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Identification of a conserved set of cytokinin-responsive genes expressed in the fruits of *Prunus persica*

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Abstract

Ethylene plays an essential role in the ripening of peach, a climacteric stone fruit. However, several studies have shown that other phytohormones may alter fruit organoleptic properties, nutritional value, and overall quality. Although cytokinin levels are highest during fruit set and early stages of fruit development, exogenous cytokinin application on the late stages of peach fruit development (post-lignification stage) alters the peach fruit ripening indicators and delays ripening. To better understand the molecular mechanisms by which cytokinin alters fruit ripening and fruit quality traits, we identified a conserved set of 26 peach cytokinin-regulated orthologs of the Arabidopsis cytokinin-regulated genes. All but two of these genes show expression in ripe peach fruits. The peach cytokinin-regulated genes map throughout the *Prunus persica* genome, located on all eight chromosome-scale pseudomolecules. However, eight of these orthologs are located on pseudomolecule 1, while five orthologs are on pseudomolecule 2. Gene Ontology enrichment analyses revealed that many of these genes are associated with metabolic processes. The region upstream of the transcription start site contained Cytokinin Response Elements (CKREs) in 77% (20/26) of these putative cytokinin-regulated genes. The cytokinin responsiveness of eight of these genes (*PpeBPH1, PpeCKX4, PpeCPK28, PpeCYP18-2, PpeHSP90-7, PpeNRP2, PpeNIP1-1,* and *PpePCRP1*) was confirmed by RT-qPCR analyses of ripe peach fruits treated exogenously with trans-zeatin for one hour. Therefore, by using a comparative genomic analysis between peach and Arabidopsis, we have identified a conserved set of cytokinin-regulated genes expressed in peach fruits that respond to exogenous application of trans-zeatin and, based upon the conserved cis-regulatory regions, are most likely regulated by the cytokinin Type-B Response Regulators.

Keywords Peach · Cytokinin-regulated genes · Trans-zeatin · Fruit · Peach transcriptome · Peach genome

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Introduction

Phytohormones and the cross-talk among these phytohormones participate actively in the temporal and spatial growth and differentiation during fruit development. Hormone profiling for the phytohormones auxins, cytokinins, and gibberellins have revealed elevated endogenous levels of these phytohormones at early stages of fruit development in fruits such as peach, strawberry, sweet cherry and tomato (Mariotti et al. 2011; McAtee et al. 2013; Kumar et al. 2014; Teribia et al. 2016). At later stages of fruit development, the endogenous levels of these phytohormones decrease while other phytohormones such as ethylene and abscisic acid increase in both climacteric and non-climacteric fruits (McAtee et al. 2013; Teribia et al. 2016).

Among the phytohormones that are associated with fruit development and ripening, the least characterized at the molecular level is cytokinin. Studies in tomato have

demonstrated that at the early stages of fruit development when there is active cell division, endogenous cytokinin levels are elevated. These levels of cytokinin decrease as the fruit matures and ripens (McAtee et al. 2013; Kumar et al. 2014). However, analyses of the endogenous cytokinin levels in the ripening tomato mutant, *rin* (ripening inhibitor), suggests that this hormone may play a role in fruit ripening. Furthermore, exogenous application of the cytokinin *N*-(2-Chloro-4-pyridyl)-*N'*-phenylurea (CPPU) on apple fruits alters ripening indicators such as firmness, acidity, soluble solids as well as fruit weight and size (Stern et al. 2006). Similar results occur in grape, pear, kiwi, and sweet cherry (Famiani et al. 2007; Zhang and Whiting 2013). These results indicate that exogenous application of cytokinin alters fruit maturity in climacteric and non-climacteric fruits, suggesting that cytokinin may play a role in fruit ripening.

Peach [*Prunus persica* (L.) Batsch] is a hardwood tree species that belongs to the commercially important Rosaceae family. This stone-fruit producing diploid species, with eight chromosomes and a genome size of about 230 Mb/haploid (Verde et al. 2013), has been considered a model species for the *Prunus* genus (including peaches, sweet cherries, and plums) (Shulaev et al. 2008; Carrasco et al. 2018). Over the past decade, the international Rosaceae community has developed a diverse array of molecular tools that is helping to identify QTLs, eQTLs, and candidate genes associated with fruit quality parameters desirable to the consumers, as well as those traits beneficial for the growers (highly productive cultivars, resistance to disease, extended post-harvest life and different harvest dates to prolong the period of fruit production) (Carrasco et al. 2013; Cirilli et al. 2016; Nuñez et al. 2019; Carrasco-Valenzuela et al. 2019).

Previously, we have reported the identification of the cytokinin signaling and homeostasis gene families in *Prunus persica* (Immanen et al. 2013). However, the molecular basis underlying the physiological effect of cytokinin, cytokinin signaling, or cytokinin homeostasis on fruits and during fruit ripening is still poorly understood.

In the model plant *Arabidopsis thaliana,* the molecular basis of cytokinin response is better understood. Meta-analyses in this plant have revealed a conserved set of cytokinin-responsive genes (Brenner et al. 2012; Bhargava et al. 2013; Brenner and Schmülling 2015). These studies summarize a decade of transcriptomic analyses in this plant, presenting a set of genes that are consistently up- or down-regulated by cytokinin in different assays in *Arabidopsis thaliana*. Furthermore, these meta-analyses have revealed the molecular mechanisms that may control the crosstalk between hormone signaling pathways.

