



Tandem transcription factors *PpNAC1* and *PpNAC5* synergistically activate the transcription of the *PpPGF* to regulate peach softening during fruit ripening

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Abstract

Peach fruit rapidly softens after harvest, a significant challenge for producers and marketers as it results in rotting fruit and significantly reduces shelf life. In this study, we identified two tandem genes, *PpNAC1* and *PpNAC5*, within the *sr* (slow ripening) locus. Phylogenetic analysis showed that *NAC1* and *NAC5* are highly conserved in dicots and that *PpNAC1* is the orthologous gene of *Non-ripening (NOR)* in tomato. *PpNAC1* and *PpNAC5* were highly expressed in peach fruit, with their transcript levels up-regulated at the onset of ripening. Yeast two-hybrid and bimolecular fluorescence complementation assays showed *PpNAC1* interacting with *PpNAC5* and this interaction occurs with the tomato and apple orthologues. Transient gene silencing experiments showed that *PpNAC1* and *PpNAC5* positively regulate peach fruit softening. Yeast one-hybrid and dual luciferase assays and LUC bioluminescence imaging proved that *PpNAC1* and *PpNAC5* directly bind to the *PpPGF* promoter and activate its transcription. Co-expression of *PpNAC1* and *PpNAC5* showed higher levels of *PpPGF* activation than expression of *PpNAC1* or *PpNAC5* alone. In summary, our findings demonstrate that the tandem transcription factors *PpNAC1* and *PpNAC5* synergistically activate the transcription of *PpPGF* to regulate fruit softening during peach fruit ripening.

Key message

PpNAC1 and *PpNAC5* directly bind to the *PpPGF* promoters, synergistically activate its transcription to regulate peach fruit softening.

Keywords Peach · Ripening · *NAC* family · Transcriptional regulation · Fruit softening

Introduction

Peach [*Prunus persica* (L.) Batsch] is a fruit crop of economic importance and celebrated for its distinct flavor and nutrient content. However, peach fruits have a limited shelf

life and are harvested before physiological ripening, resulting in inferior fruit quality (Eduardo et al. 2011). The ability to regulate fruit ripening could not only extend the shelf life but also allow strategic planning of the marketing season. As a climacteric fruit, peach ripening is influenced by various factors, with the plant hormone ethylene serving as a critical regulator. Ethylene exerts an effect on multiple physiological responses throughout peach ripening (Tatsuki et al. 2007, 2013; Monti et al. 2016). Furthermore, several transcription factors, including some in the *NAC* family, have been identified and functionally characterized as important regulators of fruit ripening (Lü et al. 2018; Gu et al. 2019; Wang et al. 2021; Dai et al. 2023; Cheng et al. 2023).

In tomato, the *Non-ripening (NOR)* gene, encoding a *NAC* transcription factor, serves as a pivotal regulator of

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fruit ripening. The *SINOR* loss-of-function mutant exhibited disrupted pigment accumulation, abnormal fruit softening and an extended shelf life (Gao et al. 2020). Similarly, the silencing of *NOR-like1* suppressed tomato fruit ripening and, *NOR-like1* directly interacted with the promoters and positively regulated the expression of several genes involved in processes related to tomato ripening, including ethylene biosynthesis, color change, and cell wall metabolism (Gao et al. 2018). In the climacteric fruit melon, *CmNAC-NOR* directly induces the expression of *CmACS5*, *CmNCED3*, and *CmZDS1* to promote fruit ripening (Wang et al. 2022a, b). In apple, *MdNAC18.1* has been identified as an indicator of apple harvest date, and influences fruit firmness at harvest and after harvest (Migicovsky et al. 2021). Transgenic introduction of *MdNAC18.1* into the tomato *nor* mutant complemented the ripening deficiency observed in the *nor* tomato (Migicovsky et al. 2021). Furthermore, the NAC transcription factor *RIPENING INDUCING FACTOR (RIF)*, which is an orthologue of *NOR*, plays a crucial role in the regulation of ripening in strawberry, a non-climacteric fruit. Knockout mutations of *FvRIF* result in a complete blockade of fruit ripening (Martin-Pizarro et al. 2021; Li et al. 2023). Moreover, *FvRIF* modulates anthocyanin biosynthesis and fruit softening by directly regulating the expression of related core genes (Li et al. 2023). Collectively, these studies demonstrate that *NOR* and its orthologous genes play vital roles in both climacteric and non-climacteric fruit ripening.

In peach, *PpNAC1* (Prupe.4G187100), which shares homology with *NOR*, has been identified and shown to be a key participant in the ethylene synthesis positive feedback loop during peach fruit ripening. *PpNAC1* exerts its influence by directly binding to the promoter regions of the ethylene synthesis genes *ACS* and *ACO*, triggering their expression (Tatsuki et al. 2007, 2013; Lü et al. 2018). Additionally, *PpNAC1* also serves as an activator of biosynthesis of flavor-related volatile compounds and of anthocyanin metabolism during peach fruit ripening (Zhou et al. 2015; Cao et al. 2021; Jin et al. 2022). These findings collectively underscore the roles played by *PpNAC1* during peach fruit ripening. However, fruit ripening involves dramatic and complex changes beyond the generation of volatiles and anthocyanin, and the precise contribution of *PpNAC1* to other ripening-associated changes remains unclear. Furthermore, previous investigations have reported that *PpNAC1* is located in the quantitative trait locus (QTL) *qMD4.1*, which controls maturity date (Pirona et al. 2013). In conjunction with *PpNAC1*, another member of the NAC transcription factor family, *PpNAC5* (Prupe.4G186800), is also located in the *qMD4.1* locus and has been considered to be a candidate gene for maturity date (Nuñez-Lillo et al. 2015). Notably, a 9-bp insertion in the coding sequence of *PpNAC5* has been consistently associated with the early-ripening phenotype (Pirona et al. 2013; Guo et al. 2020). However,

the mechanisms by which *PpNAC5* regulates fruit ripening remain unknown.

