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•Special topic•

Genome-wide identification of abscisic acid (ABA) receptor pyrabactin resistance 1-like protein (PYL) family members and expression analysis of *PYL* genes in response to different concentrations of ABA stress in *Glycyrrhiza uralensis*

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[ABSTRACT] As abscisic acid (ABA) receptor, the pyrabactin resistance 1-like (PYR/PYL) protein (named PYL for simplicity) plays an important part to unveil the signal transduction of ABA and its regulatory mechanisms. *Glycyrrhiza uralensis*, a drought-tolerant medicinal plant, is a good model for the mechanism analysis of ABA response and active compound biosynthesis. However, knowledge about *PYL* family in *G. uralensis* remains largely unknown. Here, 10 *PYLs* were identified in *G. uralensis* genome. Characterization analysis indicated that *PYLs* in *G. uralensis* (*GuPYLs*) are relatively conserved. Phylogenetic analysis showed that *GuPYL1−3* belongs to subfamily I, *GuPYL4−6* and *GuPYL10* belong to subfamily II and *GuPYL7−9* belongs to subfamily III. In addition, transcriptome data presented various expression levels of *GuPYLs* under different exogenous ABA stresses. The expression pattern of *GuPYLs* was verified by Quantitative real-time polymerase chain reaction (qRT-PCR). The study proved that *GuPYL4*, *GuPYL5*, *GuPYL8* and *GuPYL9* genes are significantly up-regulated by ABA stress and the response process is dynamic. This study paves the way for elucidating the regulation mechanism of ABA signal to secondary metabolites and improving the cultivation and quality of *G. uralensis* using agricultural strategies.

[KEY WORDS] *Glycyrrhiza uralensis*; Abscisic acid; Pyrabactin resistance 1-like (PYR/PYL) protein family; Gene expression; Signaling pathway; Stress responses

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Introduction

Abscisic acid (ABA), an important plant endogenous hormone with a sesquiterpene structure, plays prominent roles not only in controlling various cellular processes, including stomatal movement, seed dormancy, seedling growth and leaf senescence, but also in regulating plant responses to abiotic and biotic stresses, such as drought, cold, extreme heat, salinity, waterlogging, anoxia, ammonium poisoning, pathogen infection and the parasitism of other plants [1-3]. As the most widely recognized ABA receptor family among various types of ABA receptors, the pyrabactin resistance1/PYR1-like/regulatory components of ABA receptor (PYR1/PYL/RCAR) proteins (PYLs for simplicity) play an important role in responses to various stresses, particularly drought stress [4, 5]. *PYLs* can inhibit the activity of 2C serine/threonine protein phosphatase (*PP2C*) by binding with ABA and then activating the sucrose non-fermenting 1-related protein kinase (*SnRK*) subfamily 2 (*SnRK2s*). Then, the ABA signaling pathway can be transmitted downward and the drought resistance of plants can be improved [6, 7]. A total of 14 members of the *PYL* family in *Arabidopsis thaliana* were identified and named *PYR1* to *PYL1−13* [4, 5]. Then, the

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PYL family genes of many plants, such as *Oryza sativa* (12) [8, 9], *Vitis vinifera* (6) [10], *Lycopersicon esculentum* (14) [11], *Hevea brasiliensis* (21) [12], *Anemone vitifolia* (27) [13], *Nicotiana tabacum* (29) [14], and *Brassica napus* (46) [2], have been identified. In most species, *PYL* genes are categorized into three subfamilies and the functions of several *PYL* genes have been verified.

Among leguminous plants, *Glycyrrhiza uralensis* is one of the most valuable species. Its roots and rhizomes have been widely used worldwide as herbal medicine and natural sweetener. Modern pharmacological research has indicated that *G. uralensis* exhibits pharmacological effects, such as antibacterial, antiviral, anti-inflammatory, anti-cancer and antioxidation activities; it enhances immune regulation and has high values for drug development and application [15]. Previous studies have shown that ABA plays an important role in the accumulation of secondary metabolites and exogenous ABA can increase the accumulation of such metabolites in *G. uralensis* [10]. Hence, identifying the ABA receptor family is essential for studies on the mechanism of secondary metabolites and plant resistance and molecular breeding in *G. uralensis*.

