ISSN 1021-4437, Russian Journal of Plant Physiology, 2022, Vol. 69:91. © Pleiades Publishing, Ltd., 2022.

RESEARCH PAPERS

Genome-Wide Identification, Gene Structure, and Expression Analyses of the *NtPP2C* Gene Family in *Nicotiana tabacum* in Response to Low Temperature, Salt, and Drought Conditions

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Received January 27, 2022; revised February 18, 2022; accepted February 25, 2022

Abstract—In plants, members of the large *PP2C* gene family regulate development, growth, and responses to stress exposure. While the tobacco genome is now readily available, there have been few analyses regarding the evolution, expression, and function of *PP2C* family genes in tobacco plants. Herein, a genome-wide identification strategy was used to explore *PP2C* gene structures, motifs, and expression patterns in different *Nicotiana tabacum* L. tissues, with their expression further being evaluated via real-time PCR under a range of stress conditions. In total, 183 *NtPP2C* genes were identified within the tobacco genome, and phylogenetic analyses revealed these genes to harbor similar gene structures and conserved motifs, and to be clustered within the same clade. The tissue-specific expression patterns of *NtPP2C* genes from Clade A were additionally characterized. Notably, *NtPP2C11* and *NtPP2C12* exhibited the highest levels of expression, while *NtPP2C18* and *NtPP2C19* transcripts were largely undetectable. Specific NtPP2C genes were found to be upregulated in response to low temperature, salt, and drought treatment conditions. Of these, *NtPP2C11* and *NtPP2C12* were significantly upregulated in response to these treatments, suggesting that they may play important roles in regulating plant responses to exogenous stressors. Overall, these data provide a robust foundation for further functional analyses of NtPP2C genes in tobacco.

Keywords: *Nicotiana tabacum*, genome-wide, *NtPP2C* gene family, tobacco, abiotic stress

DOI: 10.1134/S1021443722050065

INTRODUCTION

Sessile plants are exposed to a range of stressors over the course of growth and development, including abiotic stressors such as temperature changes and varying salt levels, as well as biotic stressors such as pathogens and insect bites [1, 2]. To persist under these adverse conditions, plants adopt diverse responses to mitigating and managing these different forms of stress. Reversible protein phosphorylation is integral to many key stress response signaling pathways involved in plant adaptation to particular environments. Such phosphorylation is regulated by protein kinases (PKs) and protein phosphatases (PPs), which are respectively responsible for phosphorylating and dephosphorylating target protein serine (Ser), threonine (Thr), and tyrosine (Tyr) residues [3]. PPs are broadly classified into three categories based on their substrate specificity, including Ser/Thr phosphatases (STPs), protein Tyr phosphatases (PTPs), and dual-specificity phosphatases [4]. These STPs are further subdivided according to their amino acid sequences, metal ion dependence, and sensitivity to compounds including cyclosporine A and okadaic acid into the phosphoprotein phosphatase (PPP) and phosphoprotein metallophosphatase (PPM) categories. PPPs include type 1 (PP1), type 2A (PP2A), type 4 (PP4), type 5 (PP5), type 6 (PP6), type 7 (PP7), and type 2B (PP2B) PPs, while PPMs are further subdivided according to their dependence on Mg^{2+} or Mn^{2+} into type-2C protein phosphatases (PP2Cs) and pyruvate dehydrogenase phosphatase [5].

Exhibiting a high degree of evolutionary conservation, PP2Cs are present in plants, animals, archaea, bacteria and fungi. These PPs play a wide range of roles in the context of stress response signaling and the negative regulation of stress-inducible PK cascades [6]. Up to 76 PP2C-type phosphatases have been described in *Arabidopsis thaliana*, with these proteins being clustered into 10 groups, with six failing to fit into these clusters [7]. Of these, the majority of Group A PP2Cs regulate the abscisic acid (ABA) pathway via ABA-dependent binding to the ABA receptor PYR1/PYL thus promoting SnRK2 release [8–12]. When ABA is not present, the ABA receptor does not interact with Group A PP2Cs, which instead interact with SnRK2, inhibiting its activation. Recent research

shows that PP2C in abscisic acid signalingpathway is the center of the common hormone regulatory network [13].