To further decipher the role that cytokinin may play at the molecular level in peach fruits and during fruit ripening, this study identified a conserved set of cytokinin-regulated gene orthologs in *Prunus persica.* The expression of these orthologs and variations in their transcript levels in response to exogenous application of cytokinin (trans-zeatin) in ripe peach fruits were analyzed. Furthermore, to determine if these genes may be under the control of Type-B response regulators, the upstream regulatory regions of these genes were analyzed for Cytokinin Response Elements (CKRE).

Materials and methods

Plant materials and cytokinin treatment

Physiologically mature peach fruits (c.v. 'September Sun' grafted onto Nemaguard rootstocks) were harvested during the 2014–2015 season from a commercial farm (Santa Helena) in Graneros, in the "General Libertador Bernardo O'Higgins Region of Chile (Latitude: $-34°04'7.07''$ S, Longitude: $-70°43'38.89''$ W). Fruits from three peach trees (with similar vigor, fruit load, and phytosanitary conditions) were used for subsequent analyses. Physiologically mature fruits were transferred to the laboratory, hand sectioned (~ 5 mm thick), and incubated in a solution containing 10 nM trans-zeatin in 10 nM Sodium Phosphate buffer for 1 h, as has been described previously in *Populus trichocarpa* (Immanen et al. 2016). Control samples were similar sections from the same fruits incubated for 1 h in the buffer. Samples were subsequently frozen in liquid nitrogen and stored at -80 °C for future RNA extraction and RT-qPCR analyses. The transcriptome database contained transcripts from ripe peach mesocarp (c.v. Sweet September), 2010–2011 season treated exogenously with trans-zeatin as described above.

Identification of putative peach cytokinin-regulated genes

Cytokinin-regulated genes identified in the meta-analyses performed by Brenner et al. (2012), Brenner and Schmülling (2015) and Bhargava et al. (2013) were used as query sequences to search the CDS on the *Prunus persica* V2.1 genome (Verde et al. 2017) in the Phytozome database V12.1.6 (https://phytozome.jgi.doe.gov) (Goodstein et al. 2012) using tBLASTn (Overbeek et al. 1999). The best hits of this search (considering an e-value $\leq 10^{-5}$ and a protein identity $\geq 60\%$) were then used as queries for a tBLASTx analysis against the Arabidopsis CDS database (Berardini et al. 2015) (https://www.arabidopsis.org). Both BLASTs were performed under the substitution matrix BLOSUM62 (https://www.ncbi.nlm.nih.gov/Class/FieldGuide/BLOSUM62.txt) using CLC genomics workbench v9.5.3 (https://digitalinsights.qiagen.com/products-overview/analy

sis-and-visualization/qiagen-clc-genomics-workbench/). Genes with positive best hits in the bidirectional blast analyses were considered putative peach cytokinin-regulated genes.

Pseudochromosome mapping of putative peach cytokinin-regulated genes

The locations of the putative peach cytokinin-regulated genes on the pseudochromosomes were positioned based upon the positions of the initiation codons provided by the peach genome V2.1. The maps of the pseudochromosomes with the putative peach cytokinin-regulated genes were drawn using MapChart (https://mapchart.net/) and edited with Inkscape v0.92 software (https://inkscape.org/release/inkscape-0.92.4/) to indicate the transcriptional orientation marks.

Gene ontology annotation enrichment analysis and functional analysis of peach genes

Supporting functional annotation of the putative cytokinin-regulated genes in peach was obtained by searching for the digital annotations for these genes in the Phytozome V12.1.6 (Goodstein et al. 2012) and UniProt (www.uniprot.org) (The UniProt Consortium 2018) databases. Additional evidence was obtained by identifying conserved protein families and domains in InterProScan (https://www.ebi.ac.uk) (Jones et al. 2014; Mitchell et al. 2019). This accumulated evidence was then used, together with AMIGO 2 (https://amigo.geneontology.org/amigo) (Ashburner et al. 2000), to assign a GO term (The Gene Ontology Consortium 2018) for each putative peach cytokinin-regulated gene. The conceptual maps representing the Gene ontology annotation enrichment analysis were created for the parental GO term "Biological Process," "Molecular Functions," and "Cellular Component," using the information available at QuickGO (https://www.ebi.ac.uk/QuickGO/) (Binns et al. 2009).

In silico promoter analysis

The 2000 bp region immediately upstream of the transcription start site of each putative peach cytokinin-regulated gene was used to perform the in silico promoter analyses. These regions were extracted from the genomic sequence (*Prunus persica* genome v2.1, Verde et al. 2017) using the CLC genomics workbench v9.5.3. Bioinformatic analysis of the promoter region of these genes was performed in the RSAT plant's platform (https://rsat.eead.csic.es/plants/) (van Helden 2003; Nguyen et al. 2018). The matrix-scan tool was used (https://rsat.eead.csic.es/plants/matrix-scan_form.cgi) to identify cytokinin-responsive regulatory motifs (Ramireddy et al. 2013). The position weight matrices (PWM)

databases used were JASPAR (https://jaspar.genereg.net/; Sandelin 2004; Khan et al. 2017) and Arabidopsis PBM (Franco-Zorrilla et al. 2014) databases, which contain 12 PWM for six Type-B response regulators (ARR1, ARR2, ARR10, ARR11, ARR14, and ARR18). These analyses were performed as recommended by Turatsinze et al. (2008), using as a background model the *Prunus persica* genome, and a Markov chain order of 2. The binding site motif predictions were performed on both strands, considering a Weight score > 1 and a p value < 0.0001. The results of these analyses were visualized using the TOUCAN2 program (https://homes.esat.kuleuven.be/~saerts/software/toucan.php; Aerts et al. 2005).

