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Cloning and Expression Analysis of *NAC2* Gene of Potato (*Solanum tuberosum L*)

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Abstract NAC is one of the unique transcription factor families in plants, which plays an important regulatory role in response to abiotic stress. In this study, a NAC transcription factor gene *StNAC2* was cloned in potato 'Yunshu 505' by RT-PCR. Sequence analysis showed that the ORF of *StNAC2* gene was 876 bp, encoding 291 amino acids, including a conserved Nam domain with a molecular weight of 33.585 12 kD, a theoretical isoelectric point of 7.04, an instability coefficient of 50.33 and an average hydrophilic number of -0.660. Protein prediction results showed that *StNAC2* protein is an unstable hydrophilic protein with no signal peptide and transmembrane domain, which can be expressed in both cytoplasm and mitochondria. Homology comparison and phylogenetic tree analysis showed that *StNAC2* gene is closely related to *Capsicum annuum* and *Nicotiana tab*acum. Spatio-temporal expression results showed that the expression of *StNAC2* gene was different in tissue, the expression of *StNAC2* gene was higher in leaves than in stems. The expression of *StNAC2* gene was induced under cadmium stress, and reached the maximum value at 50 mg/kg, but decreased significantly after being treated with 100 mg/kg cadmium, indicating that the expression of *StNAC2* gene was inhibited under high concentration stress and *StNAC2* gene could involve in cadmium stress. The results lay the foundation for dissecting the molecular mechanism of *StNAC2* gene involved in Cd stress response.

Keywords Potato (*Solanum tuberosum*); *StNAC2*; Transcription factor; Gene cloning; Expression analysis

Cadmium stress has significant effects on plant growth and development, yield, and quality (Ismael et al., 2019). When plants are subjected to stress, they will start the defense regulation of adverse environment by regulating the expression of related genes. Transcription factors can regulate the expression of downstream genes by binding to the cis-acting elements of specific genes, thus enhancing the tolerance of plants to stress (Chen et al., 2019). It has been found that NAC transcription factor (NAM, ATAF1/2, CUC2) is one of the plant-specific regulatory proteins (Zhu et al., 2014), which plays an important role in plant growth and development processes such as seed germination, lateral root formation, leaf senescence, cellulose synthesis, and secondary cell wall growth (Balazadeh et al., 2010; Yang et al., 2011a; Zhao et al., 2014). Overexpression of *PopNAC122* gene in poplar (*Populus L*) reduced height growth and showed reductions in cell size and number (Grant et al., 2010). While the overexpression of *MpSNAC67* gene in banana (*Musa nana Lour*) showed reductions in leaf area of transgenic banana lines (Tak et al., 2018). At the same time, some studies have shown that overexpression of *TaNAC47* in *Arabidopsis thaliana* can improve the tolerance of transgenic plants to drought, salt and freezing stress (Zhang et al., 2015). Both *SlNAC1* and *SLNAM1* in tomato (*Lycopersicon esculentum*) were induced by salt stress (Yang et al., 2011b). Overexpression of *CmNAC14* in melon (*Cucumis melo* L.) increased the sensitivity of melon seedlings to salt stress and inhibited the growth of melon seedlings under salt stress (Wei et al., 2016). By constructing *HaNAC2* transgenic tobacco and studying its biological functions, it was found that the expression of *HaNAC2* increased the resistance of transgenic tobacco to drought, salt and cold stress (Ren, 2016). The expression analysis

of *StNAC72* gene in potato after drought and rewatering showed that *StNAC72* gene may be involved in the signal transduction process of drought and water stress (Gong et al., 2016). The analysis of NAC transcriptome data of potato by high-throughput sequencing showed that *StNAC 072* and *StNAC 101* were orthologs of stress response *Arabidopsis thaliana* to dehydration response 26 (RD26) (Singh et al., 2013). It has also been reported that the regulation of plants in osmotic stress was controlled by regulating the expression of *StNAC262* gene in potato (Zhang et al., 2018). Although NAC transcription factor has been widely studied in different crops, the response of this gene to heavy metals is still unclear.

