Increasing Expression of *PnGAP* and *PnEXPA4* Provides Insights Into the Enlargement of *Panax notoginseng* Root Size From Qing Dynasty to Cultivation Era

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Root size is a key trait in plant cultivation and can be influenced by the cultivation environment. However, physical evidence of root size change in a secular context is scarce due to the difficulty in preserving ancient root samples, and how they were modified during the domestication and cultivation stays unclear. About 100 ancient root samples of *Panax notoginseng*, preserved as tribute in the Palace Museum (A.D. 1636 to 1912, Qing dynasty), provided an opportunity to investigate the root size changes during the last 100 years of cultivation. The dry weight of ancient root samples (∼120 tou samples, tou represents number of roots per 500 g dry weight) is 0.22-fold of the modern samples with the biggest size (20 tou samples). Transcriptome analysis revealed that *PnGAP* and *PnEXPA4* were highly expressed in 20 tou samples, compared with the 120 tou samples, which might contribute to the thicker cell wall and a higher content of lignin, cellulose, and callose in 20 tou samples. A relatively lower content of dencichine and higher content of ginsenoside *Rb*<sub>1</sub> in 20 tou samples are also consistent with higher expression of ginsenoside biosynthesis-related genes. *PnPHL8* was filtrated through transcriptome analysis, which could specifically bind the promoters of *PnGAP*, *PnCYP716A47*, and *PnGGPPS3*, respectively. The results in this study represent the first physical evidence of root size changes in *P. notoginseng* in the last 100 years of cultivation and contribute to a comprehensive understanding of how the cultivation environment affected root size, chemical composition, and clinical application.

Keywords: root size, cultivation, GPI-anchored, expansin, *Panax notoginseng*, cell wall

INTRODUCTION

Plant root size is a key trait for improving water and nitrogen uptake efficiency. In cultivation, temperature (Teskey and Hinckley, 2010; Chathurika et al., 2018), precipitation (Barraclough et al., 2010; Ghaffari et al., 2021), light transmittance (Cheon et al., 2004; Kuang et al., 2015), fertilization (Ba Racleough et al., 2010; Goodsman et al., 2010), and agrotypes
(Hiroyoshi et al., 2004; Chen, 2017) can lead to deformation of plant root phenotype. Except for the environmental factors, the genotype is the main factor determining the root size, and a number of quantitative trait loci (QTL) or genes associated with root size have been identified (Jeong et al., 2013; Tamirisa et al., 2014; Yao et al., 2014; Cheng et al., 2016; Ding et al., 2016). It was reported that glycosylphosphatidylinositol (GPI)-anchored protein (GAP) has diverse function on root architecture, by affecting cell wall architecture (Macmillan et al., 2010), cell elongation (Niu et al., 2018), cytoderm thickness, and content of lignin, cellulose, and callose (Liu, 2009; Bundy et al., 2016; Zhao et al., 2020). High temperature decreased the expression of OsGAP18, leading to a thinner cell wall (Zhao et al., 2020), while several GAPs were prominently upregulated during cold acclimation (Daisuke et al., 2016) and nitrogen supply (Engelsberger and Schulze, 2012). Expansin (EXP) also plays significant role in root architecture, and the expression of EXPs could be induced under cold acclimation, water stress, and higher application of fertilizer (Bian, 2006; Kozbial et al., 2010; Li et al., 2013, 2019b; Sun, 2013; Han et al., 2014; Ren et al., 2019).

_Punax notoginseng_ is a popular functional food and traditional medicine, whose benefits are considered to be represented by the root size and ginsenoside content. The main root of _P. notoginseng_ cultivated at higher altitude is significantly larger (Zheng et al., 2014). Root diameter is directly proportional to light transmittance within limit (Kuang et al., 2014; Wang et al., 2018a), and the application of phosphorus and potassium can increase root weight and promote root thickening (Wang et al., 2008; Zhang et al., 2008). A suitable soil texture possesses great fertilizer preserving capability and abundant mineral elements, promoting the growth of root system (Cui et al., 2005; Li et al., 2016).

