SpARF4 reduces cadmium accumulation by negatively regulating SpABCG14 and SpACO4 in the cadmium/zinc hyperaccumulator Sedum plumbizincicola

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Abstract

Root development and apoplastic transport are respectively important for cadmium (Cd) absorption and transportation, which profoundly influence Cd bioremediation. However, molecular mechanisms underlying the two processes are not completely understood. In this study, we demonstrated that auxin response factor 4 (SpARF4) from a Cd hyperaccumulator Sedum plumbizincicola was a negative regulator for these processes. SpARF4 positively regulated by auxin was highly expressed in xylem. Overexpression of SpARF4 significantly decreased vessel area and declined lignin content of S. plumbizincicola. Meanwhile, less adventitious roots were found, and lateral root development was delayed in transgenic plants. Furthermore, ethylene production and auxin transportation were impaired. More importantly, SpARF4 negatively regulated Cd content of xylem saps and aerial tissues. Combining dual-LUC reporter, Y1H and qRT-PCR assays, SpARF4 was a repressor for two downstream genes (SpABCG14 and SpACO4) which influenced vascular bundle development and ethylene production, respectively. PIN1, 2, 3, 7 were downregulated and slowed down local auxin accumulation rate, which suspended root development. These results indicate that SpARF4 can decelerate Cd transportation rate from roots to aerial parts and reduce Cd content of aboveground tissues by delaying the root development and decreasing vessel area.

Introduction

Two different attitudes are adopted by people toward cadmium (Cd) accumulation in plants. On one hand, Cd accumulation should be decreased in crops (especially the edible portions) for ensuring food security (Sasaki et al., 2012; Rizwan et al., 2016; Lu et al., 2019). On the other hand, plants used for bioremediation should accumulate more Cd in their aerial parts for removing Cd contamination (Ali et al., 2013; Evangelou et al., 2015; Luo et al., 2016). Cd absorption and transportation of plants are involved in both cases. Therefore, understanding the molecular mechanism underlying Cd absorption and transportation is crucial for ensuring food security and environmental restoration.

Root morphology can response to Cd stress (Daud et al., 2009), which may lead to a change in Cd absorption (Li et al., 2009). For example, in hyperaccumulating-ecotypes*Sedum alfredii* (HE, a famous Cd hyperaccumulator), Yang et al. (2004) pointed out that although the maximum root length was significantly reduced when Cd levels >50 $\mu mol \cdot L^{-1}$, more LRs were grown (Yang et al., 2004). Most importantly, Cd

absorption was simultaneously increased. In line with this idea, it is also reported that apoplastic pathway via LRs contributed to Cd accumulation (Tao et al., 2016). In this study, our attention is focused on root development, especially lateral roots (LRs) and adventitious roots (ARs). Because LRs are mainly relevant with Cd absorption of plants (Redjala et al., 2011; Tao et al., 2016; Xie et al., 2019), while ARs are related with LR numbers.

Auxin plays crucial roles in initiation of ARs and LRs, especially its transportation and local accumulation (Casimiro et al., 2001; Vidoz et al., 2010; Sukumar et al., 2013; Du and Scheres, 2017). Auxin response factors (*ARFs*), as important components for auxin pathway, are thus significant for root development (Okushima et al., 2007; Lee et al., 2019). To date, largely based on studies of Arabidopsis, *ARF2, 3, 4, 5, 7, 6, 8, 17* and*19* have been proved to be associated with root development (Wilmoth et al., 2005; Gutierrez et al., 2009; De Smet et al., 2010; Marin et al., 2010; Xi et al., 2018). In addition, the root initiation seems to be depended on expression levels of *ARFs* . For example,*miR167* downregulates *GmARF8* to increase LRs (Wang et al., 2015). Overexpression of *AtARF17* decreased the amount of ARs (Sorin et al., 2005). A regulatory module (containing *ARF4*) has dramatic impacts on LRs development and remains highly conservative between different species (Marin et al., 2010; Hobecker et al., 2017; He et al., 2018). *ARF4* is a repressor for LRs in normal condition (Marin et al., 2010). But,*ARF4* was downregulated and released LRs restriction in adverse situation (He et al., 2018). However, as a typical transcription factor, how *ARF4* regulates its downstream genes to control LRs development requires further investigation.

Notably, most of auxin which induces LRs initiation is originated from young shoot organs (Aloni et al., 2006), suggesting the importance of polar auxin transportation for LRs development. Intercellular transport of auxin is mainly mediated by*PIN, ABCB* and *AUX/LAX* families (Geisler et al., 2005; Paponov et al., 2005; Péret et al., 2012). Over the past decades, numerous studies were focused on *PIN* genes, including their evolution, expression locations and functions (Blilou et al., 2005; Paponov et al., 2005). Up to date, eight *PIN* genes are identified in *Arabidopsis* (Paponov et al., 2005). Among them, *PIN1, 2, 3, 4, 7* control root patterns and their mutants exhibited developmental retardation or even deficiency of LRs (Benková et al., 2003), suggesting*PINs* are necessary for LRs. Moreover, expression levels of them could be regulated by ethylene (Lewis et al., 2011). Therefore, adverse environments (such as Cd stress) which induce ethylene promote more LRs emergence, indicating that LRs development seems to be an important strategy for stress adaptation of plants (Yang et al., 2004; He et al., 2005). However, in turn, excessive ethylene is produced with the increase of stress time, which blocks LRs initiation (Xiong et al., 2006; Negi et al., 2008). Normal LRs development are processing in the presence of ethylene within a certain range. In other words, the factor which represses ethylene production under abiotic stress should be crucial for LRs growth. Thus, it is necessary to uncover the mechanism inhibiting ethylene production. Meanwhile, different factors (*ARFs* , *PINs* and ethylene) can all inhibit LRs, what is the relationships between them ?

After Cd accumulated in roots, timely transportation of Cd to aerial tissues is an important strategy for protecting roots. More efficient Cd transportation is a most striking difference between*Sedum plumbizincicola*(previously known as HE) and *S. alfredii* (known as NHE, non-hyperaccumulator of Cd) (Lu et al., 2008), even though the two species have very closely genetic relationships (Jin et al., 2008). Up to data, apoplastic and symplastic pathway are responsible for Cd transportation (Luo et al., 2016). Most studies are concentrated on symplastic pathway (Park et al., 2012; Liu et al., 2017). However, recent studies demonstrated that the apoplastic pathway not only exerts their roles in Cd transportation (Tao et al., 2019) but also in Cd sequestration (ZHANG et al., 2010). Nevertheless, the molecular mechanism underlying this pathway has been elusive.

