

Article

The Peroxidase12 Gene Regulates Morphogenesis of the Daughter Root of *Aconitum carmichaelii* Debx. by Promoting Lignin Synthesis

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Simple Summary

Research on the diterpenoid alkaloids of *Aconitum carmichaelii* Debx. has been relatively extensive, yet systematic studies on the development of its daughter roots remain limited. The daughter root serves as both the direct source of the medicinal material “FuZi” and the vegetative propagation material, and its morphogenesis directly affects the yield and quality of the medicinal products. This study focused on key factors regulating the morphology of the daughter root and found that the expression of the peroxidase gene *AcPRX12* was consistently higher in non-swollen regions than in swollen regions of the daughter root, with its expression pattern aligning with the trend of lignin accumulation. The full-length transcript of *AcPRX12* was obtained using RACE technology, and bioinformatics analysis suggested that the encoded protein is likely involved in lignin biosynthesis. Heterologous expression of *AcPRX12* in *Arabidopsis* significantly increased lignin content and inhibited plant growth, further confirming that this gene influences organ morphology by regulating lignin synthesis.

Abstract

Although the diterpenoid alkaloids of *Aconitum carmichaelii* Debx. have long been a research focus in phytochemistry and pharmacology, systematic studies on the growth and development of its daughter roots remain limited, yet this process critically determines the yield and quality of the medicinal material. This study utilized the Jiangyou-derived daughter root of *A. carmichaelii* as experimental material. Quantitative real-time polymerase chain reaction (qRT-PCR) and total lignin quantification demonstrated that both the expression level of *AcPRX12* and total lignin relative content were consistently higher in the non-swollen (PB) parts than in the swollen (P) parts of the daughter roots. The complete cDNA sequence of the *AcPRX12* (with a full length of 1357 bp and encoding 350 amino acids) was obtained by rapid amplification of cDNA ends (RACE). Bioinformatics analysis identified *AcPRX12* as an extracellular class III peroxidase containing a secretory peroxidase domain, and further predicted its strong binding affinity for syringaldazine, an S-type lignin monomer analog. In addition, the heterologous expression of *AcPRX12* in *Arabidopsis thaliana* resulted in a significant increase in lignin content, which inhibited plant growth, as evidenced by shorter roots, thinner stems, smaller leaves, and shorter siliques. Collectively, these results support a model in which *AcPRX12* promotes lignin biosynthesis to modulate daughter root development, ultimately shaping its distinctive tapered morphology. In conclusion, our findings propose a lignin-mediated regulatory mechanism for daughter root development controlled by *AcPRX12*, offering a key gene resource and a theoretical basis for understanding its morphogenesis.



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Keywords: *Aconitum carmichaelii* Debx.; AcPRX12; lignin synthesis; daughter root morphogenesis

1. Introduction

Valued primarily for its roots, the medicinal plant *A. carmichaelii* (Ranunculaceae) yields two key products: the processed mother root and daughter root, known as “Chuanwu” and “Fuzi” in the Chinese Pharmacopeia, respectively. In China, this species has been used for over 2000 years and cultivated for more than 1000 years. The daughter root of *A. carmichaelii* is a storage organ formed by the swelling of an adventitious root from the mother root, which also functions as its asexual reproductive structure. Interestingly, once detached from the mother root, the daughter root of *A. carmichaelii* can establish as an autonomous plant, generating adventitious roots downward and differentiating a shoot bud upward. To produce the traditional medicinal materials Chuanwu and Fuzi, the plant is propagated in Chinese cultivation areas using its daughter roots as planting stock. In particular, Jiangyou, Sichuan Province, is the geo-authentic producing area of Fuzi. Furthermore, modern pharmacological research has identified diverse activities in Fuzi, including cardiac strengthening, anti-inflammatory, analgesic, and immunoregulatory effects, which are primarily mediated by its characteristic alkaloids [1–3]. Thus, increasing research attention has been paid to alkaloids in recent years, particularly their chemical composition, biosynthesis, and mechanisms of toxicity [4–6]. However, research on the developmental regulation of the daughter roots remains limited. Most studies have been predominantly descriptive at the anatomical level or focused on preliminary candidate gene screening, lacking in-depth functional validation. But in fact, the morphogenesis of the daughter root directly determines the yield and quality of the medicinal material. Analyzing the developmental patterns of the daughter root is crucial for regulating its morphology and thereby ensuring a sustainable resource supply.

The roots of *A. carmichaelii* are storage roots, similar to those of crops like sweet potato and carrot. Research on sweet potato has shown that adventitious roots with five or more primary xylem ridges develop into tuberous roots. In contrast, only four ridges typically remain as non-thickening fibrous roots [7]. This pattern corresponds to anatomical observations in *A. carmichaelii*; roots with three to four primary xylem ridges and higher central lignification exhibit restricted radial expansion, while those with four to seven ridges and lower lignification can further differentiate into tuberous roots [8]. Lignification, the process of lignin deposition in cell walls, is known to suppress storage root development. In sweet potato, for instance, it inhibits the swelling of adventitious roots into starch-storing tubers and promotes the formation of slender “pencil roots” [9]. Exogenous gibberellin application enhances lignification and lignin deposition in sweet potato roots, reducing lateral root number and length, as well as storage root number and diameter [10]. Similarly, gibberellin increases lignification in carrot roots and restricts their radial expansion [11]. Collectively, these findings indicate that lignin plays a critical regulatory role in the morphogenesis of storage roots.