The *A. thaliana* Group A PP2C family consists of nine members including *ABI1*, *ABI2*, *HAB1*, *HAB2*, *AHG1*, *AHG3*/*AtPP2CA*, *HAI1*, *HAI2*, and *HAI3* [14]. The dominant mutations in ABI1 and ABI2 were identified as being ABA-insensitive through mutant screening efforts [15], and were found to function as negative regulators of the ABA signaling pathway [16]. *ABI1* and *ABI2* account for approximately half of all ABA-induced PP2C activity, indicating other PP2C genes also regulate these signaling responses [17]. The *ABI1* and *ABI2* homologs *HAB1* and *HAB2* are both ABA inducible, and *HAB1* and *HAB2* overexpression in *A. thaliana* has been found to enhance ABA tolerance, whereas the knockout of these genes yielded the opposite phenotype [18, 19]. The *ahg3* and *ahg1* mutants of the *AHG3* and *AHG1* genes were found to be hypersensitive to ABA, salt, and osmotic stress during the germination stage whereas they did not exhibit any developmental changes in mature plants [20, 21]. Of the remaining Group A PP2Cs (*HAI1*, *HAI2,* and *HAI3*), HAI1 has been found to interact with SnRK2. Single *HAI1, HAI2,* or *HAI3* mutants did not exhibit any apparent phenotypes, whereas double or triple mutants remained insensitive to ABA during the germination stage of growth despite being ABA-sensitive in the post-germination stage [22].

While there have been many studies of Arabidopsis PP2Cs, there have been relatively few analyses of this protein family in tobacco (*Nicotiana tabacum*). Recently, the *AtPP2CA* homolog *NtPP2C1* was shown to be drought-inducible, whereas its expression was suppressed by reactive oxygen species (ROS) exposure and heat stress [23]. Moreover, the overexpression of *OsBIPP2C1* in tobacco plants increased their resistance to tobacco mosaic virus (TMV) and *Phytophthora infestans* as well as their tolerance to salt and osmotic stress [24]. As a valuable model plant species, tobacco generally exhibits drought tolerance. The roles of PP2C family members in this context, however, are incompletely understood. Due to the important roles PP2Cs play in tobacco growth, development, it is meaningful to identify the members of PP2C family and characterize the member(s) responding to abiotic stresses. Our results provide a foundation for futurefunctional analysis of the PP2C gene family in stressresponses in *Nicotiana tabacum*.

MATERIALS AND METHODS

Plant materials and growth conditions. Tobacco (*Nicotiana tabacum*) 'Yunyan87' was utilized to profile NtPP2C gene expression analysis. Tobacco seeds were maintained by the Yunnan Academy of Tobacco Agricultural Sciences (Yunnan, China). Initially, a 40% bleach solution was applied to disinfect the surfaces of tobacco seeds for 10 min, after which they were washed thrice using sterile distilled water and sown in pots. Tobacco seedlings were grown in a growth chamber with a 16-h light/8-h dark photoperiod under continuous white light (75 mol/m^2 s) at 28°C day/23°C dark. Standard management practices including regular watering were employed for all seedlings. The 8-week-old tobacco seedlings were exposed to different treatments, including drought, low temperature, and salt. For drought treatment, the whole seedlings were slowly pulled out of the pots and kept under the air. Samples were harvested at 0, 1, 6, 12 h. For the low temperature treatment, the seedlings were kept at 8°C for 0, 1, 6, 12 h. For salt treatment, the seedlings were irrigated with 300 mmol/L NaCl and samples were harvested at 0, 1, 6, 12 h. When untreated plants had reached the blooming stage, samples including roots, stems, leaves, and flowers were collected and snap-frozen using liquid nitrogen. All samples for expression analysis contain three biological replicates.

Phylogenetic relationship, motif, and gene structure analyses. Putative NtPP2C protein sequences were identified using HMMER based on the China tobacco genome database V 2.0. AtPP2C sequences were downloaded from the NCBI databases. ClustalW was used to align the NtPP2C and AtPP2Cprotein sequences, with MEGA 7.0 (https://www.megasoftware.net/) being utilized to construct an unrooted phylogenetic tree via the neighbor-joining method with 1000 bootstrap replicates. cDNA and gDNA sequences for individual NtPP2C genes were compared to define gene structures (http://gsds.cbi.pku.edu.cn/). Conserved NtPP2C protein motif predictions were made using MEME (http://meme.nbcr.net/meme3/mme.html), with the InterPro database (http://www.ebi.ac.uk/interpro) being used for the functional identification thereof.

Real-time PCR. In total, SuperScript III Reverse Transcriptase (Invitrogen, USA) was used based on provided directions to generate cDNA from 2 μg of total RNA per sample in a 20 μL reaction volume with an Eppendorf Mastercycler thermocycler (Eppendorf AG, Germany) and the following thermocycler settings:predenatureat 95°C for 15 sec; followed by 40 cycles of denature at 95°C for 10 sec, mealting at 60°C for 30 sec and elongation at 70°C for 15 sec. Next, 60 μL of deionized water and 20 μL cDNA were combined, after which 1 μL of this diluted cDNA mixture was utilized for qPCR analyses performed in a 20 μL volume using a SuperRealPreMix Plus SYBR Green Kit (TIANGEN Biotech, China) based on provided directions. These reactions were conducted using an Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (ThemoFisher Scientific, USA) and the following thermocycler settings: 15 min at 95°C; 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 32 s. A melt curve (95°C for 15 s, 60°C for 1 min, 95°C for 15 s) was then used to confirm reaction specificity, with one melt peak being evident for each sample. Relative gene expression (log2FC) was

