

# Systematic Identification and Analysis of NAC Gene Family in Moso Bamboo (*Phyllostachys edulis*)

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## Research article

**Keywords:** NAC, Microsynteny analysis, Quantitative real-time PCR, Subcellular localization, Transcriptional activity

**Posted Date:** March 20th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-18155/v1>

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# Abstract

**Background:** NAC (NAM/ATAF1/2/CUC2) gene family is a large plant-specific transcription factor family, which is implicated in many functions, such as morphogenesis, the thickness formation of secondary cell walls as well as biotic and abiotic stress and more. In moso bamboo (*Phyllostachys edulis*), 94 PeNACs have been identified and three members are predicted to relate to the secondary cell wall. However, there were few studies on moso bamboo NAC genes under stress.

**Results:** In this study, we re-identified 165 PheNACs with the latest moso bamboo genome data and divided them into 12 subfamilies using NAM domains. Gene structure and motif distribution manifested the NAC gene family was fairly conserved. Evolutionary analysis showed that the segmental duplication played a significant role in the expansion of NAC genes and the relationship between moso bamboo and *Brachypodium distachyon* was closest than between moso bamboo and other four species (*Arabidopsis thaliana*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays*). Based on the promoter analysis of the 27 NAC members in A subfamily, quantitative real-time PCR exhibited these genes reacted differently under drought, high salt, abscisic acid and methyl jasmonate treatments. Finally, we selected out four potential stress-associated genes (PheNAC001, -056, -080 and -100) and found they all localized in the tobacco nucleus and had transcriptional activity in yeast.

**Conclusions:** These preliminary results provide valuable information for mining potential resistance NAC genes and lay theoretical basis for breeding new stress-resistant varieties in moso bamboo.

## Background

Bamboo is a fast-growing plant with many applications for industrial manufacturing, medical materials, ecological landscapes and cultural appreciations in many Asian and African countries [1]. According to the data, approximately 2.5 billion people depend on bamboo economically and the annual international bamboo trade volume exceeds 2.5 billion US dollars. In china, moso bamboo (*Phyllostachys edulis*) occupies ~ 73.76% of the total bamboo-planting area (4.43 million ha), which creates enormous woody products including furniture, paper and musical instruments, and also brings great benefits [1, 2]. In 2013, the first report released 2.05 Gb of moso bamboo draft genome containing 31987 predicted genes, followed by the publication of the supplementation on chromosome-level reference and alternative splicing atlas in 2018, which was helpful for the classification, evolution and functional analyses of moso bamboo genes [1, 2].

Moso bamboo, like other plants, is also forced to encounter multiple adverse environmental stresses throughout the life time, such as drought, cold and high salinity etc. To adapt to these unfavorable conditions, moso bamboo has been and is constantly undergoing evolutionary mechanisms at physiology, biochemical and molecular levels [3]. Generally, when plants are subjected to external stress, they will be induced the production of pivotal enzymes and metabolic proteins, such as dehydrins and late embryogenesis abundant (LEA) proteins [4]. Meanwhile, the receptors on the cell membrane sense

the stress stimulation and convert it into the signal for intracellular transmission to mediate the expression levels of some responsive genes called regulatory proteins [5]. These regulatory genes will further modulate the expression levels of downstream genes by binding to DNA fragments in the promoter region of the target genes, for instance transcription factors (TFs): AP2/ERF [6], bZIP [7, 8], WRKY [9], MYB [10] and NAC [11] have been identified in rice and Arabidopsis.

For plant-specific transcription factor NAC, one NAM domain is generally located at the N-terminus of the gene and has ~ 150 amino acids [12], which can be further divided into 5 sub-domains (A-E): the ACD sub-domains are relatively conservative; the B and E sub-domains are divergent [13]. The domains from ANAC019-(1-168), NAC2/ORE1-(1-165), ATAF1-(1-165) and VND7-(1-163) can be combined with CGT[GA] or CGT core sequences in vitro [14]. Comparatively, the diverse C-terminal transcription regulation region (TRR) can activate or suppress the transcription of multiple target genes, for instance ANAC019 [14, 15]. Although the C-terminal sequence is variable, simple repeat regions rich in acidic amino acids, prolines, serines, and threonines are necessary for transcriptional activation [13]. Also, the area might have transmembrane motifs (TMs), which anchor to the plasma membrane and might relate to signal perception and conduction [15].

To date, researchers have paid more and more attention to NAC genes due to their numerous functions. Accumulating functional reports have showed many NAC genes in rice play key roles in biotic and abiotic stress containing SANC1/OsNAC9 [16], SNAC2/OsNAC6 [17], SNAC3/ONAC003 [18], OsNAC5 [19], OsNAC9 [20], OsNAC10 [21], OsNAP/ONAC058 [22] and OsNAC066 [23]. The overexpression of SNAC1, SNAC2 or ONAC066 in transgenic plants could markedly improve the tolerance to dehydration, cold and salt stresses. Nevertheless, transgenic rice plants of overexpressing the root-specific OsNAC5, OsNAC6, OsNAC9, or OsNAC10 display significant enhancement in drought tolerance. In Arabidopsis thaliana, ANAC019/055/072 have been proven to improve plant tolerance to drought stress by binding to the ERD1 promoter [15]. And ANAC019 and ANAC055 may function as transcription activators to regulate JA-induced expression of defense genes by the anac019 anac055 double mutant and transgenic plants overexpressing of ANAC019 or ANAC055 [24]. The salt-induced AtNAC2/ANAC092 can also integrate the environmental stress and endogenous stimuli to modulate plant lateral root development [25]. Similarly, ATAF1 is a negative regulator of defense responses against both *Pseudomonas syringae* pv. tomato DC3000 and *Botrytis cinerea* [26], and it also directly regulates abscisic acid (ABA) biosynthetic gene NCED3 by binding its promoter in vivo [27]. Until now, increasing evidences have uncovered NAC genes have great functions not only in biotic and abiotic stress, but also in many other aspects such as cell division [28], the formation and maintenance of sepals and stamens (floral organs) [12], the shoot apical meristem developments [29], leaf senescence [30], flowering time control [31], mediating hormone signaling pathways [32] and starch synthesis [33]. Besides these, NAC genes also participate in secondary cell wall (SCW) biosynthesis in plants. For example, VND6/7 (vascular-related NAC domain) causes an inhibition of secondary wall thickening in vessels [34]. AtXND1 (xylem NAC domain 1) negatively regulates terminal secondary wall biosynthesis [35]. Even, overexpression and RNAi transgenic cotton lines have been verified GhFSN1 positively partakes SCW biosynthesis and modification of fibers [36].

For *Phyllostachys edulis* gene PeSNAC1 (PH01001309G0120), ectopic expression in *Arabidopsis thaliana* suggests it together with ped-miR164b participates in the regulation of organ boundaries and salinity and drought stresses [37]. In recent years, moso bamboo has received considerable attention in various fields, including its chromosome-level reference genomes and alternative splicing atlas [1], rhizome-related alternative splicing and alternative polyadenylation [38], the phylogeny of *Phyllostachys* [39] and more. Although 94 NAC genes has been identified in moso bamboo and predicted three members (PeNAC8, PeNAC36 and PeNAC73) were related to the secondary cell wall [40], we use the newly issued moso bamboo genome data to re-identify NAC family genes, analyze their basic characteristics, the evolutionary patterns and the expression levels under different stress treatments to quickly find key genes association with stress in this study. Therefore, these results are helpful to understand the characteristics of NAC genes and lay the foundation for revealing moso bamboo internal molecular mechanisms under stress.

## Materials And Methods

### Data collection and gene identification

First, the latest data about *Phyllostachys edulis* including CDS (coding sequence), peptide sequences, genome sequence and scaffold location information were downloaded from the website ([http://gigadb.org/dataset/view/tdsourcetag/s\\_pcqq\\_aiomsg/id/100498/File\\_sort/name/File\\_page/2](http://gigadb.org/dataset/view/tdsourcetag/s_pcqq_aiomsg/id/100498/File_sort/name/File_page/2)). Then the NAC protein sequences from other species (*Arabidopsis thaliana*, *Oryza sativa*, *Brachypodium distachyon*, *Sorghum bicolor* and *Zea mays*) including 116 *AtNACs* [41], 143 *OsNACs* [42], 114 *BNACs* [43], 180 *SbNACs* [44], 144 *ZmNACs* [45] (some protein sequences were not found at <https://phytozome.jgi.doe.gov/pz/portal.html>) were used for blast ( Basic Local Alignment Search Tool) in moso bamboo latest peptide database with E value  $\leq 1e-10$  [46]. Next, the obtained results were verified to the existence of NAM domain (PF02365) in pfam website (<http://pfam.xfam.org/search>). Finally, all predicted proteins were further checked for the complete NAM domain using NCBI website (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Gene length and GC counts were calculated by Fasta Stater TBtools [47]. And the basic properties of amino acids including length, molecular weight (MW) and isoelectric point (pI) were determined at [https://web.expasy.org/compute\\_pi/](https://web.expasy.org/compute_pi/). Subcellular localization and the membrane-bound were respectively predicted by EuLoc (<http://euloc.mbc.nctu.edu.tw/>) and PROTTER (<http://wlab.ethz.ch/protter/start/>).

### Phylogenetic tree of *PheNACs* and *OsNACs* domains

After determining the specific location of the NAC protein domain using pfam website, the conserved domain sequences of 143 *OsNACs* and 165 *PheNACs* were accurately extracted from their complete amino acid sequence by tbtools software [47]. Then an unrooted tree was constructed using MEGA6 program by the neighbor-joining method with 100 bootstrap values, whereas the single phylogenetic tree of *PheNACs* was set to 1000 bootstrap values [48]. Moreover, multiple alignments were also carried out through DNAMAN software [49].

## Gene structure and conserved motifs

In order to more clearly see the specific positions of exons, introns and domains on genes, GSDS (<http://gsds.cbi.pku.edu.cn/index.php>) [50, 51] was employed to visualize the gene structure. Meanwhile, the *PheNAC* protein sequences were submitted to MEME online (<http://meme-suite.org/tools/meme>) with following parameters: maximum number of motifs 10; minimum motif width 6; maximum motif width 200 [52].

