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NAC072 Interacts with HB12, HAT9, and MYBR1 in a Temporal Regulatory Network Controlling Peach Fruit Development

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Abstract

Fruit development is a complex process that involves the interplay of different biological processes carefully coordinated to control fruit quality traits. The peach fruit development consists in four recognized growth stages (S1–S4). The second stage (S2) is characterized by the endocarp hardening process and is followed by the second exponential growth phase (S3), where an increase in fruit size is produced by a rapid cell expansion. A nectarine genotype incapable of ripening and described as a slow ripening phenotype was identified and selected as a good model for studying peach fruit development. Slow ripening fruit remained firm, green, and exhibited no rise in CO_2 or ethylene production rates blocking fruit development at S3 stage. The transcription factor NAC072 has been proposed as a key regulatory element involved in both the slow ripening and the harvest date phenotypes. However, the regulatory mechanisms by which NAC072 produces these phenotypic changes are still unknown. Using a transcriptomic approach between normal and slow ripening individuals with a transcription factor–gene target interaction database, a NAC072 regulatory network was constructed, identifying putative direct and indirect NAC072 fruit development-related elements. Three transcription factors were identified along with NAC072 in early stages of fruit development, two homeobox-leucine zippers (HB12 and HAT9), and one MYB transcription factor (MYBR1). In addition, we determined that the NAC072 transcriptional regulatory network promotes phenylpropanoids biosynthesis and cell wall remodeling to develop fruit growth, seed development, and softening, probably through hormonal signaling pathways involving abscisic acid and gibberellic acid.

Keywords Harvest date · Transcriptomics · Transcription factor · Fruit quality · Fruit development · Gene network analysis

Introduction

Fruit development is a complex process that involves the interplay of biological processes including softening, fruit growth, seed development, sugar accumulation, and acidity reduction carefully coordinated to control fruit quality traits such as harvest date, color, aroma, size, and flavor (Ozga and Reinecke 2003; Pirona et al. 2013). Several studies on harvest date have determined that it is a critical parameter for consumers and producers in terms of agro-industrial performance to cover and extend the marketing season (Pirona et al. 2013), and also because it has a pleiotropic

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effect controlling the development of other fruit quality traits such as softening, sugar accumulation, and acidity degree (Cantín et al. 2010).

A wide range of transcription factors associated with different aspects of peach fruit development have been reported, for example, the softening process is regulated by the transcription factor SEP1 (Li et al. 2017), while flavor development has been related to both the transcription factor ERF61 promoting linalool synthesis (Wei et al. 2022) and the transcription factor NAC1 regulating the synthesis of short-chain volatiles (Jin et al. 2022). On the other hand, the regulation of ethylene biosynthesis associated to peach fruit ripening has been related to the transcription factors NAC.A59 and ERF.A16 (Guo et al. 2021), ERF113 (Wang et al. 2022), HB.G7 (Gu et al. 2019), or NAP9 (Dai et al. 2023). There are many published examples that associate transcription factors to some fruit quality traits at the ripening stage; however, there are few examples of key transcription factors that could arrest fruit development or control the harvest date phenotype in peach. In tomato (*Solanum lycopersicum*), a range of well-characterized transcription factors have been associated to fruit development. The most studied examples of master regulatory transcription factors include the MADS-box transcription factor defined as ripening-inhibitor MADS-RIN (Vrebalov et al. 2002), the mutation in the NAC2-related non-ripening NAC-NOR transcription factor (Kumar et al. 2018), and the colorless non-ripening SPL-CNR (Manning et al. 2006). However, despite the fact that these transcription factors have been described as master regulators of fruit development, like previous examples, they act in late stages of fruit development associated with ethylene production at the onset of fruit ripening.

A nectarine genotype originated from 'Fantasia' incapable of ripening and described as slow ripening (SR) phenotype was identified (Brecht et al. 1984) and selected as a good model for studying peach fruit growth in the early stages of fruit development. Slow ripening fruit remained firm, green, and did not show an increase in CO_2 or ethylene production rates (Giné-Bordonaba et al. 2020). When comparing the seed and mesocarp development between 'Fantasia' and SR phenotypes, it was evidenced that mesocarp development of SR individuals seems to be blocked at S3 stage (Bonghi et al. 2011). Previous studies have suggested that this trait is controlled by a single recessive gene (*sr*) (Ramming 1991) that colocalizes on chromosome 4 with the harvest date candidate gene NAC domain containing protein 72 (Eduardo et al. 2015).

The transcription factor NAC072 has been described in *Arabidopsis thaliana* as a drought-induced transcription factor involved in chlorophyll degradation, participates in sucrose metabolism and mono- and disaccharide accumulation (Kamranfar et al. 2018). Also, functions as a transcriptional activator in ABA-inducible gene expression under abiotic stress (Fujita et al. 2004) and mediates the crosstalk between drought stress and brassinosteroid (BR) signaling pathway by acting as an antagonist of BR (Ye et al. 2017). It has been reported that the two orthologs of NAC072 in tomato *SlNAC072* (Solyc12g013620.2.1) and *SlNAC019* (Solyc07g063410.3.1) were differentially regulated by ABA and JA mediating stomatal closure and reopening during pathogen infection (Du et al. 2014). In peach, the transcription factor NAC072 was first identified as a candidate gene for harvest date control by QTL analysis (Prinoa et al. 2013; Romeu et al. 2014; Nuñez-Lillo et al. 2015). Two genomic variations were found in this transcription factor that could explain the harvest date phenotype, a heterozygous InDel of 9 pb in the third NAC072 exon that segregates for this trait (Pirona et al. 2013) and a 26.6 kbp deletion that removes the entire NAC072 gene from the peach genome (Nuñez-Lillo et al. 2015). Genotyping of the NAC072 deletion in two peach populations and 18 peach varieties was published (Meneses et al. 2016), identifying a high correlation between the absence of NAC072 and the SR phenotype. Subsequently, a transcriptomic analysis between normal and slow ripening individuals was performed considering different stages of fruit development from 37 days after bloom (DAB) to harvest (120 DAB) identifying significant differences in key biosynthetic genes related to abscisic acid and gibberellin pathways in the early stages of fruit development probably regulated by NAC072 (Nuñez-Lillo et al. 2021). Despite all the evidence demonstrating the role of the transcription factor NAC072 in harvest date and slow ripening phenotypes, the regulatory mechanisms by which NAC072 produces these phenotypic changes are still unknown.

With the advances in high-throughput sequencing technologies and the growth in databases of transcription factors–target genes (TF-TG) interactions such as chromatin immunoprecipitation sequencing (ChIP-seq) and DNA affinity purification sequencing (DAP-seq), *in silico* gene regulatory networks have become a powerful tool that allows the identification of key transcription factors that regulate an organism reaction to changes in its environment (Karlebach and Shamir 2008). In this way, by analyzing the transcriptome of normal and slow ripening individuals in the early stages of fruit development, where the transcription factor NAC072 displays its expression peak, the aims of this work were (i) to identify the direct and indirect target genes of NAC072 through network analysis to understand the mechanisms by which the absence of the NAC072 gene produces the slow ripening phenotype and (ii) by using early-, middle-, and late-harvested peach varieties to correlate the expression differences in the NAC072 transcription factor and its target genes with the harvest date phenotype.