RNA extraction and RT-qPCR analysis

Total RNA was extracted from peach fruits using a modified version of the method described by Meisel et al. (2005). Samples were ground in liquid nitrogen using an IKA A11 basic grinder ((model A-11, IKA, USA). Genomic DNA traces were eliminated by using DNase TURBO™ (Thermo Fisher Scientific) treatment, according to the manufacturer's specifications. The reverse transcription reaction was performed on 2 µg of total RNA, using the First Strand cDNA Synthesis System Kit (Thermo Fisher Scientific), according to the manufacturer's specifications.

Relative quantifications of peach transcripts were determined by real-time PCR (RT-qPCR) using three biological replicates with two technical replicates for each biological replicate. Primers were designed using the Vector NTI Express® Designer V10.3 software, verifying the absence of secondary structures. Additionally, a BLAST was performed against the peach genome to confirm that these primers align only with the genes of interest. The efficiency of the primers for a qRT-PCR reaction was determined by the LinRegPCR program (Ruijter et al. 2009). The list of the primers used in this study can be found in Supplementary Table S1.

The RT-qPCR reaction was performed on the QIAGEN Rotor-Gene Q, using the Rotor-Gene Q Series software version 2.1.0. The reactions were carried out using the Fast Plus system EvaGreen® qPCR Master Mix (Biotium) according to the manufacturer's specifications. RT-qPCR analyses were performed using the conditions recommended in "Minimum information established for qRT-PCR experiments" (MIQE, Bustin et al. 2009) and "Golden Rules of Quantitative PCR" (Udvardi et al. 2008). The efficiency of the primers was taken into account in the gene expression analyses, as indicated by Pfaffl (2001). *UBQ10* was used as a reference gene (Tong et al. 2009). Results are expressed as the ratio between transcript levels in cytokinin treated samples versus the untreated samples (fold change). The significant differences were calculated with the FRIEDMAN test for the distribution of nonparametric data without homogeneity of

variances (Karlen et al. 2007) in the InfoStat software (https://www.infostat.com.ar/) when the p value ≤ 0.05. All the graphs of this work were made with the GraphPad software Prism version 6.0e (https://www.graphpad.com/scientific-software/prism/).

Results

Identification of cytokinin-regulated gene orthologs in *Prunus persica*

The Arabidopsis "Cytokinin-regulated genes" described by Bhargava et al. (2013) and Brenner and Schmülling (2015) (Supplementary Table 2) were used to identify putative cytokinin-responsive genes in peach, *Prunus persica* genome

(V2.1) (Verde et al. 2017). A bidirectional BLAST between the Arabidopsis "Cytokinin-regulated genes" and the *Prunus persica* genome revealed 26 putative peach cytokinin-regulated genes (Table 1). Twenty of these putative peach cytokinin-regulated genes were assigned the same gene symbol as their Arabidopsis orthologs, but with the standard nomenclature prefix for *Prunus persica (Ppe)* added (Jung et al. 2015). The remaining six peach genes, whose Arabidopsis orthologs do not have a gene symbol, were named "Putative Cytokinin responsive protein" (*PCRP*): *PpePCRP1, PpePCRP2, PpePCRP3, PpePCRP4, PpePCRP5, PpePCRP6*.

The 26 putative peach cytokinin-regulated genes are distributed throughout the peach genome (Fig. 1). The chromosome-scale pseudomolecule 1 (Pp01) contains a total of eight of these genes, including a cluster of four genes between 30.4 and 33.5 Mb, as well as two genes located