## Scaffold location, duplication relationship and selection pressure

First, an analytical view for 165 *PheNACs* were mapped in offline MapInspect. Then, we used Blastp and MCScanX to examine duplication genes [53]. The paralogous pairs were visualized using Amazing Super Circos; and orthologous pairs between moso bamboo and the other five plants were displayed using Dual Synteny Plotter (<https://github.com/CJ-Chen/TBtools>). Even all the non-synonymous substitution rate ( $K_a$ ) and the synonymous substitution ( $K_s$ ) values of homologous pairs were also measured by TBtools [47]. In genetics, the  $K_a/K_s$  ratio is an indicator of selective pressure.  $K_a/K_s > 1$  means positive selection;  $K_a/K_s = 1$  implies neutral evolution and  $K_a/K_s < 1$  represents purifying selection [54]. Using the formula  $T = K_s / 2\lambda$  ( $\lambda = 6.5 \times 10^{-9}$ ), divergence time was also calculated [2].

## Gene ontology annotation

165 *NAC* protein sequences were loaded into Blast2GO program, then blasted, mapped and annotated to obtain GO terms, which were mainly divided into three categories: biological process, cellular component and molecular function [55].

## Cis-acting element analysis

The upstream 2-kb promoter region of *PheNAC* genes were captured from moso bamboo genome sequences and upload to the website (<http://planttfdb.cbi.pku.edu.cn/>) to detect cis-acting regulatory elements [56].

## Plant materials, RNA extraction and quantitative Real-time PCR (qRT-PCR)

Moso bamboo seeds were collected from Guilin of Guang Xi Province in China, which were identified by the Guilin Forestry Bureau. Then moso bamboo seedlings were cultivated for 8 weeks in  $25 \pm 2$  °C greenhouse photoperiod for 16 h light/8 h dark. And 200 mM NaCl [43], 100  $\mu$ M methyl jasmonate (MeJA) [54] and 100  $\mu$ M abscisic acid (ABA) [50] were sprayed on the leaves. For drought stress, 20% PEG6000 [44] were selected to irrigate the roots. The samples for RNA extraction were collected at 0, 1, 3, 6, 12 and 24 hours after treatments.

RNA isolation of moso bamboo leaves were manipulated with RNAiso Plus (TaKaRa Code No.9108) according to the manufacturer's protocol. Then the cDNA was synthesized using thePrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa Code No. 6210A) as the instructions described. The reverse

transcription reaction was at 30 °C for 10 min followed by 42 °C for 60 min. In addition, Gene primers were designed using Primer Premier 5.0 software, and moso bamboo CDS library was uploaded manually on the NCBI website to check the primer specificity (Additional file 8: Table S6). The intrinsic membrane protein 41 (*TIP41*) was used as a reference gene [57, 58]. The qRT-PCR program was set to 95 °C for 30 s; 40 cycles of 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 10 s.

### Subcellular Localization and Transcriptional Activity

The pCAMBIA1305 vector with GFP protein driven by 35S was inserted by CDS with stop codon removed, the fusion vectors were transferred into EH105 strains respectively (BIO-RAD, Hercules, CA, USA) for injection into *Nicotiana benthamiana* to observe the fusion protein location using confocal laser scanning microscope (CarlZeiss LSM710). In addition, the *PheNACs* were cloned and independently insert into the pGBKT7 vector (Clontech, Palo Alto, CA, United States), which was used to detect whether the genes had transcriptional activity in yeast. The positive control pGBKT7-53 + pGADT7-T, the negative control pGBKT7 and pGBKT7-PheNAC were respectively transferred to Y2HGold strains (The competent cells purchased from Shanghai Weidi biotechnology Co., Ltd.) according to the instructions. The positive strains were cultured on double deficiency medium (SD/-Trp/-Leu) and the other two groups were grown on single deficiency selection medium (SD/-Trp). Afterwards, we move them to triple deficiency medium (SD/-Trp/-His/-Ade/X- $\alpha$ -Gal) at 30°C for 3-5 days.

## Results

### Identification and classification subfamily of moso bamboo NAC genes

To acquire *NAC* members in moso bamboo, 697 *NAC* protein sequences including 116 *AtNACs*, 143 *OsNACs*, 114 *BNACs*, 180 *SbNACs* and 144 *ZmNACs* were used as guidance for BLASTP in moso bamboo protein database (Additional file 3: Table S1). Correspondingly, the number of collected moso bamboo protein sequences was 261, 269, 264, 273 and 264. We then obtained 279 non-repeating genes by merging and filtering, and further eliminated redundancy and fragmentation. Only 165 members containing the complete NAM domain sequences were named *PeNAC001-165* based on their chromosome scaffold location for the subsequent sequence alignment and phylogenetic analysis. In the integrated phylogenetic tree constructed with the domains of 165 *PheNACs* and 143 *OsNACs*, the identified 165 proteins were roughly divided into 14 groups (SNAC, ANAC34, OMNAC, TIP, NAM/CUC3, ONAC1, NAC22, SND, NEO, ONAC7, ONAC4, ONAC3, NAC1 and ONAC6) according to previous report (Nuruzzaman et al. 2010) (Fig. 1). However, when drawing phylogenetic trees using moso bamboo protein sequences, only 12 subfamilies appeared and we renamed these subfamilies again from A to L because 6 members (*PheNAC034*, *-047*, *-78*, *-102*, *-053* and *-062*) in ONAC7 group were divided into B subfamily and *PheNAC143* belonging to NEO group was reclassified to C subfamily (The remaining subfamily members remained unchanged) (Fig. 2A). Of the 12 subfamilies, there were 5 subfamilies with over 20 genes: subfamily A, B, D, E and H. And the least gene subfamily C had only 3 members which contained the largest protein *PheNAC008* (907 amino acids). Coincidentally, *PheNAC064* had the shortest

sequence in protein and gene lengths (141aa, 514bp). For gene GC counts, *PheNAC160* and *PheNAC162* were the largest and smallest genes, accounting for 69% and 39%, respectively. The protein MWs were from 15.82 (*PheNAC064*) to 99.32 kDa (*PheNAC008*), and the pIs ranged from 4.53 (*PheNAC111*) to 9.89 (*PheNAC006*). Unexpectedly, except for *PheNAC160* in the cytoplasm, the remaining 164 *PheNACs* were predicted to be located in the nucleus (Additional file 4: Table S2). In addition to *PheNAC030* and *PheNAC096*, there were also 10 *PheNACs* with a membrane-bound motif at their own C-terminus, 8 of which were from H subfamily (Fig. S1).

### Gene structure and protein motifs for *PheNACs*

To better understand the structural characteristics of moso bamboo *NAC* gene family, exon/intron structure and motif composition were examined. We counted 9 kinds of exon number (1, 2, 3, 4, 5, 6, 7, 9 and 11). Among these, the *NAC* genes with three exons were 120 members (72.73%) and with four exons had 16 (9.7%). *PheNAC008* had the most exons (11) and *PheNAC097* contained an entire exon. And each gene had only one NAM domain basically distributed on the first two exons. In subfamily H and J, some genes had longer UTR (untranslated region) (Fig. 2B). Additionally, the genes in a common cluster shared the similar gene structure and motif compositions. For example, the members in subfamily L had three exons, even their lengths were so close and the formation and order of motifs seemed to be completely identical. Surprisingly, 134 members (81.2%) of nine subfamilies shared the motif 1, 6, 4, 3, 5, 2 and 7. And the motif 1 represented the NAM domain, which was why each *NAC* gene had it. Moreover, the seven members in subfamily E, their motif compositions were quite consistent and they all contained motif 8 and 10. And motif 9 was discovered in three members *PheNAC095*, *-096* and *-097*, suggesting they might play special functions (Fig. 2C).

### Gene distribution and evolutionary pattern analysis of *PheNACs*

A total of 165 *PheNACs* were mapped onto 23 moso bamboo scaffolds and the highest frequency was observed on scaffold 13 (14, 8.48%), followed by scaffold 14, 15, 16 (12, 7.27%), 3 and 21 (10, 6.06%). And the six scaffolds contained no less than 10 *PheNACs*, whereas scaffold 1 was unoccupied by any (Fig. 3A). For *NAC* gene family evolution, different duplication modes, including 2 pairs of proximal duplication genes, 5 pairs of transposition duplication and 6 pairs of tandem duplication genes, were investigated in moso bamboo genome (Fig. 3A). Most remarkable, 142 segmental duplication gene pairs (involving 145 *PheNACs*) were also detected, with Ks values ranging from 0.062 to 1.179 (Fig. 3B, Additional file 5: Table S3). Based on previous reports, moso bamboo had undergone two whole-genome duplication (WGD) events and the time of two events was very close (peak Ks: 0.499 ~ 1.556, recent peak Ks: 0.197 ~ 0.382). Therefore, we roughly attributed 63 genes to derive from the first WGD event (Ks values of 53 pairs were in the range of 0.499 ~ 1.556) and the remaining 82 *NAC* members might result from the recent WGD event [59]. Meanwhile, we noticed 42 *NAC* genes correspond to 3 paralogs and 32 members had 2 homologous genes in moso bamboo genome (Additional file 5: Table S3), which might prove moso bamboo genome had experienced two WGD events. Additionally, the ka/ks ratios of 150

paralogous pairs (Phe-Phe) were lower than 1 (5 paralogous pairs have no Ks value), implying a purifying selection for these *PheNACs* (Fig. 3C).

### Microsynteny analysis of NAC genes between moso bamboo and other five grass species

To further explore the evolutionary relationships of *NAC* genes between moso bamboo and other five species (*Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays*), collinear pairs were conducted by BLASTP and MCScanX (Multiple Collinearity Scan). The corresponding numbers of collinear gene pairs between moso bamboo and other five grass species were 13, 177, 215, 202 and 240 respectively. And the *NAC* genes involved were 9 *AtNACs*, 70 *BNACs*, 83 *OsNACs*, 78 *SbNACs* and 108 *ZmNACs*. As for Ks, *Arabidopsis thaliana* (Phe-At) only reserved a Ks value; 80 Ks values were concentrated in 0.4-0.5 (Phe-Bd) and the peak values of 118 Ks were in the range of 0.3-0.5 (Phe-Os); for *Sorghum bicolor* (Phe-Sb) and *Zea mays* (Phe-Zm), the Ks values were both concentrated in 0.4-0.6 (Fig. 4B). And the average Ks values between moso bamboo and these four species (except *Arabidopsis thaliana*) ranged from 0.563 (Phe-Bd) to 0.613 (Phe-Zm), suggesting the evolutionary distance of moso bamboo and *Brachypodium distachyon* was closest, because moso bamboo diverged from *Brachypodium distachyon* (Phe-Bd), *Oryza sativa* (Phe-Os), *Sorghum bicolor* (Phe-Sb) and *Zea mays* in turn were 46.9, 48.6, 58.5 and 64.6 million years ago (MYA) (Peng et al., 2013). Also, apart from one pair with 1.0745 (*PheNAC160/Bradi5g13300.1*), all Ka/Ks ratios of homologous gene pairs were less than 1, representing purified evolution (Fig. 4C, Additional file 6: Table S4).

