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Analysis genome of *Pseudotaxus chienii* reveals insights into the origin and evolution of taxane biosynthesis

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Abstract

As a sister genus to *Taxus*, *Pseudotaxus* holds significant importance for studying the origin and evolution of the taxane biosynthesis pathway. However, the reference genome of *Pseudotaxus chienii* is yet unavailable. Here, we report a chromosome-level genome assembly of *P. chienii* (15.6 Gb). We show that *P. chienii* only possesses a partial taxane pathway, which terminates before taxane 2 α -*O*-benzoyl transferase (TBT), a crucial enzyme responsible for the production of 10-deacetylbaccatin III. With the emergence of the *Taxus* genus, the limitation posed by the lacking of functional TBT is overcome, allowing for the extension of the existing taxane biosynthesis pathway into a complete Taxol biosynthesis. The protein structure of metal ion catalysis sites in taxadiene synthase (TS) is conserved across the *Pseudotaxus* and *Taxus* genera, providing potential sites for enhancing TS activity through enzyme engineering. This comparative genomic analysis contributes to our understanding of the origin and evolution of taxane biosynthesis within the Taxaceae family.

Introduction

Taxaceae, a well-known family of non-flowering conifers with considerable medicinal and ecological value, is primarily distributed across Asia, North America, and Europe ¹. Plants of the genus *Taxus*, which belong to the Taxaceae family, have gained prominence for their secondary metabolite, paclitaxel (also named as Taxol), a chemotherapeutic agent applied in the treatment of various cancers ². Due to its low abundance in natural *Taxus* tissues, the price of Taxol has been steadily increasing ^{3,4}. Therefore, studying the origin and evolution of *Taxus*-specific Taxol biosynthesis is of great significance for future research.

Phylogenetic analysis speculated that Taxaceae *sensu lato* comprises six extant genera: *Amentotaxus*, *Austrotaxus*, *Pseudotaxus*, *Taxus*, *Torreya*, and *Cephalotaxus* ⁵. *Pseudotaxus chienii*, belonging to the monotypic genus *Pseudotaxus*, is an endangered conifer endemic to China ⁶. The iconic feature of the *P. chienii* tree is the peculiar white aril and stomatal bands on the backlit surface of leaves ^{1,7}. As an old rare tree, *P. chienii* is an ideal material for studying environmental adaptation and evolution. The *Pseudotaxus* genus, which has one living representative, diverged from its sister genus, *Taxus*, approximately 54-65 million years ago (MYA) (Palaeocene or Eocene), making it an evolutionary relict ^{7,8}. Evolutionary analyses of *P. chienii* genus provided insights into the mechanisms driving speciation for conifers ⁹.

Diterpenoids, comprised of several isoprene structural cores, are derived from geranylgeranyl diphosphate (GGPP) ¹⁰. Most diterpenoids share a common upstream biosynthetic pathway known as the 2-C-methyl-D-erythritol phosphate (MEP) pathway, which supplies essential precursors. They depend on diterpene synthases (diTPSs) to initiate the species-specific diterpenoid biosynthesis pathway ¹¹. The functional diversity of diterpenoids is attributed to the core sequences of diTPS, which are categorized into three distinct types: class I (DDXXD/E motif), class II (DXDD motif), and bifunctional class I/II diTPSs ¹². In gymnosperms, bifunctional diTPSs are the

predominant type, in contrast to the mono-functional diTPSs found in angiosperms¹³. For example, AgAS from *Abies grandis*, GbLPS from *Ginkgo biloba*, and SmCPSKSL1 from *Selaginella moellendorffii* are typical bifunctional diTPSs¹⁴⁻¹⁶. Despite being a gymnosperm, the taxane-4(5),11(12)-diene derived from *Taxus* plants is synthesized directly by a class I diTPS, taxadiene synthase (TS)¹⁷. Once the taxane core is formed, a series of taxoid hydroxylases, including the 2 α -, 5 α -, 7 β -, 9 α -, 10 β -, and 13 α -hydroxylases, are involved in modifying the core¹⁸. The origin and evolution of such a complex pathway for synthesizing Taxol in *Taxus* plants remain unknown.

Since the first isolation of Taxol from the stem bark of *T. brevifolia*, researchers have also detected taxanes in other plant species¹⁹. Taxol and its precursor, baccatin III, were identified in the leaves of *Cephalotaxus hainanensis*, an important member of the Taxaceae family²⁰. Anticancer taxanes, including 10-deacetylbaccatin III, baccatin III, cephalomannine, and paclitaxel, were detected in Turkish Hazelnut (*Corylus avellana* L.)²¹. However, it remains unclear whether the Taxol biosynthesis pathway in *P. chienii* is fully established. Recently, chromosome-level genomes of *Taxus* and *Torreya* have been published in succession, deepening our understanding of Taxaceae plants²²⁻²⁵. Recently, the coding genes for the last several key enzymes, such as TOT1, T9a oxidase, TCPR, and CYP1, were cloned, which improved the Taxol biosynthesis pathway²⁶⁻²⁹.

In this study, we assemble the genome of *P. chienii*. Our comparative genomic analysis contributes to our understanding of the origin and evolution of taxane biosynthesis and provides insights into the genome structure and organization of the Taxaceae family.

Results

Visualization of metabolite locations in the stems and leaves of *P. chienii*

P. chienii is a rare gymnosperm with limited research, and its economic value remains unclear. Exploring the medicinal active ingredients of *P. chienii* is essential for the effective and protective utilization of this ancient and rare species, which has a long history. Given that taxanes, iconic

metabolites of the *Taxus* genus, are a type of diterpenoid compound, MALDI-IMS was employed to detect various potential diterpenoids in the leaves and stems of *P. chienii* (**Fig. 1a**). The optical images of its leaf and stem sections are shown in **Fig. 1b**. The MALDI-IMS analysis produced a series of colored maps comprising a total of 9,999 data points, indicating the tissue-specific accumulation of active ingredients (**Fig. 1c** and **Supplementary Data 1**). After database matching, most metabolites were annotated and categorized into various KEGG pathways (**Supplementary Fig. 1**).

All identified metabolites were separated into five principal components (PCs) associated with different stem and leaf tissues. For the stems, four major tissues, including endodermis (PC1), epidermis (PC2/5), xylem (PC4), and pith (PC3), were visualized (**Supplementary Fig. 2a-b**). To identify stem tissue-specific accumulated metabolites (SAMs), clustering analyses identified 1,512 endodermis-, 3,237 epidermis-, 1,494 pith-, and 1531 xylem-SAMs. Additionally, 390 metabolites accumulated in both epidermis and xylem (**Supplementary Fig. 2c**). For the leaves, three major tissues, including epidermis (PC1), mesophyll (PC2/3/5), and bundle sheath (PC4), were visualized (**Supplementary Fig. 3a-b**). To identify leaf tissue-SAMs, clustering analyses identified 1833 epidermis-, 5201 mesophyll-, and 1248 bundle sheath-SAMs. Additionally, 336 metabolites accumulated in both epidermis and mesophyll (**Supplementary Fig. 3c**).

Several active diterpenoids were detected using the MS-Imaging analysis method (**Supplementary Data 2**). In the stems, geranyl-PP, a core precursor for terpenoid synthesis, and 3D,7D,11D-phytanic acid were predominantly accumulated in the epidermis; while ζ -carotene epoxide, presqualene-PP, and taxine B were highly concentrated in the xylem. Additionally, erinacine D, resiniferatoxin, jesaconitine, and azadirachtin exhibited a non-tissue-specific accumulation pattern (**Fig. 1d**). The proportions of different active ingredients in the stems and leaves are shown in **Fig. 1e**.

