Article

The NAC-like transcription factor CsNAC7 positively regulates the caffeine biosynthesis-related gene yhNMT1 in Camellia sinensis

Wenhui Ma1, Xin Kang1, Ping Liu1, Kexin Shen1, Yuanyuan Zhang1, Xiaorong Lin1, Bin Li1,2* and Zhongzheng Chen1,2,*

1College of Food Science, South China Agricultural University, 483 Wushan Street, Tianhe District, Guangzhou, Guangdong, 510642, China
2Guangdong Provincial Key Laboratory of Nutraceuticals and Functional Foods, South China Agricultural University, Guangzhou, Guangdong, 510642, China
*Corresponding authors. Email: bli@scau.edu.cn, zhongzhengch@scau.edu.cn

Abstract

Caffeine is an important functional substance and is abundant in tea plant, but little is known about how its biosynthesis is regulated by transcription factors. In this study, the NAC-like transcription factor-encoding gene CsNAC7, which is involved in caffeine synthesis, was isolated from a Yinghong 9 cDNA library using a yeast one-hybrid assay; this gene comprises 1371 bp nucleotides and is predicted to encode 456 amino acids. The expression of CsNAC7 at the transcriptional level in tea shoots shared a similar pattern with that of the caffeine synthase gene yhNMT1 in the spring and summer, and its expressed protein was localized in the nucleus. Assays of gene activity showed that CsNAC7 has self-activation activity in yeast, that the active region is at the N-terminus, and that the transient expression of CsNAC7 could significantly promote the expression of yhNMT1 in tobacco leaves. In addition, overexpression or silencing of CsNAC7 significantly increased or decreased the expression of yhNMT1 and the accumulation of caffeine in transgenic tea calli, respectively. Our data suggest that the isolated transcription factor CsNAC7 positively regulates the caffeine synthase gene yhNMT1 and promotes caffeine accumulation in tea plant.

Introduction

Tea [Camellia sinensis (L.) O. Kuntze] is rich in functional metabolites and is highly consumed worldwide. Constituting the main functional metabolites, alkaloids include caffeine, theobromine and theophylline. Caffeine is the main component of tea alkaloids, accounting for 2%–4% of tea dry weight, and has been verified to have many beneficial effects, such as causing refreshed feelings, reducing high blood pressure and reducing anxiety [1–3]. Therefore, caffeine has received much more attention due to its unique physiological activity, and various related studies, including studies on its biosynthesis mechanism, have also been conducted in depth.

Caffeine was first found in tea plant in the early 1820s, and its main synthesis pathway is xanthosine (XR) → 7-methylxanthosine (7-mXR) → 7-methylxanthine (7-mX) → theobromine (Tb) → caffeine (Cf); N-methyltransferase (NMT) plays a catalytic role in the three-step methylation reaction in this pathway [4]. The first NMT gene cloned from tea leaves, TSC1, exhibits a two-step transmethylation activity, including catalysis of 7-mX to Tb and Tb to Cf [5]. Subsequently, more NMT genes, including TCS2, PCS1, PCS2, ICS1, ICS2 and yhNMT1, have been isolated from Camellia ptilophylla, Camellia irrawadiensis and Camellia sinensis respectively [6, 7]. Previous studies have shown that NMT expression in tea plant not only is closely related to caffeine content but also has typical spatiotemporal specificity, suggesting that NMT gene expression is complex and may be regulated by specific factors [8, 9].

Transcription factors (TFs), important regulatory proteins, have been proven to play a key role in the metabolism of alkaloids, and many TFs, including AP2/ERF, WRKY, bHLH, Myb-like, bZIP, TFIIIA zinc finger and AT hook TFs, are involved in the biosynthesis of alkaloids by regulating the expression of related synthase-encoding genes [10]. In Catharanthus roseus, the biosynthesis of vincristine alkaloids has been verified to be regulated by TFs targeting STR and TDC gene expression, including CrWRKY1, CrMYC1, ORCA2 and ORCA3 TFs [11–14]. In tobacco, nicotine biosynthesis has been confirmed to be regulated by TFs including NbbHLH1, NtMYC2a and NtMYC2b targeting synthase-encoding genes [15, 16]. NAC, a plant-specific TF, has been found in dozens of plant species, such as maize, apple and Coffea canephora [17–21]. Studies have shown that
NACs are widely involved in plant metabolism, including basic physiological development, secondary metabolite synthesis and environmental stress responses [22–25]. In apple, MdNAC52 promotes the biosynthesis of anthocyanins and proanthocyanidin [19]. In tobacco, NtNAC-R1 has been shown to regulate the biosynthesis of nicotine [26]. Recently, transcriptome analysis of tea varieties with different caffeine contents showed that NACs are related to the biosynthesis of purine alkaloids [27]. Nevertheless, no studies have revealed how the synthesis of caffeine is regulated by transcription factors in tea plant.

In this study, Yinghong 9 tea plants were selected, and the transcription factor CsNAC7 was cloned using yeast one-hybrid (Y1H) assays via the promoter of yhNMT1 used as bait. The cloned TFs showed 99.12% homology with the NAC gene registered in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/) GenBank database for tea. To determine the function of CsNAC7 in caffeine accumulation by which the expression of the caffeine synthase gene yhNMT1 is regulated in tea plant, the spatiotemporal expression of CsNAC7 and yhNMT1 was determined by real-time fluorescence quantitative PCR (qRT–PCR), and the transcriptional activation of CsNAC7 was analyzed by using both transient expression and yeast two-hybrid technology. Moreover, CsNAC7 was overexpressed and silenced in tea calli, and the changes in yhNMT1 expression and caffeine content in transgenic calli were also determined. Our work and results are of great significance to further understand the molecular mechanism of caffeine metabolism in terms of TFs in tea plant.