### Gene Ontology Annotations

To clarify the function of the 165 *PheNACs*, the gene ontology (GO) was performed and gained 624 GO terms which were divided into three main categories: biological process, cellular component and molecular function (Fig. 5). Both 104 items were implicated in bio-synthetic process and cellular nitrogen compound metabolic process, and 52 were commented on transcriptional regulation, DNA-templated. Totally, the three entries occupied 81.5% in biological process. Meanwhile, we found 11 items were related to positive regulation of response to salt stress (GO: 1901002), 8 involved in positive regulation of response to water deprivation (GO: 1902584), and 4 responded to cold (GO: 0009409). Actually, the 11 salt-stress genes included the drought and cold-stress genes. In addition, it was unexpected that only one gene (*PheNAC041*) was interpreted to participate in the cell wall organization or bio-genesis, which was not consistent with the previous report (Shan et al., 2019). However, 125 terms in cellular component were classified into 5 kinds, 120 of them were annotated nucleus (GO: 0005634). Two gene were noted with membrane (*PheNAC074* and *PheNAC125*, GO: 0016020) and one was plasma membrane (*PheNAC096*, GO: 0061617), which signified the three genes might play an important role in membrane composition. Among total 180 notes of molecular function, 170 terms were assigned to involve binding: 150 were associated with DNA binding, regulation of transcription; 15 were related to sequence-specific DNA (GO: 0043565) and 5 genes (*PheNAC003*, *-063*, *-064*, *-080* and *-100*) might concern protein binding (GO: 0005515). The details were seen in Additional file 7: Table S5.

### Promoter analysis of SNAC subfamily

To explore the potential functions of *NAC* genes, the promoter region of 27 members gathered subfamily A was investigated in the first 2000bp of the translation initiation site (ATG). Promoter elements were mainly divided into three categories: plant growth and development, abiotic and biotic stress and phytohormone responses.

First and foremost, light responsive elements Sp1, Box 4 and G-Box total 268 accounted for 82.46%, of which 10 or more were detected in 13 members (*PheNAC052*, -127, -90, -003, -63, -032, -045, -080, -100, -018, -103, -94 and -130). And the promoter elements of endosperm expression (GCN4\_motif) and meristem expression (CAT-box) were also observed among 6 genes and 16 members, respectively. For O2-site (zein metabolism) and RY-element (seed-specific regulation), their numbers were 15 and 12, separately. In the second category, both ARE and GC-motif were associated with anaerobic induction. The former was identified within 24 *PheNACs* with a total of 51, while the latter had 24 out of 15 members. The low-temperature responsiveness elements (LTR) appeared 34 times among 27 *PheNACs* with 6 for *PheNAC016*, and TC-rich repeats occupied 7. And MBS, the MYB binding site involved in drought-inducibility was also identified in 15 members (Fig. 6A). The abscisic acid responsiveness element ABRE ranked the first in the third category and *PheNAC080* held the maximum number (22), followed by 94 responsive to MeJA cis-acting elements (CGTCA-motif). And other hormone-related elements including salicylic acid element, gibberellin response elements (P-box, TATC-box and GARE-motif) and auxin-responsive elements (AuxRR-core, TGA-box and TGA-element) were also noticed no more than 3. In addition, three kinds of cis-acting elements (ABRE, CGTCA-motif and MBS) were shown the specific location in the promoter area. We could find 70% ABRE elements were scattered within the first 1000bp, and some were even concentrated in the first 500bp, for instance *PheNAC003*, -032, -045, -80 and -100 (Fig. 6B).

### The expression levels of SNAC genes under different treatments

Based on the analysis of cis-acting elements and the published related articles, 27 *NAC* genes were selected for qRT-PCR at five time points under the treatments of drought and high salt, ABA and MeJA. After 20% PEG6000 treatment, the expression of 15 genes were obviously increased (Fig. 7A), of which 8 had peaked over 10 times than the control group (0h), even *PheNAC016* was up-regulated over 40-fold at 24h. Interestingly, almost these up-regulated genes reached their maximum at 12h. Conversely, those *NAC* genes that rapidly decreased after stress treatment were also noted, such as *PheNAC077*, -094, -80 and -110. The strangest behavior was *PheNAC056*, whose expression was first strongly suppressed at 3h, then induced up-regulated about 7-fold at 6h, and finally down-regulated. For salt stress, *PheNAC064*, -050, -103, -077, -052, -130, -94, and -056 manifested as consistently high expression at all observation time points, the highest expression levels of the last 5 members were more than 20 times. However, the genes that were slightly up-regulated at the initial time point of observation, but in the later period, some tended to express normally, including *PheNAC003*, -063, -045, -035, and some exhibited higher expression, such as *PheNAC061*, -080 and -001. In addition, *PheNAC048*, -016, -090, -085, -015, -032 and -100 showed significant downregulation and the latter three members tended to normal expression in the later stages (Fig. 7B). Unexpectedly, the transcription levels of some *PheNACs* were strongly inhibited by exogenous

ABA containing *PheNAC048*, *-016*, *-003*, *-103*, *-080*, *-032*, *-100* and *-110*. And some genes (*PheNAC094*, *-130*, *-001*, *-052*, *-061*, *-085*, *-015* and *-127*) were significantly induced, especially the highest expression level of *PheNAC001* was more 80 times than that of the control group. Moreover, with the extension of the observation time, some up-regulated genes gradually approached the control value, concerning *PheNAC090*, *-106*, *-135*, *-050*, *-064*, *-035*, *-063*, *-045* and *PheNAC077* (Fig. 8A). In the MeJA treatment, the majority of NAC genes were severely suppressed (Fig. 8B). The high expression of certain genes was not significant, such as *PheNAC035*, *-032*, *-018*, *-063*, *-056*, *-085*, *-110*, *-127* and *-064*. Yet three members (*PheNAC061*, *-135* and *-077*) presented obviously high expression level. Meanwhile, the performance of *PheNAC100* attracted our attention, which was repressed at 3 hours, then intensely induced up-regulation at 6h, then severely suppressed, and finally induced up-regulated about 4 times, *PheNAC016* and *PheNAC015* did the unstable performance as well.

### Subcellular Localization and Transactivation Activity of Four NAC Genes

It is reported that a large proportion of NAC proteins are located in nucleus [60, 61], and the EuLoc website predicted the 164 PheNACs were in the nucleus. Based on GO annotations, cis-acting elements and expression patterns analysis, *PheNAC001*, *-056*, *-080* and *-100* were picked for next subcellular localization and transcriptional activity analysis. The CDS without stop codon of four *PheNACs* independently fused with GFP constructs and the GFP control driven by CaMV 35S promoter were expressed in *Nicotiana benthamiana* leaves. The result showed the GFP signal was distributed throughout the cell with the 35S::GFP proteins, and *PheNAC001*, *-056*, *080* and *-100* proteins were clearly localized in the nucleus, as predicted by the website (Fig. 9).

To investigate the transcriptional activity of the four PheNACs, pGBKT7-PheNACs, the positive control plasmids containing pGBKT7-53 and pGADT7-T, and the negative control plasmid pGBKT7 were independently transformed into the Y2HGold yeast strain. All of these transformants could grow and displayed visible white colonies on the SD/-Trp medium. In the SD/-Ade/-His/-Trp/X- $\alpha$ -Gal medium, the yeast cells with PheNACs and the positive control grew well and turned blue. In contrast, the negative control did not grow on this medium (Fig. 10). Thence, the four NAC fusion constructs could activate the transcription of the His3 and LacZ reporter genes, demonstrating they had self-transcription activity in yeast strains.

## Discussion

### Sequence features

As a plant-specific large transcription factor family, 165 *NAC* members were identified and divided into 12 subfamilies based on the report [42]. First, we found the NAM domain with average length 128bp in pfam was shorter as described 150bp in other articles [62]. Hence, we manually aligned the sequences outside the NAM domain and found ~50bp following by NAM domain was a certain degree of conservatism. For one subfamily, their NAM domain sequences were quite conserved, whereas great differences existed between different subfamilies (Additional file 2: Fig. S2). As transcription factor, the function of *NAC*

genes is mainly dependent on the NAM domain which can combine the promoter region specific cis-acting element of different downstream genes or interacts with other proteins. Generally, *NAC* genes mainly bind to the NACRS with core sequences CACG or CATGTG sequence [16, 63]. As increasing reports, more *NAC* genes could combine with different DNA fragments. *Arabidopsis* transcriptional repressor CBNAC could bind to CBNACBS with a GCTT core sequence but not the CACG sequence [31]. SNAC1 could also recognize an ABA responsive element (ABRE) from *OsbZIP23* promoter to monitor the expression level of several ABA signaling genes [64]. Thence, we speculated *NAC* genes could bind different DNA sequences to participate in different pathways due to distinct NAM domains. On the other hand, *NAC* gene function need to be controlled by upstream genes. MdHY5 interacted with the G-box from *MdNAC52* promoter to affect the *MdNAC52* transcription and Anthocyanin and proanthocyanidin (PA) synthesis [65]; *AdNAC6* and *AdNAC7*, the targets for miR164, were both significantly up-regulated by exogenous ethylene in various fruit [66]. For the 165 *NAC* promoter regions, which contains various cis-acting elements such as Sp1, GT1-motif, G-Box and Box 4 involved in light responsive elements, abscisic acid responsiveness element (ABRE) and the MeJA-responsiveness regulatory elements (TGACG/CGTCA-motif), MYB binding site involved in drought-inducibility (MBS) and low-temperature responsiveness elements (LTR) and many more, suggesting they might perform different functions at different stages (statistics but not shown).

In addition, the majority of *NAC* genes in moso bamboo and other species contained two introns, indicating that the family was structurally conservative (Fig. 2) [43]. Strikingly, the members of subfamily F, *PheNAC096/097* and *PheNAC095/096* were two pairs of paralogous genes, but the three members had different numbers of introns: *PheNAC095*(1), *PheNAC096*(8), *PheNAC097*(0), indicating *PheNAC095* and *PheNAC097* might lose introns during evolution process. The changes in gene structure might cause gene function change. In comparison with *PheNAC066*, *PheNAC054* and *PheNAC057* of subfamily G had one less intron. And *PheNAC066* lacked motif 6 and contained two GO terms, whereas *PheNAC054* and *PheNAC057* were short of motif 4 but had 4 GO terms, which might explain that their function had changed (Fig. 2B, C, Additional file 7: Table S5).