Genome assembly and annotation

To explore the chromosome-level genome of *P. chienii*, twigs from a wild *P. chienii* tree were collected for genome sequencing (**Fig. 2a**). A total of 652.4 Gb of Illumina clean data was obtained. The *P. chienii* genome was estimated to be 16.8 Gb (~ 39x) based on 19-mer analyses (**Supplementary Fig. 4a and 4b**). By using 586.5 Gb PacBio HiFi reads, a 15.6 Gb non-redundant assembly was generated, comprising 954 contigs with a contig N50 of 65.6 Mb and a GC content of 35.2% (**Supplementary Table 1**). Using 2.34 Tb Hi-C clean data, a total of 15.5 Gb (99.0%) of the assembled sequences were anchored on 12 pseudochromosomes ranging in size from 0.8 Gb to 1.5 Gb (**Fig. 2b and Supplementary Fig. 4c**).

To evaluate the completeness and accuracy of the assembly, the Illumina short reads and transcriptome data were aligned to the assembly. The result showed mapping rates of 99.33% and 98.58%, respectively. BUSCO evaluation using the protein model revealed that 85.2% (1,376 out of 1,614) of the core embryophytic genes were identified in the *P. chienii* genome, showing relatively high completeness of genome assembly among gymnosperms (**Supplementary Table 2**). The LAI value was estimated to be 10.1, which is larger than the proposed standard for a reference-level assembly ($LAI > 10$)³⁰, indicating a high quality of the chromosome-level assembly of *P. chienii*.

Genome annotation yielded 35,404 protein-coding genes with an average mRNA length of 29,725 bp were annotated. The average CDS length is 1,060 bp, and the average number of exons per gene is 4.2 (**Supplementary Table 3**). A total of 32,166 (90.9%) completed genes could be functionally annotated by searching against various known databases (**Supplementary Data 3**).

Analysis of repeat sequence

The genome size of *P. chienii* is much larger than that of the closely related *Taxus* species (~10 Gb). We identified a greatly high percentage of repetitive sequences (86.12%) in the *P. chienii*

genome, with transposable elements (TEs) and tandem repeats accounting for 82.73% and 3.39% of the genome, respectively. Most of the Long Terminal Repeat Retrotransposon (LTR-RT) expansions in gymnosperms happened between 25 to 7 MYA. The estimation of the insertion time showed that the LTR-RT expansion in *P. chienii* experienced an extremely long period, the burst of LTR-RT insertions range from about 60 to 10 MYA, with the accumulation reaching a peak at about 23 MYA (**Fig. 2c**). These results suggested that continuous insertions of LTR-RTs leads to the giant genome of *P. chienii*. LTR-RTs comprise the majority of those TEs (82.06%), with Gypsy and Copia contributing 40.87% and 9.04% of the total, respectively (**Fig. 2d** and **Supplementary Table 4**). The results of tandem repeat sequence analysis are shown in **Supplementary Table 5**.

Whole genome duplication (WGD)

A synteny analysis between *P. chienii* and two other Taxaceae species (*T. grandis* and *T. mairei*) found that 86% and 81% of genes in *P. chienii* had one-to-one syntenic orthologs in *T. mairei* and *T. grandis*, respectively, while only 1% and 3% of genes in *P. chienii* had one-to-more syntenic orthologs in *T. mairei* and *T. grandis*, respectively (**Supplementary Fig. 5a** and **b**). These results indicated the lack of a recent WGD event in *P. chienii*. We next tried to seek evolutionary relics of WGD in *P. chienii* by detecting paralogous syntenic gene blocks. However, we only detected 60 syntenic blocks including 672 genes (~2.0 % of all genes), further supporting the absence of recent WGD.

Syntenic comparison and gene family evolution

Homologous syntenic blocks between *P. chienii* and other Taxaceae species were identified. The results showed that nearly perfect macro-syntenic is observed among the three *Taxus* species, suggesting a high degree of similarity in their genomic structures. Although macro-syntenic is also present between *P. chienii* and the *Taxus* species, extensive chromosomal rearrangements and intra-chromosomal inversions exist. The reorganization of the genome is even more evident when comparing the genomic structures of *P. chienii* and *T. grandis* (**Supplementary Fig. 5c**). These

results coincide with the phylogeny of these species. We identified a core set of 10,419 gene families shared among these species, along with 5,977 gene families specific to *P. chienii* (**Fig. 2e**). To investigate the evolutionary processes of gene families in *P. chienii*, we identified 20,986 orthologous groups across 16 land plant species, including 10 gymnosperms, 5 angiosperms, and 1 lycophyte (**Fig. 2f**). Phylogenetic analysis and molecular dating using 184 single-copy genes indicated that *P. chienii* diverged from *Taxus* around 48.3 million years ago. Furthermore, we found 190 expanded and only 1 contracted gene family in the common ancestor of the Taxaceae family. The extinct ancestors of *Pseudotaxus* and *Taxus* exhibited expansions and contractions of 73 and 38 gene families, respectively. The *Taxus* lineages uniquely contain 170 expanded and 32 contracted gene families, which may be related to their diversification compared to other Taxaceae species (**Fig. 2f**). *P. chienii* demonstrated an expansion of 161 gene families, many of which contained conserved domains linked to catabolic processes, responses to abiotic stimuli, secondary metabolic processes, and reproduction.

Identification of functional genes

GO analysis revealed a great number of environmental adaptation-related genes, including 275 defense response-related genes, 237 herbicide response-related genes, and 147 oxidative stress response-related genes (**Supplementary Fig. 6a**). Moreover, many metabolism-related genes, including 342 carbohydrate metabolic process-related genes, 194 lipid metabolic process-related genes, and 114 glutathione metabolic process-related genes (**Supplementary Fig. 6b**). Our study provides sufficient candidates to improve the metabolic flux and enhance environmental adaptation of *P. chienii*. It is worth mentioning that 587 genes were grouped into a taxane biosynthesis-related GO term “paclitaxel biosynthetic process”, suggesting a potential biosynthetic ability for taxanes in *P. chienii* (**Supplementary Fig. 6c** and **Supplementary Data 4**).

A total of 591 putative TF genes belonging to 10 major metabolism-related TF families were identified in *P. chienii* (**Supplementary Data 5**). To investigate the evolutionary processes of TF

families in the Taxaceae family, we identified an average of 1,075 and 1,877 TFs consisting of ten families in ten gymnosperm and eight angiosperm genomes, respectively. This indicated a 1.8-fold and a 3.2-fold increase compared to *P. chienii* (**Supplementary Fig. 7**). This suggests that the genome of *P. chienii* is relatively ancient and that copy number expansion of these TF families has occurred in other plant species.

Origin and evolution of the taxane biosynthesis pathway

We observed that the genome of *P. chienii* contains many genes involved in the taxane biosynthesis, making *P. chienii* an ideal species for investigating the evolution of the Taxol biosynthetic pathway. A comprehensive search of the *P. chienii* genome revealed a complete MEP pathway, suggesting that *P. chienii* has the capacity to provide precursors for taxane biosynthesis. A nearly complete Taxol biosynthesis pathway has been published recently^{26,31}. In the *P. chienii* genome, a partial Taxol biosynthesis pathway was identified. It is interesting that neither *TBT* nor *BAPT* genes were detected (**Fig. 3a**). Similar to the *Taxus* genus plants, most of the genes involved in the taxane biosynthesis pathway in *P. chienii* are arranged into two gene clusters located on Chr 9 and 11, respectively (**Fig. 3b**).

Furthermore, we found that 14 identified taxane biosynthesis-related genes were located in the 142.04-245.71 Mb region of chromosome 9 and the 16.56-101.93 Mb region of chromosome 11, respectively (**Fig. 3b** and **Supplementary Data 6**). Previous study showed that all these corresponding genes are distributed on the 195.41-486.53 Mb region of chromosome 10 of *T. wallichiana*²³. Given the conserved genome structures between *P. chienii* and *T. wallichiana*, as well as their phylogenetic relationship, it appears that the taxane biosynthesis gene-enriched regions of chromosomes 9 and 11 in *P. chienii* have undergone translocations with inversions, aligning with the corresponding region of chromosome 10 in *T. wallichiana* (**Fig. 3c**). Despite evolutionary divergence, *P. chienii* and *T. wallichiana* still share an ancestral lineage, suggesting that taxane biosynthesis pathway has a common origin.