Fruit softening is one of the most important physiological changes during peach fruit ripening. The rapid softening of harvested peaches makes them susceptible to rotting and significantly shortens their shelf life, thereby limiting potential markets (Qian et al. 2021). It has been reported that the peach fruit texture and flesh adhesion to the pit are closely related traits controlled by the *F-M* locus in linkage group 4 (Dettori et al. 2001; Dirlwanger et al. 2006; Ogundiwin et al. 2009). In addition, two pectinases, namely *Pp-endoPGM* (PGM) and *Pp-endoPGF* (PGF), have been identified as key regulators of distinct fruit texture and flesh adhesion phenotypes across various peach accessions (Peace et al. 2005; Gu et al. 2016).

The slow ripening (SR) trait is a mutation that inhibits the normal fruit ripening process in peach. This trait is controlled by a single locus *sr*, which has been mapped on linkage group 4 (G4) and overlaps with the *qMD4.1* locus (Eduardo et al. 2015; Meneses et al. 2016; Nuñez-Lillo et al. 2015). A deletion of 26.6 kb in the *sr* locus correlates with the SR trait/maturity date, and the gene *PpNAC5* was located in the deleted sequence (Eduardo et al. 2015; Nuñez-Lillo et al. 2015). In this study, we conducted an analysis of the 26.6-kb deleted sequence and found that it contains not only the *PpNAC5* gene but also the entire promoter sequence of *PpNAC1*. The tandem arrangement of these two genes was highly conserved in dicots. Furthermore, our findings demonstrate that both *PpNAC1* and *PpNAC5* play positive roles in fruit softening during ripening. Moreover, *PpNAC1* physically interacted with *PpNAC5* to form a protein heterodimer, resulting in greater *PpPGF* transcription.

Materials and methods

Plant materials

The peach cultivars used in this study were 7-year-old trees maintained at the Fruit Tree Germplasm Repository of Henan Agricultural University (Henan Province, China). Fruit samples from the early-ripening variety ‘FeiYu’ and the late-ripening peach variety ‘JinQiuHongMi’ were collected 15 days before (– 15) to 182 days after full bloom (DAFB). The two freestone melting peach cv. ‘YuMeiRen’ and ‘HuangShuiMi’ were collected at four development stages, including 30, 61, 74, and 87 DAFB for ‘YuMeiRen’ and 35, 54, 85, and 100 DAFB for ‘HuangShuiMi’. The fruits were immediately frozen in liquid nitrogen and stored at – 80 °C. Three biological replicates, each consisting of five fruits from different trees, were used for the experiments.

Nicotiana benthamiana seedlings were grown in pots under a 16/8 h photoperiod condition in a growth chamber at 25 °C for 4 weeks, and the leaves were subsequently used for further tests.

Methods

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using the FastPure® Universal Plant Total RNA Isolation Kit (Vazyme, Jiangsu, China), followed by the synthesis of first-strand cDNA using the HiScript® III RT SuperMix for qPCR(+ gDNA wiper) (Vazyme, Jiangsu, China) according to the manufacturer's instructions. qRT-PCR reactions were conducted in a total reaction volume of 10 µL, containing 5 µL of SYBR Green I Master Mix, 0.5 µM of each primer, and 200 ng of template cDNA, using the SYBR® Green PCR kit (Takara, China). Amplification was carried out on an ABI Prism 7500 FAST Sequence Detection System (Applied Biosystems, USA). The peach *PpTEF2* (Prupe.4G138900) and tobacco *β-tubulin* (U91564) genes were selected as reference genes (Tong et al. 2009; Schmidt and Delaney 2010). The primer sequences used for qRT-PCR are listed in Table S1. Three biological replicates were performed for each analysis.

Gene isolation and promoter cloning

The full-length Coding DNA Sequence (CDS) of each gene was isolated from the cDNA of peach cv. 'HuangShuiMi' using primers designed based on the reference sequences from the peach genome version 2.0 (https://phytozome-next.jgi.doe.gov/info/Ppersica_v2_1). Homologous genes used for phylogenetic analyses in other species were obtained from the NCBI database. Sequence alignments and phylogenetic analyses were performed using MEGA7.

Yeast two-hybrid (Y2H) assay

The coding sequences of *PpNAC1* (for amino acids 1–285), *SINAC1* and *MdNAC5* were cloned into pGBKT7, while the coding sequences of *PpNAC5*, *SINAC5* and *MdNAC1* were fused to pGADT7. The resulting AD and BD plasmids were co-transformed into the Y2H-gold yeast strain (TSC0502, Beijing Tsingke Biotech). P53 and 53-BD + AD were used as positive control and negative control, respectively. The interaction between the proteins was analyzed on SD/-Trp/-Leu (-TL) and SD/-Trp/-His/-Leu/-Ade (-THLA) media supplemented with X-α-gal medium (Clontech, Mountain View, CA, USA). Primers used for cloning in this study are listed in Table S1.

Bimolecular fluorescence complementation (BiFC) assay

The full-length CDSs of *PpNAC1* and *PpNAC5* were cloned into the pNC-Ecn and pNC-Enn vectors, respectively. These constructs were then introduced into *A. tumefaciens* GV3101 (pSoup). After incubation, the bacteria were collected and resuspended in infiltration buffer to an OD₆₀₀ = 0.8. The bacterial suspensions harboring *pNC-Ecn-PpNAC1* and *pNC-Enn-PpNAC5* were mixed at a ratio of 1:1 (v:v) and infiltrated into tobacco leaves. The green fluorescent protein (GFP) signals were visualized using a confocal microscope (Nikon, Japan).

Transient transformation in peach fruit

For silencing experiments, a 235-bp cDNA fragment of *PpNAC1* and a 343-bp cDNA fragment of *PpNAC5* were amplified and then individually inserted into the virus vector pTRV2 to generate the Virus-induced gene silencing (VIGS) constructs.

The recombinant plasmids, along with empty plasmid (pTRV1 and pTRV2) as control, were transformed into *A. tumefaciens* GV3101 and incubated at 28 °C until an OD₆₀₀ of 0.6. The bacteria were then suspended in infiltration buffer (10 mM MES + 10 mM MgCl₂ + 150 µM AS, pH5.6). For the VIGS experiment, the culture containing the recombinant plasmids pTRV1 and pTRV2 was mixed at a ratio of 1:1, and 500 µL of this culture was infiltrated into both sides of peach fruit growing on trees of 'YuNongMiXiang' at the end of the S3 developmental stage, following the protocol described by Wang et. al (2022). A total of 100 peach fruits were infiltrated, and the fruits were harvested to detect gene expression levels and measure fruit firmness 2 weeks after the injection.

