Cloning, Expression and Sequence Analysis of *PbeHSFA1d* in *Pyrus betulifolia*

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Keywords: *Pyrus Betulifolia*, *Pbehsfa1d* Gene, Gene Cloning, Abiotic Stress, Expression Analysis.

Abstract: Heat shock protein is a kind of transcription factors widely existing in plants, which plays a major role in regulating plant growth and responses to environmental stress. In order to study the sequence characteristics of *HSF* gene from pear and its response to abiotic stress, *PbeHSFA1d* gene was isolated from *Pyrus betulifolia* by RT-PCR. Sequence analysis of *PbeHSFA1d* was carried out by bioinformatics software, and real time quantitative PCR was used to detect the expression level of *PbeHSFA1d*in different tissues and its response patterns to various abiotic stresses. The results reflected that the ORF of *PbeHSFA1d* was 1548 bp in length, 516 amino acids were encoded, and there were 52 phosphorylation sites in the predicted protein, but the glycosylation site was not found. Domain analysis showed that PbeHSFA1d contained a conserved N-terminal DNA binding domain (DBD), a bipartite oligomerization domain (HR-A/B), an activation domain (AHA), a nuclear localization signal (NLS) and a nuclear export signal (NES), which are typical structural features of plant HSF protein family. Moreover, PbeHSFA1d was close to MdHSFA1d, PaHSF8, PpHSF8 and PdHSFA1d in genetic relationship. PbeHSFA1d was localized to the nucleus by subcellular localization. The results of RT-PCR demonstrated that the expression of *PbeHSFA1d* in stem was higher than that in flower, leaf and root, and the transcript levels of *PbeHSFA1d* were induced by high temperature, dehydration, low temperature and salt. The results of this study will provide a theoretical basis for further exploration of the function in abiotic stress of *PbeHSFA1d*.

1 INTRODUCTION

During the last decades, heat has gradually become an important environmental factor seriously affecting plant growth and yield with global warming. Therefore, plants have formed a series of molecular and physiological mechanisms to resist environmental stress in the long-term evolution process (Gall 2015). As an important regulator of gene expression, transcription factors participate in a set of plant protection mechanisms under environmental stresses, indicating that transcription factors play an essential role in improving plant stress resistance, such as bZIP, NAC, bHLH and HSF (Tang 2020).

The response of plants to heat is a extremely complex process, which involves a wide range of genes that related to abiotic stress, signal transduction, material transport, photosynthesis, protein metabolism and carbohydrate metabolism gene (Tian 2021). Since the first plant *HSF* (heat shock transcription factor) gene was cloned from tomato, there are 21, 52, 25 and 30 members in Arabidopsis, soybean, rice and maize, respectively (Yoshida 2011). As a large transcription factor family in plants, HSF is the core regulator of heat stress response genes in higher plants (Qiao 2015). HSF could specifically recognize and bind heat shock elements, and then activate the expression and transcription of downstream heat shock protein genes to produce heat shock proteins, so as to improve the heat resistance of plants. HSF has five typical functional domains: DBD, HR-A/B domain, NLS, NES and AHA (Li 2020). HSF can be further divided into A, B and C types in plants, and A-type HSF contains AHA structure, which mostly plays a positive regulatory role in the response to heat stress, while most of B-type HSF play a negative regulatory role in the response to heat stress. Several studies have shown that HSF could respond not only to heat, but also to cold, drought, oxidative stress and salt

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(Tian 2021, Liu 2021). HSFA1b enhances heat tolerance in wheat and Arabidopsis through jasmonate signalling pathway and OPR3 (Tian 2020). AtHSFA2 is involved in drought and oxidative stress in Arabidopsis (Nishizawa 2006). In addition, AtABI3 positively regulates the expression of AtHSFA9 by directly binding to its promoter, but AtHSFA9 is negatively regulated by AtIAA27 (Kotak 2007).

So far, although many HSF transcription factors that respond to plant development and abiotic stress have been identified, but most of these studies focus on Arabidopsis and crops, and there are still few studies on HSF transcription factors in woody plants. *Pyrus betulifolia* is a heat-tolerant species, making it an ideal source to isolate gene. In this study, the full length of *PbeHSFA1d* of *Pyrus betulifolia* was cloned by reverse transcription PCR. The sequence analysis was carried out by bioinformatics software, and the tissue expression level and the pattern of expression to abiotic stress were studied by quantitative PCR, in order to prove the function and stress resistance mechanism of *PbeHSFA1d.*

2 MATERIALS AND METHODS

2.1 Plant Materials

Healthy and uniform seedlings were collected from 60-day-old *Pyrus betulifolia*, which were exposed to abiotic stresses of low temperature (4℃), high temperature (40℃), dehydration and salt (200 mmol·L^{-1} NaCl).

