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ARTICLE

Overexpression of the *LcPIN2* and *LtPIN2* Gene in *Arabidopsis thaliana* Promotes Root Elongation

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ABSTRACT

The auxin polar transporter, PIN-FORMED 2 (PIN2) plays an important role in root development. However, it remains unclear whether *PIN2* genes form two *Liriodendron* species, *L. chinense (*LcPIN2*)* and *L. tulipifera (*LtPIN2*)*, are both involved in root development and whether and to what extent these two genes diverge in function. Here, we cloned and overexpressed *LcPIN2* and *LtPIN2* in A*rabidopsis thaliana* wild-type (WT) and *Atpin2* mutant. Phylogenetic and sequence analysis showed a small degree of differentiation between these two *Liriodendron PIN2* genes. Tissue-specific gene expression analysis indicated that both *Liriodendron PIN2* genes were highly expressed in roots, implying a potential role in root development. Finally, heterologous overexpression of *LcPIN2* and *LtPIN2* in *Arabidopsis* both significantly increased the root length compared to wild-type and empty vector. Furthermore, the root length defect in *Atpin2* was complemented both by *LcPIN2* and *LtPIN2*. However, heterologous overexpression of *LcPIN2* and *LtPIN2* cannot rescue the defect in root gravitropism of *Atpin2* mutants. Taken together, our findings unravel *PIN2* genes from the magnoliids plant *Liriodendron* were functionally conserved with AtPIN2 in the dicotyledonous plant *Arabidopsis* in regard to the regulation of root length, but not root gravitropism. This study also provides a potential target for genetic improvement of the root system in these valuable forest trees *Liriodendron*.

KEYWORDS

Liriodendron; *PIN2*; auxin polar transport; root development

1 Introduction

Auxin is a class of endogenous hormones that play important roles in almost every aspect of plant growth and development [1–4]. Auxin controls many aspects of plant growth and development [5–8]. Membrane transport processes [9], such as secretion and transmembrane transport [10], influence plant growth and development by establishing concentration gradients of auxin, such as apical dominance [11] and root elongation [12–14]. The auxin activity in various biological processes is mainly determined by its biosynthesis, transport, and signaling [15–18]. Among them, directional auxin transport mediated by auxin influx and efflux carriers helps to establish an auxin concentration gradient, regulating embryo development, root patterning, organ formation, etc. [19–21]. For auxin import, AUXIN1/LIKE-AUX1

(AUX1/LAX) proteins are the major auxin influx carriers, whereas PIN-FORMED (PIN), PIN-LIKES (PILS) and ATP-BINDING CASSETTE subfamily B (ABCB) proteins are major auxin efflux carriers that function in auxin export [22,23]. A total of eight PIN proteins were identified in *Arabidopsis thaliana*, five of which AtPIN1-4 and 7 are localized at the plasma membrane and act as auxin efflux carriers whereas the other three AtPIN5, 6, and 8 are localized in the endoplasmic reticulum (ER) and facilitate intracellular auxin movement between the cytosol and ER [24,25].

PIN-mediated auxin polar transport plays a crucial role in establishing a directional auxin flow in the root [13,22,26]. Specifically, auxin is first transported to the root tip by PIN1, 3, and 7 in the stele and then laterally transported by PIN3 and 7 to the epidermis where PIN2 guides auxin flow upwards to the root elongation zone [19,27–29]. Thus, *PIN2* is involved in the proper root development via establishing a directional auxin flow in the root. As a result, mutations in PIN2 led to gravitropic root growth by affecting the redistribution of auxin from the stele toward the root elongation zone in *A. thaliana* [28]. And in rice, the *pin2-1* and *pin2-2* exhibited curly root phenotypes and altered lateral root formation patterns due to the defect in proper auxin distribution. Endogenous overexpression of *PIN2* contributed to aluminum resistance both in *A. thaliana* and *Oryza sativa* [30]. In addition, overexpression of *OsPIN2* in *O. sativa* caused less sensitivity in root response to phosphate deficiency and N-1-naphthylphthalamic acid treatment [31].

Liriodendron, an ancient relict genus, is comprised of two woody plants, i.e., *L. tulipifera* and *L. chinense* [32]. *L. chinense* is naturally distributed in the east of Asia while *L. tulipifera* grows in the east of North America, comprising a pair of vicarious species with a well-known classical intercontinental disjunct distribution [33,34]. Although this species pair has been diverged since 10–16 million years ago [35], they are similar in morphological and phenological characteristics in general and interspecific hybrids with obvious heterosis were created by artificial pollination approaches. Genome-wide of the *PIN* gene family has been identified using the genome of *L. chinense* and *LcPIN2* was highly expressed in roots [36–38]. However, the potential role of *LcPIN2* in roots and whether *PIN2* genes form *L. chinense* and *L. tulipifera* diverge in function is still largely unclear. Here, we explored the above issues by cloning the full-length sequence of *PIN2* genes from *L. chinense (*LcPIN2*)* and *L. tulipifera (*LtPIN2*)* and overexpressing them in *A. thaliana*.

2 Materials and Methods

2.1 Gene Identification in Liriodendron Species

The sequence of *LcPIN2* was obtained from the previous report [37]. The *Arabidopsis* PIN2 protein sequence was downloaded from The Arabidopsis Information Resource (TAIR) [39]. Then, AtPIN2 was used as a query to search the protein database of *L. tulipifera* (unpublished data) using the NCBI Blastp program [40]. The candidate *PIN* gene from *L. tulipifera* was further submitted to the TAIR database for searching potential homologous genes, resulting in *AtPIN2*. Thus, *LcPIN2* and *LtPIN2* sequences were used as references to design primers for gene cloning.

2.2 Phylogenetic Analysis and Gene Sequence Analysis

The PIN2 protein sequences of ten species (*Solanum tuberosum*, *Salix purpurea*, *Theobroma cacao*, *Vitis vinifera*, *Anacardium occidentale*, *Corymbia citriodora*, *Cucumis sativus*, *Citrus sinensis*, *Glycine soja*, *Carya illinoinensis Pawnee*) were downloaded from Phytozome (https://phytozome-next.jgi.doe.gov/) [41]. The PIN2 protein sequence was analyzed using Blastp in the National Center for Biotechnology Information (NCBI). The PIN2 protein structure was analyzed using DNAMAN (v6.0.3.40). We use TMHMM to predict the transmembrane domains of PIN2 proteins. The phylogenetic tree of *PIN2* was reconstructed using the Neighbor-Joining algorithm with default parameters and a bootstrap value of 1000, using MEGA 7.0 software [42].

2.3 RNA Extraction and cDNA Synthesis

The total RNA was isolated from *L. chinense* and *L. tulipifera* tissue by using the FastPure Plant Total RNA Isolation Kit of Vazyme following the instructions of the manufacturer (Nanjing, China). RNA was

quantified by measuring absorbance at 260 nm, and its integrity was checked through denaturing agarose gel electrophoresis. The cDNA (complementary DNA) was synthesized from 500 ng of the total RNA by using the Evo M-MLV RT Premix (Accurate Biology, Changsha, China) for RT-qPCR according to the manufacturer's protocol.

2.4 Quantitative Real-Time PCR Analysis

The plant materials used in the quantitative analysis of *PIN2* expression across different tissues were collected from the Baima Forest Farm of Nanjing Forestry University (119.18E, 31.61N). Different tissues were obtained from adult plants and immediately frozen in liquid nitrogen and then stored in a refrigerator at −80°C (We took the tissue from 15-year-old trees). The expression of the *PIN2* gene in specific tissues, i.e., root, stem, leaf, petal, pistil, and stamen, of *L. chinense* and *L. tulipifera* were quantified by RT-qPCR analysis. Specific primers were designed according to the sequences of *LcPIN2* and *LtPIN2* genes, and quantitative experiments were performed with three biological replicates and three technical replicates. The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak method).

