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Full Length Article

Isolation and Characterization of *PlNAC2* in Herbaceous Peony (*Paeonia lactiflora*)

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Abstract

NAC domain transcription factors play an important role in the formation of secondary cell walls (SCW) in plants. Although NAC family transcription factors have been cloned in many plants, no studies have been reported in herbaceous peony. In order to further explore and utilize NAC gene, this study cloned *PlNAC2* from the stem of herbaceous peony cultivar 'Xixia Yingxue' based on the transcriptome data of the stem, and found that it had 1,109 nucleotides, encoded 313 amino acids and contained a typical NAC conserved domain. Phylogenetic tree analysis showed that *PlNAC2* had the highest homology with *QlNAC2*. The expression patterns showed that *PlNAC2* existed in the entire stage of stem growth of herbaceous peony, and its expression level gradually decreased with the development of stem. At the same time, the gene was widely expressed in different tissues of herbaceous peony cultivar 'Hongyan Zhenghui', with the highest expression in stems, followed by leaves and roots, and the lowest expression was in flowers, suggesting that *PlNAC2* might play a negative regulatory role in the formation of SCW. This study lays the foundation for further exploration of the physiological functions of *PlNAC2*.

Keywords: Herbaceous peony; *PlNAC2*; Clone; Expression patterns

Introduction

Herbaceous peony (*Paeonia lactiflora* Pall.) is a perennial herbaceous plant of the Paeoniaceae family. It is a traditional flower in China and has a cultivation history of nearly 4,000 years, which was watched in the palace in the Xia Dynasty (Lu *et al*. 2017). According to historical records, tree peony and herbaceous peony are both known as "King of the flowers" and "Premier of the flowers"; both of them mean wealth. But compared with tree peony, the quality of herbaceous peony cut flowers is better. In recent years, herbaceous peony has been quite popular in the fresh cut flower market because of its high ornamental value including erect flower stem, elegant flower postures and various colors (Tang *et al*. 2018). In the production of herbaceous peony cut flowers, the upright strength of the flower stem is an important indicator of the quality of cut flowers. Existing studies have shown that the stem strength of plants is closely related to factors such as plant height, stem thickness, flower weight and flower stem (Xia *et al*. 2018). At present, there are few reports about the molecular regulation mechanism of the stem strength of herbaceous peony. Therefore, studying the molecular mechanism of the stem strength of herbaceous peony and enhancing the stem strength have great significance for the growth and development of herbaceous peony and the quality of cut flowers.

NAC transcription factor family is one of the largest transcription factor families found in the plant genome (Kang *et al*. 2012). According to genome-wide analysis, at least 151 and 117 NAC family transcription factors were found in *Oryza sative* and *Arabidopsis thaliana* (Nuruzzaman *et al*. 2010). *Populus trichocarpa* contains at least 163 members (Hu *et al*. 2010), and at least 152 members have been found in *Glycine max* and *Nicotiana tabacum* (Rushton *et al*. 2008; Le *et al*. 2011). It is reported that 48 and 45 members of NAC family transcription factors were found in the EST database of *Hordeum vulgare* and *Citrus limonia*, respectively (Christiansen *et al*. 2011; de Oliveira *et al*. 2011). As a large family, NAC transcription factors have a variety of biological functions, and are mainly involved in plant growth and development, stress resistance, disease resistance regulation, secondary growth regulation, and hormone signal transduction (Wang and Zhang 2018). Existing studies have shown that multiple NAC genes can promote the formation of secondary cell walls (SCW) in fiber cells, such as *NST1/2/3* (NAC secondary walls thicken

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promoting factor) and *VND6*/*7* (vascular-related NAC domain) (Kubo *et al*. 2005), while other NAC genes inhibit the SCW formation in fiber cells. For example, over-expression of *AtNAC012* in *Arabidopsis* could slightly thicken the cell walls of xylem ducts, but strongly inhibit the production of fiber cells' SCW (Ko *et al*. 2007).