Lignin, an aromatic polymer and a principal component of vascular plant cell walls, is incorporated into the plant cell wall structure. Its biosynthesis involves repeated free-radical coupling reactions between newly synthesized lignin monomers (primarily the H, S, and G types) and existing cell wall polymers or oligomers. This process is catalyzed by laccases (Lac) and/or peroxidases (Prx) [12–16]. Among the at least four classes of peroxidases in higher plants, class III peroxidase (EC 1.11.1.7) is a plant-specific secretory enzyme involved in diverse physiological processes, including lignification, defense responses, and development [17]. Functional studies in *Arabidopsis thaliana* underscore the key role

of peroxidases in lignin synthesis. For example, the lignin content in *AtPrx72* knockout mutants is significantly reduced compared to the wild type [18]. *AtPRX64* contributes to lignin biosynthesis in stems, and overexpression of *AtPRX64* in transgenic tobacco increases total lignin content [19,20]. Together, these findings demonstrate that peroxidases play a significant role in the plant lignin biosynthetic pathway.

This study focused on *AcPRX12*, a peroxidase gene differentially expressed in the lignin synthesis pathway during the daughter root development. Using the daughter roots of Jiangyou *A. carmichaelii* as material, we employed qRT-PCR to profile *AcPRX12* expression in the P and PB root sections across developmental stages, concurrently measuring their total lignin relative content using the thioglycolic acid method. Then, we obtained the full-length cDNA of *AcPRX12* via RACE and conducted a bioinformatic functional analysis. Finally, heterologous expression in *Arabidopsis thaliana* provided preliminary verification of its role in promoting lignification, laying a foundation for elucidating the molecular mechanism underlying the daughter root morphogenesis.

2. Materials and Methods

2.1. Plant Material Source and Processing

2.1.1. The Daughter Root Material

Aconitum carmichaelii Debx. plants were sourced from Puzhao Village, Jiangyou City, Sichuan Province, China (31°43'55" N, 104°41'42" E; altitude: 547 m). Daughter roots were collected from early April to mid-June 2023. Based on their diameter (*d*), roots were categorized into five developmental stages (S1–S5): S1 ($1 < *d \leq 4$ mm), S2 ($4 < *d \leq 10$ mm), S3 ($10 < *d \leq 20$ mm), S4 ($20 < *d \leq 30$ mm), and S5 ($30 < *d \leq 50$ mm). For each stage, three biological replicates were prepared, each comprising four plants. All roots were washed, surface-sterilized, and dissected to remove the basal junction and lateral roots. They were then separated into the P and PB regions (except S1), flash-frozen in liquid nitrogen, and stored at -80 °C for subsequent analysis.

2.1.2. Arabidopsis Material and Growth Conditions

Wild-type *Arabidopsis thaliana* ecotype Col-0, obtained from the National Engineering Laboratory for Northwest Endangered Medicinal Materials Resources and Development at Shaanxi Normal University, was used for heterologous expression. Seeds were surface-sterilized and sown on 1/2 Murashige and Skoog (MS) medium. Plants were grown in a growth chamber under controlled conditions: 22 °C, a 16 h light/8 h dark photoperiod, and 65% relative humidity. After approximately two weeks, seedlings at the 4–6 true leaf stage were transferred to pots containing a 3:1:1 (v/v/v) mixture of peat soil, perlite, and vermiculite, and maintained under the same growth conditions for subsequent transformation experiments.

2.2. qRT-PCR Analysis

The P and PB root segments from developmental stages S1 to S5 of *A. carmichaelii* were used as experimental materials. Total RNA was extracted separately from each segment using the RNeasy Pure Polysaccharide Polyphenol Plant Total RNA Extraction Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. RNA integrity was confirmed by clear 28S and 18S bands on electrophoresis, and its high quality was indicated by an OD_{260}/OD_{280} ratio between 1.8 and 2.1. First-strand cDNA was synthesized from the extracted RNA using the RT Easy™ II Kit (With gDNase) (FORE GENE, Chengdu, China).

Quantitative reverse transcription PCR (qRT-PCR) was performed using the Real-time PCR Easy™-SYBR Green I Kit (FORE GENE, Chengdu, China). Each reaction was run in triplicate. The relative expression level of *AcPRX12* was calculated using the $2^{-\Delta\Delta Ct}$ method, with ACT (*A. carmichaelii* ACT [21], *Arabidopsis thaliana* ACT [22]) for normalization.

The primers used for qRT-PCR are listed in Supplementary Table S2. The One-Way ANOVA was employed for comparisons among multiple groups (Figures 2B and 5E).

2.3. Determination of the Relative Content of Total Lignin

The relative content of total lignin was determined using the acetyl bromide method, which is based on the principle that lignin derivatives solubilized in acetyl bromide yield a characteristic absorption peak at 280 nm, with absorbance intensity proportional to lignin concentration. The assay was performed according to the method described by Bonawitz et al. [23]. Lignin content was expressed as absorbance units at 280 nm per milligram of fresh weight (A_{280} nm/mg FW). For comparisons between two groups (Figure 5D), the *t*-test was used; for comparisons among multiple groups (Figure 2C), one-way ANOVA was employed.

2.4. Total RNA Extraction and RACE Cloning of AcPRX12

Total RNA was extracted from the daughter roots of the S3 stage using the method described in Section 2.2. RNA concentration and purity were measured with a spectrophotometer, and integrity was assessed by agarose gel electrophoresis (1.2% gel, 0.5× TBE buffer, 130 V for 25 min).

