SpHDZ40*. Following 200 μ M Cu exposure, *SpHDZ6*, *SpHDZ7*, *SpHDZ8*, *SpHDZ29*, and *SpHDZ41* were markedly upregulated, whereas *SpHDZ2*, *SpHDZ11*, *SpHDZ15*, *SpHDZ20*, *SpHDZ22*, *SpHDZ23*, *SpHDZ39*, and *SpHDZ45* exhibited significant downregulation relative to control levels (Fig. 6B). Those results suggested that different *SpHDZs* may response to different abiotic stress.

SpHDZ39 and SpHDZ41 function as transcription factors

SpHDZ39 and SpHDZ41 were predicted to be localized in the nucleus based on WOLF PSORT analysis (Table S1), and this prediction was experimentally confirmed through transient expression assays in tobacco leaves (Fig. 7A). Constructs expressing SpHDZ39-GFP or SpHDZ41-GFP were co-expressed with the nuclear marker TksARF3-mCherry in leaf epidermal cells. The observed overlap of fluorescence signals confirmed that both proteins were localized in the nucleus.

To further examine their DNA-binding capacity, yeast one-hybrid assays were conducted. Yeast cells co-transformed with pHIS2-MA0573 or pHIS2-MA0008 and pGADT7-rec2-SpHDZ39 grew normally on TDO medium (Fig. 7B). In contrast, yeast cells carrying the mutant versions of these sequences or the negative control failed to grow under the same conditions. Similar binding behavior was observed for SpHDZ41 (Fig. 7C), indicating that both transcription factors specifically recognize and bind these DNA motifs. Taken together, those results showed that both SpHDZ39 and SpHDZ41 function as a transcription factor.

Overexpression of SpHDZ39 and SpHDZ41 improved Cd tolerance in *A. thaliana*

We generated *A. thaliana* plants transgene overexpressing *SpHDZ39* (*SpHDZ39OE*) and *SpHDZ41* (*SpHDZ41OE*) (Fig. 8A). Segregating T₃ plants from two independent *SpHDZ39OE* and *SpHDZ41OE* plants were subjected to 75 μ M Cd treatment. Both *SpHDZ39OE* and *SpHDZ41OE* plants exhibited stronger growth compared with wild-type plants (Fig. 8B). The average root length of *SpHDZ39OE* plants ranged from 2.2 ± 0.2 cm to 2.4 ± 0.3 cm, while wild-type plants showed an average of 1.8 ± 0.2 cm (Fig. 8C). *SpHDZ41OE* plants displayed longer roots ranging from 4.2 ± 0.3 cm to 4.9 ± 0.2 cm, compared with 4.0 ± 0.1 cm in wild type (Fig. 8C). Fresh weight measurements also indicated enhanced biomass. *SpHDZ39OE* plants reached 1.4 ± 0.1 mg/plant to 1.6 ± 0.2 mg/plant, whereas wild-type plants averaged 1.2 ± 0.2 mg/plant (Fig. 8D). *SpHDZ41OE* plants showed values between 1.7 ± 0.2 mg/plant to 1.9 ± 0.2 mg/plant compared with 1.5 ± 0.1 mg/plant in wild type (Fig. 8D). Taken together, those results suggest

that *SpHDZ39* and *SpHDZ41* enhance Cd tolerance in *A. thaliana* by promoting root growth and biomass accumulation.

Discussion

HDZ is an evolutionarily conserved transcription factor that have been identified in Arabidopsis, rice, maize, soybean, wheat, and tomato (Henriksson et al., 2005; Kim et al., 2008; Chen et al., 2014; Zhang et al., 2014; Yue et al., 2018). In this study, we present the first comprehensive identification and characterization of 46 *SpHDZs* in the *S. portulacastrum* genome through systematic bioinformatics analysis.

The gene number in *S. portulacastrum* is equal to that in *Arabidopsis*, suggesting possible evolutionary and functional parallels between the two species. From an evolutionary perspective, the identical number of *HDZs* may reflect conserved gene retention, with minimal gene loss or expansion across lineages. This pattern supports the notion that the *HDZs* has undergone strong evolutionary constraints, preserving its core structure across species. Functionally, the conserved gene number implies that similar molecular mechanisms may operate in both species to regulate development and environmental adaptation. This finding lays the groundwork for future investigations into the molecular mechanisms underlying stress tolerance in *S. portulacastrum*. Previous studies have indicated that the abundance of *HDZs* are not directly related to genome size but is shaped by tandem and segmental duplication events (Zhao et al., 2011). Phylogenetic and domain structure analyses classified the 46 identified genes into four subfamilies, with HDZ I containing the largest number of members. This classification is consistent with results in other plants, supporting the evolutionary conservation of the *HDZs*.

Gene structure and motif composition analyses revealed that members of the HDZ I subgroup contain highly conserved HD and LZ domains. In contrast, HDZ III and IV subfamilies include START domains, and HDZ III genes additionally contain MEKHLA domains at their C-terminus, consistent with patterns reported in other species. The structural features vary significantly among subfamilies, suggesting potential functional divergence. Subcellular localization prediction showed that most *SpHDZs* are localized in the nucleus, while a few are present in the cytoplasm or chloroplast. Transient expression assays confirmed that *SpHDZ39* and *SpHDZ41* are nuclear proteins, with DNA-binding activities supported by yeast one-hybrid assays. These results indicate that these genes likely function as transcriptional regulators.

Collinearity analysis revealed 57 syntenic gene pairs between *S. portulacastrum* and *Arabidopsis*, including 38 pairs involving *HDZs*. This indicates limited gene family expansion and suggests a relatively stable evolutionary trajectory for the HDZ family in *S. portulacastrum*.