Taxusin is one of potential end products of taxane biosynthesis pathway in *P. chienii*

Untargeted metabolomic analysis was conducted to detect the variations in the metabolic profiles of three typical Taxaceae species (**Supplementary Fig. 8a** and **Supplementary Data 7**). A PCA indicated clear separation among the metabolomes of *P. chienii*, *T. wallichiana*, and *T. grandis* (**Supplementary Fig. 8b**). Based on their annotations, 8,780 metabolites were identified (**Supplementary Fig. 8c**).

To elucidate the completeness of the Taxol biosynthesis pathway in three closely related Taxaceae species, we compared the genomes of *P. chienii*, *T. wallichiana*, and *T. grandis* ^{23,25}. The complete MEP pathway exists in all three selected Taxaceae species (**Fig. 4a**). TBT catalyzes the conversion of 2-debenzoyl-7,13-diacetylbaccatin III to 7,13-diacetylbaccatin III ³². TOT1 (from *T. mairei*), also named CYP1 (from *T. media*), catalyzes an oxidative rearrangement in paclitaxel oxetane formation ^{26,28,29}. The absence of TBT and TOT1 (also known as CYP1 or CYP725A55) may be a significant reason why *P. chienii* is unable to synthesize 10-deacetylbaccatin III and the subsequent intermediates following 10-deacetylbaccatin III. Certainly, there may be another pathway that consumes an amount of 10-deacetylbaccatin III or baccatin III, making them undetectable. Moreover, the *T2OH* and *T7OH* genes were identified in *P. chienii* (**Supplementary Table 6** and **Supplementary Fig. 9**). A feeding experiment showed that the activities of T2OH and T7OH from *P. chienii* were similar to those from *T. mairei* (**Supplementary Fig. 10**). The quality control parameters and raw spectra were showed in **Supplementary Table 7** and **Supplementary Fig. 11-15**. Considering the presence of both T2OH and T7OH, taxusin can be hydroxylated into 2 α ,7 β -dihydroxytaxusin ³³. Taxusin and its analogues are highly acetylated intermediates in an alternative route of the Taxol biosynthesis pathway ²⁶. We hypothesized that blocking at TBT and TOT1 (CYP1) might redirect metabolic flow towards taxusin or its analogues instead of Taxol (**Fig. 4b**). No homologous genes for *TS* were identified in *T. grandis*, indicating that this species lacks the taxane biosynthesis pathway.

As expected, taxusin is highly accumulated in *P. chienii*, particularly in its stems, while it is minimally accumulated in *T. wallichiana*, and undetected in *T. grandis*. 10-Deacetylbaicatin III is highly accumulated in *T. wallichiana* and undetected in both of *P. chienii* and *T. grandis* (**Fig. 4c**). LC-MS/MS analysis confirmed that the content of taxusin in the twigs of *P. chienii* is 3.5-fold higher than that in *T. wallichiana* (**Fig. 4d** and **e**). Several other taxoids, including Taxol, 10-deacetyltaxol, and 10-deacetyl-7-xylosyltaxol C, were only detected in *T. wallichiana* through untargeted metabolomic analysis (**Fig. 4f**). Taxusin can serve as a precursor for Taxol synthesis because of its exceptionally high content in *P. chienii*.

Functional identification of the *TS* genes in *P. chienii*

To date, *Taxus* is the only genus among gymnosperms known to produce Taxol, although all plants possess the TPS enzymes, which catalyze isoprenoid cyclization reactions to produce a variety of natural products³⁴. The TS enzymes catalyze the initial key step of taxane biosynthesis from GGPP. Phylogenetic analysis has revealed that TPSs from gymnosperms form a distinct clan, while TPSs from angiosperms are grouped into two separate branches, consisting of Class I and Class II TPSs (**Fig. 5a**).

To confirm the function of *PcTS1* in the initial step of taxane biosynthesis pathway, pBWA(V)HS-*PcTS1* vector was constructed and genetic transformed into *N. benthamiana* to produce transgenic seedlings (**Fig. 5b**). The leaf samples expressing *PcTS1* were extracted using hexane and analyzed via GC-MS. As expected, we a peak at a RT of 11.875 mins in samples expressing *PcTS1* and *TwTS1*, respectively (**Fig. 5c** and **Supplementary Fig. 16**). The mass spectrum matched with the analogue of taxadiene, eicosane (**Fig. 5d**). Similar to its homologous gene in *Taxus*, the heterologous expression of *PcTS1* can also be utilized for the heterologous production of taxadiene in tobacco.

Protein structure analysis of TPSs from Taxaceae

Interestingly, TPSs from the Taxaceae family differ from those found in other gymnosperms, as they belong to Class I, while TPSs from other gymnosperms are classified as bifunctional. Multiple sequence alignment analysis showed that the TPSs from other gymnosperms contain both of the DXDD and DDXXD/E motifs, as well as the (N,D)DXX(S,T)XXXE motif; however, TPSs from Taxaceae family lack the characteristic DXDD motif (**Fig. 5e**). A previous study reported the X-ray crystal structure and substrate binding sites of the TS from the Pacific yew (*T. brevifolia*)³⁵. Using the known 3p5p.1.A model as a template, the 3D structure and substrate/Mg²⁺ binding sites of TS1 from *P. chienii* were predicted using SWISS-MODEL program. The amino acid residues forming DDXXD/E motif, such as 668-D, 669-D, 670-M, 671-A, and 672-D, and (N,D)DXX(S,T)XXXE motif, such as 812-N, 813-D, and 820-E, were surrounding the Mg²⁺ binding sites, suggesting that the DDXXD/E and (N,D)DXX(S,T)XXXE motifs are essential for catalytic activity, whereas the DXDD motif is not (**Fig. 5f**). The 3D structures of six typical TPSs, OcCPSsyn and ScCPS from Class II, GrTPS4 and AtEKS from Class I, and PsTPS_LAS and AgAS from bifunctional TPS group, were analyzed (**Fig. 5g**). TM-score analysis demonstrated that PcTS1 exhibit a greatest structural similarity to the bifunctional TPSs and a lowest structural similarity to the Class II TPSs (**Supplementary Table 8**).

CYP super-family in *P. chienii*

CYP superfamily-mediated oxygenation plays crucial roles in the regulation of plant secondary metabolism. Genes from the CYP725 clan have been reported to participate in Taxol biosynthesis³⁶. In *P. chienii*, 548 genes from the CYP superfamily were identified and categorized into 52 clans, with 62 of these genes belonging to the CYP725 clan (**Supplementary Fig. 17** and **Supplementary Data 8**). Phylogenomic analysis demonstrated that several CYP Clans, including CYP725, CYP728, CYP867, and CYP98, were greatly expanded in the Taxaceae family compared to nine other representative species, which encompass gymnosperms, angiosperms, and bryophytes (**Fig. 6a** and **Supplementary Data 9**). The genes from the CYP725 clan are primarily distributed in Chr 9 (24 genes) and Chr11 (37 genes), suggesting that the CYP725 clan has

undergone large-scale expansion due to tandem duplication events (**Fig. 6b**).

Expression response to MeJA treatment

Under MeJA treatment, a total of 3,338 and 4,191 DEGs were identified at 24 h and 48 h, respectively (**Supplementary Fig. 18a**). Most of the DEGs were regulated by MeJA treatment at both 24 h and 48 h (**Supplementary Fig. 18b**). GO enrichment analysis revealed that the MeJA-responsive genes were significantly enriched in the GO terms ‘paclitaxel biosynthetic process’ and ‘taxoid 14-beta-hydroxylase activity’ (**Supplementary Fig. 18c**).

