Genome-Wide Identification and Expression Analysis of the PAL Gene Family in Foxtail Millet (Postprint)

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Date: 2021-07-20T00:00:00+00:00

Abstract

The Phenylalanine ammonia-lyase (PAL) gene family participates in the phenylpropanoid metabolic pathway and plays an important role in plant stress responses by regulating the synthesis of disease-resistant secondary metabolites. To elucidate the expression patterns of the foxtail millet PAL gene family under stress conditions, this study employed bioinformatics methods for identification and expression analysis. The results revealed that foxtail millet contains 11 PAL genes, which can be classified into three subfamilies in the phylogenetic tree, with SiPAL7 evolving as an independent branch. Protein domain construction demonstrated that all PAL gene family members contain a conserved PAL domain. Promoter analysis showed that PAL genes harbor cis-acting elements responsive to hormones, stress, and various other factors, indicating their extensive involvement in diverse biological regulatory processes. qRT-PCR results indicated that most foxtail millet PAL family genes are inducibly expressed, with expression levels showing significant variations under different light conditions and different genes exhibiting distinct response patterns, suggesting that the foxtail millet PAL gene family plays an important role in light-regulated responses. Foxtail millet PAL genes are highly conserved, broadly responsive to various abiotic stresses, and display expression specificity. These findings provide a reference for elucidating the role of the PAL gene family in regulating foxtail millet resistance and stress response processes.

Full Text

Genome-wide Identification and Expression Analysis of the PAL Gene Family in Foxtail Millet

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Abstract

Phenylalanine ammonia-lyase (PAL) is a key enzyme in phenylpropanoid metabolism that plays a crucial role in plant stress responses by regulating the synthesis of disease-resistant secondary metabolites. To elucidate the expression patterns of the PAL gene family in foxtail millet under stress conditions, this study employed bioinformatics approaches to conduct genome-wide identification and expression analysis. The results revealed that foxtail millet contains 11 PAL genes, which can be divided into three subfamilies in the phylogenetic tree, with SiPAL7 evolving independently as a separate branch. Protein domain analysis showed that all PAL family members contain conserved PAL domains. Promoter analysis indicated that PAL genes harbor cis-acting elements responsive to hormones, stress factors, and other regulatory signals, suggesting their broad involvement in diverse biological processes. qRT-PCR results demonstrated that most foxtail millet PAL family genes exhibit inducible expression patterns, with expression levels varying significantly under different light conditions and displaying distinct response modes among different genes. This indicates that the foxtail millet PAL gene family plays an important role in light-regulated responses. The PAL genes are highly conserved, widely respond to various abiotic stresses, and show expression specificity. These findings provide a reference for revealing the role of the PAL gene family in regulating stress resistance and stress responses in foxtail millet.

Keywords: foxtail millet, PAL gene family, bioinformatics analysis, protein structure, expression analysis

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Introduction

Phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenylpropanoid metabolic pathway, participating in the synthesis of lignin and phenolic compounds, and is widely present in various plants (Fraser & Chapple, 2011; Gai et al., 2016). Koukol and Conn (1961) first extracted PAL protein from barley (*Hordeum vulgare*), and subsequent studies successfully isolated and purified PAL proteins from other higher plants such as potato (*Solanum tuberosum*) (Joos & Hahl, 1992) and tobacco (*Nicotiana tabacum*) (Reichert et al., 2009).