Cultivation also affects the content of ginsenosides and amino acids, as well as the transcriptional level of corresponding biosynthetic genes in _P. notoginseng_. High precipitation inhibits the accumulation of total ginsenosides, while low temperature induced upregulation of HMGR, SS, and SE and increased the ginsenoside content (Liu et al., 2016b; Ma et al., 2021). Increasing the concentration of potassium, nitrogen, magnesium, and calcium brings a remarkable boost to the ginsenoside content, in a certain range (Konsler et al., 1990; Yu et al., 2001). As a key enzyme in dencichine biosynthesis, activity of serine acetyltransferase (SAT) can be decreased by drought stress (Ahmad et al., 2016; Yang et al., 2021).

The cultivation of _P. notoginseng_ can be tracked back to _Jiaqing_ year in _Qing_ dynasty, which is around A.D. 1800 (Wu, 1963). The Palace Museum (Beijing, China) has abundant and well-preserved _P. notoginseng_ samples from _Qing_ dynasty, and those ancient root samples were tributes from Guangxi or Yunnan provinces to the emperor, which represented the best quality of _P. notoginseng_ at that time. The cultivation environments of _P. notoginseng_ showed a great improvement toward planting temperature and humidity (Hua, 1967; He and Deng, 1981; China Association of Chinese Medicine., 2019), light transmittance (Chen, 1958; Huang et al., 2007; China Association of Chinese Medicine., 2019), soil type (He and Deng, 1981; China Association of Chinese Medicine., 2019), and fertilizer application (Chen, 1958; He and Deng, 1981; China Association of Chinese Medicine., 2019) during past 100 years, with root size also showing an enlargement since 1950s (Jin et al., 1996; Xu, 2016). Tributes from _Qing_ dynasty ought to be an excellent material to investigate how long cultivation influenced root development.

In this study, we compared the root length, diameter, and weight of _P. notoginseng_ from the Palace Museum (Beijing, China) and modern market and performed detailed RNA-seq, UPLC-QQQ-MS, and desorption electrospray ionization analyses of two types of root size (SRW samples, 120 _tou_ with small root weight, representing substitutes for _Qing_ dynasty tribute; and LRW samples, 20 _tou_ with large root weight, representing for highest benefits in modern market). We used these datasets to explore how domestication and cultivation lead to deformation of _P. notoginseng_ root size and to investigate the gene-level regulatory mechanisms that control a better-architected cell wall obtained in LRW samples, providing guidance on artificial cultivation of _P. notoginseng_.

**MATERIALS AND METHODS**

**Plant Materials**

About 100 root samples of _P. notoginseng_ in _Qing_ dynasty were obtained from The Palace Museum, Beijing. Six types of dry root samples with different sizes (20 _tou_, 40 _tou_, 60 _tou_, 80 _tou_, 120 _tou_, and >120 _tou_, _tou_ represents the number of roots per 500 g dry weight) were purchased from Anhui Tienho Herbal Source Company. Fresh root samples were collected in Wenshan, Yunnan Province, and stored at −80°C (Zheng et al., 2017). According to the drying rate (Xu, 2016), 21 fresh roots of _P. notoginseng_ were divided into two groups: (1) Seven LRW samples that have fresh weight of 14.27 ± 2.54 g and are equivalent to 20 _tou_ samples, and (2) 14 SRW samples that have fresh weight of 5.17 ± 0.62 g and are equivalent to 120 _tou_ samples (Supplementary Table 1).

**Analysis of RNA Sequencing Profiles**

Raw data from transcript database of _P. notoginseng_ (Zheng et al., 2017) were used for further analysis. Clean data were obtained by removing reads with low quality from raw data. Filtered reads were aligned to the _P. notoginseng_ genome (Jiang et al., 2020) using STAR (Valencia, 2014) and counted using RSEM (Dewey and Li, 2011). Differentially expressed genes (DEGs) were obtained by comparing the gene expression in LRW and SRW _P. notoginseng_ using DESeq2 (Love et al., 2014). Genes with padj below 0.05 and log2 (fold change) >1 were considered as DEGs.

**Microscope Observation**

Root material was cut into 3-mm tissue samples and dehydrated with a gradient of 70, 80, 95, and 100% of ethanol. The tissue was treated with xylene twice for cell permeabilization and then was soaked and embedded with paraffin. After that, the tissue was cut into 5-μm slice, which was heated and dewaxed in water. Microsection was observed using an Olympus BX51 microscope. The diameter of vessel and the thickness of cell wall were measured using DP2-BSW software. The diameters of vessels in...
one field of view were measured and averaged, and six fields of view of each five biological replicates were obtained for t-test. Measurement of cell wall thickness in each tissue was same as that of the vessel diameter, and the thickness of 10 cells in one field of view was measured and averaged.

