Cloning and Expression Analysis of the Saussurea medusa Aerenchyma Formation-Related Gene SmPAD4 Postprint

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Abstract

Aerenchyma is an adaptive structure in Saussurea medusa for coping with extreme environments, and its formation is typically accompanied by programmed cell death (PCD). The death of cells and the formation of aerenchyma are usually regulated by the PAD4 gene (Phytoalexin deficient 4), but the mechanism by which PAD4 regulates aerenchyma formation in Saussurea medusa remains unclear. In this study, using Saussurea medusa as experimental material, the aerenchyma formation-related gene SmPAD4 was cloned using homologous cloning and RACE technology. Its sequence, phylogenetic evolution, expression, and subcellular localization were analyzed, and its promoter was amplified using hi-1IL PCR technology to explore the function of this gene in environmental adaptation. The results showed: (1) The full-length cDNA of SmPAD4 gene is 2,047 bp (GenBank accession number OR766038), including a 1,866 bp open reading frame encoding 621 amino acids, with a molecular formula of (C3163H4906N848O910S26). This protein is a basic hydrophilic unstable protein. (2) Phylogenetic tree analysis revealed that SmPAD4 has the highest amino acid sequence similarity with CcPAD4 from Cynara cardunculus. (3) A 1,049 bp promoter sequence of SmPAD4 was amplified, containing cis-acting elements such as light-responsive elements, hypoxia-responsive elements, drought-responsive elements, and auxin-responsive elements. (4) Real-time quantitative PCR analysis showed that SmPAD4 was expressed in roots, stems, and leaves, with the highest expression level in leaves; under UV and hypoxia stress, SmPAD4 expression was upregulated in leaves and stems but downregulated in roots. (5) Subcellular localization demonstrated that SmPAD4 is distributed in the nucleus, cell membrane, and chloroplast. This study indicates that the SmPAD4 gene possesses unique protein domains and responds to both hypoxia and UV environmental stresses, playing an important role in aerenchyma formation

and response to abiotic stress, providing a theoretical basis for further investigation of the role of SmPAD4 gene in the environmental adaptation process of Saussurea medusa.

Full Text

Cloning and Expression Analysis of the Aerenchyma-Related Gene SmPAD4 in Saussurea medusa

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Abstract

Aerenchyma is an adaptive structure in Saussurea medusa that enables survival in extreme environments, and its formation is typically accompanied by programmed cell death (PCD). The PAD4 gene (Phytoalexin Deficient 4) plays a crucial role in regulating both cell death and aerenchyma formation, yet the specific mechanism by which SmPAD4 controls aerenchyma development in S. medusa remains unclear. This study employed homologous cloning and RACE technology to isolate SmPAD4, a gene associated with aerenchyma formation, from S. medusa. We conducted comprehensive analyses of its sequence characteristics, phylogenetic relationships, expression patterns, and subcellular localization, and amplified its promoter region using hiTAIL-PCR to investigate the gene' s function in environmental adaptation.

Our results revealed: (1) The full-length SmPAD4 cDNA spans 2,047 bp (GenBank accession OR766038), containing a 1,866 bp open reading frame that encodes 621 amino acids with a molecular formula of $C_{3163}H_{4906}N_{848}O_{910}S_{26}$. The predicted protein is basic, hydrophilic, and unstable. (2) Phylogenetic analysis demonstrated that SmPAD4 shares the highest amino acid sequence similarity with CcPAD4 from Cynara cardunculus. (3) We successfully amplified a 1,049 bp promoter sequence containing multiple cis-acting elements, including light-responsive elements, hypoxia-response elements, drought-response elements, and auxin-response elements. (4) Real-time quantitative PCR analysis showed that SmPAD4 is expressed in roots, stems, and leaves, with the highest expression in leaves. Under UV and hypoxia stress, SmPAD4 expression was upregulated in leaves and stems but downregulated in roots. (5) Subcellular localization revealed that SmPAD4 is distributed in the nucleus, cell membrane, and chloroplast. These findings indicate that SmPAD4 possesses unique protein domains and responds to both hypoxia and UV stress, playing an important role

in aerenchyma formation and stress responses. This study provides a theoretical foundation for further investigation of SmPAD4' s function in the environmental adaptation of S. medusa.

