Transcription factor PpNAC1 and DNA demethylase PpDML1 synergistically regulate peach fruit ripening

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Abstract

Fruit ripening is accompanied by dramatic changes in color, texture, and flavor and is regulated by transcription factors (TFs) and epigenetic factors. However, the detailed regulatory mechanism remains unclear. Gene expression patterns suggest that PpNAC1 (NAM/ATAF1/2/CUC) TF plays a major role in peach (Prunus persica) fruit ripening. DNA affinity purification (DAP) seq combined with transactivation tests demonstrated that PpNAC1 can directly activate the expression of multiple ripening-related genes, including ACC synthase1 (PpACS1) and ACC oxidase1 (PpACO1) involved in ethylene biosynthesis, pectinesterase1 (PpPME1), pectate lyase1 (PpPL1), and polygalacturonase1 (PpPG1) related to cell wall modification, and lipase1 (PpLIP1), fatty acid desaturase (PpFAD3-1), and alcohol acyltransferase1 (PpAAT1) involved in volatiles synthesis. Overexpression of PpNAC1 in the tomato (Solanum lycopersicum) nor (nonripening) mutant restored fruit ripening, and its transient overexpression in peach fruit induced target gene expression, supporting a positive role of PpNAC1 in fruit ripening. The enhanced transcript levels of PpNAC1 and its target genes were associated with decreases in their promoter mCG methylation during ripening. Declining DNA methylation was negatively associated with increased transcripts of DNA demethylase1 (PpDML1), whose promoter is recognized and activated by PpNAC1. We propose that decreased methylation of the promoter region of PpNAC1 leads to a subsequent decrease in DNA methylation levels and enhanced transcription of ripening-related genes. These results indicate that positive feedback between PpNAC1 and PpDML1 plays an important role in directly regulating expression of multiple genes required for peach ripening and quality formation.

Introduction

Fruit ripening is a complex biological process that involves hormone synthesis, pigment accumulation, texture change and softening, and production of flavor-related compounds, including soluble sugars, organic acids, and aroma volatiles (Seymour et al. 2013). Ethylene plays a pivotal role in fruit ripening and development of quality traits of climacteric fruits, such as tomato (Solanum lycopersicum), peach (Prunus persica), apple (Malus domestica), kiwifruit (Actinidia delicosa), and banana (Musa acuminata). The fruit ripening process involves an elaborate regulatory network of interacting hormones, transcription factors (TFs) and epigenetic factors (Giovannoni et al. 2017; Chen et al. 2020a). Although ripening involves a major positive change in attractiveness and nutritional value, it also increases susceptibility to pathogens and major economic losses. Therefore, a systematic and comprehensive understanding of ripening is important for fruit quality regulation, flavor improvement, and food security.
The plant-specific NAC (NAM/ATAF1/2/CUC2) TF family plays an important role in the regulation of fruit ripening in both climacteric and nonclimacteric fruits. In climacteric tomato, NAC-NOR (NAC nonripening) TF can regulate the expression of SIA1CS2, encoding ACC synthase, and affect ethylene synthesis. Moreover, NAC-NOR also regulates fruit softening and flavor formation by regulating the expression of SIPL (pectate lyase), which is related to cell wall metabolism, and SIILOX (lipoxigenase C), SIHPL (hydroperoxide lyase), SIADH2 (alcohol dehydrogenase), which are related to the synthesis of fatty acid derived volatiles (Gao et al. 2020, 2022). In addition, tomato SINAC1, SINAC4, SINOR-like1, and SINAM1 (NAM, no epical meristem) have also been shown to regulate the fruit ripening process (Ma et al. 2014; Zhu et al. 2014; Gao et al. 2018, 2021). Additionally, AdNAC6/7 in kiwifruit (Wang et al. 2020a), MaNAC1/2, MaNAC42, and MaNAC029 in banana (Shan et al. 2020; Yan et al. 2021; Wei et al. 2022), FaRIF/FvRIF (ripening inducing factor) in strawberry (Fragaria spp.) (Martin-Pizarro et al. 2021; Li et al. 2023) and PaVNR6 in sweet cherry (Prunus avium) (Qi et al. 2022) have been demonstrated to regulate fruit ripening by activating the expression of genes involved in cell wall degradation, pigment formation, and the synthesis of flavor-related compounds.

Although there are some similarities in ripening mechanisms, for example in softening and color change in different fruits (Karlova et al. 2014), the fleshy fruit ripening process has evolved on at least three separate occasions and fruits can be derived from different flower parts (Karlova et al. 2014; Liu et al. 2020). Ethylene is predominantly involved in causing ripening in climacteric fruits whereas ABA appears to play this role in nonclimacteric strawberry (Chen et al. 2020a; Li et al. 2022; Perotti et al. 2023). Recently, the role of TFs such as MADS-RIN (MADS-box-ripening inhibitor), NAC-NOR, and CNR (colorless nonripening), once considered hub or “master regulators” of ripening in tomato, has been revised because of differences in the phenotypes of naturally occurring and CRISPR mutants (Gao et al. 2019, 2020a; Li et al. 2018, 2020a) and they are no longer considered as “master regulators” (Wang et al. 2020b). The fruitENCODE project suggested that the ripening process in climacteric apple and pear (Pyrus pyrifolia) fruits is regulated by an MADS positive feedback loop, whereas in peach, a PpNAC1 positive feedback loop, but not a MADS TF, regulates fruit ripening by activating the expression of ACC synthase1 PpACS1 and ACC oxidase1 PpACO1 (Lü et al. 2018). These observations suggest that there are differences in the regulatory mechanisms of the ripening process in different climacteric fruits, and therefore it is essential to explore the unique ripening regulatory mechanisms of each fruit in order to understand fruit quality regulation and flavor improvement. For these reasons, and in order to extend our knowledge beyond tomato, we carried out a detailed analysis of the ripening mechanism in peach.

Besides transcriptional regulation, DNA methylation has an important regulatory effect on fruit ripening and flavor quality (Farinati et al. 2017; Tang et al. 2020). Studies in model plants have shown that high methylation of gene promoters usually leads to the silencing of genes (Zhang et al. 2018). For example, during tomato (Ailsa Craig) fruit ripening, the differentially methylated regions (DMRs) were mainly enriched in the upstream 5’ region of genes, and the average methylation level in this region gradually decreased (Zhong et al. 2013). Moreover, the promoter region of ripening-associated genes, including SIPG2a (polygalacturonases) and SIPSY1 (phytoene synthase), also showed demethylation during wild-type (WT) fruit ripening compared to cnr mutant (Zhong et al. 2013). These results indicated an important role of DNA methylation for fruit ripening. In addition, RNAi silencing or knockout of DNA demethylase SIDML2 results in hypermethylation of ripening-related genes, thereby inhibiting their expression and consequently affecting fruit ripening (Liu et al. 2015; Lang et al. 2017). A recent study in tomato revealed that NAC-NOR could bind and activate SIDML2 expression to promote the expression of ripening-related genes, and that the expression of NAC-NOR is regulated by DNA methylation (Gao et al. 2022).