In this study, a NAC transcription factor gene *StNAC2* was excavated and cloned from the previous transcriptome sequencing data of our group using potato 'Yunshu 505' as the material. The coding sequence characteristics of *StNAC2* were analyzed, and the gene expression patterns in different tissues and different concentrations under Cd stress were studied, so as to provide a theoretical basis for revealing the molecular mechanism of *StNAC2* response to stress in potato.

1 Results and Analysis

1.1 Cloning and sequence analysis of *NAC2* gene of potato (*Solanum tuberosum* L)

Potato leaf cDNA was used as template for PCR amplification. The amplified products were detected by 1% agarose gel electrophoresis, and the segment of about 800 bp was obtained (Figure 1), which was consistent with the expected. The target segment was recovered and purified, ligated with pMD19-T vector, and transformed into competent cells of *E. coil* DH5α. The positive clones of the transformed products were screened by colony PCR and sent to Sangon Biotech (Shanghai) for sequencing. Sequencing analysis showed that there was a complete ORF with a length of 876 bp encoding 291 amino acids (Figure 2). There was a highly conserved NAM domain between the 22nd and 390th amino acids at the N-terminal, indicating that *StNAC2* belonged to the NAC family transcription factor. ProtParam predicted the physical and chemical properties of StNAC2 protein were as follows: the relative molecular weight of StNAC2 protein was 33.58 512 kD, the theoretical isoelectric point (pI) was 7.04, the molecular formula was $C_{1513}H_{2297}N_{397}O_{443}S_{14}$, the fat coefficient was 63.68, and the instability coefficient was 50.33. StNAC2 was an unstable hydrophilic protein with the average hydrophilicity (GRAVY) of -0.660. Subcellular localization predicted that *StNAC2* was mainly located in the cytoplasm and mitochondria, and there was no signal peptide and transmembrane domain. It was speculated that the protein was a non-secretory protein.

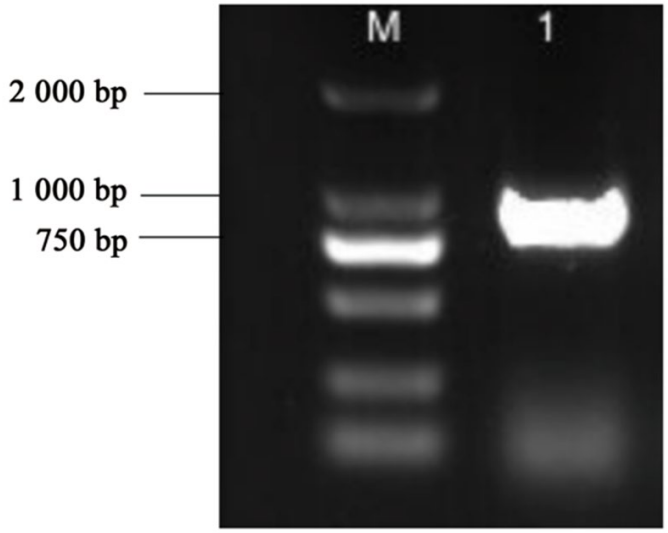

Figure 1 *StNAC2* PCR amplification product
Note: M: DL 2000 Marker; 1: PCR product