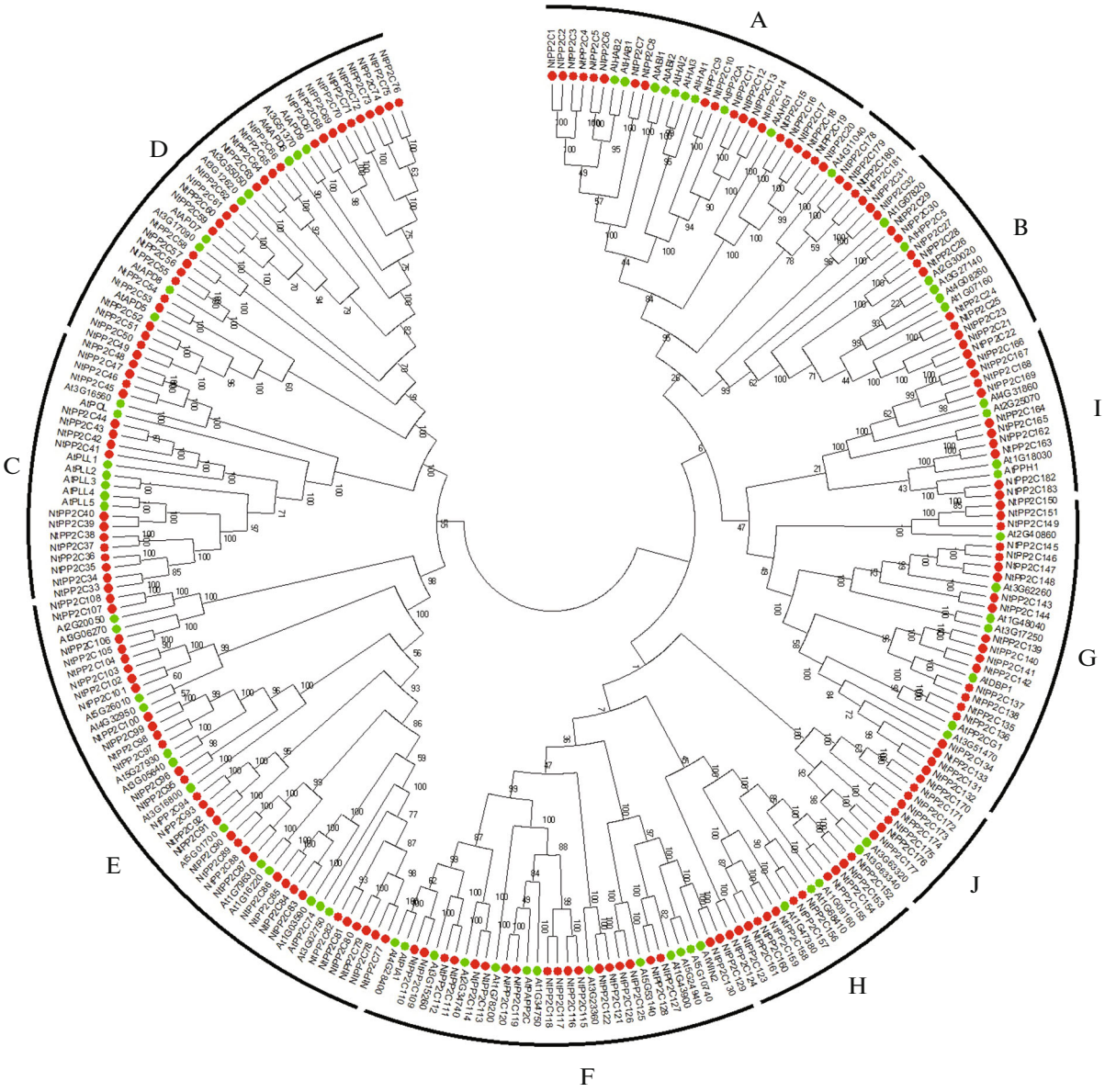

Fig. 1. Phylogenetic analysis of PP2C proteins from *Arabidopsis* and cultivated tobacco. NtPP2C proteins were classified into 11 clades (clades A–K), which were differentiated by different colors. AtPP2C and NtPP2C proteins were labeled with green and red circles, respectively.

calculated via the $2^{-\Delta\Delta CT}$ approach, with 26S rRNA gene being used for normalization. CT values were averaged from three technical replicates. Primers used in these analyses are compiled in Table S1.