**Cell Wall Component Measurement**

Sulfuric acid hydrolysis method was used to determine the lignin content (Xiong et al., 2005; Chen et al., 2010). LRW and SRW samples were freeze-dried and ground into powder. About 100 mg of powder was weighed and extracted with 1% acetic acid solution twice. The precipitate was soaked in a mixture of ethanol and diethyl ether (1:1) for three times and evaporated to residue. About 3 mL of 72% (w/v) sulfuric acid was mixed up with the precipitate and stood for 16 h at room temperature. Then, 10 mL of distilled water was added and placed in boiling water bath for 5 min. After cooling, 5 mL of distilled water and 0.5 mL of 10% (w/v) barium chloride solution were added, and the residue was washed with distilled water subsequently. About 10 mL of 10% (w/v) sulfuric acid solution and 10 mL of 0.1 mol \( \cdot \) L\(^{-1} \) potassium dichromate solution were added to the residue and heated in boiling water for 15 min. The supernatant after cooling was transferred to flask and then mixed up with 5 mL of 20% (w/v) KI solution and 1 mL of 0.5% (w/v) starch solution for titration. The titrant was 0.2 mol \( \cdot \) L\(^{-1} \) of sodium thiosulfate.

Determination of cellulose was performed using enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions (Jiangsu Jingmei Biological Technology Co. Ltd., China. Item number, JM-110113P1). Homogenized LRW and SRW samples were extracted in 900-\( \mu \)L PBS buffer (pH 7.4), and then, supernatant was obtained by centrifugation at 2,000 rpm for 20 min. Microtiter plates were coated with purified cellulose antibody. After 5 times dilution, the sample solution was added to coated micropore, which subsequently bound with HRP-labeled cellulose antibody at 37°C for 30 min. Tetramethylbenzidine (TMB) was added as substrate and then incubated in the dark at 37°C for 10 min. The reaction was terminated using 1 mol \( \cdot \) L\(^{-1} \) of sulfuric acid, and the absorbance value was determined at 450 nm. Calibration curve was obtained using cellulose standard solution of 400, 200, 100, 50, and 25 ng \( \cdot \) L\(^{-1} \).

Callose determination was performed according to the published method (Khle et al., 1985) with some modification. Briefly, 100 mg of fresh plant materials was washed with ethanol for three times to eliminate autofluorescence, ground in liquid nitrogen, and extracted with 1 mL of 1 mol \( \cdot \) L\(^{-1} \) NaOH at 80°C for 15 min. After centrifugation (10,000 \( \times \) g, 15 min), the supernatant was mixed with 0.1% (w/v) aniline blue to produce a violet-red color. Then, 1 mol \( \cdot \) L\(^{-1} \) of glycine/NaOH buffer (pH 9.5) was added and incubated at 50°C for 20 min, and then at room temperature for 20 min. Fluorescence was recorded using a HITACHI F-7000 spectrophotometer (Tokyo, Japan) with the following parameters: excitation wavelength of 400 nm, emission wavelength of 510 nm, and slit width of 10 nm. Calibration curve was obtained using β-1,3-glucan in 1 mol \( \cdot \) L\(^{-1} \) of NaOH.

**qRT-PCR Analysis**

Total RNA was extracted from root of *P. notoginseng*, using the Plant RNA Purification Reagent (Invitrogen, USA), according to the manufacturer’s instructions. qRT-PCR was performed on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland), with primers listed in Supplementary Table 2.

**Determination of Ginsenosides Using UPLC-QTRAP-MS/MS**

The ginsenoside content was measured as described previously (Liu et al., 2020), with some modifications. *P. notoginseng* samples were first ground into powder, and 0.1 g powder was weighed accurately into 5-mL centrifuge tubes and extracted with 2 mL of 70% ethanol solution. The tubes were then sonicated for 30 min at room temperature. The supernatant was collected after being centrifuged for 10 min at 13,000 g. The test solution was obtained by filtering the supernatant through 0.22-μm Millipore filter.