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1       ATGGTGGAATTGCAATTTCCTGCTGGATTTCGATTCCATCCAACTGATGAAGAGCTTGTG
1        M  V  E  L  Q  F  P  A  G  F  R  F  H  P  T  D  E  E  L  V
61      ATGCACTATTTATGTCGAAAATGCGCCTCACAGCCGATTGCTGTTCCGATTATAGCTGAA
21       M  H  Y  L  C  R  K  C  A  S  Q  P  I  A  V  P  I  I  A  E
121     ATTGACCTCTACAAGTATAATCCATGGGATCTACCTGATTTGGCTTTGTATGGTGAGAAA
41       I  D  L  Y  K  Y  N  P  W  D  L  P  D  L  A  L  Y  G  E  K
181     GAGTGGTATTTCTTTTCGCCGCGAGATCGGAAGTATCCGAACGGTTCACGGCCGAATCGA
61       E  W  Y  F  F  S  P  R  D  R  K  Y  P  N  G  S  R  P  N  R
241     GCTGCGGGAAATGGATATTGGAAGGCGACGGGTGCGGATAAGCCGATTGGTCGTCCAAAA
81       A  A  G  N  G  Y  W  K  A  T  G  A  D  K  P  I  G  R  P  K
301     TCGATGGGAATTAAGAAAGCTTTGGTGTTTTACGCAGGCAAAGATCCGAAAGGAGAAAAA
101      S  M  G  I  K  K  A  L  V  F  Y  A  G  K  D  P  K  G  E  K
361     ACAAATTGGATTATGCACGAATATCGACTTGCTCATGTTGATCGATCTGCTCGTAACAAG
121      T  N  W  I  M  H  E  Y  R  L  A  H  V  D  R  S  A  R  N  K
421     AACAATAGCTTAAGACTTGACGATTGGGTTTTATGTCGAATCTACAATAAGAAGGGTACA
141      N  N  S  L  R  L  D  D  W  V  L  C  R  I  Y  N  K  K  G  T
481     GTTGAGAAGAATCAACTGAATATTCGAAAAATGAATGTTGAAATGTCGCCGGCGGTGAGT
161      V  E  K  N  Q  L  N  I  R  K  M  N  V  E  M  S  P  A  V  S
541     GAAGGTGATGTAAAGCCGGAGATTATGCCGTTTTCAGTTTCAACAAAGCCGTCTTCGACG
181      E  G  D  V  K  P  E  I  M  P  F  S  V  S  T  K  P  S  S  T
601     TCCTACCACGTTTACAATGACTTCACGTACTTTGCCTCATCGGATTCTCTCCCGAAACTT
201      S  Y  H  V  Y  N  D  F  T  Y  F  A  S  S  D  S  L  P  K  L
661     CACACCGATTCCAGTTGCTCGGAGCACGTACCTTCGCCGGAGTTCACATGCGAGAAGGAA
221      H  T  D  S  S  C  S  E  H  V  P  S  P  E  F  T  C  E  K  E
721     GTTCAGAGCGAACCGAAGGTGAAACTGAGTGAGTGGGAAAAAACTGCCCTTGATTTTCCG
241      V  Q  S  E  P  K  V  K  L  S  E  W  E  K  T  A  L  D  F  P
781     TTTAATTACACAGATGCCACTACTTCTGAGTTGCAGAATTGTTATGAGCTGTCACCGCTA
261      F  N  Y  T  D  A  T  T  S  E  L  Q  N  C  Y  E  L  S  P  L
841     CAAGATATATTCATGTATCTGCAAAAGCCGTTTTAA
281      Q  D  I  F  M  Y  L  Q  K  P  F  *
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Figure 2 Nucleotide and amino acid sequence of *StNAC2*

1.2 Analysis of StNAC78 protein structure and evolution

It is predicted by SOPMA that the secondary structure of StNAC2 protein was mainly random coil structure (65.64%), followed by α-helix (16.84%) and extension strand (14.78%) (Figure 3A). SWISS-MODEL was used to construct the three-dimensional structural model, and the similarity with the database model was 74.53% (>50%), indicating that it can be used as a structural model for further analysis (Figure 3B).