Materials and Methods

Plant Material

An F2 population with 151 siblings from the self-pollination of the nectarine cultivar 'Venus' [*Prunus persica* (L.) Batsch cv. 'Venus'] was used. This cultivar produces freestone melting yellow-fleshed nectarines and it was obtained from the intra-specific cross between 'Stark Red Gold' and 'Flamekist' at the INIA-Rayentué facilities (VI Region, Chile). The 'Venus' x 'Venus' population (VxV) consists of 7 year-old trees grown on GxN rootstock in an experimental orchard located at 34°19'S latitude and 70°50'W longitude (INIA-Rayentué). This population was obtained with the objective of studying fruit quality traits such as soluble solids content, mealiness, and harvest date in a research work already published by our group. In addition, derived from the previous research, 25 % of the individuals belonging to the V×V population displayed the slow ripening phenotype produced by a 26.6 kbp deletion in the genome of these individuals (Nuñez-Lillo et al. 2015). Previous analyses determined that the differences between normal and slow ripening phenotypes in the V×V population begin at 51 days after bloom (DAB) (Núñez-Lillo et al. 2021). In this sense, fruit mesocarp tissue of two siblings (one early ripening and one slow ripening individuals) were ground and frozen in liquid nitrogen at three fruit developmental stages defined at 37, 51, and 65 DAB (T1, T2, and T3, respectively) considering three biological replicates per developmental stage (three fruits randomly chosen for each biological replicate).

To evaluate expression levels of candidate genes, three contrasting varieties for harvest date phenotype were used to perform RT-qPCR validations. The early-harvested 'Rebus' variety (100 DAB), the middle-harvested 'Venus' variety (142 DAB), and the late-harvested 'Late Red Jim' variety (170 DAB) were evaluated. These cultivars produce yellow-flesh nectarines and consist of 7 year-old trees grown on "Nemaguard" rootstocks from the University of Chile Peach Improvement Program (Rinconada, Metropolitan Region, Chile) located at 33°30′ S latitude and 70°49′ W longitude. The studied sampling time points evaluated by RT-qPCR in these three varieties correspond to 30, 44, 65, 72, and 79 DAB (P1, P2, P3, P4, and P5, respectively).

Differential Expression Analysis Between Normal and Slow Ripening Individuals

Transcriptomic data files used in this work were previously published (Núñez-Lillo et al. 2021) and are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA730315. Total RNA was extracted from 100 mg of frozen fruit mesocarp using a Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Luis, USA) and evaluated according to the protocol described by Núñez-Lillo et al. (2021). RNA libraries were constructed from 1 µg of total RNA using the TruSeq® RNA Library Prep Kit v2 (Illumina Inc., San Diego, CA, USA) and sequenced in a HiSeq2500 with Macrogen's service in paired-end mode. The details of each sample sequenced used in this work, in addition to the number of sequenced, filtered, and aligned reads are shown in Table S1. Raw sequencing data files were evaluated with FASTQC software (Andrews 2010) and read quality trimming and filtering process was made using Flexbar v3.5.0 (Dodt et al. 2012). The STAR aligner software v2.7.10 (Dobin et al. 2013) was used to align filtered reads against *Prunus persica* reference genome v2.1 (Verde et al. 2013). For each library, the *featureCounts* function from the R package Rsubread v2.8.1 (Liao et al. 2019) was applied to assign expression values to each uniquely aligned fragment. A Principal Component Analysis (PCA) was performed using all expressed genes between normal and slow ripening individuals with the R package ggfortify v0.4.14 (Tang et al. 2016) with the *autoplot* function. Differential expression analysis was carried out using the R package edgeR v3.36.0 (Robinson et al. 2010) implementing a statistical method based on a generalized linear model (GLM) approach (McCarthy et al. 2012). Unlike the work of Núñez-Lillo et al. (2021), the differential expression analysis performed in this research work was not performed comparing each developmental stage independently, but rather both genotypes were compared using the three development points at the same time. Thus, through this analysis, only one list of differentially expressed genes between normal and slow ripening samples was obtained. Differentially expressed genes were plotted in a volcano plot using the R package EnhancedVolcano v1.14.0 (Blighe et al. 2022) using a $p\ value < 0.01$ and a $|\log_2 FC| > 1.0$ and scaled expression values were plotted in a color scale heatmap using the R package pheatmap v1.0.12.

Network Analysis

Network analyses were made using the ConnecTF platform (Brooks et al. 2021) available at https://connectf.org. ConnecTF is a database that compiles ChIP-seq and DAP-seq results containing information on more than 400 transcription factors and more than 3.5 million TF–TG interactions in *Arabidopsis thaliana*, and when it is used with gene expression information, it allows the identification of master regulatory transcription factors in specific processes together with their possible target genes, and even the interaction with secondary transcription factors and indirect targets

(Brooks et al. 2021). The list of differentially expressed genes was used as "Target Gene List" and "Filter TFs" to identify transcription factors candidates with DAP-seq information in the ConnecTF database for network construction. The *Target List Enrichment* tool was used to identify the significance of each transcription factor by comparing the target gene list and queried analyses to filter them considering only transcription factors with $p\ value < 0.01$. Additionally, the candidate transcription factors were filtered considering a minimum normalized expression of 5 counts per million mapped reads (CPM) to construct the network. Finally, network construction was made using Cytoscape software v3.9.1 (Shannon et al. 2003).

Expression profiles of candidate transcription factors were plotted with Prism v9.5.0 software (GraphPad Software Inc., San Diego, CA, USA) and used to classify and order the candidate transcription factors. Two-way ANOVA tests were used to identify significant differences between normal and slow ripening samples comparing biological replicate mean values for each developmental stage ($p\ value < 0.01$).

Candidate Gene Validation in Contrasting Peach Varieties for Harvest Date Phenotype by RT-qPCR

Total RNA was extracted from 100 mg of frozen fruit mesocarp using a Spectrum™ Plant Total RNA kit (Sigma-Aldrich) following the manufacturer's instructions and stored at – 80 °C. Extracted RNA was quantified with a Qubit™ 4.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using a Qubit™ RNA BR assay kit and RNA integrity was assessed by capillary electrophoresis using a Fragment Analyzer™ System (Agilent Technologies, Santa Clara, CA, USA) with the RNA kit DNF-471-0500 (15nt). The RNA quality number (RQN value) was used to identify RNA integrity. RNA samples with an RQN value beyond 7.0 were used for the following steps.

Transcript levels of four selected differentially expressed transcription factors (*PpeNAC072*, *PpeHB12*, *PpeHAT9*, and *PpeMYBR1*) were analyzed by RT-qPCR in three contrasting varieties for harvest date phenotype ('Rebus', 'Venus,' and 'Late Red Jim') and five fruit developmental points (P1, P2, P3, P4, and P5). For cDNA synthesis, 1 µg of total RNA was first treated with DNase I (Thermo Fisher Scientific) and the Superscript First-Strand Synthesis for RT-PCR (Thermo Fisher Scientific) was used according to the manufacturer's instructions. RT-qPCR amplification reactions were performed in a total volume of 10 µL. The reaction mixture contained 1 µL of templated cDNA, 0.25 mM primers (0.25 µL Forward, 0.25 µL Reverse), 0.2 µL ROX, 3.3 µl nuclease-free water, and 5 µL SYBR Green PCR intercalating dye (Roche® Life Science, Basel, Switzerland) as a fluorescent indicator. Reactions were performed using three biological and two technical replicates for each evaluation point in the AriaMx Real-time PCR System (Agilent Technologies). Relative expression was calculated using the housekeeping *PpeRPII* as reference gene (Tong et al. 2009). Data were plotted and analyzed in Prism v9.5.0 (GraphPad Software Inc). Statistical analyses were performed using a two-way ANOVA test considering the three peach varieties and five evaluation points as factors. Significant differences were identified comparing replicate mean values between varieties for each evaluation point ($p\ value < 0.01$).

