Insights into the plateau adaptation of *Salvia castanea* by comparative genomic and WGCNA analyses

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**Highlights**

- *Salvia castanea* evolved the special underground stem with "trunk-branches" developmental pattern.
- Significantly expanded and contracted OGGs, SSGs, and PSGs contribute to morphogenesis and QTP adaptation of *S. castanea*.
- Tomenta of leaves and periderm of underground stem are like armors protecting *S. castanea* against QTP environment.
- Secondary metabolites mainly RA and T-IIA realize the functions of armors.
- NAC29 and TGA22 were identified as key TFs involved in regulating T-IIA and RA biosynthesis.

**Abstract**

**Introduction:** *Salvia castanea*, a wild plant species is adapted to extreme Qinghai-Tibetan plateau (QTP) environments. It is also used for medicinal purposes due to high ingredient of tanshinone IIA (T-IIA). Despite its importance to Chinese medicinal industry, the mechanisms associated with secondary metabolites accumulation (i.e. T-IIA and rosmarinic acid (RA)) in this species have not been characterized. Also, the role of special underground tissues in QTP adaptation of *S. castanea* is still unknown.

**Objectives:** We explored the phenomenon of periderm-like structure in underground stem center of *S. castanea* with an aim to unravel the molecular evolutionary mechanisms of QTP adaptation in this species.

**Methods:** Morphologic observation and full-length transcriptome of *S. castanea* plants were conducted. Comparative genomic analyses of *S. castanea* with other 14 representative species were used to reveal its phylogenetic position and molecular evolutionary mechanisms. RNA-seq and WGCNA analyses were conducted.
Introduction

The Qinghai-Tibet Plateau (QTP) of China is the highest plateau in the world with an elevation of about 3,000–5,000 m [1–3]. This region is characterized by extreme climatic conditions, including intense ultraviolet (UV) radiation, low oxygen and low temperature. Salvia castanea (Lamiaceae) is a wild plant species, which primarily grows in QTP regions with no reported artificial cultivation [4,5]. Although the evolution of Salvia genus has been studied to some extent [6], development of S. castanea and its adaptations to extreme QTP environment have not been reported. Another species in this genus, S. miltiorrhiza (Danshen) is widely cultivated for extracting the active ingredient of tanshinone IIA (T-IIA) from its root tissues [7]. Significantly higher levels of T-IIA and rosmarinic acid (RA) in wild S. castanea make it attractive to medicinal industry [8]. However, accumulation mechanisms of high secondary metabolites (mainly T-IIA and RA) in S. castanea are yet to be explored.

Plant secondary metabolites (PSMs) can be especially affected by environmental factors [9]. Drought could increase the accumulation of secondary metabolites (salvianolic acid B and T-IIA) in S. miltiorrhiza [10]. Dynamic changes in plant secondary metabolites during UV acclimation were reported in Arabidopsis thaliana [11]. Temperature could change the contents of various PSMs [12], as revealed that low-temperature stress could trigger the secondary metabolic pathways of phenylpropanoid and anthocyanin in maize seedlings [13]. Therefore, PSMs could exert long-term effects on plant growth and survival under stressful environments [14]. Plant secondary metabolism is the result of plant adaptation to ecological environment in long-term evolution, and PSMs provide material basis for plants to against adverse environment [15,16]. In this study, we focused species S. castanea accumulates two kinds of PSMs (Tanshinones and RA), which have relatively clear functions. Tanshinones e.g. dihydrotanshinone I (DT-I), cryptotanshinone (CT), T-I and T-IIA, are lipid-soluble diterpenoid quinone compounds. Plants synthesize these compounds via mevalonate (MVA) pathway in cytoplasm and via 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway in plastids [17,18]. Tanshinone IIA is a pharmacologically important constituent, which shows anti-hypoxic effect for altitude sickness [19], and prevents myocardial ischemia injury in human [20]. T-IIA also protects cells from oxidative damage by regulating reactive oxygen species (ROS) [21]. Similarly, RA is a natural antioxidant [22], which inhibits lipid peroxidation in situ [23] and protects cells from ionizing radiations [24]. Due to anti-hypoxic, anti-inflammatory properties of T-IIA and -oxidant character of RA, we propose that S. castanea adapts to extreme QTP environments through high accumulations of these secondary metabolites.

As a main T-IIA synthesis site, it is interesting to understand morphogenesis of underground tissues in S. castanea. Special environment could affect the species-specific morphogenesis such as aerial roots of Ficus microcarpa in tropics or subtropics [25], adventitious roots of lotus in water [26], underground stem of Welwitschia mirabilis in Africa desert [27]. The underlying mechanisms of these specific morphogenesis are complex due to the interactions between environment and endogenous factors. In recent decades, there were some important progresses regarding to plant morphogenesis. Continuous organ initiation and outgrowth in plants rely on proliferation and differentiation of stem cells, and several candidate genes for genetic improvement of cultivated Chinese medicinal plant Danshen.