*ABCG14*in *Arabidopsis* is a crucial gene to profoundly influence the apoplastic pathway, because it is related with vessel cell development and lignin biosynthesis (Ko et al., 2014; Zhang et al., 2014; Zhao, 2016). On one hand, vessel cells are relevant with inorganic ion transportation. On the other hand, although the functions of lignin *in vivo* in binding Cd were not clear, it exhibits extraordinary abilities for Cd absorption *in vitro* experiment (Demirbas, 2004; Harmita et al., 2009). Moreover, lignin biosynthesis genes can response to Cd stress (van de Mortel et al., 2008). Thus, ABCG14 has a potential ability to affect Cd bioremediation,

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however, regulatory mechanism about it remains largely unknown. In this study, *S. plumbizincicola* and *S. alfredii*were chosen as materials to identify gene functions. *SpABCG14*whose protein domain remained highly conserved with *ABCG14* was isolated from *S. plumbizincicola* (Fig. S1) and many binding sites associated with various transcription factors were found in its promoter (Fig. S2), such as *ARFs* binding element, AuxRE. It revealed that*SpABCG14* may be controlled by these transcription factors. Among them, *ARFs* are selected to understand whether and how *ARF*members could modify *SpABCG14* expression to influence apoplast pathway.

Therefore, our main aims in this study are i) to choose the candidate gene from *ARF* family by bioinformatics analysis and to verify the relationship between it and *SpABCG14* ; ii) to analyze its functions in root development; iii) to identify its influence on Cd transportation and accumulation.

Materials and methods

Plant materials and growth conditions

Materials of *S. plumbizincicola* and *S. alfredii* were collected as previously described (Liu et al., 2017). Some of them were treated for tissue culture. Shoots of other *S. plumbizincicola*were cut and used in the hydroponic treatment for two weeks. The rooted clones were transplanted in nutrient soils and cultured for 6 months before Cd treatment with 16-hour light/8-hour dark cycles. After that, the suitable shoots were harvested and rooted in nutrient solutions containing Murashige and Skoog (MS) for two weeks. These rooted plants were treated with 400 μM $CdCl_2$ for 0, 0.5, 1, 2, 4, 8 and 12h, respectively.

Identification of *ARF* genes using genome data

Protein sequence data of *S. plumbizincicola* and other species (including *Kalanchoe laxiflora* , *Kalanchoe fedtschenkoi* ,*Arabidopsis thaliana* , *Populus trichocarpa* ,*Selaginella moellendorffii* , *Zea mays* , *Oryza sativa* ,*Marchantia polymorpha* , *Ostreococcus lucimarinus* ,*Chromochloris zofingiensis*) were downloaded from Phytozome (*https://phytozome.jgi.doe.gov/pz/portal.html#!bulk?org=Org_Athaliana*). ARF genes were screened from these protein data on Pfam platform using Auxin_resp domain (PF06507.12) and the sequences with E-values equal or more to 1E-5 were discarded. After that, all of genes in this study were named, according to the conservation between them and their homologous genes in *Arabidopsis* . Then, if two or more genes have the same homologous gene in *Arabidopsis* , they were named 1, 1.2 etc.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs were extracted from roots, stems and leaves of above Cd-treatment plants (three biological replicates per treatment) using an RNA extraction kit (RNAprep Pure Plant Kit, TIANGEN, Dalian, China). Next, first-strand cDNAs were produced using a cDNA synthesis kit (PrimeScript RT Master Mix, TAKARA, Beijing, China). These cDNAs were utilized for qRT-PCR using TB Green reagent (TB Green Premix Ex Taq, TAKARA, Dalian, China). The primers of *ARF* genes were designed at the website PRIMER3 (*http://primer3.ut.ee/*). All primers including the reference gene (*UBC9*) were listed in Table 1S.

Analysis of Phylogenetic, positively selected sites, functional divergence and coexpression network

ARF protein sequences of *S. plumbizincicola* , *K. laxiflora*and *K. fedtschenkoi* were renamed after blasted in TAIR (*https://www.arabidopsis.org/*). Then, protein sequences alignment were made using the method of Mafft (*http://mafft.cbrc.jp/alignment/software/*) and Muscle (*https://www.ebi.ac.uk/Tools/msa/muscle/*). After that, phylogenetic tree was conducted by MEGA-X (Kumar et al., 2018) with the bootstrap 1000. Then the phylogenetic tree was applied in the software PAML (Yang, 2007) for positively selected sites calculation and the alignment file was produced by MEGA-X, with the method of clustal W (codons). Meanwhile, the software DAMBE was used for format conversion. M0, M3 and M7, M8 models were respectively applied for assuming variable selected pressure, according to the method of Liu et al. (2018) (Liu et al., 2018). Functional divergence analysis was made using the software of DIVERGE v3.0 (Gu, 2011). Coexpression network were conducted by Cytoscape software, based on the transcriptome data (Han et al., 2016). The

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corresponding genes related to hub genes were thus identified and then promoter cis-elements of these genes were further analyzed, combined with the data of transcriptome and genome, using TBtools and PlantCARE.

Plasmid construction

The open reading frame and 2 kb promoter of *SpARF4* were amplified from cDNA and DNA of *S. plumbizincicola* , respectively. These products were cloned into pDONR222 and then were recombined into pK2GW7.0 and pMDC164 to generate *35S:SpARF4* and*ProSpARF4:GUS* , respectively. Meanwhile, the full coding sequence of *SpARF4* was assembled into the vectors of pGADT7-Rec (for Yeast one-hybrid assay, Y1H) and pGreenII 62-SK (for dual-LUC reporter system) using the sites of *Sma* I/*BamH* I and*EcoR* I/*Hind* III, respectively. Full-length promoter sequences (2kb) of *SpABCG14* and *SpACO4* were amplified from*S. plumbizincicola* and inserted into the vector of pGreenII 0800-LUC using S*al* I/*BamH* I to form$SpABCG14_{pro}$*:LUC* and$SpACO4_{pro}$*:LUC* . Meanwhile, to demonstrate the availability of our system, 35S promoter was also cloned into pGreenII 0800-Luc to form *35S:LUC* , as the efficiency of 35S promoter was stronger than that of plant promoters. All primers were listed in Table 1S.

Plant transformation

Explants for shoot induction in transgenic experiment were calluses of*S.plumbizincicola* and leaves of *S.alfredii* . The constructs containing *35S:SpARF4* and *ProSpARF4:GUS* were introduced into EHA105 *Agrobacterium tumefaciens* strain by electroporation, respectively. Subsequently, the *35S:SpARF4* was used for infecting *S. plumbizincicola* and *S. alfredii* . Meanwhile, the *ProSpARF4:GUS* was used for infecting *S. plumbizincicola* , and *ProDR5:GUS* vector was introduced into*S. alfredii* . The *A. tumefaciens* -infected method was performed as described before (Liu et al., 2017), with minor modifications. Differentiation medium: MS + 2 $mg{\cdot}L^{-1}$6-benzylaminopurine (6-BA) + 0.3 $mg{\cdot}L^{-1}$1-naphthaleneacetic acid (NAA); rooting medium: 1/2 MS + 2 $mg{\cdot}L^{-1}$ 3-indolebutyric acid (IBA). The use of antibiotics is varied with different vectors. The concentrations of kanamycin and hygromycin were 30 $mg{\cdot}L^{-1}$ and 20 $mg{\cdot}L^{-1}$, respectively. After that, the well-rooted wild-type and transgenic plants were transplanted into soils and grown for further detection and experiments.