Results
cDNA library construction and candidate TFs of yhNMT1 via Y1H assays

To reveal the expression of the caffeine synthase gene yhNMT1 regulated by TFs in tea plant, a cDNA library was constructed, followed by TF screening via Y1H assays. For construction of the cDNA library, the promoter of yhNMT1 was amplified, digested with Hind III and Xho I, cloned, and then inserted into the pAbAi vector pretreated with Hind III and Xho I. The recombinant PNMT1-AbAi vector was selected as a bait vector and transformed into the Y1H Gold strain. Total RNA was extracted from the shoots of Yinghong 9 plants and subjected to SMART RT–PCR. Then, SMART-obtained cDNA was separated by a Chroma column, which yielded more than 500 bp of ds-cDNA. The purified ds-cDNA and the linearized pGADT7-Rec prey vector were cotransformed into a new host yeast strain using a ClonExpress II One Step Cloning Kit (Takara, Tokyo), and a cDNA library was generated. The calculated capacity of 2.7 x 10^11 [6] CFU, which was higher than the required 1.0 x 10^10 [6] CFU, verified the success of the cDNA library.

Moreover, to identify TFs by screening the constructed cDNA library, an experiment to determine the minimum concentration of AbA inhibiting the growth of a new host yeast strain with the bait vector PNMT1-AbAi was conducted. Our results showed that the new developed host yeast strain could not grow on SD/–Leu media plus 150 ng.mL^-1 AbA (Fig. 1A); thus, a higher concentration of AbA (200 ng.mL^-1) was selected for addition to SD/–Leu media for TF screening from the cDNA library (Fig. 1B). As the screening results showed, one colony grew on the selected media.

Analysis of the TF-encoding gene CsNAC7 screened from the cDNA library

Colonies growing in SD/–Leu plus AbA were selected, and the recombinant pGADT7 plasmid was isolated followed by sequencing. Our data showed that the cDNA that was integrated into the pGADT7 vector had a complete open reading frame (ORF) containing 1371 bp nucleotides and encoding a predicted 456 amino acids, with a molecular weight of 49.77 kD. The isolated gene shared a NAC domain and 99.12% homology with NAC domain-containing protein 7-like isoform X2 (Camellia sinensis) (accession No. XP_028085347.1) from the tea genome registered in the NCBI database, and we designated the isolated gene as CsNAC7 (accession No. MT882349). Moreover, the sequence alignment results showed that CsNAC7 shared high amino acid sequence identity with Actinidia chinensis var. Chinesis AcNAC (45.2%), Vitis vinifera VvNAC29 (38.81%), Vitis riparia VrNAC92 (38.81%) and V. vinifera VuNAC30 (38.38%) (Fig. 2A). Further phylogenetic analysis showed that CsNAC7 from Yinghong 9 exhibited a high evolutionary relationship with NAC proteins from Gramineae and woody plant species registered in the NCBI GenBank database, especially Actinidiaceae (A. chinensis), which was assigned to Group 1 (Fig. 2B and Supplemental Table S2).

CsNAC7 Acts as a Transcription Activator in Yeast AH109

To determine whether the CsNAC7 gene has self-activation activity and which region of the gene has an activation effect, assays of transcriptional activators were conducted in yeast AH109 cells. Based on the results of our bioinformatics analysis, full-length CsNAC7 and two truncated fragments, including a 900 bp N-terminal sequence and 468 bp C-terminal sequence fused to the GAL4-binding domain, were amplified and inserted into the pGBK7 vector, and the recombinant vectors were designated as NAC7, NAC7ΔN and NAC7ΔC (Fig. 3A). The three recombinant vectors and the negative control vector pGBK7 were transformed into yeast AH109 cells, which were then inoculated onto the designated media (Fig. 3B). The results showed that all the strains could grow in SD/–Trp media; however, only two strains with NAC7 and NAC7ΔN could grow in SD/–Trp-His-Ade media, and the color of the colonies turned blue when X-a-gal was added to the media. Our data confirmed that the CsNAC7 gene has self-activation activity and that the activation region is located at the N-terminus.
Transient expression of CsNAC7 in tobacco reveals its TF role

To explore whether CsNAC7 has a regulatory effect on yhNMT1, a reporter vector $P_{\text{NMT1}}$-GUS and effector construct pCaMV35S-CsNAC7 were constructed, and then the two constructed vectors and positive-control plasmid pCAMBIA1301 (pCaMV35S-GUS) were transformed into Agrobacterium EHA105, which were subsequently injected into tobacco leaves (Fig. 4A). The results of GUS histochemical staining and GUS activity determination of the injected leaves showed that GUS activity was the highest in the positive control; moreover, the GUS activity in leaves cotransformed with the reporter vector ($P_{\text{NMT1}}$-GUS) and effector construct (pCaMV35S-CsNAC7) was significantly higher than that of the reporter vector alone (Fig. 4B-C), which indicated that the expression of CsNAC7 driven by the yhNMT1 promoter had a significant effect on the expression of GUS in tobacco leaves.

Expression analysis of CsNAC7 and yhNMT1 in tea shoots

Different parts of tea shoots (including the buds, the first leaf and the second leaf) in different seasons (spring, summer and autumn) were collected (Fig. 6A), and the expression of CsNAC7 and yhNMT1 was measured by qRT–PCR (Fig. 6B). Our results showed that CsNAC7 and yhNMT1 exhibited similar expression changes in different parts of tea shoots in the spring and summer. However, in autumn, the expression pattern of CsNAC7 was different from that of yhNMT1, and the expression of yhNMT1 gradually decreased from the buds to the second leaf; nevertheless, the expression of CsNAC7 in the first leaf decreased significantly from summer to autumn, and the expression levels in the buds, first leaf and second leaf were the same, with no significant difference.