Keywords: Saussurea medusa, SmPAD4, aerenchyma, expression analysis, hypoxia stress, UV stress

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Saussurea medusa (Asteraceae) is a perennial herbaceous plant endemic to the Qinghai-Tibet Plateau, where it faces extreme environmental pressures including high altitude, hypoxia, and intense UV radiation. The well-developed aerenchyma in its roots, stems, and leaves represents a key adaptive mechanism for survival under these harsh conditions. The PAD4 gene is known to play a critical role in plant programmed cell death and aerenchyma formation, yet the molecular mechanisms underlying abiotic stress responses in S. medusa remain poorly understood. Investigating SmPAD4, a gene associated with aerenchyma formation, is therefore essential for elucidating the unique adaptive strategies of alpine plants.

PAD4 functions as a signal transduction molecule mediated by R (resistance) genes and represents a key regulator of plant immune responses, PCD control, and aerenchyma formation, playing vital roles in both biotic and abiotic stress responses. PAD4 responds to various abiotic stresses—including high light, UV radiation, drought, and cold—primarily through secondary messengers such as salicylic acid (SA), reactive oxygen species (ROS), ethylene (ET), and other signaling molecules. Studies in Arabidopsis and woody plants have demonstrated PAD4' s involvement in regulating PCD, cell wall synthesis, seed yield, biomolecule production, and water use efficiency. Extensive research has shown that PAD4 forms specific hubs with LSD1 (Lesion Simulating Disease 1) and EDS1 (Enhanced Disease Susceptibility 1) to modulate plant cell death and adaptation to both biotic and abiotic stresses. In Arabidopsis, AtPAD4 mutations disrupt SA, ET, and ROS homeostasis, thereby interrupting adaptive responses and cell death signaling. Under hypoxic conditions, AtPAD4 is negatively regulated by AtLSD1.1 to participate in ethylene-mediated lysigenous aerenchyma formation. In rice, altering nitrogen levels modulates OsPAD4 expression patterns to regulate PCD-induced aerenchyma formation. In grapevine, VvPAD4 forms stable molecular complexes with VvEDS1 to adapt to abiotic stress and regulate cell death. In soybean, GmPAD4 is essential for defense signal transduction and participates in regulating cell death and biotic stress responses.

To date, research on PAD4-mediated aerenchyma formation and regulation has primarily focused on Arabidopsis, rice, and tobacco, while its functional role in S. medusa' s stress adaptation remains unclear. Therefore, this study used naturally growing S. medusa from the Qinghai-Tibet Plateau as experimental material to clone SmPAD4 and its promoter using molecular biology techniques. We examined the gene' s expression under UV and hypoxia stress to analyze

the relationship between gene function and ecological adaptation, providing scientific evidence for understanding the molecular adaptation mechanisms of S. medusa and functional ecology of alpine plants.

1.1 Plant Materials and Treatments

Plant materials and seeds of S. medusa were collected from the Qilian Mountains in northeastern Qinghai-Tibet Plateau (Xining, Qinghai, 101°41 E, 37°36 N). Young leaves were harvested during the flowering stage for gene cloning and promoter amplification. Seeds were germinated and grown in the laboratory until they developed two true leaves (approximately 70 days), after which they were subjected to UV and hypoxia stress treatments following established protocols. Samples were collected at 1, 2, 4, 6, and 12 hours post-treatment for tissue-specific expression analysis.