2.5 Plant Materials and Growth Conditions

A. thaliana Columbia ecotype (Col-0) wild-type (WT) plants were provided by Prof. Thomas Laux (Signalling Research Centres BIOSS and CIBSS, Faculty of Biology, University of Freiburg, Germany). The *Atpin2* mutant was derived from Arashare Corporation (https://www.arashare.cn/index/Product/index.html) and the company storage number is AS016. Seeds were sown on 1/2 MS medium after disinfection and then stored at 4°C for 3 days for vernalization. Next, they were transferred into a homoeothermic incubator (22°C) with a 16/8 h (light/dark) photoperiod. When seedlings reached the four leaves stage, they were transferred to the mixed matrix with peat soil, vermiculite, and perlite ratio of 5:1:1 in a greenhouse.

2.6 Heterologous Overexpression in A. thaliana

pBI121 was used to construct overexpression vectors of *Liriodendron PIN*s, i.e., *35S:* LcPIN2 *and* 35S: *LtPIN2*, which were confirmed to be correct after Sanger sequencing. The constructed overexpression vectors were respectively introduced into Agrobacterium GV3101, and the positive strains were screened by PCR through the medium containing kanamycin resistance. After that, the agrobacterium solution was inoculated in an LB medium. After centrifugation, the strains were collected, and the optical density value reached 0.6 by shaking the bacterial solution with 5% sucrose solution. Using *Arabidopsis* plants in good condition, we cut off the pod, leaving the bud unopened. After that, the bacterial solution was used to infect the *Arabidopsis* inflorescence, soak for 45 s, hide from light for 24 h, then grow normally, then collect mature seeds. The positive plants were screened by 1/2MS medium containing Kan,PCR, and RT-qPCR.

2.7 Phenotypic Identification of Transgenic A. thaliana

The collected T1 generation seeds of 35S: *LcPIN2* and 35S: *LtPIN2* were sterilized and placed in a 1/2MS medium containing Kana for screening. After a week, the positive plants were transferred to the nutrient soil and cultured in a light incubator. A week later, leaf DNA was extracted for PCR verification, and RNA was extracted for qRT-PCR to determine the expression level of the *PIN2* gene. After normal culture, T2 generation seeds were obtained, T2 generation seeds were cultured to T3 generation, and then T3 generation seeds were used to observe the phenotype.

3 Result

3.1 Gene Cloning, and Sequence Analysis of Liriodendron PIN2 Genes

LtPIN2 gene was identified using *LcPIN2* and *AtPIN2* as queries to search the protein database of *L. tulipifera* (unpublished data) by the NCBI blast program. Two pairs of primers were designed to clone the predicted gene sequences. The length and sequences of *LcPIN2* and *LtPIN2* genes were further determined by Sanger sequencing. Results showed that the length of *LcPIN2* and *LtPIN2* was both 1,848 bp in length, encoding 616 amino acids. The molecular weight of *LcPIN2* and *LtPIN2* was

predicted to be 66,771.05 Da and 66,771.18 Da, respectively, and the isoelectric point was 9.49 and 9.55, Grand average of hydropathicity (GRAVY) was 0.063 and 0.062, respectively (Table 1). The prediction results of subcellular protein showed that both *Liriodendron* PIN2 belonged to the integral membrane protein, which is consistent with the auxin output function of PIN proteins.

Table 1: The basic information of *LcPIN2* and *LtPIN2*

Gene name	CDS (bp)	Peptide (aa)	Mw (Da)	PI	GRAVY	Subcellular localization
LcPIN2	1848	616	66771.05	9.49	0.063	Integral membrane protein
LtPIN2	1848	616	66771.18	9.55	0.062	Integral membrane protein

3.2 Phylogenetic Analysis of Liriodendron PIN2 Genes

We used DNAMAN to perform multiple sequence alignment of two *Liriodendron* PIN2 together with PIN2 proteins from *Arabidopsis* and the other ten species. Extremely conserved regions were found at both ends of the protein sequences, referring to the transmembrane domain (Fig. 1). Further analysis confirmed the transmembrane domain of *LcPIN2* and *LtPIN2* (Figs. 2B and 2C), both with five transmembrane structures at the N-terminal and four transmembrane regions at the C-terminal. Phylogenetic analysis showed that *Liriodendron* PIN2 proteins were closely clustered together (Fig. 2A). Furthermore, motif analysis indicated that two *Liriodendron* PIN2 proteins shared almost the same motif arrangement except the missing motif 5 located at the C-terminal compared with PIN2 proteins from plant species (Fig. 2D).

3.3 Liriodendron PIN2 Genes Were Both Highly Expressed in Roots

We quantified the tissue expression profiling of *LcPIN2* and *LtPIN2* across different tissues, i.e., root, stem, leaf, petal, pistil, and stamen, in *L. chinense* and *L. tulipifera*, respectively (Fig. 3). The results showed that both *LcPIN2* and *LtPIN2* were highly expressed in roots compared with other tissues. And, in *L. tulipifera*, the expression level of *LtPIN2* was also relatively high in the pistil with no similar results in *L. chinense*. These results implied that *Liriodendron PIN2* genes might both function in the root development, reflecting the functional conservation. However, *LcPIN2* and *LtPIN2* have diverged at least in tissue-specific expression patterns.

3.4 Overexpressing Liriodendron PIN2 Genes Promotes Root Elongation in A. thaliana

To explore the potential role of *Liriodendron PIN2* genes in root development, we overexpressed *LcPIN2* and *LtPIN2* in *A. thaliana*. We used kanamycin to screen positive transgenic lines. The transgenic lines were further confirmed by qRT-PCR (Fig. S1), indicating an obvious heterogeneous overexpression of *LcPIN2* or *LtPIN2* compared to WT and empty vector (Fig. 4B). We did not find significant changes in root density of lateral roots of *A. thaliana* in overexpressed strains (Fig. S2). Overexpression of *LcPIN2* and *LtPIN2* significantly increased the root length compare to WT and empty vector whereas no significant difference was found between WT and empty vector (Fig. 4). Since we performed these two experiments independently, we removed potential batch effects and then compared the effects of two *Liriodendron PIN2* genes in promoting root elongation. Results showed that no significant difference existed between the root length of p35S: *LcPIN2* and p35S: *LtPIN2*. To investigate whether *LcPIN2* and *LtPIN2* promote the function of wild-type *A. thaliana* differently, we calculated and compared the root length of wild-type *A. thaliana* overexpressing *LcPIN2* and *LtPIN2* (Fig. 4D). The results showed that strain 1 overexpressing *LcPIN2* was significantly different from other strains, but there was no significant difference between strains 2 and 4 and lines overexpressing *LtPIN2*. So we determined that *LcPIN2* and *LtPIN2* may be consistent in their ability to promote wild-type *A. thaliana*. In summary, we concluded that there was no significant difference between *LcPIN2* and *LtPIN2* in promoting the root length of wild-type *A. thaliana*.