Pot experiment and determination of Cd concentration in plants

The overexpression lines (OE) of *S. plumbizincicola* with highest-expression level of *SpARF4* (at least six lines) were cultured for 14 d using basic nutrient solution (Yang et al., 2004). Then, these plants were transplanted into plastic pots filled with 2 kg soil and watered every seven days (photoperiod 16h light/8h dark, day/night temperature 26/20 *C). The wild-type lines (WT) followed the same processes. After three months, these plants were cultured but without water for 14 d. After that, these plants were supplied with 400 $\mu mol{\cdot}L^{-1}$ Cd solution for 48 h. Aerial tissues of WT and OE were collected for measuring Cd concentration and three biological repeats. At the same time, removing the first drop, other xylem saps of these plants were collected in four hours using injectors and three biological repeats. Cd concentrations of these samples was determined as previously described (Liu et al., 2017).

Measurement of IAA concentration and lignin content

Plants of *S.plumbizincicola* were grown hydroponically for eight weeks. Then, these plants were divided into two groups which treated by 0 and 400 $\mu mol{\cdot}L^{-1}$ $CdCl_2$ for seven days, respectively. The aboveground tissues of these plants were collected at the 0, 1, 4 and 7 d, respectively, for determination of lignin concentrations. Meanwhile, the samples which were cultured for seven days under Cd stress were prepared for IAA measurement. The determination IAA concentration was conducted as described preciously (Zhao et al., 2019). Lignin content was measured using the method of Redox titration (Pengcheng et al., 2008). In detail, the dried samples were soaked using 1% acetic acid solution and then the mixed solution containing ethanol and ether (volume ratio, 1:1). The sediments were evaporated to dryness in boiling water bath and treated by 72 % sulfuric acid. Distilled water was used to clean the sediments. After that, 10 % sulfuric acid and 0.1 mol L^{-1} potassium dichromate solution were added and the system was placed into boiling water bath. Then, adding 20 % KI and 0.5 % starch solution to the mixture and using 0.2 mol L^{-1}sodium thiosulfate for

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titration.

Y1H assay

The coding region of *SpARF4* was inserted into the vector of pGADT7-Rec (Clontech) to produce pGADT7-SpARF4. A 20-bp fragment (as a unit) whose center was AuxRE motif (TGTCTC) was cloned from the promoters of *SpABCG14* and *SpACO4* , respectively. Then four tandem copies of the two units were constructed and cloned into pHIS2 vectors to produce pHIS2-ABCG14 and pHIS2-ACO4, respectively (Liu et al., 2015). The two pairs (pGADT7-SpARF4/pHIS2-ABCG14 and pGADT7-SpARF4/pHIS2-ACO4) were cotransformed into AH109 (yeast strain) using LiAc-PEG3350 method. SD-Leu-Trp plates were used for transformants selection and SD-Leu-Trp-His plates supplemented with 30 μM 3-AT (3-amino-1,2,4,-triazole) were utilized for testing the interactions. Three biological repeats and three technical repeats for this process. The primers were contained in Table 1S.

Transient transcriptional activities assay

The transient transcriptional activity assays were performed as described previously (Liu et al., 2019), with minor modifications. The corresponding vectors were constructed as stated above. Lumazone imaging system (Mag Biosystems, USA) was used for image acquisition of luciferase *in vivo* . The images processing was accomplished by the software of ImageJ (*https://imagej.net/Welcome*). qRT-PCR were conducted for testing the expression level of *LUC*and *Ren* . At least three biological repeats and technical repeats for each assay.

Histochemical analyses

β-glucuronidase (*GUS*) staining was performed as described previously (Han et al., 2012). Phloroglucinol staining was conducted following the standard protocols (Zhang et al., 2012). All tissues sections were 30-μm-thick, which observed and photographed using LEICA DM4000B/DFC450 and M165FC/DFC550. Vessel diameters and cross-section area of fifty vessel cells were measured by software of autoCAD (*https://www.autodesk.com.cn/*). After that, statistical analysis of these data was performed by SPSS 20.0.

Auxin and ethylene inhibitors treatment

The selection and application of NPA (naphthylphthalamic acid, auxin transport inhibitor), AVG (aminoethoxyvinylglycine, auxin synthesis inhibitor) and PZA (pyrazinamide, ethylene biosynthesis inhibitor) were referred to published studies (Scanlon, 2003; Soeno et al., 2010; Sun et al., 2017). In details, transgenic lines of *ProSpARF4:GUS* which were cultured under hydroponic conditions for 2 weeks were treated by 10 μM NPA and 40 μM AVG for 7 d and 3h, respectively. The seedings of*S. alfredii* were cultured in MS medium in the presence or absence of 100 μM PZA for 14 d. Three biologicals.

Results

Lignin content was increased and IAA was decreased under Cd stress

As *ABCG14* are related with lignin biosynthesis (Ko et al., 2014), while the functions of lignin in vivo about Cd accumulation were not so clear. lignin concentration in dry weight (DW) was measured during Cd stress. Meanwhile, Cd concentration in lignin was also tested. The results indicated that lignin concentration of DW under Cd stress was significantly higher than that without Cd treatment (Fig. 1a, $P < 0.01$). The concentration of lignin continued to be increased during Cd stress, even though, the increase did not reach a significant level (Fig. 1a, $P < 0.05$). Interestingly, there was an obviously difference in Cd concentration of lignin during Cd stress, indicating that slight changes in lignin can significantly enhance Cd content in DW (Fig. 1b, $P < 0.01$). Thus, genes related with lignin biosynthesis may trigger the variation in Cd content.

In order to clarify the roles of *ARFs* under Cd stress, we need to identify the influence of Cd on auxin, as *ARFs* functions were depended on auxin level (Li et al., 2016). The results showed that IAA was seriously repressed in root (Fig. 1c, $P < 0.01$). similar trend was also found in stem (Fig. 1c, $P < 0.05$). however, IAA content in leaf seemed not be affected by Cd treatment (Fig. 1c).