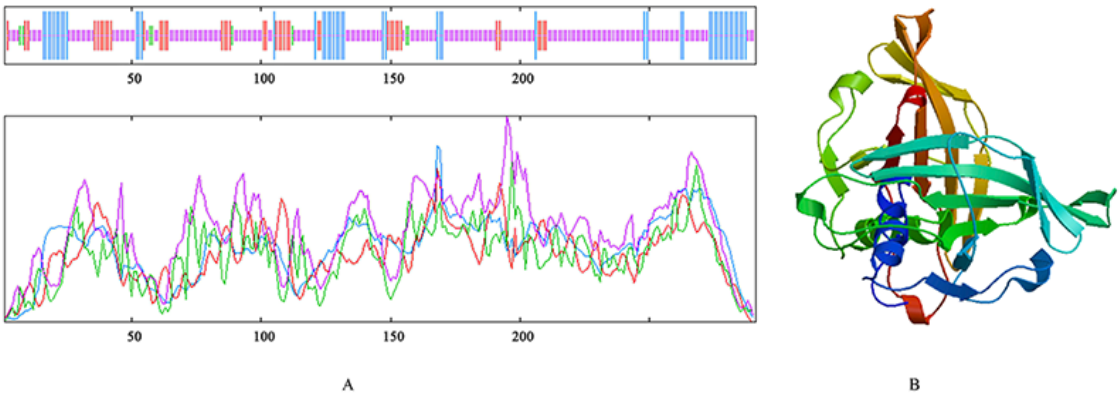

Figure 3 Prediction of secondary structure (A)and tertiary structure of *StNAC2* protein(B)

Note: Blue: α-helix; Red: Extension strand; Green: β-turn; Purple: Random coil

The amino acid sequences of StNAC2 were compared with other plants on NCBI (Figure 4), and the protein sequences with high homology among species were selected to construct the phylogenetic tree (Figure 5). The results showed that the amino acid sequences of potato StNAC2 protein were highly consistent with those of *Capsicum annuum* L, *Nicotiana tabacum* L, *Ricinus communis* L, *Petunia hybrida*, *Diospyros kaki* Thunb, *Camellia sinensis*, *Pistacia chinensis* Bunge, *Gossypium spp*, *Gossypium hirsutum* Linn, *Populus euphratica*, *Jatropha curcas* L, and *Hevea brasiliensis* published on GenBank, and the similarity was more than 70%, indicating that NAC2 protein is highly conservative in the process of species evolution. Phylogenetic tree showed that potato StNAC2 had the closest relationship with pepper protein, with a similarity of 85.67%. It was also clustered with *Nicotiana tabacum* and belonged to Solanaceae plants, indicating that the protein source homology between the same family and genus was higher, and it was speculated that the protein function was the most similar.