## Evolutionary relationships

In moso bamboo genome, the number for 165 *NAC* genes is a little more than 118 in *Brachypodium distachyon*, 151 in rice, 145 in sorghum and 152 in maize, which may be because moso bamboo genome (1.91Gb) contains more protein-coding genes (51074) than these monocotyledonous plants [1]. Certainly, *NAC* gene family has also been widely introduced in many dicotyledonous plants such as *Arabidopsis thaliana* (116) [41], potato (110) [67], soybean (152) [68], *Populus trichocarpa* (163) [69] and Asian pears (185) [70] etc. This different numbers might be not only correlated with the size of plant genomes but also related to gene duplication. The genomes of *Arabidopsis thaliana* (125Mb, 25498 protein coding genes) [71], potato (844Mb, 39031) [72], poplar (385Mb, 45555) [73], Asian pears (527Mb, 59552) [74] and soybean (1.025 Gb, 52051) [75] are smaller than that of moso bamboo and the total number of protein-coding genes varies widely. On one hand, potato has experienced two rounds of whole-genome triplication events (WGT) [72]; *Arabidopsis thaliana* and soybean have undergone three genome

duplication events, including two doublings and one triple [76]; in *Populus*, apart from a triploid event, there is a single “salicoid” duplication event (P-duplication) [73]; Asian pears is similar to poplar, with one doubling and one triple [59]. Moso bamboo undergoes only two whole-genome doubling events, finally leading to an unbalanced gene number in different species. On the other hand, Asian pears, like moso bamboo, segmental duplication seemed to make a greater contribution in NAC gene expansion (Fig. 3A and B). We also identified 18 *OsNAC* genes involved in segmental duplication and 36 members associated with tandem duplication [42]. And in *Brachypodium distachyon*, both tandem duplicated and segment duplicated genes reached 22 *BNACs* [43]. In potato, 20, 27, 10 and 46 *StNACs* were discovered to be segmental, tandem, proximal and dispersed duplicated, respectively [67]. Therefore, different patterns of gene duplication contributed differently to *NAC* family gene amplification in diverse species.

As for *Ks* values of *NAC* orthologous pairs between moso bamboo and *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays* (Phe-Bd, Phe-Os and Phe-Sb, Phe-Zm), the distribution situations were very similar to that of the entire genome (Fig. 4B, Supplementary Figure 11 from [2]), suggesting some *PheNACs* had appeared before separation from the four species. On the basis of previous article, the divergence time of moso bamboo isolated from *Brachypodium*, rice, sorghum and maize was approximately 46.9, 48.6, 58.5 and 64.6 MYA [2]. And the *Ks* peak of *NAC* orthologous pairs between moso bamboo and the four species were all clustered at 0.4-0.5, with corresponding average 0.564 (Phe-Bd), 0.573 (Phe-Os), 0.598 (Phe-Sb) and 0.613 (Phe-Zm) (Additional file 6: Table S4), meaning the closer relationships with moso bamboo were in turn *Brachypodium distachyon* > *Oryza sativa* > *Sorghum bicolor* > *Zea mays*. This is exactly consistent with the conclusions previously reported [2]. Additionally, in terms of *Ka/Ks* ratios, the values of 32 paralogous pairs (Phe-Phe) containing 43 *PheNACs* (Fig. 3C), 20 pairs (Phe-Bd), 26 pairs (Phe-Os), 26 pairs (Phe-Sb) and 34 orthologous pairs (Phe-Zm) were between 0.5 and 1 (Fig. 4C), signifying these genes were moving forward the direction of positive selection to adapt to the environment [77].

### **NAC genes response to stresses**

The *NAC* functions have been well studied in rice [41, 42]. Based on the N-J phylogenetic tree constructed using the domains from 165 *PheNACs* and 143 *OsNACs*, *NAC* genes that clustered together are predicted to have similar potential functions (Fig. 1). In *SNAC* group, there are six *OsNACs* with known functions: *SNAC1* (LOC\_Os03g60080.1), *SNAC2/OsNAC6* (LOC\_Os01g66120.1), *OsNAC3* (LOC\_Os07g12340.1), *OsNAC5* (LOC\_Os11g08210.1) and *OsNAC10* (LOC\_Os12g03040.1), which have been reported to be associated with biotic and abiotic stress, especially for *OsNAC6* (LOC\_Os01g66120.1). The gene aggregated with *PheNAC080* and *PheNAC100* and was induced by cold, high salt, drought and ABA [78]. Over-expressing *OsNAC6* in rice exhibited increased tolerance to dehydration and high-salt stresses, as well as blast disease [79]. And the transgenic rice enhanced plant drought tolerance by affecting its direct targets NICOTIANAMINE SYNTHASE to promote the accumulation of the metal chelator NA [80]. Therefore, we speculated that *PheNAC080* and *PheNAC100* might also have similar functions and regarded them as the study focus. However, their performances were always the opposite under drought and salt treatments. The transcription level of *PheNAC080* was suppressed under drought, but

*PheNAC100* was high expression at the observed time points (Fig. 7A). Under salt treatment, *PheNAC080* showed high expression at 12 and 24h yet *PheNAC100* presented low expression (Fig. 7B). Thus, their specific functions still need further experiments to explain. In addition, plant hormone ABA and Jasmonic acid (JA) are key regulators of stress signaling networks [81]. When plants suffer from drought or high salt stress, ABA will be generated to control stomatal closure by the SnRK/PP2C signaling pathway and endogenous jasmonic acid (JA) levels generally increase [81-83]. In this study, after ABA-treated, *PheNAC080* and *PheNAC100* were both low expression, although the former held the most ABRE elements (22) and the latter had 13 ones in their own promoter region. Meanwhile, we also found *PheNAC001* was strongly induced by exogenous ABA and it had 8 ABRE elements (Fig 6 & 8A). The most noticeable is the varied performance of *PheNAC100*, whose expression was down-regulated first, then suddenly induced up-regulated nearly 20 times, then down and up again. *PheNAC056* has the most CGTCA-motif elements (9), whereas its expression level was slightly increased but not significant under MeJA. And *OsNAC10* were annotated as the homologous gene of *PheNAC001* and *PheNAC056*, whose overexpression improves rice drought tolerance and grain yield (Jeong et al., 2010). And a grapevine gene *VaNAC17* was strongly induced exogenous ABA and methyl jasmonate (MeJA), whose over-expression in *Arabidopsis* plants enhanced drought tolerance by regulating endogenous JA biosynthesis and ROS scavenging [84]. Thus, these genes are also predicted to have similar functions with great possibility. Finally, we also proved that these four genes are located in the nucleus of tobacco cell and have transcriptional activity in yeast, which could explain the general properties as transcription factors.

## Conclusion

In this study, we carefully re-identified the NAC members in moso bamboo using the latest genome data. After comprehensive investigation and analysis of these NAC genes, qRT-PCR assays showed the expression level of four genes (*PheNAC001*, -056, -080 and -100) were induced to extent degree under different stresses, and they were all localized in the nucleus and both had transcriptional activity. To clarify the biological functions and related pathways of these four genes, more experiments need to perform. Thus, this study provides a useful reference for the functional research of NAC family genes.

## Additional Files

**Additional file 1: Fig S1.** The specific location of membrane-bound motif protein.

**Additional file 2: Fig S2.** The sequence Alignment of 165 *PheNAC* NAM domains from 12 subfamilies (A-K).

**Additional file 3: Table S1.** NAC gene ID name of five species (*Arabidopsis thaliana*, *Brachypodium distachyon*, Rice, *Sorghum bicolor* and maize).

**Additional file 4: Table S2.** The detailed information of 165 *PheNACs*.

**Additional file 5: Table S3.** Ka, Ks and Ka/Ks values and duplication types of paralogous pairs (Phe-Phe).

**Additional file 6: Table S4.** Ka, Ks and Ka/Ks values of orthologous pairs between moso bamboo and *Arabidopsis thaliana*, *Brachypodium distachyon*, rice, *Sorghum bicolor* and maize.

**Additional file 7: Table S5.** Details of 165 *PheNACs* GO ontology.

**Additional file 8: Table S6.** The specific primers of quantitative real-time PCR for 27 *PheNACs*.

**Additional file 9: Table S7.** The detailed information of motif 1-motif 10.

## Abbreviations

NAC: NAM, ATAF1/2 and CUC2; LEA: late embryogenesis abundant; TRR: transcription regulation region; TMs: transmembrane motifs; PstDC3000: *Pseudomonas syringae* pv. tomato DC3000; SCW: secondary cell wall; VND: vascular-related NAC domain; XND1: xylem NAC domain 1; CDS: coding sequence; MW: molecular weight; pI: isoelectric point; Ka: non-synonymous substitution rate; Ks: synonymous substitution; MeJA: Methyl Jasmonate; ABA: abscisic acid; UTR: untranslated region; MYA: million years ago; GO: gene ontology; LTR: low-temperature responsiveness elements; ABRE: abscisic acid responsiveness element; MBS: MYB binding site involved in drought-inducibility; JA: jasmonic acid.

## Declarations

### Ethics approval and consent to participate

The seeds of moso bamboo were collected from Guilin in Guang Xi Province, China. And the seeds were provided and identified by the Guilin Forestry Bureau. In addition, all the materials of moso bamboo used and analyzed were available for non-commercial purpose. This article did not contain any studies with human participants or animals performed by any of the authors.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### Funding

This work was supported by the National Natural Science Foundation of China (31670672), the National Science and Technology Support Program (2015BAD04B0302) and the Graduate Innovation Fund from Anhui Agricultural University (2020ysj-55).

### Author contributions

YMG conceived and wrote the manuscript, HLL participated in organizing figures and tables, LW designed and performed experiments, RX collated and analyzed the experimental data, YNS collected plant materials and assisted to complete the writing of this paper. YX the correspondent author designed the main frame and provided advice on writing methods of this article. All authors read and approved the manuscript.

## Acknowledgments

We thank the members from the Crop Stress Resistance Breeding and Laboratory of Modern Biotechnology for the help in this study.

## Availability of data and materials

The genome sequences of moso bamboo were downloaded from the website ([http://gigadb.org/dataset/view/id/100498/File\\_sort/name?tdsourcetag=s\\_pcqq\\_aiomsg](http://gigadb.org/dataset/view/id/100498/File_sort/name?tdsourcetag=s_pcqq_aiomsg)) and these moso bamboo *NAC* gene IDs were listed in Additional file 4: Table 2. And the *NAC* proteins sequences of *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays* were obtained from Phytozome database (<https://phytozome.jgi.doe.gov/pz/portal.html>) and these IDs were listed in Additional file 3: Table 1.