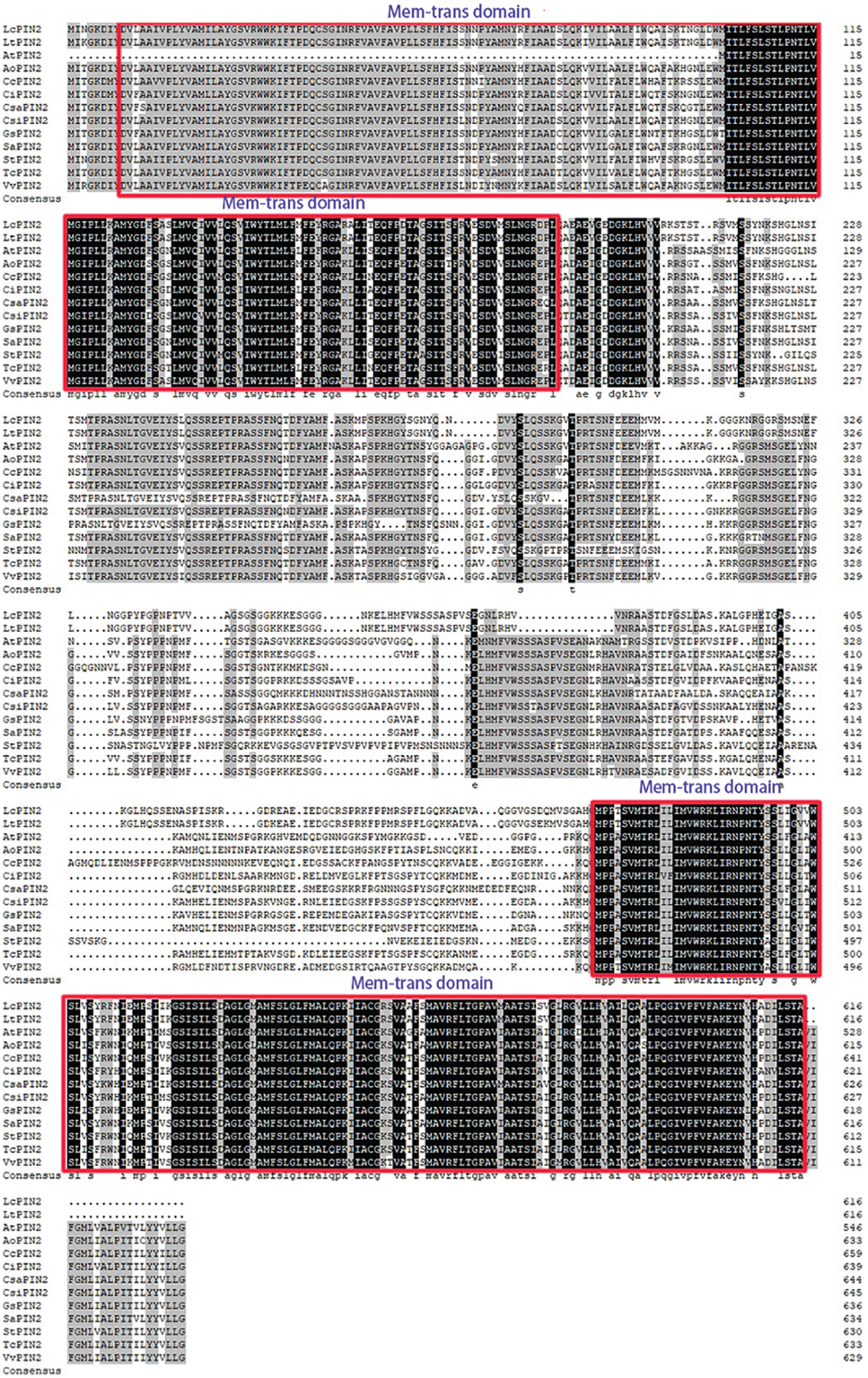

Figure 1: *LcPIN2* and *LtPIN2* genes amino acid sequence multiple alignments. The red box shows the transmembrane domain. *Anacardium occidentale* (*Ao*), *Arabidopsis thaliana* (*At*), *Corymbia citriodora* (*Cc*), *Carya illinoinensis* (*Ci*), *Cucsa sativus* (*Csa*), *Citrus sinensis* (*Csi*), *Glycine soja* (*Gs*), *Salix purpurea* (*Sp*), *Solanum tuberosum* (*St*), *Theobroma cacao* (*Tc*), *Vitis vinifera* (*Vv*)