Overexpression and silencing of CsNAC7 affects caffeine accumulation in tea Calli

To further elucidate the effect of the TF CsNAC7 on the expression of yhNMT1 and caffeine accumulation in tea plant, recombinant vectors pCAMBIA1031-35SN-CsNAC7 and RNAi-CsNAC7 for overexpression and silencing of CsNAC7, respectively, were constructed by inserting the CsNAC7 gene into pCAMBIA1031-35SN and pYLRNAi-35S, respectively. Genetic transformation was conducted via Agrobacterium infection of tea calli, which involved the use of newly generated calli resistant to hygromycin (Fig. 7A-D). Genomic DNA was extracted from the resistant calli and used as template for HPT gene amplification, and the PCR results verified the success of the transgene. Then, the transgenic calli were designated as P7–1 and R7–1, which corresponded to overexpression and silencing of CsNAC7, respectively (Supplemental Figure S2).

Total RNA was extracted from the transgenic calli, and the expression levels of yhNMT1 and CsNAC7 were
Figure 2. Analysis of the transcription factor CsNAC7 isolated from tea plant. (A) The amino acid sequence of CsNAC7 and alignment with other NAC homologs from Actinidia chinensis var. chinensis (AcNAC; accession No. F5S31359.1), Vitis vinifera (VvNAC29 and VvNAC30; accession No. RVX15250.1 and XP_002283395.1), and Vitis riparia (VrNAC92; accession No. XP_034675869.1). The conserved domain of CsNAC7 (amino acids 18–119) is indicated by the red line. (B) Phylogenetic analysis of CsNAC7.

detected by qRT–PCR (Fig. 7E). Our results showed that the expression of CsNAC7 in P7–1 was 6 times higher than that in non-transgenic CK calli, and the expression level of the caffeine synthase gene yhNMT1 was increased by 2.46 times compared with that in the control, indicating that overexpression of the CsNAC7 gene in P7–1 transgenic calli increased the expression of the yhNMT1 gene.

In contrast, the expression of CsNAC7 and yhNMT1 in R7–1 was decreased by 60% and 46%, respectively, compared with that in the control, indicating that the silencing of CsNAC7 in transgenic calli decreased the expression of yhNMT1. Moreover, caffeine accumulation in calli was determined by HPLC (Supplemental Figure S1), and the content of caffeine in P7–1 increased to 1398 μg g⁻¹ and
Figure 3. Analysis of the role of the CsNAC7 gene as a transcriptional activator in yeast AH109 cells. (A) Conserved domain analysis of the CsNAC7 gene and schematic diagram of the recombinant vector used for activation analysis. Diagram of the conserved domain of the CsNAC7 gene; pGBK77, vector used as a negative control; NAC7, full-length CsNAC7 fused to a GAL4 DNA-binding domain and inserted into the pGBK77 vector; NAC7AN, 900 bp N-terminal sequence of the CsNAC7 gene fused to the GAL4 DNA-binding domain; NAC7AC, 685 bp C-terminal sequence of the CsNAC7 gene fused to the GAL4 DNA-binding domain. (B) The growth of yeast cells transformed into different recombinant vectors in designated media. SD−Trp, different transformed yeast strains growing in SD−Trp media; SD−Trp−His−Ade/X−a-gal, different transformed yeast strains growing in SD−Trp−His−Ade media plus X−a-gal. The b colonies show the reporter gene MEL1 activated by inserted fragments, and the added X−a-gal hydrolyzed to the blue product 5-bromo-4-chloro-indigo.

Figure 4. Analysis of GUS staining and GUS activity in transiently transformed tobacco leaves. (A) Tobacco plants used for transient expression. (B) GUS histochemical staining of tobacco leaves. (C) GUS enzyme activity determination in tobacco leaves. Negative, normal tobacco leaves used as negative controls; positive, tobacco leaves transformed with the pcAMBI A1301 vector, serving as positive controls; PNMT1, tobacco leaves transformed with the reporter vector pNMT1-GUS; PNMT1 + CsNAC7, tobacco leaves cotransformed with the reporter vector pNMT1-GUS and effector construct pCaMV35S-CsNAC7; the asterisks show that the values are significantly different from that of PNMT1 (**, p < 0.01; *, p < 0.05). Reached 1.28 times that in the control (1094 μg g−1); nevertheless, the content of caffeine in R7–1 decreased to 358 μg g−1 and was reduced by 67% (Fig. 7F). Therefore, overexpression of CsNAC7 promoted the expression of yhNMT1 and the accumulation of caffeine, whereas inhibition of CsNAC7 decreased the expression of yhNMT1.
and the accumulation of caffeine in the calli of Yinghong 9. In conclusion, the transcription factor CsNAC7 positively regulates the yhNMT1 gene and promotes caffeine accumulation in calli.

Discussion

Alkaloids are important secondary chemical substances in plants, and their biosynthesis is mainly catalyzed by synthetase genes. Previous studies have shown that many transcription factors are involved in the regulation of plant alkaloid synthase genes. The biosynthesis of terpenoid indole alkaloids in C. roseus was inhibited by the transcription factors CrGBF1 and CrGBF2 by inhibiting the expression of the strictosidine synthase gene [28]. A similar phenomenon was found in C. roseus, in which light-induced biosynthesis of vindoline was regulated by the transcription factor CrPIF1 by inhibiting CrGATA1 gene expression [29]. On the other hand, transcription factors also promote the synthesis of plant alkaloids. In C. roseus, overexpression of the transcription factors ORCA3 and G10H significantly increased the accumulation of monoindole indole alkaloids [14]; overexpression of CrERF5 can increase the expression of key genes in the biosynthesis pathway of monoterpenoid indole alkaloids and upregulate the synthesis of bisindole alkaloids [30]. In tobacco, the transcription factors ERF189, ORCA3 and AtERF13 have been found to activate PMT genes related to nicotine and tropane alkaloid biosynthesis [31]; the transcription
factor NtWRKY-R1 integrates JA and IAA signals and regulates the expression of nicotine synthesis-related genes [32]. In addition, transcriptome analysis showed that 549 transcription factor genes belonging to the MYB, bHLH, NAC, bZIP, WRKY, GRAS and other families were involved in the regulation of nicotine biosynthesis in tobacco after topping was performed [33]. Caffeine is one of the most important alkaloids in tea plant, and little is known about the regulation of caffeine biosynthesis by transcription factors. Transcriptome analysis of four tea varieties with high, normal and low caffeine contents showed that NAC, bHLH, WRKY, GRAS and MYB TFs were related to the biosynthesis of purine alkaloids, but the regulatory network of caffeine biosynthesis in tea is still unclear [27]. In this study, we cloned the NAC TF CsNAC7 from tea plant and confirmed its regulatory role in the expression of the caffeine synthase gene. Overexpression or silencing of CsNAC7 in tea calli leads to a significant
increase or decrease, respectively, in yhNMT1 expression and accumulation.