RESULTS

Tobacco NtPP2C Phylogenetic Relationships

In total, 183 *NtPP2C* genes were identified in the most recent version of the tobacco genome by HMMER (see Supplemental protein sequences S1, S2, S3), with these genes being numbered *NtPP2C1–NtPP2C183* based on an evolutionary tree constructed using the *AtPP2C* and *NtPP2C* proteins. To better understand the evolutionary relationships among *NtPP2C* proteins, an unrooted phylogenetic tree was constructed using the MEGA7 software via the neighbor-joining method (1000 bootstrap replicates). This tree incorporated 76 AtPP2C [7] and 183 NtPP2C proteins, which were clustered into 11 clades (clade A-K; Fig. 1). Most of these clades included between 4 and 32 NtPP2Cs, with 40 NtPP2Cs genes in clade E, accounting for 21.9% of the total number of NtPP2Cs. In addition, clade E contained 28 AtPP2C proteins. In contrast, clade K contained the fewest PP2C proteins, incorporating just four tobacco PP2Cs. While PP2C proteins

Table 1. The distribution of PP2Cs in differentspecies

Sugroup of PP2C	Arabidopsis	Rice	*Medicago truncatula*	*Triticum aestivum*	Strawberry	*Brachypodium distachyon*	*Gossypium hirsutum*	*Brassica rapa*	Tobacco
A	9	10	9	15	10	8	18	15	20
B	6	3	3	3	3	4	10	7	12
C	7	6	7	6	4	5	12	10	16
D	9	11	19	10	8	9	38	16	28
E	13	12	12	12	9	8	27	25	32
F	13	11	14	12	6	11	23	25	22
G	6	8	6	6	7	6	17	9	21
H	3	7	7	8	3	7	12	6	10
I	2	12	4	7	2	7	10	4	8
J	2	1	1	1	0	1	0	1	8
K	0	6	4	9	7	12	10	6	0
L	0	0	3	2	2	2	4	4	0
M	0	0	0	1	0	3	0	0	0
Single branch	6	3	5	4	1	3	0	3	6
Total number	76	90	94	96	62	86	181	131	183

were relatively evenly distributed in tobacco and Arabidopsis, tobacco PP2Cs were more closely associated with other tobacco proteins, rather than with Arabidopsis proteins. Based on this analysis, the tobacco genome harbors more PP2C members than most other plants which generally contain no more than 100PP2C genes, as is the case for *Arabidopsis thaliana* (76 PP2Cs) [7], rice (90 PP2Cs) [25], alfalfa (94 PP2Cs) [26], *Riticum aestivum* L. (95 PP2Cs) [27], strawberry (62 PP2Cs) [28], and *Brachypodium distachyon* (86 PP2Cs) [29], although both soybean and *Brassica napus* harbor more than 100 PP2C family members [30, 31] (Table 1).

NtPP2C Gene Structures

To explore *NtPP2C* gene conservation and diversity, *NtPP2C* gene CDS and gDNA sequences were compared to assess gene structures (Fig. 2). There was substantial variability with respect to the number of introns observed within *NtPP2C* genes, with the majority from clade A-E harboring fewer than four introns, while most *NtPP2C* genes from clades F-K contained more than five introns. Most *NtPP2C* genes within the same clade exhibited similar splice forms and exons of similar length. *NtPP2C* genes that were closely related to one another harbored introns of similar length. Overall, these results suggested that *NtPP2C* genes are highly evolutionarily conserved.

NtPP2C Protein Conserved Motif Analysis

To gain further insight regarding the evolution of NtPP2C proteins, conserved motifs within these proteins were analyzed using MEME (Fig. 3). This approach revealed that all NtPP2Cs harbored the key PP2C functional domain, which consisted of motifs 1, 2, 3, 4, 6, and 7. Of the 10 identified motifs, motifs 1–4 and 6 were the most highly conserved, as they were present in all analyzed NtPP2Cs other than NtPP2C7, NtPP2C105–108, NtPP2C121–124, NtPP2C131, NtPP2C134, and NtPP2C167. Motif 5 and motif 7 were primarily present within members of clade C and clade D, while motif 8 was restricted to clade D. Motifs 9 and 10 were distributed across all clades other than clade C and clade D.

Expression Profiles of Clade A NtPP2C Genes under Stresses

Plant PP2C genes have been reported to be highly responsive to a range of stressors. For example, Arabidopsis PP2C genes from the well-studied clade A are known to be highly stress-inducible. To assess the possible functional roles of tobacco clade A *NtPP2C*, we exposed tobacco plants to various stressors and assessed *NtPP2C* expression patterns via qPCR (Fig. 4). This analysis revealed that most clade A *NtPP2C* genes exhibited similar expression patterns in response to low-temperature exposure. At 1 h after cold treatment, the expression of all genes were dramatically down-