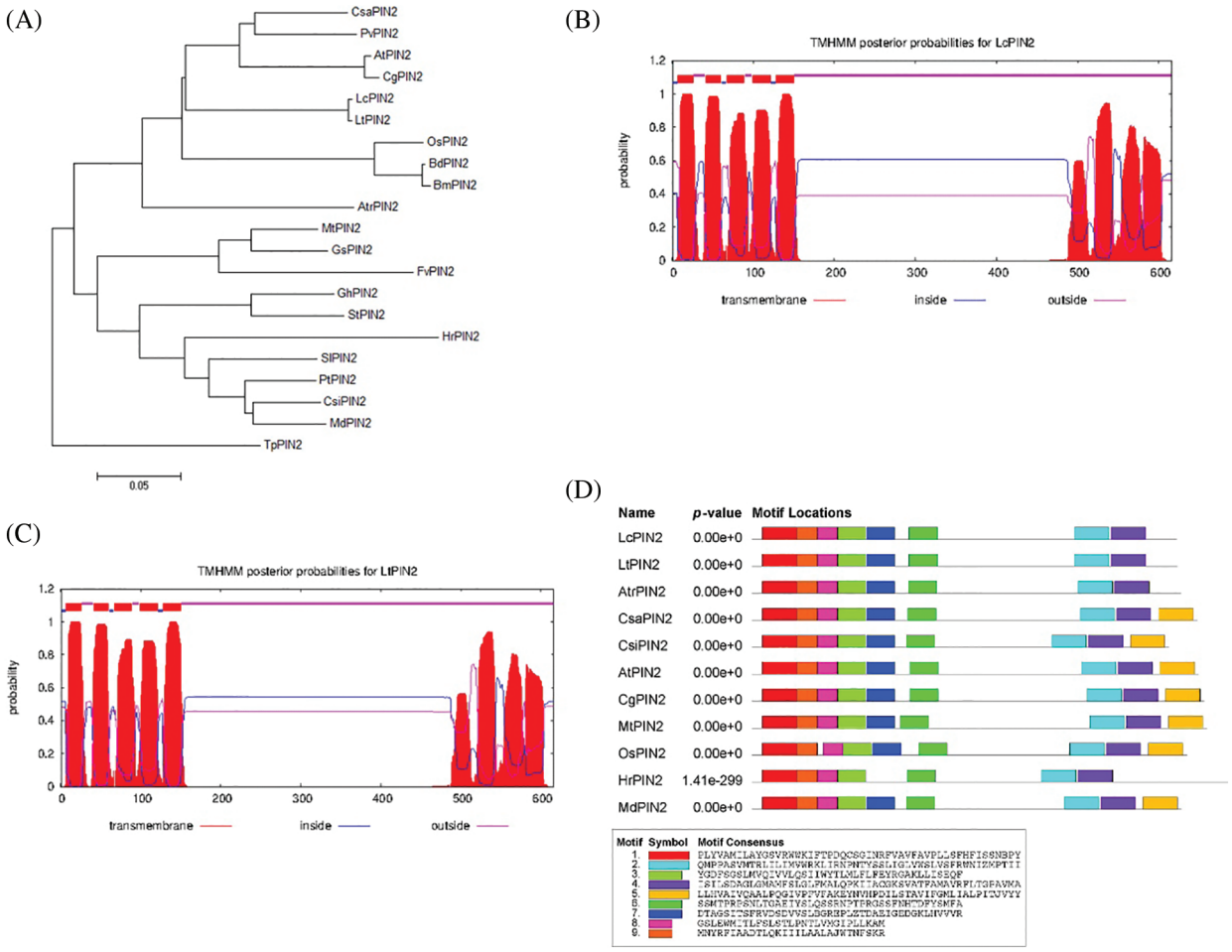

Figure 2: Evolution and structural analysis of LcPIN2 and LtPIN2. (A) Phylogenetic tree of PIN2 from *Cucumis sativus* (*Csa*), *Phaseolus vulgaris* (*Pv*), *Arabidopsis thaliana* (*At*), *Capsella grandiflora* (*Cg*), *Oryza sativa* (*Os*), *Brachypodium distachyon* (*Bd*), *Brachypodium mexicanum* (*Bm*), *Liriodendron chinense* (*Lc*), *Liriodendron tulipifera* (*Lt*), *Amborella trichopoda* (*Atr*), *Thuja plicata* (*Tp*), *Medicago truncatula* (*Mt*), *Glycine soja* (*Gs*), *Citrus sinensis* (*Csi*), *Fragaria vesca* (*Fv*), *Gossypium hirsutum* (*Gh*), *Hordeum vulgare* (*Hv*), *Populus trichocarpa* (*Pt*), *Solanum lycopersicum* (*Sl*), *Malus domestica* (*Md*), *Solanum tuberosum* (*St*), *Triticum aestivum* (*Ta*), *Zea mays* (*Zm*). (B) Prediction of the transmembrane domain of the *LcPIN2* gene. (C) Prediction of the transmembrane domain of the *LtPIN2* gene. (D) Motif analysis of *LcPIN2* and *LtPIN2*

3.5 Overexpressing Liriodendron PIN2 Genes Rescue Defects in Root Length, But Not Root Gravitropism, of Atpin2 Mutants

The phenotype of the *Atpin2* mutant is a loss of gravitropism and a decrease in the amount of root elongation [28,43]. To determine whether the *Liriodendron PIN2* gene has the function of restoring the gravitropism of the *Atpin2 mutant*, we overexpressed the *LcPIN2* and *LtPIN2* genes in the *A. thaliana* mutant. After qRT-PCR identification (Figs. 5B and S3) and T3 generations are produced by self-crossing, it was found that the overexpressed *Atpin2* mutant under vertical culture conditions, the roots of *Atpin2* mutant did not return to gravity, and the roots remained curled without a definite downward growth direction (Figs. 5A and 5C). We found no significant change in lateral root density in overexpressed *AtPIN2* (Fig. S4). Therefore, we believe that *LcPIN2* and *LtPIN2* do not restore gravitropism in *Arabidopsis Atpin2* mutants. The root length of the *Atpin2* mutant was measured 18 days after the mutant grew (Fig. 5D, Table S1). It was found that the root of the *Atpin2* mutant overexpressing *LcPIN2* and *LtPIN2* genes was longer than that of the WT, and the change was statistically significant

after statistical analysis. This result is similar to that of the wild-type *A. thaliana* with overexpression of the *Liriodendron PIN2* gene. To investigate whether *LcPIN2* and *LtPIN2* promote the function of the *Atpin2* mutant differently, we calculated and compared the root length of the *Atpin2* mutant overexpressing *LcPIN2* and *LtPIN2* (Fig. 5D). *LcPIN2* and *LtPIN2* genes were overexpressed in the *Atpin2* mutant, and statistical analysis showed that there was no significant difference between overexpressed *LcPIN2* and *LtPIN2* in *A. thaliana*. In summary, we concluded that there was no significant difference between *LcPIN2* and *LtPIN2* in promoting the root length of the *Atpin2* mutant.

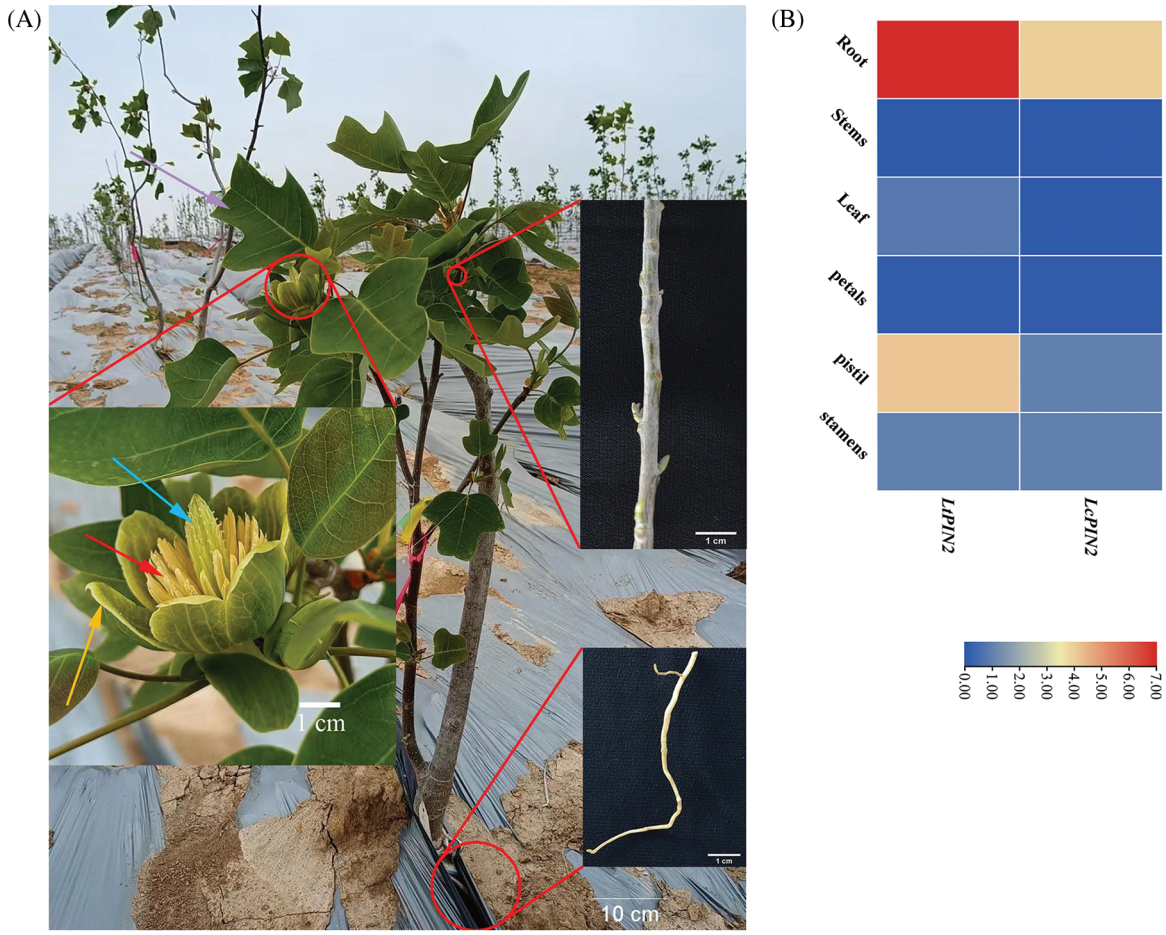

Figure 3: Expression patterns of *LcPIN2* and *LtPIN2* across different tissues. (A) Schematic diagram of *Liriodendron* sampling site. The root, stem, and flower are shown in enlarged pictures. The purple arrow represents the leaf, the cyan arrow represents the pistil, the red arrow represents the stamen and the yellow arrow represents the petal. (B) Gene expression patterns of *LcPIN2* and *LtPIN2* in different tissues of *L. chinensis* and *L. tulipifera*, respectively