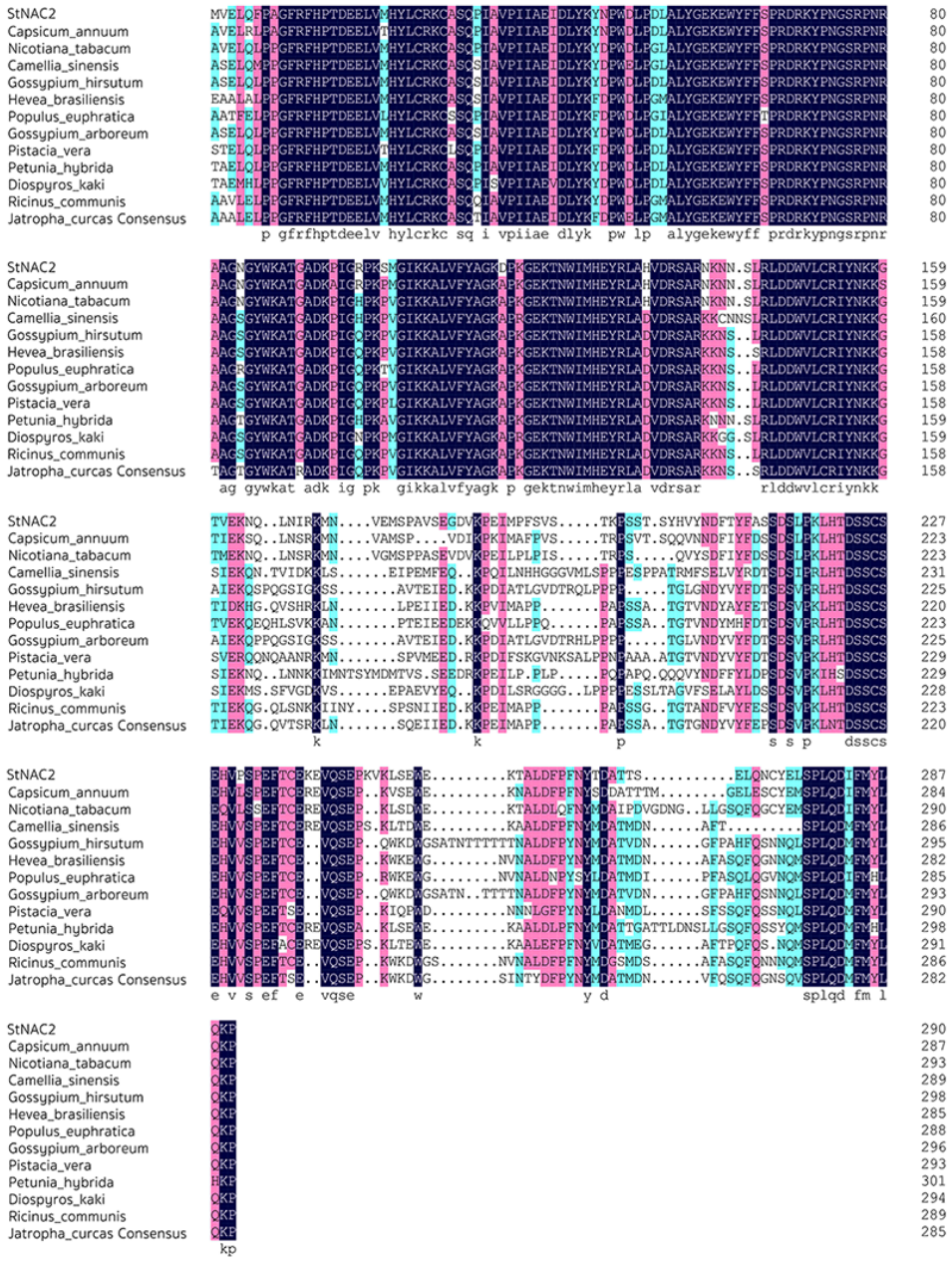

Figure 4 Multiple alignment of *StNAC2* amino acid sequence with other plants

Note: Black shadow indicates the conservative NAC domain in the sequence. Other sequences are marked by red and blue shadow according to the degree of conservatism. The consistent sequences are listed below. The first line represents *StNAC2* protein