In the current study, we report the first identification of *PYL* family genes in *G. uralensis* based on whole-genome sequencing data. Then, we analyzed the characterization of these *PYL* genes, and constructed a phylogenetic tree of all *PYL* genes based on their homology with *A. thaliana*. Thereafter, all the genes were categorized into three subfamilies. Subsequently, we sequenced the transcriptome data to investigate the expression patterns of these *PYL* genes under various exogenous ABA stresses. In addition, we programmed a quantitative real-time polymerase chain reaction (qRT-PCR) to further verify the expression levels of several preferentially expressed *PYL* genes. The results may pave the way for understanding the function of *PYLs* in ABA-signaling, stress tolerance and secondary metabolite biosynthesis.

Materials and Methods

Identification and analysis of the PYL family in G. uralensis

The amino acid sequences of 14 *AtPYLs* found in the *A. thaliana* genome were used as queries to identify *PYL* genes families in the *G. uralensis* genome *via* the protein basic local alignment search tool (BLASTP) [16] with an *E*-value threshold of 1×10^{-5}. Redundant genes were removed using DNAMAN version 6 (Lynnon Biosoft, USA, https://www.lynnon.com/dnaman.html), and APOLLO [17] was used to correct the coding DNA sequence (CDS), DNA sequence and protein sequences information of *PYLs*.

The ExPaSy website (http://web.expasy.org/) was utilized to determine the properties of *PYLs*, including the number of amino acids, molecular weight (MW), theoretical isoelectric point (pI), instability index (II), aliphatic index (AI) and grand average of hydropathicity (GRAVY). The conserved domains in the identified *PYLs* were confirmed *via* the PFAM website (http://pfam.xfam.org/search/batch). Multiple Em for Motif Elicitation (MEME) version 5.1.1 (http://meme-suite.org/) was adopted to identify the motifs [18]. The *E*-value was less than 2×10^{-30}, and repetitions were of any number. The gene structure was analyzed on the basis of codon and genomic sequences using the Gene Structure Display Server (GSDS 2.0) website (http://gsds.cbi.pku.edu.cn/) [19].

Phylogenetic analysis of PYLs

To determine the evolutionary relationships of the *PYL* family, the *PYL* full-length protein sequences of *A. thaliana* and *G. uralensis* were aligned using ClustalW (UCD, Dublin, Ireland) [20], and the sequence alignments were utilized to construct a maximum likelihood (ML) phylogenetic tree *via* MAGE 6.0 [21]with 1000 bootstrap replicates and the Jones-Taylor-Thornton (JTT) model.

Expression analysis of GuPYL genes in response to different ABA stress levels

The test field is located in Beijing Medical Botanical Garden in Beijing City. In this experiment, 96 2-year-old *G. uralensis* plants were sprayed on August 26, 2018 (9 : 00 am−9 : 00 pm). To monitor the expression levels of *GuPYLs* in different concentrations of ABA (Sigma A1049, USA) treatments, the top leaves of *G. uralensis* plants were sprayed with 10, 25, 50, 100 mg·L^{-1} ABA and 20% PEG4000. Leaves sprayed with water were used as control. Plant leaves were collected at 3, 6 and 12 h after treatment. Then, a total of 48 samples were immediately frozen in liquid nitrogen and stored in a −80 °C freezer for RNA isolation. Total RNA was extracted using the RNA Pure Plant Kit's protocol (TIANGEN DP441, Beijing, China). A high-throughput sequencing technology was conducted to sequence the transcriptome and TBtools software (CJ-Chen, China) was used to draw a heat map of transcriptome data. In addition, complementary DNA (cDNA) was obtained using the M-MLV reverse transcriptase synthesis system (Promega, Madison, WI, USA) following the instructions in the Promega kit. Samples of cDNA were used to verify gene expression levels through qRT-PCR experiments. The specific primers of *GuPYL* genes were designed using the Primer5 program (PREMIER Biosoft International, CA, USA) and their specificity was checked *via* BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table S1). The *ACTIN* gene was used as internal control. The results were calculated on the basis of at least three biological replicates.

Statistical analysis

We calculated the relative gene expression levels based on the $2^{-\Delta\Delta Ct}$ method using Excel (Microsoft, USA) and gene relative expression patterns was analyzed by SPSS (IBM, USA).