Measurement of fruit firmness

Peel firmness and flesh firmness were measured using a TA-XTPlusC (Stable Micro Systems, UK). The texture analyzer was set with the following parameters: test depth of 5 mm, pre-test speed of 1 mm s⁻¹, test speed of 1 mm s⁻¹ and post-test speed of 10 mm s⁻¹. A stainless-steel probe with a diameter of 5 mm was used for the measurements. Each side of the fruit was measured three times. Measurements were taken at 48-h intervals, and 10 fruits were tested for each treatment.

Yeast one-hybrid (Y1H) assay

The full-length CDSs of *PpNAC1* and *PpNAC5* were cloned into pB42AD vector as prey. Different lengths of the *PpPGF* promoter were cloned into pLacZi vector as bait using the

SE Seamless Cloning and Assembly Kit (Beijing ZOMAN-BIO, China). The Y1H assay was performed following the protocol described in a previous study (Zhang et al. 2022). The primer sequences used for the Y1H assay are provided in Table S1.

Dual-luciferase (LUC) reporter assays

About 1.8-kb promoter regions of *PpPGF* were amplified and cloned into pGreen II 0800-LUC reporter vectors. The constructs of *pSAK277-PpNAC1* and *SAK277-PpNAC5* under the CaMV35S promoter were used as effectors. For the transient expression assay, the empty vector (EV) *pSAK277*, the effector and reporter vectors were separately transformed into *A.tumefaciens* GV3101 and incubated. After collection, the bacteria were suspended in solution (10 mM MgCl₂ + 10 mM MES + 150 μM AS, pH5.6) to an OD₆₀₀ of 0.8. The effector and the reporter were mixed at ratios of 4:1 or 2:2:1 and statically incubated for 3 h at room temperature. Then, the mixture was infiltrated into four-week-old tobacco leaves. The LUC and REN luciferase activities were measured using the Dual-Luciferase Reporter Assay Kit (Vazyme, China). The LUC signal intensity was also analyzed using the NightSHADE LB 985 in vivo imaging system (Berthold Technologies, Bad Wildbad, Germany). Three biological replicates with a mixture of 6 infiltrated leaves each replicates were performed for four different vector mixtures.

Statistical analysis

The data were presented as means and standard errors calculated from at least three biological replicates. Statistical analysis was conducted using Student's *t*-test within SPSS version 21. Statistical significance is indicated with single or double asterisks at $P < 0.05$ and $P < 0.01$, respectively.

Results

The tandem genes *PpNAC1* and *PpNAC5* were located in the maturity date locus and were highly conserved in dicots

A 26.6-kb deletion in the *sr* locus was previously reported to co-localize with the SR and maturity date traits, and within this deleted region, *PpNAC5* has been identified as a candidate gene for this trait (Eduardo et al. 2015; Nuñez-Lillo et al. 2015). In this study, the 26.6-kb deleted sequence was further analyzed and found to include not only the *PpNAC5* gene but also the entire promoter region of *PpNAC1* (Fig. 1a). This finding raises the possibility that both the

PpNAC1 and *PpNAC5* genes may be candidate genes for SR /maturity date.

To further determine the functional roles of *PpNAC1* and *PpNAC5*, an orthologous gene comparison was conducted across multiple species. The analysis showed that *PpNAC1* is the ortholog of *NOR* and *Nor-like* in tomato and *CmNAC-NOR* in melon (Fig. 1b) (Gao et al. 2018; Wang et al. 2022a, b). The tandem arrangement of *NAC1* and *NAC5* in the genomes of various taxa revealed that monocotyledons solely possess *NAC1*, whereas dicotyledons carry both *NAC1* and *NAC5*, but with variable lengths of their intergenic intervals (Table S2). Remarkably, the tandem configuration of *NAC1* and *NAC5* in dicots exhibits a notable evolutionary conservation (Table S2). These results suggest an integrated role of these tandemly arranged genes in modulating peach fruit ripening.

Transcript levels of *PpNAC1* and *PpNAC5* are increased at the onset of peach fruit ripening

To further investigate the roles of *PpNAC1* and *PpNAC5* in peach fruit development and ripening, the transcript levels of these two genes were assessed during fruit development in the early-ripening cultivar 'Feiyu' and late-ripening cultivar 'JinQiuHongMi'. The transcriptional profiles of *PpNAC1* and *PpNAC5* were similar during fruit development in each cultivar, although the timing was shifted between the early- and late- ripening peach (Fig. 2a and b). At the preliminary stages of fruit development (S1 and S2), transcript abundance for both genes was relatively low. Significant increases in gene expression were observed concomitant with the initiation of ripening (S3), reaching peak expression levels prior to a subsequent decline during the ripening phase (S4). Additionally, tissue-specific expression analysis revealed higher expression levels of *PpNAC1* and *PpNAC5* in the fruit than in other tissues like stem, mature leaves and blooming flowers (Fig. S1). These results suggest an involvement of *PpNAC1* and *PpNAC5* in regulation of the ripening of peach fruits. Furthermore, a distinct peak in expression was observed at the S1 to S2 transition in both the early and late- ripening varieties. This expression peak indicates a potential role for these two genes not only in ripening stages but also in the initial phases of fruit development.