Peach (P. persica L. Batsch) is a climacteric fruit of the Rosaceae family, widely cultivated worldwide, with total production of ~25 million tons (Food and Agriculture Organization, 2021, https://www.fao.org/), and the ripening process is characterized by rapid softening, accumulation of sugars, decrease in acidity and dramatic change in aroma from green to fruity notes (Lombardo et al. 2011; Wang et al. 2016). Peach fruit softening is mainly related to the degradation of pectin in the cell wall. According to the fruit firmness and texture characteristics, peach cultivars are usually classified as melting flesh (MF) or nonmelting flesh (NMF). In general, MF peaches used in this study soften rapidly in the late stages of ripening, whereas NMF peaches show slow softening and maintain flesh firmness (Gu et al. 2016). The presence or absence of the endo-polygalacturonases PpPG1 and PpPG2 control the MF or NMF traits of peach flesh (Gu et al. 2016; Qian et al. 2022), and their expression levels are closely related to fruit softening in MF peaches. In addition, pectin methylsterase (PME) and pectate lyase (PL) also play important roles in peach softening (Zhu et al. 2017). Soluble sugars, organic acids, and volatile organic compounds (VOCs) together determine fruit flavor and influence the degree of consumer satisfaction. Recently, a tonoplast sugar transporter (TST) encoding gene PpTST1, which belongs to the major facilitator superfamily (MFS), with a dual function in sugar accumulation and organic acid content reduction, was identified by genome-wide association studies (GWAS) (Peng et al. 2020a; Wang et al. 2022). The expression profile of PpTST1 was consistent with the sugar accumulation pattern. Volatile esters and lactones are the main source of fruity and peachy notes, and positively impact consumers’ sensory preferences (Bianchi et al. 2017). Free unsaturated fatty acids are the main precursors of peach fruit VOCs and their synthesis is catalyzed by stearoyl-acyl carrier protein desaturase (SAD), fatty acid desaturase (FAD), and lipase (LIP) (Li et al. 2020b; Jin et al. 2022). Alcohol acyltransferase PpAAT1 catalyzes the
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final step in the synthesis of esters and lactones (Peng et al. 2020b; Cao et al. 2021). As these fruit ripening processes are progressively elucidated, their transcriptional and epigenetic regulation are receiving increasing attention.

Our previous studies showed that PpNAC1 could activate the expression of PpFAD3-1 and PpAAT1 to regulate the synthesis of fatty acid-derived volatiles; meanwhile, overexpression of PpNAC1 in the tomato nor mutant could restore the ethylene synthesis and pigment production (Cao et al. 2021; Jin et al. 2022), indicating that PpNAC1 plays an important regulatory role in fruit ripening. However, the detailed regulatory mechanisms leading to fruit softening and flavor formation are still unclear. Whole genome bisulfite sequencing (WGBS) analysis of peach fruit DNA methylation levels in the fruitENCODE project revealed CG and CHG hypomethylation and CHH hypermethylation changes in the gene regions during peach ripening. In addition, the hypomethylation levels in the promoter region of PpACS1 and PpPL1 indicated that DNA methylation plays an important role in peach fruit ripening (Lü et al. 2018). Conversely, storage at 0 °C for 7 d induced whole-genome DNA methylation in peach fruit, negatively influencing fruit ripening and flavor quality and the transcript level of a DNA demethylase PpDML1 was negatively associated with CG, CHG, and CHH methylation levels (Duan et al. 2022).

Here, we used DAP-seq and dual-luciferase reporter (DLR) assay to identify ripening genes targeted and activated by PpNAC1, including those involved in ethylene synthesis, pectin metabolism, sugar transport, and VOCs synthesis. By combining with WGBS sequencing, it was revealed that PpNAC1 also activates the expression of PpDML1 and this promotes the decrease in DNA methylation level of ripening-related genes, increases their transcription, and decreases the DNA methylation level of PpNAC1.

Results

Changes in ethylene release, texture, and flavor during peach fruit ripening

During the ripening stages of peach fruit (Fig. 1A) ethylene synthesis increased approximately 100-fold from stages S3 to S5, and softening measured by the decline in firmness decreased substantially (Fig. 1B). At the same time, the content of total organic acids decreased significantly, e.g. citric acid and quinic acid (Fig. 1C). Content of total sugars was increased with fruit ripening, despite a transitory decline in monosaccharides (glucose and fructose) in the early stages (Fig. 1C). The content of C6 aldehydes and alcohols with “green” odor decreased, whereas the content of volatile esters (hexyl acetate, Z-3-hexenyl acetate, and E-2-hexenyl acetate) and lactones (γ-decalactone, δ-decalactone, and γ-undecanoylactone), which contribute to the “fruity” and “peachy” odor, increased (Fig. 1D). Exogenous ethylene treatment accelerated fruit softening and promoted the synthesis of esters and lactones, while 1-MCP, a specific inhibitor of ethylene perception and action, inhibited fruit softening and volatiles synthesis (Fig. 1E).

NAC family member PpNAC1 is associated with peach fruit ripening

Peach contains 115 NAC members, and the phylogenetic analysis showed that there were nine members clustered together with other known ripening-related NACs, including PpNAC1 and PpNAC2, which have similarity (56.4% and 48.8% at the amino acid sequence, respectively) to tomato NAC-NOR (Fig. 1F; Supplemental Fig. S1). RNA-seq analysis showed that the transcript levels of PpNAC1 increased during fruit ripening and remained at high levels post-harvest, while the transcript levels of PpNAC4 and PpNAC5 showed an opposite pattern (Fig. 1G). Other NAC members were present at relatively low abundance. Exogenous ethylene treatment increased the PpNAC1 transcripts compared to the control and 1-MCP treatment (Fig. 1H).

Based on the important regulatory role of MADS-RIN in tomato, the transcript levels of MADS family members in peach were analyzed. Ten members were homologous to MADS-RIN (Supplemental Fig. S2A), four of which showed relatively high transcript levels but were negatively correlated with the ethylene production during peach ripening, while the other six members were barely expressed (Supplemental Fig. S2, B and C). Therefore, together with ripening-related expression in the present and our previous study (Cao et al. 2021), we selected PpNAC1 as a candidate TF to explore the regulatory mechanism of peach fruit ripening.