Although NAC family transcription factors have been cloned in many plants, so far, no study has been reported on NAC transcription factor in herbaceous peony. In view of this, the aim of this study was to clone *PlNAC2* in herbaceous peony, perform sequence comparison of *PlNAC2* protein with other proteins and construct phylogenetic tree. Also, we predicted protein property, secondary structure and tertiary structure of *PlNAC2*, and analyzed its spatio-temporal expression patterns.

Materials and Methods

Preparation of RNA and cDNA synthesis

The stems of the herbaceous peony cultivar 'Xixia Yingxue' from S1 (Stage 1: flower-bud stage) to S3 (Stage 3: full-flowering stage) and the roots, stems, leaves and flowers of 'Hongyan Zhenghui' were used as the materials. Total RNA of herbaceous peony samples were extracted by the CTAB method. The reverse transcription was performed by using 5×M-MLV Buffer (TAKARA, Dalian, China) reverse transcription kit, and the cDNA was synthesized according to the instructions.

Cloning of *PlNAC2* in herbaceous peony

Based on *PlNAC2* sequence obtained from the transcriptome data, a pair of primers was designed and selected for final PCR amplification (Table 1). The total volume of the RT-PCR reaction system was 25 μL, containing 12.5 μL High enzyme, 2 μL cDNA, 1 μL for each of the forward primers and reverse primers, and 8.5 μL ddH_2O. The reaction program was pre-denaturation at 98°C for 2 min. PCR was performed: 40 cycles at 98°C for 10 s, 50°C for 15 s and 72°C for 30 s by using a thermal cycler. The Agarose Gel DNA Recovery Kit (Tiangen, Beijing, China) was used to purify and recover the PCR products and then ligated it with the pMD18-T (Tiangen, Beijing, China) vector. Finally, the identified positive recombinants were sent to Qingke Biotechnology Co., Ltd for sequencing.

Bioinformatics analyses

Software for bioinformatics analysis were listed in Table 2. BLAST was used to identify the homology of nucleic acid and protein sequences. ORF Finder was used to find open reading frames and translate it into protein sequences. Based on the sequence alignment of *PlNAC2* with other homologous sequences, a phylogenetic tree was constructed by using MEGA 5.0. ProtParam was used to predict the physical and chemical properties of *PlNAC2* protein. Signal P 5.0 Server was used to predict signal peptide. TMHMM 2.0 Server was used to analyze the transmembrane domain of *PlNAC2* protein. The online software SOPMA was used to infer the secondary structure of *PlNAC2* protein. The online software ProtScale was used to predict the hydrophobicity/hydrophilicity of the amino acid sequence of *PlNAC2* protein. SWISS-MODEL workspace was used to simulate the tertiary structure of *PlNAC2* protein.

Quantitative real-time PCR

Based on *PlNAC2* sequence obtained from the transcriptome data, a pair of primers was designed to analyze the distribution of *PlNAC2* during the stem growth of 'Xixia Yingxue' and the distribution of *PlNAC2* in the roots, stems, flowers and leaves of 'Hongyan Zhenghui' (Table 1). The PCR mixture contained 1 μL cDNA, 10 μM forward and reverse primers 0.5 μL each, 4.3 μL ddH_2O and 6.3 μL SYBR Master Mix (TAKARA, Dalian, China). The reaction program was pre-denaturation at 95°C for 30 s, 40 cycles included 95°C denaturation for 5 s, 50°C annealing for 30 s, and 72°C extension for 30 s. The comparative CT method calculated the relative quantification of *PlNAC2* expression level. The cDNA samples to be tested were set up in triplicate, and the data analysis was performed by using $2^{-\Delta\Delta Ct}$ method.