Table 1 Putative cytokinin-regulated genes in *Prunus persica*

Gene Symbol	Gene model	Description	Arabidopsis[1]	BLAST (Ppa:At)[2] e-value	% Id	BLAST (At:Ppa)[3] e-value	% Id
PpeACO5	Prupe.3G209900	1-Aminocyclopropane-1-carboxylate oxidase 5	AT1G77330	5.19E-33	70.71	9.97E-36	70.71
PpeBPH1	Prupe.1G344900	BTB/POZ PROTEIN HYPERSENSITIVE TO ABA 1	AT1G50280	0.00	70.30	0.00	70.30
PpeCKX4	Prupe.7G208400	Cytokinin oxidase 4	AT4G29740	0.00	73.68	0.00	73.68
PpeCPK28	Prupe.7G168400	Calcium Dependent Protein kinase 16-related	AT5G66210	0.00	87.72	0.00	85.92
PpeCRF2	Prupe.5G114100	Cytokinin response factor 2	AT4G23750	1.34E-45	94.92	3.14E-30	82.26
PpeCYP18-2	Prupe.8G161100	Peptidyl-prolyl *cis–trans* isomerase CYP18-2	AT2G36130	2.86E-10	84.44	2.86E-10	84.44
PpeEXP1	Prupe.2G263600	Expansin-A1	AT1G69530	1.44E-121	75.16	1.44E-121	75.16
PpeFLS1	Prupe.1G502700	Flavonol synthase	AT5G08640	2.15E-72	80	1.26E-120	70.45
PpeGA3OX1	Prupe.1G111900	Gibberellin 3-oxidase 1	AT5G08640	2.15E-72	80	1.26E-120	70.45
PpeGIS3	Prupe.1G314000	Zinc finger protein GIS3	AT1G68360	4.75E-33	100	2.26E-30	100
PpeHIPP06	Prupe.8G165600	Heavy metal-associated isoprenylated plant protein 6	AT5G03380	9.21E-04	76.92	9.21E-04	76.92
PpeHSP90-7	Prupe.1G363900	Endoplasmin homolog (HEAT SHOCK PROTEIN 90.7)	AT4G24190	0.00E+00	87.66	0	93.42
PpeIAA17	Prupe.8G232200	Auxin-responsive protein IAA (IAA)	AT1G04250	1.65E-57	69.49	1.65E-57	69.49
PpeNIP1-1	Prupe.2G247300	NOD26-like major intrinsic protein 1	AT4G19030	4.00E-02	75.76	0.04	82.61
PpeNRP2	Prupe.5G191400	NAP1-related protein 2	AT1G18800	5.63E-98	87.5	2.78E-40	80
PpePAP8	Prupe.1G337700	Purple acid phosphatase 8	AT2G01890	6.59E-20	81.82	1.96E-107	70.41
PpePCRP1	Prupe.1G118700	Adenine nucleotide alpha hydrolases-like	AT3G62550	4.80E-01	92.31	4.80E-01	92.31
PpePCRP2	Prupe.1G217100	None predicted	AT1G28100	2.76E-96	84.76	1.59E-56	84.75
PpePCRP3	Prupe.2G017800	Unknown function	AT5G11420	1.31E-22	67.67	1.31E-22	67.67
PpePCRP4	Prupe.5G207300	Protein LURP-one-related 1	AT5G41590	4.74E-42	70.76	1.20E-41	81.82
PpePCRP5	Prupe.5G072800	Bifunctional inhibitor	AT3G22142	7.19E-05	73.53	7.19E-05	70.69
PpePCRP6	Prupe.6G310300	Unknown function	AT5G46230	4.51E-08	66.26	4.51E-08	66.26
PpePER45	Prupe.6G091600	L-ascorbate peroxidase 2	AT4G30170	1.23E+00	92.31	1.23E+00	86.67
PpePG	Prupe.4G262200	Polygalacturonase	AT3G15720	1.26E-05	84.85	1.26E-05	84.85
PpeRAP2.3	Prupe.2G272400	Ethylene-responsive transcription factor RAP2-3	AT3G16770	2.58E-18	72.37	2.58E-18	72.37
PpeUGT79B8	Prupe.2G085000	Glucuronosyl transferases	AT2G22930	3.36E-05	68.8	3.36E-05	68.8

The putative orthologs of the Arabidopsis cytokinin-regulated genes were identified in the peach genome (V2.1) by performing a bidirectional BLAST. The gene description of the peach cytokinin-regulated genes are based upon the description of their Arabidopsis orthologs. Similarly, the peach gene symbols are adaptations of the Arabidopsis ortholog gene symbols. The six peach genes whose Arabidopsis orthologs do not have gene symbols were named "Putative Cytokinin responsive protein" one through six (*PCRP1–PCRP6*)
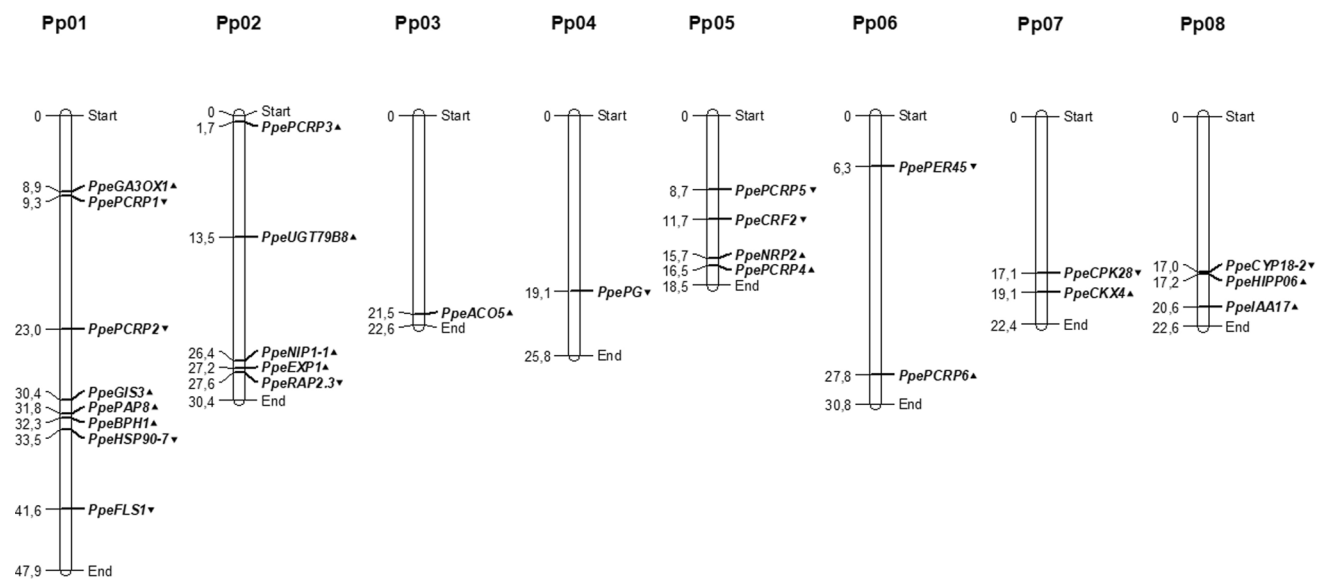
Fig. 1 Location of the putative cytokinin-regulated genes on the *Prunus persica* chromosome-scale pseudomolecules (V.2.1). The numbers on the figure represent megabase pairs (Mb) of the given chromosome-scale pseudomolecules. Triangles represent the orientation of the transcripts

between 8.9 and 9.3 Mb. Pseudomolecule 2 (Pp02) contains a total of five genes, with three of these genes clustering between 26.4 and 27.6 Mb. Pseudomolecule 5 (Pp05) contains four genes with two genes clustering at the end of the pseudomolecule, between 15.7 and 16.5 Mb. Three genes are mapped to Pseudomolecule 8 (Pp08), two of which are very tightly linked (17.0–17.2 Mb). Pseudomolecules 6 and 7 (Pp06 and Pp07) each have two genes that map to them. Finally, there is a single gene on both Pseudomolecules 3 and 4 (Pp03 and Pp04).