As plant-specific transcriptional regulators, NACs are widely involved in the regulation of plant secondary metabolite synthesis. In *A. chinensis*, three transcription factors, AaNAC2, AaNAC3 and AaNAC4, are involved in monoterpenoid biosynthesis by binding to the promoter of *AaTPS1* [34]. In *V. vinifera*, two NAC genes probably participate in the regulation of anthocyanin accumulation [35]. In peach, *PpNAC1* acts as a transcriptional activator of *PpMYB10.1*, regulating the synthesis of anthocyanin pigment [36]. In *Arabidopsis thaliana*, *VaNAC17* is involved in jasmonic acid biosynthesis by upregulating the transcription of the LOX3, AOC1 and OP3 genes [37], and overexpressing *ANAC046* significantly increases the expression of suberin biosynthesis-related genes [38]. Interestingly, the NAC-like transcription factor *CsNAC7* isolated from Yinghong 9 tea plant in our study proved to be involved in the caffeine biosynthesis regulatory network by activating *yhNMT1* expression.

How to isolate and scientifically determine the functional activity of TFs is of great significance for understanding the synthesis of metabolites in organisms. Y1H assays combined with transient expression technology is generally used to study metabolic regulation. In sweet cherry, Y1H assays revealed that the transcription factor *PaMADS7* could directly bind to the *PaPG1* promoter, which is involved in fruit softening [39]. In apple, *MdLUX* and *MdPCL*-like transcription factors were found via Y1H assays to bind to the promoter of the anthocyanin biosynthesis-related gene *MdF3H* [40]. In *Taraxacum antungense*, a Y1H assay proved that the transcription factor *TaWRKY14* binds to the W-box of the *TaPAL1* promoter, which is related to the accumulation of chlorogenic acid [41]. In this study, Y1H technology was also adopted to screen TFs from Yinghong 9 tea plant with the use of the promoter of *yhNMT1* as bait, and as a result, the transcription factor *CsNAC7* was successfully screened on the basis of its ability to bind to the promoter. On the other hand, transient expression using GUS as a reporter is often used to reveal the regulatory function of transcription factors. In soybean calli, GUS activity analysis showed that the TF *GmERF6*, a transcriptional repressor, downregulated the expression of *AtPDF1.2* and *AtPR4* [42]. In *Nicotiana benthamiana* leaves, GUS activity analysis revealed that the TF *CrGATA1* isolated from *C. roseus* could strongly activate the expression of *D4H* [29]. In tobacco, GUS activity analysis demonstrated that the TFs *ORCA3* and *AtERF13* could promote the expression of *PMT2* [31]. In our study, transient expression in tobacco leaves was successfully adopted, and the TF *CsNAC7* was verified via GUS staining and GUS activity analysis to activate the expression of *yhNMT1*.

Transgenic plant technology provides a good platform for gene functional research. However, some species are difficult to regenerate, which limits the application of transgenic technology to reveal gene function. Callus, which is induced in vitro and exhibits metabolic characteristics similar to those of cultivated plants [43–45], provides a new platform for revealing the function of genes and has been successfully used in sugarcane, apple, grapevine, *carica papaya* and melon [46–50]. TFs are important proteins in plants, and functional research via callus platforms in which transgene technology was used has also been conducted. In chrysanthemum, the regulatory gene *elf5B1* was introduced into calli, and transgenic calli with high temperature tolerance [51] were obtained. In blueberry, overexpression of the transcription factor *VcMYB4a* in calli enhanced the sensitivity of transgenic calli to salt, drought, cold, freezing, and heat stress [52]. Similarly, in grapefruit, overexpression of the transcription factor *CsPIF8*, which was isolated from *Citrus sinensis* calli, increased the tolerance of transgenic calli to cold [53]. Moreover, reports have shown that overexpression of the transcription factor *CmAG11* in Chinese chestnut calli could enhance somatic embryogenesis [54]. Tea plants are perennials used for beverages, and regeneration in vitro is difficult due to the abundance of secondary metabolites, which conversely hinders functional research on tea genes [55]. To date, the function of only the TCS1 gene isolated from tea plant has studied through transgenic plant regeneration technology in tea [56]. Fortunately, our previous study verified that transgenic calli induced from tea leaves could be employed to reveal gene function [57]. In the present study, a transgene was generated in tea calli induced from Yinghong 9 by using overexpression and silencing technology, and it was found that the transcription factor *CsNAC7* could positively affect *yhNMT1* expression and the accumulation of caffeine in the transgenic calli, which verified the regulatory function of *CsNAC7* in caffeine metabolism in tea. In addition, the established transgenic overexpression and silencing methods in our research will help to reveal the functions of other genes involved in the development and metabolism of tea plant. Overall, our data demonstrated that the synthesis of caffeine is regulated by transcription factors as well as by caffeine synthase genes, which indicates the complexity of the molecular mechanism underlying caffeine metabolism in tea plant (Fig. 8).