Expression analysis showed that only the *P. chienii* CYP725 subfamily genes were specifically up-regulated compared to the other CYP subfamilies by MeJA treatments (**Fig. 6c**). Furthermore, the majority of the identified taxane biosynthesis-related genes were significantly up-regulated by MeJA treatment (**Fig. 6d**), suggesting that taxane biosynthesis was induced by MeJA treatment. LC-MS/MS analysis confirmed that the content of taxusin in the MeJA-treated twigs of *P. chienii* is 4.7- and 4.4-fold higher than that in the CK twigs (**Fig. 6e, f** and **Supplementary Table 9**).

To elucidate the genetic basis of the MeJA-induced expression patterns of CYP725 subfamily genes and other identified taxane biosynthesis-related genes, we extracted their 1500-bp promoter sequences from the *P. chienii* genome. We counted the average numbers of two reported MeJA-responsive *cis*-elements, G-box (CACGTGG) and G-box-like (AACGTG), in the promoters of CYP genes and CYP725 genes in both the ‘+’ and ‘-’ directions, respectively. The average numbers of these *cis*-elements in the promoters of all protein coding genes were treated as controls. The average number of G-box (+) and G-box (-) in the CYP725 promoters were 0.23 and 0.08, respectively, higher than those in the CYP promoters (0.07 and 0.06). The average numbers of G-box-like (+) and G-box-like (-) in the CYP725 promoters were 0.33 and 0.47, respectively, also significantly higher than those in the CYP promoters (0.14 and 0.16). Interestingly, the above MeJA-responsive *cis*-elements were greatly enriched in the promoters of the identified taxane

biosynthesis-related genes (**Fig. 6f**). This suggests a possible explanation for the increased expression levels of CYP725 family genes and taxane biosynthesis-related genes under MeJA treatment.

Transcription regulation of taxusin biosynthesis

The biosynthesis of active ingredients in medicinal plants is coordinated by various complex transcriptional regulatory networks³⁷. We identified 78 MeJA responsive TFs by integrating genomic and transcriptomic data (**Supplementary Data 10** and **Supplementary Fig. 19a**). Most of the MeJA responsive TFs were significantly up-regulated by the MeJA treatment, indicating their important roles in the MeJA-induced accumulation of active ingredients in *P. chienii* (**Supplementary Fig. 19b**). Pearson correlation analysis revealed several taxane biosynthesis-related TF genes (**Supplementary Fig. 19c**). Four important *cis*-elements, including ABRE and G-box (bHLH binding elements), MBE (MYB binding element), and W-box (WRKY binding element), were identified in the promoter regions of the taxane biosynthesis-related genes (**Supplementary Fig. 19d**). Our data suggest that MYB12, MYB13, bHLH18, and WRKY6 may serve as potential regulators involved in the regulation of taxane biosynthesis in *P. chienii*.

Discussion

P. chienii, commonly known as white-berry yew, has a limited geographical distribution in several southern provinces in China³⁸. *Taxus* is the sister genus of *Pseudotaxus*, which is well-known for its capability of producing anti-cancer active ingredient Taxol and has been extensively studied in recent years^{26,39}. The natural populations of *P. chienii* are relatively dispersed throughout China, exhibiting reproductive isolation among them. In our previous study, we reported the presence of 10-deacetylbaccatin III and paclitaxel in *P. chienii* samples collected from a wild population located in Mount Tianmu (30.33° N/119.42° E, Hangzhou, Zhejiang, China)⁴⁰. However, paclitaxel was not detected in *P. chienii* samples reported in the present study, which were collected from a wild population located in Nanjianyan Scenic Resort (28.36° N/119.09° E, Lishui, Zhejiang,

China). We speculated that we might have mistakenly collected some *Taxus* specimens and considered them as *P. chienii* due to the lacking of experience in distinguishing *Taxus* and *Pseudotaxus* for our previous study⁴⁰. Phenotypes of *Taxus* and *Pseudotaxus* are highly similar. It's critical to sample the plant materials in fall when the colors of the fruits are developed, which are the critical character to distinguish *Taxus* and *Pseudotaxus*. Since the plant materials used for the current study coming from different batch of sampling, we are confident that the spatial distribution of various types of active compounds reported in the current study is reliable (**Fig. 1**).

Although the complete genomes of various Taxaceae plants are publicly available, the reason why Taxol is synthesized exclusively in *Taxus* species remains unclear²²⁻²⁴. Our study presents the complete genome of *P. chienii* (**Fig. 2a-b**), a relict conifer endemic to China that belongs to a monotypic genus closely related to *Taxus*⁴¹. The genome size of *P. chienii* ($2n$) is approximately 15.6 Gb, which is larger than the known genomes of *Taxus* species, such as *T. mairei* (10.2 Gb), *T. wallichiana* (10.9 Gb), and *T. yunnanensis* (10.7 Gb)²²⁻²⁴. WGD is a dramatic genomic event that supplies a source of genetic material, thereby increasing both the complexity and size of the genome in angiosperms⁴². However, the occurrence of WGD events in gymnosperms has been a subject of controversy⁴³. Previous studies have reported that a recent WGD event has happened during the evolution of *T. mairei*, whereas no obvious WGD was observed for *T. wallichiana*^{22,23}. Our data also indicated the absence of a recent WGD event in the *P. chienii* genome (**Supplementary Fig. 5c**), suggesting that the complexity of WGD within gymnosperms, particularly within the Taxaceae family, is noteworthy⁴⁴. Most of the LTR-RT expansions in the *Taxus* genomes happened between 24 to 8 MYA²²; however, the expansion in the *P. chienii* genome experienced an exceptionally long duration, ranging from approximately 60 to 10 MYA (**Fig. 2c-d**). Continuous insertions of LTR-RTs have enlarged the size of *P. chienii* genome⁴⁵.

The origin and evolution of the taxane biosynthesis pathway have consistently been a focal point in plant secondary metabolism⁴⁶. Previous research pointed out that the loss of essential gene

families, particularly the orthologues of *TS*, results in the absence of Taxol and its analogues in *T. grandis*²⁵. Evolutionary analysis suggests that *T. grandis* is the oldest plant in the Taxaceae family, based on five selected species with complete genome (**Fig. 2f**). Following the emergence of the Taxaceae family, *P. chienii* has evolved TPS with taxadiene synthase activity and diverged from *T. grandis* approximately 48.3 MYA. Although *P. chienii* has entered the initial stages of taxane biosynthesis, it possesses only a partial pathway for Taxol biosynthesis, which terminates before the production of the enzyme responsible for synthesizing 10-deacetylbaccatin III, known as TBT⁴⁷. Approximately 24.3 to 25.6 MYA, the evolution of the *Taxus* genus formed an independent branch, diverging from the *Pseudotaxus* lineage (**Fig. 2f**). With the formation of the *Taxus* genus, the limitation caused by the absence of TBT was addressed, leading to the extension of the existing taxane biosynthesis pathway in *P. chienii* and resulting in a complete Taxol biosynthesis pathway (**Fig. 3**).

In *P. chienii*, the incomplete Taxol biosynthetic pathway results in the accumulation of an acetylated taxane, taxusin (**Fig. 4a-b**), which exhibits minimal anti-tumor activity⁴⁸. Taxol contains only two acetylated sites (C4 and C10), whereas taxusin contains four acetylated sites (C5, C9, C10, and C13)⁴⁹. Previous study has demonstrated that taxusin can be efficiently deacetylated by treatment with K₂CO₃ in THF/MeOH at 0°C for 3 days³³. Consequently, taxusin was selected as an industrial substrate for the production of taxoids due to its high natural abundance in yew heartwoods⁵⁰. Our study showed that *P. chienii* contains a large amount of taxusin and is a good raw material for industrial production of Taxol (**Fig. 4e-f**). Undoubtedly, *P. chienii* has both ecological and medicinal value.

In *Taxus* plants, taxane biosynthetic genes are arranged in one or two gene clusters, which is also observed in *P. chienii*²²⁻²⁴. Taking *T. wallichiana* as an example, several taxane biosynthesis-related gene clusters were located in Chr10, which expanded on this chromosome through tandem duplication²³. We verified genomic rearrangements between the Chr9/11 of *P. chienii* and the

Chr10 of *T. wallichiana* (**Fig. 3**). These results confirm a common origin between the taxane biosynthetic pathway of *P. chienii* and the Taxol biosynthetic pathway of *T. wallichiana*.