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SpARF4 as a center in coexpression network underwent the positive selection

Total 247 *ARF* genes in 11 species from four categories plants (phycophyta, moss, gymnosperm and angiosperm) were identified using pfam platform (PF06507.12), according to the published genome data (*https://phytozome.jgi.doe.gov/pz/portal.html*). The results revealed that *ARF* numbers were increased with plant evolution, and most of them were concentrated in terrestrial plants (Fig. 2A). More importantly, further analysis indicated that *ARFs*may be involved in environmental stress responses. For example, promoter analysis showed that many elements related with abiotic stress (such as drought-inducibility element) were found in promoters of 23*SpARFs* in *S. plumbizincicola* (Fig. S3). In summary, we assumed that *ARF* gene family seemed to be relevant with the process of plant terrestrial adaptation and play crucial roles in protecting plants from abiotic stress. In order to verify this conclusion, we tested expression levels of *SpARFs* under Cd stimuli. The results revealed that expression patterns of *SpARFs*were changed during Cd stress, which indicated that *SpARFs* can response to Cd treatment (Fig. S4).

After analyzing *ARFs* in different species (Fig. 2A), we focused our attention on Crassulaceae family. *ARFs* in *K. laxiflora* , *K. fedtschenkoi* and *S. plumbizincicola* were used for constructing phylogenetic tree to analyze the functional divergence and adaptive selection (Fig. 2B). Only *S. plumbizincicola* in chosen three species was Cd hyperaccumulator. The results indicated that these *ARFs* were divided into four classes (Fig. 2B). Functional divergence analysis showed that the LRT values between the I class and other three classes reached a significant level (Table S1, P <0.05), indicating that Type I divergence sites may be distributed in the class I, during the evolution. Further evidence suggested that abundant positive selection sites were found in class I which containing *SpARF3* and *SpARF4* (Fig. 2B, Table S2). Furthermore, coexpression network containing five hub genes (*SpARF4, 8, 9, 16.2 and 19.2*) was made using the transcriptome data under Cd stress (Fig. S5). The five genes were divided into two categories, according to the amount of shared related genes (Fig. S5A, B). Thus, from the point of our view, *SpARF4* could be an ideal candidate gene, as it was a hub gene and underwent positive selection. Over 1000 genes in coexpression network were related with *SpARF4* . Among of them, expression levels of 201 genes were changed during Cd stress (Correlation coefficient between gene expression and stress time >0.9 or <-0.9). In this study, our attention was mainly focused on three kinds of genes (transcription factors, transporters and phytohormone pathway). Finally, 11 genes including*SpABCG14* and *SpACO4* were identified (Fig. 2C, the red letters).

SpARF4 positively regulated by auxin mainly expressed in vessel cells of xylem

Expression sites of genes were closely associated with their functions. To investigate *SpARF4*expression patterns, 2kb promoter sequences was assembled into a*GUS* vector (pMDC164) (Fig. S6) and transformed into*S. plumbizincicola* . The results revealed that GUS activities were detected in phloem and xylem (Fig. 3A, red arrows) and stronger signals were focused on vessel cells (Fig. 3B, the red arrow), which were similar to the expression locations of *ABCG14* (Ko et al., 2014), suggesting that there were possibilities for the relationship between*SpARF4* and *SpABCG14* .

In general, genes in auxin pathway were controlled by auxin which was influenced by abiotic stress (Iglesias et al., 2010). Thus, in order to clarify the roles of *SpARF4* in Cd stress, we need to identify the response of *SpARF4* to auxin. NPA and AVG were used to examine the relationship between *SpARF4* and auxin, as NPA can inhibit auxin transportation but not repress auxin biosynthesis while AVG can block auxin biosynthesis. We found that GUS signals were mainly focused on roots center in normal condition (Fig. 3C-1; Fig. S7A, B). Moreover, the signals were not only distributed in root tips but also in other parts (Fig. 3C-1, the red arrow). In terms of LRs, *GUS* was mainly expressed in mature LRs (Fig. S7C, D) and the bases of immature LRs (Fig. 3C-2, the red arrow). Intriguingly, under the influence of NPA, *GUS* was only expressed at root tips (Fig. 3D-1, the red and blue arrow). Little signal can be observed in LR bases (Fig. 3D-2, the blue arrow). In sharp contrast, *GUS* expression was hardly detected in neither mature nor immature roots, with the treatment of AVG (Fig. 3E-1, E-2, the blue arrow). These results suggested that*SpARF4* expression can be induced by auxin.

SpARF4 influences Cd transportation and accumulation by changing plant architectures

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In order to clarify the functions of *SpARF4* , it was introduced into *S. plumbizincicola* and*S. alfredii* under the control of CaMV 35S promoter, respectively.

In leaf, overexpressing *SpARF4* made leaves distorted and asymmetric, especially in highest-expression lines of *S. alfredii* (Fig. 4A). In addition, the petiole of OE became wider than that of WT (Fig. 4A). Then, these leaves were soaked in ethanol to identify the causes for the phenotypic changes. The results showed that an unambiguous midvein was observed in WT leaves (Fig. 4A, III, the red arrow) and the distribution of lateral veins was stronger symmetry (Fig. 4A, III). However, two midveins can be observed in OE leaves (Fig. 4A, IV, the red arrow and the blue arrow), which may be the reason for a wider petiole. Moreover, the second midvein was wider and run parallel to the first midvein (Fig. 4A, IV). Given that these morphological alterations accorded with typical phenotypes of *PIN1* mutation which were conserved in dicots (Mattsson et al., 1999; Sawchuk et al., 2013), we next tested *PIN1* expression in WT and OE. The results showed that *PIN1* expression was indeed seriously decreased in OE (Fig. 4A, V, $P < 0.05$), indicating that alteration of auxin transportation and distribution may be the reason for leaves phenotypic changes.

In terms of stem, as *ABCG14*regulated lignin content and vessel cell numbers while *SpARF4* may control *SpABCG14* (Fig. 2C), overexpression of *SpARF4* could influence the lignification degree and vessel development of transgenic plants. For this reason, stem sections of OE were made and then stained by phloroglucinol-HCl. At the same time, lignin contents were also tested to verify this assumption. The results indicated that vessel numbers and diameters in *S. plumbizincicola* were all seriously decreased in OE (Fig. 4B, I and II; Fig. S8A, B). Furthermore, there were highly significant decreases in vessel areas and lignin contents (Fig. 4B, III, VI). More than itself of variation in vessels, we focused on whether these changes could modify Cd transportation. The xylem saps of WT and OE were collected at the same time under Cd stress and testing Cd content of them. The results showed that no significant difference in Cd concentration was observed in xylem saps of WT and OE (Fig. S9A). However, the collected saps volume and Cd contents of OE were significantly lower than these of WT (Fig. S9B, $P < 0.01$; Fig. 4B, V, $P < 0.05$). Except for this, we also found that Cd accumulation in OE aerial parts were obviously lower than that of WT (Fig. 4B, VI, $P < 0.01$).