PpNAC1 physically interacted with *PpNAC5*

NAC transcription factors are known to form homo- and heterodimers. The evolutionary conservation of the tandem *NAC1/5* gene arrangement and their synchronized expression profiles in peach fruit suggest a potential for direct interaction between *PpNAC1* and *PpNAC5*. Yeast two-hybrid (Y2H) assays were carried out to confirm this hypothesis. Since it has been reported that full-length *PpNAC1* shows

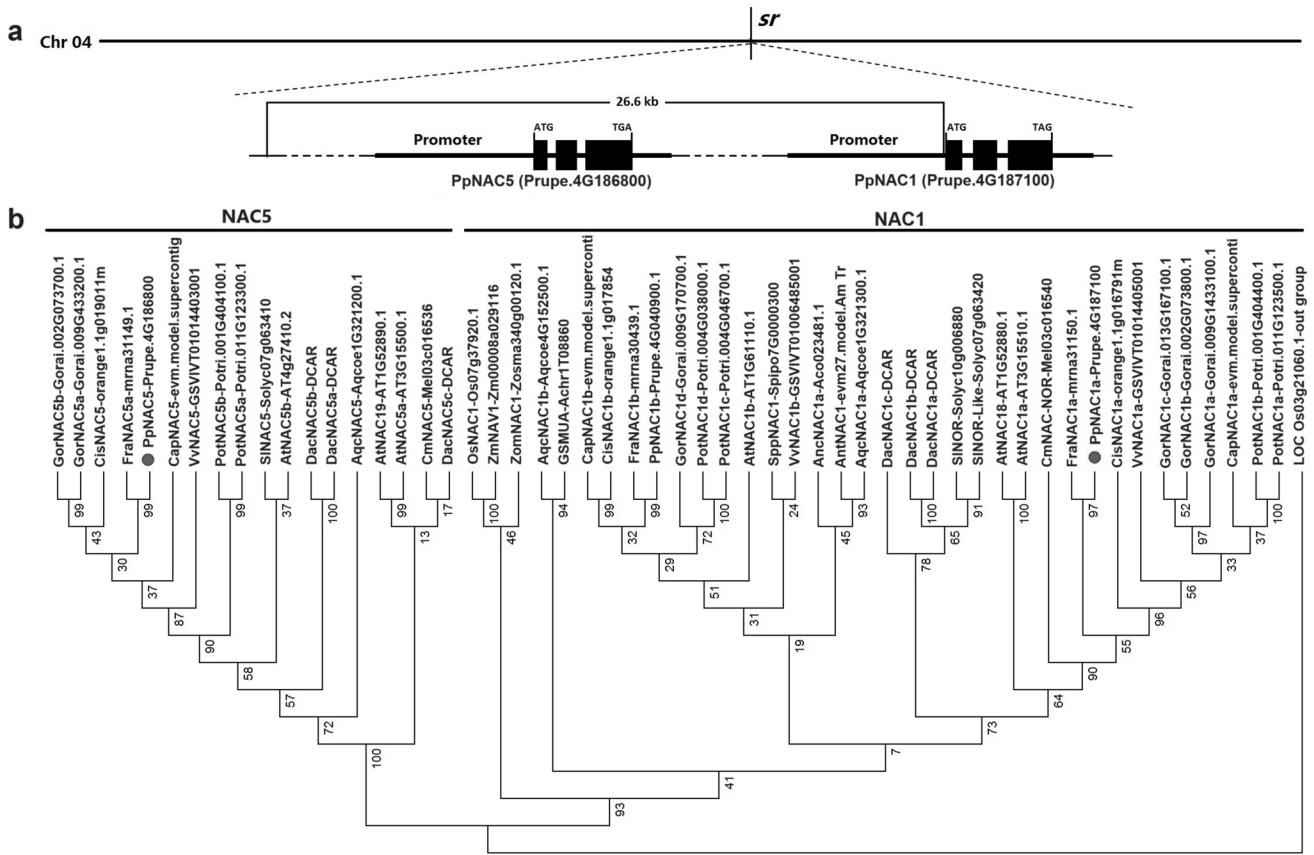


Fig. 1 Location of *PpNAC1* and *PpNAC5* in the maturity date locus. **a** The tandem genes *PpNAC1* and *PpNAC5* were located in the SR locus. All of *PpNAC5* and promoter of *PpNAC1* were located within

a 26.6-kb sequence that is deleted in the SR genotype. **b** Phylogenetic analyses of *PpNAC1* and *PpNAC5* with their homologous genes in other dicotyledonous species

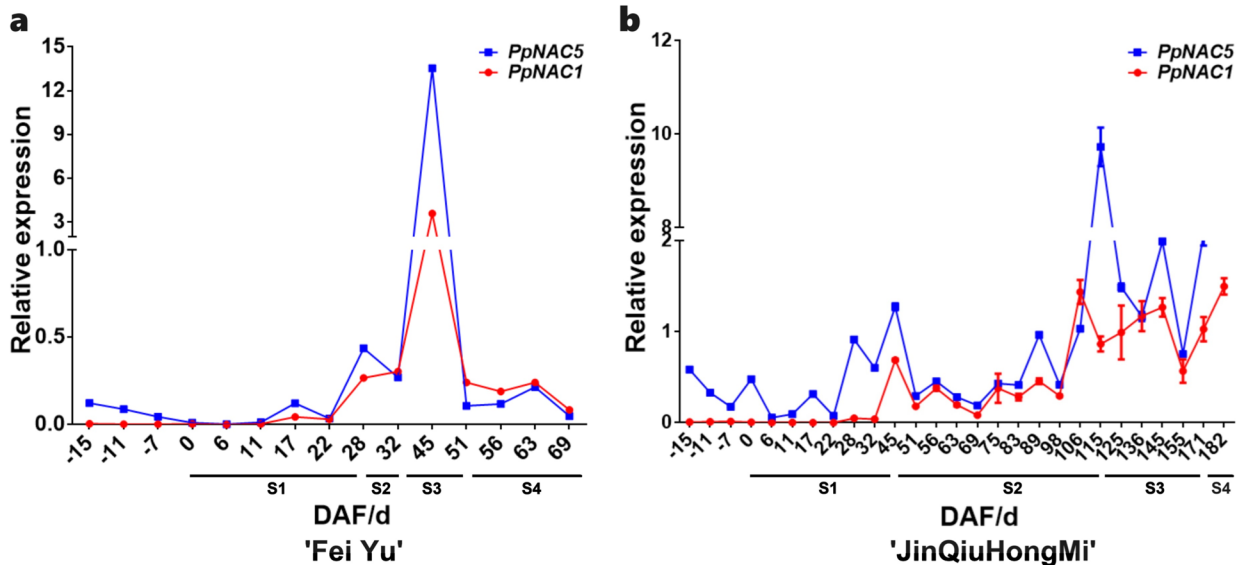


Fig. 2 Transcript levels of *PpNAC1* and *PpNAC5* during peach fruit development and ripening. **a** Transcript levels of *PpNAC1* and *PpNAC5* in the early-ripening peach cultivar 'Fei Yu'. **b** Transcript levels of *PpNAC1* and *PpNAC5* in the late-ripening peach cultivar

'JinQiuHongMi'. The fruit development stage was divided into four periods (S1, S2, S3 and S4; identified with black lines) according to the days after full bloom (DAFB). The minus days represent days before full bloom

strong self-activation, a truncated *PpNAC1*, encoding the amino acids 1 to 285, was used according to the previous report (Zhou et al. 2015) for the protein interaction analysis. The results revealed an interaction between *PpNAC1* and *PpNAC5* (Fig. 3a). The interaction was further confirmed *in planta* via BiFC assays in tobacco leaves (Fig. 3b).