A gene ontology enrichment analysis was performed to gather additional information to support the functional annotation of the 26 cytokinin-regulated genes in peach (Fig. 2, Table 2). InterProScan analysis was used to obtain additional evidence about the functional domains coded in these genes (Table 2).

The putative cytokinin-regulated genes in peach are classified into four terms associated with GO "Biological Process." These include "Metabolic process" (GO:0008152) (11/26 genes, 42.3% of the putative cytokinin-regulated genes identified); "Cellular process" (GO:0009687) (4/26 genes, 15.4% of the cytokinin-regulated genes identified); "Localization" (GO:0051179) (2/26 genes, 7.7% of the putative cytokinin-regulated genes identified), and "Response to Stimulus" (GO:006950) (2/26 genes, 7.7% of the putative cytokinin-regulated genes identified) (Fig. 2a). However, eight genes (30.7% of the putative cytokinin-regulated genes identified) do not have predicted GO biological processes, including five of the six genes whose Arabidopsis orthologs do not have gene symbols (*PpePCRP2, PpePCRP3, PpePCRP4, PpePCRP5, PpePCRP6*).

The two genes annotated with the child term "Localization" are associated with transport (*PpeNIP1-1* and *PpeHIPP06*). *PpeNIP1-1* is an aquaporin, and *PpeHIPP06* presents a heavy metal associated domain (Table 2). There are 11 genes (42.3% of the putative cytokinin-related genes identified) related to metabolic processes (*PpeGA3OX1, PpeUGT79B8*) including "Protein phosphorylation" (*PpeCPK28*), "Carbohydrate Metabolism" (*PpePG*), "Regulation of transcription" (*PpeIAA17, PpeRAP2-3, PpeCRF2*) and "Oxidation–reduction processes" (*PpeACO5, PpeFLS1, PpePER45, PpeCKX4*). There are two genes annotated as being in "Response to stimulus," including "Response to stress" (*PpePCRP1*) and "Oxidative stress" (*PpePER45*). Whereas, three genes are associated with "Cellular Processes," including "Cell wall organization" (*PpeEXP1*), "protein folding" (*PpeHSP90-7*), and "nucleosome assembly" (*PpeCYP18-2*).

In terms of GO "Molecular Function," 20 (76.9%) of these putative cytokinin-regulated genes are sub-classified into four different child terms: "Catalytic activity" (GO:0003824) (10/26, 38.5% of the putative cytokinin-related genes identified); Transcription regulator activity / DNA transcription factor activity (GO:0003700) (3/26, 11.5% of the putative cytokinin-related genes identified); "Binding" (GO:0005488) (9/26, 34.6% of the putative cytokinin-related genes identified); and "Transporter activity" (GO:0005215) (1/26, 3.8% of the putative cytokinin-related genes identified). However, six genes (23% of the putative cytokinin-related genes identified) do not have predicted molecular functions (Fig. 2b, Table 2). The genes related to DNA-binding transcription factor activity include
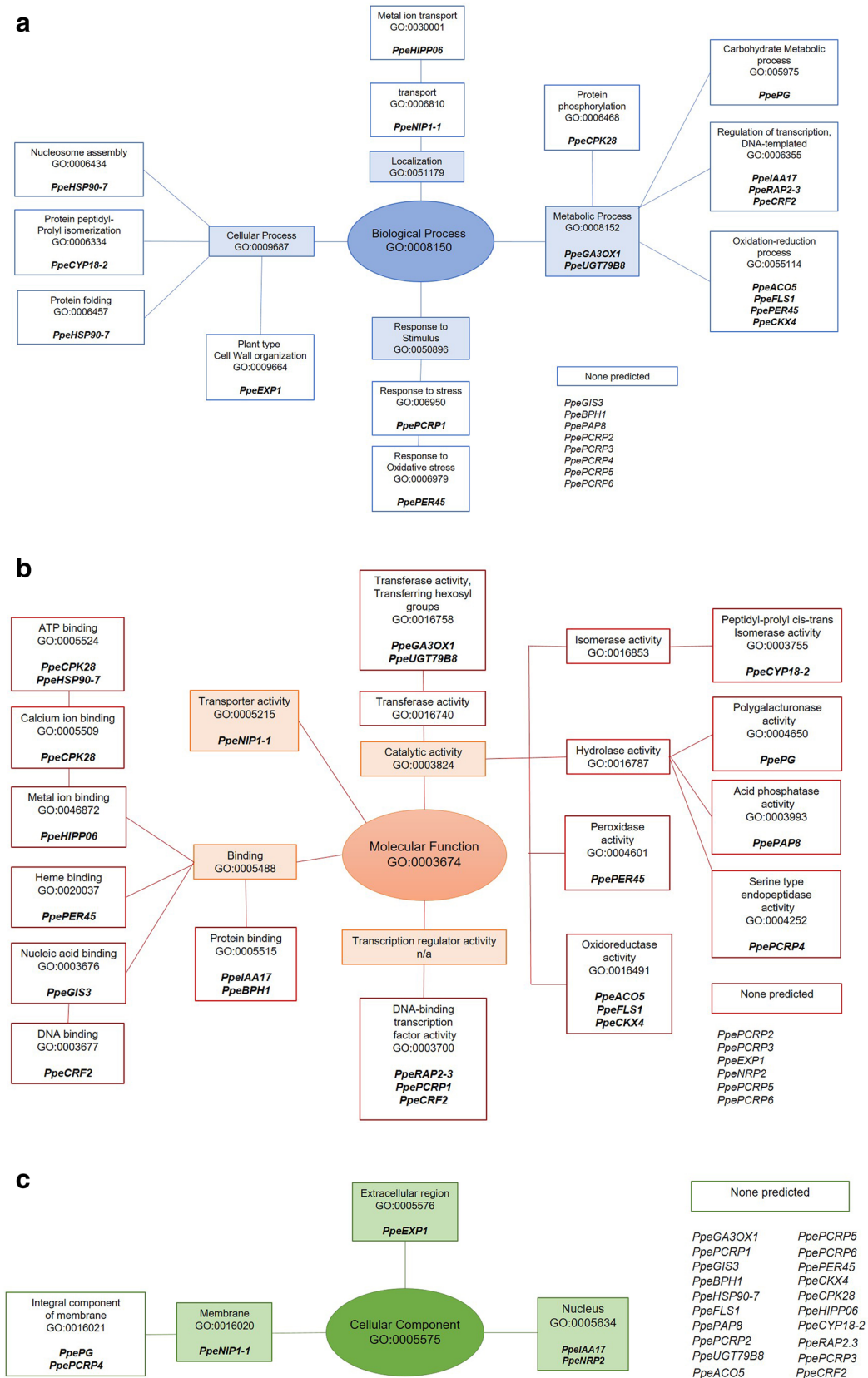
Fig. 2 Gene ontology enrichment analysis of the putative cytokinin-regulated genes in *Prunus persica*. Conceptual map of functional annotation analysis by Gene Ontology-based for "GO" terms and InterProScan analysis for 26 genes of interest. These putative peach cytokinin-response genes are classified according to **a** "Biological Process," **b** "Molecular Functions," or **c** "Cellular Component"