1.2 Cloning of Full-Length SmPAD4 cDNA

PAD4 coding sequences from Asteraceae species including Cynara cardunculus, Lactuca sativa, Helianthus annuus, and Erigeron canadensis were downloaded from NCBI. Degenerate primers were designed using DNAMAN5.0 after sequence alignment . Total RNA was extracted from field-collected S. medusa leaves using the MiniBest Universal RNA Extraction Kit. First-strand cDNA was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa). Based on the conserved fragment amplified with degenerate primers, two nested 3 -RACE and two 5 -RACE specific primers were designed . RACE experiments were performed using the SMARTer® RACE 5 /3 Kit, with PCR products analyzed by 1% agarose gel electrophoresis. Fragments of expected size were recovered and subjected to blue-white screening, with positive colonies sequenced by a commercial service. The full-length SmPAD4 cDNA was assembled from the nested PCR sequences and the conserved fragment, then verified by designing primers based on the complete sequence.

1.3 Bioinformatics Analysis of SmPAD4

The open reading frame and encoded product were identified using ORF Finder. Physicochemical properties including isoelectric point, molecular weight, and instability index were analyzed using ProtParam (http://web.expasy.org/protparam/). Conserved domains were identified using Pfam (http://pfam.xfam.org/), while secondary and tertiary structures were predicted using SOPMA (http://pbil.ibcp.fr) and SWISS-MODEL (http://swissmodel.expasy.org/), respectively. Homologous sequences were obtained using NCBI Blastp and DNAMAN5.0 for sequence alignment. A phylogenetic tree of SmPAD4 and PAD4 proteins from other plants was constructed using MEGA11 based on high homology sequences. Subcellular localization was predicted using WoLF PSORT (https://www.genscript.com/wolf-psort).

1.4 Expression Analysis of SmPAD4

For tissue expression analysis under natural conditions, RNA was extracted from field-collected S. medusa tissues (roots, stems, leaves) and reverse-transcribed into cDNA. For stress treatments, laboratory-grown seedlings were subjected to UV and hypoxia stress, with samples collected at 1, 2, 4, 6, and 12 hours. Following established methods, UPL7 was used as the reference gene and qRT-PCR primers were designed . Three biological replicates were performed for each treatment using SYBR Premix Ex Taq II according to the manufacturer' s protocol. The reaction conditions were: 95°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Expression levels were calculated using the 2^(-ΔΔCt) method and analyzed using SPSS 26, Excel 2021, and Origin 2021.

1.5 Promoter Amplification of SmPAD4

Three nested downstream specific primers were designed based on the cloned SmPAD4 genomic sequence . High-concentration DNA was extracted using the MiniBEST Universal DNA Extraction Kit. The SmPAD4 promoter was amplified using hiTAIL-PCR through pre-amplification, first-round, and second-round PCR reactions. The amplified promoter sequence was analyzed for cis-acting elements using PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

1.6 Subcellular Localization of SmPAD4

Primer Premier 5 was used to identify SacI and XbaI restriction sites in SmPAD4, and adapter primers were added to synthesize PAD4-2300F and PAD4-2300R . The CDS region was amplified by PCR, and both the PCR product and pCAMBIA2300-GFP vector were double-digested. The fusion expression vector PAD4-2300 was constructed using T4 DNA ligase via homologous recombination. The construct was transformed into E. coli DH5α competent cells by heat shock, and positive clones were selected and verified by colony PCR. The recombinant plasmid was transformed into Agrobacterium GV3101 by liquid nitrogen freeze-thaw method and used to infect Nicotiana benthamiana leaves for transient expression. After 2–3 days, leaves were observed using an FV10-ASW laser confocal microscope with 2300-GFP empty vector as the control.

Results

2.1 Cloning of Full-Length SmPAD4 cDNA

RT-PCR amplification yielded a 574 bp conserved fragment, while 3 RACE and 5 RACE produced fragments of 1,150 bp and 1,359 bp, respectively [Figure 1: see original paper]. The complete SmPAD4 cDNA is 2,047 bp (GenBank accession OR766038), containing a 1,866 bp open reading frame encoding 621 amino acids. The full-length cDNA was successfully verified.