Materials and methods

Plant materials

The wild samples of annual and perennial S. castanea were collected in November 2020, from Jade Dragon Snow (Yulong) Mountain region (100°15′E, 27°9′N, 3052 m) in Lijiang city, Yunnan Province, China (Fig. 1a). Meteorological data from 1979 to 2020 were obtained from http://www.wheata.cn in Yulong Naxi autonomous county (100°25′E, 27°25′N), location close to the sampling site. For the morphological study, root cross and vertical sections were prepared within 1 mm of thickness, and soaked in 70% formaldehyde-acetic acid-ethanol fixative (FAA) for paraffin sli-
Software Caseviewer 2.3 was used for the observation of parafin section. For the analyses of species evolution and high secondary metabolites accumulation, samples were divided into different tissues (root, stem, leaf and petiole), which were immediately immersed into liquid nitrogen for further PacBio SMRT sequencing analysis. The leaf, periderm, phloem and xylem tissues from both annual and perennial plants, total eight tissues were collected for the next-generation sequencing and active ingredient analyses with three biological replicates. These tissue samples were termed as: annual leaf (A-leaf), annual periderm (A-periderm), annual phloem (A-phloem), annual xylem (A-xylem), perennial leaf (P-leaf), perennial periderm (P-periderm), perennial phloem (P-phloem), and perennial xylem (P-xylem). Annual S. castanea is the plant germinated from the seed. Perennial S. castanea is the plant sprung up from the previous root because S. castanea withers in winter, leaving the roots alive in the ground.

PacBio and illumina sequencing and data analysis

Total RNAs from four different tissues (root, stem, leaf, and petiole) were extracted using RNAprep Pure Plant Kit DP441 (TIANGEN, Beijing, China) following the manufacturer’s procedure. The PacBio single-molecule real-time libraries were constructed in Personal Biotechnology Co., Ltd. Shanghai, China (PacBio Sequel II platform, USA) according to Yue et al. [37]. The longest transcript was selected as unigene after the iterative isoform-clustering (ICE) and CD-HIT software removing redundant sequences. Functional annotations of unigenes were performed in six public databases (NR, Pfam, Egg-nog, Swissprot, GO and KEGG). The genes were mainly named according to the annotation of Swissprot database. For more than one gene from S. castanea identified as the homologues of same known gene deposited in Swissprot database, we use ‘1’, ‘2’ and ‘3’ or ‘a’, ‘b’ and ‘c’ to number them. The classification of TF families was defined following the Plant Transcription Factor Database.

RNA sequencing was performed on an Illumina HiSeq 2500 platform (USA), and 150 bp paired-end reads were generated. The raw transcriptome sequencing data have been deposited in the NCBI under the accession number PRJNA800258. After quality control, the clean reads were mapped to a full-length transcriptome using Bowtie2 [38]. To estimate unigene expression levels, the number of mapped clean reads for each gene was determined and normalized for calculating fragments per kilobase transcript length per million fragments mapped (FPKM) value using RNA-Seq with Expectation-Maximization (RSEM) (v1.2.15) [39]. Detailed information of RNA sequencing and mapping is summarized in Tables S1-2. Gene expression profiles for biological replicates were assessed using principal component analysis (PCA) (Fig. S1). Fold change (FC) and binomial tests were used to detect differentially expressed unigenes (DEGs) between samples. DESeq was employed to calculate the false discovery rate (FDR). In this study, $P < 0.05$ and $|\log_2 FC| > 1$ were set as the thresholds to identify the DEGs. We used a hypergeometric test with an FDR correction method using topGO and KEGG pathway for enrichment analysis.

Weighted gene co-expression network analysis (WGCNA) was performed using WGCNA (V1.69) package in R (V3.6.1) [40]. All genes were hierarchically clustered using topological overlap-based dissimilarity measure and a gene dendrogram was generated based on Topological Overlap Matrix (TOM). Gene expression profile of each module identified by gene dendrogram was calculated to test the association with each plant effective component.

Fig. 1. Geographic information and morphological characteristics of Salvia castanea. (a) Geographical location of our experimental sample, S. castanea, from Jade Dragon Snow (Yulong) Mountain (100°15'E, 27°9'N, 3,052 m), Lijiang city, Yunnan Province, China. The map was downloaded from the website of http://bzdt.ch.mnr.gov.cn. (b) Morphological characteristics of S. castanea. (c) Features of upper and lower surfaces of S. castanea leaves by microscope.
In co-expression network, the edge weight (ranging from 0 to 1) of any two genes connected was determined based on their topology overlap measure. The networks were visualized using Cytoscape (v.3.8.2).

Phylogenetic tree reconstruction and divergence time estimation

The evolution of S. castanea was analyzed by identifying homologous genes from 15 presentative plant species, including 3 Lami-ales, 3 Monocotyledons, 7 Dicotyledons and 2 outgroup species (Table S3). Unigenes, predicted as protein-coding genes by OrfPre-dictor [41], were used for further analysis. Predicted polypeptides and proteomes of the above 15 species were clustered into ortho-logous gene groups (OGGs) using OrthoFinder (v2.2.7) [42]. For species tree reconstruction, we selected those one-to-one ortholog genes among the 15 species. MAFFT (v7.1) [43] was used for mul-tiple alignment of protein sequences of each single-copy homologous gene. Low-quality single-copy regions were removed by TrimAl (v 1.4) [44]. A phylogenetic tree was constructed by using PROTGAMEAFT model with RAxML (v 8.2) [45]. Maximum like-lihood (ML) method was used all the sites for bootstrap analysis with 1,000 replicates for the reliability of interior branches. We set Amborella trichopoda as an outgroup. Internal node time interval of different species was calibrated according to the Timetree database (http://www.timetree.org/) [46], and Bayesian method was used to estimate the divergence time of species by using mcmctree implemented in PAML (v4.9) [47]. CAFÉ (v3.1) [48] was used to accurately estimate the expansion and contraction of OGGs. We further conducted a positive selection analysis for the one-to-one OGGs according to Li et al. [49].