Identification of PpNAC1 target genes by DAP-seq and RNA-seq

The DAP-seq technique was developed to identify the genome-wide binding location of TFs and derive the sequence motifs of the TF-binding site (TFBS) (Bartlett et al. 2017). Here, we identified the target gene of PpNAC1 by DAP-seq (Supplemental Table S1), and a total of 9,238 binding sites were obtained on the genome by two technical replicates (Fig. 2A). The binding sites of PpNAC1 were mainly located in the 500 bp region upstream of the transcription start sites (TSS) (Fig. 2B); 24.3% of PpNAC1 binding sites were located in promoter region (2 kb upstream of TSS) of 2,245 genes and therefore potentially involved in regulating their expression (Fig. 2C; Supplemental Data Set 1). Based on the DAP-seq results, MEME (Multiple EM for Motif Elicitation) analysis revealed that ACG(T/C)(A/C) is the binding site for PpNAC1 TF (Fig. 2D). The binding site motif is also observed in other NACs over multiple fruit crops, including kiwifruit and banana (Nieuwenhuizen et al. 2015; Wei et al. 2022). These results indicated a conserved motif for NAC binding in fruit. Considering that TFs regulate gene transcription mainly by binding to the promoter region, these genes were selected as candidates for further analysis. Gene Ontology (GO) enrichment analysis revealed that genes with promoters binding PpNAC1 are involved in primary
metabolic processes of fruit, such as organic acid and sucrose metabolism, secondary metabolic processes, such as phenylpropane metabolism and lactone metabolism, hormone metabolism and response, and are also related to fruit development and defense response (Fig. 2E).

To further screen candidate target genes regulated by PpNAC1 that were associated with fruit quality during peach ripening, expression of 2,245 genes with NAC binding sites was examined based on RNA-seq data. Subsequently, weighted gene co-expression network analysis (WGCNA) was performed to mine PpNAC1 target genes related to ethylene production, fruit softening, volatile contents, sugar and acid contents. For the five modules produced by the WGCNA, the transcript levels of genes in the turquoise module were positively correlated with ethylene production and content of lactones and sucrose, and the transcript levels of genes in the brown module were positively correlated with the content of Z-3-hexenyl acetate and sucrose. Moreover, the transcript levels of genes in these two modules were significantly positively correlated with PpNAC1 (Fig. 2F).

The gene co-expression networks showed that the turquoise module contained the genes related to ethylene synthesis, such as PpACS1 and PpACO1; genes related to volatiles synthesis, such as PpLIP1, PpFAD3-1, PpAAT1, genes related to fruit softening, such as PpPME1, PpPL5 and PpPG1, and genes related to sugars metabolism, such as PpNINV3 and PpTST2 (Fig. 2G). In addition, the brown module contained the genes required for fruit softening, including...
Figure 2. Genome-wide analysis of PpNAC1 binding sites and target genes using DAP-seq and RNA-seq. A) Overlap of PpNAC1 binding peaks in two technical replicates of DAP-seq. B) PpNAC1 binding sites are enriched in the region proximal to the TSS. C) Distribution of peaks in different regions of target genes. D) Potential binding motifs of PpNAC1 according to DAP-seq. E) GO enrichment analysis of PpNAC1 target genes. F) WGCNA analysis of PpNAC1 target genes during fruit ripening. The asterisks represent significant correlation at the level of significance (*P < 0.05, **P < 0.01, Pearson correlation analysis). G) Gene co-expression networks obtained by WGCNA.
pectate lyase PpPL1, PpPL2 and expansin gene PpEXP2, and genes related to sucrose metabolism, such as PpTST1.

**PpNAC1 regulates ethylene synthesis and fruit softening**

To clarify the regulatory effects of PpNAC1 on ethylene production and fruit softening, genes related to these processes were screened out from the turquoise and brown modules for further study. RNA-seq analysis and RT-qPCR results showed that the transcript levels of genes related to ethylene synthesis and signal transduction increased during fruit ripening, consistent with the pattern of ethylene production, while the transcript levels of PpACS1, PpACO1, and PpEETR2 were induced by ethylene and repressed by 1-MCP (Fig. 3A; Supplemental Fig. S3). The transcript levels of PpPME1, PpPL1/2/5, and PpPG1/2, which are involved in pectin degradation, also increased with fruit ripening and were induced by ethylene (Fig. 3B; Supplemental Fig. S3), resulting in fruit softening. The DLR assay was used to verify the transcriptional activation effects of PpNAC1 on these potential gene targets, and the results showed that PpNAC1 could activate the promoter of ethylene-related PpACS1 (3-fold), PpACO1 (3-fold), and PpERF1 (5-fold); whereas, it could also activate the promoter of PpPME1 (5-fold), PpPL1 (10-fold), and PpPG1 (13-fold) (Fig. 3C).

DAP-seq analysis showed that the PpNAC1 binding peaks were located in the promoter regions of PpACS1 (~340 bp upstream of TSS), PpACO1 (~650 bp upstream of TSS), and PpERF1 (~650 bp upstream of TSS), which contain the conserved PpNAC1 binding motifs (Fig. 3D). Similarly, the DAP-seq peaks in the promoter region of PpPME1 (~380 bp upstream of TSS), PpPL1 (~450 bp upstream of TSS) and PpPG1 (~340 bp upstream of TSS) also contain the PpNAC1 binding site ACGTA (Fig. 3D). Electrophoretic mobility shift assay (EMSA) with PpACS1, PpACO1, and PpPL1 promoters was performed to verify the DAP-seq results. The probes were designed based on the sequence in the DAP-seq peaks. The recombinant PpNAC1 protein could bind to the biotin-labeled probes (Biotin-P) and cold probes (Cold-P) compete with the binding; while the mutated cold probe (Mutant-P) could not bind to PpNAC1 and thus did not affect the binding of the labeled probe (Fig. 3E). The EMSA results confirmed that PpNAC1 could bind to the NACBS in the promoter regions of PpACS1, PpACO1, and PpPL1 (Fig. 3E). These results suggest that PpNAC1 plays an important regulatory role in ethylene synthesis and the fruit softening process by activating the transcription of related genes during fruit ripening. In addition, the above results also demonstrate the value of DAP-seq for identifying TF-regulated target genes.

**PpNAC1 regulates genes required for volatiles synthesis and sugar transport**

The turquoise and brown modules in the gene co-expression networks of PpNAC1 targets contains genes related to the synthesis of volatiles from the fatty acid pathway and genes related to sucrose metabolism and transport, suggesting an important regulatory role of PpNAC1 in fruit flavor formation (Fig. 2G). RNA-seq analysis showed that the transcript levels of PpFAB1 (fatty acid biosynthesis), PpSAD1/2 (steroyl-acyl carrier protein desaturases), PpFAD2 (ω-6 fatty acid desaturase), PpFAD3-1 (ω-3 fatty acid desaturase), and PpLIP1 (lipase), which are involved in the free fatty acid synthesis, were increased during fruit ripening; while the transcript levels of PpAAAT1 (alcohol acyl transferase), which catalyzes the synthesis of esters and lactones, was also increased and positively correlated with PpNAC1 transcripts (r = 0.84, P < 0.05) (Fig. 4A). The transcription of most of the volatile synthesis-related genes was induced by ethylene and repressed by 1-MCP. Furthermore, the transcript levels of PpNINV3 (neutral invertase) and PpTST1/2 (tonoplast sucrose transporter), which are involved in sugar metabolism and transport, were increased during fruit ripening (Fig. 4B), suggesting that the expression of these genes may be also regulated by PpNAC1. The results of the DLR assay showed that PpNAC1 significantly activated the promoter of PpSAD1 (3-fold), PpFAD3-1 (20-fold), PpLIP1 (6-fold), and PpAAAT1 (12-fold) (Fig. 4C), and it also activated the promoter of PpTST1 (6-fold), which has been demonstrated to affect the content of sugars and organic acids in peach (Wang et al. 2022).