In root, compared with WT, *SpARF4* seriously repressed ARs growth (Fig. 4C, I and II). This inhibition mainly showed in two aspects: one was ARs numbers (Fig. 4C, III) and another was the maximum root length (Fig. 4C, III-2). Given that *PIN1* was declined in leaves of OE (Fig. 4A, V), we speculated that the two phenomena may be due to alteration in auxin transportation and distribution of OE. In order to verify this assumption, the auxin reporter gene vector, *DR5:GUS* , was introduced into WT and OE, respectively. When local auxin accumulation (LAA) and LRs were observed on fibrous roots of WT (Fig. 4C, IV, the white arrow), GUS signals were still uniformly distributed on the same-length fibrous roots of OE and no LR was observed (Fig. 4C, IV-2). Moreover, GUS signals in WT root tips were not only distributed in auxin-produced sites, but also spread to surrounding areas (Fig. 4C, V). Whereas, *GUS* was only expressed in auxin-produced sites of OE root tips (Fig. 4C, V-2). Taken together, these results strongly indicated that *SpARF4* could delay the rate of LAA to influence root development through disturbing auxin transportation and distribution.

SpARF4 alters auxin transportation by indirectly regulating *PINs*

SpARF4 may affect *PIN* genes to change auxin transportation and distribution, which was concluded by phenotypic analysis of leaves and roots. To further verify this conclusion, qRT-PCR was made to detect mRNA levels of *PINs* (*PIN1* , *PIN2* , *PIN3* and*PIN7*) in roots. As we expected, *PINs* transcripts were seriously repressed in OE (Fig. 5A, $P < 0.01$). Intriguingly, promoter analysis showed that no AuxRE element was found in upstream regulatory sequences of *PINs* (examined by Plantcare,*http://bioinformatics.psb.ugent.be/webtools/plantcare/html/*; Table 2S). Therefore, we tended to consider that *SpARF4*indirectly regulate these *PINs* instead of a direct way.

When measuring ethylene production of WT and OE under Cd stress, we found that ethylene was produced at the beginning of Cd treatment in both WT and OE (Fig. 5B). After that, ethylene production of WT

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continued to increase, while little increase was found in OE during Cd treatment. Given that ethylene was an important regulator for *PIN* genes, we cultured WT with supplement of PZA and then tested *PINs* expression in roots. Interestingly,*PINs* of WT in this situation were highly similar to these of OE (Fig. 5C). According to these results, we suspected that *SpARF4* indirectly controlled *PINs* by changing ethylene production. Moreover, *ACO4* processing ACC to ethylene was also detected in coexpression network (Hu et al., 2018) (Fig. 2C, the red letter), indicating that *ACO4* may be a media between *SpARF4* and ethylene.

SpARF4 negatively controls the transcripts of*SpABCG14* and *SpACO4*

According to the above analysis, substantial evidences promoted us to explore whether *SpARF4* could directly regulate *SpABCG14* and *SpACO4* .

Combining dual-LUC reporter (Fig. 6A), qRT-PCR and Y1H assays, we found that *SpARF4* can directly negatively regulate *SpABCG14* and*SpACO4* . There were three evidences supporting our conclusion. Firstly, at the protein level, when using *Agrobacterium tumefaciens* (GV1301) carrying pGreenII 62-SK (EV), pGreenII 62-SK-*SpARF4* (*SpARF4*),*SpABCG14*$_{pro}$*:LUC* and*SpACO4*$_{pro}$*:LUC* to infect *Nicotiana benthamiana* leaves (Fig. 6B, C), we found that luminescence intensities of *35S:LUC* were stronger than these of*SpABCG14*$_{pro}$*:LUC* (Fig. 6B, 1 and 2), suggesting that our system was available. Interestingly, when adding *SpARF4* , the transient transcriptional activity of *SpABCG14* promoter was significantly repressed (Fig. 6B, 2 and 3). At the mRNA level, adding*SpARF4* made relative mRNA level of *Luc* lower than that of adding EV (Fig. 6D, $P < 0.05$), suggesting that *SpARF4* can negatively control *SpABCG14* . This conclusion was applied equally to the relationship between *SpARF4* and *SpACO4* (Fig. 6C, E). Secondly, overexpression of *SpARF4* significantly downregulated *SpABCG14* and *SpACO4* in OE, in comparison with these of WT (Fig. 6F and G, $P < 0.05$). Thirdly, Y1H assay indicated that SpARF4 rescued the auxotrophic phenotypes of yeast (Fig. 6H), suggesting that *SpARF4* can directly bind to AuxRE elements in promoters of *SpABCG14* and *SpACO4* .

Discussion

Cd absorption, transportation and sequestration are crucially important for Cd bioremediation. *ABCG14* plays roles in vessel cell development and lignin biosynthesis (Zhao, 2016), which is relevant with Cd transportation and sequestration. In this study, we demonstrated that*SpARF4* was an upstream gene to negatively regulate*SpABCG14* (Fig. 6B). The following evidences are provided to support our conclusions: firstly, *SpARF4* was prominently expressed in vessel cells of xylem (Fig. 3A); secondly, *SpARF4* can directly bind to the AuxRE motif of *SpABCG14* promoter in Y1H and negatively regulate *SpABCG14* expression in transient transcription assay (Fig. 6 B, H); thirdly, by contrast with WT, the transcript of *SpABCG14* was seriously declined in OE (Fig. 6F). Besides, genetic phenotypes of OE with declined *SpABCG14* expression exhibited smaller vessel areas (Fig. 4B, II and III) and lower lignin content (Fig. 4B, VI), which were consistent with the*ABCG14* mutant phenotypes (Ko et al., 2014; Zhang et al., 2014). In this study, pot experiment was conducted under Cd stress, which may effectively simulate actual scene of bioremediation. The results indicated that *SpARF4* can negatively regulate Cd transportation rate of xylem saps and Cd concentration of OE aerial tissues (Fig. 4B, V and VI).

Dubrovsky et al. (2008) demonstrated that LAA was a necessary and sufficient signal for LRs formation (Dubrovsky et al., 2008). Another study pointed out that root excision promoted IAA transportation and local accumulation to drive ARs formation (Sukumar et al., 2013). These results emphasize the importance of LAA for root development while genes affecting LAA are related with root development. Here, we demonstrated that *SpARF4* disturbed ethylene biosynthesis by regulating *SpACO4* (Fig. 5B; Fig. 6C, G, H) and repressed the growth of ARs and LRs (Fig. 4C). Similar to our results, LRs initiation hypothesis points out that an increase of local IAA concentration triggers ethylene production during vessel differentiation of roots, which blocks the normal IAA transportation to form LAA (Aloni et al., 2006). This hypothesis highlights the roles of ethylene in LAA during LRs formation. It has been shown in other studies (Ivanchenko et al., 2008). In a nutshell, *SpARF4* is a bridge between auxin and ethylene to function in root development by changing auxin transportation.