HPLC analysis of active ingredients

Eight tissues including leaf, periderm, phloem and xylem from annual and perennial S. castanea were separately analyzed for active ingredients contents (i.e. T-IIA, T-I, DT-I, CT, RA and salviano-lidic acid B (SAB)). The samples were dried at 60 °C to constant weight, 20 mg ground powder (passed through 60-mesh sieve) and then mixed with 70% methanol for ultrasonic pretreatment for 30 s, 1 cycle; 95 °C for 30 s, 1 cycle; 95 °C for 5 s and 58 °C for 30 s, 40 cycles according to the method of Xu et al. [50]. The 2-Act model, was selected as a reference. PCRs were conducted under the following conditions: 3 min at 95 °C; 30 cycles of 95 °C for 30 s, 1 cycle; 58 °C for 30 s, 1 cycle; 72 °C for 30 s, 1 cycle. The amplified products were visualized using agarose gel electrophoresis. The mean ± standard error (SE) of the results were presented as mean ± standard error (SE).

Results

Morphology and special underground stem developmental pattern

S. castanea species used in this study grew in Yulong Mountain region (Fig. 1a). Meteorological data nearby the sampling site from 1979 to 2020 presented in Table S5. It could be summarized as hypoxia, intense UV light and cold. The oblong ovoid leaves of this species were basal grown, and densely tomentose in the upper and lower surfaces (Fig. 1b, c). During flowering season from July to October, quadrangular stem would bolt from basal leaves and blossom chestnut verticillasters (Fig. 1b). Interestingly, upper lip of flower was also densely villous. All these special characteristics contributed greatly to the adaptation of plateau environment.

Interestingly, underground morphology of this species was special, as multi-branches of tree shrank and buried in the ground. Based on morphological appearance, it was hard to distinguish underground stem from main root (Fig. 2a). However, differentiation of underground stem from bottom up was clearly visible from the cross section of the annual root. The central part of underground stem gradually split to generate periderm-like tissues to surround new generated tissues, leaving the hollow structure in the middle of underground stem. During the development, central part radially separates into several symmetric small “branches” with independent xylem structures (Fig. 2a). The periderm might act as an outer coat or armor closely surrounding the new generated branch-like structures, and it regenerated continuously with the developing underground stem. Paraffin cross-sections unraveled more exquisite numerous branch-like structures, with the differentiation of underground stem from bottom to up (Fig. 2c-h).

Therefore, we proposed a “trunk-branches” developmental model in the underground stem of this species. According to this model, we could easily explain the bifurcating phenomena in Fig. 2e-h, which presented first 3 bifurcations, then it continues to regenerate 4 bifurcations, 5 bifurcations, 6 bifurcations and so on as the underground stem developed. Fig. 2i-j showed the vertical section of underground stem in S. castanea. Interestingly, Fig. 2j revealed the bifurcating process of xylem, which led to generate multiple branch-like structures in underground stem. This xylem trend in longitudinal section further confirmed our hypothesized “trunk-branches” developmental model in the underground stem of S. castanea. This model could also explain why periderm-like structure generated in the center of underground stem. Indeed, it was the newly generated periderm of small bifurcations squeezed together. Considering the basal leaves and special developmental pattern, we confirmed the upper part of main root was indeed underground stem hidden in the soil. However, this branching phenomenon was not found in the roots of cultivated Danshen.

Differentiation of perennial underground stems was more complex than the annuals (Fig. 2b). In perennial underground stems, these branch-like tissues twisted and intertwined to form the perennial underground stem structures, generating more periderm-like tissues in the inner of underground stem in S. castanea (Fig. 2b). The underground stem paraffin cross-sections of perennial S. castanea, showed multiple xylem structures were squeezed together (Fig. 2k-l). However, the outmost periderm was not completely split, and it appeared to be as squeezed branches of a tree without spatial distribution. Vertical section of underground stem in perennial S. castanea (Fig. 2m), and paraffin vertical section (Fig. 2n) further confirmed presence of multiple and complex xylem vessels. Thus, the underground stem of S. cas-
Fig. 2. The underground stem morphological characteristics and developmental progress of *Salvia castanea*. (a) Annual underground stem of *S. castanea*, and its different cross-sections from down to up. Fig. 2c-h presented more exquisite paraffin cross-sections, revealing the gradual differentiation to form branches-like structures i.e. 3 branches, 4 branches, 5 branches, 6 branches and so on with the growth of underground stem. Fig. 2i-j were the vertical sections of underground stem, especially Fig. 2j uncovered the bifurcating process of xylem to generate multiple vessel tissues. The core of underground stem was like the results of programmed cell death, and generated the periderm-like structures in the center of underground stem to protect the new generated branches-like structures. (b) Perennial underground stem of *S. castanea* and cross-sections from down to up. Fig. 2k-l were the paraffin cross-sections of perennial underground stem of *S. castanea*, with the multiple xylem structures squeezed together. Fig. 2m was the vertical section of *S. castanea*, presenting multiple xylem vessels with more periderm-like structures in underground stem. Fig. 2n was the paraffin vertical section of perennial underground stem in *S. castanea*. 
tanea is like multiple branches of a tree shrunk, squeezed together and hidden underground.