DAP-seq analysis showed that the PpNAC1 binding sites ACGTA or ACGCA were located in the promoter regions of PpSAD1 (~90 bp upstream of TSS), PpFAD3-1 (~470 bp upstream of TSS), PpLIP1 (~1,000 bp upstream of TSS), and PpAAAT1 (~350 bp upstream of TSS), respectively, coinciding with the binding peak in DAP-seq (Fig. 4D). The PpTST1 promoter contained three NAC binding sites (NACBS) as serial repeats 150 bp upstream of the TSS, resulting in a strong binding signal (Fig. 4E). EMSA assays of PpFAD3-1, PpAAAT1, and PpTST1 promoters were used to verify the DAP-seq results, which confirmed that PpNAC1 could bind to the NACBS in the promoter regions of PpFAD3-1, PpAAAT1, and PpTST1, which is consistent with the DAP-seq results (Fig. 4F). The above results indicated that PpNAC1 could activate the expression of flavor-related genes and regulate the synthesis of sugars, organic acids, and volatiles during fruit ripening.

**PpNAC1 regulates fruit softening and flavor in peach and transgenic tomato**

Transient overexpression in peach callus and transgenic tomato was used to verify the regulatory effects of PpNAC1 on fruit ripening, softening, and flavor formation. Overexpression of PpNAC1 in peach callus promoted the transcription of the above-mentioned target genes involved in ethylene synthesis, pectin degradation, and volatiles synthesis (Fig. 5A).

Overexpression of PpNAC1 in nor tomato mutants could also essentially restore the ethylene synthesis and other gene expression defects of tomato nor mutants and promote fruit ripening (Fig. 5B). Compared with nor fruits, the firmness of transgenic tomato fruits was significantly decreased,
Figure 3. PpNAC1 regulates fruit ripening and softening by activating the transcription of genes related to ethylene synthesis and pectin metabolism. A) Transcript levels of genes related to ethylene synthesis and signal transduction. SAM, S-adenosyl-L-Met; SAMS, SAM synthase; ACC, 1-amino-cyclopropane-1-carboxylic acid; ACS, ACC synthase; ACO, ACC oxidase; ETR, ethylene receptor; CTR1, constitutive triple-response1;
and the transcript levels of pectin degradation genes SJP1 and SJP2a were significantly induced. The transcript levels of SITST1/3 in transgenic tomato were up-regulated, accompanied by a decrease in organic acid content and a slight increase in sucrose content (Fig. 5C; Supplemental Fig. S4A). In addition, the transcript levels of SISAD1, SIFAD3, and SIAAT1, which are involved in the synthesis of fatty acid-derived volatiles, were significantly up-regulated in transgenic tomatoes, along with the increased content of fatty acid-derived VOCs (Fig. 5C; Supplemental Fig. S4B). These results confirmed the critical role of PpNAC1 in the regulation of fruit ripening, softening and flavor formation, and indicate a degree of sequence and functional conservatism in different climacteric fruits.

DNA methylation levels in the promoter region of PpNAC1 target genes were negatively associated with transcript levels during fruit ripening

DNA methylation has an important regulatory effect on fruit ripening and flavor quality (Farinati et al. 2017; Tang et al. 2020). Whole genome methylation levels of the three contexts (CG, CHG, and CHH) showed that mCG levels were the highest and tended to decrease with fruit ripening; mCHG levels also decreased in the early stage of development and then remained stable; while mCHH levels were the lowest and did not show a clear pattern of change (Fig. 6A). DNA methylation levels in different regions of genes showed that mCG levels in the upstream (−2 kb) and downstream (2 kb) regions gradually decreased with fruit ripening, and mCHG levels were significantly higher in S1 fruit than in other stages (Fig. 6B) suggesting the important role of DNA methylation in regulating gene expression.

Analysis of DMRs in S4 and S1 stages yielded 29,466 mCG, 25,277 mCHG, and 59,934 mCHH-type DMRs, respectively, mainly located in gene promoters and intergenic regions. The KEGG pathway of mCG-type differentially methylated genes (DMGs) was enriched for plant hormone signal transduction, phenylpropanoid metabolism, sucrose metabolism, and fatty acid metabolism (Supplemental Fig. S5). DNA methylation levels are mainly determined by DNA methyltransferases and DNA demethylases. Our previous study indicated the 5′-flanking regions of gene promoters and intergenic regions.

PPNAC1 and DNA methylation are associated with peach fruit ripening and flavor formation

A recent study in tomato showed that NAC-NOR can activate the transcription of SIDI2L, and affect DNA methylation levels of flavor-related genes, and thus regulate fruit ripening (Gao et al. 2022). Here, overexpression of PpNAC1 in nor mutant fruit promoted the expression of SIDI2L, indicating a conserved function between PpNAC1 and tomato NAC-NOR (Fig. 7A). Moreover, overexpression of PpNAC1 in peach callus promoted the expression of PpDML1 (Fig. 7B). DLR and EMSA assays verified that PpNAC1 could directly bind to the PpDML1 promoter to activate its transcription (Fig. 7, C and D). Notably, the mCG methylation level in...
Figure 4. PpNAC1 regulates fruit flavor by activating the transcription of genes related to VOCs synthesis and sugar transport. A) Transcript levels of genes related to the synthesis of fatty acid-derived VOCs. FAB, fatty acid biosynthesis; SAD, stearoyl-acyl carrier protein desaturase; FAD, fatty acid desaturase; LIP, lipase; LOX, lipoxygenase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; AAT, alcohol acyl transferase. B) Transcript levels of genes related to sugar metabolism and transport. NINV, neutral invertase; TST, tonoplast sugar transporter. The different letters in the heatmap indicate significant differences at $P < 0.05$ level (one-way ANOVA, Tukey’s HSD). C) Analysis of the activation effect of PpNAC1 on the target genes by DLR assay. Error bars indicate SE ($n = 6$). The asterisks represent significant differences compared to the empty vector (set to 1.0) as control at the significance level (*$P < 0.05$, **$P < 0.01$, Student’s t-test). D, E) PpNAC1 binding sites in the target gene identified by DAP-seq. The region of the gene body and upstream 2 kb are shown, the arrows indicate the 5’ to 3’ direction. The dotted lines indicate the positions of PpNAC1 binding motif. F) Validation of PpNAC1 binding to target genes by EMSA. The red letters represent the conserved binding motif of PpNAC1 and the underlines represent the mutated regions.
the promoter region of PpNAC1 decreased with fruit ripening and was negatively correlated with its transcript level ($r = -0.88$) (Fig. 6F), suggesting that its transcription may be regulated by PpDML1-mediated methylation. These results suggest that, as found in tomato, there is a positive feedback loop between PpNAC1 and PpDML1 in peaches, which coordinate regulatory fruit ripening.