Cis-element analysis of promoter regions showed that *SpHDZs* are associated with a wide range of regulatory functions, including hormone response, abiotic and biotic stress responses, developmental control, and light signaling (Zhou et al., 2025). Notably, drought-related elements were more frequently enriched in the HDZ III and IV subfamilies. This contrasts with patterns reported in other plants, where

HDZ I genes typically play central roles in drought response (Li et al., 2022). For example, in *Arabidopsis*, AtHB7 and AtHB12, which belong to HDZ I, are involved in ABA-mediated regulation of stomatal movement and osmotic balance under drought conditions. This divergence suggests that, in *S. portulacastrum*, HDZ III and IV genes may have evolved to compensate for or replace some of the functions typically attributed to HDZ I genes. Given that *S. portulacastrum* naturally inhabits saline environments, this may represent a unique adaptation shaped by long-term exposure to salt stress. In *Zea mays*, overexpression of *ZmHDZ9* enhances drought resistance in transgenic *Arabidopsis* (Qiu et al., 2022), further supporting the idea of functional plasticity within HDZ subfamilies. These findings raise the possibility that HDZ III and IV genes in *S. portulacastrum* may have been selectively co-opted for stress regulation. Further functional studies are needed to confirm this hypothesis.

Transcriptome data under NaCl treatment revealed 13 *SpHDZs* responsive to salt stress. Among these, *SpHDZ1* and *SpHDZ30* belonged to the HDZ II subgroup, while the remaining 11 genes were classified under HDZ I. This pattern aligns with findings in soybean, where most salt-induced *HDZs* also belong to subgroup I and II (Chen et al., 2014). It has been reported that sodium ions stimulate V-ATPase activity, promoting the accumulation of soluble sugars in vacuoles and chloroplasts to maintain osmotic balance (Yi et al., 2014). These processes enhance salt tolerance in *S. portulacastrum*. While both sodium ions and chloride ions are toxic to most plants (Adams & Shin, 2014; Lutts & Lefèvre, 2015), in this halophyte, chloride ions appear to be the main contributor to toxicity, while sodium ions have been shown to promote shoot development and leaf succulence (Wang et al., 2012). Under NaCl, Cd, and Cu treatment, multiple *SpHDZs* exhibited altered expression. Notably, *SpHDZ6* from subgroup I was upregulated in response to all three treatments, suggesting a central role in multistress tolerance, consistent with previous findings regarding HDZ I function in salt adaptation (Li et al., 2022). qRT-PCR results showed that *SpHDZ39* expression declined progressively under Cd stress, while *SpHDZ41* initially increased at day 7, followed by a decrease over time. A similar regulatory pattern has been observed for *Oshox22*, which influences ABA biosynthesis and mediates stress responses in rice (Zhang et al., 2012). Conversely, overexpression of *JcHDZ21* has been shown to increase salt sensitivity in *Arabidopsis* (Tang et al., 2023), indicating that HDZ function can vary significantly depending on genetic context. Additional genes such as *SpHDZ4*, *SpHDZ14*, *SpHDZ24*, and *SpHDZ29* also responded to multiple stress treatments, although their precise functions remain unclear. Further experimental validation is required to explore their roles in regulating plant responses to environmental challenges. These genes may offer valuable targets for enhancing crop resistance to Cd stress through genetic improvement strategies.

Conclusions

We identified 46 *SpHDZs* in *S. portulacastrum*, distributed unevenly across 21 chromosomes. Based on gene structures and conserved motifs, these *SpHDZs* were classified into four subgroups, each associated with distinct domain features. All duplication events identified were attributed to whole-genome or segmental duplication, and 39 duplicated gene pairs were detected. Among these, nearly all gene pairs had Ka/Ks ratios below one, indicating that purifying selection has maintained their sequence conservation. Promoter analysis revealed the presence of 703 *cis*-regulatory elements, which were

categorized into four functional types related to light response, hormone signaling, environmental stress, and developmental regulation. These elements were distributed unevenly among the gene subgroups, suggesting potential functional divergence. Expression profiling identified twelve *SpHDZs* involved in salt stress responses and twelve others responsive to heavy metal treatment. Further verification using qRT-PCR confirmed that *SpHDZ39* and *SpHDZ41* showed altered expression patterns under Cd treatment. Subcellular localization predictions showed that most SpHDZs are targeted to the nucleus, with a few located in the chloroplast or cytoplasm. Experimental validation further confirmed that SpHDZ39 and SpHDZ41 act as nuclear transcription factors, as demonstrated by localization and yeast one-hybrid assays. Overexpression of these genes in *A. thaliana* enhanced Cd tolerance, supporting their role in stress regulation. These findings contribute to a deeper understanding of the molecular mechanisms underlying abiotic stress adaptation in *S. portulacastrum*. The identified genes offer promising targets for enhancing Cd tolerance in crops through genetic engineering and molecular breeding strategies.

Declarations

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Author Contributions

S. H. and T. C. initiated and designed the project, S. H., T. C., Y. L., X. W., F. F., Y. L., Y. Y., G. Z., Z. N., and Y. W. reviewing the referenced articles and preparing the Figures and tables, S. H. and T. C. writing and revising the manuscript. All authors approved the final version of the text.

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Data Availability Statement

Data will be made available on request.

Ethical approval and consent to participate Not applicable.

Consent for publication All authors have read and approved the final version of the manuscript and consent to its submission for publication. The authors affirm that this work is original and has not been published previously, nor is it currently under consideration for publication elsewhere.

Competing interests The authors declare no competing interests.