Based on a partial *AcPRX12* sequence obtained from our group's transcriptome data, gene-specific primers (see Supplementary Table S2) were designed for RACE. Both 5'- and 3'-RACE were performed using the SMARTer® RACE 5' / 3' Kit (Takara, Mountain View, CA, USA). RACE products were cloned, and positive clones were sequenced by Sangon Biotech (Shanghai, China). The full-length cDNA sequence was assembled from the 5'- and 3'-RACE sequences using DNAMAN software (version 6.0.3.99).

To clone the full-length sequence (excluding the poly(A) tail), primers were designed at the 5'- and 3'-ends (see Supplementary Table S2). The amplified fragment was seamlessly cloned into the pUC19 vector using MonClone™ Hi-Fusion Cloning Mix V2 (Monad, Wuhan, China), transformed into *Escherichia coli* DH5α competent cells (Sangon Biotech, Shanghai, China), and verified by colony PCR and sequencing. Correct clones were preserved for subsequent use.

2.5. Bioinformatics Analysis and Functional Prediction of AcPRX12

Bioinformatic analysis of *AcPRX12* was performed using the following online tools and databases:

- The **NCBI ORF Finder** <https://www.ncbi.nlm.nih.gov/orffinder/> (accessed on 25 November 2025) was used to identify the open reading frame (ORF) and deduce the amino acid sequence.
- The **SOPMA server** https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html (accessed on 26 November 2025) was employed for secondary structure prediction.
- Physicochemical properties and hydrophobicity were predicted using **ProtParam** on the ExPASy server (<https://www.expasy.org>, accessed on 29 November 2025).
- Subcellular localization was predicted via **WoLF PSORT** <https://wolfpsort.hgc.jp/> (accessed on 29 November 2025).
- Conserved domains were identified using the **CD-Search tool** on the NCBI website <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi> (accessed on 29 November 2025).
- Homology modeling of the *AcPRX12* protein structure was performed using the **SWISS-MODEL workspace** <https://swissmodel.expasy.org/interactive> (accessed on 29 November 2025).

- For comparative analysis, peroxidase (PRX) protein sequences from *Arabidopsis thaliana* were retrieved from TAIR <https://www.arabidopsis.org/> (accessed on 30 November 2025). Multiple sequence alignment was conducted with Clustal Omega <https://www.ebi.ac.uk/tools/msa/clustalo/> (accessed on 31 November 2025). and visualized using ESPript 3 <https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi> (accessed on 31 November 2025).
- Molecular docking with lignin monomer analogs was performed using <https://cadd.labshare.cn/cb-dock2/php/index.php> (accessed on 31 November 2025). The 3D structures of the analogs were obtained from PubChem <https://pubchem.ncbi.nlm.nih.gov/> (accessed on 31 November 2025).

2.6. Heterologous Expression of AcPRX12 in Wild-Type *Arabidopsis thaliana*

2.6.1. Gene Cloning and Vector Construction

The full-length coding sequence (CDS) of *AcPRX12* was amplified via high-fidelity PCR using 2× High Fidelity PCR Master Mix (Sangon Biotech, Shanghai, China), with total RNA from the swollen part of S3-stage aconite roots as the template. The PCR product was purified using the MiniBEST Agarose Gel DNA Extraction Kit (Takara, Mountain View, CA, USA) and then seamlessly cloned into the plant expression vector pCambia3300 using MonClone™ Hi-Fusion Cloning Mix V2 (Monad, Wuhan, China). The recombinant plasmid and the empty vector (control) were each introduced into *Agrobacterium tumefaciens* strain GV3101 (Sangon Biotech, Shanghai, China).

2.6.2. Plant Transformation and Transgenic Line Selection

Transgenic *Arabidopsis thaliana* (ecotype Col-0) was generated via the *Agrobacterium*-mediated floral dip method. Primary transformants (T0) were selected on 1/2 MS solid medium containing 50 µg/mL kanamycin and 20 µg/mL phosphinothricin (PPT). Positive lines were confirmed by PCR using leaf tissue (primers listed in Supplementary Table S2). Plants were propagated under the growth conditions described in Section 3.1, and selection was continued until T3 lines were obtained.

2.6.3. Phenotypic Analysis

Homozygous T3 plants overexpressing *AcPRX12* (OE) and empty vector controls (EV) were used for subsequent analysis. For phenotypic observation, the aerial parts of ~2-month-old soil-grown plants and the roots of ~1-month-old MS medium-grown seedlings were examined. The relative total lignin content in various tissues was determined using the acetyl bromide method as described in Section 2.3. Each experiment included at least three biological replicates, with each replicate consisting of no fewer than three individual plants. All reagent sources and primers are detailed in Supplementary Tables S1 and S2, respectively. The *t*-test was used for comparisons between two groups (Figure 6F–H).

3. Results

3.1. Down-Regulation of the Peroxidase Gene *AcPRX12* During the Daughter Root Swelling

Based on previous anatomical findings, transcriptome sequencing was performed on the daughter roots at three key developmental stages, defined by diameter (*d*): 1 mm < *d1* < 2 mm, 4 mm ≤ *d2* < 10 mm, and 10 mm ≤ *d3* < 20 mm. Analysis revealed that the phenylpropanoid biosynthesis pathway—the central route for lignin synthesis—was one of the key pathways significantly enriched with differentially expressed genes. Within this pathway, peroxidase genes generally showed a down-regulated expression trend (Table 1). By focusing on the differentially expressed peroxidase genes common to all three comparison groups (d2 vs. d1, d3 vs. d1, and d3 vs. d2), six core candidates

were identified. Among these, Cluster-5683.29757, which displayed the most pronounced down-regulation across the three comparisons, was selected as the candidate for further functional study (Figure 1). Annotation of the transcriptome data indicated that this transcript shared the highest sequence similarity with peroxidase 12-like (LOC113357213) from *Papaver somniferum* in the NT database. Following the convention for homologous gene nomenclature, the peroxidase gene corresponding to Cluster-5683.29757 was designated *AcPRX12*.