regulated, then up-regulated at 6 h with higher expersion levels than 0 h for most genes tested. Following salt treatment for 1 h, all clade A *NtPP2C* genes were significantly downregulated with the exception of *NtPP2C10*, which was slightly downregulated. Following salt treatment for 6 and 12 h, however, these clade A *NtPP2C* genes were upregulated, with *NtPP2C1*, *NtPP2C3*, *NtPP2C4,* and *NtPP2C15* being slightly upregulated and having recovered to baseline levels, whereas *NtPP2C5–NtPP2C14,* and *NtPP2C16–NtPP2C18* were markedly upregulated. Under these salt stress conditions, *NtPP2C3–NtPP2C14* genes were upregulated following treatment for 1 h or 6 h, but were downregulated after 12 h. *NtPP2C1*, *NtPP2C15*, and *NtPP2C16* remained slightly downregulated following treatment. *NtPP2C17* and *NtPP2C18* genes were significantly upregulated up to 12-fold after salt treatment. Under drought conditions, most clade A *NtPP2C* genes were downregulated after 1 h, after which they were upregulated. These increases in transcript levels for many of these genes, including *NtPP2C3,* and *NtPP2C5–NtPP2C10,* were more pronounced under drought conditions as compared to levels following salt exposure after 1 h. Certain *NtPP2C* genes exhibited reduced expression at 6 h post-treatment, including *NtPP2C12–NtPP2C18.*

NtPP2C Tissue-Specific Expression Patterns

To gain additional insight regarding the potential functional roles of *NtPP2C* genes, an analysis of clade A *NtPP2C* family member expression in root, leaf, stem, and flower tissue samples from tobacco plants was performed (Fig. 5). *NtPP2C11* and *NtPP2C12* were found to be the most highly expressed of the analyzed genes, while *NtPP2C1* and *NtPP2C8* exhibited intermediate expression levels, and *NtPP2C17* and *NtPP2C18* were expressed at the lowest levels. *NtPP2C1* was primarily expressed in stems and leaves, while the *NtPP2C8* gene was expressed at high levels in leaves and flowers, and *NtPP2C9* and *NtPP2C10* were specifically expressed in flowers. *NtPP2C13–NtPP2C15* were expressed at relatively high levels in roots and flowers, whereas *NtPP2C16* was abundantly expressed in stems and flowers. Owing to their high degree of sequence similarity, we were unable to differentiate between *NtPP2C1* and *NtPP2C2* transcripts. *NtPP2C19* of *NtPP2C20* expression levels were below the detection threshold.

Fig. 2. Tobacco *NtPP2C* family gene structures. Exon-intron analyses of tobacco *NtPP2C* genes were conducted using GSDS 2.0. Exons and introns are respectively shown using rectangles and lines. Exon as and introns for each gene are shown to scale.

DISCUSSION

Plants are exposed to a wide range of abiotic stressors including low temperatures, drought, and salt, all of which can influence yields. To mitigate these stressors, plants employ a variety of survival strategies [32–34]. PP2C family, a key factor in ABA signaling pathway, is integral to many plant stress responses to salt, low temperatures, and drought conditions [6, 13, 26, 35–40]. While many studies of plant PP2Cs have been performed to date, tobacco PP2Cs have not been examined in detail. In this analysis, we comprehensively analyzed tobacco PP2Cs, with a focus on their identification, phylogenetic analysis, conserved motifs and