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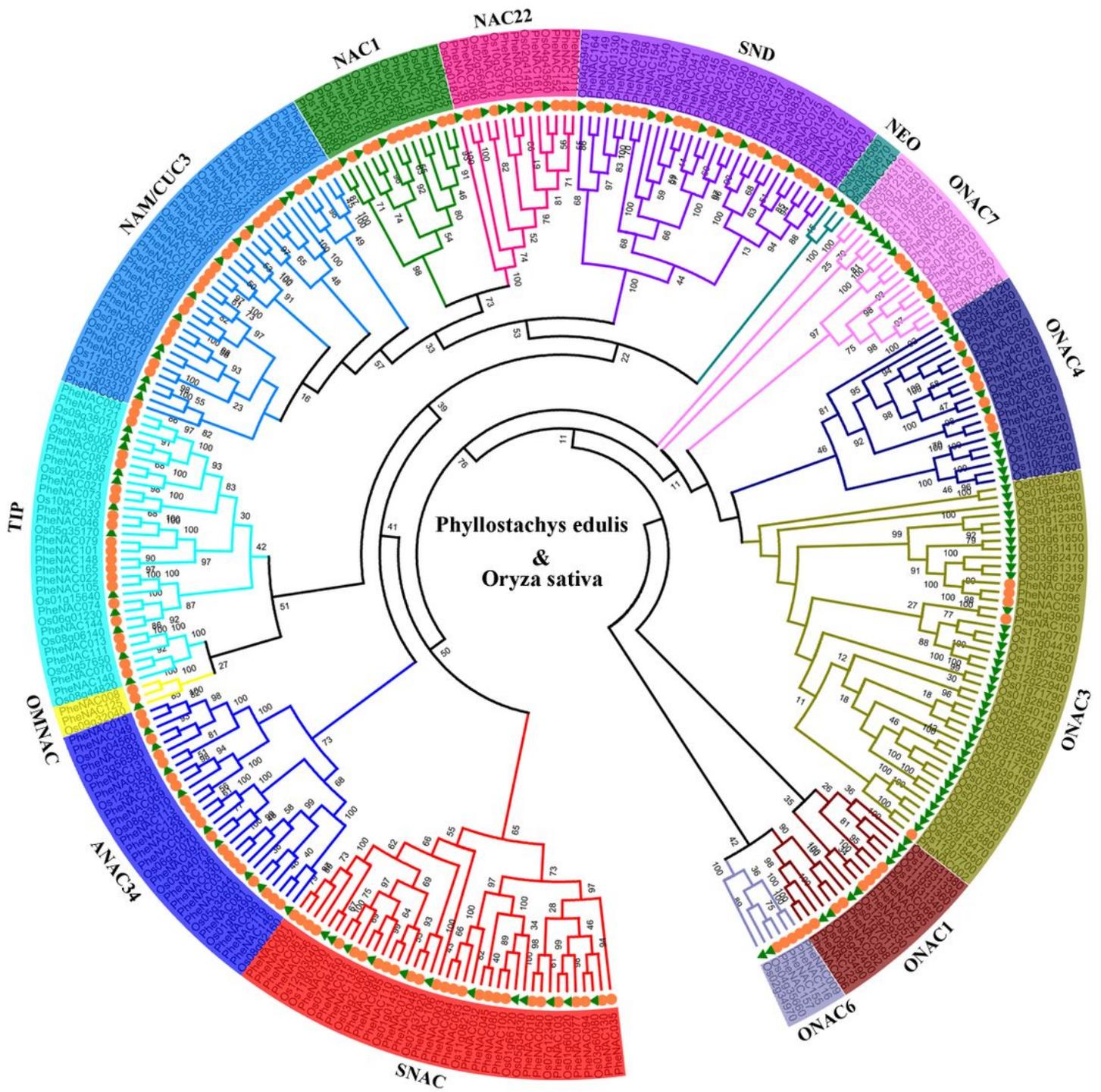
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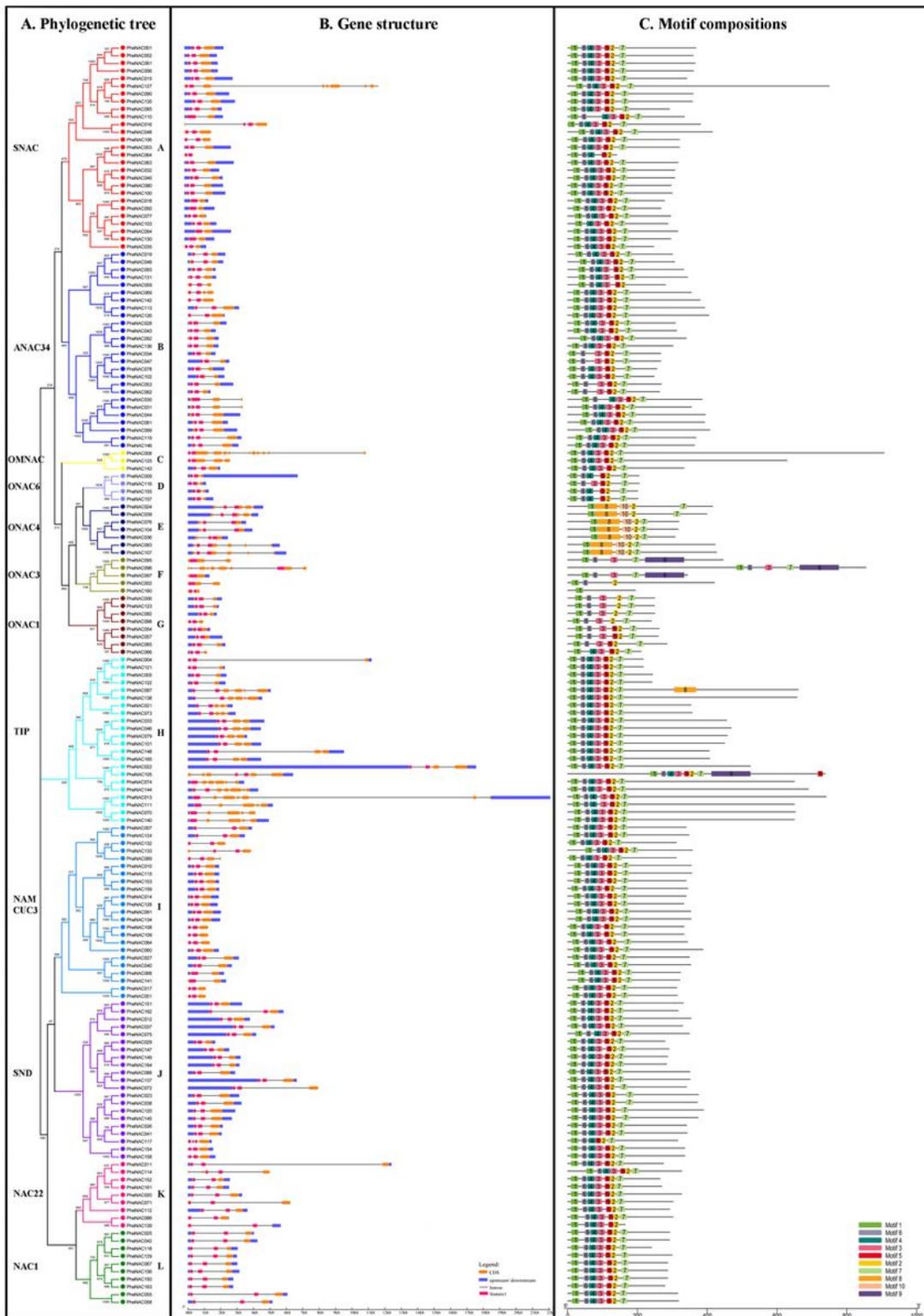
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## Figures



**Figure 1**

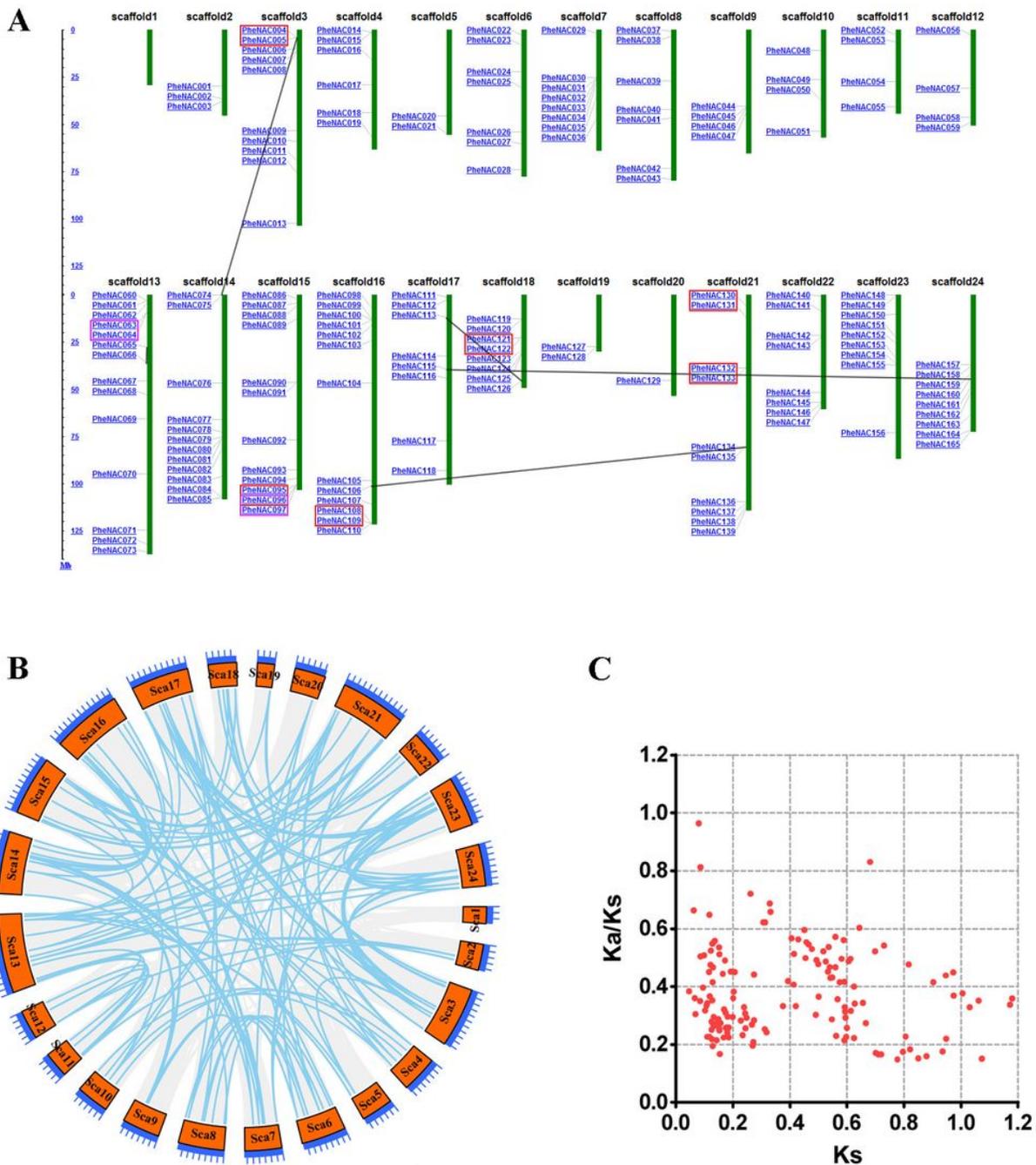
Phylogenetic tree of the domains from 165 PheNACs and 143 OsNACs. The phylogenetic tree of the NAM domain sequence was constructed using the neighbor-joining method with 100 bootstrap replicates. And the PheNAC members were divided into 14 distinct groups, which were marked with different colors, respectively.