Variations of a 9-base pair insertion/deletion (INDEL) in the *PpNAC5* coding sequence have been associated with the maturation date of peach fruits (Pirone et al. 2013; Guo et al. 2020). To delineate the impact of different *PpNAC5* allelic variants on its interaction with *PpNAC1*, Y2H assays were employed. These assays demonstrated that the

allele of *PpNAC5* with the 9-base pair segment exhibited an enhanced interaction with *PpNAC1* compared to the one with the deletion (*PpNAC5**) (Fig. 3c).

The highly conserved tandem arrangement of *NAC1* and *NAC5* across different dicotyledonous species implies a functional conservation between the two genes. Additional Y2H assays confirmed the physical interaction between the two NAC proteins encoded within tomato and within apple (Fig. 3d). These results indicated this protein–protein interaction is likely preserved across various dicot plants. The implication of these findings is that *PpNAC1*

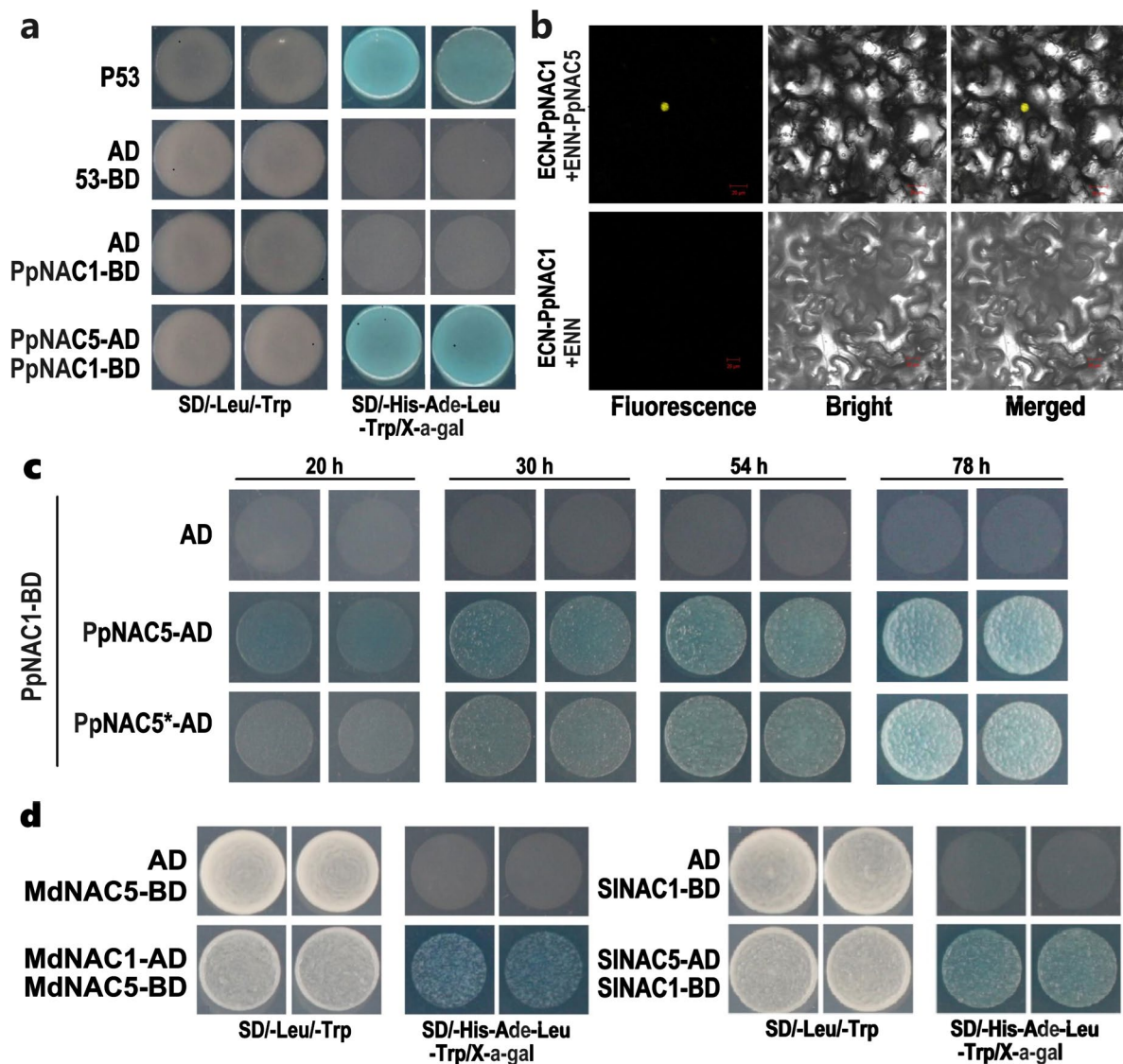


Fig. 3 *PpNAC1* physically interacted with *PpNAC5*. **a** Yeast two-hybrid analyses confirmed the interaction between *PpNAC1* and *PpNAC5*. The pGBKT7-53+pGADT7-RecT (P53) is a positive control, and pGBKT7-53+pGADT7 (AD+53-BD) is a negative control. **b** BiFC assay of the interaction between *PpNAC1* and *PpNAC5*. ECN-PpNAC1+ENN is used as a negative control. **c** Y2H analyses confirmed the interaction between *PpNAC1* and *PpNAC5* (*PpNAC5* without the 9-base pair segment was identified with an asterisk). **d** Y2H analyses confirmed the interaction between the two tandem genes *NAC1* and *NAC5* in apple and in tomato

and *PpNAC5* may operate synergistically in the regulatory network governing fruit ripening.