2.2 Stress Treatments

Temperature stresses were carried out by placing the seedlings in a growth incubator at the set 4℃or 40℃ for 0, 6, 12, 24, 48 h, respectively. Before dehydration and salt treatment, the roots of seedlings were placed in a beaker containing distilled water, which were grown in a growth chamber at 25°C for 1 day, had a 16-h light/8-h dark photoperiod. For the dehydration treatment, the seedlings were put on the dry filter papers dried at 25°C in an artificial climate chamber, with relative humidity of 40.0% for 0, 0.5, 1, 3 and 6 h. Salt treatment was applied by transferring the seedlings to flasks with 200 mmol·L^{-1} NaCl solution for 0, 6, 12, 24 and 48 h. At least 50 seedlings were used in each treatment, and the fully expanded leaves were collected from randomly 3-4 seedlings at set time points. All the samples of each treatment were mixed and put into the liquid nitrogen frozen immediately, then stored at -80°C for further experiments.

2.3 Gene Isolation

Based on the pear genome database, a sequence with a complete opening reading frame, high degree of similarity to Md*HSFA1d* was obtained, named as *PbeHSFA1d*. The 2000 bp upstream of the transcription start site of P*beHSFA1d* gene was selected as the promoter sequence. Based on the two sequences, specific primers GSP1 and GSP2 were designed for amplifying the gene and promoter by using cDNA and DNA in the leaves of *Pyrus betulifolia* as templates, respectively. The PCR mixture in a total 20 μL reaction volume, contained 100 ng cDNA, 10 μL I-5^{TM} 2X High-Fidelity Master Mix, 0.4 μM of a pair of specific primers and nuclease-free water. PCR was performed by a program as follows: initial denaturation at 98°C for 1 min, 35 cycles of 98°C for 10 s, 56°C for 15 s, 72°C for 90 s, and 72°C for 10 min. The PCR product of gene cloning was purified and cloned into pCAMBIA1300 vector to generate a fusion construct (pCAMBIA1300-PbeHSFA1d) and sequenced in Sangon. The promoter of *PbeHSFA1d* product was subcloned into pMD-18T vector and sequenced in Sangon.

2.4 Sequence Analysis of PbeHSFA1d

The molecular weight and isoelectric point (pI) were calculated by Expasy (http://web.expasy.org/compute_pi/); The cis acting elements in the promoter that related to environmental stress was detected by the online tool of Plant CARE; Subcellular localization was analyzed by software Softberry; The secondary and three-dimensional structures of PbeHSFA1d protein were predicted by software Sopma and SWISS-MODEL (Waterhouse 2018), respectively; The protein modification patterns were analyzed by software Netphos 3.1 server and Dictyglyc 1.1 server; The multiple alignments of the deduced amino acid sequence were carried out by the Clustal W program (Jin 2017); The protein functional domain was analyzed by software Pfam; Phylogenetic tree was constructed by the Maximum Likelihood method using MEGA 6.0.

2.5 Analysis of PbeHSFA1d Gene Expression Characteristics

Based on the confirmed PbeHSFA1d sequence, a pair of specific expression primers (GSP3) were designed. Quantitative Real-Time PCR (qRT-PCR) was performed to analyze the expression levels in different tissues and expression patterns under various abiotic stresses of *PbeHSFA1d*, the primer of internal reference gene was GSP4. The PCR solution in a total 20 μL reaction volume, contained 10 ml of SYBR-Green PCR Master Mix, 0.25 mM of each primer, 100 ng of cDNA, and nuclease-free water. Quadruple qPCR was carried out in a LightCycler 480 Real-Time PCR System, the PCR reaction conditions were as follows: 95 °C for 5 min, then 40 cycles of 94 °C for 10 s, 60 °C for 30 s, 72 °C for 30s, and 72 °C for 3 min. Relative gene expression levels of the amplified products were normalized to *Pyrus* TUB-b2 to minimize variation in the cDNA template and the relative gene expression data were calculated by the $2^{-\Delta\Delta CT}$ method.