Yang et al. (2017) found that PAL primarily exists as a homotetramer in the cytoplasm and chloroplasts, playing an important role in plant defense systems through multi-level regulatory mechanisms including transcriptional regulation. Promoter deletion experiments have shown that MYB transcription factors can regulate PAL at the transcriptional level by binding to specific regions in the PAL promoter (Yang et al., 2017). PAL can affect fruit quality by regulating the synthesis of HCA-type phenolics and flavonoids (Zhang et al., 2018). Additionally, PAL is extensively involved in plant responses to pathogen infection. Yang et al. (2019) demonstrated that PAL is closely related to disease resistance in banana, with high expression levels during *Fusarium* infection. Increased PAL activity is closely associated with enhanced production of phenylpropanoid compounds, and its activity levels vary with developmental stages, cell and tissue differentiation, and different stress stimuli. Lister et al. (1996) first discovered a significant positive correlation between PAL activity and flavonoid content in apple (*Malus pumila*) fruit. Yang et al. (2019) found that *MaPAL* is highly expressed during banana (*Musa nana*) fruit development and ripening, coinciding with high expression efficiency of secondary metabolites during this stage. Olsen et al. (2008) reported that *Arabidopsis thaliana* genes *AtPAL1* and *AtPAL2* are highly expressed under nitrogen stress and temperature fluctuations, accompanied by accumulation of flavonoid compounds.

With the development of genomics, genome sequencing of various plants has been completed, and the functions of PAL genes in plants have been studied successively. In soybean (*Glycine max*), *PAL1-1*, *PAL2-1*, and *PAL2-3* play important roles in lignin synthesis (Hou et al., 2016). *ZmPAL10* strongly responds to *Rhizoctonia solani* infection in maize (*Zea mays*) (Deng et al., 2019). *VamPAL* participates in anthocyanin accumulation in *Vitis amurensis* (Chen et al., 2018). The rapid development of genomics has made systematic analysis at the molecular level a mainstream trend in biological research (Wang et al., 2020). Foxtail millet (*Setaria italica*) is an annual Poaceae crop with a small diploid genome, strong drought resistance, short growth period, and high yield, making it an important crop for mining drought-resistant genes and understanding molecular mechanisms of stress resistance (Song et al., 2020). Foxtail millet requires less water during its growth period and is an environmentally friendly crop (Song et al., 2019). Research on targeted gene resources for stress resistance breeding has become an important component of foxtail millet yield improvement (Nadeem et al., 2020). The completion of foxtail millet genome sequencing has established a data foundation for millet research, marking the entry of foxtail millet genetic research into the post-genomics era (Bennetzen et al., 2012; Zhang et al., 2012). Although the PAL gene family is widely present in plants, few studies have reported on the foxtail millet PAL gene family. To clarify the functional mechanisms of the foxtail millet PAL gene family under stress conditions, this study used bioinformatics methods to identify the PAL gene family in foxtail millet, analyze its structural characteristics and evolutionary patterns, and construct expression profiles under abiotic stresses, providing a reference for studying the biological functions of the foxtail millet PAL gene

family.

Materials and Methods

1.1 Plant Materials The foxtail millet (*Setaria italica*) cultivar 'Zhangzagu 8' was used as experimental material. Plants were grown in pots at the farm of Hebei North University. Seedlings were exposed to natural light, darkness, red light, blue light, or far-red light for 24 hours. Young leaves were collected, snap-frozen in liquid nitrogen, and stored at -80°C.

1.2 Identification and Protein Sequence Analysis of Foxtail Millet PAL Genes The hidden Markov model file for the PAL protein domain was downloaded from the Pfam database (Pfam ID: 00221) (Finn et al., 2008). Gene stable IDs and transcript stable IDs were obtained from the Plant Genomes database. Sequences acquired from the Gramene website were screened and identified using CDD and InterProScan, with redundant sequences removed to obtain PAL protein sequences, chromosomal location information, and other gene data (Marchler-Bauer et al., 2009; Hunter et al., 2009). The ProtParam database was used to obtain amino acid numbers, isoelectric points, and other parameters for protein characterization (Song et al., 2020).

1.3 Chromosomal Localization of Genes The MG2C online tool was used to map the chromosomal locations of foxtail millet PAL genes using positional information and chromosome lengths queried from Ensembl Plants.