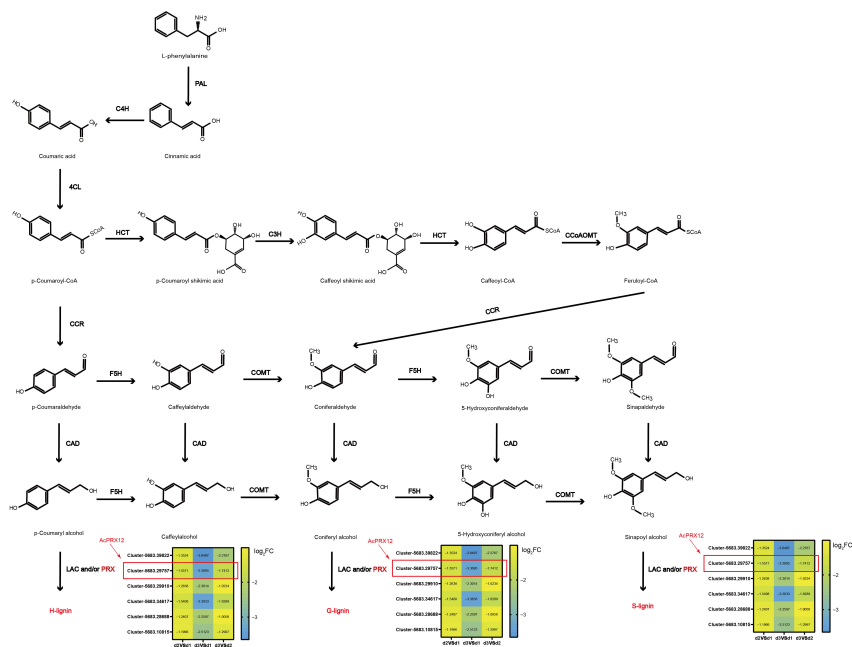


Figure 1. Lignin biosynthesis pathway and differential expression of six candidate peroxidases in three comparison groups. PAL: Phenylalanine ammonia lyase; C4H: Cinnamate 4-hydroxylase; HCT: Hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyl transferase; C3H: Coumarate 3-hydroxylase; CCoAOMT: Caffeoyl-CoA-O-methyltransferase; CCR: Cinnamoyl-CoA reductase; F5H: Ferulate 5-hydroxylase; COMT: Caffeic acid O-methyltransferase; CAD: Cinnamyl alcohol dehydrogenase; LAC: Laccase; PRX: Peroxidase.

Table 1. Peroxidase genes of the three comparisons in the phenylpropanoid biosynthesis pathway.

Comparison	Up (log ₂ FC)	Down (log ₂ FC)
d2 vs. d1	Cluster-5683.21825 (1.1094)	Cluster-5683.28688 (−1.2407) Cluster-5683.31403 (−1.4252)
		Cluster-5683.9544 (−6.7904) Cluster-5683.39822 (−1.3524)
		Cluster-14433.0 (−5.2167) Cluster-5683.36486 (−1.177)
		Cluster-5683.31270 (−1.1704) Cluster-5683.29757 (−1.5571)
		Cluster-5683.29910 (−1.2638) Cluster-5683.34617 (−1.5408)
		Cluster-5683.31269 (−1.1348) Cluster-5683.24929 (−1.0499)
		Cluster-5683.10815 (−1.1966)
d3 vs. d1	Cluster-5683.23596 (1.6299) Cluster-5683.1384 (4.6712)	Cluster-5683.32664 (−1.1159) Cluster-5683.9544 (−6.8524)
		Cluster-5683.17869 (−2.0413) Cluster-5683.39822 (−3.6497)
		Cluster-14433.0 (−6.2388) Cluster-5683.38028 (−2.1573)
		Cluster-5683.18690 (−1.4161) Cluster-5683.19847 (−2.0178)
		Cluster-5683.29910 (−2.3014) Cluster-5683.31269 (−2.1466)
		Cluster-5683.29757 (−3.3095) Cluster-5683.24049 (−3.2397)
		Cluster-5683.31364 (−1.3493) Cluster-5683.28690 (−1.9045)
		Cluster-5683.6713 (−3.0597) Cluster-5683.6714 (−2.968)
		Cluster-5683.34617 (−3.3833) Cluster-5683.37676 (−1.2798)
		Cluster-5683.53206 (−2.863) Cluster-5683.40476 (−2.3906)
		Cluster-5683.28688 (−2.2597) Cluster-5683.24929 (−1.8215)
		Cluster-5683.10815 (−2.5123) Cluster-5683.42038 (−2.8281)

Table 1. Cont.

Comparison	Up (log ₂ FC)	Down (log ₂ FC)
d3 vs. d2	Cluster-5683.23596 (1.9641)	Cluster-5683.18690 (−1.2629) Cluster-5683.39822 (−2.2767)
	Cluster-5683.31403 (1.0684)	Cluster-5683.38028 (−2.8268) Cluster-5683.29757 (−1.7412)
	Cluster-5683.1384 (5.8162)	Cluster-5683.31364 (−1.6513) Cluster-5683.28690 (−1.2015)
		Cluster-5683.29910 (−1.0234) Cluster-5683.34617 (−1.8289)
		Cluster-5683.24049 (−3.0557) Cluster-5683.37676 (−1.3556)
		Cluster-5683.40476 (−1.9012) Cluster-5683.28688 (−1.0058)
		Cluster-5683.10815 (−1.2997) Cluster-5683.42038 (−2.0205)

3.2. Spatiotemporal Expression of AcPRX12 and Total Lignin Relative Content in the Daughter Roots

To preserve freshness and integrity for RNA extraction and subsequent RACE, fresh growing roots of *A. carmichaelii* (Jiangyou) at different developmental stages were collected from April to June 2023, with detailed sample processing described in Section 2.1.1. Roots from the same growth period were sectioned transversely and, based on relative thickness and external morphology, divided into two distinct regions: P and PB (Figure 2A). These samples were then used for qRT-PCR and lignin content assays.