Results

Cloning and sequencing analysis of *PlNAC2* in herbaceous peony

The full-length sequence of *PlNAC2* was obtained by RT-PCR. The PCR product was visualized by agarose gel electrophoresis (Fig. 1), which showed that the size of product was 1,109 bp. Analysis of the sequencing results showed that the open reading frames of *PlNAC2* was 939 bp in length and encoded 313 amino acids (Fig. 2). The NCBI website was used to perform a BLAST alignment on the amino acid sequence of *PlNAC2*. The comparison results showed that the amino acid sequence of the conserved domain of *PlNAC2* had high homology with other NAC transcription factors. The structural function domain of the encoded amino acids was analyzed by using DNAMAN software. Compared with *Quercus lobata*, *Suaeda liaotungensis*, *Durio zibethinus*, *Chenopodium quinoa*, *Herrania umbratica*, *Camellia sinensis*, *Theobroma cacao*, *Vitis quinquangularis* and *A. thaliana*, the N-terminal of *PlNAC2* protein had a complete NAM (no apical meristem) characteristic domain and this sequence had a typical NAM conserved domain consisting of 155 amino acids between amino acids 6–161. This domain contained 5 sub-domains (A–E), while the C-terminal sequence had a transcriptional regulation domain with high diversity (Fig. 3). It could be inferred that *PlNAC2* belonged to the NAC family.

Table 1: Primers used for *PlNAC2* isolation and expression analysis

Gene name	Primer name	Sequence (5'→3')	Note
PlNAC2	*PlNAC2*-F	CATTCTTCGCTTCCAGAG	Full-length clone
	PlNAC2-R	CCGAAATCCTAAGACTAACA	
PlNAC2	*PlNAC2*-qRT-F	TTCAGATTTCATCCGACG	qRT-PCR
	PlNAC2-qRT-R	TTTCCCCATAAAGAGCCA	
PlActin	*PlActin*-qRT-F	GTTGCCCTTGATTACGAG	qRT-PCR
	PlActin-qRT-R	CAGCTTCCATTCCGATTA	

Table 2: Software used for bioinformatics analysis

Software	Website	Purpose
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Assemble sequences
ORF Finder	http://www.ncbi.nlm.nih.gov/gorf/gorf.html	Find ORF
MEGA 5.0	/	Construct phylogenetic tree
DNAMAN	/	Multiple sequence alignment
ProtParam	https://web.expasy.org/protparam/	Predict protein property
SignalP 5.0 Server	http://www.cbs.dtu.dk/services/SignalP/	Predict signal peptide
SOPMA	https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html	Predict secondary structure of protein
ProtScale	https://web.expasy.org/cgi-bin/protscale/protscale.	Predicting hydrophilicity
SWISS-MODEL	https://swissmodel.Expasy.org/workspace/	Predict tertiary structure of protein

Moreover, the sequence contained two nuclear localization signal sequences, which led to speculate that *PlNAC2* protein was located in the nucleus (Fig. 2). *PlNAC2* protein was longer than *Q. lobata* (293 aa), *S. liaotungensis* (302 aa), *D. zibethinus* (298 aa), *C. quinoa* (304 aa), *H. umbratica* (303 aa), *C. sinensis* (301 aa), *T. cacao* (296 aa), *V. quinquangularis* (294 aa) and *A. thaliana* (289 aa).

MEGA 5.0 software was used to construct a phylogenetic tree of *PlNAC2* protein and other protein sequences including *QlNAC2* (XP_030949572.1), *SlNAC2* (AGZ15313.1), *DzNAC2* (XP_022740811.1), *CqNAC2* (XP_021772603.1), *HuNAC2* (XP_021274745.1), *CsNAC2* (XP_028092031.1), *TcNAC2* (XP_007048529.2), *VqNAC2* (ALM02085.1) and *AtNAC2* (NP_171677.1) (Fig. 4). Among them, *PlNAC2* had a high homology with *QlNAC2*, which indicated that *PlNAC2* and *QlNAC2* were relatively closely related in evolution.

ProtParam was used to analyze the amino acid sequence encoded by *PlNAC2*, and we found that its molecular formula was $C_{1621}H_{2460}N_{426}O_{471}S_{16}$, the number of amino acid residues was 313; the relative molecular mass was 35964.90 Da, the theoretical isoelectric point was 8.89; and there were 34 amino acid residues (Asp + Glu) were negatively charged, and 40 amino acid residues (Arg + Lys) were positively charged. The instability coefficient was 45.51, which suggested that it was an unstable protein (< 40, protein is stable), and the aliphatic coefficient was 56.39. The total hydrophilic coefficient was -0.783.