2.6 Statistical Analysis

The experimental results are at least three independent replicates in this study, shown as mean ± SE. Experimental data were analyzed using SPSS software and statistical difference was compared based on Duncan's multiple range test.

Table 1: Primer sequences.

Primer	Primer Sequence (5′-3′)	Function
GSP1-F	GAGAACACGGGGGACTCTAGAAT GGAGGCTGCTAATAACAAC	Gene cloning
GSP1-R	GCCCTTGCTCACCATGGATCCCAC CCCTTTGGTCTCTGATG	
GSP2-F	AACAAATGAACGGTATTGGTAA	Promoter cloning
GSP2-R	CAATTCTTTTATTGTTCTCTGTG	
GSP3-F	GAGCATCAATCGGCGTAA	QRT-PCR
GSP3-R	CTGCACCATTGTTTGTAGCT	
GSP4-F	TGGGCTTTGCTCCTCTTAC	Internal control
GSP4-R	CCTTCGTGCTCATCTTACC	

3 RESULTS

3.1 Sequence Analysis of *PbeHSFA1d*

The target fragment was amplified by using specific cloning primers with the cDNA from the leaves of *Pyrus betulifolia* as the template, sequencing results showed that the fragment was consistent with the *PbeHSFA1d*. The full length of is 1548 bp, encoding 516 amino acids.

In order to further speculate the potential function of *PbeHSFA1d*, the cis acting elements of promoter were analyzed. The results showed that a various of cis acting elements related to environmental stress (MBS, LTR, STRE, TC-rich repeats, ARE and so on) and hormone (ABRE, TCA-element, ERE, TGACC-motif, GARE-motif, and so on) in the promoter, which suggested that *PbeHSFA1d* might play a key role in plant defense against abiotic stresses and response hormones.

3.2 Sequence and Bioactivity Analysis of PbeHSFA1d

The predicted molecular weight of PbeHSFA1d was about 57.141 kDa, the theoretical isoelectric point was 4.8, the instability index was 56.67, and the hydrophilicity coefficient was - 0.697, indicating that PbeHSFA1d belonged to unstable hydrophilic protein; The results of signal peptide analysis showed that there was no signal peptide sequence in PbeHSFA1d, which belonged to non-secretory protein; The prediction of subcellular localization showed that PbeHSFA1d was mainly located in the nucleus. The results of protein secondary structure prediction showed that PbeHSFA1d contained 32.75% α- Helix, 9.11% extension chain, 5.62% β-Corner and 52.52% random coil; The protein tertiary structure of PbeHSFA1d was analyzed by using HSF protein template (PDB ID:1fbu.1) in SWISS-MODEL software, ratio of the coverage of the constructed model and template was 50% and the amino acid coverage range was 41-116 aa.

Phosphorylation and glycosylation, as the common protein modifications, may greatly affect the protein structure and function in the process of plant growth and development. The phosphorylation analysis of PbeHSFA1d showed that 52 phosphorylation sites exceeded the probability threshold, including 40 serine sites and 12 threonine sites, and there were dense phosphorylation sites in the amino acid sequence range 290-350 aa (Figure 1). Glycosylation prediction analysis showed that there were no sites that exceed the probability threshold, which indicated glycosylation of PbeHSFA1d protein was highly likely.

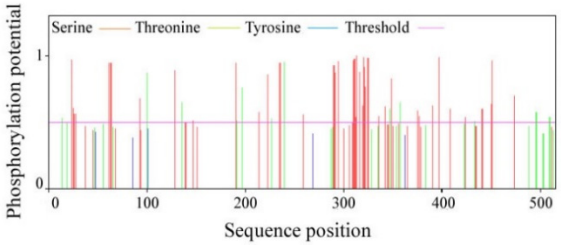

Figure 1: Prediction of phosphorylation site in amino acid sequence of PbeHSFA1d protein.

3.3 Phylogenetic and Sequence homology Analysis of PbeHSFA1d

Phylogenetic analysis showed that PbeHSFA1d had a close relationship with MdHSFA1d, PaHSF8, PpHSF8 and PdHSFA1d of Rosaceae, and clustered together; Besides that, PbeHSFA1d had a distant relationship with ZjHSF8, GmHSF33 and SsHSFA1d (Figure 2).