**Phylogenetic relationships and evolution**

A total of 40 Gb of clean reads with 18,182 unigenes generated from SMRT sequencing of *S. castanea* in this study were assessed by BUSCO (Fig. S2a). Most unigenes were distributed in 1,600–1,799 bp, with 45.14% GC and a mean length of 1,678.18 bp (Fig. S2b). Notably, 804 TFs were identified and classified into 52 distinct TF families according to full-length transcriptome of *S. castanea* (Fig. S2c). The highest number of members was found for bHLH family, followed by ERF and WRKY families (Fig. S2c).

**Species-specific genes and phylogenetic tree**

Phylogenetic analysis of *S. castanea* with other 14 representative species was performed to reveal *S. castanea* evolution (Table S3). Consequently, a total of 17,542 OGGs comprising of 355,262 genes were obtained. Among these, 102,916 genes belonging to 4,555 OGGs were shared by all the 15 species (Fig. S2d). Based on GO enrichment analysis, 8 OGGs including 24 species-specific genes (SSGs) were identified in *S. castanea*. These SSGs were enriched in QTP adaptation processes such as DNA binding, defense response, programmed cell death (PCD) (Table S6). Especially two PCD genes (*NSL1a* and *NSL1b*) might closely related to the formation of bifurcate structure in underground stem of *S. castanea* to adapt the QTP environment.

Among 4,555 shared OGGs, 212 single-copy OGGs were identified for reconstruction of a species phylogenetic tree (Fig. 3b). It showed that *S. castanea* was sister to *S. miltiorrhiza* and branched off from *S. miltiorrhiza* around 16 million years ago (MYA). Sister lineage of *A. paniculata* and *S. indicum* diverged approximately 122 MYA. Three monocotyledons, *M. acuminata*, *O. sativa*, and *Z. mays*, were grouped together, and diverged approximately 387 MYA from the other 10 dicotyledons. *A. trichopoda* and *S. moellen-dorffii* lay at the base of this tree and approximately diverged 1,023 MYA.

**Expanded and contracted OGGs**

There were 1,013 significantly expanded and 5,750 contracted OGGs identified in *S. castanea* (Fig. 3b). For the expanded OGGs, KEGG analysis showed that these genes were mainly enriched in signalosome, ubiquitin mediated proteolysis, cysteine and methionine metabolism pathways (*P* < 0.05) (Table S7). According to the biological process (BP) category of GO enrichment, the genes were mainly enriched in 33 GO terms regarding development or morphogenesis, especially root system (*GO:002622*, *P* = 9.85 × 10−5) and root development (*GO:0048364*, *P* = 9.85 × 10−5). For the molecular function (MF) category, the genes were mainly enriched in GO terms related to QTP adaptation, including 34 genes associated with oxidoreductase activity, 12 with DNA binding, 2 with nucleic acid binding and one with MAP kinase activity (*GO:0004707*, *P* = 7.86 × 10−5) (Table S8).

Domain identification revealed that significantly expanded OGGs mainly included ubiquitin, leucine-rich repeat receptor (LRR), pentatricopeptide repeat (PPR) and some other domains (Fig. 3c). There were totally 988 proteins possessing LRR domain, followed by PPR (462 proteins) and ubiquitin (289 proteins). Some other domains included extensin 2 (299 proteins), WD40 (182 proteins), pkinase (117 proteins), and AP2 (42 proteins) (Fig. 3c).

GO analyses for significantly contracted OGGs revealed 37 GO terms were associated with plant development, morphogenesis or histogenesis (Table S9). Noticeably, three *SEOB* genes were enriched in phloem or xylem histogenesis (*GO:0010087*, *P* = 8.99 × 10−3) and phloem development (*GO:0010088*, *P* = 8.99 × 10−3). Another gene *PPF1* was involved in three GO terms (*GO:0048831*, *GO:0090567* and *GO:0048367*) regarding shoot system development. Interestingly, following GO terms phloem or xylem histogenesis, phloem development and shoot development could only be observed in contracted, but not in the expanded OGGs. Thus, contracted OGGs might contribute to the specific underground stem architecture in *S. castanea*.

**Positively selected genes**

Up to 64 out of 212 single-copy genes were identified as positively selected genes (PSGs) with ω (dN/dS) > 1, and 94 amino acid sites were under positive selection (posterior probability > 0.95) (Table S10). According to annotation, two DNA repair related genes (*TYDPI* and *SWC4*) were PSGs. Ribosome related PSGs (*RS6* and *RK31*), and transporter activity related PSGs (*AB7G* and *AB3F*) were involved in the DNA damage response. Moreover, three oxidoreductase activities related PSGs (*HBD*, *DIOX4* and *HF101*), and three phosphatase activities related PSGs (*P2C19*, *F16P1* and *P2C04*) were vital in regulating ROS (Fig. 3a, Table S11).

GO annotation showed that PSG *PAC* was involved in the development of shoot system (*GO:0048367*, leaf (*GO:0048366*) and phyllophone (*GO:0048827*). PSG *CHLM* was involved in chlorophyll formation of specific leaves adapted to extreme environments (Fig. 3a, Table S12). As for MF category, 64 PSGs were enriched in ten top GO terms (*P* < 0.05), including methyltransferase activity (*GO:0008168*), transerase activity (*GO:0016741*) and iron-sulfur cluster binding (GO:0051536 etc. (Table S12). KEGG results showed 64 PSGs were mainly enriched in nucleotide excision repair (ko03420), RNA degradation (ko03018) and ribosome (ko03010) (Table S13).