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In fact, the crosstalk between auxin and ethylene is not only related with plant's development but also with their adaptation for abiotic stress (Vandenbussche et al., 2010; Su et al., 2015). For example, hormonal network containing ethylene and auxin is crucial for root architecture under osmotic stress (Rowe et al., 2016). Therefore, it is important for us to understand the interactional mechanisms between auxin and ethylene. As early as 2006,*ARF7* and *ARF19* has been reported as a cross talk between auxin and ethylene in *Arabidopsis* (Li et al., 2006). Muday et al. (2012) further emphasized the roles of *ARFs* as a bridge between auxin and ethylene (Muday et al., 2012). But, how *ARFs* play their roles between auxin and ethylene requires more explanations. Finally,*ARF7* , as an transcription activator in auxin pathway, is reported to regulate *ACO4* to influence ethylene production (Hu et al., 2018). Our study further affirmed that *ARFs* were involved in ethylene pathway by this mode (Fig. 6C, E, H). Even though, unlike*ARF7* , *ARF4* is a transcription repressor in auxin pathway (Chandler, 2016). Moreover, the coexpression network analysis (containing hub genes, *SpARF4, 8, 19.2*) showed that the three genes shared the same related genes (Fig. S6), suggesting that*SpARF4, 8, 19.2* were likely to function in the same developmental event. A line of evidences supports this hypothesis. For example,*ARF4, 8, 19* in *Arabidopsis* play their roles in LRs development, respectively (Okushima et al., 2007; Marin et al., 2010; Xi et al., 2018). Thus, it is speculated that *ARF* family members:*ARF4, 7, 8* and *19* , as important components for auxin signal transduction may function in ethylene pathway. Nonetheless, more studies are necessary to clarify the exact functions of these genes.

According to our results, we uncovered the relationships of*SpARF4* , *SpABCG14* and *SpACO4* . Fig. 7A exhibited the crosstalk between auxin and ethylene. Furthermore, based on these information, a model (Fig. 7B) was proposed to explain the following phenomena: (1) Cd content of aerial tissues was increased under Cd stress (Fig. 4B, VI); (2) More lateral roots were grown when Cd concentration was from 25 to 200 μmol·L^{-1} (Yang et al., 2004); (3) Lignification degree was enhanced during Cd treatment (Fig. 1a); (4) Ethylene was overproduced under Cd stress (Fig. 5B). Based on this model (Fig. 7B), IAA contents are decreased under Cd stress, which leads to a decline in *SpARF4* expression. This process enhances the expressions of *SpACO4* and *SpABCG14* , as *SpARF4* negatively regulate the two genes. On one hand, more ethylene is produced, which enhances *PINs* expression. LAA is accelerated and more LRs are grown. More Cd are thus absorbed by roots. On the other hand, more vessel cells are developed, which can accelerate Cd transportation through apoplastic pathway. Moreover, abundant lignin is biosynthesized to fix Cd in aerial tissues. And then, Cd accumulation is enhanced through these processes in aboveground tissues.

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Author contributiions

D.X., X.H., R.Z. designed the research. D.X., X.H. performed the experiments. Z.L., L.W., G.Q.,and W.Q. contributed analytical tools, and D.X., X.H. wrote the manuscript and coordinated its revision. R.Z. contributed reagents/materials/funds support. All authors read and provided helpful discussions, and approved the final version.

Declaration of Competing Interest

None

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Figure legends

Fig. 1 Variation of lignin and IAA under Cd stress

a and **b** indicated lignin concentration of aerial tissues and Cd concentration in lignin, respectively, during Cd stress for 0-7 d. **c** IAA concentration of different organs in WT and OE at the 7 d under Cd stress. d, day; DW, dry weight; Capital letters indicated P value <0.01 and different letters showed there was a highly significant level between different treatments while the same letter indicated no significant difference was in different treatments. *, P <0.05; **, P <0.01.

Fig. 2 bioinformatics analysis of *ARF* gene family

A ARFs number in different species. **B** Phylogenetic tree of ARF gene families in *Kalanchoe laxiflora* , *Kalanchoe fedtschenkoi* and *S. plumbizincicola* . **C** coexpression analysis of SpARF4 and the diameters of circles indicated AuxRE number in the promoters of related genes.

Fig. 3 Histological localization of *SpARF4* and strength of GUS signals treated by different inhibitors

A *ProSpARF4:GUS* signals were detected in xylem and phloem of stem. ph, phloem; xy, xylem; pi, pith.

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B Longitudinal sections showed that a stronger signal of GUS was in vessel elements.

C-1-E-1 The influence of auxin on promoter activities was tested by different inhibitors. **C-1** and **C-2** without any inhibitors; **D-1** and **D-2** treated by NPA;**E-1** and **E-2** treated by AVG. The red arrows pointed GUS signals and the blue arrows indicated that GUS signals in these sites were not detected. NPA, auxin transport inhibitor naphthylphthalamic acid; AVG, auxin synthesis inhibitor aminoethoxyvinylglycine.

Fig. 4 Morphological characters of *SpARF4* overexpression plants

A Developmental malformation of leaves were observed in*SpARF4* overexpression lines (OE, II). Compared with WT lines (WT, I), OE leaves were asymmetric and the petioles were wider than that of WT (I and II). Only one midvein was seen in WT (III, the red arrow). By contrast, a second midvein (IV, the blue arrow) was produced and parallel to the first midvein (IV, the red arrow). Moreover, lateral veins of OE were more tanglesome than these of WT (III and IV).*PIN1* expression was repressed in OE (V).

B In stem, tissue sections of WT and OE were made and stained by phloroglucinol (I and II). There was a significant difference in vessel areas between WT and OE (III). Meanwhile, lignin contents were seriously decreased in OE (VI). Cd contents in xylem saps and aboveground tissues of OE were significantly declined compared with WT (VI).

C In terms of roots, contrast with WT (I), adventitious roots were repressed in OE (II). The number of adventitious roots were decreased in OE (III) and the maximum root length of OE was also much shorter than that of WT (III-2). When local auxin was accumulated in lateral roots of WT (IV, the white arrow), auxin distribution was uniform in OE lateral roots (IV-2). GUS signal was mainly focused on auxin-production sites in OE root tips (V-2) while the signal was also observed in surrounding area of WT root tips (V).

* indicated P< 0.05, ** indicated P <0.01.

Fig. 5 The effect of *SpARF4* on *PINs* expression and ethylene production

Overexpression of *SpARF4* can highly significantly repress*PINs* (*PIN1, 2, 3* and *7*) transcripts (**A**). Ethylene in WT and OE was produced at the beginning of Cd treatment. However, little increase in ethylene production were observed in OE during Cd stress (**B**). When culturing WT plants with supplement of pyrazinamide (PZA), expression patterns of the four PINs were similar to these of OE (**C**). **, P < 0.01.