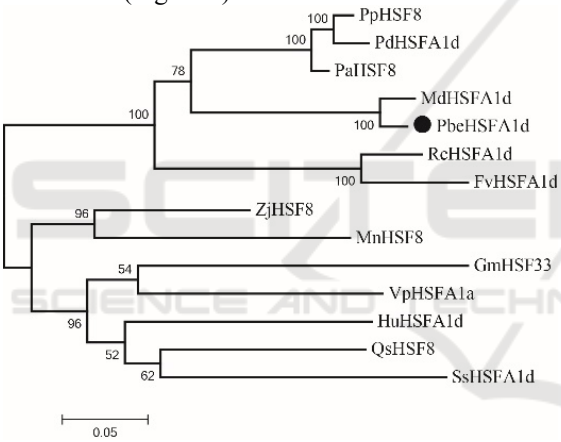

Figure 2: Phylogenetic tree of PbeHSFA1d and HSFs of other species.

Multiple sequence alignment analysis was performed between PaHSF8, PpHSF8, FvHSFA1d, RcHSFA1d, MdHSFA1d and PbeHSFA1d, the results showed that the sequence similarity respectively was 77.50%, 79.03%, 73.29%, 73.84% and 95.16%, which indicated PbeHSFA1d had high homology with these five species and including a conserved N-terminal DBD domain, a HR-A/B domain, a NLS, AHA and NES. In addition, PbeHSFA1d possessed an AHA domain which is specific to A-type HSF transcription factors in plants, all of those proved that PbeHSFA1d was a A-type HSF transcription factor (Figure 3).

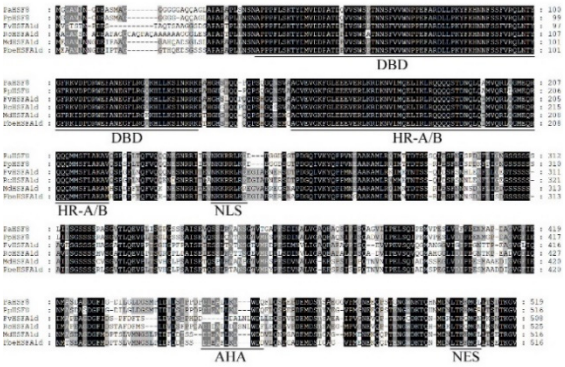

Figure 3: Multiple alignment of HSF proteins.

3.4 Tissues Specific Expression of *PbeHSFA*

HSFs were expressed in different tissues of plant, and with a certain degree of tissue specificity. Therefore, the expression levels of *PbeHSFA1d* in *Pyrus betulifolia* different tissue of flower, leaf, stem and root were analyzed (Figure 4). The result indicated that *PbeHSFA1d* was expressed in all tested tissues, with the highest transcription level in stem and the least in flower, which demonstrated the significant tissue expression specificity of *PbeHSFA1d*.

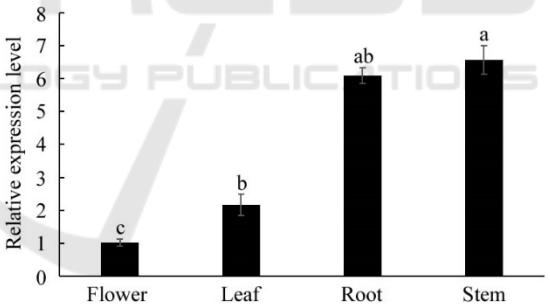

Figure 4: Expression of *PbeHSFA1d* in various tissues.

3.5 Expression Characteristics of *PbeHSFA1d* under Different Stresses

The qRT-PCR was performed to detect the tanscription levels of *PbeHSFA1d* under various treatments, including low temperature (4℃), high temperature (40℃), salt and dehydration. For all treatments, the expression of *PbeHSFA1d* was higher than the initial level after treatment. Under low temperature stress, *PbeHSFA1d* was continuously induced and reached the highest level at 24 h and declined at the last day (Figure 5A).

Under heat stress, expression profile was progressive elevated until the highest level was reached at 12 h (Figure 5B). As can be seen in Figure 5C, *PbeHSFA1d* was sharply induced within 1 h, which was 6 times that of initial level. The expression of *PbeHSFA1d* reached the maximum level at 12 h with salt treatment and then decreased continuously (Figure 5D).