UPLC was performed on a Waters ACQUITY UPLC I-Class system. Chromatographic separations were performed on an ACQUITY BEH C18 column (2.1 × 100 mm, 1.7 μm) with a flow rate of 0.5 mL·min\(^{-1} \) at 40°C. The mobile phase was composed of 0.1% of formic acid–acetonitrile (A) and 0.05% of formic acid–water (B). Gradient elution program was as follows: 0–0.5 min, 20% A; 0.5–3 min, 20–80% A; 3–3.1 min, 80–98% A; 3.1–5 min, 98% A; 5–5.1 min, 98–20% A; 5.1–8 min 20% A. The injection volume was 1 μL for each sample.

Mass analysis was performed on a ABSCIEX 6500 QTRAP mass spectrometer. Mass spectrometer was performed in a positive ion mode using multiple reaction monitoring (MRM) mode. Optimized MS/MS parameters of saponins are shown in Supplementary Table 3. Ion spray voltage was set at 5500 eV, and turbo spray temperature was 550°C. Both gas 1 and gas 2 were set at 50 psi.

**Quantitation of Dencichine Using UPLC-UV-MS**

Determination of dencichine was performed as reported with some modification (Ju et al., 2015). About 0.1 g of root power was added with 5 mL of 70% methanol solution, and the mixture was sonicated for 2 h subsequently. The supernatant was obtained through centrifugation at 12,000 \( \times \) g for 15 min and then diluted with 70% of methanol for 10 times before quantification. Dencichine was detected on a Waters ACQUITY UPLC I-Class system, equipped with a PDA detector under a UV wavelength of 213 nm. An ACQUITY BEH C18 column (2.1 × 100 mm, 1.7 μm) was used for separation, with a flow rate of 0.3 mL·min\(^{-1} \). Mobile phase A consisted of 0.05% phosphoric acid–water, while mobile phase B was methanol, and an isocratic elution of 53% A was used. Injection of samples was 1 μL.

**Yeast One-Hybrid Assay**

Y1H assay was performed as described previously (Zheng et al., 2021). The sequences of *proPnEXPA4, proPnGAP, proPnFPS, proPnCYP716A47, proPnCYP716A53v2*, and *proPnGAP* were nested PCR-amplified according to genomic sequences...
The probable binding domains of promoters were inserted into pAbAi vector as baits and were integrated into Y1HGold, and then, the minimal inhibitory concentration of aureobasidin (AbA) was tested on SD/-Uracil (Ura) plates. A pGADT7-PnPHL8 recombinant plasmid was synthesized by RuiBiotech Co. Ltd., as prey. The pGADT7-PnPHL8 construct and a blank pGADT7 were introduced into bait reporter strains, while blank pGADT7 was used as control. Positive transformants were selected on SD/-Leucine (Leu)/-Ura plates.

Electrophoretic Mobility Shift Assay

EMSA was performed as described previously (Zheng et al., 2021). The full-length cDNA of PnPHL8 was first cloned into a pMAL-c2x vector and then transformed into a Rosetta (DE3) competent cell. Prokaryotic expression was performed at 20°C for 12 h, and then, recombinant protein was purified using Amylose Resin High Flow (NEB, Ipswich, MA, USA). EMSA was performed according to the manufacturer's instructions, using chemiluminescent EMSA kit (Betoytime, Item number, GS009). Primers and probes are listed in Supplementary Table 2.

RESULTS

Transition of Root Size of P. notoginseng From Qing Dynasty to Modern Times

To investigate the morphological difference between Qing dynasty tributes and modern commodities, root length, diameter, and root weight of ancient samples (Figure 1A) from the Palace Museum and six types of root samples (20 tou, 40 tou, 60 tou, 80 tou, 120 tou, and >120 tou) bought from modern market (Figure 1B) were measured. The root of P. notoginseng in Qing dynasty had a similar diameter and dry weight with 120 tou samples, while the length of ancient samples was relatively shorter than the 120 tou samples (Figure 1C).

After 1950s, artificial cultivation techniques, for example, the application of chemical fertilizers, reduced light transmittance and temperature has been applied to the cultivation of P. notoginseng (Supplementary Table 4), and the biggest root weight increased from 6.25 to 12.5 g in 1950s (Jin et al., 1996) to 25 g at present (Liu et al., 2016a; Xu, 2016). The dry weight of Qing root samples is around 5.17 g, which is 0.22-fold of the biggest of modern samples (20 tou samples), suggesting that a transition in root size of P. notoginseng has occurred following the change in cultivation practices and environment among Qing dynasty, 1950’s, and modern time.