**Figure 2**

Phylogenetic tree, gene structure and conserved motifs of 165 PheNACs. A: The Phylogenetic tree of 165 PheNACs was built by N-J method in MEGA6.0 with 1000 bootstrap values. B: CDS, intron, upstream/downstream and NAM domain (feature 1) were presented by different colors. C: All motifs were identified by MEME using the complete amino acid sequences of 165 NAC proteins. Each motif was

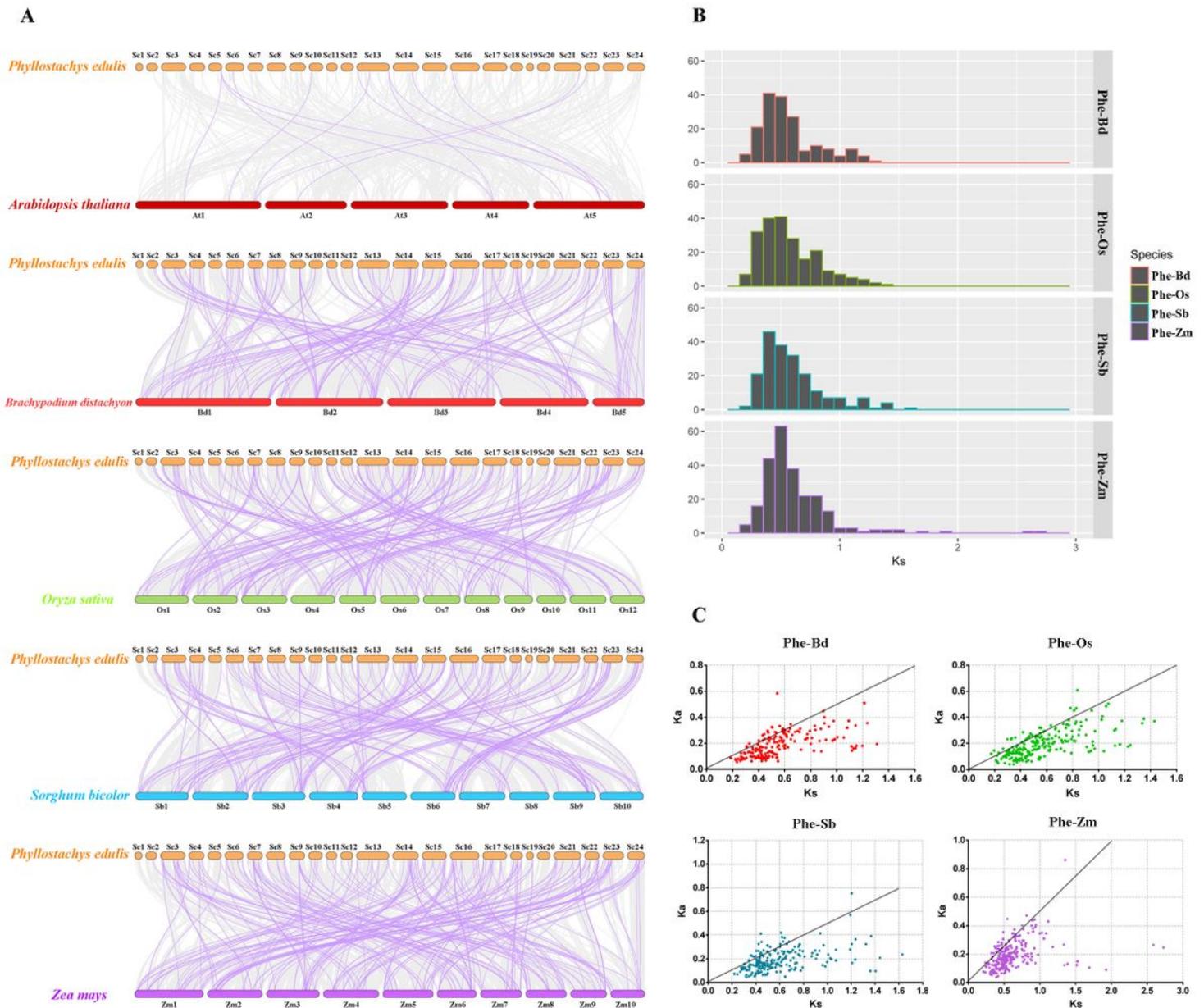
indicated with a different-colored box and numbered 1-10; motif details were listed in Additional file 9: Table S7.



**Figure 3**

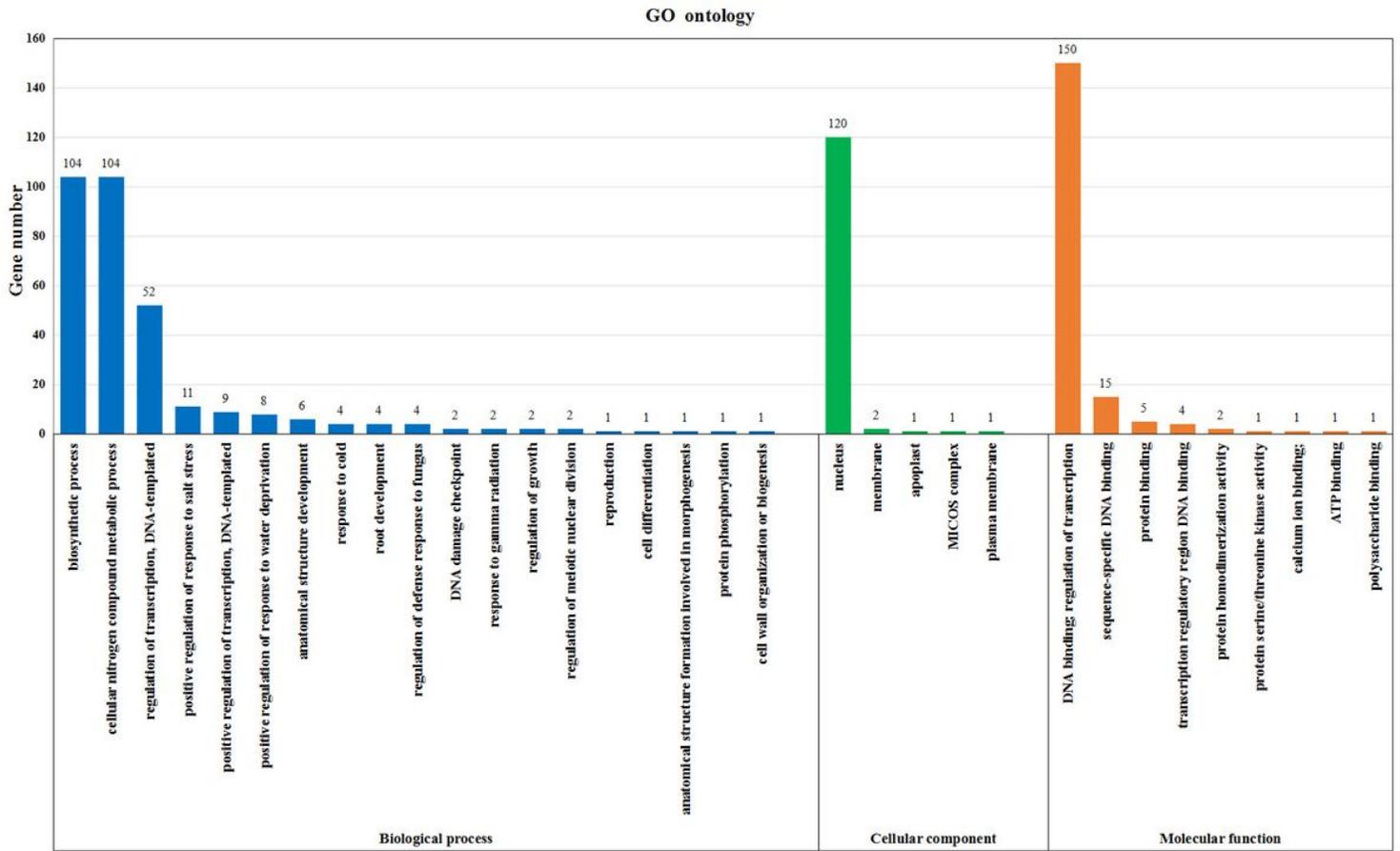
Scaffold location and gene duplication of PheNAC genes. A The 165 PheNAC genes were unevenly distributed on 23 scaffolds and scaffold numbers were indicated at the top of each vertical bar. The paralogous pairs of tandem duplication were marked with red rectangle frame, proximal duplication pairs were colored with purple rectangle frame and transposed duplication pairs were connected with gray

lines. B Orange blocks were moso bamboo scaffolds and the segmental duplicated pairs were connected with blue lines. C The distribution of Ks and Ka/Ks of 150 paralogous pairs (5 pair had no ks value).