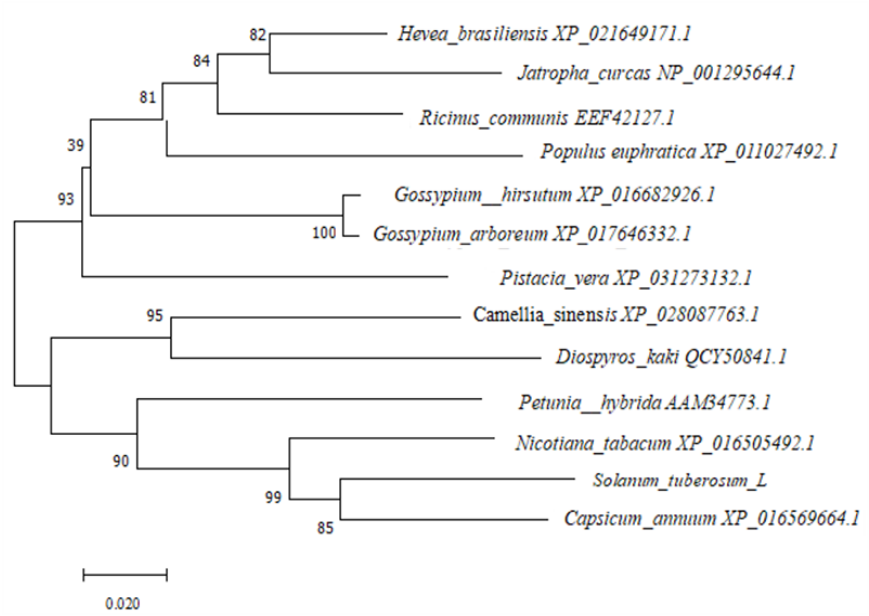

Figure 5 Phylogenetic tree of StNAC2 protein homologous sequence

1.3 Relative expression analysis of *StNAC2* gene in response to Cd stress

Under Cd stress, the expression of *StNAC2* gene in leaves was up-regulated with the increase of stress concentration and reached the maximum with the treatment of 50 mg/kg, which was 9.56 times higher than that of the control. But it was significantly down-regulated at high concentration of 100 mg/kg. The expression of *StNAC2* gene in stem was the same as that in leaves. It was significantly up-regulated under 50 mg/kg treatment, which was 1.76 times higher than that in the control, and significantly down-regulated under 100 mg/kg treatment, indicating that the expression of *StNAC2* gene increased gradually with the increase of stress concentration, but the expression of *NAC2* gene was inhibited under high concentration of 100 mg/kg stress (Figure 6). It was speculated that *StNAC2* has a certain response and regulation to Cd stress.

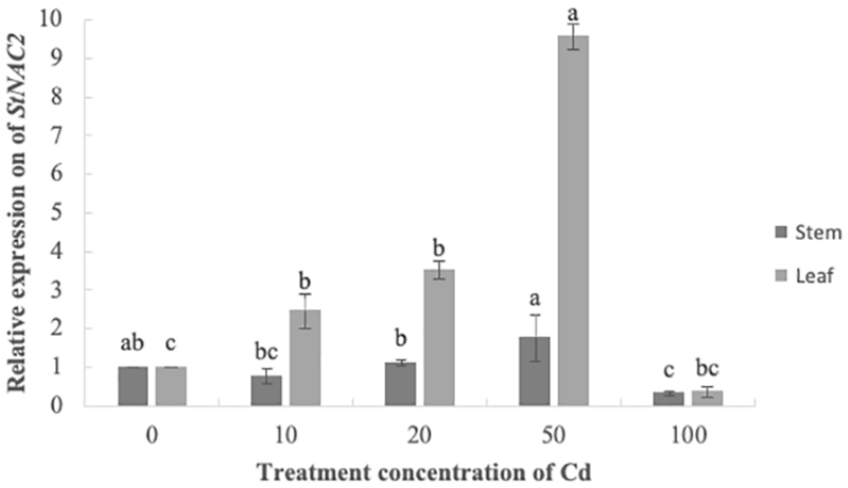

Figure 6 Expression of *StNAC2* gene in different tissues and treatments

Note: The relative expression of *StNAC2* gene in stem and leaf is significantly different(p<0.05)

2 Discussion

In this study, *StNAC2* gene was cloned from potato leaves by homologous cloning technology. Analysis results showed that the open reading frame (ORF) was 876 bp, encoding 291 amino acids, including a conserved NAM domain, belonging to the NAM subfamily of NAC family members. Prediction of subcellular localization is helpful to infer protein function (Yu et al., 2006). In this study, we predicted that StNAC2 protein was mainly