Higher Expression of GPI-Anchored Protein and Thicker Cell Wall in Root With Larger Weight

To further investigate the probable molecular mechanism behind the transition of root size, transcriptome sequencing was performed on 21 P. notoginseng root samples with two types of root size (LRW and SRW). A total of nine DEGs were identified between LRW and SRW samples, and the transcriptional levels of eight genes, namely a MYB-CC transcriptional factor, AUX/IAA, DNA ligase, ceramide glucosyltransferase, L-type lectin-domain containing receptor kinase, berberine bridge enzyme-like (BBE), β-1-3-galactosyltransferase (GALT), and E3 ubiquitin-protein ligase (RUE), were decreased in LRW samples (Table 1). Among the nine DEGs, only BBE was enriched into phenylpropanoid biosynthesis pathway in KEGG analysis, while no DEGs were enriched in GO analysis.

The expression of an uncharacterized GPI-anchored protein (GAP) was upregulated in LRW, with a 3.14-fold increase, compared with that in SRW. GAPs were reported to be associated with cell wall architecture (Macmillan et al., 2010; Niu et al., 2018), so we speculated that higher expression of PnGAP in LRW samples may lead to the thicker cell wall. We then measured the structure and component of cell wall in P. notoginseng root. Compared with SRW samples, cell wall in phloem and xylem of LRW samples was significantly thicker. An extremely significantly thicker cytoderm of vessel was detected in LRW, which was 1.41-fold of that in SRW. There was no remarkable difference of the cell wall thickness in cork and cortex between LRW and SRW samples (Figure 2A). In addition, LRW samples possessed a prominently larger vessel width than SRW samples (Figure 2B). As major components of cell walls, the content of lignin, cellulose, and callose was significantly higher in LRW samples (Figure 2C).

Expansin (EXP) and extension (EXT), which possess a membrane-binding mode of GPI-anchored, could be associated with thickening of P. notoginseng root through cell wall expansion pathway (Li et al., 2019b; Zhou, 2019). PnEXPA5 (PN022438) showed a prominently higher transcriptional level in SRW samples, while PnEXPA4 (PN017088) expressed significantly higher transcriptional level in LRW samples, which was 1.44-fold higher than that in SRW samples (Figure 3). Phylogenetic tree using 39 AtEXP and multiple sequence alignments indicated that PnEXPA4 (PN017088) shows most similarity with AtEXP4, and PnEXPA5 (PN022438) is homologous with AtEXP5, both possessing a DPBB domain and a pollen allergen domain. In addition, a WCNP domain in front of a HFD motif was found in PN017088, which is particularly owned in alpha-expansin (Supplementary Figures 1A, B). Since AtEXP4A had positively correlated with the cell wall thickness and root size (Ren et al., 2019), we speculated that PnEXPA4 (PN017088) is associated with root enlargement of P. notoginseng as an essential factor.

Higher Root Weight Was Accompanied by Higher Content of Ginsenoside Rb1 and Lower Content of Dencichine

To investigate whether the chemical composition is related to root size of P. notoginseng, we analyzed the expression of the genes related to ginsenosides biosynthesis, and the accumulation of ginsenosides and dencichine in LRW and SRW samples. In LRW, the expression level of GGPPS1 (PN000021), GGPPS3 (PN029682), GGPPS4 (PN016696), FPP (PN009896), CYP716A47 (PN011429), and CYP716A53v2 (PN006374) was
3.25-, 4.54-, 3.42-, 1.84-, 1.46-, and 1.68-fold higher than that in SRW, respectively (Figure 4A). The content of ginsenoside Rb₁ was significantly lower in SRW samples than that in LRW samples, while the content of dencichine in SRW samples was 1.28-fold higher than that in LRW samples (Figure 4B). It was reported that the content of protopanaxadiol and ginsenoside Rb₁ was considerably increased under overexpression of FPS and CYP716A47, respectively (Han et al., 2012; Yang et al., 2017; Li et al., 2019a), indicating that higher expression level of FPS and CYP716A47 led to remarkably higher content of ginsenoside Rb₁ in LRW. In addition, WGCNA showed a significantly positive correlation between transcriptional level of polysaccharides and ginsenoside Rb₁.
of PnEXPA4 and PnGGPPS3, which were positively associated with root weight (Supplementary Figures 2A,B), suggesting that LRW samples with the thicker cell wall may be related to a higher content of ginsenosides.