Table 2 Functional annotation of the "Putative *Prunus persica* orthologs of *Arabidopsis* cytokinin-regulated genes"

Gene Symbol	Gene Ontology				InterProScan	
	Gene model	Biological Process	Molecular Function	Cellular Component	Homologous superfamilies	Domains
PpeACO5	Prupe.3G209900	GO:0055114	GO:0016491	None predicted	Isopenicillin N synthase-like	Non-haem dioxygenase N-terminal domain/Oxoglutarate/iron-dependent dioxygenase
PpeBPH1	Prupe.1G344900	None predicted	GO:0005515; GO:0051082	None predicted	SKP1/BTB/POZ domain superfamily	BTB/POZ domain/NPH3 domain
PpeCKX4	Prupe.7G208400	GO:0055114	GO:0003824; GO:0016491; GO:0019139; GO:0050660; GO:0071949	None predicted	Cytokinin dehydrogenase, C-terminal domain superfamily	Cytokinin dehydrogenase 1, FAD/cytokinin binding domain
PpeCPK28	Prupe.7G168400	GO:0006468	GO:0004672; GO:0005509; GO:0005524	None predicted	Protein kinase-like domain superfamily	Protein kinase domain
PpeCRF2	Prupe.5G114100	GO:0006355	GO:0003677; GO:0003700	None predicted	AP2/ERF domain superfamily/DNA-binding domain superfamily	AP2/ERF domain
PpeCYP18-2	Prupe.8G161100	GO:0000413	GO:0003755	None predicted	Cyclophilin-like domain superfamily	Cyclophilin-type peptidyl-prolyl cis–trans isomerase domain
PpeEXP1	Prupe.2G263600	GO:0009664	None predicted	GO:0005576	RlpA-like domain superfamily/Expansin, cellulose-binding-like domain superfamily	RlpA-like protein, double-psi beta-barrel domain
PpeFLS1	Prupe.1G502700	GO:0055114	GO:0016491	None predicted	Isopenicillin N synthase-like	Non-haem dioxygenase N-terminal domain/Oxoglutarate/iron-dependent dioxygenase
PpeGA3OX1	Prupe.1G111900	GO:0008152	GO:0016758	None predicted	Isopenicillin N synthase-like	Non-haem dioxygenase N-terminal domain/Oxoglutarate/iron-dependent dioxygenase
PpeGIS3	Prupe.1G314000	None predicted	GO:0003676	None predicted	Zinc finger C2H2 superfamily	Zinc finger C2H2-type
PpeHIPP06	Prupe.8G165600	GO:0030001	GO:0046872	None predicted	Heavy metal-associated domain superfamily	Heavy metal-associated domain, HMA
PpeHSP90-7	Prupe.1G363900	GO:0006457	GO:0005524	None predicted	Histidine kinase/HSP90-like ATPase superfamily/Ribosomal protein S5 domain 2-type fold /HSP90, C-terminal domain	Histidine kinase/HSP90-like ATPase
PpeIAA17	Prupe.8G232200	GO:0006355	GO:0005515	GO:0005634	None predicted	AUX/IAA domain /PB1 domain
PpeNIP1-1	Prupe.2G247300	GO:0006810	GO:0005215	GO:0016020	Aquaporin-like	None predicted
PpeNRP2	Prupe.5G191400	GO:0006334	None predicted	GO:0005634	NAP-like superfamily	None predicted
PpePAP8	Prupe.1G337700	None predicted	GO:0003993; GO:0016787	None predicted	Metallo-dependent phosphatase-like	Calcineurin-like phosphoesterase domain, ApaH type
PpePCRP1	Prupe.1G118700	GO:0006950	GO:0003700	None predicted	Rossmann-like alpha	UspA
PpePCRP2	Prupe.1G217100	None predicted	None predicted	None predicted	Acetoacetate decarboxylase domain superfamily	None predicted
PpePCRP3	Prupe.2G017800	None predicted	None predicted	None predicted	Galactose-binding-like domain superfamily	Domain of unknown function DUF642
PpePCRP4	Prupe.5G207300	None predicted	GO:0004252	GO:0016021	Rhomboid-like superfamily	Peptidase S54, rhomboid domain