Results

Differential Expression Analysis

Transcriptome data were assessed in a principal component analysis (PCA) using all expressed genes (Fig. 1A). With components 1 and 2 it was possible to separate the different fruit developmental stages (T1, T2, and T3) of both genotypes with 85.00 % and 11.64 % of explained variance, respectively. While with 1.60 % explained variance, component 3 separated normal from slow ripening samples. No large variations were observed between replicates of each condition. To identify all genes with differences between normal and slow ripening genotypes in any of the stages of fruit development, a differential expression analysis was performed with a statistical method based on the generalized linear model (GLM) approach. Detailed information about differentially expressed genes is shown in Table S2. Figure 1B shows a volcano plot with the differentially expressed genes between normal and slow ripening samples. A total of 1055 DEG were identified with a $p\ value < 0.01$ and $|\log_2 FC| > 1.0$; 645 of them with higher expression in normal ripening samples and 410 with higher expression in slow ripening samples. Normalized expression values of all differentially expressed genes were plotted in a heatmap in which clear differences can be observed between the sequenced samples of the normal and slow ripening phenotypes (Fig. 1C). In addition, as the slow ripening phenotype is characterized by the absence of the transcription factor NAC072 throughout fruit development, significant differences are even observed from T1, however, the most significant changes are found at T2, which corresponds to the NAC072 expression peak in the normal ripening samples.

Candidate Transcription Factors

Of the total 1055 differentially expressed genes between normal and slow ripening samples, 808 presented an *Arabidopsis thaliana* ortholog to perform a network analysis, among which 15 transcription factors were identified with DAP-seq information in the ConnecTF database including the transcription factor NAC072. Candidate transcription factors

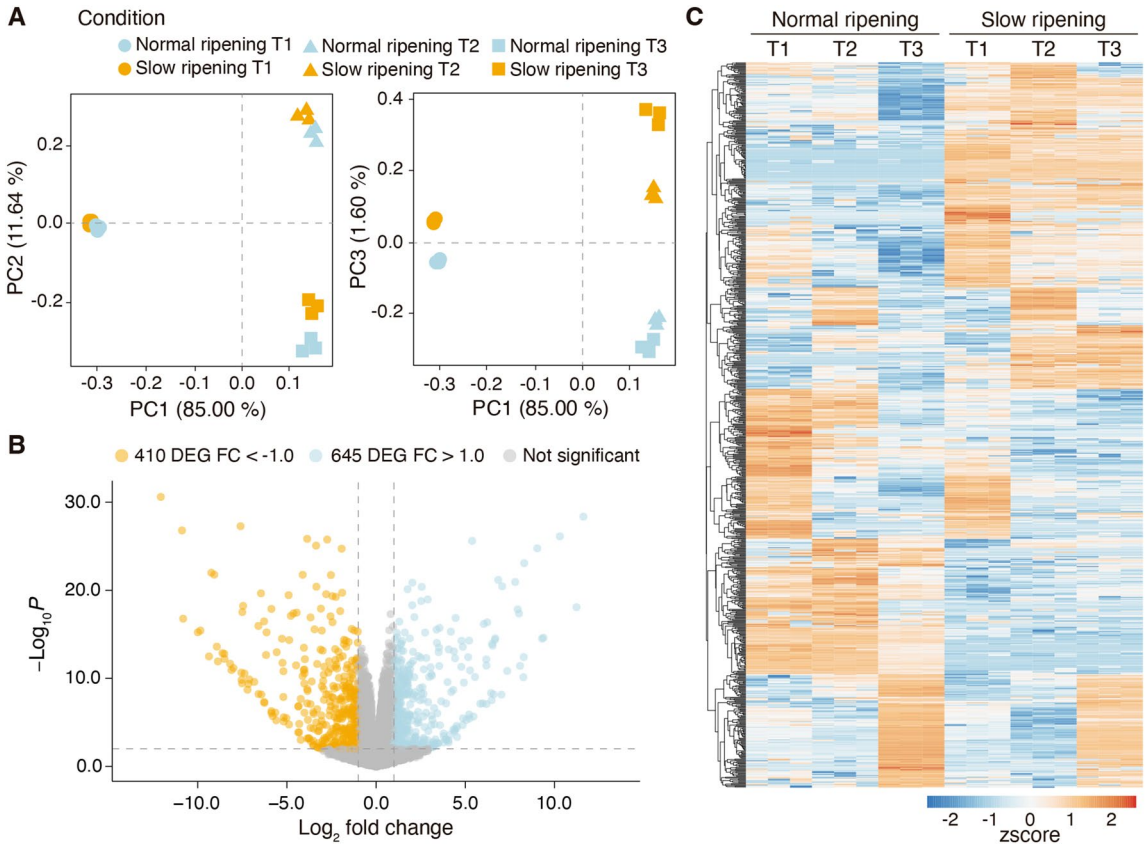

Fig. 1 Differential expression analysis between normal and slow ripening samples. **A** Principal component analysis using normalized counts of each sequenced library. Three biological replicates for each developmental stage were plotted. Normal and slow ripening samples are represented by light blue and orange symbols, respectively. The fruit developmental stages T1, T2, and T3 are represented by circles, triangles, and squares, respectively. **B** The differential expressed genes obtained comparing normal and slow ripening samples using a generalized linear model (GLM) approach were represented in a volcano plot. Candidate genes were selected with a *p value* < 0.01 and $|\log_2 FC| > 1.0$. **C** Differentially expressed genes between normal and slow ripening phenotypes are plotted in a blue-orange scale heatmap with a 'ward.D2' clustering method. Expression values are scaled considering the mean centered divided by standard deviation. Each column represents the expression of three replicates for each individual selected

with *Prunus persica* and *Arabidopsis thaliana* geneID and gene name are listed in Table 1. The influence of each transcription factor (calculated as the percent of target genes from the total list of differentially expressed genes) corresponded to NAC072 (502 targets; 62.13 %), followed by HB12 (475 targets; 58.79 %), MYBR1 (440 targets; 54.46 %), HAT9 (382 targets; 47.28 %), AZF3 (370 targets; 45.79 %), NAC043 (322 targets; 39.85 %), WRKY28 (322 targets; 39.85 %), CBF4 (305 targets; 37.75 %), MYB73 (256 targets; 31.68 %), HHO3 (247 targets; 30.57 %), ERF017 (236 targets; 29.21 %), WRKY40 (207 targets; 25.62 %), RRTF1 (149 targets; 18.44 %), RAP2.11 (109 targets; 13.49 %), and LOB (59 targets; 7.30 %).