SignalP 5.0 Server was used to analyze the signal peptide of *PlNAC2* protein. As shown in Fig. 5 that no signal peptide existed in *PlNAC2* protein. And TMHMM 2.0 Server was used to analyze the transmembrane domain of *PlNAC2* protein. As shown in Fig. 6, there was no obvious transmembrane domain in *PlNAC2* protein. The online software SOPMA was used to infer the secondary structure of *PlNAC2* protein. The results showed that this protein was mainly random coil (66.13%), and contained

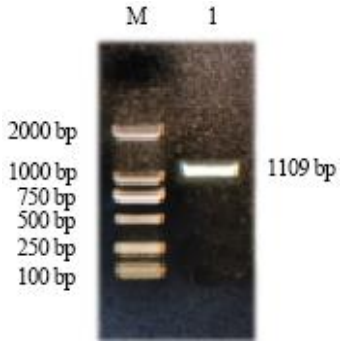

Fig. 1: Amplification of *PlNAC2*
M: 2 kb DNA Marker; 1: *PlNAC2* cDNA result

19.17% alpha helix, 2.56% beta turn and 12.14% extended strand and other structures (Fig. 7). The online software ProtScale was used to predict the hydrophobicity/hydrophilicity of the amino acid sequence of *PlNAC2* protein. The results showed that the proportion of hydrophilic amino acids in the protein was greater than proportion of hydrophobic amino acids. It could be inferred that this protein was hydrophilic (Fig. 8).

The SWISS-MODEL workspace was used to simulate the tertiary structure of *PlNAC2* protein. The prediction result showed that the similarity between *PlNAC2* protein sequence and the template sequence was 0.54, and the identity was 70.73% (Fig. 9). It could be inferred that the protein was a NAC protein, which was consistent with the target gene.

Tissue distribution of *PlNAC2* in herbaceous peony

qRT-PCR (quantitative real-time PCR) was used to detect the distribution of *PlNAC2* during the growth and development of herbaceous peony and different tissues. And the results showed that *PlNAC2* existed in the whole process

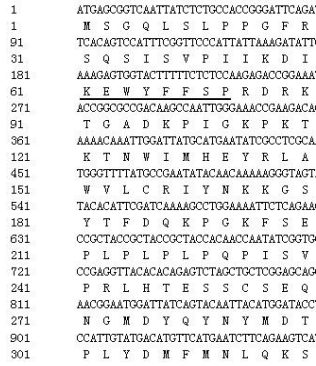

Fig. 2: Nucleotide and amino acid sequence of *PlNAC2*
Putative nuclear localization signal are shown by underline