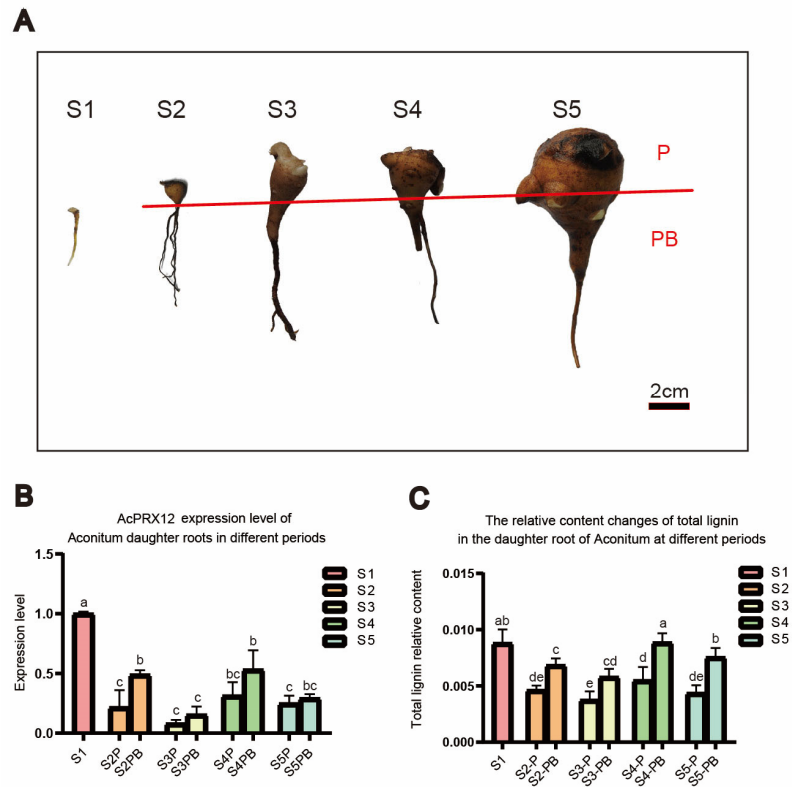


Figure 2. The daughter root sampling stages and the determination of *AcPRX12* expression levels and relative total lignin content in the P and PB sections. (A) Size of the daughter roots at different stages. (B) Expression levels of *AcPRX12* in the P and PB sections of the daughter roots at different stages. (C) Determination of total lignin relative content in the P and PB sections of the daughter roots at different stages. Results are presented as units of A280 nm/mg FW; In the bar graph, different lowercase letters above the bars indicate significant differences between groups ($p < 0.05$), while the same lowercase letters indicate no significant difference, $p > 0.05$.

Throughout root development, *AcPRX12* transcript levels exhibited a fluctuating trend, declining from S1 to S3, rising from S3 to S4, and falling again from S4 to S5. Spatially, expression was invariably elevated in the PB region compared to the P region at each stage (Figure 2B). Notably, the temporal variation in total lignin content mirrored the expression

3.4. Bioinformatics Analysis and Functional Prediction of AcPRX12

Structural analysis predicted AcPRX12 to be an extracellular protein (37.79 kDa, pI 6.21) featuring a canonical secretory peroxidase domain (aa 35–329). This domain harbors four essential functional sites for peroxidase activity (Figure 4C), with the heme-binding and active sites showing high conservation among related plant enzymes (Figure 4A). Moreover, the protein's secondary structure is rich in α -helices (39.71%) and coils (47.14%). Furthermore, it shows 88% sequence identity to a peroxidase from *Aquilegia coerulea* (A0A2G5C2B8_AQUCA). Phylogenetic analysis with known AtPRXs placed AcPRX12 in the same clade as AtPRX64, suggesting a potential functional similarity (Figure 4B). Crucially, molecular docking simulations demonstrated a predicted binding affinity between AcPRX12 and lignin-related compounds, strongest for syringaldazine (−7.5 kcal/mol) (Figure 4D), a commonly used S-type lignin monomer analog for in vitro enzyme activity verification, supporting its putative role in lignin biosynthesis.