The expression patterns of each transcription factor are shown in Fig. 2. In addition to NAC072, five other transcription factors (HB12, HAT9, HHO3, NAC043, and WRKY28) showed higher expression levels in normal ripening fruit. The other nine transcription factors (MYBR1, WRKY18, MYB73, AZF3, CBF4, ERF17, RRTF1, LOB, and RAP2.11) increased their expression in the absence of NAC072.

Of the transcription factors with higher expression in normal ripening individuals, HB12 and HAT9 showed significant differences from T2, thus, they were considered as early-regulated transcription factors and possibly with a NAC072 more direct regulation, while HHO3, NAC043, and WRKY28 only showed significant differences in T3 and were considered late-regulated transcription factors. On the other hand, of the transcription factors with higher expression in slow ripening samples, only RAP2.11 did not present significant differences at T2 (Fig. 2), thus, being the only one considered as a late-regulated transcription factor.

Table 1 Slow ripening candidate transcription factors and their orthologs in *Arabidopsis thaliana* for gene regulatory network construction

Ppersica ID	*Ppersica* Description	*Athaliana* ID	*Athaliana* Description	Score	Similarity (%)	Symbol
Prupe.4G186800	NAC domain-containing protein 72	AT4G27410	NAC domain-containing prtein	372	83.5	NAC072
Prupe.2G194400	Homeobox-leucine zipper ATHB-12	AT2G46680	Homeobox 7	206	80.6	HB12
Prupe.6G213800	Homeobox-leucine zipper HAT9	AT4G37790	Homeobox-leucine zipper	296	73.7	HAT9
Prupe.1G306300	MYB family transcription factor	AT1G25550	MYB-like transcription factor	322	71.2	HHO3
Prupe.2G196600	NAC domain-containing protein 43	AT2G46770	NAC domain-containing protein	405	68.4	NAC043
Prupe.4G075400	WRKY transcription facor 28-related	AT4G18170	WRKY DNA-binding protein 28	205	61.9	WRKY28
Prupe.1G441700	MYB -like DNA-binding domain	AT5G67300	MYB domain protein R1	189	67.9	MYBR1
Prupe.1G393000	WRKY transcription factor 18-related	AT1G80840	WRKY DNA-binding protein 40	228	66.0	WRKY18
Prupe.8G134900	MYB transcription factor	AT4G37260	MYB domain protein 73	263	69.1	MYB73
Prupe.1G424300	Zinc finger protein AZF3-related	AT1G27730	Salt tolerance zinc finger	191	60.2	AZF3
Prupe.2G289500	Dehydration-responsive element	AT5G51990	C-repeat binding factor 4	234	78.0	CBF4
Prupe.7G194400	Ethylene-responsive transcription factor	AT1G19210	Integrase-type DNA-binding	187	66.2	ERF017
Prupe.8G125100	AP2 domain	AT4G34410	Redox responsive transcription factor	130	68.8	RRTF1
Prupe.5G167800	Protein lateral organ boundaries	AT5G63090	Lateral organ boundaries domain	239	81.2	LOB
Prupe.5G213800	SHN Shine, DNA binding	AT5G19790	Related to AP2 11	102	85.7	RAP2.11

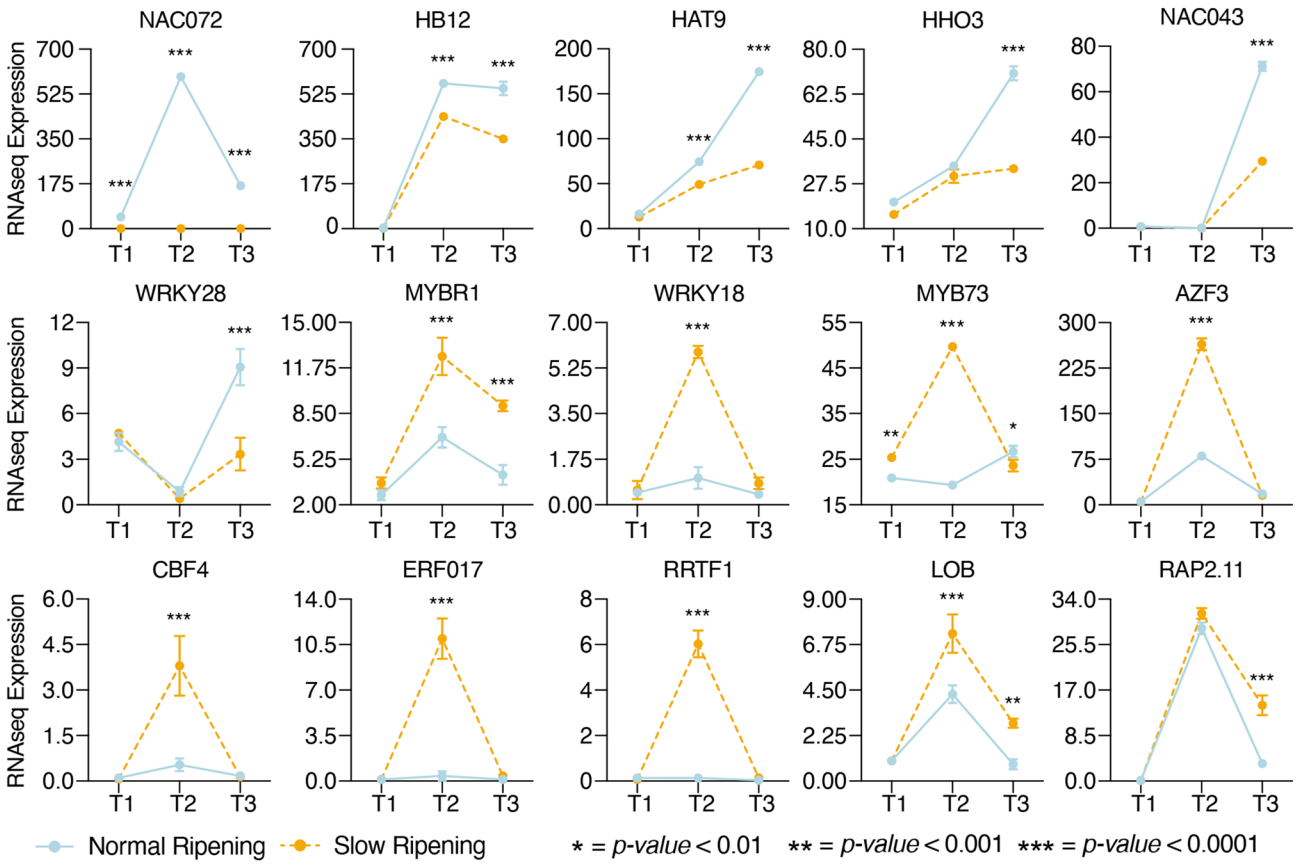

Fig. 2 RNAseq normalized expression patterns of candidate transcription factors for slow ripening regulation control. Normalized expression data of 15 transcription factors were plotted. Normal and slow ripening expression values are represented by light blue continuous lines and orange dashed lines, respectively. Two-way ANOVA test comparing the mean expression values of three biological replicates in each fruit development stage between normal and slow ripening samples are represented by asterisk