Investigating terpene synthases, which catalyze isoprenoid cyclization reactions, offers valuable insights into the evolution of plant-specific terpenoid biosynthesis in terrestrial plants ³⁴. Diterpenoid biosynthesis can be initiated by three distinct enzymes: Class I TPSs, Class II TPSs, and bifunctional Class I/Class II TPSs, all utilizing GGPP as a precursor ⁵¹. Bifunctional TPSs have been identified in gymnosperms and lycophytes, such as *Abies grandis*, *Abies balsamea*, *Ginkgo biloba*, and *Selaginella involvens* ¹⁵. The initial committed step in taxane biosynthesis involves the cyclization of linear GGPP to form taxa-4(5),11(12)diene, catalyzed by TS ⁵². A previous study has indicated that TS protein in *T. brevifolia* is a Class I TPS ⁵¹. The classification of TPS proteins is primarily based on the presence or absence of two motifs: DXDD and DDXXD/E. It is noteworthy that the known TSs from the Taxaceae plants, which lack the classic “DDXD” motif, are classified as Class I TPSs rather than bifunctional TPSs, indicating a distinctive characteristic of the Taxaceae family within gymnosperms (**Fig. 5a**). Comprehensive sequence analysis showed that the protein sequence surrounding the core DXDD motif, which is the iconic sequence of Class II TPSs, has a high similarity between TPSs from Taxaceae and other gymnosperms, indicating its conserved role during the evolution process. In the future, this peculiar phenomenon needs further attention of researchers.

X-ray crystal structure analysis showed that a TS protein from *T. brevifolia* possesses a complete class I-type C-terminal domain and a vestigial class II-type N-terminal domain, suggesting it activates the isoprenoid substrate by ionization, rather than protonation ³⁵. Using the 3p5p.1.A model as a template, the structure of PcTS1 is closer to the Class I type TPSs than to the bifunctional TPSs present in gymnosperms, indicating that it may be an evolutionary transitional form. Previous studies uncovered the function of the DDXXD and (N,D)DXX(S,T)XXE motifs as metal-binding sites in class I-type terpenoid cyclase ^{35,53}. In PcTS1, the residues D668 and D669

of the “DDXXD” motif, along with N812, D813, and E820 of the (N,D)DXX(S,T)XXXE motif, are predicted to be located the divalent cation binding site (**Fig. 5b-c**). This observation suggests that the structure of the metal ion catalysis site is conserved among TS enzymes across the *Pseudotsuga* and *Taxus* genera ⁵². Heterologous expression of *PcTS1* produced taxadiene in tobacco, confirming its complete functionality as TS (**Fig. 5e-g**).

In *Taxus* plants, several CYP725 subfamily genes were identified to be candidates for Taxol biosynthesis ²². Comparative genomic analysis showed that CYP725 is a gymnosperm specific CYP subfamily, indicating its specialized functions in the secondary metabolism of gymnosperm ⁵⁴. In plants, many members of CYP family showed high inducibility in response to MeJA ⁵⁵. In *Taxus* cell suspension cultures, MeJA elicitation is an effective strategy to induce and enhance biosynthesis of Taxol ⁵⁶. RNA-seq analysis demonstrated that MeJA treatment significantly up-regulated the expression of CYP725 genes, implying a potential coordinated regulatory pattern in their expression. Gene expression is regulated by various *cis*-acting elements, and the distribution of these elements within the plant genome is uneven. Consequently, genes that are clustered together may be influenced by the same regulatory elements and display similar expression patterns. Most of the CYP725 genes are situated within a very restricted genomic region (**Fig. 6**), which may account for the consistent response of CYP725 to MeJA treatment. In *Taxus*, several TFs belonging to the MYB, bHLH, and WRKY families were identified as regulators involved in the regulation of Taxol biosynthesis ^{57,58}. Co-expression analysis identified a number of MeJA responsive TFs, such as *MYB12*, *MYB13*, *bHLH18*, and *WRKY6*, providing a valuable resource outlining the regulation of taxane biosynthesis.

In conclusion, analysis of the high-quality chromosome-level genome assembly of *P. chienii* presented here reveals insights into the origin and evolution of taxane biosynthesis in the Taxaceae family. *P. chienii*, belonging to an older genus than *Taxus*, possesses only a partial pathway for Taxol biosynthesis, as it lacks a key enzyme known as TBT. This deficiency results in the

accumulation of taxusin, a highly acetylated form of taxane. With the emergence of the *Taxus* genus, the limitations imposed by the absence of this transferase have been overcome, resulting in a complete biosynthetic pathway for Taxol. Protein structure analysis showed that the metal ion catalysis sites TS are conserved across both the *Pseudotaxus* and *Taxus* genera.

Methods

Plant material

The tissues of *Pseudotaxus chienii* (specimen ID: ZQBX) were collected from the Nan Jian Yan Scenic Area (28.0°20.0'39.19"N, 119.0°6.0'0.97"E, Altitude:1197 m) in Zhejiang Province, China, in November 2022, for genome sequencing. The distinguishing feature of *P. chienii* is the presence of a white aril and two distinct white stomatal bands on the underside of mature leaves.

MS imaging (MSI) analysis

Young leaves and stems of *P. chienii* were sheared into 20- μ m sections on the MALDI-2 conductive slide using a freezing microtome (Leica CM1950). The sections were vacuum dried with N₂ gas. The tissue sections were dried and kept in -80°C for long-term preservation after vacuum packaging. The MALDI-2 special matrix containing 2,5-dihydroxybenzoic acid and 2,5-dihydroxyacetophenone was purchased from Bruker company. Prior to MS analysis, a MALDI-2 special matrix was sprayed onto the slides using a TM-sprayer (HTX Technologies) with the default parameters. No signal of the matrix background was detected. The size of MALDI-2 chip is 2.5 x 7.5 cm. We placed the leaf and stem materials on the same chip to ensure comparability between them.

Compounds in the slides were visualized using a timsTOF fleX MALDI2 (Bruker) equipped with a smartbeam 3D laser operating at 10 kHz. MSI was conducted in both of positive and negative ion modes across a m/z range of 50-1500. The spatial resolution of MSI was set to 20 μ m and the laser frequency was adjusted to 10 Hz. MS imaging data were firstly normalized using the Root

Mean Square method and processed with SCiLS Lab software (ver. 2021c, Bruker, Billerica, USA). Metabolites were identified by comparing the measured m/z values (with an accuracy of < 0.006 Da) against an in-house standard database and the Bruker Library MS-Metabobase 3.0 database. Receiver Operation Characteristic analysis and Student's t test analysis were applied to determine the significance of differences between the samples. Corrected p value < 0.01 were used to screen significant changed metabolites.

Genome sequencing

Genomic DNA was isolated from the twigs of *P. chienii* using the classical cetyl trimethyl ammonium bromide method. After quality checking, the genome was surveyed through the Illumina NovaSeq 6000 platform using a paired-end library (read length of 150 bp, insert size of 350 bp).

A HiFi SMRTbell library was constructed using the SMRTbell Prep Kit 3.0 (Pacific Biosciences of California) and sequenced on the PacBio Revio sequencing platform at Wuhan Benagen Tech Solutions Company Limited (Wuhan, China). The consensus HiFi reads were generated using circular consensus sequencing software (ver.6.4.0) with default parameters. The Hi-C library was prepared using fresh twigs of *P. chienii*. Briefly, the tissues of *P. chienii* were fixed with a 2% formaldehyde. Nuclei were extracted and then digested by restriction enzyme DPN II. The end of digested fragments was labeled with biotin and ligated randomly with T4 DNA ligase (NEB) and reverse crosslinked using proteinase K (NEB). Then, DNA fragments were sequenced on the Illumina NovaSeq 6000 platform.