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Figures

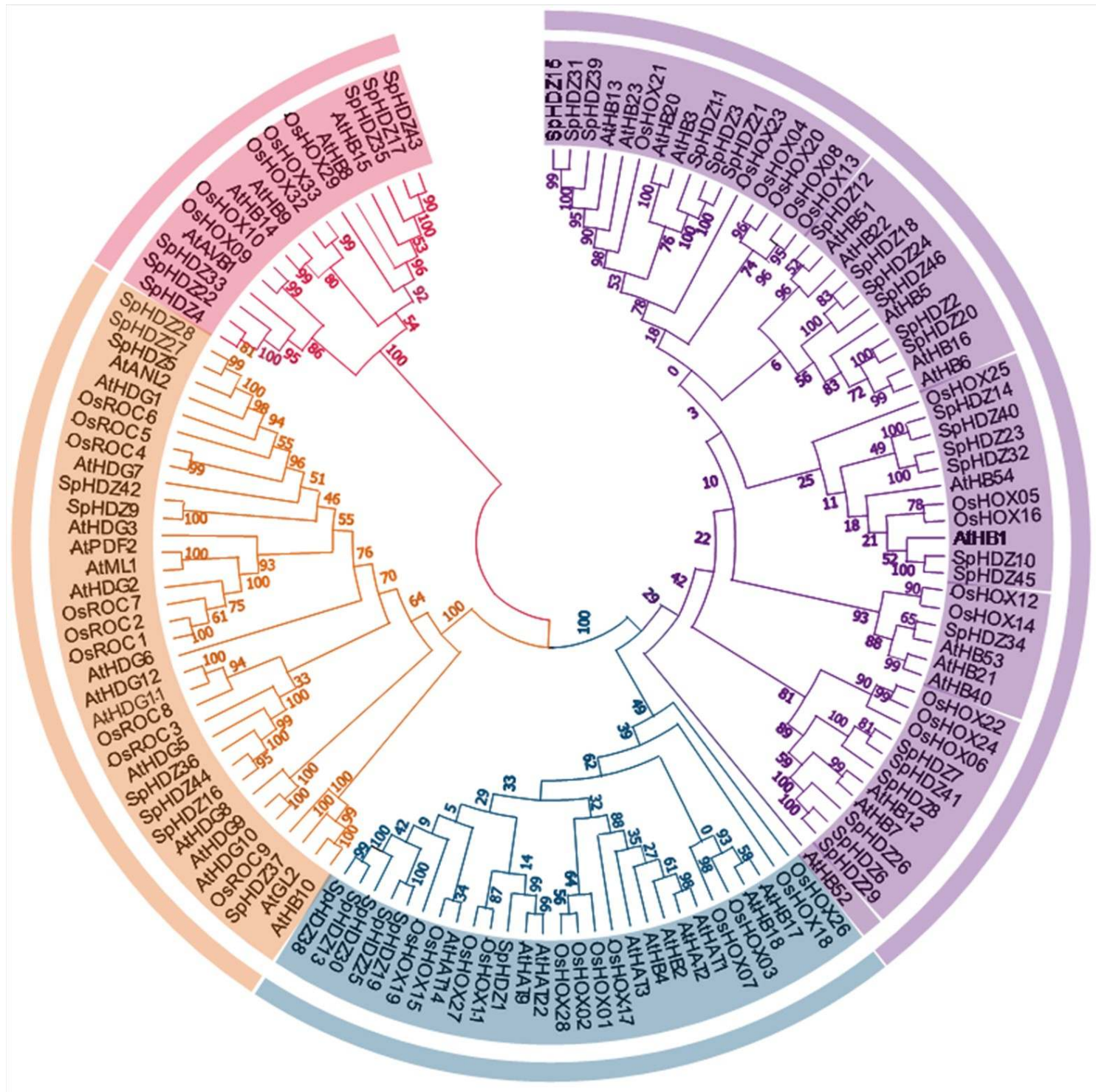


Figure 1

Phylogenetic analysis of HDZs in *S. portulacastrum*, *A. thaliana* and *O. sativa*. The phylogenetic tree was constructed *via* the Neighbor Joining method with 1000 bootstrap replicates by MEGA7. The number on the node indicates the supported percentage. Subclades are indicated by different colors.