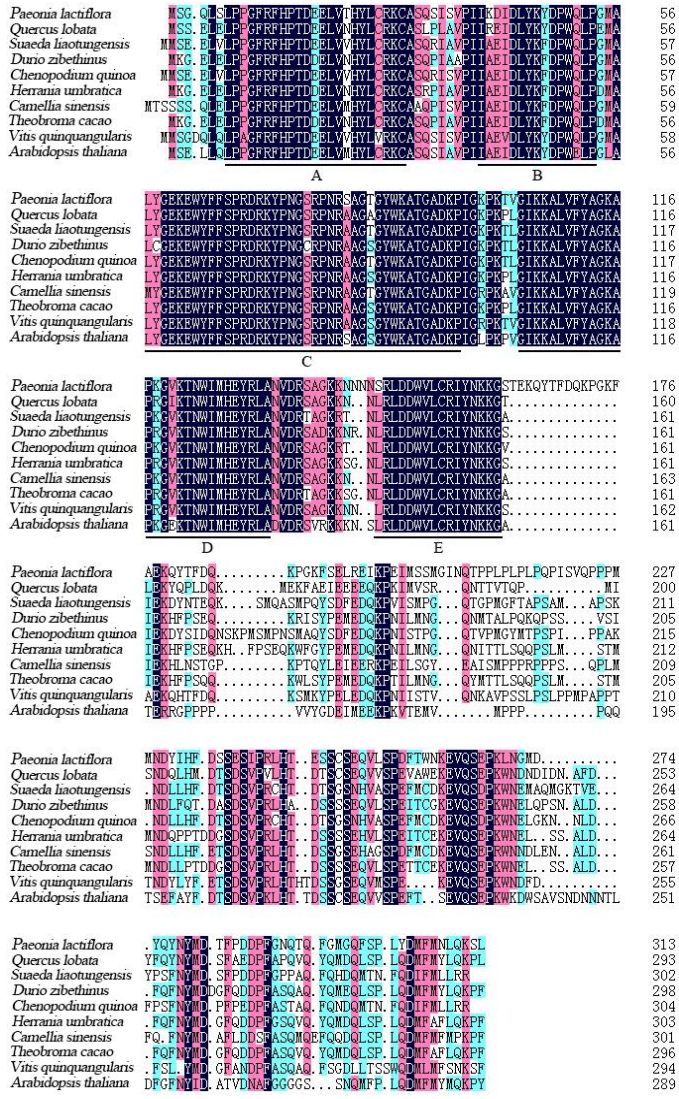

Fig. 3: Alignments of NAC domain between *PlNAC2* and other NAC proteins
Conserved sub-domains A to E are shown by underline in the NAC domain

of stem growth and development in 'Xixia Yingxue'. The expression of *PlNAC2* gradually decreased with its development. Among different tissues in 'Hongyan Zhenghui', *PlNAC2* was highly expressed in stems, followed by leaves and roots, and was the lowest in flowers (Fig. 10).