**Potential mechanisms of underground stem formation**

RNA-seq was conducted to identify the candidate genes regulating underground stem formation in *S. castanea*. Detected genes could be classified into three categories: plant stem cells related genes (Fig. 4a), phytohormone related genes (Fig. 4b), and branching and root development related genes (Fig. 4c). Auxin was important for plant morphogenesis, and *PIN* genes were mainly responsible for auxin transportation. In this study, significantly up-regulated *PIN1A* gene in A-xylem and P-xylem would promote the formation of branch-like structure in underground stem (Fig. 4a). However, some LRR-RLKs genes (*BAM1b*, *BAME2*, *CLV1*) and most of *AGL* genes were significantly depressed in A-xylem and P-xylem as compared with other tissues. Interestingly, *WOX8* gene was stably induced in all tested tissues (Fig. 4a). Therefore, potential mechanisms of underground stem formation in *S. castanea* could be concluded as LRR-RLKs, mainly BAM, RPK, CLV and CRN, regulated the stable expression of *WOX8*, reconstructed its own CLV–WUS negative-feedback loop which was crucial to maintain the stem cell homeostasis (Fig. 4e).

Phytohormones such as ethylene, auxin and abscisic acid (ABA) were closely related to the morphogenesis of *S. castanea*. For example, ethylene biosynthesis related gene *ETR2a* in A-xylem and P-xylem, and auxin signal related genes *AX22D2* in A-leaf, A-xylem, P-leaf and P-xylem were significantly triggered as compared with other tissues. Auxin related gene *RAC3* was highly induced in all tested eight samples. *ANX1* gene in ABA signal pathway was induced in all root tissues (Fig. 4b). Strigolactone branching related gene (*MAX1*) and root development related genes (*PMTT* and *REV1*) were highly expressed in A-xylem and P-xylem as compared with other tissues, indicating their crucial functions in regul-
Genes involved in QTP adaptation of *S. castanea*

Cold adaptation related genes *RCI2A1* and *RCI2A2* were significantly up-regulated in perennial periderm, phloem and xylem tissues. Cold adaptation in leaf tissues could be regulated by *GOLS1*, which was significantly expressed in all tested tissues, especially in xylem tissues. In MAPKK cluster, M3K3A was significantly up-regulated in all tested tissues, indicating its important functions in QTP adaptation of *S. castanea*.

Seven genes related to DNA repair were differentially induced in all tested 8 tissues (Fig. 4d). For instance, *DR102* was highly induced in all tested tissues, especially in A-leaf and P-leaf. However, DNA damage repair related genes *DR100a* and *DR100b* were depressed in both A-leaf and P-leaf, implying their vital functions in responding to DNA damage (Fig. 4d, e). Ubiquitin (Ub)-proteasome system (UPS) related genes were highly expressed in all tested tissues, especially in A-leaf and P-leaf. Ubiquitin (Ub) proteasome system (UPS) related genes were highly expressed in both A-leaf and P-leaf, implying their vital functions in responding to DNA damage (Fig. 4d, e).

Genes involved in the biosynthesis of tanshinones

Four tanshinones were mainly distributed in underground tissues (Fig. 5a). Content of each tanshinone was significantly higher in periderm than that in other tissues, no matter in A-periderm or P-periderm. For instance, DT-I, CT, T-I and T-IIA content of A-

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**Fig. 3.** *Salvia castanea* phylogeny and its QTP adaptation mechanism from comparative genomic analyses. (a) Positively selected genes closely related to the morphogenesis and QTP adaptation. The genes shown here were mainly named according to the annotation of Swissprot database. (b) Phylogenetic position of *S. castanea* relative to other 14 representative plant species. The branch lengths of phylogenetic tree were scaled to estimated divergence time, and rhombus nodes indicated the fossil calibration times used for setting the upper and lower bounds of the estimates. Tree topology was supported by posterior probabilities of 1.0 for all nodes. Moreover, 95% credibility intervals were marked to indicate the estimated posterior distributions of divergence time. The numbers of significantly expanded (green) and contracted (red) OGGs were presented on each branch. (c) The number of protein members from significantly expanded OGGs containing ubiquitin, LRR, PPR and some other domains. The domains were identified according to the Pfam database. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Periderm were 2.97, 2.38, 3.28, 2.07 folds that of A-phloem and A-xylem (sum content of these two tissues), respectively. Each tanshinone content in P-periderm was significantly higher than that in A-periderm except DT-I without significant difference. Interestingly, content of each tanshinone in A-xylem was significantly lower than that in P-xylem, especially for T-IIA which was undetectable in A-xylem tissue. However, the content of each tanshinone in A-phloem was significantly higher than that in P-phloem (Fig. 5a). For example, DT-I, CT, T-I and T-IIA in A-phloem was 2.60, 1.64, 1.77, 1.92 folds that in P-phloem.

Majority of genes associated with tanshinone biosynthesis pathways (MVA and MEP) in S. castanea were highly expressed (Fig. 5b). Expressions of 251, 130, 251 and 193 genes were significantly correlated with CT, DT-I, T-I and T-IIA contents, respectively (Fig. 5c). Totally, there were 63 TF genes were identified from 103 shared genes (Fig. 5c,d). WRKY family counted the most member number among all these TFs, all of them were negatively correlated with the contents of four tanshinones. Interestingly, NAC17 and NAC98 had opposite correlations with the contents of four tanshinones.