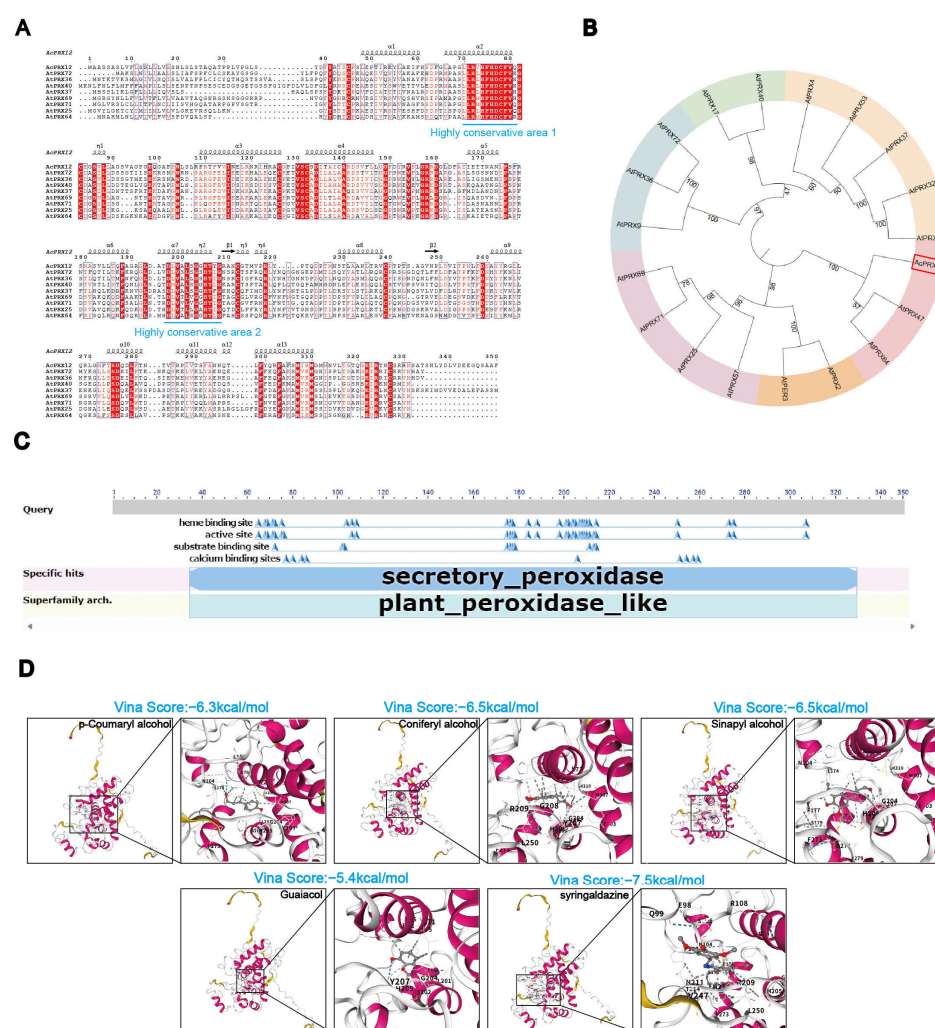


Figure 4. Bioinformatic analysis of the AcPRX12 protein. (A) Sequence alignment of AcPRX12 with Arabidopsis PRX proteins. The secondary structure corresponding to the AcPRX12 protein is shown at the top, where α , β , and η represent alpha-helix, beta-sheet, and 310-helix, respectively. Identical residues are highlighted with a white font on a red background, highly similar residues are shown in red font on a white background, and non-conserved residues are displayed in black font on a white background. Blue boxes indicate regions of similarity. Highly conserved areas 1 and 2 correspond to the functional sites of heme binding and the catalytic site, respectively, as shown in panel (C). (B) Phylogenetic tree of AcPRX12

and functionally characterized PRX proteins from Arabidopsis. The red box indicates the position of the protein encoded by the target gene *AcPRX12* in this study. (C) Prediction of conserved domains in the *AcPRX12* protein. (D) Molecular docking results of the *AcPRX12* protein with lignin monomers and their analogs. A higher absolute value of the Vina Score indicates greater binding stability and higher affinity.

3.5. Heterologous Expression of *AcPRX12* in *Arabidopsis thaliana*

Transgenic Arabidopsis overexpressing *AcPRX12* (OE) were compared to empty vector (EV) controls using selected T3 lines. Wild-type (WT) Arabidopsis seeds failed to germinate on 1/2 MS solid medium containing PPT, whereas both OE and EV T3 homozygous lines grew normally (Figure 5A). Plants that survived on selective medium were subsequently propagated and genotyped at the DNA level. PCR amplification confirmed the presence of the *AcPRX12* transgene in OE lines and the Bar selectable marker in EV lines (Figure 5B,C). These results verified that all plants used for subsequent lignin quantification and phenotypic analysis were positive transgenic individuals. The phenotypic observation results show that OE plants were growth stunted, featuring short roots, thin stems, small leaves, and short siliques (Figure 6B–E). Among them, the stems and siliques of Arabidopsis thaliana have more obvious phenotypic differences (Figure 6G,H). Concomitantly, the total lignin relative content was higher in all OE tissues, with stems > siliques > roots ≈ leaves in both groups (Figure 5D). This lignin accumulation pattern of OE plants correlated with *AcPRX12* transcript levels, which were highest in stems and siliques (Figure 5E).