Genome assembly

The raw data of short reads was filtered to remove adapters and low-quality reads using fastp software (ver. 0.21.0)⁵⁹. The genome size and heterozygosity were estimated using Jellyfish v2.2.10 and GCE (ver.1.0.0) with K-mer method⁶⁰. The Hifi sequences were first assembled into

contigs using hifiasm (ver. 0.16.1) ⁶¹, followed by two rounds of polishing using Pilon with short reads ⁶². Purge_dups (ver. 1.2.5) was used to eliminate redundant Haplotigs ⁶³. For pseudochromosome level scaffolding, clean reads of the Hi-C data were aligned to the pre-assembled genome using the HICUP software (ver. 0.8.0) to obtain unique mapped paired reads ⁶⁴. The preliminary assembly of the draft genome sequence was anchored onto chromosomes using ALLHiC (ver. 0.9.8) ⁶⁵. Finally, a chromosome-level assembly of *P. chienii* was obtained through manual curation using Juicebox (ver. 1.11.08) ⁶⁶. To estimate the assembly completeness, clean Illumina reads were mapped onto the reference sequence using BWA software (ver. 0.7.17) ⁶⁷. BUSCO program (ver. 4.1.2) was used to evaluate the integrity of genome assembly based on lineage-specific profile library embryophyta_odb10.2024-01-08 ⁶⁸.

Gene prediction and annotation

The gene structure of the *P. chienii* genome was predicted using three strategies, including transcript mapping, ab initio gene prediction, and homologous gene alignment ⁶⁹. For transcriptome-based prediction, full-length cDNA transcripts were first aligned to the reference sequence using Minimap2 (ver. 2.17) assembled with Stringtie2 (ver. 2.1.5) ^{70,71}, and gene models were then predicted using TransDecoder (ver. 5.7.0) (<https://github.com/TransDecoder/TransDecoder>). For homology-based gene prediction, peptide sequences from *T. wallichiana*, *T. yunnanensis*, *T. mairei*, and *T. grandis* were aligned to the *P. chienii* genome using tblastn (ver. 2.13.0) and gene models were predicted using Exonerate (ver. 2.4.0) ⁷². For ab initio prediction, Augustus and GlimmerHMM were used with a custom training set from transcriptome and protein-based gene model ^{73,74}. At last, all the results from the above three distinct methods were combined and integrated to produce a high-confidence gene structure by Maker (ver. 2.31.10) ⁷⁵.

For functional annotation, the protein encoding sequences of *P. chienii* were searched against the several protein databases, including NR (ver. 2022-03-09), UniProt and TrEMBL (ver. 2022-03-

09), and Kyoto Encyclopedia of Genes and Genomes (KEGG) using BLASTp program. The Pfam domain and Gene Ontology (GO) classification was performed using InterProScan (ver. 5.55-88.0)

76.

Prediction of repeat sequence

To identify repeat sequences in the *P. chienii* genome, we first generated a *de novo* repeat sequence library using RepeatModeler (ver. 2.0.4) ⁷⁷. LTR elements were identified using LTR_FINDER and LTRharvest (ver. 1.6.0) ^{78,79}. The above two libraries were then combined with the Repbase library and used as the input data for RepeatMasker (ver. 2.0.4, <https://www.repeatmasker.org/>). The TE Protein type repeat sequences were predicted using RepeatProteinMask (ver. 4.1.5) in the RepeatMasker software. Tandem repeat prediction was performed using software Look4TRs ⁸⁰. To calculate the insertion time (T) of LTRs, the following formula was used:

$$T = K (\text{nucleotide distance}) / 2r (\text{nucleotide substitution rate}) \quad (1)$$

where the r for gymnosperm is 2.2×10^{-9} substitutions per year per synonymous site and the K value was calculated using Distmat from the EMBOSS (ver. 6.5.7.0) package ⁸¹. The LTR Assembly Index was calculated using LTR_retriever (ver. 2.9.0) ³⁰.

Synteny and whole-genome duplication analysis

To investigate genome collinearity, syntenic gene pairs between *P. chienii*, *T. yunnanensis*, *T. wallichiana*, *T. mairei*, and *T. grandis* were identified using JCVI (ver. 0.84) with default parameters ⁸². For whole-genome duplication analysis, an all-versus-all analysis with BLASTp was performed using all proteins sequences of *P. chienii* with an e-value cutoff of 10^{-5} , query coverage of 0.3, and an alignment threshold of 100 amid acids. The syntenic regions with collinearity of paralog pairs were detected by MCScan X with default parameters ⁸³. Nucleotide substitutions (Ks) value of each syntenic gene pair was calculated using the PAML program (ver. 4.9 package) ⁸⁴.

Phylogenetic analysis and genomic comparison

The longest transcripts of each coding gene from 15 representative species, including *Arabidopsis thaliana*, *Vitis vinifera*, *Oryza sativa*, *Nymphaea colorata*, *Amborella trichopoda*, *Taxus yunnanensis*, *Taxus wallichiana*, *Taxus mairei*, *Torreya grandis*, *Fokienia hodginsii*, *Gnetum montanum*, *Pinus densiflora*, *Ginkgo biloba*, *Cycas panzhihuaensis*, *Selaginella moellendorffii*, and *P. chienii* were used to construct gene families using program OrthoFinder (ver. 2.09) ⁸⁵. Phylogenetic analysis was conducted using IQ-TREE (ver. 2.1.3) with the best model selected by ModelFinder ⁸⁶. The resultant phylogenetic tree was applied to calculate the divergence time by the MCMCTREE program from the PAML (ver. 4.9 package) ⁸⁴. Divergence times between *O. sativa* and *G. biloba* (326.4 - 336.8 million years ago [Mya]), *G. montanum* and *C. panzhihuaensis* (275.3 - 316.4 Mya), and *A. trichopoda* and *N. colorata* (179.9 - 205.0 Mya) were collected from Timetree (<http://timetree.org/>) for use as fossil calibration points. The expansion and contraction of gene families were identified using CAFE (ver. 4.2.1) ⁸⁷.

Untargeted metabolomic profiling

The stem and leaf samples of different Taxaceae trees, including *P. chienii*, *T. wallichiana*, and *T. grandis* (50 mg each sample, *N* = 6), were ground with 800 μ L pre-cooled methanol solution (50%) in a tube. The tissue samples were added with 500 μ L of pre-cold chloroform/methanol/H₂O (v:v:v, 1:3:1) and subjected to ultrasonication for 5 min at 4°C. The chromatographic separation of tissue sample was carried out using a SCIEX UPLC system (Applied Biosystems, Foster City, USA) with an ACQUITY BEH Amide column (2.1 \times 100 mm, 1.7 μ m particle size; Waters, Milford, USA). The flow rate in column was 0.4 mL/min and the working temperature was 35°C ⁸⁸.

An efficient MS/MS SCIEX Triple-TOF-5600 plus system (Applied Biosystems) was used to detect metabolites eluted by the reversed-phase separation. The MS data pretreatments, such as peak picking, peak grouping, retention time (RT) correction, and adduct annotation, were

conducted using XCMS software. The MS data was converted into mz.XML format file and identified by checking the RT and m/z values. The metabolite was annotated by matching the exact m/z value of each MS ion to the KEGG database. The molecular formula of each presumptive compound was validated by the isotopic distribution according to an in-house MS library. The intensity of each ion peak was calculated by software metaX. Wilcoxon test was conducted to analyze the metabolic differences between two sample groups. The P value was adjusted and corrected by false discovery rate (FDR) with Benjamini–Hochberg method. A VIP cut-off value of 1.0 was used to select important features.

Identification of taxane biosynthesis-related genes

The full sequences of taxane biosynthesis-related proteins were downloaded from the published *Taxus* genomes and used as baits to search against the *P. chienii* genome²²⁻²⁴. Homologous genes in *P. chienii* were obtained by using OrthoFinder ver.2.09 with default parameters⁸⁹. Furthermore, important gene classes, including the CYP (Pfam ID: PF00067), TRF (Pfam ID: PF02458), and TPS (Pfam ID: PF03936 and PF00432) families, were identified by performing HMM searches. To identify the gene with complete function domain, a gene body containing a continuous long gag (N >10) in the multiple sequence alignment was removed. Multiple sequence alignment analysis was performed using ClustalOmega program (<http://www.clustal.org/>).