Based on the results of this study, we developed a regulatory model for the role of PpNAC1 and PpDML1 in fruit ripening (Fig. 7E). A positive feedback loop involving PpNAC1 activates the transcription of genes related to ethylene synthesis and in turn its expression is induced by ethylene. Furthermore, in addition to regulating fruit softening and flavor formation by inducing ethylene production, PpNAC1 could directly activate the transcription of genes related to pectin metabolism, VOC synthesis, and sugar transport during fruit ripening, thus regulating the fruit softening and flavor formation. In addition, PpNAC1 activates the transcription of PpDML1, reduces the mCG methylation level of ripening-related genes, which enhances their expressibility. In turn, the transcript level of PpNAC1 is also increased by PpDML1-mediated DNA demethylation, representing a second positive feedback loop promoting ripening (Fig. 7E).

**Discussion**

Here, we focused on the role of PpNAC1 in the regulation of expression of genes determining multiple processes that are
Figure 6. Whole genome methylation profiling and promoter region methylation levels of genes related to fruit ripening and flavor formation. A) Whole genome methylation levels of mCG, mCHG, and mCHH during peach fruit ripening. B) DNA methylation levels of mCG, mCHG, and mCHH in the different regions (upstream 2 kb, gene body and downstream 2 kb) surrounding the genes. C) Transcript levels of genes involved in DNA methylation. 

(continued)
crucial for peach ripening including ethylene synthesis, fruit softening, and flavor formation. The results indicate a central role for PpNAC1 in regulating ethylene synthesis, the expression of ripening genes encoding pectin-modifying enzymes, such as PpPME1, PpPL1, and PpPG1 to promote fruit softening, as well as the transcription of PpSad1, PpLip1, PpFad3-1, and PpAat1, required for volatiles synthesis, and the expression of the PpTST1, for vacuum sugar storage. We also explored the role of DNA methylation in the regulation of gene expression associated with fruit ripening and a positive feedback regulatory loop between PpNAC1 and DNA methylation mediated by the DNA demethylase PpDML1. The present study supports the proposed synergistic regulatory relationship between TFs and DNA methylation proposed in tomato and highlights the general role of NACs in directly regulating genes involved in multiple aspects of fruit ripening and quality formation.

Potential regulatory effects of other NACs and MADS TFs in peach ripening

The findings of the present study and recently published results (Cao et al. 2021; Gao et al. 2022) indicate strong similarities in the function of NAC genes in tomato and peach. In addition to NAC-NOR, SINOR-like1 and SINAC1/4 also participate in the regulation of tomato fruit ripening (Gao et al. 2018, 2021). Therefore, we cannot exclude the possibility that other members of peach NAC family may also have functions in affecting fruit ripening. For instance, PpNAC2, which has high similarity with PpNAC1, might make a contribution, although it has a relatively low transcript level in ripening peach fruit (Fig. 1G); PpNAC4 and PpNAC5, which show a decreased expression pattern during fruit development, have high sequence similarity to SINAC1 and SINAC4 that are associated with tomato fruit ripening (Ma et al. 2014; Zhu et al. 2014). In addition, two other NACs (Prune.4G053300 and Prune.2G326000) in Cluster II also showed ripening-related expression patterns (Supplemental Fig. S1). Furthermore, it remains to be established whether other TFs, such as MADS-RIN, which is required for full ripening in tomato (Li et al. 2019, 2020a), also play a role in peach. There are 81 MADS TF genes in peach, and only 10 members clustered together by sequence homology with tomato MADS-RIN based on phylogenetic analysis (Supplemental Fig. S2A). For these MADS TFs, PruneFUL4 (Prune.5G20850) and PruneSEP1 (Prune.3G249400) have been suggested to be related to peach fruit ripening (Zhang et al. 2022) and softening (Li et al. 2017), respectively. However, these conclusions were based solely on the expression pattern without other experimental evidence. Although the expression levels of these 10 members were negatively associated with ethylene production (Supplemental Fig. S2C), a contributory role for MADS TFs in peach fruit ripening cannot be ruled out.

PpNAC1 targets genes associated with synthesis and signaling of ethylene and other hormones

PpNAC1 activates the expression of genes involved in ethylene synthesis (PpACO1) and downstream signaling (PpERF1) by binding to their promoters to regulate fruit ripening (Fig. 3). A previous study by Liu et al. (2018) in the fruitENCODE project only verified that PpNAC1 regulates ethylene synthesis by ChIP-seq and transient overexpression in tobacco (Nicotiana benthamiana). Recently, DAP-seq has been successfully applied to studying the TF function in horticultural plants, such as apple (Falavigna et al. 2021), grape (Vitis vinifera) (D’Incà et al. 2021; Orduña et al. 2022), banana (Wei et al. 2022; Wu et al. 2022; Yang et al. 2022a; Zhu et al. 2023), and tomato (Song et al. 2022). Our study, integrating DLR, DAP-seq, EMSA, homologous transient overexpression, and tomato mutant complementation, extends this characterization and shows that PpNAC1 regulates ethylene synthesis in peach (Fig. 6). In this respect, there is a strong similarity with tomato NAC-NOR, SINOR-like1, SINAM1, SINAC1 (Ma et al. 2014; Gao et al. 2018, 2020, 2021), MaNAC1/2 in banana (Shan et al. 2012, 2020), and AdNAC2/3/6/7 in kiwifruit (Wu et al. 2020; Fu et al. 2021), which have all been shown to promote ethylene synthesis and regulate fruit ripening.

GO analysis showed that PpNAC1 targets were also enriched in genes involved in ABA, Aux/IAA metabolism and response processes (Fig. 2E). Gene transactivation analysis by DLR assay demonstrated that PpNAC1 could activate the expression of indole-3-acetic-acid-amido synthase PpGH3-4 (~4-fold) (Supplemental Fig. S7). Previous studies in peach have shown that the induction of Aux/IAA is required for ethylene production at the onset of fruit ripening and is important for the regulation of fruit softening (Tatsuki et al. 2013; Ma et al. 2023). NACs have also been implicated in regulating fruit ripening by affecting signal transduction of other hormones, in addition to ethylene. For instance, ethylene, auxin, ABA, JA, etc., can jointly regulate fruit ripening through complex synergistic or antagonistic effects (Kumar et al. 2014; Li et al. 2021). Tomato SINOR-like1 and
SINAC19 TFs synergistically regulate ethylene synthesis and ABA signal transduction during fruit maturation (Kou et al. 2016; Yang et al. 2021) and AdNAC2 and AdNAC3 mediate crosstalk between JA and ethylene in ripening kiwifruit (Wu et al. 2020). Our present study suggests that PpNAC1 plays an important role in the synergistic regulation of IAA and ethylene, but the mechanism of this complex interaction requires further study.