1.4 Protein Analysis and Phylogenetic Tree Construction ProSite and Clustal X online software were used to align PAL protein domain sequences. A neighbor-joining (NJ) phylogenetic tree was constructed using MEGA6.0 software with the Poisson model (bootstrap value: 1,000) (Sigrist et al., 2010; Larkin et al., 2007). The same method was used to construct a phylogenetic tree for 54 PAL family members from *Brachypodium distachyon*, *Sorghum bicolor*, *Setaria viridis*, and other species (default bootstrap value).

1.5 Motif Identification and Protein Structure Prediction Motif patterns of foxtail millet PAL proteins were obtained from the MEME website (minimum width: 60, maximum width: 200) (Bailey et al., 2009). Weblogo was used to analyze PAL domain motifs (stacks per line: 100). The SWISS-MODEL website was used to predict three-dimensional structures and helix patterns based on the most frequent conserved sequences at each gene locus (Kiefer et al., 2009). The same approach was applied to PAL proteins from *Brachypodium distachyon*, rice, and other species for comparative structural analysis.

1.6 Homologous Collinearity Analysis of PAL Genes Based on PAL phylogeny, orthologous segments between foxtail millet and rice (*Oryza sativa*)

were identified. The genome comparison function of Ensembl Plants was used to obtain synteny maps of PAL homologs between foxtail millet and rice (Wang et al., 2012). Using chromosomal locations and gene positions, collinearity maps were drawn in Adobe Illustrator CS4 to analyze syntenic relationships.

1.7 Gene Structure Analysis of PAL Genes Sequence information for foxtail millet PAL genes was obtained from Phytozome. The GSDS2.0 online database was used to analyze coding sequences and generate intron-exon structure models (Hu et al., 2015). The 1,500 bp upstream region of PAL start codons was retrieved from the foxtail millet genome database, and cis-acting elements were analyzed using PlantCARE and visualized with GSDS2.0 (Lescot et al., 2002).

1.8 Expression Pattern Analysis of Foxtail Millet PAL Genes Expression data for 11 genes under various treatments were obtained from Phytozome, including leaves under strong light for two weeks, buds under strong light for one week, dark-induced aerial tissues, red light-induced aerial tissues, normal light-induced roots, drought-induced roots, urea-induced roots, and panicles under strong light. Heatmaps were generated using TBtools (Chen et al., 2020).

1.9 RT-qPCR Analysis of Foxtail Millet PAL Genes Under Different Light Qualities Primers were designed using DNAMAN with *Actin* as the reference gene. Total RNA was extracted using a plant RNA kit (Tiangen Biotech), cDNA was synthesized using a FastQuant RT Kit, and PCR amplification was performed on an Agilent 3000P real-time PCR system. The reaction conditions were: pre-denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s. Each sample was analyzed in triplicate, and data were processed using the 2^(-ΔΔCt) method.

Results

2.1 Identification of the Foxtail Millet PAL Gene Family Using CDD and InterProScan software, gene stable IDs and transcript stable IDs were detected and identified from the Plant Genes 63 genome (Pfam ID: 00221), with missing data checked and redundant sequences removed to obtain foxtail millet PAL gene sequences. A total of 11 PAL family genes were identified in foxtail millet and designated *SiPAL1* through *SiPAL11* (Table 1). The 11 PAL protein sequences showed minor differences: amino acid lengths ranged from 698 aa (SiPAL1, SiPAL8) to 891 aa (SiPAL7), open reading frame lengths from 2,142 bp (SiPAL3) to 4,610 bp (SiPAL2), molecular masses from 74.99 kD (SiPAL8) to 95.01 kD (SiPAL7), isoelectric points from 5.82 (SiPAL5) to 6.52 (SiPAL6), and exon numbers from 1 (SiPAL3, SiPAL4, SiPAL5) to 6 (SiPAL7). Table 1 reveals that five genes located on chromosome 1 (*SiPAL1-SiPAL5*) share similar protein-coding characteristics and are tightly clustered, while three genes on

chromosome 7 (*SiPAL9-SiPAL11*) are also closely arranged but exhibit greater variation in their encoded protein features. All PAL proteins have isoelectric points between 5.82 and 6.52, indicating that phenylalanine ammonia-lyase has acidic characteristics and likely functions in weakly acidic environments. Except for *SiPAL7* (with 6 exons), the PAL family genes contain relatively few exons, suggesting that 10 of the PAL family genes may have similar functions.