**Materials and methods**

**Plant materials and growth conditions**

*C. sinensis* Yinghong 9 is a tea variety bred from a single plant selected from an inbreeding population of large-leaf Yunnan species. The caffeine content of Yinghong 9, a common tea variety, is similar to that of other tea plant varieties, but Yinghong 9 is rich in tea polyphenols, is suitable for the production of black tea and is widely planted in Guangdong Province [58]. New shoots of Yinghong 9 were collected from tea plantations at South China Agricultural University, Guangzhou, China. Tea calli were induced from Yinghong 9 as previously reported [45].
Figure 8. Working model of CsNAC7 involvement in caffeine biosynthesis. The caffeine biosynthesis pathway is xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine. The two dashed pink arrows indicate the two-step methylation reaction in the caffeine biosynthesis pathway performed by the yhNMT1 protein. The two dashed green arrows indicate that tea leaves and tea calli share the same caffeine biosynthesis pathway. The three red solid arrows indicate that CsNAC7 upregulates the expression of yhNMT1 and increases the accumulation of caffeine in tea calli.

**Construction of a complementary DNA (cDNA) library and screening candidate TFs via Y1H**

The pAbAi vector (Clontech) was selected for construction of the bait plasmid. The promoter of yhNMT1 was amplified from the cloned plasmid with the specific primers P_NMT1-F and P_NMT1-R together with the action of restriction endonuclease Hind III and Xho I recognition sequences at the 5′ end, respectively (Supplemental Table S1). The amplified product was treated with Hind III and Xho I and ligated into the pAbAi vector, which was pretreated with Hind III and Xho I, and grown on synthetic dropout (SD)/-Ura media; the strains were transformed with the bait recombinant vector PNMT1-AbAi, yielding bait yeast/PNMT1-AbAi strains. Then, the strains resistant to aureobasidin A (AbA) were grown in SD/-Ura media plus 0 ng.mL⁻¹, 25 ng.mL⁻¹, 50 ng.mL⁻¹, 75 ng.mL⁻¹, 100 ng.mL⁻¹, 125 ng.mL⁻¹ and 150 ng.mL⁻¹ AbA. Moreover, a total of 800 mg of leaves was crushed in liquid nitrogen, and total RNA was extracted using Plant RNA Purification Reagent (Takara, Tokyo) according to the manufacturer’s instructions. First-strand cDNA was synthesized using a SMARTer® PCR cDNA Synthesis Kit, and amplification was conducted using the Advantage R2 PCR Kit (Clontech) according to the manufacturer’s instructions to obtain SMART cDNA, followed by separation with a Chroma spin TE400 column and purification of fragments greater than 500 bp. The purified cDNA was mixed with the pGADT7-Rec prey vector, transformed into the yeast strain AH109 and grown on SD/-Trp media. The transformants growing on SD/-Trp media were selected, diluted in 100 μL of water and inoculated onto SD/-Trp-His-Ade plates. After culture at 30°C for 3–5 days, 4 mg/mL X-a-gal solution was added to the growing yeast colonies until selected single colonies were covered by the X-a-gal solution. Then, the color change of selected single colonies was observed to evaluate the transactivation activity.

**Sequence analysis of CsNAC7**

The candidate transcription factor gene CsNAC7 was analyzed using online tools of the NCBI database. The isoelectric point and molecular weight of the CsNAC7-deduced protein were predicted using ProtParam, multiple sequence alignment was analyzed by DNAMAN, and a phylogenetic tree comprising CsNAC7 and other registered NAC transcription factor genes from related plant species was constructed by MEGA X.

**Analysis of the transcriptional activation function of CsNAC7 in yeast AH109**

Based on the analysis of the conserved domains of the CsNAC7 gene, the full-length ORF (1371 bp), N-terminal 900 bp sequence and C-terminal 468 bp sequence of CsNAC7 were amplified from the recombinant plasmid by three specific pairs of primers (Supplemental Table S1). Then, the three purified PCR products were cloned into a linearized pGBK7 vector (Clontech) pretreated with EcoR I and BamH I using a ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd.), and recombinant vectors designated as pCsNAC7, pNAC7ΔN and pNAC7ΔC were obtained. The recombinant vectors were transformed into the yeast strain AH109 and grown on SD/-Trp media. The transformants growing on SD/-Trp media were selected, diluted in 100 μL of water and inoculated onto SD/-Trp-His-Ade plates. After culture at 30°C for 3–5 days, 4 mg/mL X-a-gal solution was added to the growing yeast colonies until selected single colonies were covered by the X-a-gal solution. Then, the color change of selected single colonies was observed to evaluate the transactivation activity.
**Transient expression of CsNAC7 in tobacco leaves**

The promoter of yhNMT1 was amplified from the cloned plasmid with the specific primers p1301-F and p1301-R (Supplemental Table S1), which had restriction endonuclease Hind III and Nco I recognition sequences at the 5’ end. The amplified product was treated with Hind III and Nco I and ligated into the pCAMBIA1301 vector, which was pretreated with Hind III and Nco I, to obtain the reporter vector P\textsubscript{NMT1}-GUS. In addition, full-length CsNAC7 was amplified with the specific primers pR1101-F and pR1101-R (Supplemental Table S1), cloned and ligated into the pR1101 AN vector pretreated with EcoR I and Sal I using a ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd.) to obtain the effect vector pCaMV3SS-CsNAC7. The effect vector and reporter vector were transformed into Agrobacterium EHA105. The infection solution with the two strains of Agrobacterium tumefaciens at a ratio of 1:1 and an OD600 of 0.6 (adjusted by dilution of a solution consisting of 10 mmol·L\(^{-1}\) MES, 150 μmol·L\(^{-1}\) AS and 10 mmol·L\(^{-1}\) MgCl\(_2\)) was prepared and injected into tobacco leaves, and GUS histochemical staining and determination of GUS enzyme activity were conducted on the injected leaves 3 days later in darkness. For GUS staining, the tobacco leaves were cut into 0.5 cm\(^2\) pieces and soaked in GUS staining solution for 24 hours at 37°C, followed by washing with 75% ethanol. The GUS enzyme activity was detected with a plant GUS activity assay kit from FCNCSTechnology Laboratories. Untreated tobacco leaves were used as negative controls, and tobacco leaves transformed with pCaMV3SS-GUS were used as positive controls.