Results

Genome-wide identification and characterization analysis of PYLs in G. uralensis

In this study, 14 *AtPYL* genes were used as reference to detect the *PYL* family in *G. uralensis* genome sequence databases. A total of 10 *PYL* genes were identified in the genome

databases and named *GuPYL1−10* on the basis of their orthologous similarity to the 14 *AtPYLs* following the methods described by Mohanta *et al*. [22]. The analysis of the physical properties of 10 *GuPYL* genes showed that these genes are relatively conserved (Table 1). The gene length of *GuPYLs* ranged from 537 to 7259 bp, and CDS varied from 537 to 5276 bp. The protein sequences of *GuPYLs* possessed 178−223 amino acids. The MWs of *GuPYLs* ranged from 20.11 to 24.36 kDa. The pIs varied from 5.37 to 8.29, with an average of 6.15. With the exception of *GuPYL5*, the pI values of all the *GuPYLs* were less than 7.00 and all the *GuPYLs* were rich in acidic amino acids. Only the II values of *GuPYL1*, *GuPYL2*, *GuPYL3*, and *GuPYL6* were less than 40, i.e. stable proteins, and the remaining values were unstable proteins. The AI values were between 76.59 and 96.86. The average hydrophobic index of all the family members was negative, indicating that all family members were hydrophilic proteins.

Table 1 List of 10 *GuPYL* genes and their basic characterizations

Gene Name	AA size	Gene (bp)	CDS (bp)	Exon	MWs (kDa)	PI	II	AI	GRAVY
PYL1	211	669	669	1	23.24	5.52	29.78	76.59	−0.436
PYL2	178	537	537	1	20.11	5.37	39.27	85.79	−0.278
PYL3	178	537	537	1	20.14	5.37	39.52	85.79	−0.254
PYL4	221	666	666	1	23.58	6.58	49.86	80.18	−0.217
PYL5	223	672	672	1	23.95	8.29	42.72	86.86	−0.097
PYL6	195	588	588	1	21.31	6.39	33.32	87.38	−0.184
PYL7	185	7259	5276	3	20.86	6.23	46.08	86.81	−0.436
PYL8	219	4789	941	5	24.34	5.84	50.57	96.85	−0.205
PYL9	188	3353	1500	3	21.13	6.38	44.09	96.86	−0.346
PYL10	185	558	558	1	20.45	5.49	52.67	92.16	−0.124

Phylogenetic relationship and gene structures of GuPYLs

To detect the evolutionary relationship of *GuPYLs*, we constructed an ML phylogenetic tree for the 10 *GuPYLs* and 14 *AtPYLs*. The result (Fig. 1) showed that the *GuPYLs* are divided into three subfamilies (I–III). *PYL1−3* belongs to subfamily I, *PYL4−6* and *PYL10* belong to subfamily II and *PYL7−9* belong to subfamily III. Overall, subfamily I is composed of three members, subfamily II has four *PYLs*, and subfamily III has three *PYLs*. *GuPYLs* that are grouped under the same subfamilies may perform the same functions.

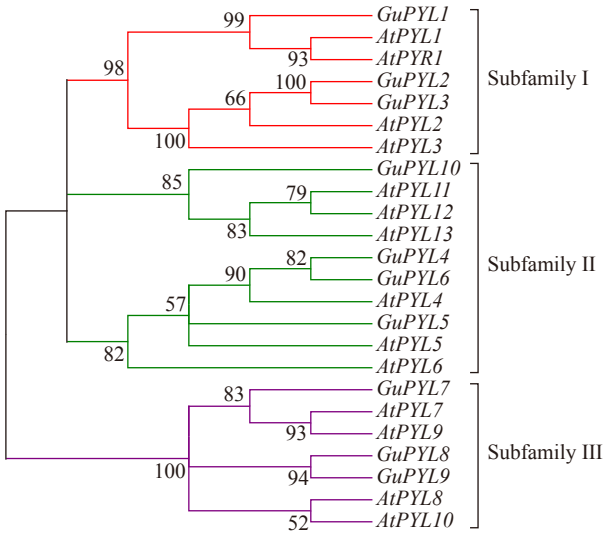

Fig. 1 Phylogenetic relationships of *PYL* genes in *G. uralensis* and *A. thaliana*. In total, 10 *GuPYLs* from *G. uralensis* and 14 *AtPYLs* from *A. thaliana* were included to construct an ML tree, which was divided into three groups. Node labels represent values for bootstrap support

Fig. 2 shows the *PYL* gene structures based on the exon-intron model produced using the GSDS software. The result showed that only subfamily III has introns. In this subfamily, *GuPYL7* and *GuPYL9* have two introns and *GuPYL8* has four introns, and thus, three genes are remarkably longer than others. The result indicated that the similarity of the *GuPYL* gene structure is consistent with the phylogenetic relationship and the genes in the same subfamilies are highly conserved.