Homology modeling of TPS family members

The 3D models of TPS proteins were built using SWISS-MODEL program (<https://swissmodel.expasy.org/>). The protein 3D structures were predicted based on the published crystal structure of *T. brevifolia* TS protein (PDB ID: 3p5p.1, resolution: 2.25 Å)³⁵. For each TPS family member, the sequence alignments between template and target sequences were performed by Clustal Omega (<http://www.clustal.org/>). The model with the lowest overall energy was selected as the final structure of the target protein. The PBD files of selected TPS proteins were generated and download from SWISS-MODEL program. TM score was analyzed using alphafold3 online

tool with referring PBD file.

MeJA treatment and RNA-seq analysis

P. chienii cutting seedlings were used for MeJA treatment and RNA-seq analysis. For MeJA treatment, 100 μ M MeJA ethanol solution was sprayed onto the leaves of *P. chienii* seedlings and inoculated for 24 h and 48 h, respectively. Leaves inoculated with ethanol were harvested as the controls. RNA extraction, cDNA library construction, Illumina sequencing, sequence alignment, and quality evaluation were performed. Differentially expressed genes (DEGs) were analyzed using DESeq2 program according to criteria: the FDR < 0.05 and absolute fold change ≥ 2 ⁹⁰.

Promoter analysis

The 1500-bp promoters of 581 CYP superfamily genes and 16 taxane biosynthesis pathway genes were extracted from the *P. chienii* genome. The promoter sequences were scanned using the PlantCARE software (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). Two MeJA-related cis-elements, including G-box (CACGTGG) and G-box like (AACGTG), and four TF binding elements, including ABRE (ACGTG), G-box (CACGAC and TAACACGTAG), MBE (CAACTG and CAACCA), W-box (TTGACC), were screened and visualized by TBtools⁹¹.

Transient expression in *N. benthamiana* and substrate feeding experiments

The full-length sequences of *T2OH* (PchiChr11G298480.1) and *T7OH* (PchiChr11G298490.1) from *P. chienii*, and *T2OH* (ctg2120_gene.6) and *T7OH* (ctg2120_gene.1) from *T. mairei* were cloned into pBWA(V)HS vectors and transferred into *Agrobacterium* (strain GV3101). *Agrobacterium* strains containing the gene constructs were grown in 10 mL of LB with antibiotics (50 mg/L kanamycin and 25 mg/L rifampicillin) for 16 h at 28 °C and 200 rpm, then cells were centrifuged at 2000 g for 10 min and the supernatant was removed. After incubation at room temperature for 2 h, infiltration was performed using a 1 mL syringe without needle on underside of 4-week-old *N. benthamiana* leaves. After 4 days, 100 μ g/mL taxusin was infiltrated into

the underside side of previously *Agrobacterium*-infiltrated leaves with a needleless 1-mL syringe. Leaves were collected after 1 day post-infiltration, extracted and analyzed by LC-MS/MS.

LC-MS/MS analysis of taxusin content

The twig samples are harvested from *P. chienii* cuttings and ground with liquid nitrogen. The sample power was added with 800 μ L of extract solution containing zirconia beads. Then, the sample solution was sonicated in ice for 25 min and kept in a constant temperature extract solution for 1 h. The entire supernatant was precisely transferred to a new clean microtube and vacuum dried.

Taxusin standard (CAS: 19605-80-2, $\geq 99.0\%$) was purchased from Shyuanye company (<https://www.shyuanye.com/>). An appropriate amount of standard solution was used to prepare a gradient calibration curve working solution. The parameters of LC-MS/MS analysis are as follows. Column: ACQUITY UPLC BEH C18 (100 \times 2.1 mm, 1.7 μ m); Mobile phase: 0.1% formic acid water solution (A solution) and methanol solution (B solution); Flow rate: 0.3 mL/min; Injection volume: 10 μ L; Column temperature: 30 $^{\circ}$ C; Ion source: ESI; Ion scanning mode: multiple reaction monitoring; Ion source temperature: 500 $^{\circ}$ C; Ion source voltage: 4500V; Collision gas: medium; Air curtain pressure: 35 psi; and atomizing gas and auxiliary gas: 50/55 psi. Multiquant 3.0.2 software was used to extract chromatographic peak area and RT. Standard of the target compound was used to correct the RT. The retention time of taxusin is 7.48 min. The transition of m/z 527.3 \rightarrow 467.2 was used for taxusin quantification and the transitions of m/z 527.3 \rightarrow 407.2 were utilized for confirmation. Limit of detection is 2 ng/mL.

Experimental validation of PcTS genes

Total RNA was isolated from the twigs of *P. chienii* using an RNeasy plant mini kit (Qiagen, Hilden, Germany). High quality RNA was used for cDNA library construction using a PrimeScript RT reagent kit. ORF of *TSI* (PchiChr11G299420.1) was amplified with Biorun Pfu PCR Mix (Up-

cagtGGTCTCacaacatggctcagccctcatttaattcagctc/

cagtGGTCTCatacatcatactttaattgggtcaataaaacttttcttatataat-Down) and ligated into pBWA(V)HS vector (Biorun Bio-tech, <https://plant.biorun.com/>). Purified construct was transformed into *Agrobacterium tumefaciens* cells (GV3101) using Eppendorf Eporator (Hamburg, Germany). After preparing a resuspension of *A. tumefaciens* with OD₆₀₀ = 0.2, the tobacco leaves were inoculated with the resuspension for 10 min⁹².

GC-MS analysis of taxadiene

For GC-MS analysis, 500 mg of transgenic tobacco leaves were harvested and ground to powder in liquid N₂. The powder was extracted with 5 mL hexane for 20 min sonication treatment. Tissue samples were extracted with mixed solution (hexane:ethyl acetate = 4:1, v/v) containing 200 ng of internal standard (eicosane, CAS: 112-95-8). After dried under a stream of N₂ gas, the samples were redissolved in 1 mL of hexane: ethyl acetate (4:1, v/v) mixed solution. Then, 1 µL of each sample solution was injected into a Trace GC Ultra gas chromatograph coupled to ISQ mass spectrometer (Thermo Scientific). Separation was performed with an HP5ms column (30 m × 250 µm × 0.25 µm) in splitless mode⁹³. Taxadiene was detected according to the peak area of the internal standard. All analyses were conducted using three biologically independent samples.

Data availability

Genome assembly has been deposited in GenBank under accession GCA_053640655.1 [https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_053640655.1]. The genomic raw sequencing data have been deposited in NCBI Sequence Read Archive (SRA) database under accession PRJNA1205499 [<https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA1205499>]. The transcriptomic raw sequencing data generated and analyzed during the current study are available in NCBI Gene Expression Omnibus (GEO) under accession GSE288094 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE288094>]. Genome annotation files are available at Figshare [<https://doi.org/10.6084/m9.figshare.29084240>]. The raw data of MS

imaging analysis has been uploaded to the Metaspaces database with project ID m-2025 [https://metaspaces2020.org/api_auth/review?prj=07a71fa8-5c9a-11f0-a049-b3ec65131efb&token=KzN_YV6IGpbf]. Source data are provided with this paper.

Code availability

Codes used to perform the analyses are available at Figshare [<https://doi.org/10.6084/m9.figshare.29084240>].