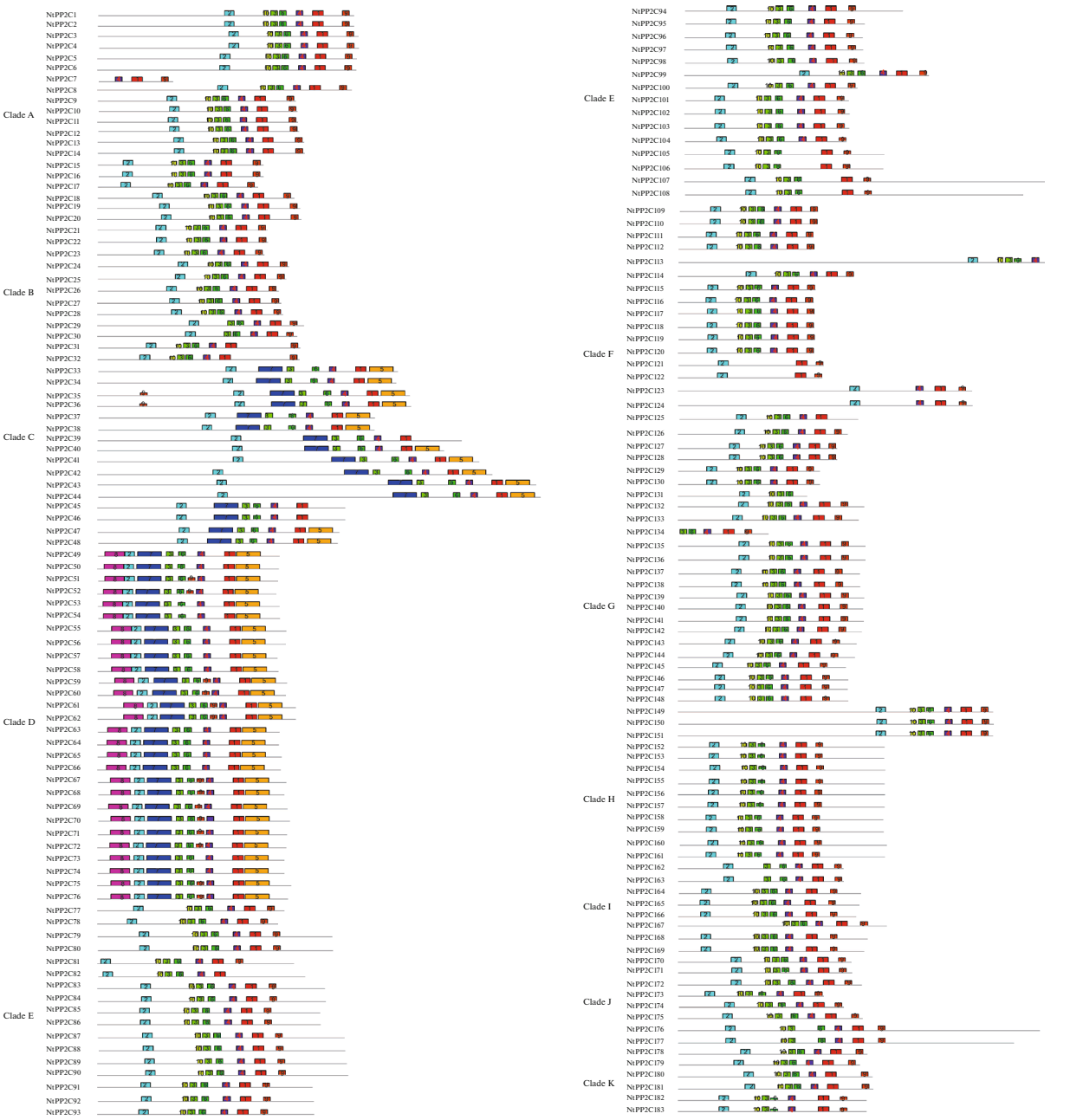

Fig. 3. Conserved tobacco *NtPP2C* gene motifs were predicted using MEME. Gray lines correspond to non-conserved sequences, while the 10 conserved motifs are indicated by different colors and numbered boxes.

gene structure identification, and expression analysis in different tissues at baseline and under stress conditions.

The PP2C gene family is among the largest plant gene families and has been studied in detail in several species, such as *Arabidopsis thaliana*, rice, alfalfa. Herein we identified 183 *NtPP2C* family genes encoded in the tobacco genome (Fig. 1). Prior studies have reported that vascular plants harbor higher numbers of PP2C family genes as compared to lower plants, suggesting that the expansion of this gene family may be associated with the environmental adaptation of these plants. The number of tobacco*NtPP2C* genes identified herein is higher than in any other plants to date, potentially owing to the genome duplication in tobacco as an allotetraploid plant. The high abundance of these *NtPP2C* genes in tobacco also suggests that these plants may have evolved to adapt to diverse environmental conditions.

Prior studies have employed classification schemes grouping PP2C proteins into 11, 12, or 13 clades [26, 29, 30]. Our analyses clustered the *NtPP2C* gene fam-