PpNAC1 regulates cell wall degradation, promoting fruit softening

Fruit softening is an important characteristic of peach fruit ripening, especially in MF peaches, and is regulated by ethylene (Tatsuki et al. 2013; Qian et al. 2022). However, there are few studies on the transcriptional regulatory mechanisms governing peach softening. The MADS-box TF PpSEP1 could bind to the promoter of PpPG2 and accelerate fruit softening.
(Li et al. 2017), while PpERF2 delays softening by repressing the expression of PpPG1 (Wang et al. 2019). Our study demonstrated that PpNAC1 can promote fruit softening by inducing ethylene synthesis and also directly activate the expression of PpPME1, PpPL1, and PpPG1 (Fig. 3), which are involved in pectin degradation and fruit softening (Shi et al. 2023). This role of PpNAC1 is in general agreement with studies in tomato (NAC-NOR, SINOR-like1, and SINAC4) (Gao et al. 2018, 2020), strawberry (FaRIF) (Martin-Pizarro et al. 2021), and banana (MaNAC029) (Wei et al. 2022).

Although the mechanisms of cell wall degradation are relatively conserved in different fruits, the detailed mechanisms leading to fruit softening and their regulation vary. For example, SIPL is essential for tomato fruit softening, but not SLPG2a, whereas PGs contribute more to Rosaceae fruit softening, such as FaPpG1 in strawberry, MdPpG1 in apple, and PpPG1 and PpPpG2 in peach (Shi et al. 2023). Different TFs target different cell wall modifying genes (Shi et al. 2022), and it is essential to investigate the softening mechanisms in specific fruits. Furthermore, balancing fruit softening and flavor is essential in breeding programs to improve fruit production and quality.

**PpNAC1 regulates peach fruit flavor and aroma**

In our previous study, we demonstrated that PpNAC1 regulates the synthesis of fatty acid-derived VOCs in peach fruit by regulating the expression of the fatty acid desaturase PpFAD3-1 and the esters synthesis gene PpAAT1 (Cao et al. 2021; Jin et al. 2022). In this study, we used DAP-seq to show that PpNAC1 can directly activate the expression of PpSAD1 and the lipase PpPL1, upstream of the fatty acid pathway, thereby affecting the synthesis of flavor-related VOCs (Fig. 4). PpSAD1 is homologous to AtFAB2 in Arabidopsis thaliana (Supplemental Fig. S8A), which has been identified as an important rate-limiting enzyme that catalyzes the desaturation of saturated fatty acids (18:0-ACP) to monounsaturated fatty acids (18:1-ACP) in plants (Kachroo et al. 2007), and transient overexpression of PpSAD1 in peach fruit promotes the synthesis of fatty acid-derived VOCs (Supplemental Fig. S8B). PpPL1 is homologous to SLP1P2/8, which has been identified in tomato to be involved in the synthesis of C6 aldehyde volatiles (Li et al. 2020b). NAC TFs that regulate the synthesis of VOCs have also been demonstrated in other fruits. In kiwifruit, AaNAC2/3/4/6/7 could directly activate the expression of terpene synthase AaTPS1, promoting the synthesis of terpene aroma (Nieuwenhuizen et al. 2015; Wang et al. 2020a). In strawberry fruit, FaRIF positively regulates the expression of FaEOBII (emission of benzenoids), FaDOF2, FaEGS2 (eugenol synthase2), and FaNESI (nerolidol synthase1) related to volatile compounds synthesis (Martin-Pizarro et al. 2021). Collectively, these studies confirm that NAC family TFs play an important role in the regulatory network leading to fruit aroma quality.

In addition to VOCs, our study found that PpNAC1 could regulate the transcription of a tonoplast sugar transporter PpTST1. There are three tandem repeats of the NAC binding motif located 150 bp upstream of the PpTST1 TSS. This is significant, since a nonsynonymous G/T variation in its coding region was identified as a quantitative trait locus controlling the content of sugars and organic acids in peach, and the expression profile of PpTST1 was consistent with the sugar accumulation pattern (Wang et al. 2022). Here, we explored the regulatory mechanism of its expression. NAC TFs also regulate other aspects of sugar synthesis. For example, MaNAC67-like in banana binds to the promoters of MaBAM6, MaSEX4, MaMEX1, the key genes for starch degradation, and activates their expression (Song et al. 2019). CINAC68 in watermelon (Citrus lanatus) positively regulates sucrose accumulation during fruit ripening by directly binding to the promoter region of invertase (ClnIV) and inhibiting its expression (Wang et al. 2021). It is noteworthy that the organic acids content of PpNAC1 transgenic tomato fruit was significantly reduced (Supplemental Fig. S4), and the GO analysis of PpNAC1 target genes indicated an enrichment for genes involved in the synthesis and metabolism of organic acid (Fig. 2E), including multiple genes involved in TCA cycle (Supplemental Data Set 1), suggesting that there may be other pathways for acid regulation by PpNAC1 that deserve more in-depth investigation.

**Synergistic regulatory relationship between PpNAC1 and DNA methylation**

DNA methylation is a relatively well-studied epigenetic modification that plays an important role in regulating fruit ripening and is also an important genetic factor leading to phenotypic variation. CRISPR knockout of SdMl2 in tomato affects the DNA methylation level of the whole genome and affects fruit ripening and flavor quality (Gao et al. 2022). Here, the WGBS analysis revealed a substantial negative association between mCG methylation levels in the promoter regions and expression levels of ripening-related genes (Fig. 6E), suggesting a more important regulatory role of mCG compared to mCHG and mCHH. Lü et al. (2018) did not report any changes in DNA methylation levels in the promoter regions of PpNAC1 and PpAco1, probably due to their focus on the total methylation levels (mC). Focusing on the mCG methylation levels, the promoter regions of PpNAC1 and PpAco1 show a significantly decreased pattern in both the fruitENCODE data (Lü et al. 2018) and our data (Fig. 6F; Supplemental Fig. S6). PpDml1 is the DNA demethylase member with the highest transcript abundance in fruit, and its concentration showed a substantial negative association with the level of DNA methylation both during ripening and under cold storage (Duan et al. 2022; Fig. 6C). Due to the lack of a genetic transformation system in peach, the regulatory effect of PpDML1 on DNA methylation level needs to be further verified.

In conclusion, both ethylene-responsive TFs and DNA methylation play important roles in regulating fruit ripening and flavor quality (Fig. 7). Our results indicate that PpNAC1 regulates fruit ripening and flavor quality by both...
transcriptional regulation of target genes and by regulating epigenetic factors, such as DNA methylation. An understanding of how epigenetic mechanisms regulate fruit ripening and quality will provide novel directions to drive crop breeding (Tirnaz and Batley 2019; Dalakouras and Vlachostergios 2021; Yang et al. 2022b), and will be of great value for the improvement of both fruit texture and flavor quality in order to help meet increasing global food demand due to population growth and changes in diet.

Materials and methods

Plant materials

Peach (P. persica L. Batsch cv. Hujingmilu) fruits were harvested from the Melting Peach Research Institute of Fenghua, Zhejiang Province. The fruits were harvested at different stages: fast growth (S1), stone hardening (S2), color turning (S3), and maturity stage (S4). Peach fruits were stored at 25 °C for 3 d (S5) and 6 d (S6) to facilitate postharvest ripening. Ethylene treatment (100 μL L−1, 12 h at 20 °C) was applied to S3 stage fruit to accelerate fruit ripening, while 1-MCP (10 μL L−1) was used to inhibit fruit ripening, with fruit incubated in normal air used as control. Fruit flesh was sampled at 4 d after treatment. Fruit flesh tissue (~5 mm) was separated and frozen in liquid nitrogen and stored at −80 °C for analysis. Five peach fruits were used in each of the three replicates.