Transcription Factor Regulatory Network

Considering the early and late transcription factor regulations determined in Fig. 2 and the target genes of each transcription factor obtained from the ConnecTF database, a regulatory network was constructed with the 15 candidate transcription factors for peach ripening control (Fig. 3). According to the ConnecTF database, the NAC072 transcription factor has binding sites in 502 of the differentially expressed genes, within this group are included the transcription factors HB12, WRKY28, HHO3, CBF4, AZF3, MYB73, MYBR1, RRTF1, ERF017, WRKY18, and RAP2.11. Thus, as candidate transcription factors of the normal ripening phenotype, HB12 was selected as the only transcription factor early and directly regulated by NAC072, while HAT9, which also presents early regulation, was determined to be indirectly regulated through the transcription factor HB12. On the other hand, the late-regulated transcription factors NAC043, WRKY28, and HHO3 could be regulated by NAC072, HB12 or HAT9, or even by the interaction between them (Fig. 3, lower side). For transcription factors with higher expression on slow ripening samples, seven transcription factors were selected as direct targets of NAC072 with early regulation, CBF4, AZF3, MYB73, MYBR1, RRTF1, ERF017, and WRKY18, while LOB transcription factor was classified as early but indirectly regulated by NAC072 through WRKY18 and RAP2.11 as late-regulated transcription factor through CBF4 (Fig. 3, upper side).

In addition to TF–TG interaction information, the ConnecTF database contains induction and repression gene regulation information, this regulatory data was used to understand the complex interactions that might exist among the different selected transcription factors. Figure 3 shows the induction (green lines) and repression (red lines) regulations of the transcription factors HB12, HAT9, HHO3, and MYBR1 on their targets. The transcription factor HB12 induces the expression of its target HAT9, which in turn represses the expression of HB12 in a negative feedback. On the other hand, HAT9 also has the ability to induce the expression of the transcription factor HHO3, which in a second negative feedback can inhibit the expression of its predecessors NAC072 and HB12. In addition, HB12 and HAT9 can inhibit the expression of the transcription factors AZF3 and MYBR1 that increase their expression in the absence of the NAC072 gene. Finally, the transcription factor MYBR1 can induce the expression of NAC072 and in turn inhibit the expression of MYB73, WRKY18, and WRKY28. These results suggest that there is a very fine temporal regulation between these transcription factors to maintain a normal and correct fruit developmental process.

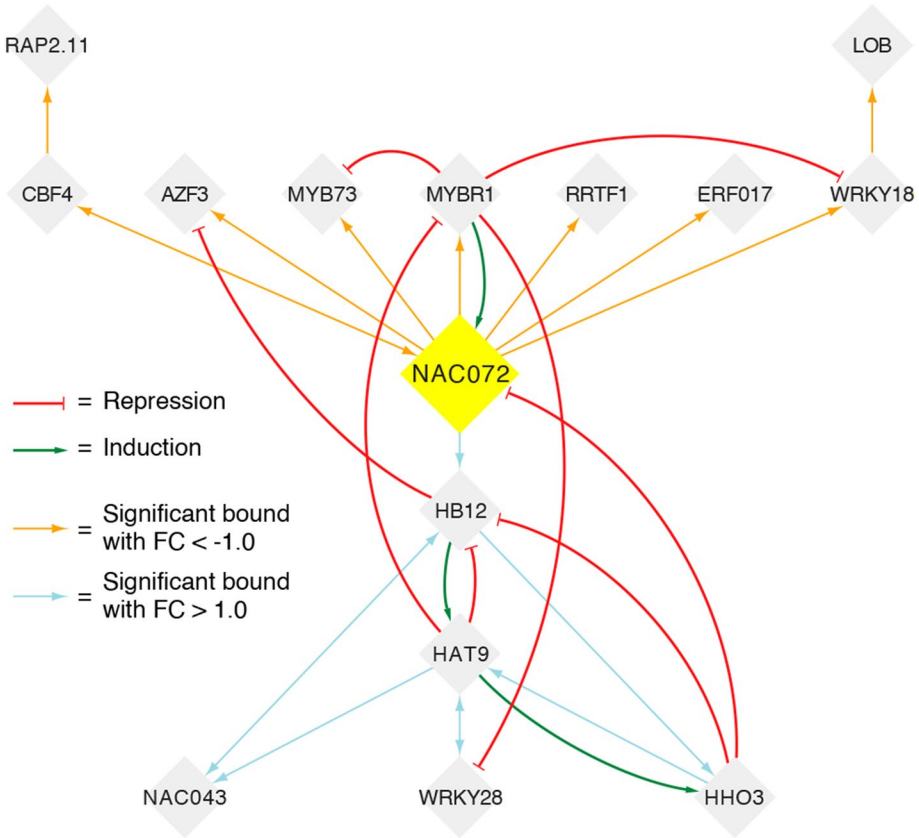

Fig. 3 Network analysis of candidate transcription factors associated with NAC072. Slow ripening regulatory network construction with 15 candidate transcription factors. Each node represents each transcription factor and each edge represents a TF–TG interaction. Red and green colored edges represent a ConnecTF database of TF-regulatory information of repression and induction, respectively. Orange and light blue colored edges represent a DAP-seq physical TF–TG interaction. Genes with higher expression in normal ripening individuals are represented by light blue colored edges, while genes with higher expression in slow ripening individuals are represented by orange colored edges (Color figure online)

NAC072 Regulatory Network

To construct the regulatory network associated with the transcription factor NAC072, all differentially expressed genes targeted by any of the candidate transcription factors in Fig. 3 were classified into early (T2) and late (T3) response genes. This classification was made considering the stage of fruit development in which each gene presented their expression peak with significant differences between normal and slow ripening samples. Differentially expressed gene classification together with the target gene list information of each transcription factor obtained from the ConnecTF database allowed us to order and build the regulatory network shown in Fig. 4. Thus, nodes directly connected to NAC072 in Fig. 4 are considered early response genes with the higher expression in T2, while all other nodes are considered late response genes with their expression peak at T3. Detailed information of all nodes and edges of Fig. 4 is shown in Table S3.

Under normal fruit development conditions (Fig. 4, light blue edges), NAC072 can directly and early regulate genes associated with the abscisic acid biosynthesis (NCED3), gibberellin biosynthetic pathway (GA3 and GA2ox4), and auxin-related genes (SAUR), while with an indirect and later regulation through its target transcription factors, an overrepresentation of genes associated with ethylene biosynthesis (SAM and ACO5), sugar metabolism (STP1, SWEET17, and SPS1F), auxins (AIR, AUX, IAA11, and IAA31), genes associated with RALF-FER signaling (RALF24, RALF33, RIC4, and ROPGAP3), and phenylpropanoids biosynthesis (PAL1, C4H, 4CL2, HCT, FAH1, FAE, and CCR1) were found. In addition, 37 genes with cell wall remodeling functions were identified, among which cellulose synthases (CESA4, IRX1, and IRX3), glucuronosyl-, galacturonosyl-, and xylosyl-transferases (FRA8, GATL1, GATL2, GAUT12, GXM2, GXM3, IRX9, IRX14, PGSIP2, and XYLT), arabinogalactan proteins (AGP19, FLA7, FLA11, and FLA12), and nucleotide-sugar biosynthesis-related genes (UGD3, GAE3, and UXS6) stood out. On the other hand, among the genes that increased their expression in slow ripening fruits (Fig. 4, orange edges), one sugar transporter (SWEET12), two genes related to terpene biosynthesis (AFS1) and five genes with functions associated with cell wall remodeling (GME, GALT10, AGP16, EXPB2, and PME) were identified. However, most of these genes displayed very low normalized expression levels compared to normal ripening identified genes.