Expression profiles analysis of GuPYL genes under various stress conditions

To investigate the expression pattern of 10 *GuPYL* genes under various exogenous ABA stresses, we determined their

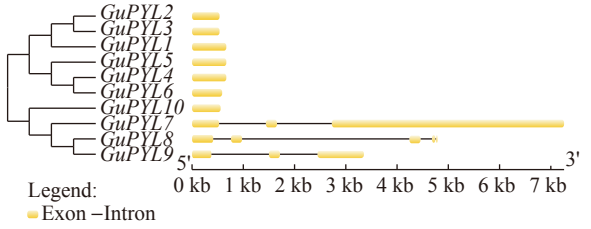

Fig. 2 Exon-intron structure of *PYL* genes in *G. uralensis* based on their phylogenetic relationships. The lengths and positions of introns and exons are shown in the figure. The yellow boxes and black lines denote exons and introns, respectively

transcriptome data and calculated their fragment per kilobase of exon model per million mapped read (FPKM) values. The results of the differential expression are shown in Fig. 3. More than half of the *GuPYL* genes have an extremely broad expression range, and barely detectable or no expression was observed for two genes (*PYL2* and *PYL7*). The *PYL4*, *PYL5*, *PYL8* and *PYL9* genes were highly expressed in all the conditions, suggesting that these genes may contribute in the regulation of ABA signaling pathways. In particular, *PYL4*, *PYL5* and *PYL8* seem capable of sensing and expressing rapidly when ABA concentration is low. Thus, we used qRT-PCR analysis to examine their transcription levels.

Expression patterns of GuPYL genes under exogenous ABA stress

The results of transcriptome data showed that *PYL4*, *PYL5*, *PYL8*, and *PYL9* genes were preferentially expressed when diverse concentrations of exogenous ABA were sprayed on the leaves of *G. uralensis*, suggesting that certain ABA receptors may specially function in leaves. To further verify the expression patterns of these genes, the *PYL4*, *PYL5*, *PYL8* and *PYL9* genes were monitored *via* qRT-PCR after treatment with 10, 25, 50 and 100 mg·L^{-1} ABA for 3, 6 and 12 h (Fig. 4). Overall, after treatment with ABA for 3, 6 and 12 h, the expression of these genes significantly increased. In particular, after treatment with 10 mg·L^{-1} ABA, the expression of *PYL4* significantly increased after 3 h, decreased slowly after 6 h and significantly increased again after 12 h. The expression of *PYL5* significantly increased after 3 h and then slowly decreased. The expression of *PYL8* and *PYL9* significantly increased after 3 h, continued increasing after 6 h and then sharply decreased after 12 h. After treatment with 25 and 100 mg·L^{-1} ABA, the expression of all four genes significantly increased after 3 h, sharply declined after 6 h and then slowly increased again after 12 h. Interestingly, when treated with 50 mg·L^{-1} ABA, the expression of all four genes did not change significantly after 3 h, sharply increased after 6 h and then sharply decreased after 12 h, suggesting that the expression levels of these genes peaked at approximately 6 h.

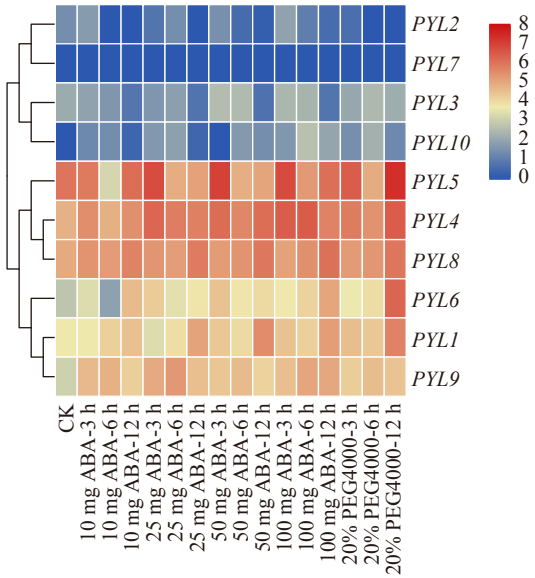

Fig. 3 Expression profiles of 10 *GuPYL* genes under various ABA stresses at different times. CK indicates the plants sprayed with water and used as control. The 3, 6 and 12 h labels indicate the time that passed after ABA treatments. The 10, 25, 50 and 100 mg·L^{-1} labels indicate the different ABA concentrations. The bar on the lower right corner represents the FPKM values, and different colors denote various expression levels