Table 2 (continued)

Gene Symbol	Gene Ontology				InterProScan	
	Gene model	Biological Process	Molecular Function	Cellular Component	Homologous superfamilies	Domains
PpePCRP5	Prupe.5G072800	None predicted	None predicted	None predicted	Bifunctional inhibitor/plant lipid transfer protein/seed storage helical domain superfamily	Hydrophobic seed protein domain
PpePCRP6	Prupe.6G310300	None predicted	None predicted	None predicted	At5g01610-like superfamily	None predicted
PpePER45	Prupe.6G091600	GO:0055114	GO:0004601; GO:0020037	None predicted	Haem peroxidase superfamily	Haem peroxidase
PpePG	Prupe.4G262200	GO:0005975	GO:0004650	None predicted	Pectin lyase fold/virulence factor/Pectin lyase fold	Parallel beta-helix repeat
PpeRAP2.3	Prupe.2G272400	GO:0006355	GO:0003677; GO:0003700	None predicted	AP2/ERF domain superfamily /DNA-binding domain superfamily	AP2/ERF domain superfamily
PpeUGT79B8	Prupe.2G085000	GO:0008152	GO:0016758	None predicted	None predicted	UDP-glycosyltransferase family

Gene Ontology and Interproscan analyses were performed on the 26 peach genes

PpeRAP2-3, *PpePCRP1*, and *PpeCRF2*. Moreover, genes *PpeCPK28*, *PpeHSP90-7*, *PpeHIPP06*, *PpePER45*, *Ppe-GIS3*, *PpeCRF2*, *PpeIAA17, and PpeBPH1* are related with "Binding" to ATP, Calcium ion, Metal ion, Heme, Nucleic acid, DNA and Protein. In "Catalytic activity," genes *PpeGA3OX1*, *PpeUGT79B8*, *PpePER45*, *PpeACO5*, *PpeFLS1*, *PpeCKX4*, *PpeCYP18-2*, *PpePG*, *PpePAP8,* and *PpePCRP4* are related with Oxidoreductase, Peroxidase, Isomerases, Endopeptidase and Hydrolase activity. Gene *PpeNIP1-1* is associated with "Transporter activity." Finally, six genes did not present an assigned GO term.

In terms of GO "Cellular Component," only six (26.9%) of these putative cytokinin-regulated genes have predicted annotations (Fig. 2c, Table 2). The majority of the putative cytokinin-regulate genes (20/26, 76.9%) do not have a predicted Cellular Component annotation. Those that do have GO "Cellular Component" annotations are classified in the child terms "Extracellular region" (*PpeEXP1*), "Nucleus" (*PpeIAA17* and *PpeNRP2*) and "Membrane" (*PpePG*, *PpeNIP1-1*, and *PpePCRP4*).

Expression of cytokinin-regulated genes in peach fruits

To determine if these putative cytokinin-regulated genes are expressed in peach fruits, RT-qPCR analyses were performed on total RNA isolated from ripe peaches. Physiological parameters of these fruits are summarized in Supplementary Table 4. Relative transcript abundance of 21 putative cytokinin-regulated genes was detected in ripe peach fruit mesocarp. Based on the transcript levels detected in ripe peach fruits, these cytokinin-regulated genes were classified into three different groups based upon the relative transcript abundance: high, medium, and low (Fig. 3). A high relative transcript abundance was detected in six putative cytokinin-regulated genes: *PpeHIPP06*, *PpePER45*, *PpeCPK28*, *PpeNRP2*, *PpeHSP90-7*, and *PpeCYP18-2* (Fig. 3a). An intermediate relative transcript abundance was detected in eight genes: *PpeCKX4*, *PpePCRP6*, *PpePCRP3*, *PpeCRF2*, *PpePG*, *PpeCRP5*, *PpeNIP1-1*, and *PpeCRP1* (Fig. 3b). A low transcript abundance was detected in seven genes: *PpePCRP2*, *PpeACO5*, *PpeBPH1*, *PpeRAP2-3*, *PpeUGT79B8*, *PpeIAA17*, and *PpeGA3OX1* (Fig. 3c). Mapped reads of these 21 genes were also detected in the peach ripe fruit transcriptome database ("Sweet September" variety) (Nuñez et al. 2019). Additional three genes (*Ppe-GIS3*, *PpeEXP1*, and *PpePCRP4*) were detected in the peach ripe fruit transcriptome database (Supplementary Table 5), but not detected in the RT-qPCR analyses (data not shown). Transcripts for *PpePAP8* and *PpeFLS1* were not detected with either analysis.