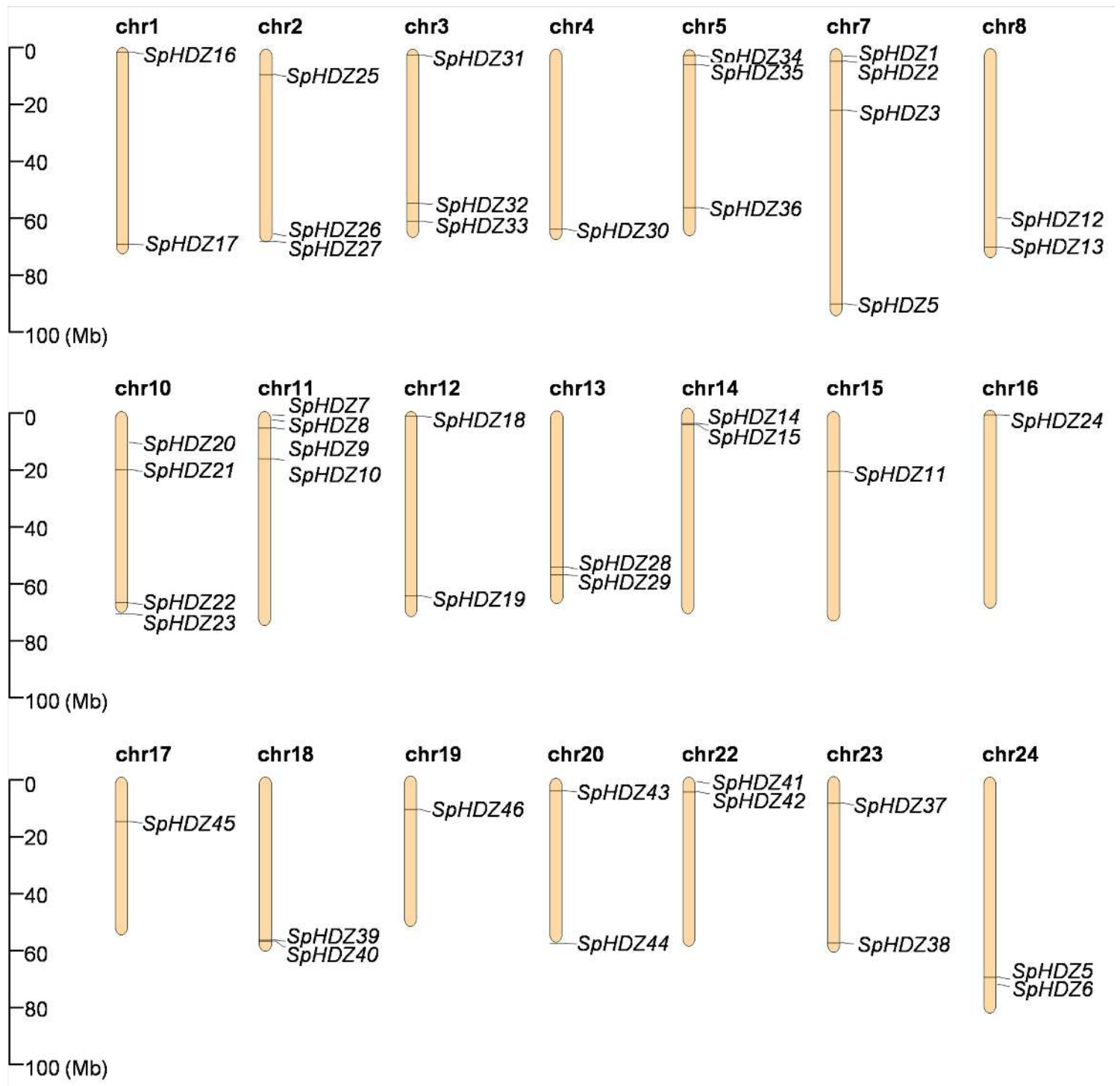


Figure 2

Chromosome distribution of *SpHDZ* genes in *S. portulacastrum*.