Totally, 29 distinct modules were identified through correlation analysis with four tanshinones contents by WGCNA analyses (Fig. 5e). Among these modules, black module (454 genes) showed the strongest correlation, followed by darkgreen module (76 genes). Among the black module, five hub genes were associated with tanshinone biosynthesis, including (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase, geranylgeranyldiphosphate synthase, cytochrome P450 CYP71D411, 1-deoxy-d-xylulose 5-phosphate reductoisomerase, and cytochrome P450 CYP76AH1. Moreover, five TFs (NAC29, WRK75, HSFB3, MYB36 and AZF2) were identified with top five edge numbers to interact with other genes regulating biosynthesis of tanshinones (Fig. 5f). Additionally, terpenoid backbone biosynthesis pathway was significantly correlated with contents of tanshinones (Table S15), indicating its importance in high accumulation of

![Image](image-url)
tanshinones in \textit{S. castanea} in response to the hypoxia environment of QTP.

**Genes involved in the biosynthesis of rosmarinic acid**

Rosmarinic acid (RA) contents in annual leaves reached 90.94 mg g\(^{-1}\), which was significantly higher than that of any other tissue (Fig. 6a). Moreover, RA content in each annual tissue was significantly higher than their corresponding perennial tissue. For instance, RA content in A-leaf was 1.76 fold that of P-leaf. The trends of SAB in different tissues were similar to those of RA, except A-xylem, which contained the highest SAB content (5.39 mg g\(^{-1}\)) among all the tested tissues. SAB content in perennial leaves was significantly higher than that of each perennial underground tissue (Fig. 6a). For instance, SAB content in P-leaf was 1.29 folds that of P-periderm.

RNA-seq indicated multiple genes were highly expressed in each step of RA biosynthetic pathway of \textit{S. castanea} (Fig. 6b). Noticeably, \textit{TAT} genes were significantly induced both in annual and perennial leaves than in other tissues, which were consistent with the trends of RA contents in different tissues. Moreover, qRT-PCR results for six \textit{P450} genes were similar with those in RNA-seq further confirming the validity of transcriptome datasets (Fig. S5).

Phenylalanine ammonia lyase (PAL) is the first enzyme of RA metabolic pathway. To further understand function of PAL family in \textit{S. castanea}, we conducted a phylogenetic analysis of PAL genes among 15 species (Fig. 6c). Results presented the number of PAL genes in \textit{S. castanea} were consistent with that in \textit{S. miltiorrhiza}. All of these PAL genes together with those counterparts from \textit{S. indicum} and \textit{A. paniculata} to form two independent clusters (5 and 6) (Fig. 6c). This indicated that the PAL genes of \textit{S. miltiorrhiza} and \textit{S. castanea} probably were evolutionarily conserved, and still played a similar role after species divergence.

WGCNA analyses identified 261 genes were significantly correlated with the accumulation of RA, among which 55 genes were TF genes (Fig. 6d,e). However, only 61 genes were significantly correlated to SAB accumulation (Fig. 6d). Further, majority of the identified TFs such as bHLH, bZIP and ERF were positively correlated with RA accumulation (Fig. 6e). Interestingly, \textit{NAC17} gene was positively and \textit{NAC98} was negatively correlated with RA accumulation. Among the identified TF genes, \textit{SRM1} belongs to MYB family and \textit{LBD37} belongs to LBD family had the highest positive and negative correlation coefficient, respectively.

Co-expression networks resulted in 32 distinct modules, among which brown module (779 genes) had a significantly positive correlation with both RA and SAB contents (Fig. 7a). Further, in brown module, hub gene with the highest edge number (658 edges) was
skp1, which was involved in root growth and auxin response [53]. Among the identified TF hub genes, TGA22, WRK33 and WRK46 were listed to be with top 3, most edge numbers (Fig. 7b). Four other modules were also positively related with RA contents, including turquoise (3,946 genes), black (330 genes), tan (111 genes) and midnightblue (89 genes) modules. Four modules were negatively related with RA contents, including red (556 genes), purple (135 genes), salmon (99 genes) and lightcyan (85 genes) modules. Moreover, two modules yellow (777 genes) and cyan (91 genes) were positively related with SAB contents (Fig. 7a). Turquoise module, with most genes and most positively related to RA accumulation, was selected for further co-expression network. Twelve TFs including WRK24, TGA21, GBF4a etc. were identified to be with most interactions to other genes. Among these TFs, WRK24 contained most edge number (3,778) (Fig. 7c). Genes in turquoise module could be classified into 10 categories with 19 pathways, which were closely related to QTP adaptation (Fig. 7d). Noticeably, mRNA surveillance pathway (ko03015) and nucleotide excision repair (ko03420) were enriched in this module (Fig. 7d, Table S16). The genes from this module were involved in several important biological processes closely related to the QTP adaptation, mainly including terpenoid metabolic, oxidation-reduction, response to ROS, cellular response to oxidative stress, response to superoxide (Fig. 7e). In addition, total 215 TFs were identified from this module genes, among which WRKY, NAC, ERF and TALE families were accounted for the top four number of genes (Fig. 7f).