Discussion

PYL genes play an important role in the ABA signaling pathway as the most direct ABA receptor [5, 23]. When plants suffer from drought, salinity and other environmental stresses, ABA content *in vivo* increases accordingly [23, 24]. Then, the ABA receptor dominated by *PYLs* senses this signal by binding with ABA regulates the expression level of downstream stress resistance genes and stimulate the synthesis of secondary metabolites to resist external stress [23]. Therefore, identifying the *PYL* family of plants and finding genes that are highly sensitive to ABA are crucial for improving the sensitivity of plants to ABA and increasing resistance to environmental stress. To date, the *PYL* gene family of many plants including *A. thaliana*, has been identified and the function of certain *PYL* genes has been determined. For example, research has proven that the *PYL9* gene plays an important role in promoting resistance to drought stress and leaf senescence [25, 26]. The *PYL8* gene can promote lateral root growth by enhancing the activities of MYB77-dependent transcription [27]. The overexpression of the *PYL4* gene can enhance the drought tolerance of *A. thaliana* [28]. Moreover, [29] reported that the overexpression of the *PYL9* gene in *Artemisia annua* not only enhances ABA sensitivity and drought tolerance, but also artemisinin content after ABA treatment, suggesting that ABA treatment and the overexpression of the ABA receptor can play an important role in the research on stress resistance and secondary metabolite synthesis of plants, particularly medicinal plants.

Licorice, as one of the most generally consumed Chinese herbal medicines worldwide, is widely used in the pharmaceutical, food service, cosmetic and health product industries. The root of *G. uralensis* is currently the primary source of licorice in the market. In accordance with the Chinese Pharmacopoeia, glycyrrhizin and liquiritin are the two major active ingredients in licorice. Previous studies [30, 31] have indicated that moderate drought stress or exogenous ABA spraying can dynamically change the accumulation of effective components in *G. uralensis*, suggesting that the molecular mechanism of ABA-dependent drought stress is significantly related to the accumulation of active components in *G. uralensis*. However, knowledge of identified *PYL* genes and the roles of most *PYL* genes in *G. uralensis* remain limited.

In this report, we first identified a total of 10 *PYL* genes in the genome of *G. uralensis*, which is less than those in oth-