Chromosomal distribution analysis showed that chromosome 1 contains five tightly arranged PAL genes, representing the highest number on any chromosome, while chromosome 3 harbors three clustered PAL genes. No other chromosomes exhibit clustered PAL gene distribution (Table 1, Figure 1 [Figure 1: see original paper]). PAL genes are unevenly distributed across chromosomal regions, with cluster distributions observed on chromosomes 1 and 7. Using the Gbrowse function of Phytozome to compare the positional relationships between clustered PAL gene family members and flanking protein-coding genes, and referencing Holub' s (2001) definition of gene clusters, the results suggest that the foxtail millet PAL gene family may have expanded through tandem duplication.

2.2 Construction of the Foxtail Millet PAL Protein Phylogenetic Tree Using protein sequence alignment files, a neighbor-joining phylogenetic tree of foxtail millet PAL proteins was constructed (Figure 2 [Figure 2: see original paper]). Based on the tree topology, the proteins can be divided into three groups. Groups 1 and 2 contain equal numbers of proteins with similar branch distributions, and some PAL proteins show bootstrap values reaching 100. This topological pattern suggests that these two groups of genes may have been acquired through duplication or perform similar functions. Clustering analysis revealed that proteins with the same PAL domain and those derived from the same gene amplification or duplication events group together; for example, SiPAL6 and SiPAL9, derived from dispersed duplication, cluster together. Additionally, SiPAL7 forms a separate branch, indicating that SiPAL7 may have a different evolutionary origin or trajectory.

2.3 Evolutionary Relationships of PAL Proteins Across Species To reveal the evolutionary relationships of the foxtail millet PAL gene family, MEGA6.0 software was used to construct a phylogenetic tree of 54 PAL proteins from six monocot and dicot species (Figure 3 [Figure 3: see original paper]). The analysis included nine PAL proteins from *Brachypodium distachyon*, eight from sorghum, eleven from *Setaria viridis*, ten from rice, and five from *Arabidopsis thaliana*. The results showed that PAL proteins from some species exhibited familial clustering within seven phylogenetic groups, and PAL proteins from different taxonomic classes also showed high homology.

2.4 Domain Analysis of Foxtail Millet PAL Proteins ProSite analysis of foxtail millet PAL protein domains revealed that all PAL proteins contain conserved PAL domains. SiPAL7 protein additionally contains HtRna and HGTP

domains, where the HtRNA domain may regulate aminoacyl adenylate synthesis and the HGTP domain may be involved in related protein synthesis (Figure 4 [Figure 4: see original paper]). This study also found that SiPAL7 has the longest amino acid sequence and the most protein domains, likely related to its highest number of exons (six), implying it contains more genetic information. Although SiPAL11 also has a relatively long amino acid sequence, it contains only the PAL domain, suggesting it may possess some low-complexity protein domains. MEME analysis of foxtail millet PAL protein domains showed that the amino acid composition of the PAL binding domain is relatively conserved.

2.5 Three-Dimensional Structure and Sequence Analysis of Foxtail Millet PAL Proteins SWISS-MODEL was used to analyze the most frequent conserved motifs at each gene locus and predict the 3D structures of foxtail millet PAL proteins. The results showed that the PAL 3D structures exhibit numerous helices and folds with symmetrical organization (Figure 5 [Figure 5: see original paper]A). Superpose Version 1.0 was used to compare PAL protein 3D structures across species, where smaller values indicate greater structural similarity. Table 2 shows that the smallest difference occurs between *Setaria viridis* and foxtail millet (0.38), consistent with their close phylogenetic relationship, while the largest difference occurs between foxtail millet and *Brachypodium distachyon* (3.93), corresponding to their different taxonomic classifications. The 3D structures revealed that most PAL proteins have symmetrical tertiary structures, indicating similar structural units, which from an evolutionary perspective suggests duplication and fusion events in PAL gene evolution.