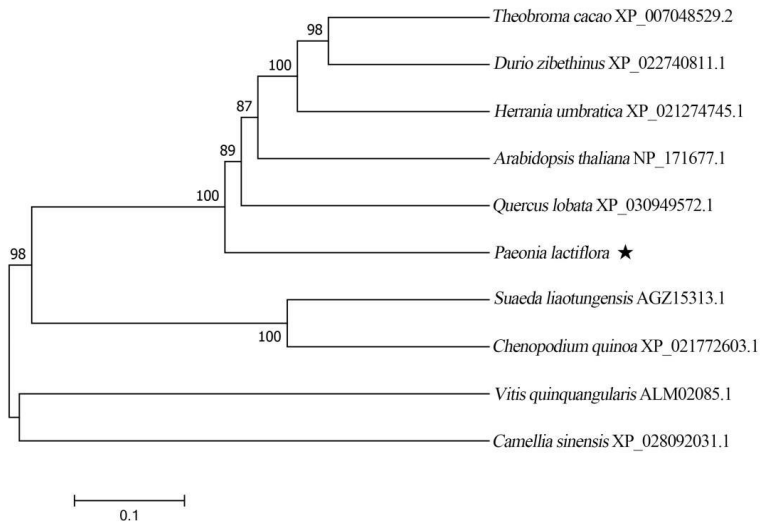

Fig. 4: Phylogenetic tree of *PlNAC2* and some other NAC proteins

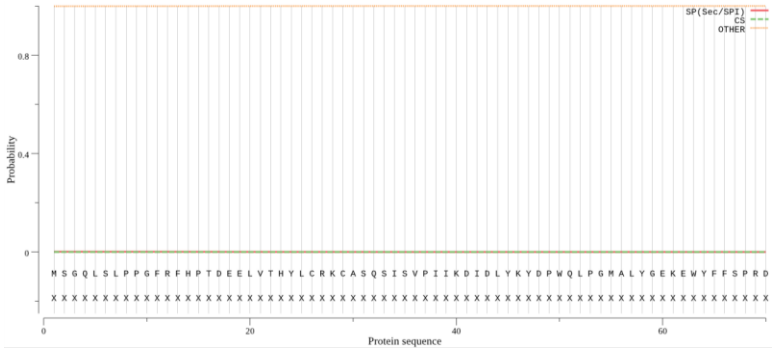

Fig. 5: Prediction of signal peptide of *PlNAC2* protein

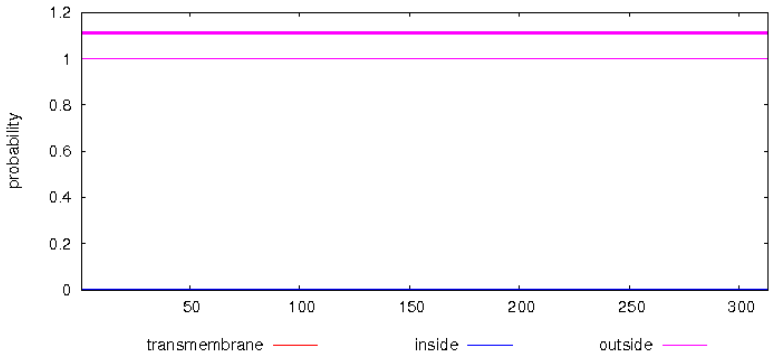

Fig. 6: Prediction of the transmembrane domain of *PlNAC2* protein

Discussion

This study evaluated *PlNAC2* and its distribution in the tissues of herbaceous peony to determine whether *PlNAC2* plays a role in the formation of SCW. Based on the transcriptome data (Xia 2019), the full-length sequence of *PlNAC2* was obtained by RT-PCR. It proved that *PlNAC2* product was 1,109 bp in size and encoded 313 amino acids. The relative molecular mass was 35964.90 Da and the theoretical isoelectric point was 8.89. It was an unstable hydrophilic protein without signal peptide and transmembrane domain.

NCBI website was used for analysis and multiple sequence alignment. The results showed that the amino acid sequence of the conserved domain of *PlNAC2* had high homology with other NAC transcription factors. Among them, *PlNAC2* had the highest homology with *QlNAC2*. In addition, the N-terminal of *PlNAC2* protein

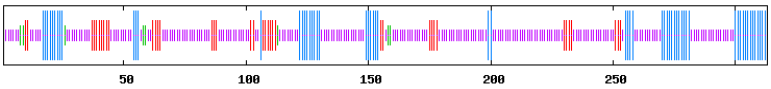

Fig. 7: Prediction of the secondary structure of protein of *PlNAC2* protein
Blue: Alpha helix; Red: Extended strand; Green: Beta turn; Purple: Random coil

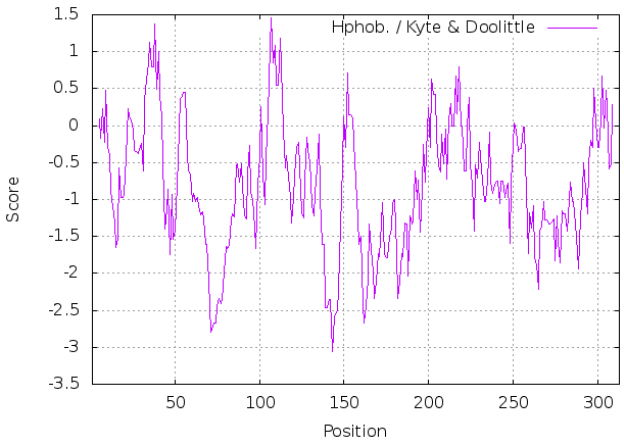

Fig. 8: Hydrophilicity/hydrophobicity analysis of *PlNAC2* protein

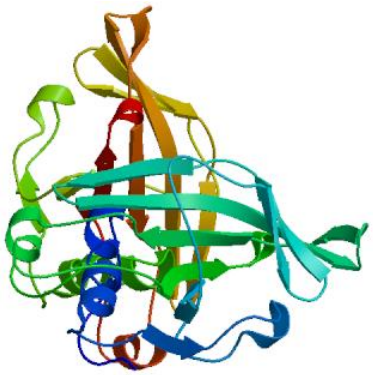

Fig. 9: Prediction of the tertiary structure of protein of *PlNAC2* protein

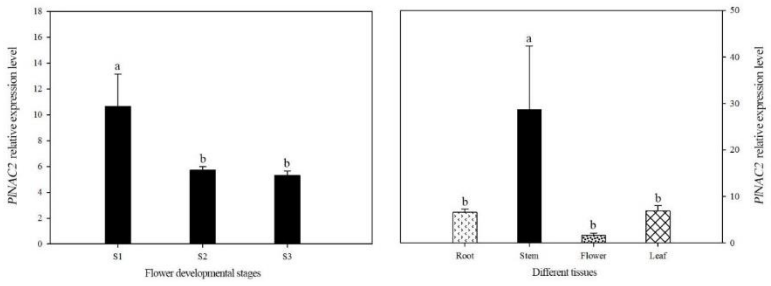

Fig. 10: The expression patterns of *PlNAC2*
S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = pigmented stage, S3 (Stage 3) = full-flowering stage. The values represented mean ± SE, and different letters marked significant differences ($P < 0.05$)

had a complete NAM characteristic domain which meant that *PlNAC2* belonged to the NAC family. *PlNAC2* protein contained two nuclear localization signal sequences. It was initially determined that this gene was located in nucleus.