4 Discussion

PIN proteins, as the major auxin efflux carriers, function in the auxin polar transport, thus playing an important role in the various biological process [22]. In plant roots, the combined action of several PINs constructs a bidirectional auxin flow in the root which affects the root development [22]. Here, we cloned two *PIN2* genes from two intercontinental vicariate species in *L. chinense* and *L. tulipifera*. Phylogenetic and sequence analysis indicated that these two genes showed a small degree of differentiation at least in the levels of protein sequence, motif, and domain (Figs. 1 and 2). Furthermore, the relatively high expression in roots suggested a conserved function of these two *Liridoendron PIN* genes (Fig. 3). However, the tissue expression analysis also indicated a potential functional divergence in expression intensity in roots and

expression in pistil between *LcPIN2* and *LtPIN2* (Fig. 3). Further heterologous overexpression in *Arabidopsis* confirmed the conserved fucntion of these two *Liridoendron PIN2* genes in promoting root elongation (Figs. 4 and 5). This is opposite to the phenotype of overexpression of *OsPIN2* in rice which led to an obvious inhibition in root length [31,44]. This might be the reflection of the evolutionary divergence since *Lirirodenron* belongs to the magnoliids which have diverged from other flowering plants during early angiosperm evolution [36]. Previous studies have shown that the *PIN2* gene in *A. thaliana* can compensate for the geotropism of *Atpin2* deletion [45]. To further study the function of *Liriodendron PIN2*, we overexpressed *LcPIN2* and *LtPIN2* in the *Atpin2* mutant. The results showed that *LcPIN2* and *LtPIN2* could not compensate for the loss of geotropism in the mutant, but they could promote the root growth of the *Atpin2* in terms of root length, indicating that *Liriodendron PIN2* could compensate for the reduced root length of the *Atpin2* mutant. Therefore, the *PIN2* gene can partially compensate for the missing phenotype of the *Atpin2* mutant. We compared the effects of *LcPIN2* and *LtPIN2* on root elongation of WT and *Atpin2* mutants, and found that *LcPIN2* and *LtPIN2* had similar effects on root elongation, indicating that *LcPIN2* and *LtPIN2* are highly conserved and have similar functions.

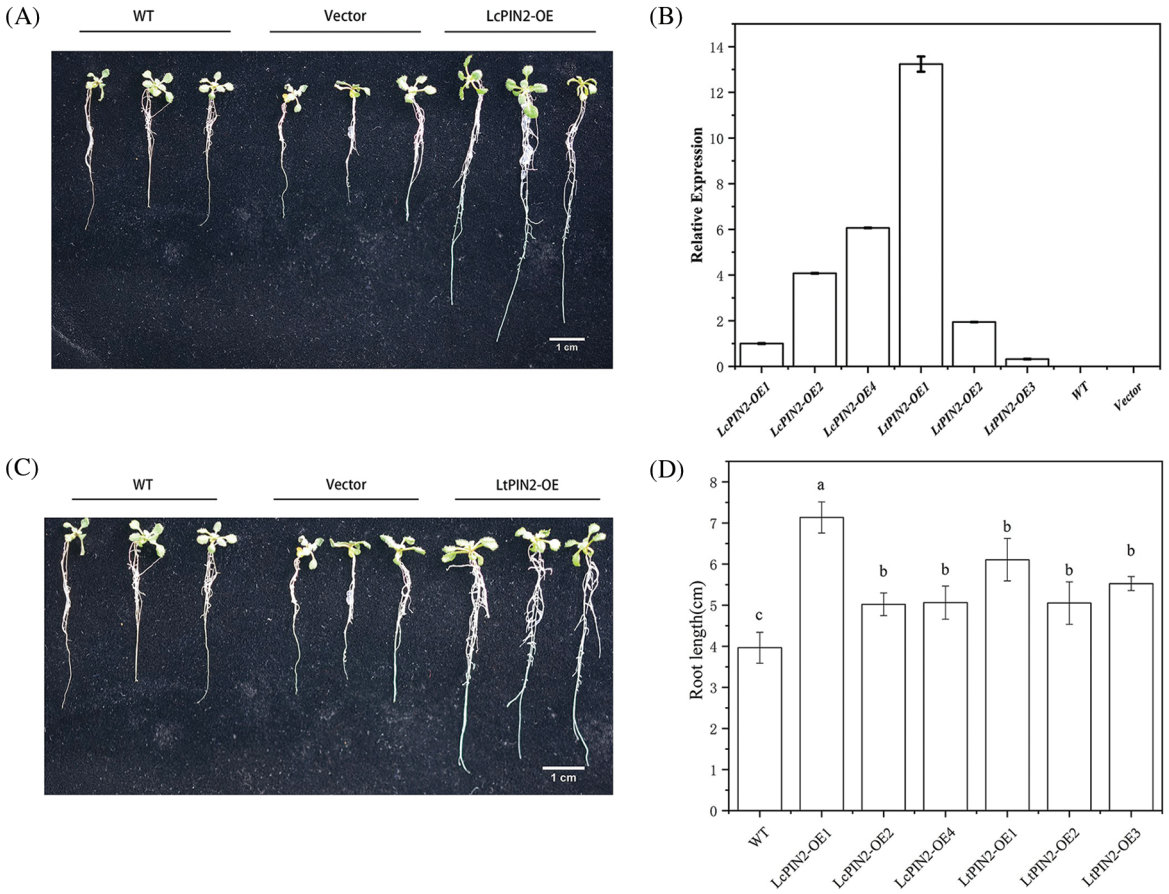

Figure 4: Overexpression of *PIN2* promotes root growth in *A. thaliana* plants. (A) Representative photographs of *A. thaliana* wild type (WT), *LcPIN2-1*, *LcPIN2-2*, and *LcPIN2-4* plants, grown for 12 d on plates. (B) Expression of *PIN2* gene in transgenic wild *A. thaliana*. The *PIN2* gene expression of LcPIN2-OE1 was 1. (C) Representative photographs of *Arabidopsis* wild-type (WT), *LtPIN2-1*, *LtPIN2-2*, and *LtPIN2-3* plants, grown for 12 d on plates. (D) Comparison of root length of wild-type *A. thaliana* overexpressing *LcPIN2* and *LtPIN2* genes. The three plants on the left are wild-type *Arabidopsis*, the three plants in the middle are no-load controls, and the three plants on the right are *35S: PIN2* transgenic *A. thaliana*. Bar = 1 cm. All experiments were normalized using *A. thaliana Actin* as an internal reference, and three independent experiments were performed. The error bars in the charts indicate the standard deviation from the mean of each triplicate treatment