2.6 Homologous Collinearity Analysis of PAL Genes Between Foxtail Millet and Rice The synteny function of Ensembl Plants was used to obtain collinearity maps of PAL homologs between foxtail millet and rice. Based on chromosomal locations and gene positions, collinearity maps were drawn (Figure 6 [Figure 6: see original paper] shows representative members). The results showed that all 11 foxtail millet PAL family members have collinear genes in rice. For example, the rice chromosome 2 genomic region containing *Os02g0626100* and *Os02g0626400* shows synteny with the foxtail millet chromosome 1 genomic block; the rice chromosome 5 region containing *Os05g0150900* shows synteny with the foxtail millet chromosome 3 block; similarly, the rice chromosome 4 region containing *Os04g0518100* and *Os04g0518400* shows synteny with the foxtail millet chromosome 7 block.

2.7 Gene Structure Analysis of PAL Genes GSDS2.0 was used to analyze and visualize foxtail millet PAL sequences, generating intron-exon structure models (Figure 7 [Figure 7: see original paper]A). Most *SiPAL* genes contain only one intron (*SiPAL1*, *SiPAL2*, *SiPAL6*, *SiPAL8*, *SiPAL9*, *SiPAL10*, *SiPAL11*), and these genes show similarity in exon structure and size, suggesting they may perform similar functions in plant biochemical processes. *SiPAL3*, *SiPAL4*, and *SiPAL5* share the same exon structure but lack introns. *SiPAL7*

possesses the most introns (five).

Cis-acting element regions obtained from PlantCARE analysis were visualized to generate distribution maps (Figure 7B). The results revealed that PAL family genes contain various hormone signaling response elements and environmental signal response elements. The most widely distributed element is the jasmonic acid response element, present in all PAL genes, suggesting PAL genes participate in plant disease resistance through response to endogenous hormones. The least common element is the endosperm expression element, found only in *SiPAL9*. Other types of cis-acting elements, such as stress response and light response elements, are unevenly distributed across *SiPAL* genes. These results demonstrate that PAL family genes play important roles in foxtail millet development, disease resistance, and stress responses.

2.8 Expression Analysis of Foxtail Millet PAL Genes Expression data for 11 genes under various treatments were obtained from Phytozome and visualized as heatmaps using TBtools (Figure 8 [Figure 8: see original paper]). All PAL genes showed detectable expression levels. *SiPAL1*, *SiPAL2*, *SiPAL8*, and *SiPAL10* exhibited significantly higher expression than other genes in buds under strong light for one week, dark-induced aerial tissues, red light-induced aerial tissues, normal light-induced roots, drought-induced roots, urea-induced roots, and panicles under strong light. Only *SiPAL2* showed weak expression in leaves under strong light for two weeks, while other genes were barely expressed. *SiPAL3*, *SiPAL6*, and *SiPAL9* showed significantly lower expression across different foxtail millet tissues. These results indicate that PAL family genes are inducibly expressed, with expression enhanced or suppressed under drought or strong light conditions, but with differences among individual genes.

2.9 RT-qPCR Analysis of Foxtail Millet PAL Genes Under Different Light Qualities To further investigate the role of PAL family genes in light-regulated responses in foxtail millet, plants were treated with different light qualities. As shown in Figure 9 [Figure 9: see original paper], under red light, *SiPAL1*, *SiPAL8*, and *SiPAL5* were upregulated, while other genes showed varying degrees of downregulation compared to the control. Under blue light, *SiPAL1*, *SiPAL8*, and *SiPAL10* were upregulated, while *SiPAL7* showed no significant change. Under dark treatment, *SiPAL1*, *SiPAL8*, and *SiPAL2* were upregulated, while other genes were downregulated, with *SiPAL6* showing extremely low expression. Under far-red light, *SiPAL1*, *SiPAL8*, *SiPAL2*, *SiPAL10*, *SiPAL5*, and *SiPAL11* were upregulated. The significant expression changes of PAL family genes under different light qualities demonstrate their important role in foxtail millet photomorphogenesis, with *SiPAL1* and *SiPAL8* being the primary members.