localized in cytoplasm and mitochondria through subcellular localization, and the protein did not contain signal peptide and no transmembrane domain. Through the comparison of similar amino acid sequences among species, it was found that the similarity of NAC2 protein with *Capsicum annuum*, *Nicotiana tabacum*, *Petunia hybrida*, *Diospyros kaki* Thun, *Camellia sinensis*, *Pistacia chinensis* Bunge, *Gossypium spp*, *Gossypium hirsutum* Linn, *Populus euphratica*, *Ricinus communis*, *Jatropha curcas* and *Hevea brasiliensis* was more than 70%, indicating that NAC2 protein was highly conserved. Phylogenetic tree analysis showed that StNAC2 had the highest similarity with pepper protein, with the similarity of 85.67%. StNAC2 was clustered with tobacco, which belonged to Solanaceae plants, indicating that the protein source homology was higher, and the protein function was the closest between the same family and genus. Tissue specificity analysis by fluorescence quantitative PCR showed that *StNAC2* gene was expressed in stems and leaves under Cd stress, but there was significant different. The expressions of *StNAC2* gene in stems and leaves were reached the maximum with the treatment of 50 mg/kg, which were 1.76 times and 9.56 times higher than those of the control, respectively. The expression of *StNAC2* gene decreased rapidly under 100 mg/kg treatment in stems and leaves, indicating that the *StNAC2* gene was induced by Cd stress, and the expression increased with the increase of concentration. But the gene expression was inhibited under high concentration of 100 mg/kg stress. It was speculated that *StNAC2* has a certain response and regulation to Cd stress. The results of this study were similar to those of Du et al. (2020). The expression pattern of *Secale cereale AmeNACs* in stem tissue induced by Cd was more than 5 times higher than that of the control, and the expression was significantly inhibited when the final concentration was 100 μmol/L. As one of the largest transcription factors in plant family, NAC family members are mostly inducible transcription factors (Zong et al., 2019). In recent years, it has made some progress in identifying potential stress-related genes that can improve the tolerance of plants to abiotic stress. Transgenic rice plants overexpressing *OsNAC6* showed slow growth and low reproductive yield, and good tolerance to dehydration and high salt stress, strong resistance to rice blast as well (Nakashima et al., 2007). The overexpression of tobacco *SlNAC35* promoted root growth and development under drought and salt stress, and improved resistance to bacterial pathogens (Wang et ai., 2016). The overexpression of *TaNAC2* enhanced the tolerance of Arabidopsis to drought, salt and freezing stresses (Mao et al., 2012). The expression level of *CSNAC019* gene in cucumber was significantly up-regulated under 300 μmol/L Cd stress. Based on the current studies on NAC transcription factor response to abiotic stress, most studies focused on drought resistance and salt tolerance (Hong et al., 2016), cold resistance (Zhao et al., 2016; Yang et al., 2015), heat resistance (Shahnejatbushehri et al., 2012), germination rate (Li et al., 2016), proliferation lateral root growth, leaf relative water content, cell membrane stability, total chlorophyll content, proline and soluble sugar content etc. (Pandurangaiah et al., 2014). Few studies were conducted on resistance to heavy metal stress. Therefore, the research on NAC transcription factor response to Cd stress was also particularly important.

In this study, the potato *StNAC2* gene was preliminarily analyzed, which provided basic theoretical knowledge for the follow-up development of gene function. The specific function and mechanism of action of *StNAC2* gene are still unclear. It is necessary to further verify its function and clarify its mechanism through experiments. In the later stage, overexpression vector and gene silencing vector were constructed to further study the molecular mechanism of *StNAC2* gene in response to Cd stress through genetic transformation, and further verify the regulatory mechanism and function of *StNAC2* gene in response to Cd stress.

3 Materials and Methods

3.1 Experimental materials and treatments

The seeds of potato 'Yunshu 505' were collected from the Experimental Base in Weining County, Guizhou Province (103°36'~104°45'E, 26°36'~27°26'N) and carried out pot experiment. The concentrations of Cd stress were set as 0, 10, 20, 50 and 100 mg/kg, respectively, and three pots were planted in each treatment. The pots were evenly mixed into the soil a week to be deposited, the potato was planted. The daily water and fertilizer management was carried out, and the plants grew until the functional leaves were fully expanded for sampling. Then it was quickly frozen with liquid nitrogen and stored in an ultra-low temperature refrigerator (−80°C) for further use. Plant total RNA extraction kit, purification recovery kit, PCR amplification enzyme, DNA Marker,

Loading Buffer, PCR amplification enzyme were purchased from TransGen Biotech. *E.coli* DH5α competent cells, cloning vector pMD19-T vector and *Taq* DNA polymerase were purchased from Takara Bio (Beijing). The primers were synthesized with the help of Sangon Biotech (Shanghai).