Candidate Gene Validation by RT-qPCR in Peach Varieties with Contrasting Harvest Dates

In order to identify the correlation of the slow ripening candidate transcription factors in the regulation of harvest date phenotype, four transcription factors including NAC072 were measured by RT-qPCR in three peach varieties with contrasting harvest dates at five sampling points distributed between the early peach fruit development stages S1 and S2 (primer information of candidate genes is shown in Table S4). Figure 5 shows the expression patterns identified for the selected transcription factors (NAC072, HB12, MYBR1, and HAT9). The transcription factor NAC072 presented significant differences at P2 and P5 with higher expression levels in the early variety 'Rebus' and no significant differences were identified between the middle- and late-harvested varieties. In the case of the transcription factor HB12, higher expression was also observed in the early variety, but only with significant differences at P5. In both cases, for NAC072 and HB12 at point P5, an inverse correlation was observed because the late-harvested genotype 'Late Red Jim' with the longest harvest date displayed the lowest level of expression on both transcription factors, while the early-harvested genotype 'Rebus' with the shortest harvest date presented the highest expression levels. On the other hand, in the case of MYBR1, significant expression differences were identified at P3, immediately after the first NAC072 expression peak, presenting the higher value in the late-harvested variety, while HAT9 presented a similar MYBR1 expression pattern, but with its peak of expression at P4. Therefore, a direct correlation was observed between the harvest date and the expression of MYBR1 in P3 and HAT9 in P4 because the late harvest genotype 'Late Red Jim' presented a higher expression of these transcription factors and the early harvest genotype 'Rebus' presented the lowest expression values.

Discussion

NAC072 Hormonal Regulation in Early Stages of Fruit Development

The results obtained through the NAC072 network analysis allow us to associate this transcription factor in the hormonal regulation of abscisic acid (ABA) and gibberellic acid (GA) through the direct regulation of the nine-cis-epoxycarotenoid dioxygenase 3 (NCED3), the ent-kaurene oxidase (GA3), and the gibberellin 2-β-dioxygenase 4 (GA2ox4) genes (Fig. 4).

In the case of ABA, a phytohormone synthesized from β-carotene characterized by being involved in stomatal closure, abiotic signaling, seed dormancy and has also been described as having a key role promoting fruit ripening related to fruit softening and skin color changes (Kuhn et al. 2021a), a high correlation between NCED expression levels and ABA content has been reported, being a key gene for this process (Frey et al. 2012; Gavassi et al. 2021).

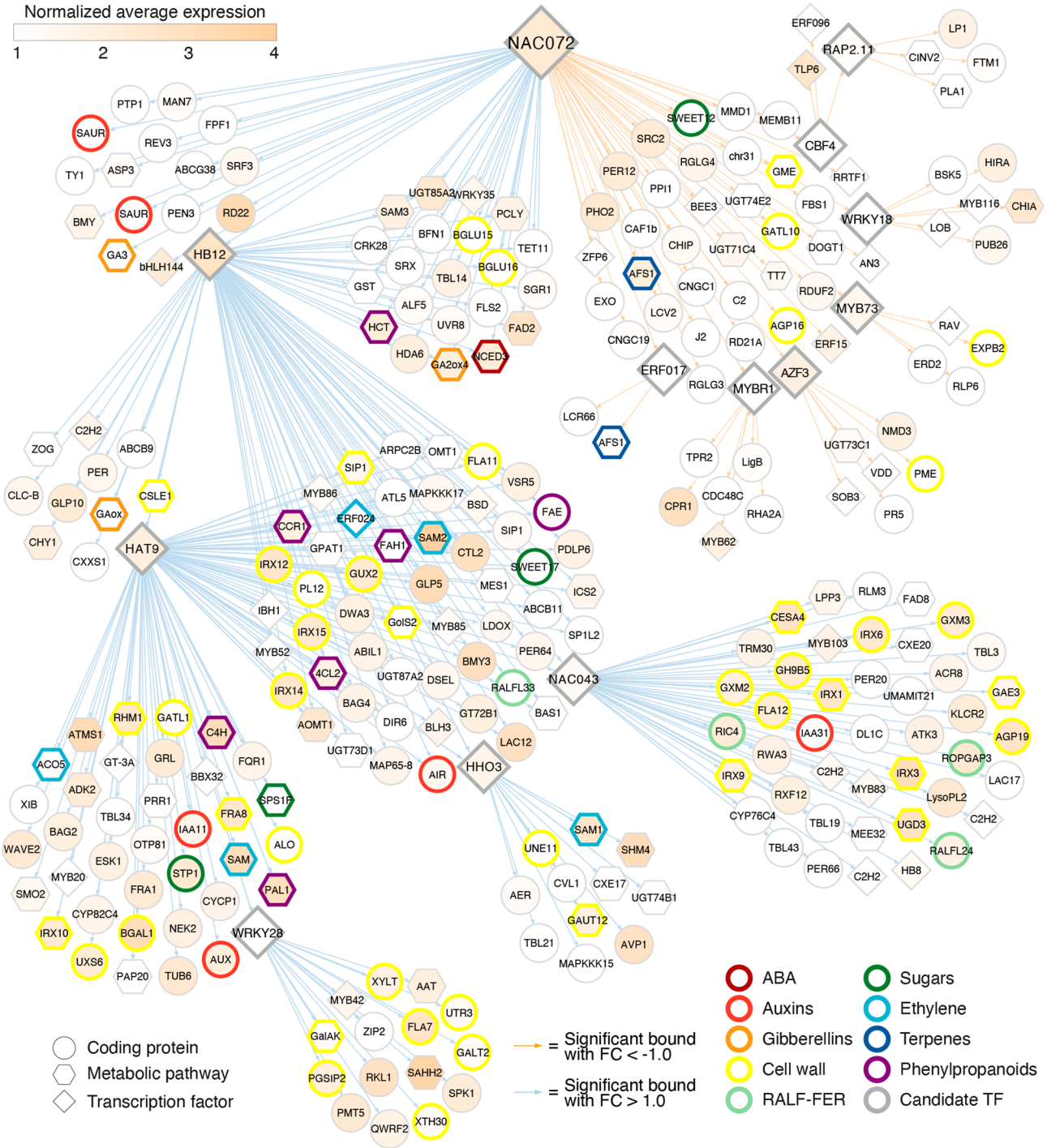

Fig. 4 Slow ripening regulatory network. Representation of most informative genes associated with slow ripening regulatory network. Each node represents a differentially expressed gene and each edge represents a DAP-seq gene association. The length of the edges was adjusted to represent the network in an ordered way, short or long edges do not represent differences between nodes. Orange scaled colored nodes correspond to normalized expression values between normal and slow ripening comparison. Genes with higher expression in normal ripening individuals are represented by light blue colored edges and genes with higher expression in slow ripening individuals are represented by orange colored edges. Nodes with colored border correspond to genes associated with metabolic pathways or signaling (Color figure online)