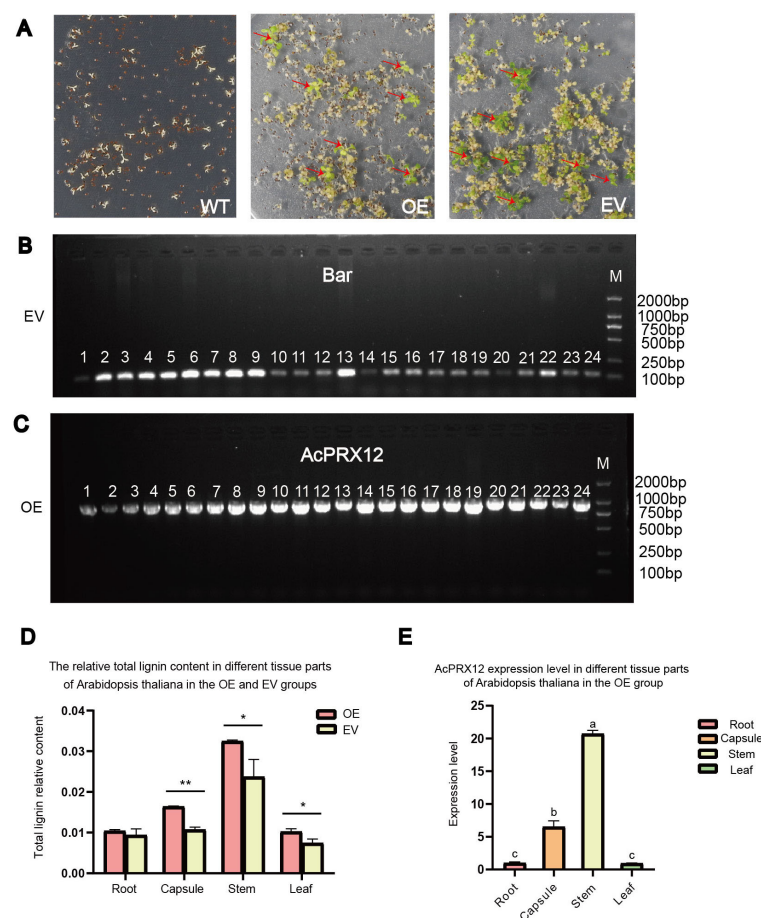


Figure 5. Screening results of T3 generation Arabidopsis transgenic plants. (A) Growth of wild-type Arabidopsis seeds and T3 generation seeds (obtained after screening post *Agrobacterium tumefaciens* infection) on PPT-containing 1/2 MS solid selective medium. The red arrows indicate the positive plants. (B) DNA-level detection of the Bar gene fragment in T3 plants from the EV (empty vector)

lines. (C) DNA-level detection of the AcPRX12 CDS in T3 plants from the OE (overexpression) lines. (D) Relative total lignin content in different tissues of OE and EV Arabidopsis plants. Results are presented as units of A280 nm/mg FW (* $p < 0.05$, ** $p < 0.0001$). (E) Expression levels of the AcPRX12 gene in different tissues of OE plants. Different lowercase letters above the bars indicate significant differences between groups ($p < 0.05$).

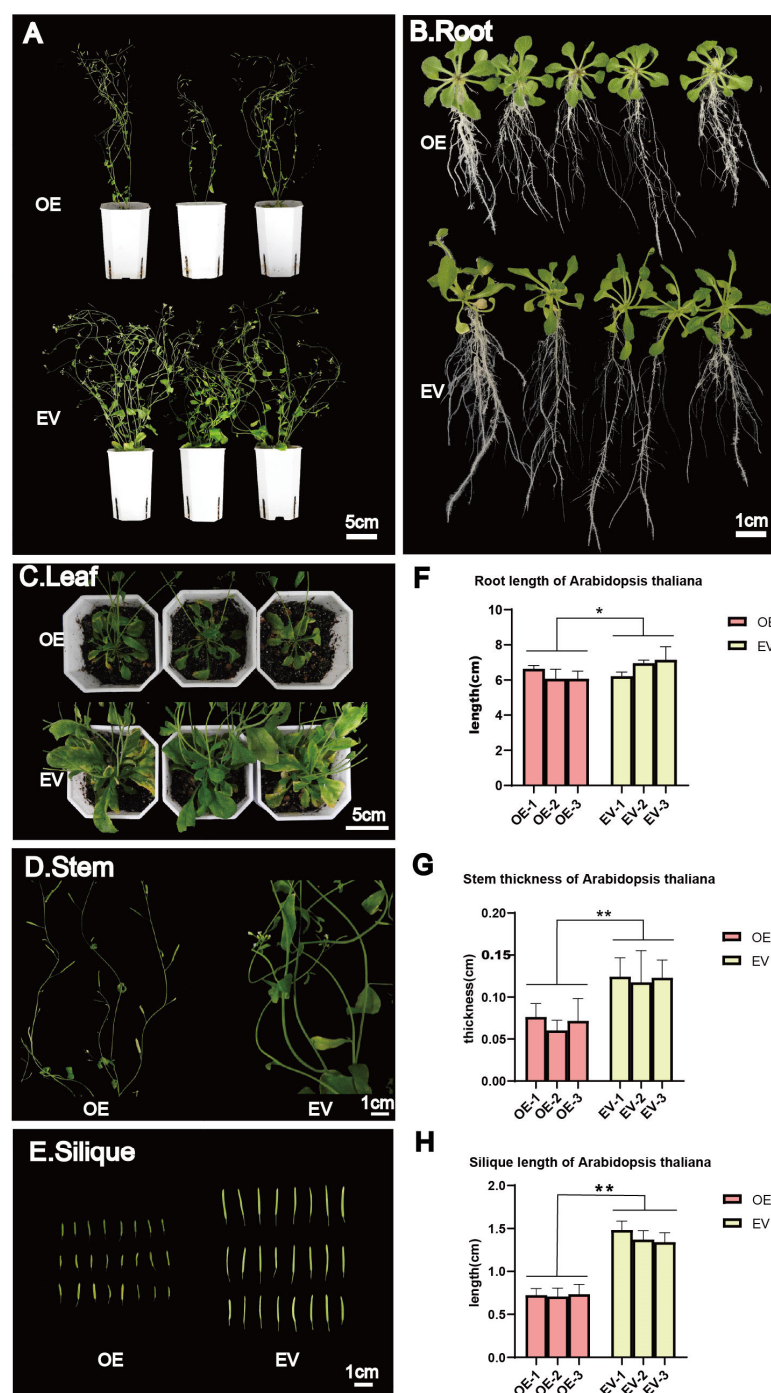


Figure 6. Phenotypic observation and quantification of T3 generation *Arabidopsis* plants. (A) Overall growth phenotype of *Arabidopsis* plants. (B) Root growth phenotype. (C) Leaf growth phenotype. (D) Stem growth phenotype. (E) Silique growth phenotype. (F) Quantification of root length (* $p < 0.05$). (G) Quantification of stem diameter (** $p < 0.0001$). (H) Quantification of silique length (** $p < 0.0001$).