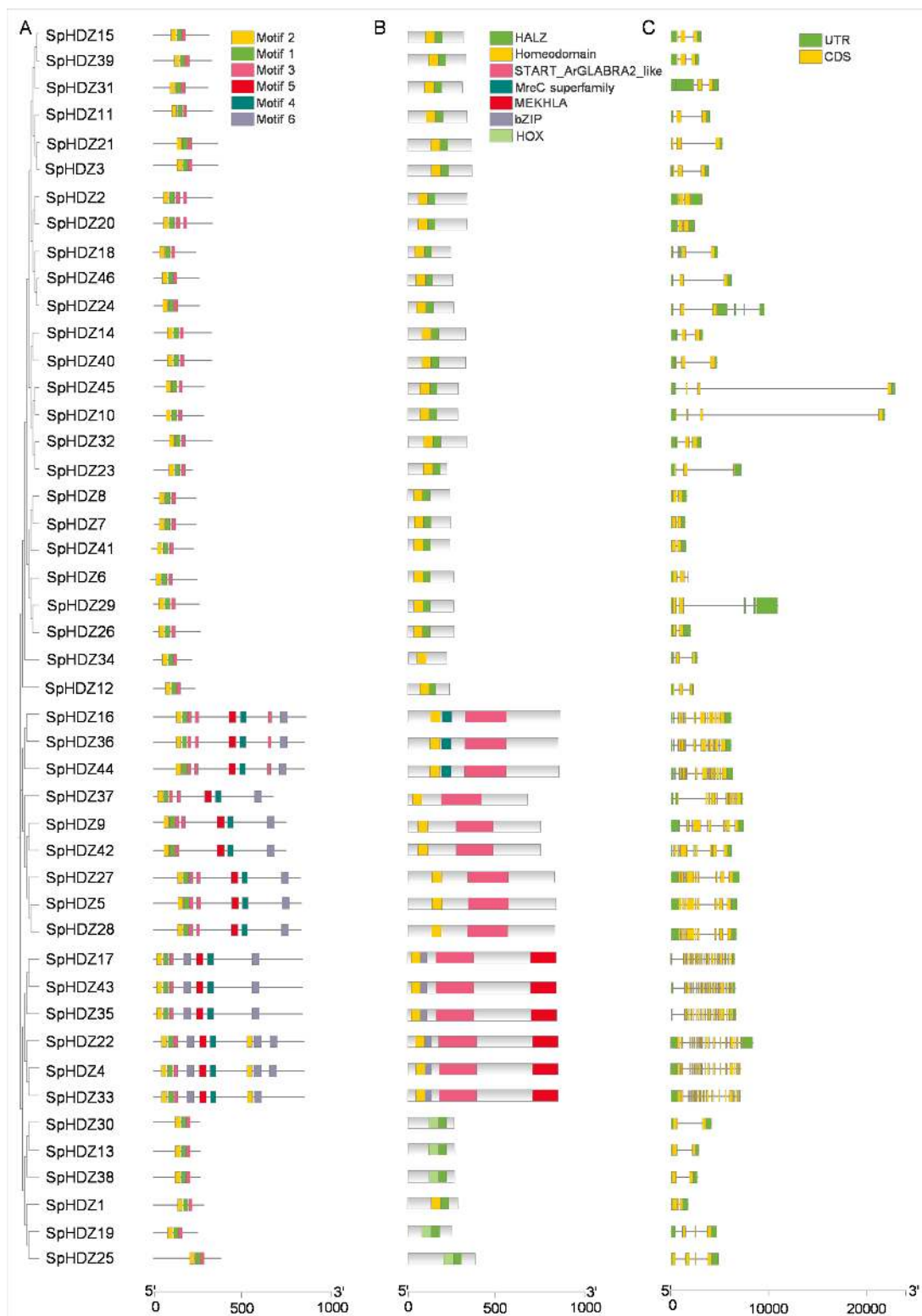


Figure 3

Gene and protein structure of 46 SpHDZs. A. Motifs in the detected 46 SpHDZs. B. Conserved structural domains in the 46 SpHDZs. C. Gene structure analysis of the 46 *SpHDZs*. The horizontal coordinate represents the length of the gene/amino acid sequence.

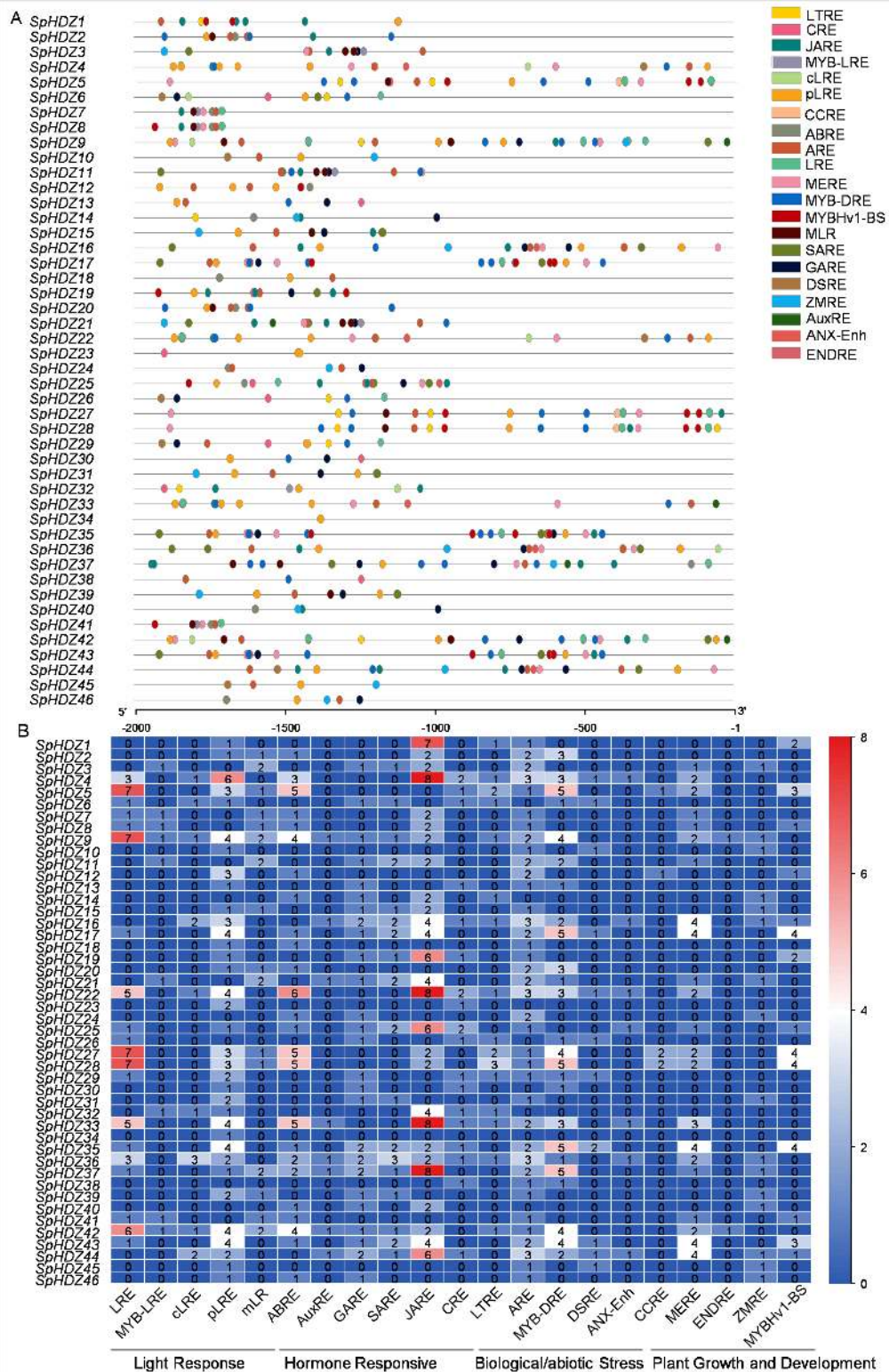


Figure 4

Predicted *cis*-elements in the promoter regions of *SpHDZs*. A. Category of *cis*-elements in *SpHDZs* promoter. B. Number of *cis*-elements in *SpHDZs* promoter.