Discussion

Environment enforce the adaptive evolution

Environment variations played a key role in plant morphogene-sis and adaptive evolution. This has been observed in several plant species i.e. Welwitschia mirabilis in Africa desert[27], Crucihimalaya himalalica in QTP [54] and Hordeum spontaneum in QTP [55]. Living organisms evolve and adapt to the harsh environment through positive selection. For example, W. mirabilis has evolved an underground stem, however, inner structure of this stem is yet to be explored.

In this study, we studied underground stem structure and proposed the “truck-branches” development model in S. castanea for adapting to harsh QTP environment (Figs. 1, 2, Table S7). Our study suggested that S. castanea branched off from cultivated S. miltiorrhiza around 16 MYA (Neogene, Fig. 3), when QTP could receive more rains from the Indian ocean monsoon. With the uplift of this plateau, the environment of QTP became extreme [1,2,5], and S. castanea began to evolve specific underground stem structure.
These severe environmental stresses have also triggered a cascade of events now visible in *S. castanea*, such as induction of species-specific genes, expanded and contracted OGGs, and PSGs. Different species had their own strategies in response to different environment even if on the same QTP [49,54]. One important strategy was inducing the MAPK pathway in plants in response to various environmental stresses. In our studied *S. castanea*, functions of induced MAPK genes were closely related to QTP adaptation. For instance, *MPK3a* and *MPK3b* genes had the clear functions in response to UV-B, cold, and freezing [56–58]. *M2K2a* and *M2K2b* genes were with oomycete pathogen resistance [59] and cold acclimation [60], *M2K9* could give cellular response to hypoxia [61]. *M3K3A* and *M3K5G* could resist to both bacterial and fungal pathogens [62], and adapt to the QTP wild environment. These MAPK genes might be generated by alternative splicing, and have important functions in response to the harsh environment. Therefore, our findings would provide insights into the evolution of *S. castanea*, and improve our understandings of survival strategy in response to extreme environment.

**PCD genes and PSGs contribute to the morphogenesis and QTP adaptation**

Programmed cell death (PCD) genes have been reported to play crucial roles in plant development [63,64]. PCD process accompanied by DNA degradation has been studied in different plant development processes such as endosperm development [65], leaf morphogenesis [66], and vessel differentiation [67,68]. Therefore, PCD was vital in plant cell fate, organ differentiation and further morphological establishment. In our study, two PCD genes (*NSL1a* and *NSL1b*) were included in 24 species-specific genes among studied 15 species (Fig. S8). This indicated these two PCD genes might contribute to formation of branch-like structures in the underground stem of *S. castanea*. Our hypothesized “trunk-branches” developmental model indicated the phenomenon of bifurcations in the underground stem might be controlled by the program, implying the crucial function of these two PCD genes. This model also easily explained why some periderm-like structures were found in the center of underground stem. That was indeed the newly generated periderm surrounding the new bifurcations, and inner multiple xylem vessels were squeezed together forming the special underground stem structure in *S. castanea*.

As we know, PSGs were evolved under driving force of environment, therefore these genes always play important roles in adaptation of specific environment. Among the 64 identified PSGs of *S. castanea* (Fig. 3a, Table S12), *DIOX4* was found to be 2-oxoglutarate-dependent dioxygenase gene, which has been reported critical for chilling stress tolerance [69]. Thus, this PSG *DIOX4* in *S. castanea* might contribute greatly to QTP adaptation in response to cold winter. Another PSC PAC has been linked with leaf and chloroplast development in *A. thaliana* [70,71]. The molecular function PAC protein has been further investigated to facilitate...
biogenesis of 50S ribosomal subunit [71], implying its important functions in regulating morphogenesis of S. castanea, especially for densely tomentose leaves. Iron-sulfur cluster has been reported it could act as sensor of gases i.e. oxygen and nitric oxide, catalyze oxidation-reduction, and conduct DNA repair [72,73]. In this study, 64 PSGs enriched in iron-sulfur cluster binding GO term, indicating these PSGs were functional to deal with oxidative or DNA damage induced by QTP stresses. Moreover, nucleotide excision repair pathway reported in ectothermic snakes and C. himalaica in response to QTP stresses [49,54], was also enriched in our results. Antioxidative enzymes could be triggered to confront ROS induced by environment stresses [20,50,74]. Thus, our identified oxidoreductase activity related PSGs (HBD, DIOX4, HF101) play crucial roles in scavenging unregulated ROS in S. castanea. Therefore, 24 species-specific genes, especially two PCD genes, combined with 64 PSGs contributed greatly to the QTP adaptation in S. castanea. Two PCD genes (NSL1a and NSL1b), some PSGs i.e. DIOX4, PAC, HBD, DIOX4, HF101 might be the important candidate genes responsible for survival of the plant in the harsh climatic condition.

Expanded OGGs contributed to morphogenesis and QTP adaptation

Proteins from significantly expanded OGGs contained LRR domain (Fig. 3c), which is an important member of CLV/WUS negative-feedback loop and regulates organ initiation, species morphogenesis and stem cell homeostasis [25,28,29,52]. LRR could regulate the cell fate, plant development and evolution innovation [25,33,75]. Therefore, these LRR domain in S. castanea might contribute greatly to formation of special underground stem. CLV3 is an important feedback signal gene in CLV/WUS loop to depress the expression of WOX gene [29]. However, CLV3 was undetectable in S. castanea. It might be due to lack of high-quality genome information, or related to, maintain stable expressions of WOX8 gene for homeostasis of stem cells in different tissues. Synergism of WOX8, auxin transport protein PIN and some important phytohormone realized the morphogenesis of special underground stem in S. castanea. This speculated mechanism has been proved in other species such as aerial roots in Ficus microcarpa [25], and lotus adventitious roots (ARs) [26]. Therefore, WOX8 gene was essential in S. castanea, and could be a candidate gene responsible to special morphogenesis and QTP adaptation of this species.