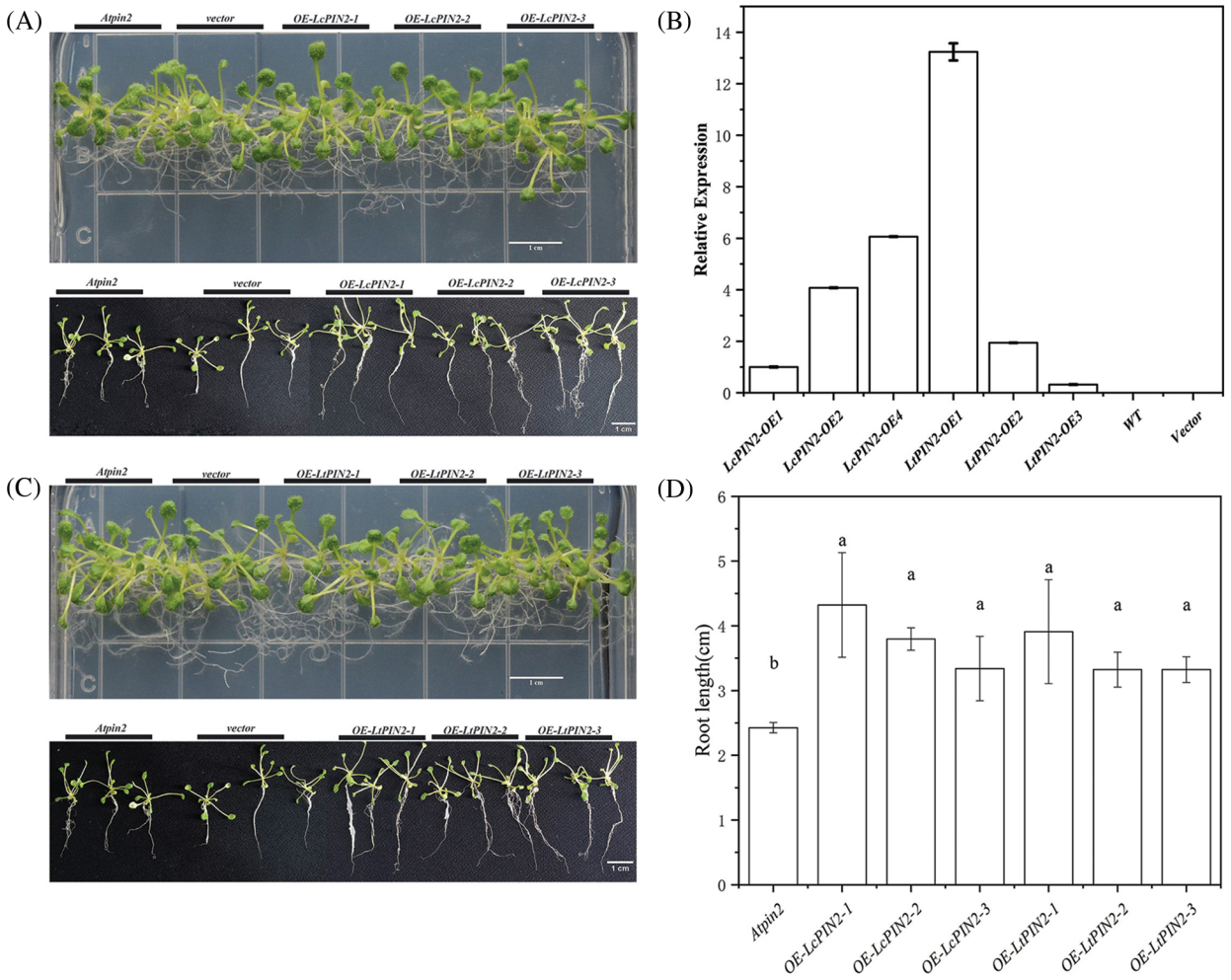

Figure 5: Overexpression of *PIN2* promotes root growth in *A. thaliana* plants. (A) Representative photographs of *Atpin2* mutant, different strains that overexpress *LcPIN2* (OE-LcPIN2-1, OE-LcPIN2-2, OE-LcPIN2-3), grown for 18 d on plates. (B) Expression of *PIN2* gene in transgenic *Atpin2* mutant. The *PIN2* gene expression of OE-LcPIN2-1 was 1. (C) Representative photographs of *Atpin2* mutant, different strains that overexpress *LtPIN2* (OE-LtPIN2-1, OE-LtPIN2-2, OE-LtPIN2-3), grown for 18 d on plates. (D) Comparison of root length of *Atpin2* mutant overexpressing *LcPIN2* and *LtPIN2* genes. The three plants on the left are *Atpin2* mutants, the three plants in the Second group on the left are no-load controls, and the three plants on the right are *35S: PIN2* transgenic *A. thaliana*. Bar = 1 cm. All experiments were normalized using *A. thaliana Actin* as an internal reference, and three independent experiments were performed. The error bars in the charts indicate the standard deviation from the mean of each triplicate treatment

5 Conclusion

In this study, the *LtPIN2* gene was first reported, and the similarities and differences between two *Liridoendron PIN* genes, *LcPIN2* and *LtPIN2*, were illustrated. Heterologous overexpression in *Arabidopsis* suggested that these two *Liriodendron PIN2* genes both functioned in promoting root elongation with no obvious functional divergence. Overexpression of *LcPIN2* and *LtPIN2* in the *Atpin2* mutant indicated that the *PIN2* gene of *Liriodendron* could partially compensate for the *Atpin2* mutant

phenotype. Thus, our study provides the first insights into how *LcPIN2* and *LtPIN2* genes may be used in the improvement of the root system in *Liriodendron*.

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Author Contributions: Z. D. H. and J. H. C. were the lead investigators of this research program. Z. D. H. and Z. J. C. designed the experiments and coordinated the project. Z. J. C. performed all the experiment works. Z. D. H. and Z. J. C. wrote and edited most of the manuscript. All authors have read and approved the final manuscript.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

References

1. Pacurar, D. I., Perrone, I., Bellini, C. (2014). Auxin is a central player in the hormone cross-talks that control adventitious rooting. *Physiologia Plantarum, 151(1),* 83–96. https://doi.org/10.1111/ppl.12171
2. Zhang, Y., Ma, Y., Zhao, D., Tang, Z., Zhang, T. et al. (2022). Genetic regulation of lateral root development. *Plant Signaling & Behavior,* 2081397. https://doi.org/10.1080/15592324.2022.2081397
3. Gu, H., Ding, W., Shi, T., Ouyang, Q., Yang, X. et al. (2022). Integrated transcriptome and endogenous hormone analysis provides new insights into callus proliferation in *Osmanthus fragrans*. *Scientific Reports, 12(1),* 7609. https://doi.org/10.1038/s41598-022-11801-9
4. Adamowski, M., Friml, J. (2015). PIN-dependent auxin transport: Action, regulation, and evolution. *Plant Cell, 27(1),* 20–32. https://doi.org/10.1105/tpc.114.134874
5. Ludwig-Muller, J. (2015). Bacteria and fungi controlling plant growth by manipulating auxin: Balance between development and defense. *Journal of Plant Physiology, 172,* 4–12. https://doi.org/10.1016/j.jplph.2014.01.002
6. Li, T., Dai, Z., Zeng, B., Li, J., Ouyang, J. et al. (2022). Autocatalytic biosynthesis of abscisic acid and its synergistic action with auxin to regulate strawberry fruit ripening. *Horticulture Research*, *9,* uhab076. https://doi.org/10.1093/hr/uhab076
7. Weijers, D. (2016). Phyllotaxis: A Matthew effect in auxin action. *Current Biology, 26(23),* R1233–R1235. https://doi.org/10.1016/j.cub.2016.10.019
8. Pattison, R. J., Csukasi, F., Catala, C. (2014). Mechanisms regulating auxin action during fruit development. *Physiol Plant, 151(1),* 62–72. https://doi.org/10.1111/ppl.12142
9. Xie, P., Cath, T. Y., Ladner, D. A. (2021). Mass transport in osmotically driven membrane processes. *Membranes, 11(1)*. https://doi.org/10.3390/membranes11010029
10. Yin, S., Duan, M., Fang, B., Zhao, G., Leng, X. et al. (2022). Zinc homeostasis and regulation: Zinc transmembrane transport through transporters. *Critical Reviews in Food Science and Nutrition,* 1–11. https://doi.org/10.1080/10408398.2022.2048292
11. Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *Plant Journal, 45(6),* 1028–1036. https://doi.org/10.1111/j.1365-313X.2006.02656.x
12. Hussain, S., Wang, W., Ahmed, S., Wang, X. T., Adnan et al. (2021). PIP2, an auxin induced plant peptide hormone regulates root and hypocotyl elongation in *Arabidopsis*. *Frontiers in Plant Science, 12*. https://doi.org/10.3389/fpls.2021.646736
13. Inahashi, H., Shelley, I. J., Yamauchi, T., Nishiuchi, S., Takahashi-Nosaka, M. et al. (2018). *OsPIN2*, which encodes a member of the auxin efflux carrier proteins, is involved in root elongation growth and lateral root formation patterns via the regulation of auxin distribution in rice. *Physiologia Plantarum, 164(2),* 216–225. https://doi.org/10.1111/ppl.12707