**PnPHL8 Had Potential to Bind With PnGAP, PnCYP716A47, and PnGGPPS3 in Vitro**

PHR transcription factor, belonging to MYB-CC family, participates in plant transcriptional responses to phosphate starvation (Wang et al., 2018b; Sega and Pacak, 2019). A PHR-like transcriptional factor PNO24679 was filtered through transcriptome analysis, which had a higher transcriptional level in SRW samples. PNO24679 contains a 1074bp open reading frame (ORF) encoding 357 amino acids. A constructed phylogenetic tree using 14 AtPHLs indicated that PNO24679 (named as PnPHL8) is homologous with AtPHL8 (Supplementary Figure 3A). Multiple sequence alignment showed PNO24679 possessing a MYB DNA-binding domain and coiled-coil domain (Supplementary Figure 3B).

We investigated whether PnPHL8 regulates genes related to root size and ginsenosides biosynthesis in vitro by Y1H method. Promoter sequences of PnGAP, PnEXPA4, PnGGPPS3, PnFPS, PnCYP716A47, and PnCYP716A53v2 were PCR-amplified, all of which except proPnFPS and proPnCYP716A53v2 contained either a P1BS binding site (GNATATNC) or a P1BS-like element (Sun, 2015). MBS domain was also existed in proPnGAP, proPnFPS, proPnCYP716A53v2, and proPnGGPPS3 (Ding et al., 2017; Mabuchi et al., 2018). Specific P1BS domain and MBS domain of proPnGAP, proPnEXPA4, proPnGGPPS3, proFPS, PnCYP716A47, and PnCYP716A53v2 were integrated into yeast, individually. After cotransformation with pGADT7-PnPHL8, we found that yeast strains carrying proPnGAP-MBS, proPnCYP716A47-P1BS, and proPnGGPPS3-P1BS could grow on SD minus leucine and uracil with aureobasidin (Figures 5A,B).

To avoid false-positive results caused by Y1H assay, we further performed EMSA to verify the interaction of PnPHL8 with the promoters of PnGAP, PnCYP716A47, and PnGGPPS3 in vitro. Fragments of proPnGAP, PnCYP716A47, and proPnGGPPS3, containing MBS or P1BS motif, were synthesized as specific probes for EMSA, while mutated probes were synthesized by replacing MBS/P1BS motif with poly-A/T. We found that MBP-PnPHL8 was able to bind to the promoter fragments of PnGAP, PnCYP716A47, and PnGGPPS3, but failed to bind to the mutated probes. Moreover, the cold competing probes, which contain high concentration of unlabeled promoter fragments, impaired the interaction between PnPHL8 and specific probes (Figure 5C). These results indicate that PnPHL8 specifically binds the promoters of PnGAP, PnCYP716A47, and PnGGPPS3 in vitro, suggesting that PnPHL8 may synergistically regulate biosynthesis of diterpenoid and triterpenoid, as well as cell wall architecture.

**DISCUSSION**

**Cell Wall Architecture Involved in Transition of Root Size of P. notoginseng**

In this study, we revealed that the root of *P. notoginseng* exhibited a faster growth rate and better-developed root system during the past 100 years. Plant possessing larger diameter of vessels usually has a faster growth rate and water carrying capacity of vessel (Ian et al., 2008). Lignin also serves as a major component of vessel, which agreed with the significantly larger vessels and a higher content of lignin in LRW samples. In LRW samples, width of root, thickness of vessels and xylem cell wall, and the content of cellulose and callose were significantly higher than that in SRW samples, which might be due to the higher expression of PnGAP and PnEXPA4 in LRW. It was reported that GAPs related to cell wall architecture were mainly expressed in xylem vessels and adjacent parenchyma cells and then functioned in secondary wall deposition (Loostra and No, 2000; Dahiya et al., 2006; Dai, 2015). In addition, the expression of EXPA4 and GAPs was positively related to the cell wall thickness, with GAPs also showing a positive correlation with the content of cellulose (Sun, 2013; Ben-Tov et al., 2015; McNair, 2015; Ren et al., 2019).