Discussion and Conclusion

PAL is a key enzyme related to plant resistance that has attracted considerable research attention. Previous studies have analyzed PAL gene families in multiple species, including maize (Deng et al., 2019), apple (Zhang et al., 2018), and upland cotton (*Gossypium hirsutum* Linn.) (Yang et al., 2017). Maize and upland cotton each contain 13 PAL genes, while apple contains 8 PAL family members, which is not substantially different from the 11 PAL genes identified in foxtail millet in this study. This suggests that PAL genes exist as small gene families without extensive amplification during species divergence. Some foxtail millet PAL-encoded proteins have similar molecular weights and isoelectric points, consistent with studies on banana (Yang et al., 2019) and *Nervilia fordii* (Huang et al., 2016), indicating similar functions among these encoded proteins.

Gene duplication events are the primary driving force for gene family expansion. This study found that foxtail millet PAL genes are distributed in clusters, a pattern also observed in apple (Zhang et al., 2018), soybean (Hou et al., 2016), and watermelon (*Citrullus lanatus*) (Dong & Shang, 2013). In watermelon, 7 of 12 PAL genes are tandemly arranged on chromosome 4, 2 are tandemly arranged on chromosome 7, and the rest are individually distributed on chromosomes 2, 3, and 8, showing high similarity to the chromosomal distribution of foxtail millet PAL genes. This suggests that tandem duplication, dispersed duplication, and segmental duplication all contribute to PAL gene family expansion (Guo et al., 2019). Collinearity analysis revealed that all 11 foxtail millet PAL family members have collinear relationships in the rice genome, indicating high conservation of PAL genes during evolution.

Based on phylogenetic tree topology, the foxtail millet PAL protein tree divides into three groups, with SiPAL7 evolving independently, suggesting lower homology with other members and possibly different origins or evolutionary trajectories. This result is consistent with studies on upland cotton (Yang et al., 2017). PAL members within the same phylogenetic branch show consistent gene structures with high conservation. However, group III member SiPAL7 exhibits greater differences in gene structure compared to other family members, possessing more introns, which implies diverse transcriptional regulatory processes within the PAL gene family. These structural differences among PAL gene family members may affect their functional activity.

Promoter analysis revealed that PAL family genes contain not only numerous abiotic stress response elements but also various types of elements such as light response and hormone response elements. Different PAL family members contain different numbers and types of elements, indicating that the PAL gene family is widely involved in different biological regulatory processes, with each PAL gene having its specific regulatory pattern.

When plants suffer from drought, high temperature, and other stresses, they rapidly produce large amounts of reactive oxygen species (ROS) that damage cellular structures. Secondary metabolites such as flavonoids produced through

the phenylpropanoid pathway have antioxidant activity to scavenge ROS (Yang et al., 2019). This study found that PAL family genes are mostly inducibly expressed, with SiPALs expression levels rapidly increasing under different stress stimuli, indicating that PAL family genes widely respond to various abiotic stresses and that secondary metabolites such as flavonoids may have high synthetic activity during abiotic stress processes. Additionally, some genes showed similar response patterns, suggesting functional redundancy among SiPALs. Light quality plays an important role in plant architecture and growth development. Fluorescence quantitative analysis results showed differential expression of PAL family genes under different light conditions. For example, SiPAL5 is highly expressed under red and far-red light, and SiPAL11 is highly expressed under far-red light. These changes indicate that PAL family genes have complex regulatory mechanisms in foxtail millet light-regulated pathways, with functional differentiation among different PAL genes.

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Note: Figure translations are in progress. See original paper for figures.