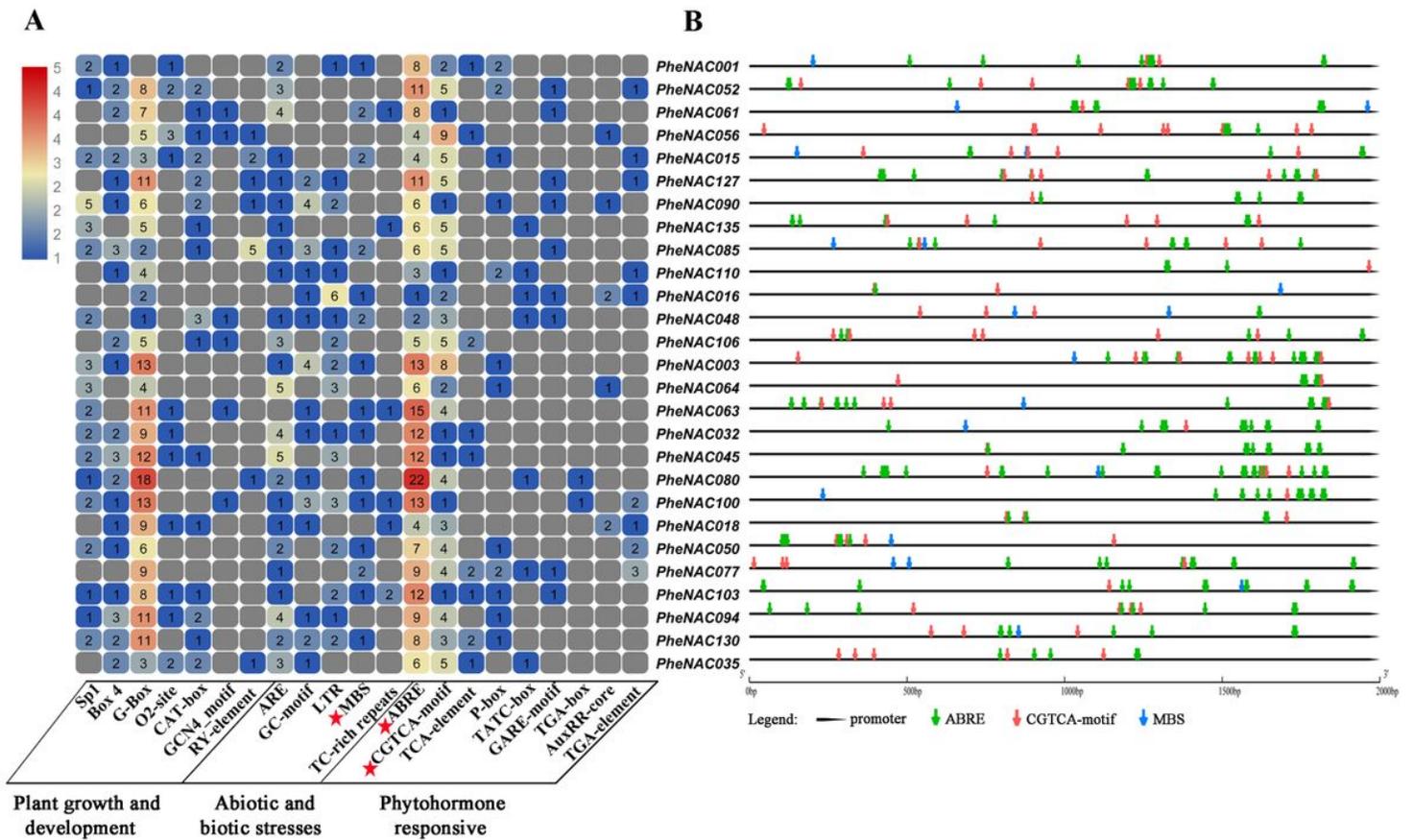
**Figure 4**

A Microsynteny analysis of NAC genes between moso bamboo and other plant species. Gray lines in the background indicated the collinear blocks within the moso bamboo genome and other species, whereas the purple lines highlighted the collinear of NAC gene pairs. B The Ks frequency distribution of moso bamboo with other four grass plants. C the distributions of Ka, Ks values of orthohomologous pairs between moso bamboo and other four species and the black lines represented slopes of  $Ka/Ks = 0.5$ . Four species were *Brachypodium distachyon*, *Oryza sativa*, *Sorghum bicolor* and *Zea mays*, respectively.



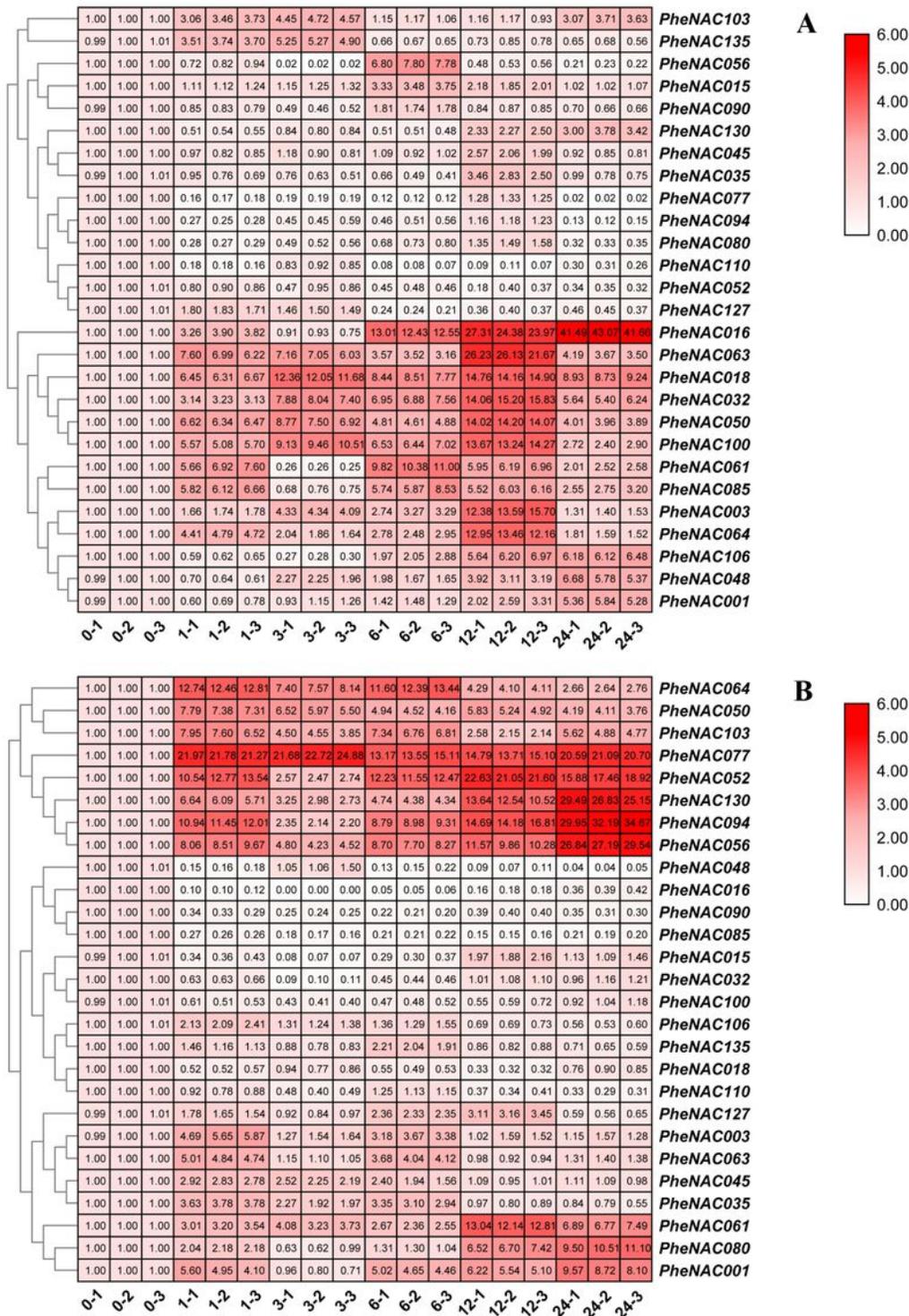
**Figure 5**

The statistics and classification of gene ontology annotation for 165 PheNACs: biological process, cellular component and molecular function.



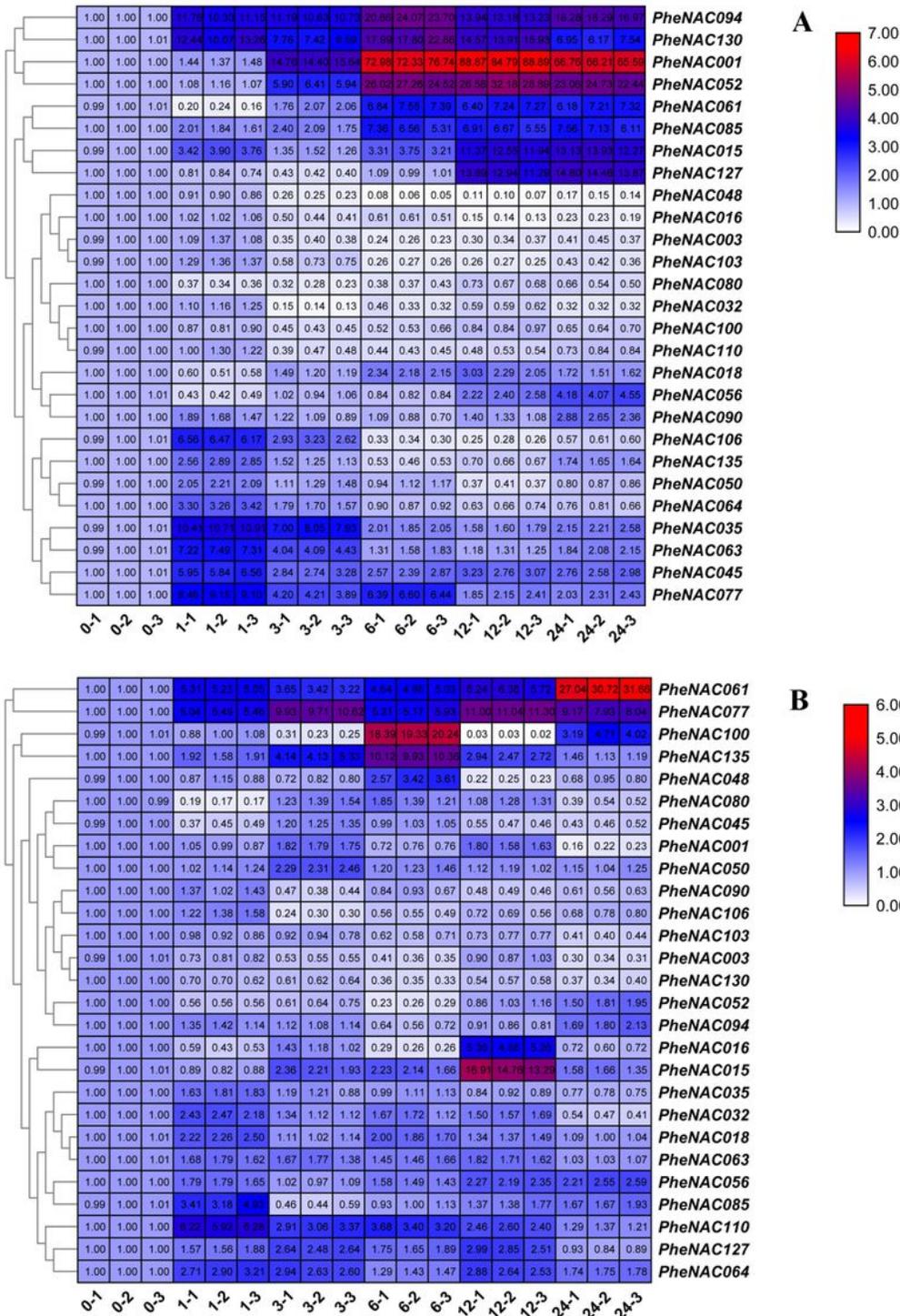
**Figure 6**

The investigation of promoter element numbers for the 27 NAC genes in A subfamily. A The numbers of different promoter elements for the 27 NAC genes were counted. B The specific locations of the three kinds of cis-acting elements (ABRE, CGTCA-motif and MBS) were depicted in the promoter region of 27 NAC genes.