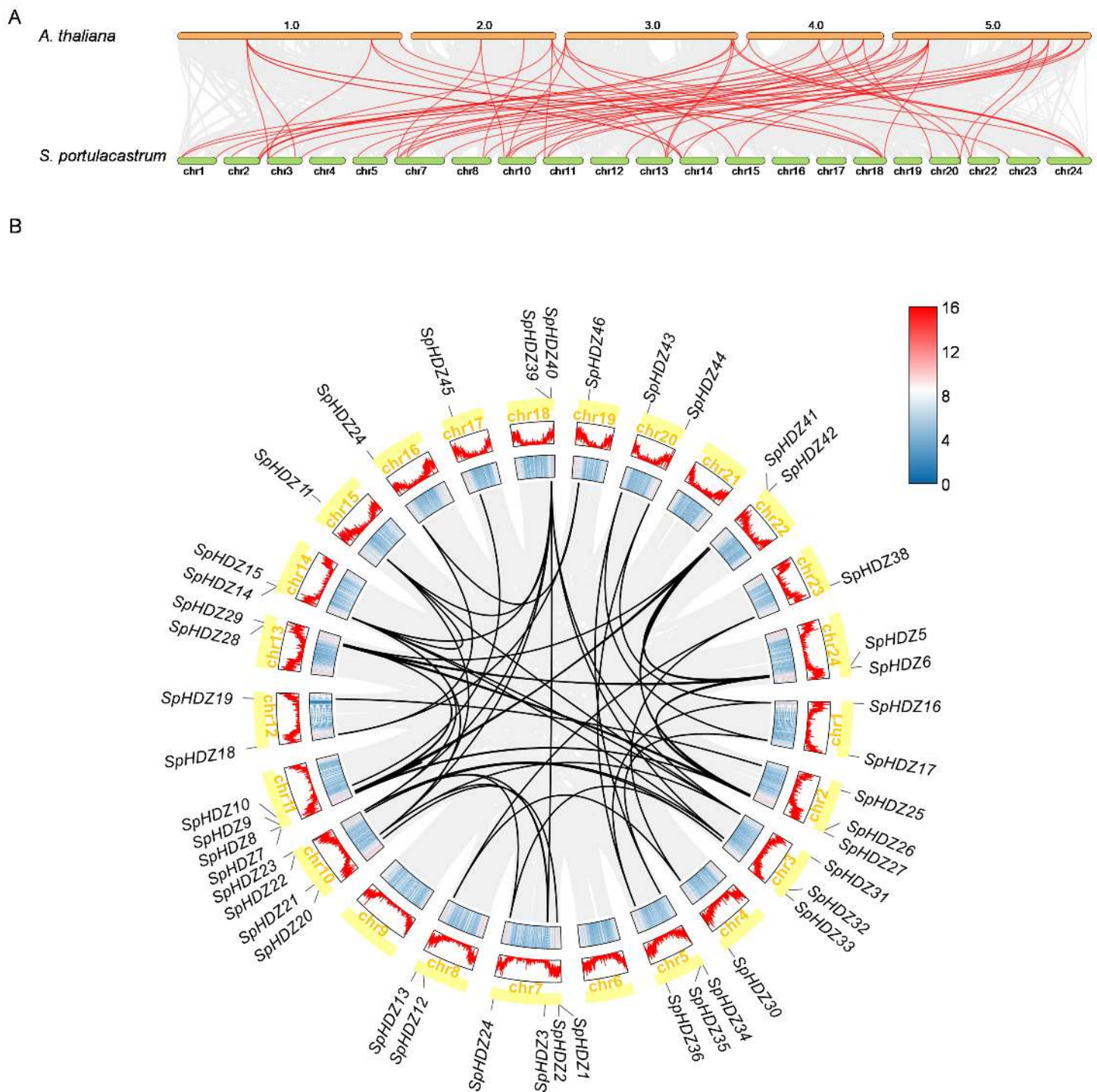


Figure 5

Collinearity analysis of *HDZs* in *S. portulacastrum* and *A. thaliana*. A. Collinearity analysis of *HDZs* between *S. portulacastrum* and *A. thaliana*. B. Synteny analysis of *SpHDZs* in *S. portulacastrum*.