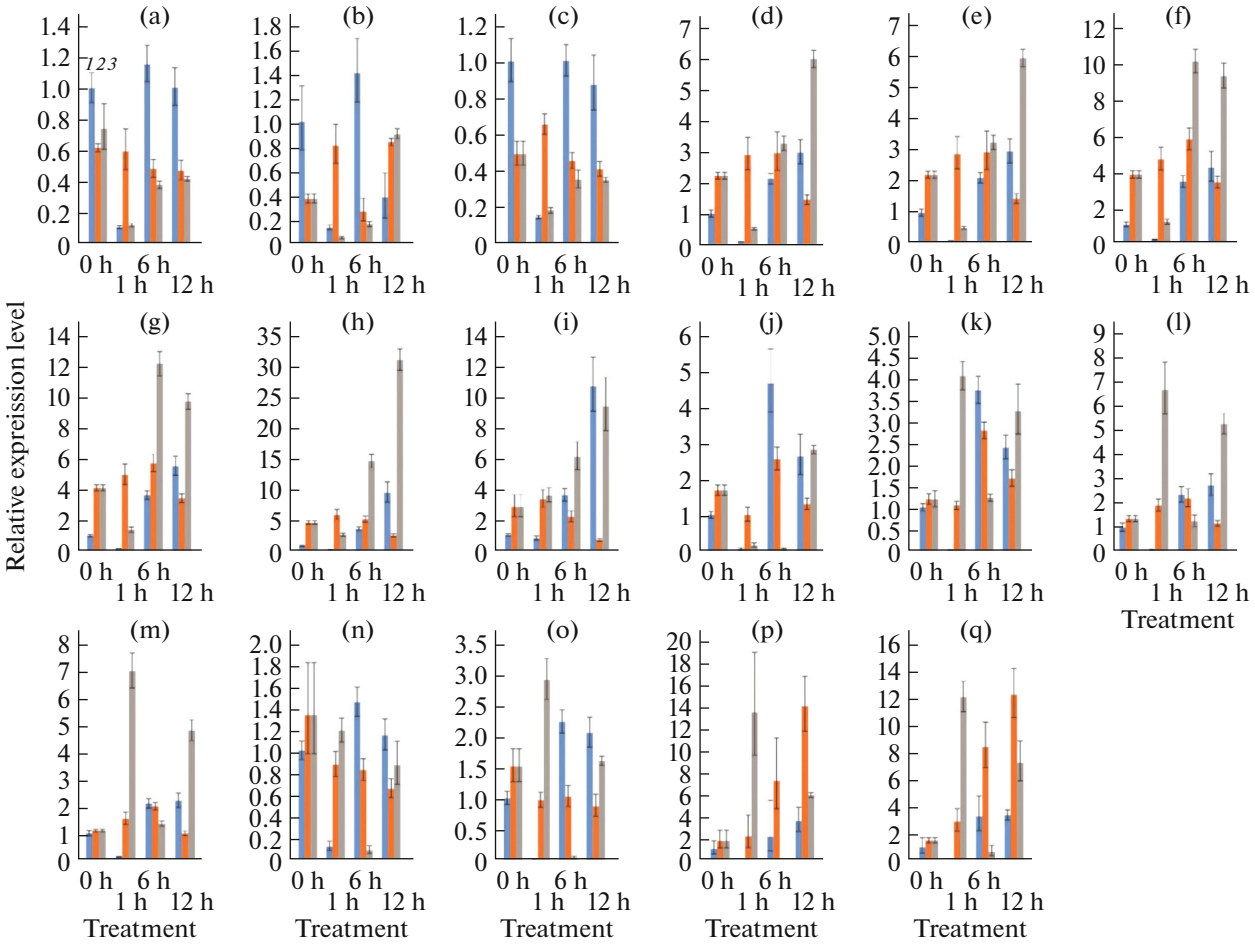

Fig. 4. Expression patterns of 17 NtPP2C Clade A genes in response to abiotic stress. The expressions at 0h were set as controls.(a)–(q) represent the expression pattern of gene *NtPP2C1*, *NtPP2C3*, *NtPP2C4*, *NtPP2C5*, *NtPP2C6*, *NtPP2C7*, *NtPP2C8*, *NtPP2C9*, *NtPP2C10*, *NtPP2C11*, *NtPP2C12*, *NtPP2C13*, *NtPP2C14*, *NtPP2C15*, *NtPP2C16*, *NtPP2C17*, *NtPP2C18* respectively. (*1*) cold; (*2*) salt; (*3*) drought.

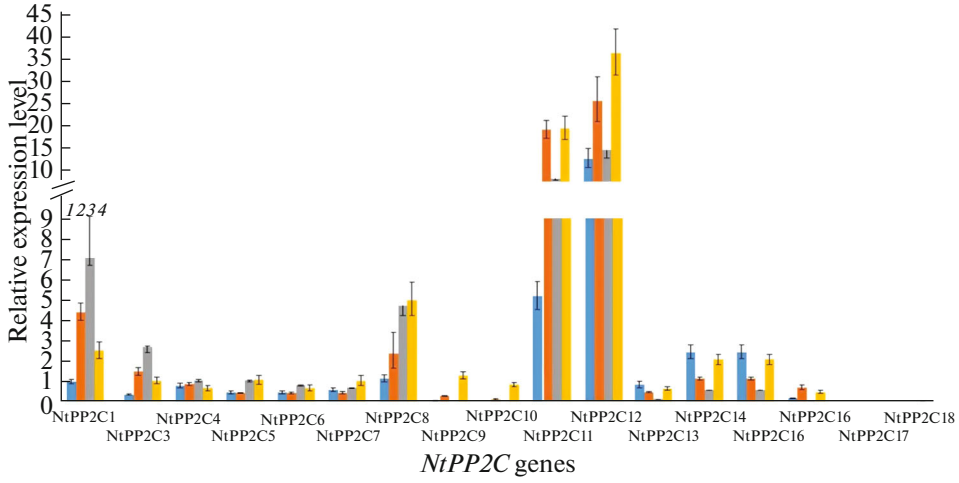

Fig. 5. Tissue specific expression profiles of 17 selected *NtPP2C* Clade A genes in tobacco. Relative transcript abundance values for 17 *NtPP2C* genes were assessed via qPCR. *26S rRNA* gene was used as a normalization control. Error bars represent the SD ($n = 3$). (*1*) Root; (*2*) Stem; (*3*) Leaf; (*4*) Flower.