2.2 Bioinformatics Analysis of SmPAD4

The SmPAD4 protein consists of 621 amino acids with a molecular formula of $C_{3163}H_{4906}N_{848}O_{910}S_{26}$, molecular weight of 70.22 kDa, isoelectric point of 8.18, and aliphatic index of 87.13. The protein lacks a signal peptide, has a grand average of hydropathicity of -0.194, and an instability index of 40.48, classifying it as a basic, hydrophilic, unstable protein. Transmembrane structure prediction revealed transmembrane regions, indicating that SmPAD4 is a transmembrane protein. Phylogenetic analysis using the Neighbor-Joining method showed that SmPAD4 clusters closely with PAD4 proteins from Cynara cardunculus (XP_{024962884}.1, XP_{024962885}.1), Lactuca sativa (XP_{023737098}.1), Erigeron canadensis (XP_{043623496}.1), and Helianthus annuus (XP_{021989681}.1), with the closest evolutionary relationship to C. cardunculus [Figure 2: see original paper]. Multiple sequence alignment revealed highest similarity with C. cardunculus [Figure 3: see original paper]. Nucleotide and amino acid sequence comparisons with other Asteraceae species showed 87.16% nucleotide identity and 87.66% amino acid identity with C. cardunculus, and over 74% nucleotide identity and 73% amino acid identity with other Asteraceae members. Subcellular localization prediction indicated nuclear, chloroplast, and cytoplasmic distribution.

SmPAD4 contains multiple phosphorylation sites [Figure 4A: see original paper], with serine phosphorylation sites being most abundant and several predicted values exceeding 0.9, suggesting phosphorylation-mediated regulation. Secondary structure prediction [Figure 4B: see original paper] revealed α-helices (314 residues, 50.56%), extended strands (51 residues, 8.21%), β-turns (18 residues, 2.9%), and random coils (238 residues, 38.33%). α-helices, extended strands, and random coils span the entire protein, while β-turns are scattered near α-helices. Tertiary structure modeling using SWISS-MODEL is shown in [Figure 4C: see original paper]. The protein' s N-terminus contains a highly conserved α/β hydrolase fold domain that is evolutionarily conserved in Asteraceae, while the C-terminus includes an EDS1-PAD4 (EP) domain essential for stable heterodimerization. This domain is characteristic of the lipase-like protein family (EDS1, PAD4, SAG101), which participates in signal transduction from cell surface and intracellular immune receptors, conferring specific domain functions to PAD4.

2.3 Promoter Amplification of SmPAD4

A 1,049 bp SmPAD4 promoter sequence was amplified using hiTAIL-PCR [Figure 5: see original paper]. PlantCARE analysis identified numerous TATA-box and CTAA-box elements, along with cis-acting elements including light-responsive elements (Box 4 and GT1-motif), hypoxia-response elements (ARE), MeJA-response elements (CGTCA-motif and TGACG-motif), drought-response elements (MBS), auxin-response elements (AuxRR-core), and WRKY transcription factor binding sites (W-box) . These findings suggest that SmPAD4 participates in regulatory mechanisms induced by light signaling, hypoxia, drought,

MeJA, and WRKY transcription factors, indicating its involvement in growth, development, and stress responses in S. medusa.

2.4 Tissue-Specific Expression of SmPAD4

Under natural conditions, SmPAD4 was expressed in roots, stems, and leaves of wild S. medusa [Figure 6: see original paper], with expression levels following the pattern: leaves > roots > stems. Leaf expression was approximately 5.3-fold higher than in stems, while root expression was about 2.5-fold higher than in stems, with all differences being statistically significant ($P < 0.05$).