14. Qi, Y. H., Wang, S. K., Shen, C. J., Zhang, S. N., Chen, Y. et al. (2012). OsARF12, a transcription activator on auxin response gene, regulates root elongation and affects iron accumulation in rice (*Oryza sativa*). *New Phytologist, 193(1),* 109–120. https://doi.org/10.1111/j.1469-8137.2011.03910.x
15. Teale, W. D., Paponov, I. A., Palme, K. (2006). Auxin in action: Signalling, transport and the control of plant growth and development. *Nature Reviews Molecular Cell Biology, 7(11),* 847–859. https://doi.org/10.1038/nrm2020
16. Tivendale, N. D., Belt, K., Berkowitz, O., Whelan, J., Millar, A. H. et al. (2021). Knockdown of succinate dehydrogenase assembly factor 2 induces reactive oxygen species-mediated auxin hypersensitivity causing pH-dependent root elongation. *Plant and Cell Physiology, 62(7),* 1185–1198. https://doi.org/10.1093/pcp/pcab061
17. Street, I. H., Mathews, D. E., Yamburkenko, M. V., Soroosh zadeh, A., John, R. T. et al. (2016). Cytokinin acts through the auxin influx carrier AUX1 to regulate cell elongation in the root. *Development, 143(21),* 3982–3993. https://doi.org/10.1242/dev.132035
18. Song, P., Xu, H., Zhang, J. X., Chen, H. T., Li, L. et al. (2021). Functional analysis of indole 3-hexanoic acid as a novel auxin from *Arabidopsis thaliana*. *Planta, 254(4)*. https://doi.org/10.1007/s00425-021-03719-9
19. Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T. et al. (2002). AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. *Cell, 108(5),* 661–673. https://doi.org/10.1016/S0092-8674(02)00656-6
20. Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D. et al. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell, 115(5),* 591–602. https://doi.org/10.1016/S0092-8674(03)00924-3
21. Liu, Y., Mu, C., Du, D., Yang, Y., Li, L. et al. (2022). Alkaline stress reduces root waving by regulating PIN7 vacuolar transport. *Frontiers in Plant Science, 13,* 1049144. https://doi.org/10.3389/fpls.2022.1049144
22. Petrasek, J., Friml, J. (2009). Auxin transport routes in plant development. *Development, 136(16),* 2675–2688. https://doi.org/10.1242/dev.030353
23. Geisler, M. M. (2021). A retro-perspective on auxin transport. *Frontiers in Plant Science, 12,* 756968. https://doi.org/10.3389/fpls.2021.756968
24. Vieten, A., Sauer, M., Brewer, P. B., Friml, J. (2007). Molecular and cellular aspects of auxin-transport-mediated development. *Trends in Plant Science, 12(4),* 160–168. https://doi.org/10.1016/j.tplants.2007.03.006
25. Naramoto, S. (2017). Polar transport in plants mediated by membrane transporters: Focus on mechanisms of polar auxin transport. *Current Opinion in Plant Biology, 40,* 8–14. https://doi.org/10.1016/j.pbi.2017.06.012
26. Zhang, M., Wang, C. P., Lin, Q. F., Liu, A. H., Wang, T. et al. (2015). A tetratricopeptide repeat domain-containing protein SSR1 located in mitochondria is involved in root development and auxin polar transport in *Arabidopsis*. *Plant Journal, 83(4),* 582–599. https://doi.org/10.1111/tpj.12911
27. Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I. et al. (2005). The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature, 433(7021),* 39–44. https://doi.org/10.1038/nature03184
28. Muller, A., Guan, C., Galweiler, L., Tanzler, P., Huijser, P. et al. (1998). *AtPIN2* defines a locus of *Arabidopsis* for root gravitropism control. *EMBO Journal, 17(23),* 6903–6911. https://doi.org/10.1093/emboj/17.23.6903
29. Friml, J., Benkova, E., Mayer, U., Palme, K., Muster, G. (2003). Automated whole mount localisation techniques for plant seedlings. *Plant Journal, 34(1),* 115–124. https://doi.org/10.1046/j.1365-313X.2003.01705.x
30. Wu, D., Shen, H., Yokawa, K., Baluska, F. (2014). Alleviation of aluminium-induced cell rigidity by overexpression of *OsPIN2* in rice roots. *Journal of Experimental Botany, 65(18),* 5305–5315. https://doi.org/10.1093/jxb/eru292
31. Sun, H., Guo, X., Xu, F., Wu, D., Zhang, X. et al. (2019). Overexpression of *OsPIN2* regulates root growth and formation in response to phosphate deficiency in rice. *International Journal of Molecular Science, 20(20)*. https://doi.org/10.3390/ijms20205144
32. Long, X. F., Weng, Y. H., Liu, S. Q., Hao, Z. D., Sheng, Y. et al. (2019). Genetic diversity and differentiation of relict plant liriodendron populations based on 29 novel EST-SSR markers. *Forests, 10(4)*. https://doi.org/10.3390/f10040334

33. Hao, R. (1995). Geographical distribution of Liriodendron chinense in China and its significance. *Journal of Plant Resources and Environment (China), 4(1),* 1–6.
34. Parks, C. R., Miller, N. G., Wendel, J. F., McDougal, K. M. (1983). Genetic divergence within the genus *Liriodendron* (Magnoliaceae). *Annals of the Missouri Botanical Garden, 70(4),* 658–666.
35. Parks, C. R., Wendel, J. F. (1990). Molecular divergence between Asian and North American species of *Liriodendron* (Magnoliaceae) with implications for interpretation of fossil floras. *American Journal of Botany, 77(10),* 1243–1256.
36. Chen, J., Hao, Z., Guang, X., Zhao, C., Wang, P. et al. (2019). Liriodendron genome sheds light on angiosperm phylogeny and species-pair differentiation. *Nature Plants, 5(1),* 18–25. https://doi.org/10.1038/s41477-018-0323-6
37. Hu, L., Wang, P., Long, X., Wu, W., Zhang, J. et al. (2021). The *PIN* gene family in relic plant *L. chinense*: Genome-wide identification and gene expression profiling in different organizations and abiotic stress responses. *Plant Physiology and Biochemistry, 162(2),* 634–646. https://doi.org/10.1016/j.plaphy.2021.03.030
38. Li, R., Pan, Y., Hu, L. F., Yang, D. J., Yuan, M. J. et al. (2022). PIN3 from liriodendron may function in inflorescence development and root elongation. *Forests, 13(4),* 568. https://doi.org/10.3390/f13040568
39. Lamesch, P., Berardini, T. Z., Li, D., Swarbreck, D., Wilks, C. et al. (2012). The Arabidopsis Information Resource (TAIR): Improved gene annotation and new tools. *Nucleic Acids Research, 40,* 1202–1210. https://doi.org/10.1093/nar/gkr1090
40. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215(3),* 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
41. Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D. et al. (2012). Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Research, 40,* 1178–1186. https://doi.org/10.1093/nar/gkr944
42. Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution, 33(7),* 1870–1874. https://doi.org/10.1093/molbev/msw054
43. Tan, C., Wang, H., Zhang, Y., Qi, B., Xu, G. et al. (2011). A proteomic approach to analyzing responses of *Arabidopsis thaliana* root cells to different gravitational conditions using an agravitropic mutant, *pin2* and its wild type. *Proteome Science, 9,* 72. https://doi.org/10.1186/1477-5956-9-72
44. Chen, Y., Fan, X., Song, W., Zhang, Y., Xu, G. (2012). Over-expression of *OsPIN2* leads to increased tiller numbers, angle and shorter plant height through suppression of *OsLAZY1*. *Plant Biotechnology Journal, 10(2),* 139–149. https://doi.org/10.1111/j.1467-7652.2011.00637.x
45. Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wisniewska, J. et al. (2006). Intracellular trafficking and proteolysis of the *Arabidopsis* auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nature Cell Biology, 8(3),* 249–256. https://doi.org/10.1038/ncb1369