**Analysis of subcellular localization**

The full-length ORF of CsNAC7 was amplified from the recombinant plasmid by a specific pair of primers (GFP-F and GFP-R) to which the base sequence of pGreen-C18-GFP was added to the 5’ end (Supplemental Table S1), and the purified PCR products were cloned and inserted into a linearized pCamV3SS-GFP vector pretreated with Hind III and Bam HI; a ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd.) to obtain the effect vector pCaMV3SS-CsNAC7. The effect vector and reporter vector were transformed into Agrobacterium tumefaciens A. tumefaciens strain EHA105. Colonies identified by PCR were selected and inoculated into Luria-Bertani (LB) liquid media that included 50 mg.L\(^{-1}\) kanamycin (Kana) and 20 mg.L\(^{-1}\) rifampin (Rif) antibiotics and incubated at 28°C for 2 min. The culture media were centrifuged at 5000 × g for 2 min to collect Agrobacterium and prepared as an infection solution by resuspension of the MS media to an OD600 value of 0.6. The induced tea calli were cut into pieces approximately 0.5 cm × 0.5 cm in size and immersed in Agrobacterium infection solution plus 100 μmol·L\(^{-1}\) acetosyringone (AS) for 20 min. The infected calli were dried on sterile filter paper and grown on MS solid media plus 100 μmol·L\(^{-1}\) AS for 3 days in the dark. Afterward, the calli were washed three times with 400 mg.L\(^{-1}\) carbenicillin solution, washed three times with sterile water, and then inoculated on MS solid media plus carbenicillin (200 mg.L\(^{-1}\)) and hygromycin (35 mg.L\(^{-1}\));
the media was replaced every 3 weeks. Approximately 60 days later, newly reproduced calli resistant to hygromycin were obtained. The newly reproduced calli were then subjected to PCR and sequencing analysis to identify transgenic calli.

Nucleic acid isolation from Calli and gene expression analysis via qRT–PCR in transgenic tea Calli

Genomic DNA and total RNA were isolated from newly reproduced calli resistant to hygromycin and nontransgenic calli (as CKs) using an All-in-One DNA/RNA Mini Prep Kit (Sangon Biotech). The screening marker gene HPT, which is resistant to hygromycin B, was amplified by the specific primers Hpt-F and Hpt-R, with the isolated genomic DNA used as template, and then sequencing of the PCR products was conducted to identify the resistant calli, which were transgenic. cDNA was prepared from isolated RNA of CsNAC7, yhNMT1 and GAPDH and subjected to qRT–PCR analysis as described in Section 2.7. Nontransgenic calli were used as negative controls, and the relative gene expression was set as 1. Each experiment was repeated 3 times with biological replicates.

Caffeine content determination in tea Calli

Tea calli were cut into small granules and dried at 50°C to a constant weight. Callus samples (0.5000 g) were accurately weighed, and 20 mL of boiling water was added for 40 min. The extract was filtered immediately and allowed to cool naturally, after which it was added to ddH2O (25 mL total volume). The content was determined by an Agilent 1200 HPLC series instrument. Isolation was accomplished on a Poroshell 120 Bonus-RP column (4.6 mm × 50 mm, 2.7 μm) maintained at 30°C. The mobile phase consisted of (A) 100% acetonitrile and (B) water plus 0.05% trifluoroacetic acid. The elution conditions were as follows: 0∼8 min, A from 0% to 9%, B from 100% to 91%; 8∼17 min, A from 9% to 17%, B from 91% to 83%; and 17∼26 min, A from 17% to 28%, B from 83% to 72%. The flow rate was 0.8 mL-min⁻¹, and the injection volume was 5 μL. The peak of caffeine was verified according to the retention time of the standard.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31770727 and No. 31270726), Science and Technology Project of Guangzhou City (No. 201607010139), Science and Technology Project of Guangdong Province (No. 2015A030302065) and Special Agricultural Industrial Technology System (CARS-19).

Author Contributions

Wenhui Ma wrote the article and performed the experiments. Xin Kang, Ping Liu and Kexin She performed the experiments. Yuanyuan Zhang and Xiaorong Lin reviewed the article. Bin Li reviewed the article and supervised the research. Zhongzheng Chen reviewed the article and designed and supervised the research.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its supplemental information files.

Conflicts of Interest

The authors declare that they have no conflicts of interest related to the contents of this article.

Supplemental Information

Figure S1. Caffeine content determination in transgenic calli and nontransgenic calli by HPLC
Figure S2. Transgene identification in resistant calli by PCR amplification of the HPT gene
Table S1. Primers used in this study
Table S2. Accession numbers of genes selected from the NCBI website (https://www.ncbi.nlm.nih.gov) for phylogenetic analysis

Supplementary data

Supplementary data is available at Horticulture Research online.

Statistical Analysis

All the results are presented as the means ± Standard Deviations of three independent biological experiments conducted three times. SPSS software was used for t-tests and one-way analysis, and the difference was significant at least when p < 0.05.

Accession Numbers

The sequence data from this article can be found on the NCBI website (https://www.ncbi.nlm.nih.gov/) under the following accession Nos.: XP_028085347.1 (NAC domain-containing protein 7-like isoform X2) and MT882349 (CsNAC7).