Under UV stress [Figure 7A: see original paper], SmPAD4 expression in roots was consistently lower than the control, reaching its lowest level at 4 h. In stems, expression increased initially then decreased, peaking at 6 h. In leaves, expression showed a similar trend, peaking at 4 h. Under hypoxia stress [Figure 7B: see original paper], root expression remained below control levels, reaching its minimum at 6 h. Stem expression fluctuated but remained above control throughout the treatment period, peaking at 12 h. Leaf expression increased initially then decreased, with maximum expression at 4 h.

Previous studies indicate that ethylene accumulation within 1–3 h under hypoxia promotes aerenchyma formation, and gene expression differences become significant by 12 h. Therefore, we analyzed SmPAD4 expression after 4 h and 12 h of stress. At 4 h [Figure 8A: see original paper], both UV and hypoxia significantly affected SmPAD4 expression across tissues. In roots, both stresses significantly reduced expression ($P < 0.05$), with UV showing the strongest effect. In stems and leaves, both stresses significantly increased expression ($P < 0.05$), with hypoxia inducing the highest expression in stems and UV inducing the highest expression in leaves. At 12 h [Figure 8B: see original paper], similar patterns were observed: both stresses significantly reduced root expression, with UV showing the strongest effect, while significantly increasing expression in stems and leaves, with hypoxia inducing the highest levels ($P < 0.05$).

2.5 Subcellular Localization of SmPAD4

Subcellular localization [Figure 9: see original paper] revealed that the PAD4-2300 fusion protein' s green fluorescent signal was primarily distributed in the cell membrane and nucleus, with partial localization in chloroplasts, indicating that SmPAD4 functions mainly in these three compartments.

Discussion and Conclusion

PAD4 is a key gene regulating plant responses to biotic and abiotic stresses and PCD, playing important roles in plant growth and development. This study cloned a 2,047 bp SmPAD4 gene from S. medusa containing a 1,866 bp CDS encoding 621 amino acids. Protein analysis revealed an aliphatic index of 87.13, classifying it as a basic, hydrophilic, unstable protein belonging to the hydro-

lase superfamily. The presence of multiple phosphorylation sites suggests regulation via phosphorylation. Domain prediction identified an α/β hydrolase domain at the N-terminus that is highly conserved in Asteraceae evolution, and a C-terminal EP domain essential for stable heterodimerization. Both PAD4 and EDS1 contain N-terminal acyl hydrolase-homologous domains that enable their interaction as partner proteins in stress responses. In seed plants, PAD4 and EDS1 form heterodimers via these homologous domains to mediate cell death and immune responses. In angiosperms, silencing PAD4' s N-terminal stable complex and co-expression studies have shown that PAD4 responds to drought, ABA treatment, and biotic stress. In Arabidopsis, expressing only the N-terminal lipase-like domain (LLD) without the C-terminal EP domain demonstrated that PAD4 can function as a bipartite protein, with LLD and EP domains playing distinct and separable roles in plant defense. The unique domain structure of SmPAD4 enables it to function as a bipartite protein responding to both hydrolytic metabolism and immune signals, thereby participating in the regulation of biotic and abiotic stress responses.

In plants, most TATA-box-containing promoters are involved in tissue-specific expression and stress responses, with AP2/ERF, bZIP, NAC, MYB, and WRKY being common promoter elements associated with pathogen defense. The SmPAD4 promoter region contains numerous TATA-box and CTAA-box elements, plus light-responsive (Box 4 and GT1-motif), hypoxia-response (ARE), MeJA-response (CGTCA-motif and TGACG-motif), drought-response (MBS), auxin-response (AuxRR-core), and WRKY binding site (W-box) cis-acting elements. Similar findings have been reported for the SikCDPK1 gene in snow lotus and the GRAS gene family in maize, where promoters containing light, hypoxia, drought, auxin, and MeJA-related elements participate in growth, development, and stress responses. These results demonstrate that SmPAD4 can respond to multiple stress signals to ensure proper plant development and adaptation, highlighting its important role in S. medusa' s adaptation to extreme environments.