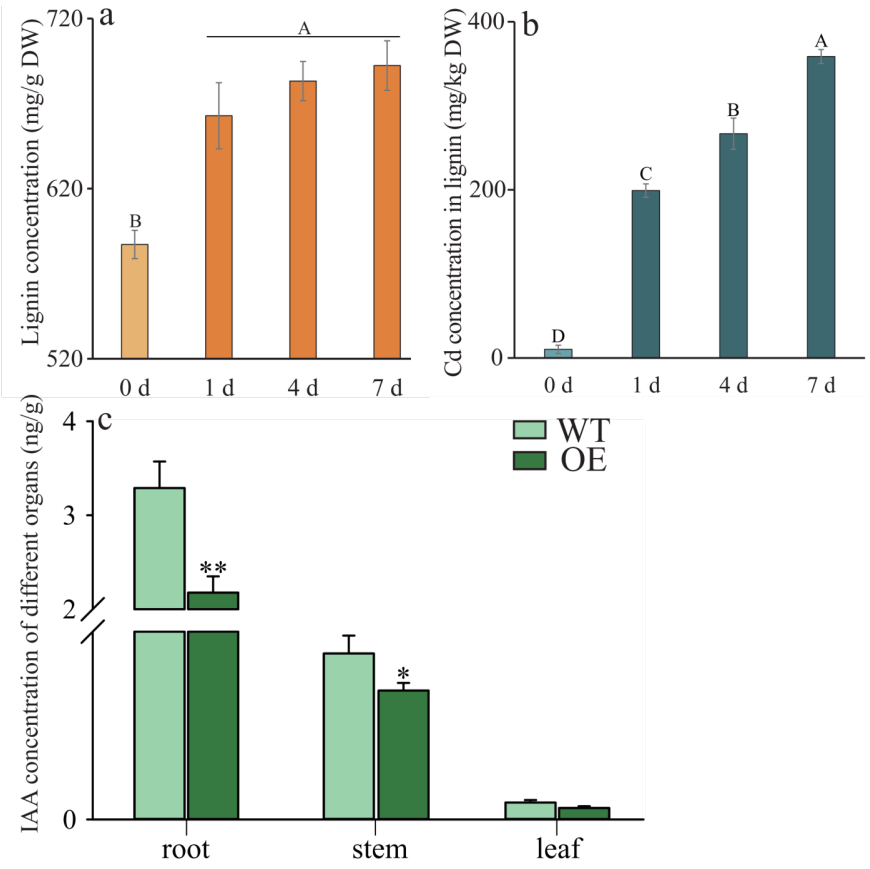
Fig. 6 *SpARF4* directly negatively regulates the expression of *SpABCG14* and *SpACO4*

The full-length sequences of *SpABCG14* and *SpACO4* promoters (2000bp) were constructed into the vector of pGreenII 0800-Luc (**A**). Transient transformation assays of *N. benthamiana*were conducted to test the effect of SpARF4 on promoter-driving activities of $SpABCG14_{pro}$ (**B**) and$SpACO4_{pro}$ (**C**). The luminescence intensity was shown by the color-scale bar; EV, empty vector pGreenII 62-SK. Relative mRNA levels of *Luc* were downregulated by*SpARF4* (**D** and **E**). Meanwhile, the expression levels of *SpABCG14* and *SpACO4* in OE were also seriously repressed in comparison with WT (**F** and **G**). Y1H assay testing interactions between *SpARF4* and promoter regions of*SpABCG14* and *SpACO4* (**H**). The medium containing 30 mM 3-AT was used for selection, which lacing Trp, Leu and His. Yeast concentration was from 0 to 10^{-3}. EV1, empty vector pHIS2; EV2, empty vector pGADT7-Rec.

Fig. 7 Model of the crosstalk between auxin and ethylene during Cd stress

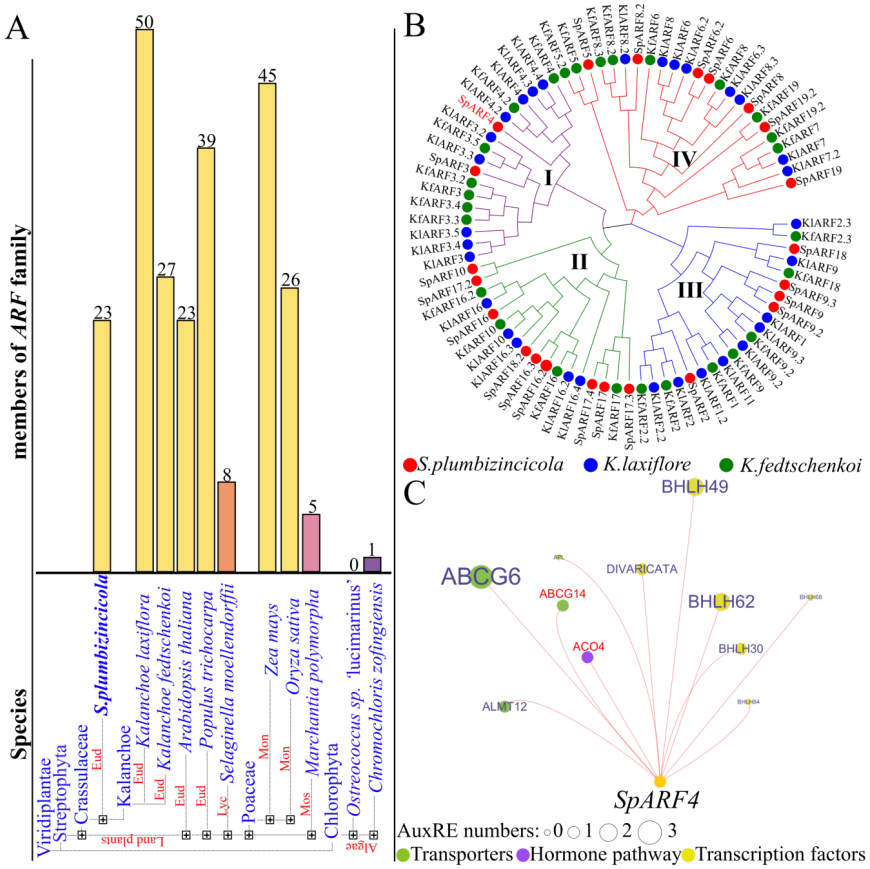
1. Regulatory relationships of components in auxin and ethylene pathway.
2. The roles of *SpARF4* , *SpACO4* and *SpABCG14* in Cd absorption, transportation and accumulation of plants under Cd stress.