4. Discussion

4.1. *AcPRX12 May Exhibit Oxidative Preference for S-Type Lignin Monomers*

Plant Class III peroxidase is a type of heme enzyme that is widely involved in various physiological processes and is mostly located outside the cell membrane [15,24–26]. In our study, as a predicted extracellular class III peroxidase containing a secretory peroxidase domain, *AcPRX12* is likely involved in lignin biosynthesis. Notably, its high sequence homology with several *Arabidopsis* peroxidases (Figure 4A,B)—particularly at the conserved heme-binding and active sites (Figure 4A)—further supports this role. Among these homologs, *AtPRX72* and *64* were confirmed to participate in lignification [20,27], *AtPRX36* functions in seed development [28], and *AtPRX40* was crucial for anther and pollen development [29]. Moreover, molecular docking indicated that *AcPRX12* displays the strongest binding affinity for syringaldazine, an S-type lignin monomer analog. Interestingly, in dicots, S-type monomers are core components of secondary cell walls, and a higher S-type proportion enhances wall rigidity and mechanical strength [30,31]. This property could underpin the asymmetric root swelling observed in *A. carmichaelii*. However, whether *AcPRX12* specifically catalyzes S-type monomers requires further validation through in vitro enzymatic assays.

4.2. *AcPRX12 Has a Potential Negative Regulatory Effect on the Growth and Development of the Daughter Root*

Lignification is known to restrict cell expansion and inhibit storage root growth [7,10,11,32]. Previous work on sweet potato shows that high lignin content correlates with slender “pencil roots”, whereas low lignin promotes tuberous swelling [9]—a pattern consistent with our observations in the daughter root. In this study, the PB region consistently exhibited higher lignin content and *AcPRX12* expression than the P region across all developmental stages of the daughter root (except S1 stage), with both parameters showing synchronous trends (Figure 2B,C). Morphologically, differences were most pronounced at the S2 stage (both *AcPRX12* expression level and total lignin relative content exhibited significant differences in the P and PB regions at this stage), when the P region appeared spherical, while the PB region remained similar to S1. Although overall root expansion during S3–S5 reduced the size discrepancy between regions (Figure 2A), the differences in which PB was higher than P region in lignin content and gene expression persisted (Figure 2B,C). To sum up, this supports a model wherein *AcPRX12*-mediated lignification locally constrains growth, contributing to the conical “thick-top, thin-bottom” morphology.

Additionally, peroxidase activity enhances plant tolerance to abiotic stresses such as high temperature and drought [33–36]. Lignification is an adaptive plant defense program triggered by high-temperature stress. Under elevated temperatures, upstream genes in the lignin biosynthetic pathway are activated. The hydrophobicity of lignin reduces water permeability across cell walls, thereby aiding plants in water retention under high-temperature conditions [37,38]. The upregulation of *AcPRX12* during S4–S5, when morphogenesis is largely complete, may reflect an adaptive shift towards promoting lignification for water conservation in the high-temperature growing environment of Jiangyou. In summary, the coordinated patterns of *AcPRX12* expression and lignin accumulation suggest that *AcPRX12* promotes lignification, thereby exerting a potential negative regulatory effect on the daughter root expansion.

4.3. *AcPRX12 Promotes Lignin Biosynthesis*

To validate its function, *AcPRX12* was heterologously expressed in *Arabidopsis thaliana*. OE groups exhibited a stunted growth phenotype, including thin stems, small leaves, and short roots and siliques (Figure 6B–E), consistent with reports that *AtPRX37* overexpression

can inhibit growth [39]. In addition, lignin quantification confirmed significantly higher levels in all OE tissues compared to EV controls, demonstrating that AcPRX12 enhances lignification, akin to the roles of *AtPRX25* and *AtPRX71* [40]. Notably, in the OE group, the expression level of *AcPRX12* was highest in stems and siliques, where the total lignin content was also elevated, and the growth restriction phenotype was most pronounced (Figure 6D,E). In addition, this aligns with the documented role of *AtPRX64* in promoting lignin synthesis in *Arabidopsis* stems. Together, these results support the hypothesis that *AcPRX12*-mediated lignification negatively regulates the daughter root development. However, current limitations include the lack of a stable genetic transformation system for *A. carmichaelii*, preventing direct endogenous validation via gene silencing/knockout. Thus, the evidence for *AcPRX12*'s role in root morphogenesis remains indirect. Furthermore, the upstream regulators and downstream targets of *AcPRX12* within the lignin pathway are unknown. Future work should employ integrated genomics and metabolomics to identify interacting factors and construct a systematic model of the molecular regulatory network controlling daughter root morphogenesis.

5. Conclusions

Our study provides a functional characterization of *AcPRX12* in *A. carmichaelii*, establishing its core role in lignin biosynthesis and proposing its potential negative regulation of root morphogenesis. This work partially addresses the knowledge gap in the molecular regulation of the daughter root development.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biology15040323/s1>: Table S1: Main reagents and strains; Table S2: Primers.

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