ily into 11 clades (Fig. 1), consistent with what has been reported in Arabidopsis. Much like Arabidopsis AtPP2Cs, tobacco NtPP2C clades D and E harbored the largest numbers of PP2C proteins. The degree of gene amplification in tobacco is similar to that in other species [25, 27, 31], suggesting that *PP2C* genes are subject to a high degree of evolutionary conservation.

Gene structure is generally reflective of evolutionary conservation and protein function. We found that *NtPP2C* genes in clades A–E harbored fewer introns, while those in clades F–K harbored a larger number of introns (Fig. 2). This intron distribution pattern is similar to that observed in other species including Arabidopsis, rice, maize, *Brachypodiumdistachyon*, and alfalfa [7, 25, 31]. *PP2C* genes in the same clade from different species generally exhibit similar gene structures, consistent with the evolutionary conservation of these genes. Many tobacco *NtPP2C* gene structures exhibited paired distributions, consistent with genome duplication. However, no paired genes were identified for some members of this gene family including *NtPP2C99*, *NtPP2C134,* and *NtPP2C177* (Fig. 2), potentially due to gene loss or an increase in intron number attributable to the insertion of other genomic fragments. These findings support the overall evolutionary differentiation of *PP2C* genes.

Members of the *PP2C* gene family harbor a single domain and a series of conserved motifs. We found motifs 1, 2, 3, 4, 6, 9, and 10 to be widely conserved among NtPP2C proteins, whereas motifs 5, 7, and 8 were restricted to clade C and D PP2Cs (Fig. 3). In prior reports, clade A PP2Cs were found to be important stress-inducible regulators of stress responses. In tobacco, 20 clade A *NtPP2C* genes were identified, and their expression patterns in different tissues and in response to different stressors were assessed. Of these genes, *NtPP2C17*, *NtPP2C18*, *NtPP2C19*, and *NtPP2C20* were largely undetectable in four tissues (Fig. 4), suggesting that they may not play functional roles therein, whereas *NtPP2C11* and *NtPP2C12* were the most highly expressed at the mRNA level (Fig. 4), suggesting their potential functional importance. *NtPP2C11* and *NtPP2C12* were found to be similar to Arabidopsis *AtPP2CA*, which is a stress response regulator that is induced by salt, drought, and cold conditions (Fig. 4). *AtPP2CA* expression has primarily been detected in leaf tissues, and its downregulation can promote accelerated plant development and enhanced resistance to freezing [36]. Consistently, we found that *NtPP2C11* and *NtPP2C12* were induced by low temperature, drought, and salt stress (Fig. 4), suggesting that they may function in a manner similar to *AtPP2CA* in the context of stress responses. Eight *NtPP2C* genes (*NtPP2C1–NtPP2C8*) were found to be expressed at relatively low levels (Fig. 5). These genes were identified as homologs of *AtABI1*, *AtABI2*, *AtHAB1*, and *AtHAB2*, indicating that more homologs are present in tobacco relative to Arabidopsis. These 8 genes were induced in response to low temperature, drought, and salt stress (Fig. 4). *AtABI1* and *AtABI2* are the most important *PP2C* genes, and dominant mutants thereof are ABA-insensitive [34], while recessive mutants exhibit ABA hypersensitivity. *AtABI1* and *AtABI2* account for roughly half of all ABA-induced PP2C activity [17]. As tobacco plants exhibit more *AtABI1*, *AtABI2*, *AtHAB1*, and *AtHAB2* homologs, they may play important roles in facilitating plant adaptation to diverse stressors.

In conclusion, we identified 183 gene members of *PP2C* family in tobacco genome by genome-wide identification strategy. Two genes, *NtPP2C11* and *NtPP2C12*, were significantly upregulated in response to low temperature, salt, and drought treatment suggesting that they may play important roles in regulating plant responses to exogenous stressors.

ACKNOWLEDGMENTS

The authors are grateful to Yunnan Academy of Tobacco Agricultural Sciences for funding for this study.

FUNDING

This work was funded by the Yunnan Academy of Tobacco Agricultural Sciences (grant nos.: 2019530000241001 and 2022530000241017).

COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving animals performed by any of the authors.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Y.P. Li., F.C. Jiao.; performed the experiments: Y.L. Gao., X.J. Chen.; analyzed the data: X.F. Wu., Y.L. Gao.; wrote the paper: Y.L. Gao., F.C. Jiao.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at https://doi.org/10.1134/S1021443722050065.

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