*P. notoginseng* is now usually cultivated at higher altitude, lower light transmittance, lower temperature, and lower precipitation condition, compared with earlier times. Lower temperature increased the gene expression level of PnGAP (Daisuke et al., 2016; Zhao et al., 2020), while cold acclimation, water stress, and phosphorus application led to higher expression of EXPA and EXT (Bian, 2006; Kozbial et al., 2010; Li et al., 2013; Han et al., 2014). The expression of EXPA4 was upregulated under green shade within limits, but was strongly inhibited under dark treatment (Peng et al., 1994; Sasidharan et al., 2009; Liu et al., 2011). The vascular diameter and vessel wall thickness showed a significant increase under drought (Xu and Chen, 2012; Aref et al., 2013). Following the transition of cultivation measures, lower temperature and drought could increase the vessel diameter and vessel wall thickness, and a faster growth
FIGURE 2 | Different tissues and cell morphology in *P. notoginseng*. SRW was an abbreviation of small root weight, while LRW represented large root weight. (A) Microscope morphology of different tissues including cork, cortex, phloem, xylem, and vessel, and comparison of the cell wall thickness. (B) Comparison of the vessel diameter of *P. notoginseng* with different root weights. (C) Determination of lignin and callose in *P. notoginseng* with different root weights. The thickness of 10 cell walls in one field of view were measured and averaged, and then in accordance with this, a total of six fields of view from five biological replicates were obtained for t-test. Measurement of the vessel diameters was same as that of the cell wall thickness, while the diameter of seven vessels was measured and averaged. Asterisks denote Student's t-test significance: *P < 0.05 and **P < 0.01.
rate and then a bigger root size of *P. notoginseng* were formed. Higher expression of *PnGAP* in vessels and xylem parenchyma contributed to a higher content of cellulose and callose, while higher expression of *PnEXPA4* led to better-architected cell wall. In addition, cell wall-mediated resistance is an important part of plant immune response system, to which the cell wall
FIGURE 4 | Expression pattern of genes related to biosynthesis of ginsenosides and content of active components. (A) Expression pattern of genes related to biosynthesis of ginsenosides in LRW and SRW groups. (B) Content of active components in LRW and SRW groups. AACT, acetyl-CoA C-acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, hydroxymethylglutaryl-CoA reductase; MVK, mevalonate kinase; MVAP, mevalonate-5-phosphate; PMK, phosphomevalonate kinase; MVAPP, mevalonate-5-phosphate; MVD, diphosphomevalonate decarboxylase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl pyrophosphate synthase; GPS, geranyl pyrophosphate synthase; FPP, farnesyl diphosphate synthase; FAPS, farnesyl diphosphate; FPP, farnesyl pyrophosphate synthase; CYP, cytochrome P450 proteins; UGT, UDP glycosyltransferase; GS, ginsenoside; NG, notoginsenoside. Genes obtained * on upper right were reported to be functional. Genes in red tag had statistical significance. Asterisks denote Student’s t-test significance: *P < 0.05, **P < 0.01, and ***P < 0.001.
FIGURE 5 | PnPHL8 binds with the promoter sequence of PnGAP, PnCYP716A47, and PnGGPPS3. (A) Structural schematics of baits and prey in yeast one-hybrid (Y1H) assay. (B) Y1H assay between PnPHL8 and PIBS domains as well as MBS domains of proPnGAP (promoter of PnGAP), proPnEXPA4 (promoter of PnEXPA4), proPnGGPPS3 (promoter of PnGGPPS3), proPnCYP716A47 (promoter of PnCYP716A47), proPnCYP716A5v2 (promoter of PnCYP716A5v2), and proPnFPS (promoter of PnFPS). Gray triangles represent dilution factor of the yeast concentration, while p53 served as a positive control. (C) EMSA among PnPHL8, proPnGAP, proPnCYP716A47, and proPnGGPPS3. GAP, glycosylphosphatidylinositol-anchored protein; PHL, phosphate starvation response transcription factor like; AbA, aureobasidin.
thickness was positively related (Aquije et al., 2010; Rachid et al., 2016), and a large root size was reportedly associated with resistance (Chloupek, 2010). The PHR transcription factor family participates in plant transcriptional responses to phosphate starvation (Wang et al., 2018b; Sega and Pacak, 2019). Lower application of phosphate in Qing dynasty might result in higher transcriptional level of PnPHL8, which subsequently influenced the expression of PnGAP and affected cell wall architecture and expansion of *P. notoginseng*. Based on this, we speculate that cell wall architecture played an important role in transition of root size from Qing dynasty to the present.