References

1. Paré W. The effect of caffeine and seconal on a visual discrimination task. J Comp Physiol Psychol. 1961;54:506–9.
2. Hartley TR, Sung BH, Pincomb GA et al. Hypertension risk status and effect of caffeine on blood pressure. Hypertension. 2000;36:137–41.
3. Mohanpuria P, Kumar V, Yadav SK. Tea caffeine: metabolism, functions, and reduction strategies. Food Sci Biotechnol. 2010;19:275–87.
4. Negishi O, Ozawa T, Imagawa H. Biosynthesis of caffeine from purine nucleotides in tea plant. Biosci Biotechnol Biochem. 1992;56:499–503.
5. Kato M, Mizuno K, Crozier A et al. Caffeine synthase gene from tea leaves. Nature. 2000;406:956–7.
6. Yoneyama N, Morimoto H, Ye CX et al. Substrate specificity of N-methyltransferase involved in purine alkaloids synthesis is dependent upon one amino acid residue of the enzyme. Mol Gen Genomics. 2006;275:125–35.
7. Ping L, Kang X, Ma W et al. Isolation and functional analysis of primer for N-methyltransferase gene associated with caffeine biosynthesis in tea plants (Camellia sinensis). Journal of tea science. 2018;38:569–79.
8. Mohanpuria P, Kumar V, Joshi R et al. Caffeine biosynthesis and degradation in tea (Camellia sinensis (L.) K.UNTZ) is under developmental and seasonal regulation. Mol Biotechnol. 2009;43:104–11.
9. Lifei X, Limbo C, Mingzhi L et al. Determination of alkaloid and analysis of gene expression in peculiar tea plant. Southwest China Journal of Agricultural Sciences. 2013;3:947–9.
10. Yamada Y, Sato F. Transcription factors in alkaloid biosynthesis. Int Rev Cell Mol Biol. 2013;305:339–82.
11. Menke FL, Champion A, Kijne JW et al. A novel jasmonate- and elicitor-responsive element in the perilipin secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J. 1999;18:4455–63.
12. Chatel G, Montiel G, Pre M et al. CrMYC1, a Catharanthus roseus elicitor- and jasmonate-responsive bHLH transcription factor that binds the G-box element of the strictosidine synthase gene promoter. J Exp Bot. 2003;54:2587–8.
13. Suttitpan T, Pattanaik S, Kulshreshtha M et al. The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in Catharanthus roseus. Plant Physiol. 2011;157:2081–93.
14. Pan Q, Wang Q, Yuan F et al. Overexpression of ORCA3 and G10H in Catharanthus roseus plants regulated alkaloid biosynthesis and metabolism revealed by NMR-metabolomics. PLoS One. 2012;7:1–14.
15. Todd AT, Liu E, Polvi SL et al. A functional genomics screen identifies diverse transcription factors that regulate alkaloid biosynthesis in Nicotiana benthamiana. Plant J. 2010;62:589–600.
16. Zhang H, Bokowiec MT, Rushton PJ et al. Tobacco transcription factors NtMYC2a and NtMYC2b form nuclear complexes with the NtAZ1 repressor and regulate multiple jasmonate-inducible steps in nicotine biosynthesis. Mol Plant. 2012;5:73–84.
17. Dong X, Jiang Y, Yang Y et al. Identification and expression analysis of the NAC gene family in Coffea canephora. Agronomy (Basel). 2019;9:670.
18. Liu M, Ma Z, Sun W et al. Genome-wide analysis of the NAC transcription factor family in Tartary buckwheat (Fagopyrum tataricum). BMC Genomics. 2019;20:1–16.
19. Sun Q, Jiang S, Zhang T et al. Apple NAC transcription factor MdNAC2 regulates biosynthesis of anthocyanin and proanthocyanidin through MdMYB9 and MdMYB11. Plant Sci. 2019;289:110286–12.
20. Nie G, Yang X, Yang Z et al. Genome-wide investigation of the NAC transcript factor family in perennial ryegrass ( Lolium perenne L.) and expression analysis under various abiotic stressors. Genomics. 2020;112:4224–4231. https://doi.org/10.1016/j.ygeno.2020.06.033.
21. Wang G, Yuan Z, Zhang P et al. Genome-wide analysis of NAC transcription factor family in maize under drought stress and rewatering. Physiol Mol Biol Plants. 2020;26:705–17.
22. Olsen AN, Ernst HA, Leggio LL et al. NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci. 2005;10:79–87.
23. Zhong R, Lee C, Ye Z. Functional characterization of poplar wood-associated NAC domain transcription factors. Plant Physiol. 2010;152:1044–55.
24. Nakashima K, Takasaki H, Mizoi J et al. NAC transcription factors in plant abiotic stress responses. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms. 2012;1819:97–103.
25. Shang X, Yu Y, Zhu L et al. A cotton NAC transcription factor GhNAC2 plays positive roles in drought tolerance via regulating ABA biosynthesis. Plant Sci. 2020;296:1–13.
26. Fu Y, Guo H, Cheng Z et al. NtNAC-R1, a novel NAC transcription factor gene in tobacco roots, responds to mechanical damage of shoot meristem. Physiologie végétale. 2013;69:74–81.
27. Zhu B, Chen LB, Lu M et al. Caffeine content and related gene expression: novel insight into caffeine metabolism in camellias plants containing low, normal, and high caffeine concentrations. J Agric Food Chem. 2019;67:3400–11.
28. Siberly I, Benhamroun S, Memelink J et al. Catharanthus roseus G-box binding factors 1 and 2 act as repressors of strictosidine synthase gene expression in cell cultures. Plant Mol Biol. 2001;45:477–88.
29. Liu Y, Patra B, Pattanaik S et al. GATA and phytochrome interacting factor transcription factors regulate light-induced vindoline biosynthesis in Catharanthus roseus. Plant Physiol. 2019;180:1336–50.
30. Pan Q, Wang C, Xiong Z et al. CrERF5, an AP2/ERF transcription factor, positively regulates the biosynthesis of bisindole alkaloids and their precursors in Catharanthus roseus. Front Plant Sci. 2019;10:1–3.
31. Shoji T, Hashimoto T. DNA-binding and transcriptional activation properties of tobacco NIC2-locus ERF189 and related transcription factors. Plant Biotechnology. 2012;29:35–42.
32. Jin W, Zhou Q, Wei Y et al. NtWRKY-R1, a novel transcription factor, integrates IAA and JA signal pathway under topping damage stress in Nicotiana tabacum. Front Plant Sci. 2018;9:2263.
33. Qin Y, Bai S, Li W et al. Transcriptome analysis reveals key genes involved in the regulation of nicotine biosynthesis at early time points after topping in tobacco (Nicotiana tabacum L.). BMC Plant Biol. 2020;20:1–15.
34. Nieuwenhuizen N, Chen X, Wang MY et al. Natural variation in monoterpene synthesis in kiwifruit: transcriptional regulation of terpene synthases by NAC and EIN3-like transcription factors. Plant Physiol. 2015;167:1243.
35. Sun L, Li S, Tang X et al. Transcriptome analysis reveal the putative genes involved in light-induced anthocyanin accumulation in grape ‘red globe’ (V.vinifera L.). gene. Gene. 2020;728:144284. https://doi.org/10.1016/j.gene.2019.144284.
36. Zhou H, Wang KL, Wang H et al. Molecular genetics of blood-fleshed peach reveals activation of anthocyanin biosynthesis by NAC transcription factors. Plant J. 2015;82:105–21.
37. Su L, Fang L, Zhu Z et al. The transcription factor VaNAC17 from grapevine (Vitis amurensis) enhances drought tolerance by modulating jasmonic acid biosynthesis in transgenic Arabidopsis. Plant Cell. 2020;30:621–34.
38. Mahmood K, Zeisler-Diehl VV, Schreiber L et al. Overexpression of ANAC046 promotes suberin biosynthesis in roots of Arabidopsis thaliana. Molecular sciences. 2019;20:6117.
39. Qi X, Liu C, Song L et al. PaMADS7, a MADS-box transcription factor, regulates sweet cherry fruit ripening and softening. Plant Sci. 2020;301:1–14.
40. Li W, Ning GX, Zuo CW et al. MYB_SH[AL]QKY[RF] transcription factors MdLUX and MdPCL-like promote anthocyanin accumulation through DNA hypomethylation and MdF3H activation in apple. Tree Physiol. 2020;41:836–848. https://doi.org/10.1093/treephys/tpaa156.