Firmness and ethylene production measurement

Fruit firmness was measured at the equator of the fruit using a TA-XT-Plus texture analyzer (Stable Micro System, UK) equipped with a 7.5 mm diameter head, according to Zhang et al. (2010). The ethylene release rate was measured using a gas chromatograph (GC) 78990B (Agilent) equipped with an FID detector and a Porapak Q column (2.0 mm × 2 m, Agilent). The temperatures of the injector, detector, and oven were 140, 230, and 100 °C, respectively.

VOC collection and GC–MS analysis

Fruit VOCs were identified and quantified according to Cao et al. (2021). Frozen peach flesh (5 g) or tomato (Micro-Tom) fruit (1 g) were ground to powder, transferred to 20 mL vials and mixed with 3 mL 200 mM ethylenediaminetetraacetic acid (EDTA) and 3 mL 20% (w/v) CaCl2. Before sealing, 30 μL of 2-octanol (0.8 mg mL−1) was added as an internal standard. Volatiles were adsorbed by 65 μm of polydimethylsiloxane and divinylbenzene (PDMS–DVB) (Supelco Co., Bellefonte, PA, USA). An Agilent 7890N GC coupled with an Agilent 5975C mass spectrophotometer (MS) (Agilent, Palo Alto, CA, USA) equipped with a DB–WAX column (0.32 mm, 30 m, 0.25 μm, J&W Scientific, Folsom, CA, USA) was applied for VOC identification. VOCs were identified by comparison of the mass spectra in the NIST Mass Spectral Library (NIST-08) and comparison of retention times of authentic standards. VOCs were quantified using the peak area of the internal standard as a reference based on the total ion chromatogram.

Soluble sugars and organic acids analysis

The content of soluble sugars and organic acids was determined by GC according to Vimolmangkang et al. (2016) with some modifications. Frozen flesh samples (0.1 g) were dissolved in 4 mL methanol and incubated at 70 °C for 15 min. After centrifugation, the supernatant was mixed with 2.2 mL of 33% (v/v) trichloromethane and centrifuged. The supernatant was mixed with 10 μL ribitol (2.0 mg mL−1) as internal standard, and dried by evaporation. The residue was dissolved in 60 μL hydroxyamine hydrochloride solution in pyridine (20 mg mL−1). Derivatization was performed by adding 40 μL BSTFA (bis(trimethylsilyl) trifluoroacetamide) + 1% TMCS (trimethylchlorosilane). Soluble sugars and organic acids were detected by GC–MS equipped with an HP–5MS column and quantified using standard curves. The temperature protocol was as follows: 100 °C for 1 min, 2.5 °C/min increased to 185 °C, then 0.35 °C/min increased to 190 °C, then 8 °C/min increased to 250 °C and maintained for 5 min, and finally, 5 °C/min increased to 280 °C and maintained for 3 min. The temperature of the injector and ion source was 250 and 230 °C, respectively.

DAP-seq analysis

The DAP-seq assay was performed as previously described (Bartlett et al. 2017) with minor modifications. Genomic DNA (gDNA) from ripening peach fruit was extracted and fragmented to an average of 200 bp by using a Covaris M220 (Woburn, MA, USA). The fragmented gDNA was purified using AMPure XP beads (Beckman Coulter, Inc., Indianapolis, IN, USA). The CD5 sequence of PpNAC1 was cloned into the pFN19K HaloTag T7 SP6 Flexi expression vector. Halo-PpNAC1 fusion protein was expressed using the TNT SP6 Coupled Wheat Germ Extract System (Promega) and captured using Magne Halo Tag Beads (Promega). The protein-bound beads were incubated with the gDNA fragments. The eluted DNA fragments were sequenced by Illumina HiSeq. Reads were mapped to the peach genome using Bowtie2 (Langmead and Salzberg 2012). MACS2 software was used to call peaks (q value < 0.05; fold enrichment > 2) and calculate the peak scores (−log10(q value)) (Zhang et al. 2008) by comparing PpNAC1 and Input. These peaks are considered as the binding sites of PpNAC1, with the higher the score, the higher the confidence. Two independent replicates were performed in the DAP-seq experiment, and peaks in two replicates are merged and annotated using Homer (Heinz et al. 2010), and peaks with distances to TSS > 0 and <2,000 bp are considered to be in the promoter region. Binding motifs in the peak region were analyzed using MEME-ChIP (Machanick and Bailey 2011), and the distribution frequency of peaks near TSS was analyzed using deepTools (Ramírez et al. 2016). PpNAC1 binding peaks were visualized by IGV. The GO analysis of PpNAC1 targets was performed using TBtools (Chen et al. 2020b).

RNA seq, RT-qPCR, and WGCNA analysis

Gene expression was analyzed using RNA-Seq and reverse transcription quantitative PCR (RT-qPCR). The RNA libraries
were transformed into Agrobacterium (GV3101). Agrobacterium cultures were grown to an OD
value of 0.75 and suspended in infiltration buffer (10 mM MES, 10 mM MgCl2, and 150 μM acetylsyringone, pH 5.6). Mixtures of TFs (1 mL) and the promoter (100 μL) were injected into N. benthamiana leaves. The activities of firefly luciferase (LUC) and renilla luciferase (REN) were measured 3 d after infiltration using dual-luciferase assay reagents (Promega). Three independent experiments with six biological replicates were performed for each TF–promoter interaction. The ratio of LUC/REN was used to evaluate the activation effect of TF on transcription from the promoter, and the LUC/REN of empty-SK to promoter-LUC was set as the calibrator to exclude the background level of transcription.

**Electrophoretic mobility shift assay**

The full-length ORF of PpNAC1 was cloned into the pET-6* HN expression vector (Clontech, Mountain View, CA, USA) with an N-terminal His-tag (primers are listed in Supplemental Table S5). The recombinant PpNAC1 protein was expressed in *Escherichia coli* BL21(DE3) pLysS (Promega, Madison, WI, USA) and purified using the His TALON gravity column (Clontech) (Cao et al. 2021). The EMSA was performed using the Lightshift Chemiluminescent EMSA kit and the Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Fisher Scientific, New York, NY, USA) according to the manufacturer’s instructions. Double-stranded synthetic oligonucleotides containing the NACBSs were labeled with biotin (bio-probe) and the unlabeled DNA fragment (cold-probe) was used as a competitor, while cold-probe with mutated NACBSs was used as mutant-probe. The probes used for EMSA are listed in Supplemental Table S6.