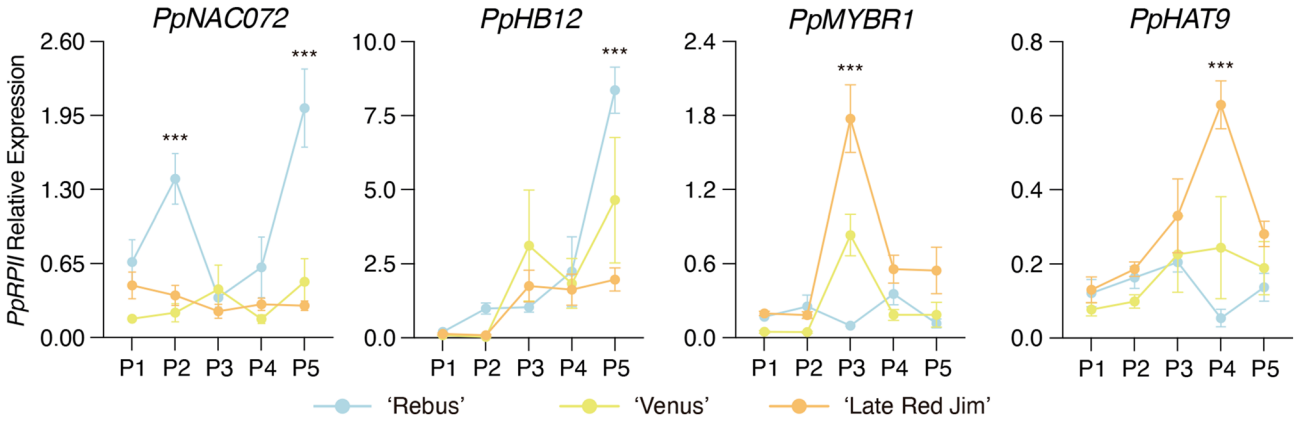

Fig. 5 Candidate gene validation in peach varieties with contrasting harvest date phenotype. Three candidate transcription factors and NAC072 were selected and validated in three peach varieties with contrasting harvest date phenotype. The early-harvested variety 'Rebus' (light blue lines), the middle-harvested variety 'Venus' (yellow lines), and the late-harvested variety 'Late Red Jim' (orange lines) were selected and used to validate candidate transcription factors in five fruit developmental points P1, P2, P3, P4, and P5. The Y-axis shows the RT-qPCR relative expression values normalized to the *PpeRPII* housekeeping gene. Two-way ANOVA test comparing the mean expression values of three biological and two technical replicates between varieties for each developmental point (***; *p value* < 0.0001) (Color figure online)

As shown in Fig. 4, higher expression of one NCED gene (NCED3) was associated with normal ripening samples in early stages of fruit development, directly regulated by the transcription factors NAC072 and HB12, suggesting that the absence of NAC072 produces an ABA deficiency in slow ripening individuals. On the other hand, like NCED3, two other ABA-related genes showed significant expression differences but did not present binding sites associated with any of the candidate transcription factors (Table S2), one phytoene synthase (PSY) and one xanthoxin dehydrogenase (ABA2), probably directly regulated by increased ABA levels reinforcing the hypothesis that there is higher ABA content in the early fruit developmental stages of normal ripening individuals.

On the other hand, GA are acidic diterpenoids and bioactive growth regulators involved in developmental process like seed germination, stem elongation, leaf expansion and trichome, flower and fruit development (Olszewski et al. 2002). GA is biosynthesized from geranylgeranyl diphosphate, a precursor of β-carotenes, and has been described as an ABA antagonist in fruit development negatively regulating ABA biosynthesis, perception, and signal transduction (Kuhn et al. 2020). Two gibberellin-related genes were found in the NAC072 regulatory network (Fig. 4) with significant differences between normal and slow ripening samples at T2 directly regulated by NAC072, one *ent*-kaurene oxidase (GA3) and one gibberellin 2-β-dioxygenase 4 (GA2ox4). On the one hand, GA3 is associated with GA biosynthesis, while GA2ox4 inhibits the GA signaling pathway by inactivating bioactive GA (Gupta and Chakrabarty 2013). These results do not allow us to determine if there is a greater biosynthesis of GA as a result of the absence of NAC072, but it is possible that the activation of the GA pathway functions as a regulator of ABA levels, however, more analyses are necessary to determine the role of GA in the early fruit development of peach trees.

NAC072 Indirect Regulation of Phenylpropanoid- and Cell Wall-Related Genes

Phenylpropanoids are organic compounds biosynthesized from shikimate pathway intermediates and are the basis for the production of a large number of secondary metabolites with diverse functions (Vogt 2010). They are a fundamental part of the plant response to biotic and abiotic stimuli and contribute substantially to plant stability and robustness against mechanical damage (La Camera et al. 2004). Seven genes related to phenylpropanoid pathway probably regulated by HB12 and HAT9 indirectly of NAC072 were found associated with normal ripening samples (Fig. 4). Among which are one phenylalanine ammonia-lyase 1 (PAL1), one cinnamate-4-hydroxylase (C4H), and two 4-coumarate:CoA ligase 2 (4CL2) associated with the first part of phenylpropanoid pathway necessary for the flavonoid, stilbenoid, and lignin biosynthesis, but also, we identify one hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), one ferulic acid 5-hydroxylase 1 (FAH1), and one cinnamoyl-CoA reductase 1 (CCR1), reported as essential genes in the lignin biosynthetic process (Zhang et al. 2016; Su et al. 2019), suggesting that the phenylpropanoid pathway in the early stages of fruit development tends to lignin biosynthesis, but it could also be the start of flavonoid biosynthesis in later stages of development, contributing to fruit color change in normal ripening samples.

On the other hand, as shown in Fig. 4, a marked gene enrichment associated with cell wall remodeling functions was identified in normal ripening compared to slow ripening fruits, genes with galactosidase, glucosidase, and expansin activity stood out for having been reported as fruit softening-related genes (Brummel et al., 2004; Hayama et al. 2006), while genes with cellulose synthase and transferase activity, arabinogalactan proteins, and nucleotide-sugar biosynthesis-related genes could be considered as new factors involved in the control of softening in early stages of peach fruit development. In addition, in tomato, pectate lyase has been described as a major contributing factor to fruit softening (Wang et al. 2018), and four pectate lyase genes with higher expression in normal ripening samples were identified. These results suggest that the absence of NAC072 in could be avoiding the softening process on slow ripening fruits.