PpNAC1 and PpNAC5 regulate peach fruit softening

To further understand the contributions of *PpNAC1* and *PpNAC5* to fruit ripening, VIGS was utilized to suppress expression of these two genes in peach fruits. Peach fruit transiently injected with TRV2-*PpNAC1* or TRV2-*PpNAC5* were harvested 15 days after infiltration, along

with control fruits injected with the empty vector TRV2 (Fig. 4a). Subsequent RT-qPCR analysis revealed a significant reduction in the transcript levels of *PpNAC1* and *PpNAC5* in the silenced fruits compared to the TRV2 controls (Fig. 4b). Post-infiltration observations revealed that both *PpNAC1*- and *PpNAC5*-silenced fruits demonstrated a significant increase in peel and flesh firmness after 7 days of storage when contrasted with fruits injected with TRV2 (Fig. 4c, d). In addition, the expression of *PpPGF*, one of the polygalacturonase (PG) genes,

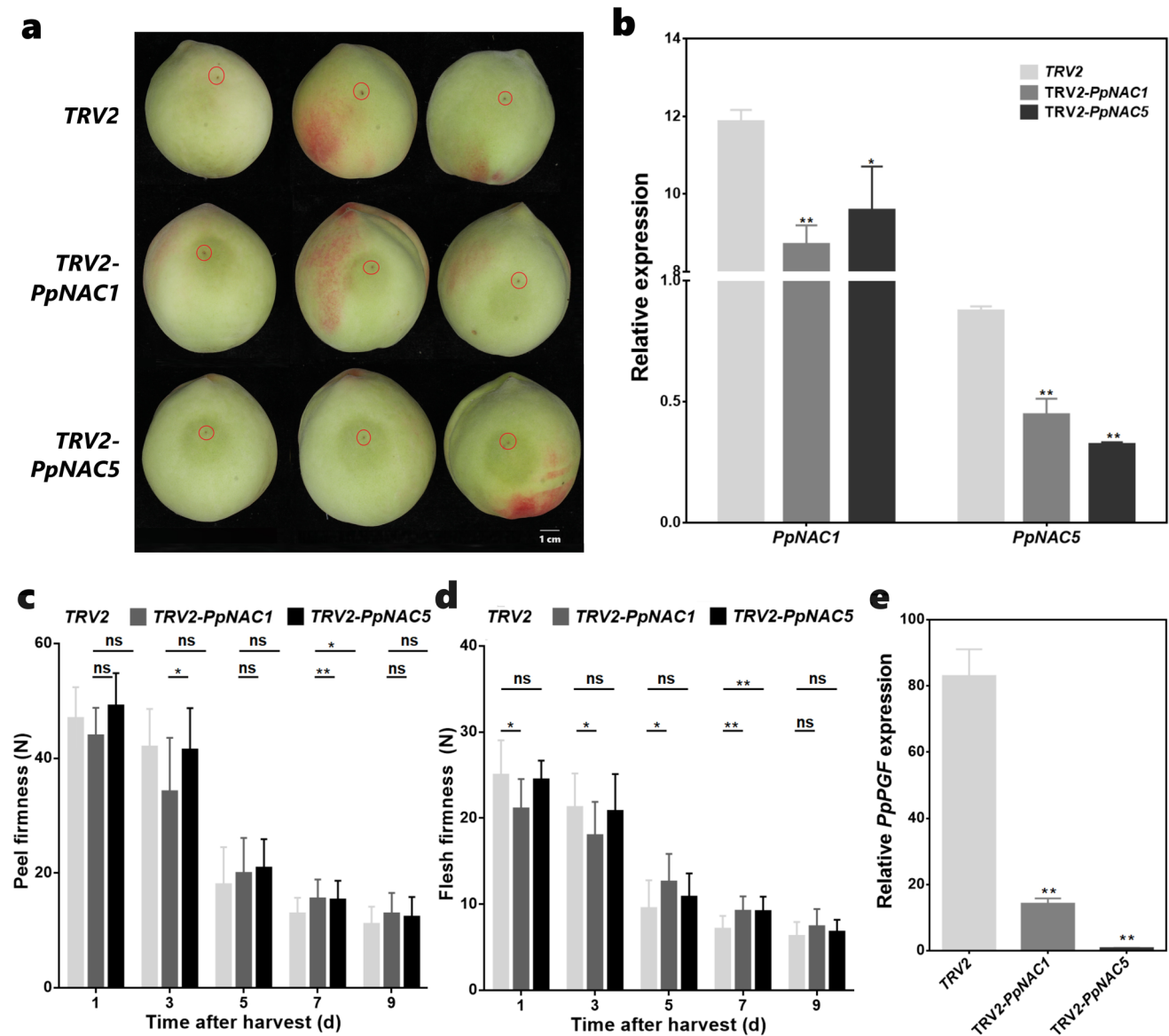


Fig. 4 Silencing of *PpNAC1* and *PpNAC5* delayed peach fruit softening. **a** Appearance of peach fruits injected with TRV2, TRV2-*PpNAC1* and TRV2-*PpNAC5*. The red circles indicate the injected sites. **b** Relative expression of *PpNAC1* and *PpNAC5* detected by RT-qPCR in the transiently silenced fruits. **c**, **d** Peel and flesh firmness of the silenced fruits at different days after harvest. Ten fruits were used

for each treatment and two injected sides of each fruit were tested. **e** Relative expression of *PpPGF* in the transiently silenced fruits. Data are the means \pm SD. The asterisks indicate statistically significant differences between the TRV2 and the silenced fruits areas, as determined by Student's *t*-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

was markedly reduced in the silenced fruits compared to the TRV2 controls (Fig. 4e). We also investigated the transcript levels of *PpPGF* together with *PpNAC1* and *PpNAC5* in different developmental stages of two free stone cultivars and found that the expression of *PpPGF* was highly expressed at S4 stage, when peaches begin to soften, which is consistent with the high expression of *PpNAC1* and *PpNAC5* at S3 and S4 (Fig. S2). These results suggest that *PpNAC1* and *PpNAC5* are involved in promoting peach fruit softening through upregulation of *PpPGF* expression.