3.2 Total RNA extraction and cDNA synthesis

The total RNA, extracted by Trizol method, was used as a template for reverse transcription into cDNA. The following components were successively added to the DEPC water-treated tubules: Total RNA 5 μL, 5 x TransScript All in-one Super Mix for PCR 4 μL, RNase-free Water 11 μL, with a total volume of 20 μL. After gently mixing and incubating at 25℃ for 10 min, 42℃ for 30 min, and 85℃ for 5 s by PCR, respectively, and the first strand cDNA was synthesized.

3.3 Cloning of *StNAC2* gene

The full-length gene and ORF sequence were found in NCBI using the NAC transcription factor coding gene ID number (LOC102585728) in transcriptome sequencing data. Primer 5.0 was used to design specific primers, fluorescent quantitative primers and *Actin* primers for amplifying the gene ORF (Table 1). Potato cDNA was used as template for PCR amplification with 25 μL PCR reaction system: cDNA 1 μL, each primer 1 μL, 2 x TransTaqPCR Super Mix II 12.5 μL, ddH_2O 9.5 μL. PCR reaction procedure was as follows: Pre-denaturation at 94℃ for 3 min, denaturation at 94℃ for 30 s, annealing at 50℃ for 30 s, extension at 72℃ for 1.5 min, 35 cycles, and finally preservation at 72℃ for 5 min, preservation at 4℃. After 1% agarose gel electrophoresis, the target band was recovered, and the PCR product was ligated to the cloning vector pMD19-T vector and transformed into *E.coli* DH5 α competent cells. After PCR identification, the positive clones were selected and sent to Sangon Biotech (Shanghai) for sequencing.

Table 1 List of PCR primer sequence

The name of the primer	Primer sequence (5′→3′)
StNAC2-F	AGTAATGGTGGAATTGCAAT
StNAC2-R	CCCCACTTAAAACGGCTT
qRT-PCR-StNAC2-F	GAGCTGCGGGAAATGGATAT
qRT-PCR-StNAC2-R	TGCCTGCGTAAAACACCAAA
Actin-F	GCTTCCCGATGGTCAAGTCA
Actin-R	GGATTCCAGCTGCTTCCATTC

3.4 Analysis of sequence biological information

The open reading frame of the cloned sequence was analyzed with the help of the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder) provided by NCBI and the amino acid sequence of *StNAC2* gene was deduced. The conserved domain of the gene was predicted with the help of the software (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) in NCBI. ProtParam (http://web.expasy.org/protparam/) was used to analyze the number of amino acids, relative molecular weight, theoretical PI value, amino acid composition, the number of positively and negatively charged residues, atomic composition, molecular formula, total atomic number, extinction coefficient, half-life, fat coefficient, instability coefficient and total average hydrophilicity of the protein. Software (http://web.expasy.org/cgi-bin/protscale/protscale.pl) was used to analyze the hydrophilicity and hydrophobicity of the StNAC2 protein. Software (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl) was used to perform the prediction of protein secondary structure. SWISS-MODEL (https://swissmodel.expasy.org/) was used to predict and draw protein tertiary structure model. DNAMAN7.0 was used for multi-sequence alignment, and MFGA7 (Neighbor-Joiningling proximity method) was used to construct the phylogenetic tree.

3.5 Expression analysis of *NAC2* gene in potato

StNAC2 fluorescent primers were designed, and *Actin* was used as reference gene (Table 1) for semi-quantitative analysis by SYBR Green method. Using 25 μL reaction system: cDNA 2 μL, forward and reverse primers 1 μL,

respectively, qPCR MasterMix 12.5 μL, ddH_2O 8.5 μL. The amplification procedure was as follows: 95°C for 3 min, 95°C for 15 s, 40 cycles, 60°C for 40 s. Three biological replicates were designed, and the expression levels of the target gene under different treatments and tissues were calculated with the help of $2^{-(\Delta\Delta ct)}$ (Bai et al., 2015). Fluorescence quantitative data were analyzed by Excel and SPSS software.

Authors' Contributions

MLL is the experimental designer and executor of this study. HGD participated in data analysis and the writing of the first draft. TWJ, HY, and LDD participated in the experimental design and the analysis of experimental results. HTB is the designer and director of the project, guiding experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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