**Plant transformation**

The full-length ORF of PpNAC1 was cloned into the Gateway entry vector pDONR207 (LT726435) with the primers listed in Supplemental Table S5. The pBlN19 vector (U09365) driven by the E8 promoter was constructed using the Gateway system. The stable transgene was carried out in tomato *nonripening* mutant in Micro-Tom background (Cao et al. 2021), and the WT fruit with normal ripening phenotype was used as a control. Agrobacterium-mediated tomato transformation was performed as described by Van Eck et al. (2006). Transformed lines were selected on kanamycin (70 mg L⁻¹). Fruits at B + 7 d from three independent transgenic T2 generations and WT plants were harvested for analysis. Three biological replicates of 10 fruits each were used for analysis. Peach callus was induced from the young fruit of “Huijingmilu” peach according to (Pérez-Jíménez et al. 2013). Transient overexpression of PpNAC1 in peach callus was performed according to Bai et al. (2019). The full-length ORF of PpNAC1 was constructed in the SUPER-1300 vector driven by the super promoter. *A. tumefaciens* GV3101 carrying the SUPER-1300-PpNAC1 construct or empty vector were resuspended in SH (Schenk & Hildebrandt) medium (13.2 g L⁻¹ SH, 20 g L⁻¹ sucrose, 1 g L⁻¹ polyvinylpyrrolidone, 100 μM acetylsyringone) (OD600 = 0.8) and infected the callus for 10 min. After 3 d coculture, the callus was transferred to SH solid medium (containing 400 mg L⁻¹ cephalosporin and 10 mg L⁻¹ hygromycin B) for 4 d, then sampled and used for gene expression analysis.

**Whole genome bisulfite sequencing**

Genomic DNA (gDNA) from peach fruits at different developmental stages was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The gDNA was digested by ultrasounds, then the fragmented gDNA was subjected to purification, end repair, addition of A to the 3’ end and addition of methylated linker, and then the DNA fragments were treated with bisulfite using ZYMO EZ DNA Methylation-Gold Kit (ZYMO, Irvine, CA, USA), and further amplified by PCR to generate a sequencing library. Qualified libraries were sequenced using Illumina HiSeqTM 2500 platform. Clean reads were mapped to the peach reference genome. Cytosine methylation (5-methylcytosine, 5mC) levels were detected using Bismark software according to our previous study (Zhang et al. 2016). The DMRs were sequenced using MOABS, requiring the presence of at least three differentially methylated sites, using Fisher’s exact test with the P-value < 0.05.
Phylogenetic tree construction
Multiple sequence alignments of the predicted full-length amino acid sequences were performed using Clustal W with default parameters. The phylogenetic tree was constructed by neighbor-joining methods with 1,000 bootstrap replications by MEGA6.0, with the matrix of the evolutionary distances calculated by Poisson correction for the multiple substitutions.

Statistical analysis
Figures were generated using Origin Pro 8 (OriginLab Corp., Northampton, MA, USA). Statistical analysis between two samples was performed using the unpaired Student's t-test (*P < 0.05, **P < 0.01). Tukey’s HSD one-way analysis of variance (ANOVA) was used to test the significance level of multiple groups; P < 0.05 was considered to indicate statistical significance (SPSS 19.0, SPSS Inc., Chicago, IL, USA).

Accession numbers
Sequence data from this study can be found in peach genome under the following accession numbers: PpNAC1, Prupe.4G187100; PpACS1, Prupe.2G176900; PpACO1, Prupe.3G209900; PpETR2, Prupe.1G034300; PpERF1, Prupe.3G032300; PpPL1, Prupe.5G161300; PpPL2, Prupe.1G060900; PpPG1, Prupe.4G262200; PpEXP2, Prupe.1G276700; PpPMET1, Prupe.7G192800; PpFAB1, Prupe.7G193900; PpSAD1, Prupe.6G365100; PpSAD2, Prupe.6G365200; PpLIP1, Prupe.1G129700; PpFAD2, Prupe.7G076500; PpFAD3-1, Prupe.6G056100; PpACX1, Prupe.5G065100; PpAAAT1, Prupe.5G018200; PpNINV3, Prupe.2G083900; PpTST1, Prupe.5G006300; PpTST2, Prupe.8G180600; PpDML1, Prupe.7G118000.

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Author contribution
X.C. and B.Z. designed the research plans; X.C. performed most of the experiments; X.L. and Y.S. performed phenotype analysis of different peach cultivars; C.Z. performed the DAP-seq analysis; C.W. performed the vectors construction; K.C. provided instruments for the experiments; D.G. contributed to manuscript preparation and editing; X.C. and B.Z. wrote the article with contributions from all the authors.

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree of NAC family members in peach and transcript levels in leaf, flower, and fruit at different stages.

Supplemental Figure S2. The phylogenetic tree of MADS family in peach, the transcript level of MADS members, and correlation with of ethylene production.

Supplemental Figure S3. Transcript levels of PpNAC1 target genes during fruit ripening by RT-qPCR and RNA-seq.

Supplemental Figure S4. Content of glucose, fructose, citric acid, and fatty acid-derived volatiles in PpNAC1 transgenic tomato.

Supplemental Figure S5. KEGG enrichment of CG, CHG, and CHH DMGs in ripening (S4) vs young (S1) fruit.

Supplemental Figure S6. IGV display of the methylation levels of mCG in the promoter regions of genes related to ethylene synthesis, fruit softening, and flavor formation obtained from the WGS data of the FruitENCODE project (Lü et al. 2018).

Supplemental Figure S7. The transcriptional activation of PpIAA1, PpGH3-4, and PpNCED1 by PpNAC1.

Supplemental Figure S8. The phylogenetic tree of SAD family and transient overexpression of PpSAD1 promotes the synthesis of fatty acid-derived VOCs.

Supplemental Table S1. Quality analysis of PpNAC1 DAP-seq.

Supplemental Table S2. Correlation of the transcript level of genes related to DNA methylation and demethylation with genome-wide DNA methylation level.

Supplemental Table S3. Correlation of the transcript level of ripening-related genes with DNA methylation level in the promoter regions.

Supplemental Table S4. Primers used for RT-qPCR in this study.

Supplemental Table S5. Primers used for expression vector construction.

Supplemental Table S6. The probe sequences of the target genes used for EMSA.

Supplemental Table S7. The Gene Bank ID of NAC members from other species.

Supplemental Data Set 1. The target genes of PpNAC1 by DAP-seq and their transcript levels during fruit ripening by RNA-seq.

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Conflict of interest statement. The authors declare no conflict of interest.

Data availability
The RNA-seq data of peach fruit at different development stages and under ethylene and 1-MCP treatment can be found in the NCBI database with accession number
PRJNA57653 and PRJNA574777, respectively. The DAP-seq data of PrpNAC1 can be obtained with the accession number PRJNAA91279. The expression levels of the PrpNAC1 target genes were listed in the Supplemental Data Set 1. The WGBS of peach fruit at different developmental stages from the fruitENCODE project can be obtained with the SRA accession number SRX3428467, SRX3428377, SRX3428376, and SRX3428378.

References


Li BJ, Grierson D, Shi Y, Chen KS. Roles of aspecific acid in regulating ripening and quality of strawberry, a model non-climacteric fruit. Hortic Res. 2022:9:huac089. https://doi.org/10.1038/s41438-022-01416-x


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