PpNAC1 and PpNAC5 regulate fruit softening by directly binding to the *PpPGF* promoter and activating its transcription

To validate the hypothesis that *PpNAC1* and *PpNAC5* modulate *PpPGF* expression, Y1H assays were conducted. About 1800-bp upstream of the *PpPGF* gene as well as four promoter fragments (*P1–P4*) were used for Y1H assays (Fig. 5a). The yeast cells co-transformed with the *PpPGF* promoter as bait and pGADT7 as prey exhibited blue colonies, indicating self-activation of the *PpPGF* promoter, while the dissected fragments showed no self-activation

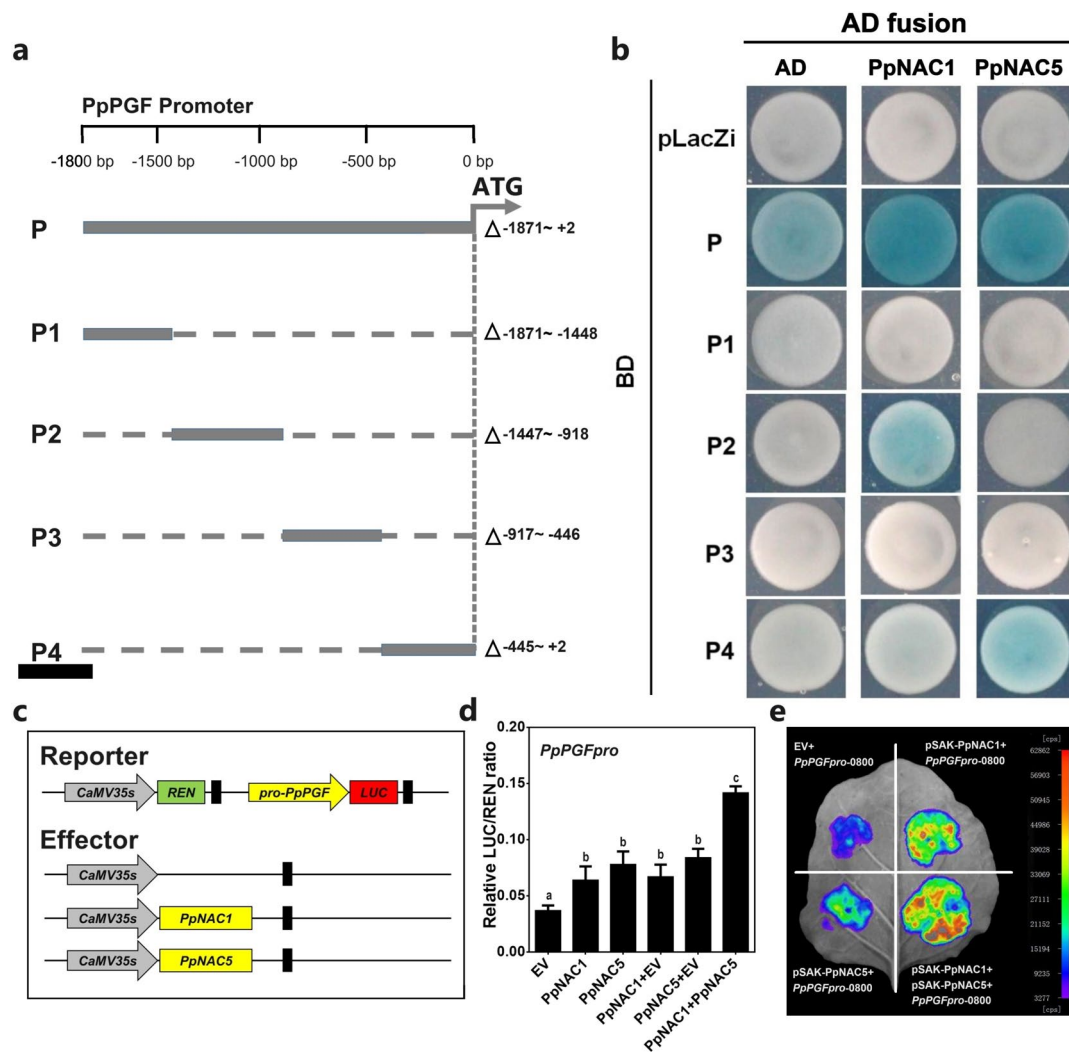


Fig. 5 *PpNAC1* and *PpNAC5* directly bind to the *PpPGF* promoter and activate its transcription. **a** Schematic diagrams of the full and four distinct *PpPGF* promoter fragments. **b** The growth and color of yeast cells co-transformed with prey (*PpNAC1*-pGADT7 or *PpNAC5*-pGADT7) and reporter constructs containing different fragments of the *PpPGF* promoter, on medium containing aureobasidin A and X-gal. **c** Schematic diagrams of reporter and effector vectors used for transformation of *N. benthamiana* leaves. **d**, **e** Dual-LUC

activation assay and LUC bioluminescence imaging in tobacco leaves co-transformed with the reporter driven by *pro-PpPGF* and the two effectors, pSAK-*PpNAC1* and pSAK-*PpNAC5*, alone or together. pGreenII 62-SK was used as a negative control. Values are the means \pm SD. The multiple comparisons were subjected to ANOVA using Duncan's test, and statistically significant differences ($P < 0.05$) were indicated by diverse lowercase

(Fig. 5b). The yeast cells co-transformed with PpNAC1 and *PpPGF* promoter fragment P2 or with PpNAC5 and fragment P4 indicated interactions (Fig. 5b). This suggested that PpNAC1 and PpNAC5 regulate *PpPGF* expression by interacting with separate regions within the *PpPGF* promoter.