Slow Ripening Candidate Transcription Factors, Direct Targets of NAC072

Two transcription factors with differential expression at T2 stage with higher levels of expression in normal ripening fruits (Fig. 2) were considered close NAC072 candidates of transcription factors associated with the correct fruit development in peach trees (HB12 and HAT9). On the one hand, the homeodomain-leucine zipper HB12 has been reported in *Arabidopsis thaliana* as a transcription factor inducible for water deficit, osmotic stress, and ABA exogenous applications (Söderman et al. 1994), and has also been described as a plant development and growth modulator that respond to abiotic stress (Ré et al. 2014) and there is no information about the effect of HB12 on fruit development, while the homeobox-leucine zipper HAT9 has been reported as drought response transcription factor (Su et al. 2013) induced by abscisic acid (Liu et al. 2016) and cytokinin signaling (Köllmer et al. 2011) in *Arabidopsis thaliana*. Also, the overexpression of HAT9 has been shown to present similar effects as treatments with exogenous ABA applications (Liu et al. 2016). On the other hand, the knockout of HAT9 ortholog in tomato (*SlABIG1*; Solyc02G091930.3.1) exhibits enhanced salinity tolerance, with higher chlorophyll content and photosynthetic capacity, root dry weight, and proline, also decreases the accumulation of ROS, malondialdehyde, and Na^+ (Ding et al. 2022). The results obtained in the network analysis (Fig. 4) together with the information obtained in literature suggest that NAC072, HB12, and HAT9 could by directly associated to ABA biosynthesis through NCED3, but it is also possible that the increase in ABA content controls the expression levels of HB12 and HAT9 indirectly of NAC072, however, regardless of whether it is induced directly or indirectly by NAC072, the results obtained suggest that HAT9 acts downstream of NAC072, HB12, or ABA signaling and could have a key role mediating the crosstalk between NAC072 and phenylpropanoid and flavonoid biosynthesis, the beginning of fruit softening process and sugar transport (Fig. 4). Furthermore, Fig. 3 shows the positive regulation of HAT9 on its target gene HHO3, both inhibiting the expression of these predecessors NAC072 and HB12 in a negative feedback, suggesting that HAT9 could be a key factor in the temporary regulation of the correct fruit development in peach trees.

On the other hand, the transcription factor MYBR1 also called MYB44 was selected as a candidate transcription factor among the nine transcription factors that increased their expression in the absence of NAC072 because it presents transcriptional regulation information in the ConnecTF database (Fig. 3). It has been reported that MYBR1 induces the expression of NAC072, and that transcription factors downstream of NAC072, such as HAT9, inhibit the expression of MYBR1, thereby in turn inhibiting the expression of NAC072. On the other hand, MYBR1 inhibits the expression of two other transcription factors that increase their expression in the absence of NAC072, MYB73, and WRKY18, which suggests that it could be a key transcription factor in the fine temporal regulatory network of NAC072.

The participation of the transcription factor MYBR1 in seed germination and abiotic stress has been described in *Arabidopsis thaliana*, being controlled by the MAPK signaling pathway through MPK3, MPK6, and MPKK4 (Nguyen et al. 2012; Persak and Pitzscheke 2013). It has also been reported that it is a negative regulator of leaf wounding response and delaying senescence, maintaining growth processes in the event of physical damage or stress, and inhibiting ABA signaling by interacting with its receptors PYL8 and PYL9 (Jaradat et al. 2013; Li et al. 2014). On the other hand, in purple-fleshed sweet potato it has been reported that MYBR1 has an inhibitory effect on anthocyanin biosynthesis by preventing the formation of the anthocyanin regulatory complex *IbMYB340-IbbHLH2-IbNAC56* (Wei et al. 2020), while in papaya, Fu et al. (2020) found that MYBR1 was a transcriptional repressor of softening and carotenoid accumulation by regulating cell wall remodeling and carotenoid biosynthesis-related genes. Finally, comparing transcriptional responses to exogenous GA_3 applications in early and midseason sweet cherry varieties, Kuhn et al. (2021b) suggest that MYBR1 expression variations may be a mechanism by which GA antagonizes the ABA signaling pathway. With all these results and background information, MYBR1 was selected as one of the genes that could be key regulators in the NAC072 transcriptional network, affecting both the slow ripening and the harvest date phenotypes in peach trees.

Relationship Between Slow Ripening Transcription Factors and Harvest Date Phenotype

The expression pattern identified for NAC072 in Fig. 5 shows two peaks of expression in the early variety 'Rebus,' which agrees with the expression pattern published by Nuñez-Lillo et al. (2021), where an individual from a segregating peach population classified as early harvest was used. On the other hand, despite a NAC072 expression difference between the middle- and late-harvested varieties is expected, there were no significant differences in the RT-qPCR analysis, possibly because the expression peak of NAC072 could temporarily be delayed between varieties with different fruit harvest dates and maybe it is necessary to analyze intermediate evaluation points to those described in this work. Regarding the results obtained for the transcription factor HB12, it is observed that in P2, despite not being significant, there is an increase in HB12 expression levels in the early-harvested variety, which accompanies the peak expression of NAC072, and which could be accentuated at an intermediate evaluation point between P2 and P3. The expression patterns of NAC072 and HB12 obtained by RT-qPCR in 'Rebus' variety have a Pearson correlation of 0.79 (Fig. S1) reinforcing the hypothesis that HB12 could be a possible direct target transcription factor of NAC072, also considering the similarities at P5 between both transcription factors.

The transcription factor MYBR1 shows an expression peak at P3 (Fig. 5) immediately after the peak of NAC072, which agrees with the transcriptional regulation model proposed in Fig. 3, where MYBR1 has a NAC072 binding site in its promoter that represses it (according to the expression values of Fig. 2). In turn, according to the information obtained from the ConnecTF database, MYBR1 is able to induce the expression of NAC072, acting as regulatory factors for each other. In this sense, both transcription factors had opposite expression patterns, NAC072 decreases its expression levels when MYBR1 expression levels increase and vice versa. Also, while high expression levels of NAC072 are associated with the early harvest date, high levels of MYBR1 are associated with late harvest. All these results turn MYBR1 as an excellent candidate for the study of fruit development and NAC072 regulation.

Finally, as expected, the peak expression of HAT9 appears later in fruit development (P4). It is probable that HAT9 was not a direct target transcription factor of NAC072 (Fig. 3), but unlike what was expected, HAT9 presented an expression pattern similar to MYBR1, with a high correlation to late-harvested fruit, so further analyses are still necessary to determine the regulation mechanisms of this model. However, as shown in Fig. 3, HAT9 could have a regulatory role in the expression of other transcription factors, for example inhibiting the expression of HB12 and MYBR1 (Brooks et al. 2019). Considering that the HAT9 expression peak was found at P4 just between the increase of MYBR1 expression (P3) and the increase of NAC072 and HB12 expression (P5), we do not rule out the possibility that HAT9 could be part of the fine transcriptional regulation of NAC072 associated with peach fruit development.

Conclusions

Using a transcriptomic analysis between slow ripening and normal fruit samples at the early developmental stages S1 and S2 in combination with transcription factor–target gene interaction data, 14 transcription factors were identified associated with the absence of the transcription factor NAC072. Using these transcription factors, a temporal regulatory network was built that could be regulating fruit development in peach trees with NAC072. In addition, the transcription factors HB12, MYBR1, and HAT9 were further evaluated in addition to NAC072 by RT-qPCR in contrasting peach varieties for the harvest date phenotype to correlate them to fruit development. In this way, the four transcription factors show expression differences between early, middle, and late peach varieties at different development points, reinforcing the hypothesis that NAC072 regulates these transcription factors in the modulation of harvest date phenotype. However, even though HB12, MYBR1, and HAT9 seem to be part of this NAC072 temporal regulatory network, the proposed analysis was built with *Arabidopsis thaliana* transcription factor–genes interaction information and does not have DNA binding sites validations in the peach genome, so further research is still necessary in a fruit model to determine how NAC072 interacts with its possible target genes HB12, MYBR1, and HAT9 regulating fruit development and the harvest date phenotype.

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Declarations

Conflict of interest The authors declare no conflict of interest

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