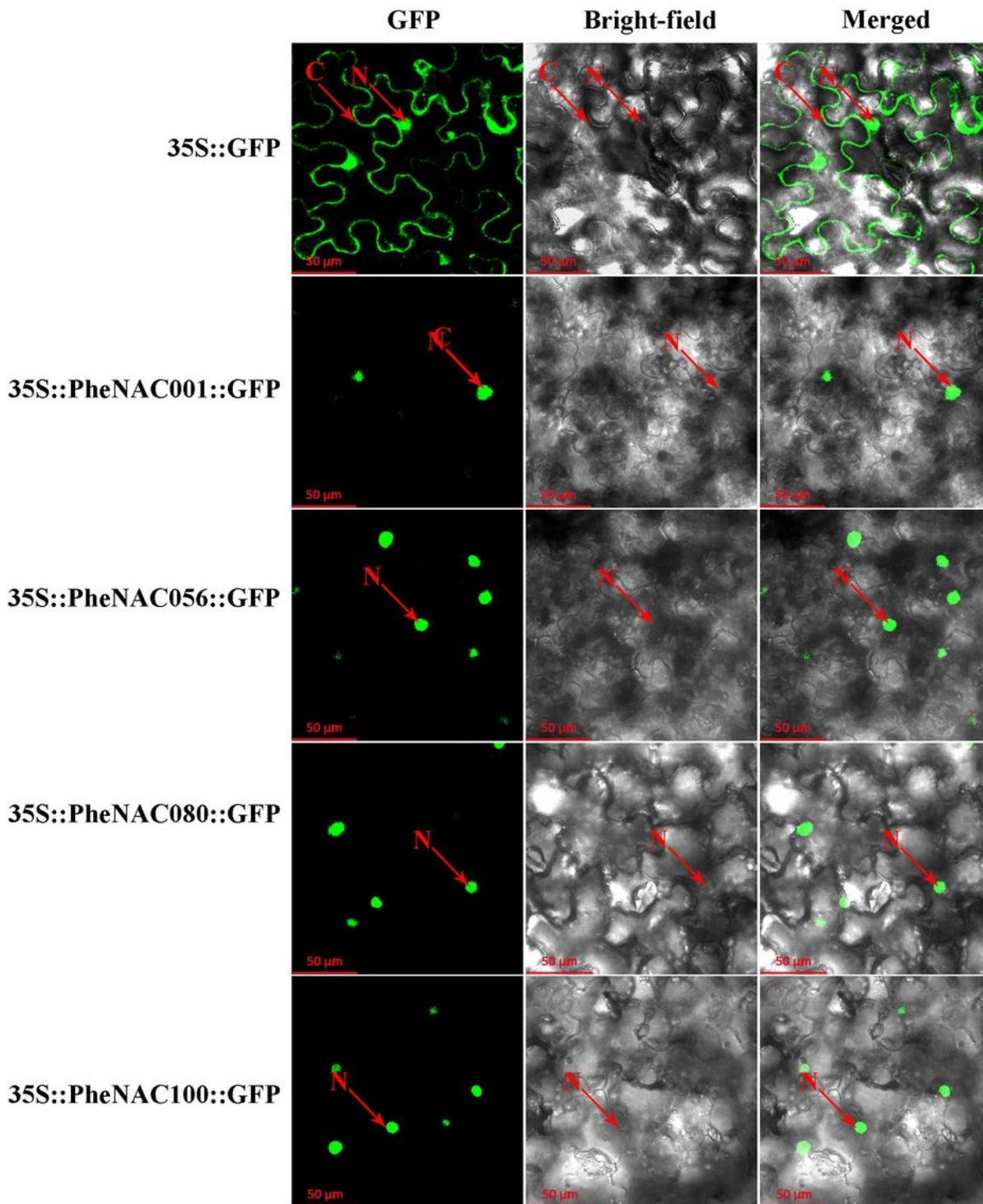
**Figure 7**

Expression analysis of PheNAC genes under 20% PEG6000 and 200 mM NaCl treatments. A The expression levels of 27 NAC members after 20% PEG6000 treatment were showed in heatmap at different time points and each sample had three replicates. The deeper the color, the higher the expression. B The expression levels of 27 NAC members after 200 mM NaCl treatment were showed in heatmap at different time points and each sample had three replicates. The deeper the color, the higher the expression.



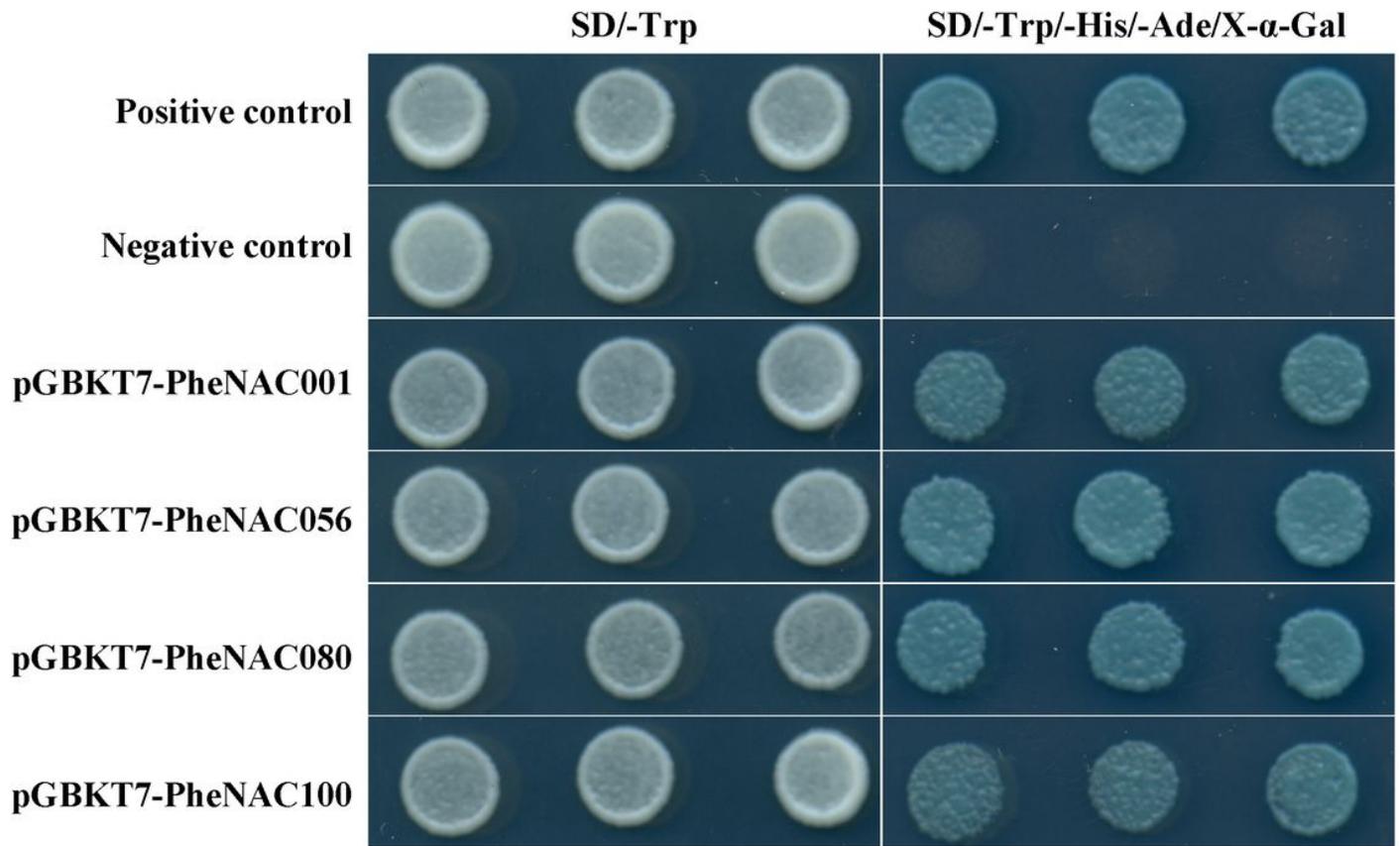
**Figure 8**

Expression analysis of PheNAC genes under 100  $\mu$ M ABA and 100  $\mu$ M MeJA treatments. A The expression levels of 27 NAC members after 100  $\mu$ M treatment were showed in heatmap at different time points and each sample had three replicates. The deeper the color, the higher the expression. B The expression levels of 27 NAC members after 100  $\mu$ M MeJA treatment were showed in heatmap at different time points and each sample had three replicates. The deeper the color, the higher the expression.



**Figure 9**

Subcellular localization of four PheNACs. The fusion proteins (35S::PheNAC001-GFP, 35S::PheNAC056::GFP, 35S::PheNAC080::GFP and 35S::PheNAC100::GFP) and the control (35S::GFP) were transiently expressed in *Nicotiana tabacum* leaves and observed under a fluorescence microscope. The red arrows indicated important areas (N stands for the nucleus and C stands for the cell membrane).



**Figure 10**

Transactivational analyses of four PheNAC proteins (PheNAC001, -056, -080 and -100) in yeast Y2HGold strain. The positive constructs, negative constructs, and fusion constructs were transformed into yeast strain and cultivated in SD/-Trp media and SD/-Trp/-His/-Ade/X-a-GAL media.

## Supplementary Files

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