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Author contributions

C.L.Y., C.J.S., and M.S.W. conceived and designed the study. R.Y.M., C.L.Y., and C.J.S. prepared the genome sequencing material. M.S.W., R.Y.M., Z.J.F., L.X.Z., and Y.B.Z. led the bioinformatics analyses. S.L.W. and H.Z.W. provided the *Taxus* and *P. chienii* materials. M.Y.Z., E.H.B., W.T.L., and Y.Z. performed the untargeted metabolome and RNA-seq analyses. R.Y.M., Y.Y.P., and H.J.M. cloned the *PcTSI* and *TwTSI* genes. M.S.W., S.G.F., X.X.Z., and C.J.S. developed the figures. M.S.W., R.Y.M., C.N.Y., C.L.Y., and C.J.S. interpreted the data and drafted the manuscript. M.S.W., C.L.Y., and C.J.S. wrote the manuscript. M.S.W., S.L.W., H.Z.W., C.L.Y., and C.J.S. revised the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Figure legends

Figure 1. Analysis of the data from timsTOF fleX MALDI-2. (a) A picture of the twig of *P. chienii*. The blue line indicated the sample site of leaf and red line indicated the sampling site of stem. (b) The optical images of the *P. chienii* leaf and stem sections. (c) Mean mass spectrogram and segmentation analysis of the *P. chienii* samples. Stem and leaf samples were performed on a single chip. (d) Imaging analysis of ten typical diterpenes in the leaves and stems of *P. chienii*. Color scale ranges from 0 to 100%. Blue indicated low accumulation level and red indicated high accumulation level. (e) Proportion of various metabolites in leaves and stems was showed by histogram. Source data are provided as a Source Data file.

Figure 2. Genomic features of *P. chienii*. (a) Pictures of a wild *P. chienii* tree used for genomic DNA sequencing. (b) Circos plot of *P. chienii* genome. Track I represents the length of the chromosomes (Gb); II and III represent GC content and gene density, respectively; IV-VI represent gene expression without or with MeJA treatment, respectively; VII-IX show the distribution of transposons, Gypsy, and Copia LTRs, respectively. These features were calculated in 10 Mb windows. Center, the syntenic regions were linked by curve lines. (c) Analysis of insertion time based on the total LTR. (d) Analysis of insertion time based on different types of LTR. (e) Venn diagram for orthologous protein-coding gene clusters in *P. chienii*, *T. grandis*, *T. mairei*, *T. yunnanensis*, and *T. wallichiana*. The number in each sector of the diagram represents the total number of genes across different comparisons. (f) Gene family expansion and contraction during the evolution of green plants. The maximum likelihood phylogeny was built with a number of low-copy orthologous groups. Numbers on branches are the sizes of expanded (red) and contracted (green) gene families at each node.

Figure 3. Origin and evolution of the taxane biosynthetic pathway. (a) The possible taxol biosynthetic pathway. The solid and dotted arrows represent the characterized and unknown

reactions, respectively. Red forks indicated the genes that do not exist in *P. chienii*. The abbreviation is as follows: taxadiene synthase (TS), taxane 5 α -hydroxylase (T5OH), taxane 13 α -hydroxylase (T13OH), taxadien-5 α -ol-*O*-acetyltransferase (TAT), *Taxus* cytochrome P450 reductase (TCPR), taxane 2 α -hydroxylase (T2OH), taxane 7 β -hydroxylase (T7OH), taxane 10 β -hydroxylase (T10OH), taxane 9-oxidase (T9OH), taxane oxetanase 1 (TOT1, also named CYP1 or CYP725A55), taxane 2 α -*O*-benzoyl transferase (TBT), 10-deacetyl-baccatin III-10-*O*-acetyltransferase (DBAT), 13-*O*-(3-amino-3-phenylpropanoyl) transferase (BAPT), taxane 2' α -hydroxylase (T2'OH), *N*-debenzoyl-2'-deoxytaxol-*N*-benzoyltransferase (DBTNBT). (b) Overview of the known genes involved in taxane biosynthesis. (c) Genome rearrangements between *P. chienii* and *T. wallichiana*. Physical and genetic maps of the 142.04-245.71 Mb region of chromosome 9 and 6.56-101.93 Mb region of chromosome 11 were extracted from the *P. chienii* genome, and the 195.41-486.53 Mb region of chromosome 10 was extracted from the *T. wallichiana* genome. Source data are provided as a Source Data file.

Figure 4. Accumulation of taxusin in *P. chienii*. (a) The number of genes encoding each key enzyme involved in the taxane biosynthesis. (b) Speculative metabolic pathway block in *P. chienii*. (c) Determination of the contents of taxusin and 10-deacetyl-baccatin III in *P. chienii*, *T. wallichiana*, and *T. grandis*. (d) MS spectra of taxusin in *P. chienii* and *T. wallichiana*. (e) The contents of taxusin in the *P. chienii* and *T. wallichiana* twigs. **** $P < 0.0001$. Data are presented as mean values \pm SD of six biological repeats. Statistical analyses were performed using one-way ANOVA, unpaired *t*-test with Welch's correction. Bar graphs were plotted using GraphPad Prism 10.6. (f) Determination of the contents of taxol and its derivatives, including 10-deacetyl-taxol, 10-deacetyl-7-xylosyltaxol C, 13-acetyl-9-dihydrobaccatin III, and cephalomannine. Source data are provided as a Source Data file.

Figure 5. Functional identification of the TS genes in *P. chienii*. (a) Phylogenetic analysis of the TPS family members in different plants. Purple stars indicated the TPS from *P. chienii*

(PcTS1/2). Green dots indicated the TPS containing Class I (DDXXD/E) motif, red dots indicated the TPS containing Class II (DXDD) motif, and yellow dots indicated the TPS containing bifunctional motif (DDXXD/E+DXDD). (b) Identification of positive transgenic tobacco expressing *PcTS1* and *TwTS1*, respectively. (c) GC–MS analysis of hexane extracts from the seedlings expressing *PcTS1* and *TwTS1*, and controls (CK), respectively. Time (11.857) indicates the target peak of taxadiene. (d) Mass spectra profile of the compound at 11.857 min matched exactly with the taxadiene mass spectra. (e) Multiple sequence alignment analysis of the DXDD and DDXXD/E motifs from different TPS proteins. (f) Structure modeling of PcTS1 by SWISS-MODEL program using 3p5p.1.A model as template. The bioactive center of PcTS1 zoom in through a red box. (g) Protein structure analysis of the typical TPSs from the Angiosperm (Class I), Angiosperm (Class II), and other gymnosperms (bifunctional TPS), respectively. Source data are provided as a Source Data file.

Figure 6. Analysis of CYP family in *P. chienii*. (a) Heat map of the number of CYP450 genes in *P. chienii* and other 10 representative plant species. Each CYP450 family is represented as a square, with the blue color representing the number of genes in the corresponding family. The heatmap scale ranges from 0 to 100. (b) Distribution of CYP725 subfamily genes on the chromosomes 9 and 11 in *P. chienii*. The color bar showed the number of CYP725 genes distributed in different chromosomes. (c) The expression pattern of CYP450 family genes under MeJA treatments. (d) The expression levels of taxusin biosynthesis-related genes under MeJA treatments. The heatmap scale ranges from 0 ~ 1000 FPKM. (e) MS spectra of taxusin in CK and MeJA treated samples. (f) The accumulation level of taxusin in the twigs of *P. chienii* under CK and MeJA treatments (24 h and 48 h). Each value is the mean±standard deviation of six biological repeats. **** $P<0.0001$ and ** $P<0.01$. Statistical analyses were performed using one-way ANOVA, unpaired *t*-test with Welch's correction. Bar graphs were plotted using GraphPad Prism 10.6. (g) The average numbers of two reported MeJA responsive *cis*-elements, G-box (CACGTGG) and G-box like (AACGTG), in the promoters of CYP genes and CYP725 genes, were counted in '+' and '-' directions,

respectively. Source data are provided as a Source Data file.

Editorial Summary

Pseudotaxus is a sister genus to *Taxus* but lacks a complete taxane biosynthetic pathway. Here, the authors assemble the genome of *P. chienii* and reveal that the presence of a functional taxane 2 α -O-benzoyl transferase in *Taxus* genus extends the existing taxane biosynthesis pathway into a complete Taxol biosynthesis pathway.

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