Supplementary Materials

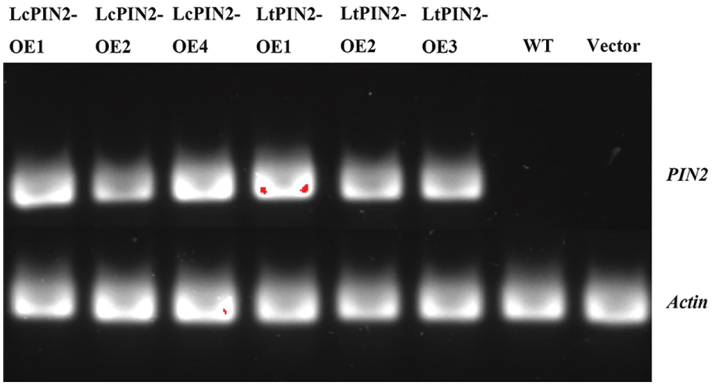

Figure S1: Semi-quantitative map of overexpression of *LcPIN2* and *LtPIN2* in *A. thaliana*

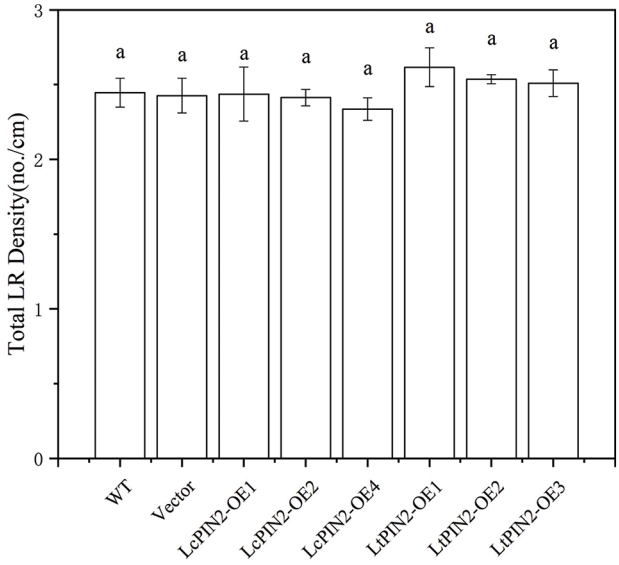

Figure S2: Lateral root density of overexpressed *LcPIN2* and *LtPIN2* in *A. thaliana*

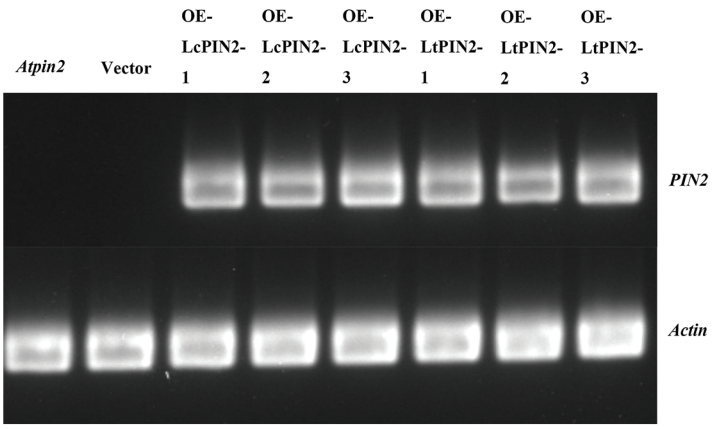

Figure S3: Semi-quantitative map of overexpression of *LcPIN2* and *LtPIN2* in *AtPIN2*

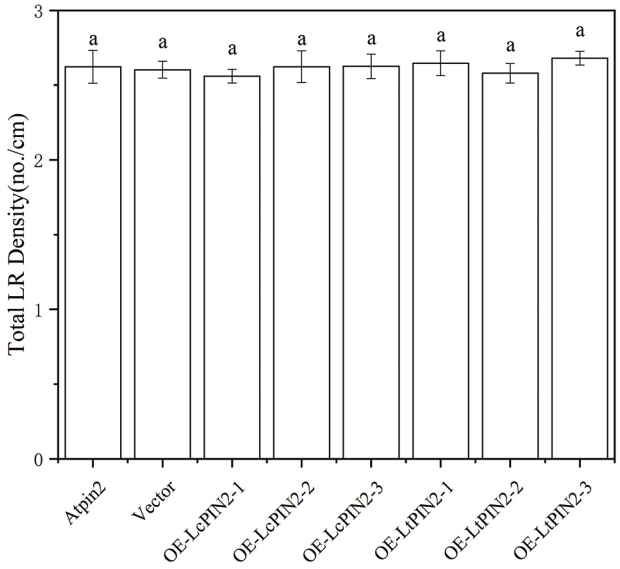

Figure S4: Lateral root density of overexpressed *LcPIN2* and *LtPIN2* in *AtPIN2*

Table S1: Gene cloning primers and experimental data

LtPIN2-F	ATGATCAAAGGCAAGGACATCTACGACG
LtPIN2-R	TTACGCCGTGCTGAGTATGTCTG
LcPIN2-F	ATGATCAATGGCAAGGACATCTACG
LcPIN2-R	CGCCGTGCTGAGTATGTCTG

	1	2	3	Mean value
WT	4.29	3.55	4.05	3.963333
Vector	3.61	3.12	3.74	3.49
OE-LcPIN2-1	6.81	7.55	7.04	7.133333
OE-LcPIN2-2	4.96	4.78	5.32	5.02
OE-LcPIN2-4	5.25	5.34	4.6	5.063333

	1	2	3	Mean value
WT	4.29	3.55	4.05	3.963333
Vector	3.61	3.12	3.74	3.49
OE-LtPIN2-1	5.52	6.31	6.49	6.106667
OE-LtPIN2-2	4.92	4.61	5.62	5.05
OE-LtPIN2-3	5.72	5.44	5.41	5.523333

	1	2	3	Mean value
Atpin2	2.51	2.35	2.42	2.426667
Vector	2.23	2.47	2.67	2.456667
OE-LcPIN2-1	4.24	5.17	3.56	4.323333
OE-LcPIN2-2	3.98	3.77	3.64	3.796667
OE-LcPIN2-3	3.91	3.12	2.99	3.34

(Continued)

Table S1 (continued)

	1	2	3	Mean value
Atpin2	2.47	2.31	2.18	2.32
Vector	2.56	2.29	2.79	2.546667
OE-LtPIN2-1	3.75	4.78	3.2	3.91
OE-LtPIN2-2	3.48	3.01	3.48	3.323333
OE-LtPIN2-3	3.12	3.33	3.52	3.323333