41. Liu Q, Zhou W, Ruan Q et al. Overexpression of TaWRKY14 transcription factor enhances accumulation of chlorogenic acid in Taraxacum antungense Kitag and increases its resistance to powdery mildew. Plant Cell. 2020;143:665–79.

42. Zhai Y, Li JW, Li XW et al. Isolation and characterization of a novel transcriptional repressor GmERF6 from soybean. Biol Plant. 2013;57:26–32.

43. Wang Y, Gao LP, WangZR et al. Light-induced expression of genes involved in phenylpropanoid biosynthetic pathways in callus of tea (Camellia sinensis L.). Sci Hortic. 2012;133:72–83.

44. Oleszkiewicz T, Klimek-Chodacka M, Milewska-Hendel A et al. Unique chromoplast organisation and carotenoid gene expression in carotenoid-rich carrot callus. Planta. 2018;248:1455–71.

45. Shi J, Zhang X, Zhang Y et al. Integrated metabolic and transcriptomic strategies to understand the effects of dark stress on tea callus flavonoid biosynthesis. Physiologie végétale. 2020;155:549–559. 10.1016/j.plaphy.2020.07.048.

46. Ferreira SJ, Kossmann J, Lloyd JR et al. The reduction of starch accumulation in transgenic sugarcane cell suspension culture lines. Biotechnol J. 2008;3:1398–406.

47. Zhang H, Chen J, Zhang F et al. Transcriptome analysis of callus from melon. Gene. 2019;648:131–138.

48. Jamaluddin ND, Rohani ER, Mohd Noor N et al. Transcriptome-wide effect of DE-ETIOLATED1 (DET1) suppression in embryogenic callus of Carica papaya. J Plant Res. 2019;132:181–95.

49. Suprun AR, Ogneva ZV, Dubrovina AS et al. Effect of spruce PjSTS1a, PjSTS2, or PjSTS3 gene overexpression on stilbene biosynthesis in callus cultures of Vitis amurensis Rupr. Biotechnol Appl Biochem. 2020;67:234–9.

50. Wei Z, Lv MJ, Wan W et al. Transformation of elf5B1 gene into chrysanthemum to gain calluses of high temperature tolerance. Biologia. 2019;74:1271–7.

51. Zhang C, Liu HC, Zhang XS et al. VcMYB4a, an R2R3-MYB transcription factor from Vaccinium corymbosum, negatively regulates salt, drought, and temperature stress. Gene-combis. 2020;757:144935.

52. He Z, Zhao T, Yin Z et al. Phytochrome-interacting transcription factor CsPIF8 contributes to cold tolerance in citrus by regulating superoxide dismutase expression. 2020;298. https://doi.org/10.1016/j.plantsci.2020.110584.

53. GAO Y, Sun JC, Sun ZL et al. The MADS-box transcription factor CmAGL11 modulates somatic embryogenesis in Chinese chestnut (Castanea mollissima Blume). J Integr Agric. 2020;19:1033–43.

54. Liu J. Research progress of tissue culture of camellia Sinensis. Journal of Ankang University. 2019;31:102–5.

55. Mohanpuria P, Kumar V, Ahuja PS et al. Producing low-caffeine tea through post-transcriptional silencing of caffeine synthase mRNA. Plant Mol Biol. 2011;76:523–34.

56. Kang X, Ping L, Wenhui M et al. RNAi silencing CsHB1 reduces the accumulation of caffeine in tea callus. Acta Horticulturae Sinica. 2020;47:1–12.

57. Chen H, Huang H, Fang H et al. Practice and understanding of industrialization development of Yinghong 9 black tea. Guangdong Tea. 2015;6:11–13.

58. Sparkes IA, Runions J, Kearns A et al. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat Protoc. 2006;1:2019–25.