LUC reporter assays and LUC bioluminescence imaging were also performed to further verify this regulation. The promoter sequence of *PpPGF* was cloned into the pGreenII 0800-LUC vector as reporter, and the CDSs of *PpNAC1* and *PpNAC5* under control of the 35S promoter were used as effectors (Fig. 5c). A significant enhancement of the LUC/REN ratio was recorded in the presence of pSAK-PpNAC1 or pSAK-PpNAC5 (Fig. 5d). Notably, simultaneous co-expression of both effectors (pSAK-PpNAC1 and pSAK-PpNAC5) with the *PpPGF* reporter vector resulted in an even greater increase in the LUC/REN ratio compared to the individual effectors or the co-expression of pSAK-PpNAC1 or pSAK-PpNAC5 with EV (Fig. 5d). These results were supported by visualization of the LUC fluorescence (Fig. 5e). Collectively, these findings demonstrate that PpNAC1 and PpNAC5 directly bind to the *PpPGF* promoter and activate its transcription, likely influencing peach fruit softening. Moreover, the evidence suggests a synergistic interaction between PpNAC1 and PpNAC5 in facilitating this transcriptional activation.

Discussion

The NAC (NAM, ATAF and CUC) proteins comprise a plant-specific transcription factor family, and several members have been identified as key regulators of fruit ripening (Olsen et al. 2005; Forlani et al. 2021; Liu et al. 2022). It has been reported that NAC proteins function through the formation of homodimers and/or heterodimers (Puranik et al. 2012). Zhou et al. (2015) demonstrated that one NAC transcription factor, BL, can interact with PpNAC1 and that the heterodimer of BL and PpNAC1, rather than BL alone, activated the transcription of *PpMYB10.1*, leading to anthocyanin pigmentation. Here, we discovered that PpNAC1 physically interacts with PpNAC5, forming a protein heterodimer and that co-expression of two proteins positively regulates a softening-related gene to a greater extent than either gene alone. This is consistent with the recently published research that PpNAC1 and PpNAC5 can form heterodimers and both function as ripening enhancers (Zhang et al. 2023). The difference is that we found that the co-expression has a higher activation activity for *PpPGF* than the genes of PpNAC1 or PpNAC5 alone, while Zhang et al. (2023) suggested that the activation activity of following PpNAC1 and PpNAC5 infiltration was significantly higher than that of PpNAC5, but lower compared with the PpNAC1. These discrepancies may be caused by the different ripening-related genes

that were directly activated. It has also been reported that PpNAC1 and DNA demethylase PpDML1 synergistically regulate peach fruit ripening by directly regulating expression of multiple genes required for peach ripening (Cao et al. 2023). Taken together, these findings suggest that *PpNAC1* serves as a central regulator modulating various metabolic pathways during peach fruit ripening either independently or through the formation of homodimers and/or heterodimers with other proteins.

Phylogenetic analyses of PpNAC1 and PpNAC5, in conjunction with their respective homologous genes, reveals that these two tandemly arrayed genes are highly conserved in dicotyledonous species. Accordingly, the physical interactions between NAC1 and NAC5 were also verified in tomato and apple (Fig. 3d), which suggested a conserved biological role of *NAC1* and *NAC5*. Extensive research has delineated the involvement of NAC1 homologs in fruit ripening. However, reports on NAC5 and its corresponding homologs remain scant. Notably, Wei et al. (2023) demonstrated that, in apple, phosphorylation of MdNAC72 (orthologous to PpNAC5) mediated by MdMAPK3 exerts influence over the softening of apple fruit during storage. Moreover, the overexpression of *AdNAC72* in kiwifruit not only enhances expression of a methionine sulfoxide reductase (*AdMsrB1*), but also increases free Met and ACC content and ethylene production rates (Fu et al. 2021). In our study, both *PpNAC1* and *PpNAC5* have regulatory effects on the softening of peach fruit. Moreover, Zhang et al. have also demonstrated the regulatory roles of *PpNAC1* and *PpNAC5* in fruit enlargement and ripening by stable or transient overexpression in tomato and strawberry, respectively (Zhang et al. 2023). All these results suggest the *NAC5* may participate in regulation of fruit ripening, thereby indicating the evolutionary conservation of the functional roles of *PpNAC1* and *PpNAC5*.

Previous research has reported that *PpNAC5* is a prime candidate gene linked to maturity date locus located on Chr. 4 (Pirone et al. 2013). A 9-bp insertion/deletion (INDEL) within the coding sequence of PpNAC5 has been correlated with phenotypic variation in maturity date, where early ripening individuals exhibit the 9-bp insertion and late ripening ones lack this segment. However, the explicit function and genetic variation of PpNAC5 in regulating developmental and ripening remained unclear. In the present study, phenotypic and molecular characterization of *PpNAC5*-knockdown transgenic peach fruits elucidated that *PpNAC5* plays a key role in accelerating the ripening of peach fruits by regulating fruit softening. Additionally, our findings suggest that the presence of the 9-bp insertion within PpNAC5 could potentiate its interaction with PpNAC1, thereby potentially contributing to the manifestation of an earlier ripening phenotype.

In addition to *PpNAC5*, *PpNAC1* is likewise posited as a key regulatory gene for peach maturity date, because

of its significant contributions to fruit ripening. Here, our investigation supports the notion that both the *PpNAC1* and *PpNAC5* are viable candidate genes for the SR /maturity date traits. Peach fruit development and ripening encompass distinct growth phases: the first exponential growth phase (S1), the onset of pit hardening (S2), the second exponential growth phase (S3), and ripening (S4) (Tonutti et al. 1991). Notably, the span of the S2 phase varies according to the cultivar, being shorter in early ripening varieties and longer in late ripening ones (Bonghi et al. 2011). In this study, both *PpNAC1* and *PpNAC5* exhibited high expression levels during the S3 and S4 phases, which suggested their roles in peach ripening. Intriguingly, our data reveal expression peaks for *PpNAC1* and *PpNAC5* at the S1 to S2 transition in both early and late ripening varieties. This transitional juncture marks a deceleration in fruit growth and precedes the lignification of the endocarp, an event synonymous with the onset of pit hardening that persists until the end of the S2 stage (Dardick et al. 2010). The increasing expression of *PpNAC1* and *PpNAC5* at the onset of the S2 stage indicated their potential roles in influencing peach fruit development and consequently modulating the maturity date of the peach fruit. It has also been verified by the recently reported that *PpNAC1* and *PpNAC5* may participate in the regulation of fruit enlargement through activating cell elongation-related genes (Zhang et al. 2023).

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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