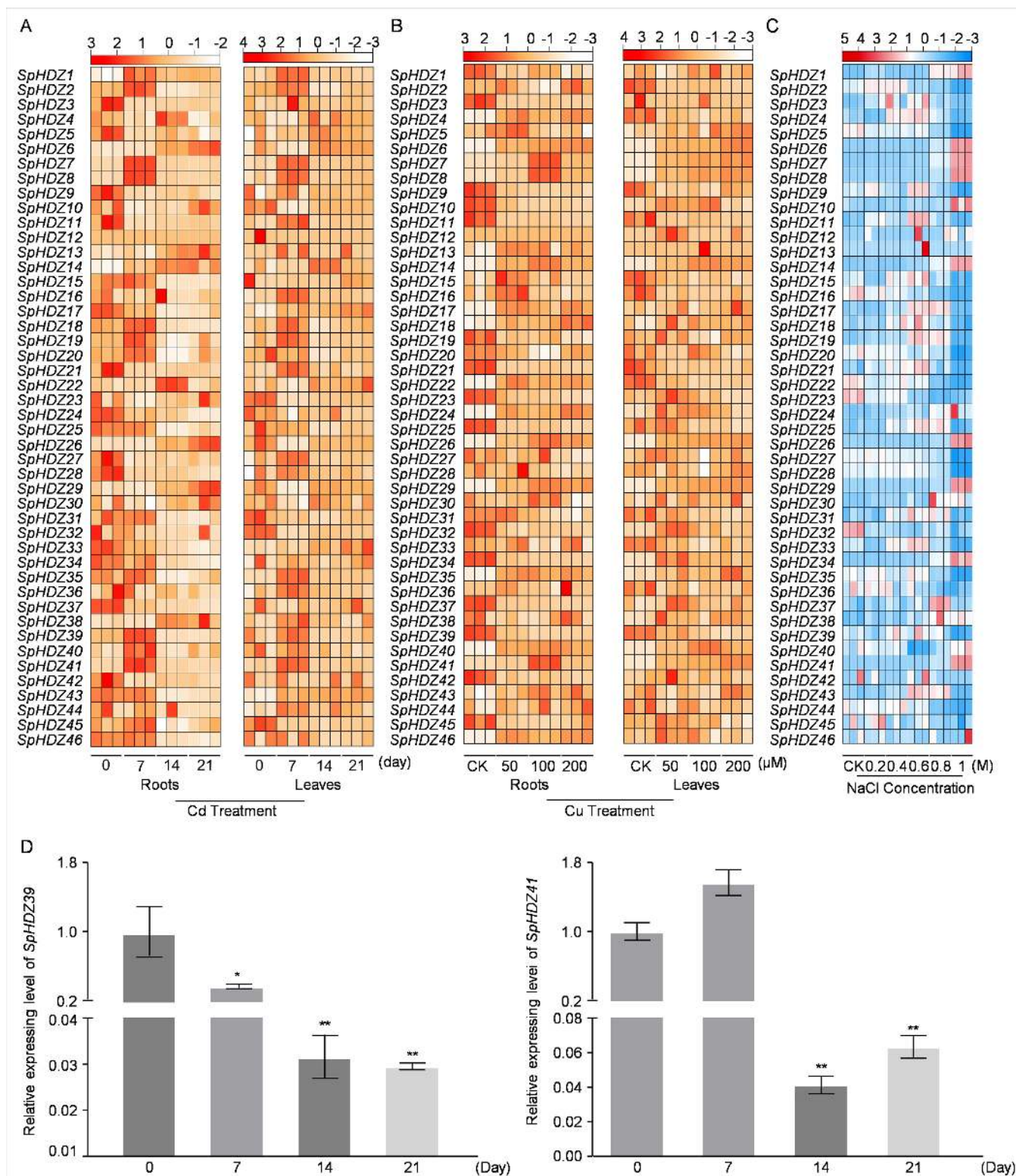


Figure 6

Expression patterns of *SpHDZs* in *S. portulacastrum* under various abiotic. A. Fold change of *SpHDZs* under Cd stress. B. Fold change of *SpHDZs* under Cu stress. C. Fold change of *SpHDZs* under salt stress. D. Relative expression patterns of *SpHDZ39* and *SpHDZ41* under Cd treatment analyzed by qRT-PCR. Asterisks indicate a significant difference between day 0 and day 7, 14, 21 Cd treatment as determined by 2-tailed Student's *t* test at ** $P < 0.01$ or * $P < 0.05$.