The N-terminal domain of the NAC family

transcription factors is highly conserved and generally consists of about 160 amino acid residues, including 5 sub-conserved structural regions (A–E) where A, C and D are highly conserved domains, while B and E are not highly conserved (Chen *et al*. 2015). *PlNAC2* had a typical NAM conserved domain consisting of 155 amino acids between the N-terminal amino acids 6–161, and this domain also contained 5 sub-domains (A–E). In addition, the C-terminal regulation region of NAC protein had a high degree of variability and specificity, conferring a variety of roles in transcription activation regulation (Wang and Dane 2013).

The NAC family transcription factors are plant-specific transcription regulation factors. Multiple NAC genes play a key role in the biosynthesis of SCW in fiber cells. VND (vascular-related NAC domain), SND (secondary wall-associated NAC domain) and NST (NAC secondary walls thicken promoting factor) are three important NAC members known to participate in the regulation of SCW synthesis (Zhou *et al*. 2014). In addition, some NAC transcription factors (such as *SND1*, *VND6* and *VND7*) that regulate transcription at higher network levels can be used as transcription switch factors to regulate the SCW synthesis of fiber cells and ductal cells in downstream transcriptional networks (Mitsuda *et al*. 2005; Huang and Li 2016). *CpSND1* isolated from *Crataegus pinnatifida* was closely homologous to *AtSND1*. The *CpSND1* up-regulated SCW biosynthesis genes and induced SCW formation in over-expressing *Arabidopsis* (Chen *et al*. 2018). In addition, some NAC transcription factors play a role in inhibiting the SCW formation. When *XND1* (xylem NAC domain) was up-regulated in fiber cells of *Arabidopsis*, the SCW formation was inhibited by regulating the activity of *NST1*. At the same time, over-expression of *XND1* might lead to short plants and loss of xylem ducts (Zhao *et al*. 2008; Zhang *et al*. 2020). *GhNAC1* in cotton had high homology with *NSTI* and *NST3*. It was specifically expressed in cotton fibers and gradually increased during the thickening period of SCW, while *GhFSN5* (fiber secondary wall-related NAC domain) played a negative regulatory role and inhibited SCW formation (Li 2011; Sun *et al*. 2020).

We used qRT-PCR to detect the spatio-temporal expression pattern of *PlNAC2* in herbaceous peony, and the results showed that *PlNAC2* existed in the entire process of stem growth. The expression level of *PlNAC2* was the highest in stems, followed by leaves and roots, and the lowest expression level was in flowers, and its expression level in stems decreased with the growth of plants, and its relative expression level was 10.66 at S1, while in S2 and S3 were 5.73 and 5.31, respectively. This result was similar to the expression patterns of *XND1* and *AtNAC012*. Thus *PlNAC2* played a down-regulating function and inhibited the SCW formation in herbaceous peony (Ko *et al*. 2007; Zhao *et al*. 2008).

Conclusion

PlNAC2 was successfully cloned and its expression patterns were studied. *PlNAC2* had 1,109 nucleotides and encoded 313 amino acids. It existed throughout the growth and development of the stem in herbaceous peony, and its expression gradually decreased with the growth of the stem. Therefore, *PlNAC2* acted as a negative regulator in the SCW formation. The results improve our understanding of how NAC family transcription factors regulate SCW formation. Whether *PlNAC2* participates in stress resistance and other functions needs further research. Cloning of *PlNAC2*, analysis of the properties and expression patterns of *PlNAC2* protein in this study will help further research and application about *PlNAC2*.

Acknowledgements

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Author Contributions

Jun Tao and Daqiu Zhao designed the experiments, Yuting Luan and Yuhan Tang performed the experiment, Xin Wang processed the data, Yuting Luan wrote and revised the paper.

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