The Variation of Chemical Components Content and Root Size of *P. notoginseng* Might Lead to Transition in Clinical Usage

Ginsenoside Rb1 and dencichine, the major compounds in root of *P. notoginseng*, show diverse pharmacological activities. Ginsenoside Rb1 has great effects on vascular endothelial function improvement (Ohashi et al., 2006), cerebral ischemia protection (Yuan et al., 2007), myocardial preservation (Zhao et al., 2010), and neuroprotection (Jin et al., 2005; Liang et al., 2010), while dencichine was used for the treatment of injury induced trauma, and its hemostatic function was proven by
clinical practice (Zhang and Yu, 2010). Modern cultivation condition, for example, lower temperature and drought, is beneficial to the accumulation of saponins in *P. notoginseng* (Konsler et al., 1990; Yu et al., 2001; Liu et al., 2016b; Ma et al., 2021). Here, we also found high content of ginsenosides Rb₁ in LRW samples. In addition, the PHL transcription factor negatively regulates secondary metabolism such as carotenoid (Lu et al., 2021), indicating that the higher expression of *PnPHL8* in SRW samples may lead to a lower content of secondary metabolism such as ginsenoside Rb₁. In contrast, the content of dencichine was lower in LRW samples, which may be resulted from the decreased activity of SAT induced by drought (Ahmad et al., 2016; Yang et al., 2021). As recorded in herbal records, *P. notoginseng* in *Qing* dynasty was mostly applied externally or prepared into powder for hemostasis (Chen, 2009). However, prescription containing *P. notoginseng* with a higher content of ginsenoside Rb₁ in recent years was mainly used to treat heart diseases and injuries (Chen et al., 2017). The change in root size, ginsenoside Rb₁ and dencichine content of *P. notoginseng* from *Qing* dynasty to modern cultivation era, may also influence the clinical usage.

Triterpenoid is reported as a regulator of cell wall biosynthesis (Jozwiak et al., 2020), and ginsenoside Rb₁ was localized to degrading primary cell wall of xylem in root of *Panax ginseng* (Yokota et al., 2011). In addition, a significantly positive correlation between the expression of *PnEXP4* and *PnGGPP3* was also observed by WGCNA, suggesting that cell wall architecture pathway and ginsenosides biosynthesis pathway may jointly participate in root enlargement of *P. notoginseng* during past 100 years.

**CONCLUSION**

From *Qing* dynasty to modern times, cultivation increased the root size and changed the content of ginsenoside and dencichine of *P. notoginseng*. In this study, we revealed that large root size of modern *P. notoginseng* should be due to the high expression of *PgGAP* and *PnEXP4*, by promoting better-architected cell walls and larger vessels. *GGPPS*, *FPS*, *CYP716A47*, and *CYP716A53v2* involved in ginsenosides biosynthesis pathway are also induced to contribute to a relatively higher content of ginsenosides, while depressed expression of SAT in LRW sample affected dencichine biosynthesis, leading to transition toward clinical efficacy from *Qing* dynasty to cultivation era. *PnPHL8* participates in transcriptional regulation of *PgGAP*, *PnCYP716A47*, and *PnGGPP3*, modulating cell wall architecture and ginsenosides biosynthesis pathway (Figure 6). Our results toward *P. notoginseng* of 2 eras separated by 100 years provided enlightenment on how long cultivation affected root size, chemical composition, and clinical usage.

**DATA AVAILABILITY STATEMENT**

The 10 *P. notoginseng* RNA-seq profiles (r9, r14, r20, r28, r29, r35, r36, r45, r48 and r55) are available in National Genomics Data Center under the GSA accession number CRA006118. The remaining 11 *P. notoginseng* RNA-seq profiles (r1, r13, r15, r18, r19, r23, r26, r32, r33, r53 and r59) have been deposited at DDBJ/EMBL/GenBank under the accession GFRX00000000 (Zheng et al., 2017).

**AUTHOR CONTRIBUTIONS**

X-MC, L-QH, and YY designed the study. M-YY, Z-YH, P-RL, HZ, and YJ performed the experiments. M-YY, Z-YH, and H-SP analyzed data. M-YY, Z-YH, and YY wrote the manuscript. All authors discussed the results and commented on the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.878796/full#supplementary-material

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