The other identified significantly expanded OGGs in S. castanea (Fig. 3c) were also closely related to QTP adaptation. Pentatri-copeptide repeat (PPR) domain has been reported to regulate editing of RNA to ensure an accurate translation of functional proteins [76]. PPR protein (WSLS) was essential for chloroplast biogenesis in rice under cold stress [77]. PPR gene TCD10 was needed for chloroplast development in rice under cold stresses [78]. PPR protein SOAR1 was reported to play crucial roles in Arabidopsis in response to drought, salt and cold stresses [79]. Thus, we suggested a crucial role of PPRs for QTP adaptation of S. castanea, especially for the cold environment. Moreover, PPR proteins were not only important for RNA editing and stress response, but also for plant growth and development [80]. PPR was also one kind of peptide, might undertake similar function of CLV3 peptide in S. castanea. Therefore, our studied species S. castanea might possess its own CLV/WUS negative-feedback loop involved PPR. PPR might contribute to not only QTP adaptation, but also special morphogenesis of S. castanea. Other identified domains from significantly expanded OGGs were also important (Fig. 3c). Extensin 2 domain has been reported to have important roles in signaling cascade in response to forthcoming challenge on QTP [81]. WD40 could form MYB-IHLH-WD40 (MBW) complex to regulate secondary metabolism [82]. Proteinase domains is regarding to leaf morphogenesis or seedling development [83,84]. AP2/ERF is key regulator connecting important phytohormonal signals to regulate plant growth and development, defense response, metabolism [85].

Moreover, Ub-proteasome system (UPS) plays vital roles in degradation of proteins, which had suffered irrecoverable injuries from QTP environment [49,54]. Our analysis presented Ub domain contained OGGs underwent a significant expansion (Fig. 3c), and this was consistent with the findings of Zhang et al. [54] who reported the adaptation mechanism of a high-altitude plant Crucichimalaya himalaica. Furthermore, most of our detected genes in UPS were highly expressed and proved the crucial roles of Ub-mediated proteolysis in QTP adaptation of S. castanea. In addition, expanded OGGs of S. castanea were enriched in phosphatidylinositol signaling system, which was critical for plant developmental processes including seed development, growth and reproduction, aging and cell responses to environmental stress [86,87]. Therefore, significantly expanded OGGs of S. castanea might play vital roles on QTP adaptation or morphogenesis of this species. It was worth to note that much more significantly contracted OGGs were observed in S. castanea as compared to other sequenced species, which may result from tissue-specific expression and lack of reference genome of our studied species S. castanea (Fig. 3b). We may infer that the actual contracted OGGs would be much fewer. Interestingly, phloem and shoot developments related OGGs were significantly contracted, indicating they were closely related to form this special underground stem structure in S. castanea.

Secondary metabolites helped to realize the QTP adaptation

Secondary metabolites were not absolutely required for plant survival, but for cell signaling, defense and antioxidant in response to environment stresses [88]. T-IIA has definite functions of anti-hypoxia, anti-oxidant and anti-inflammatory [19–21], therefore, high contents of T-IIA in S. castanea (Fig. 5a), especially in periderm could promote adaptation to hypoxic environment and protect from pathogen infection. We speculated that continuous generating branch-like structures surrounded by periderm in underground stem might be due to guaranteeing the transportation of T-IIA from periderm to inner phloem or xylem, because T-IIA could protect the plant cells against hypoxia, pathogen and so on. RA was highly antioxidative [22,23] and against UV [24], thus high accumulations of RA in leaves of S. castanea (Fig. 6a) could scavenge ROS induced by intense UV on QTP. Therefore, periderm of underground stem and tomenta of leaves were like armor protecting S. castanea against harsh environment through accumulations of specific secondary metabolites (mainly T-IIA and RA).

Our results revealed terpenoid backbone biosynthesis pathway was significantly enriched in T-IIA biosynthesis, which was consistent with the results in S. miltiorrhiza [89]. However, terpenoid backbone biosynthesis pathway was also significantly enriched in RA biosynthesis in S. castanea, indicating this might be an important pathway crossing with other metabolic pathways and regulated biosynthesis of these two metabolites. TFs regulated secondary metabolisms had been reported in various species i.e. S. miltiorrhiza [7,90], Crocus sativus [37], Arabidopsis thaliana [91] and so on. Thus, our identified TFs NAC29 and WRK75 in T-IIA biosynthesis pathway, WRKY33, WRKY24, TGA22 etc. in RA biosynthesis pathway might play crucial roles in biosynthesis of these two secondary metabolites (Figs. 5, 6). Overall, the special underground stem structure and secondary metabolism of S. castanea provide insights into the mechanisms of QTP adaptation, and further provided some candidate genes for genetic improvement of cultivated crops, especially for Chinese medicinal plant S. miltiorrhiza.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.02.004.

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