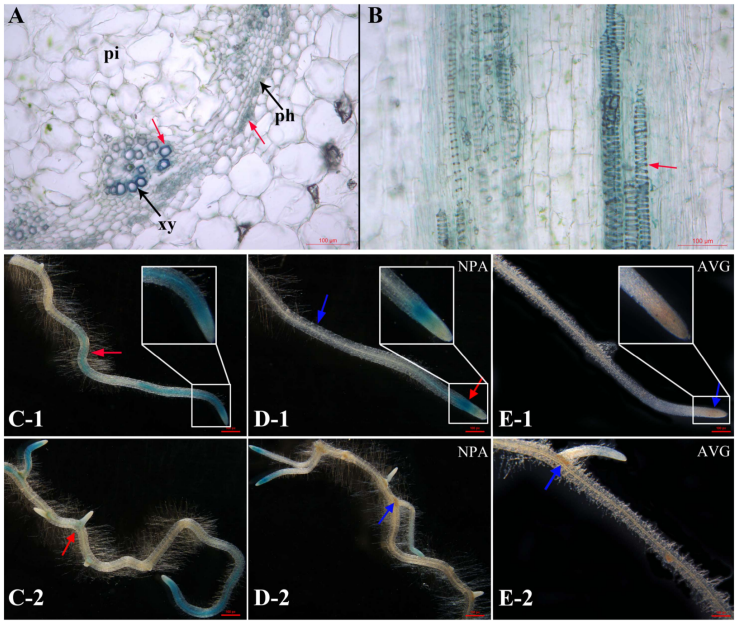
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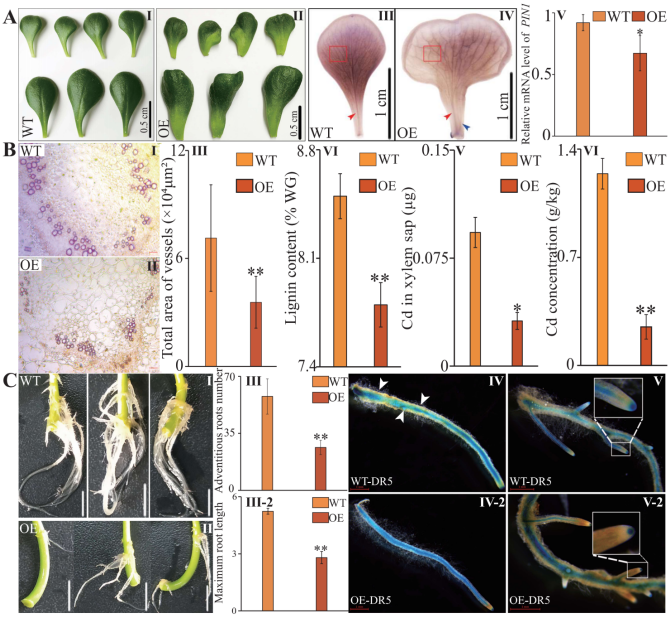
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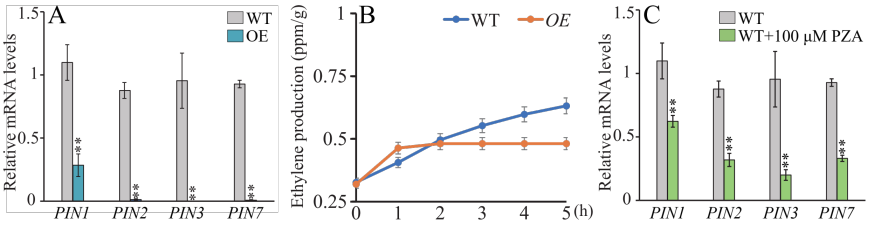
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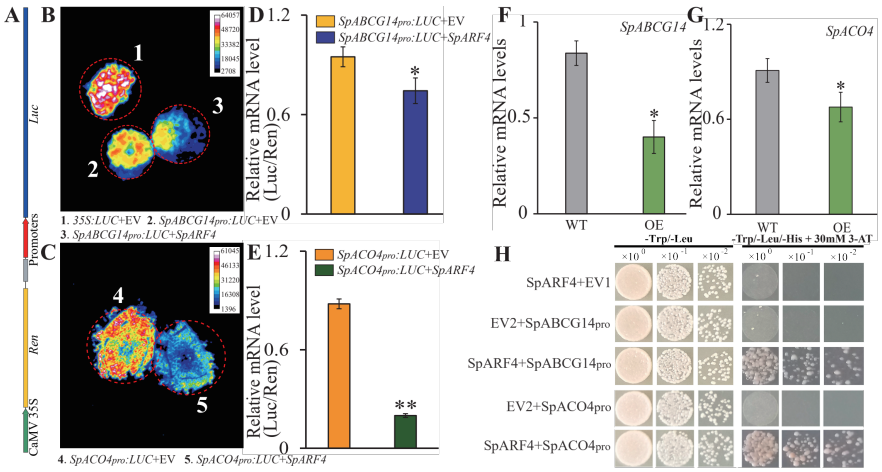


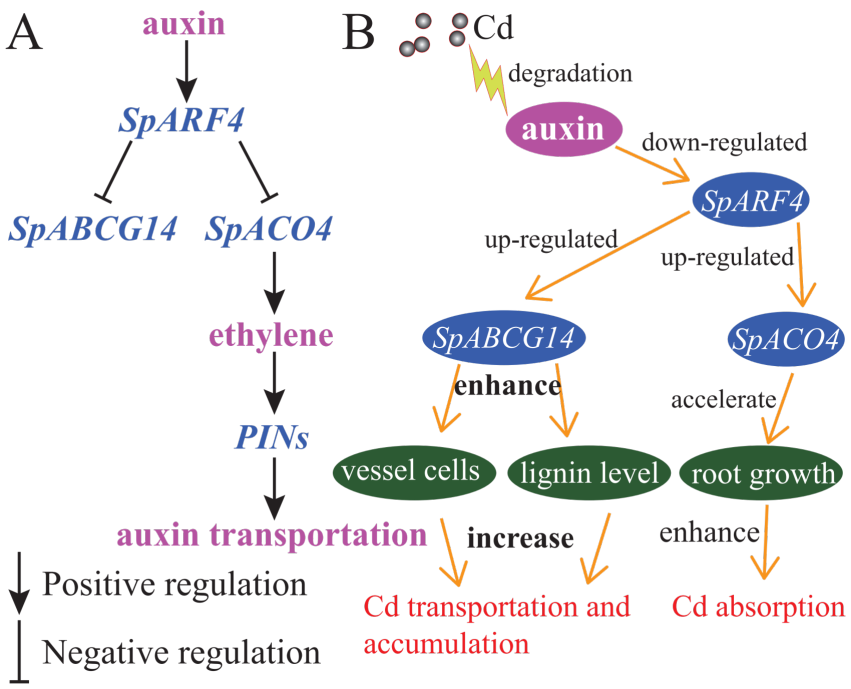


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