Protein subcellular localization is key to understanding molecular function, gene regulation, and protein-protein interactions. Our subcellular localization study using N. benthamiana revealed SmPAD4 localization in the cell membrane, nucleus, and chloroplast. This aligns with reports that the EDS1-PAD4 complex typically appears in the nucleus during biotic and abiotic stress responses. In Arabidopsis, AtPAD4 localizes to the cytoplasm and nucleus to transduce defense signals and promote PCD. In wheat, TaPAD4 similarly localizes to the cytoplasm and nucleus to participate in plant immunity. The additional chloroplast localization observed for SmPAD4 may reflect chloroplasts' role as light-responsive organelles, where PAD4 acts upstream of ethylene and ROS production in excess excitation energy (EEE) conditions to regulate PCD, photoacclimation, and overall defense signaling. PAD4 also transduces photooxidative stress signals, leading to cell death, reduced growth, and adaptive regulation. As a typical NLR signaling component, PAD4 functions in various subcellular structures including the cytoplasm, nucleus, plasma membrane, tonoplast, and endoplasmic reticulum, inducing or inhibiting ethylene production, ROS gener-

ation, callose deposition, and phytoalexin gene expression. Thus, SmPAD4' s subcellular distribution confirms its role in transducing signals from ethylene, ROS, SA, and ABA produced during stress responses to maintain normal plant growth.

As a typical alpine plant, S. medusa inhabits environments characterized by strong UV radiation and hypoxia. To investigate SmPAD4' s response to these habitat stresses, we analyzed its relative expression in roots, stems, and leaves under UV and hypoxia treatments. qRT-PCR results showed that under both stresses, SmPAD4 expression remained below control levels in roots but above control levels in stems and leaves. In Arabidopsis, AtPAD4 is upregulated under UV stress to regulate PCD, consistent with our findings. Studies on maize, wheat, and cucumber have shown that PAD4 genes regulate PCD under hypoxia. In rice, hypoxia treatment downregulates OsPAD4 expression in roots to control PCD, matching our observation that SmPAD4 expression decreases in roots under hypoxia. This likely occurs because hypoxia induces ROS responses via ethylene and auxin, leading to collaborative action of PAD4 and related genes that triggers PCD and aerenchyma formation. Our previous study on SmLSD1 in S. medusa found that UV and hypoxia stress downregulated SmLSD1 expression in leaves and stems while upregulating it in roots, showing opposite expression patterns to SmPAD4. Research has demonstrated that AtPAD4 is negatively regulated by AtLSD1 to affect PCD, suggesting that during environmental stress, SmPAD4 and SmLSD1 both respond but SmPAD4 is negatively regulated by SmLSD1 under hypoxia and UV stress to adapt to extreme environments. This is consistent with Arabidopsis studies showing AtPAD4 is negatively regulated by AtLSD1.1 to participate in ethylene-mediated aerenchyma formation, suggesting that SmPAD4 exhibits distinct expression patterns in different S. medusa tissues under stress.

After 4 h of UV and hypoxia stress, SmPAD4 expression changed significantly across tissues, with more pronounced changes in roots and leaves under UV stress. After 12 h of stress, UV stress caused greater expression changes in roots compared to hypoxia stress, indicating that SmPAD4 expression is more significantly altered under UV stress. This may be because PAD4 transduces photooxidative stress signals and acts upstream of ethylene and ROS production to regulate PCD. Our study demonstrates that SmPAD4 responds to both stresses in all tissues, negatively in roots and positively in stems and leaves, consistent with Arabidopsis studies showing AtPAD4 is negatively regulated by AtLSD1.1 to affect aerenchyma formation. This suggests that SmPAD4 exhibits tissue-specific expression patterns under stress in S. medusa. Although SmPAD4 responds to both hypoxia and UV radiation, whether aerenchyma formation is induced by hypoxia, UV radiation, or a combination of multiple alpine environmental factors remains unclear and requires further investigation.

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