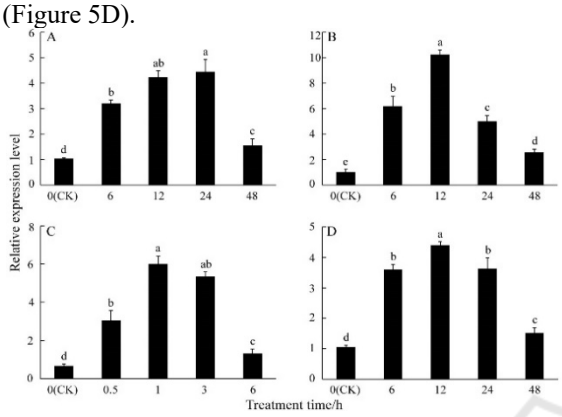

Figure 5: Relative expression pattern of *PbeHSFA1d* under various abiotic stresses.

4 DISCUSSION

With the intensification of greenhouse effect, heat stress has become one of the major limiting factors that adversely affect plant development and crop yield. Under heat stress, HSF can regulate the expression of heat shock genes and produce HSP, which is considered to be the central transcription factor to resist heat stress. Although several *HSFA1d* genes have been isolated, there are differences in tissue expression patterns and response levels under abiotic stress (Tang 2020, Ohama 2017). Identifying *HSFA1d* in different species will lay a foundation for further study of the function and application.

In this study, the *PbeHSFA1d* of *Pyrus betulifolia* was cloned, the ORF has a total length of 1548 bp, encodes 516 amino acids and contains 52 phosphorylation sites, which suggest PbeHSFA1d will play extensive role in the regulation of phosphorylated proteins (Shen 2019). Cis acting elements are mainly involved in gene expression regulation, several stress-related cis acting elements such as MBS, LTR, STRE and ARE are predicted in the *PbeHSFA1d* promoter, which are also found in the promoter of *AtHSFA1d* that in response to a variety of abiotic stresses, indicates the *PbeHSFA1d* may be involved in the pathway of resistance to environmental stress (Liu 2021). Multiple sequence alignment results show that both PbeHSFA1d and homologous genes contain the A-type HSFs key domains of DBD, HR-A/B, NLS, AHA and NES, which are also the main motifs for HSF to perform its functions (Li 2020). Evolutionary analysis show that PbeHSFA1d is closely related to MdHSFA1d, PdHSFA1d, PpHSF8 and PaHSF8, implying that PbeHSFA1d is highly conservative in the process of evolution.

HSFs are distributed in many tissues of plant and show the tissue-specific expression. In this study, the expression of *PbeHSFA1d* is higher in the stem and root, but lower in the leaf and flower, which is similar to the tissue-specific expression of PpHSF5 (Tan 2021). On the contrary, the expression of *CsHSFB2b* is higher in fruit and leaf (Zhang 2020). The difference of tissue expression among homologous genes may be related to their main physiological functions. Several studies have shown that the transcription level of *HSFs* is induced by a variety of abiotic stresses, which affect the stress resistance of plants. *AtHSFA1d* responds to chilling stress and promotes hypocotyl elongation via enhancing expression of ribosomal protein genes (Liu 2021). *MdHSFA8A* is induced by drought and modulates flavonoid synthesis (Wang 2020). Overexpression of *TaHSFA2d* and *AtHSFA2* improve salt tolerance by regulating stress response (Chauhan 2013). As a major heat stress transcription factor, AtHSFA1d improves heat tolerance by regulating related gene expression (Higashi 2013). In this study, *PbeHSFA1d* of *Pyrus betulifolia* is involved in the response to cold, dehydration, heat and salt, demonstrating that *PbeHSFA1d* may play an essential role in the response to abiotic stress. However, the action mechanism of *PbeHSFA1d* resistance to abiotic stress needs to be further explored.

5 CONCLUSIONS

In this study, PbeHSFA1d was isolated from *Pyrus betulifolia* and proved to be a A-type HSF protein by sequence analysis. PbeHSFA1d was clustered with MdHSFA1d, PaHSF8, PpHSF8 and PdHSFA1d in evolutionary relationship. The expression of *PbeHSFA1d* was the lowest in the flower and the highest in the stem, showing significant tissue expression specificity. PbeHSFA1d was induced by low temperature, heat, salt and dehydration stress, indicating that *PbeHSFA1d* may be involved in the process of resistance to abiotic stress. In brief, the present work lays a foundation for identifying the function of *PbeHSFA1d* and other more work in the future.

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