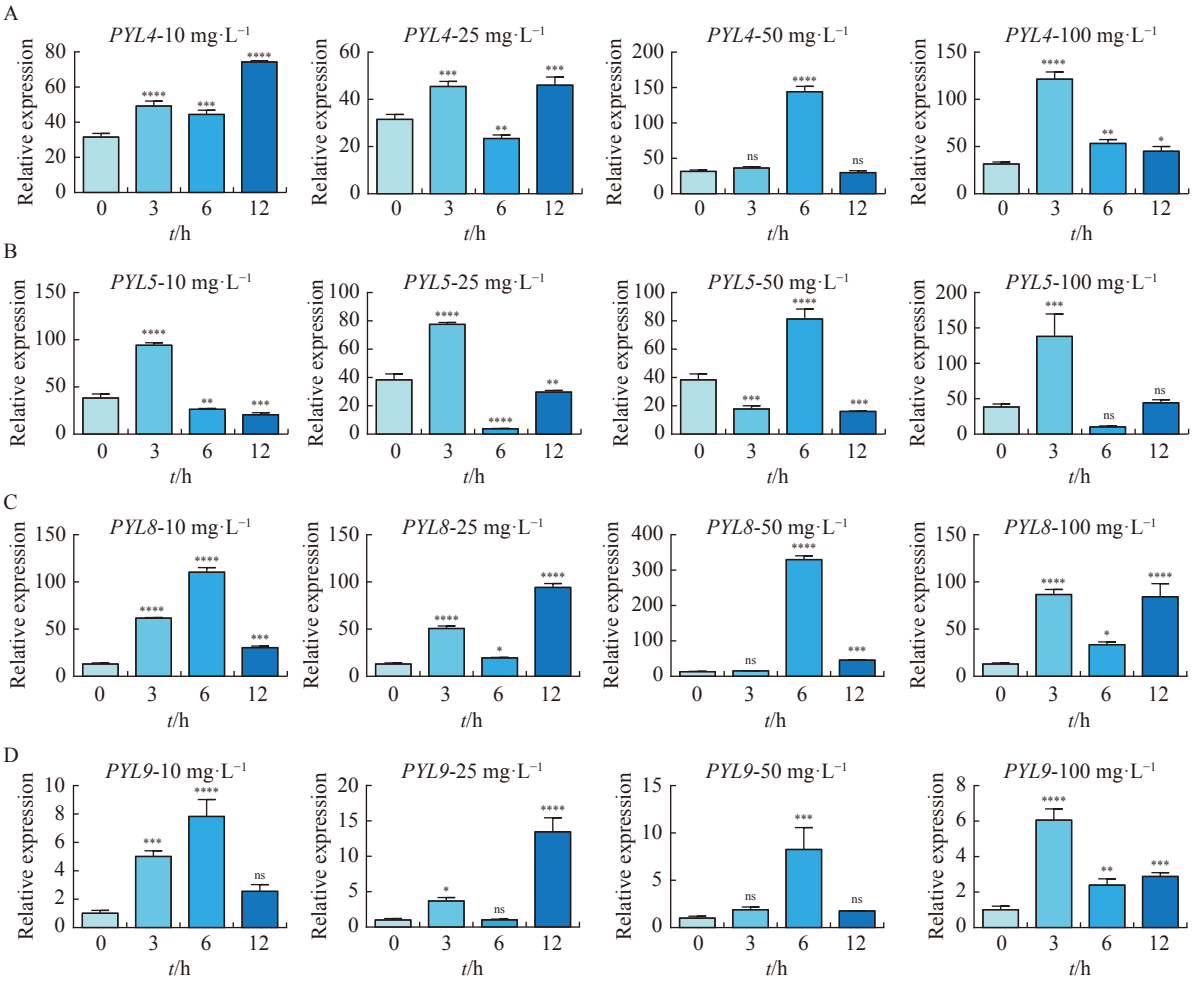

Fig. 4 Expression patterns of *GuPYL* genes in response to ABA. The relative expression of *GuPYLs* was examined after treatments with diverse concentrations of exogenous ABA for the indicated period. The *ACTIN* gene in *G. uralensis* was used as internal control. The gene expression value at 0 h was set as 1. The values are expressed as mean ± SE (n = 3), Statistical significance is assessed with Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control

er reported plants, such as *A. thaliana* (14), rice (13), soybean (23), tomato (15) and *Brassica napus* (46). The 10 *GuPYL* genes were named *GuPYL1−10* and divided into three subfamilies (I−III) in accordance with the orthologous similarity to the 14 *AtPYLs* in *A. thaliana*, suggesting similar evolutionary trajectories between *G. uralensis* and *A. thaliana*. Furthermore, the results indicated that the *GuPYL* genes, which are homologous to *A. thaliana* genes, might play similar roles in specific biological processes [30, 31]. Analysis of the physical properties of the *PYLs* in *G. uralensis* showed that these genes are highly conserved. The relationship of the exon/intron structures is closely related to the phylogenetic relationship of these genes, suggesting that *PYL* genes with similar structure in the same subfamily may perform similar biological functions.

The analysis of transcriptome data indicated that the *PYL4*, *PYL5*, *PYL8* and *PYL9* genes are highly expressed under exogenous ABA stress. This finding was also verified *via* qRT-PCR analysis. The results suggested that the *PYL4*, *PYL5*, *PYL8* and *PYL9* genes are the important ABA receptor in repose to abiotic stress which may participate in the regulation of the ABA signaling pathway in leaves of *G. uralensis* and induces the accumulation of plant secondary metabolites. In addition, the results showed that the expression level of these *PYL* genes increased significantly at 3h, decreased with the increase of ABA accumulation at 6 h, and increased again with the decrease of ABA concentration at 12 h. Therefore, we speculate that it is the negative feedback regulation mechanism of ABA receptor, which is the phenomenon that the expression of *PYL* may be inhibited when a large amount of ABA is accumulated in leaves. As highly expressed ABA receptor functional genes, the four *PYL* genes have also elicited considerable attention among other plants. Hence, we believe that the role of these genes in the ABA signal transduction pathway and the secondary metabolite synthesis in *G. uralensis* is worthy of investigation. In this study, we also found several interesting results. When ABA concentration was 50 mg·L^{-1}, the expression of each gene did not change considerably at 3 h but increased significantly at 6 h, and the change time of the gene expression differed from those of

other groups. The possible reason for this phenomenon may be the concentration of ABA, which is a special critical point that requires further study. In this work, we identified the *PYL* family in *G. uralensis* and analyzed highly expressed members, paving the path for improving the tolerance of *G. uralensis* to abiotic stresses and identifying effective components in *G. uralensis*.

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