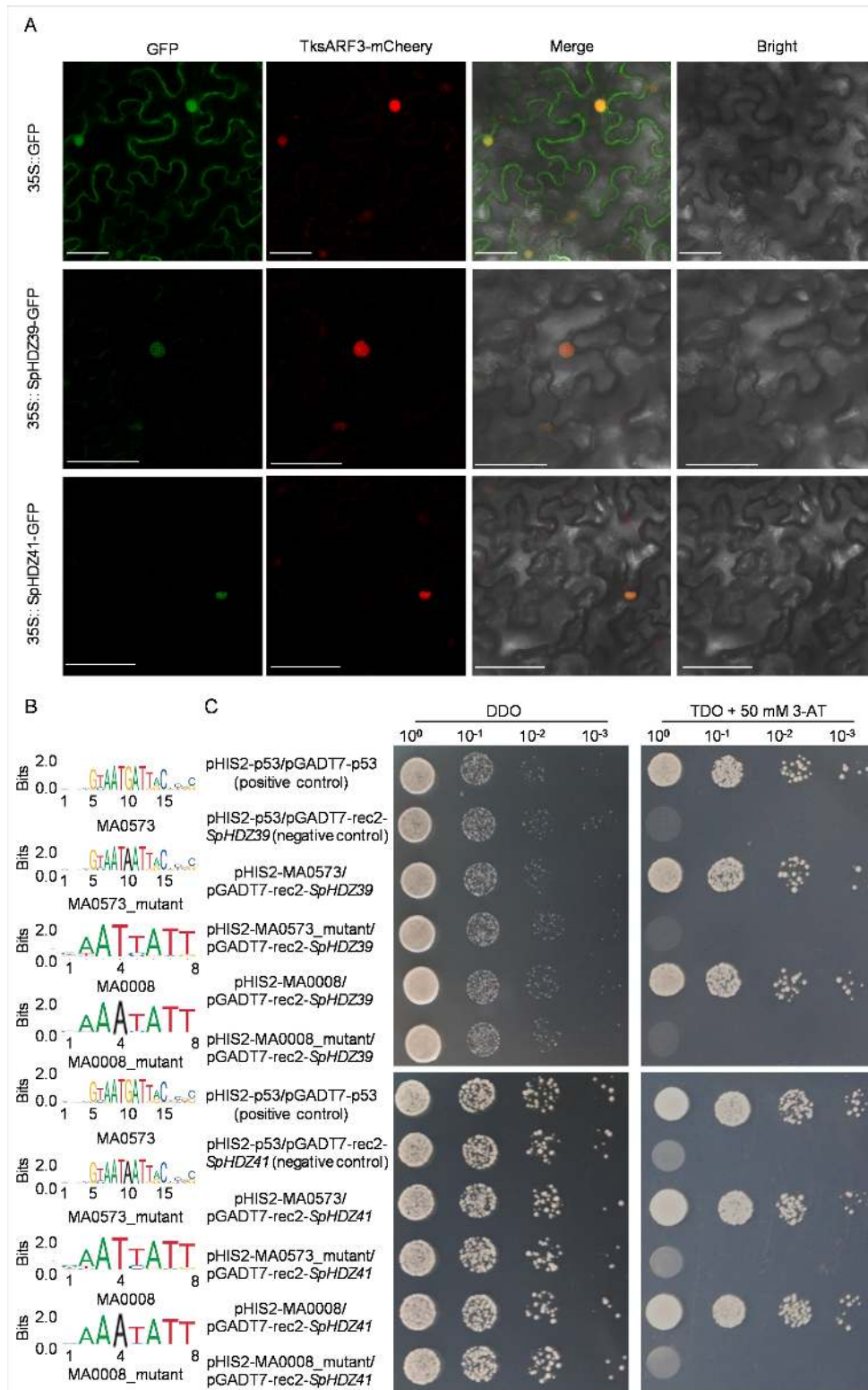


Figure 7

Transcriptional activation assay of SpHDZ39 and SpHDZ41. A. Subcellular localization of SpHDZ39 and SpHDZ41. *Taraxacum kok-saghyz* ARF3 protein fused to mCherry was used as a nucleus marker. Scale bars correspond to 20 μ m. B. Schematic diagram of MA0573 and MA0008 of SpHDZ39 and SpHDZ41 binding site. C. Analysis of the transcriptional activity of SpHDZ39 and SpHDZ41.

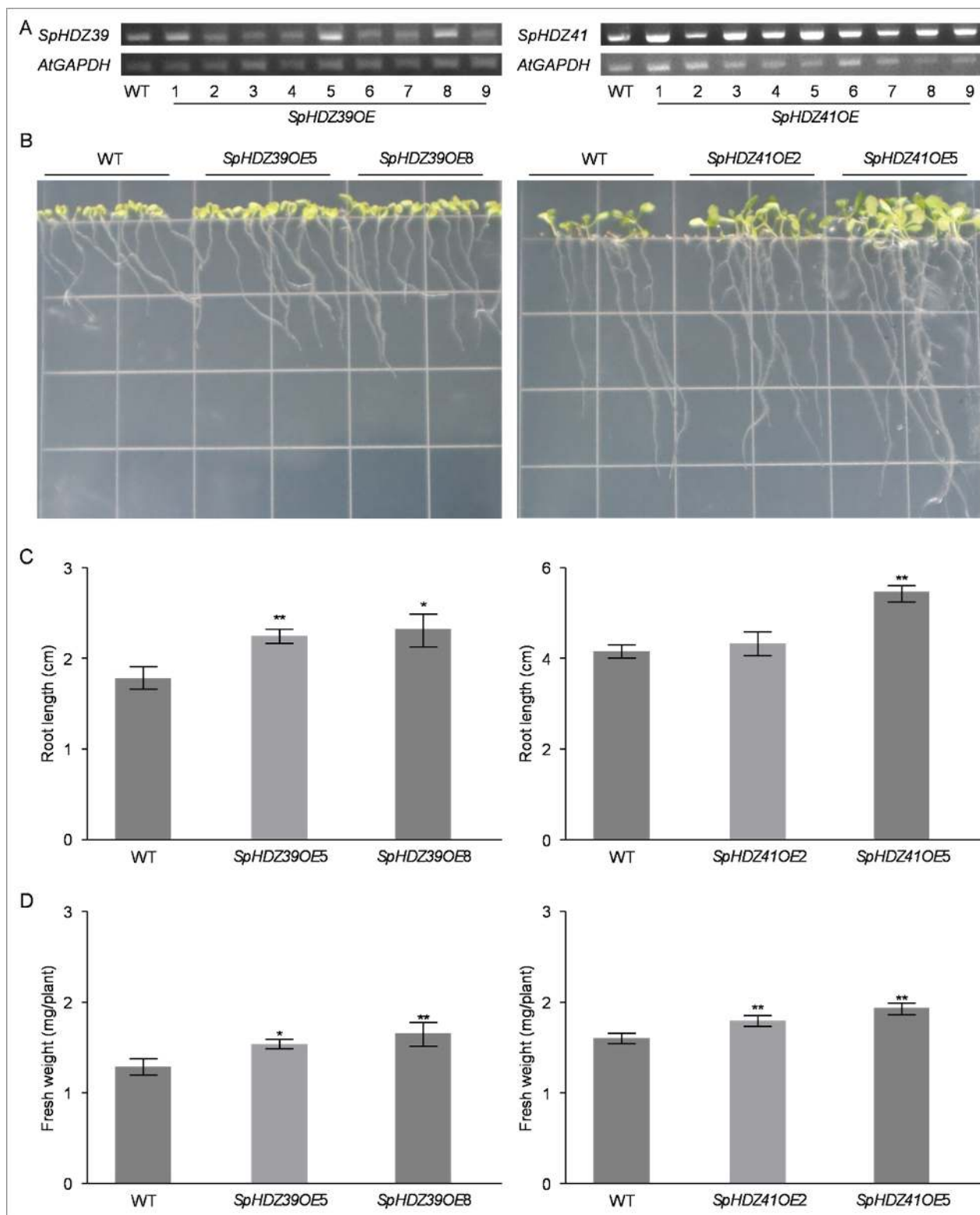


Figure 8

Overexpression of the *SpHDZ39* and *SpHDZ41* improving Cd tolerance in *A. thaliana*. A. Expression level of *SpHDZ39* and *SpHDZ41* in transgene-plants. B. Root morphology of *SpHDZ39OE* and *SpHDZ41OE* under Cd treatment. C. Root length of *SpHDZ39OE* and *SpHDZ41OE* under Cd treatment. D. Fresh weight of *SpHDZ39OE* and *SpHDZ41OE* under Cd treatment. Asterisks indicate significant difference determined by one-tail Student's *t*-test (** $P < 0.01$ or * $P < 0.05$).

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