a   **b**   **c**
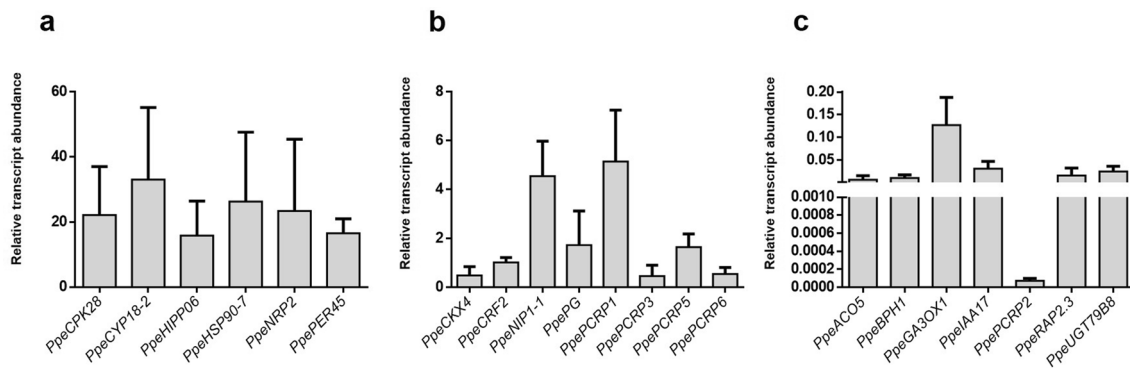
Fig. 3 Relative transcript abundance of "putative cytokinin-regulated genes" in fruit mesocarp. RT-qPCR expression analyses revealed that 21 genes of the 26 genes putative orthologs identified in the peach genome are expressed in peach fruit tissue. The graph indicates relative transcript abundance (y-axis) versus genes (x-axis), and the bars indicate average ± SEM (n = 3). **a** High transcript levels detected in ripe peach fruit mesocarp (8–60 relative transcript abundance), **b** Intermediate transcript levels detected (0.2–8 relative transcript abundance); **c** low-level transcript levels detected (0–0.2 relative transcript abundance)

Identification of Cytokinin Response Elements (CKRE) in the regulatory region of the putative peach cytokinin-regulated genes

Type-B Response Regulators are the major transcription factors that mediate cytokinin response in plants (Brenner et al. 2012). To determine if Type-B Response Regulators may regulate the expression of the putative peach cytokinin-regulated genes, the Position Weight Matrices (PWM) of the *Arabidopsis thaliana* Type-B Response Regulators (ARR1, ARR2, ARR10, ARR11, ARR14, and ARR18) (Hosoda et al. 2002; Imamura et al. 2003; Franco-Zorrilla et al. 2014; Weirauch et al. 2014; Zubo et al. 2017; Powell et al. 2019) were used to analyze the regulatory regions of the peach genes (Fig. 4a). CKREs were identified in the upstream region of the transcription start site of 20 putative cytokinin-regulated genes. Among these CKRE, potential binding sites for ARR1, ARR2, ARR10, ARR11, ARR14, and ARR18 were detected (Fig. 4b). Most of these genes have more than one binding motif and, interestingly, several of these genes have more than one putative binding site for different response regulators (Fig. 4b). Binding motifs for six different response regulators were identified in four of the putative peach cytokinin-regulated genes: *PpeACO5, PpePCRP5, PpeCKX4*, and *PpeHIPP06*. Some of these multiple binding sites were located within a very short section of the regulatory region (e.g., the regulatory region of *PpeACO5*, − 1170 through − 1162 contains binding motifs for ARR1, ARR2, ARR11, ARR14, and ARR18). The upstream regulatory regions of *PpePCRP2, PpeCRK28, PpePER45*, and *PpePCRP6* did not have any detectable CKREs.

Differential expression analysis of putative peach cytokinin-regulated genes in response to exogenous application of cytokinin

Differential expression analyses were performed between ripe peach slices incubated with 10 nM trans-zeatin or buffer-treated fruit slices (Fig. 5), to confirm the cytokinin-responsiveness of the putative cytokinin-regulated genes. These differential expression analyses revealed a significant increase in the expression of *PpeCPK28, PpeHSP90-7, PpeNRP2, PpeCKX4, PpeBPH1, PpePCRP1* (Fig. 5a–g). In contrast, one gene (*PpeNIP1-1*) decreased its expression levels significantly in response to the exogenous application of 10 nM *trans*-zeatin (Fig. 5g). The additional putative cytokinin-regulated genes presented an increased tendency in transcript levels in response to the exogenous application of 10 nM trans-zeatin for 1-h; however, these differences were not significant (Supplementary Fig. S1).

Discussion

To further decipher the molecular mechanisms by which cytokinin may affect fruit growth and development, using a comparative genomic analysis between *Prunus persica* and *Arabidopsis thaliana*, this study has identified a conserved set of cytokinin-regulated genes (Table 1) that are expressed in peach fruits (Fig. 3, Supplementary Table 5), respond to exogenous cytokinin (trans-zeatin) application (Fig. 5) and are potentially regulated by cytokinin Type-B Response Regulators (Fig. 4).

The interplay between the phytohormones auxin, gibberellin, and cytokinin is necessary for fruit set and growth (McAtee et al. 2013). The molecular mechanisms by which auxin and gibberellin affect fruit set and growth have been
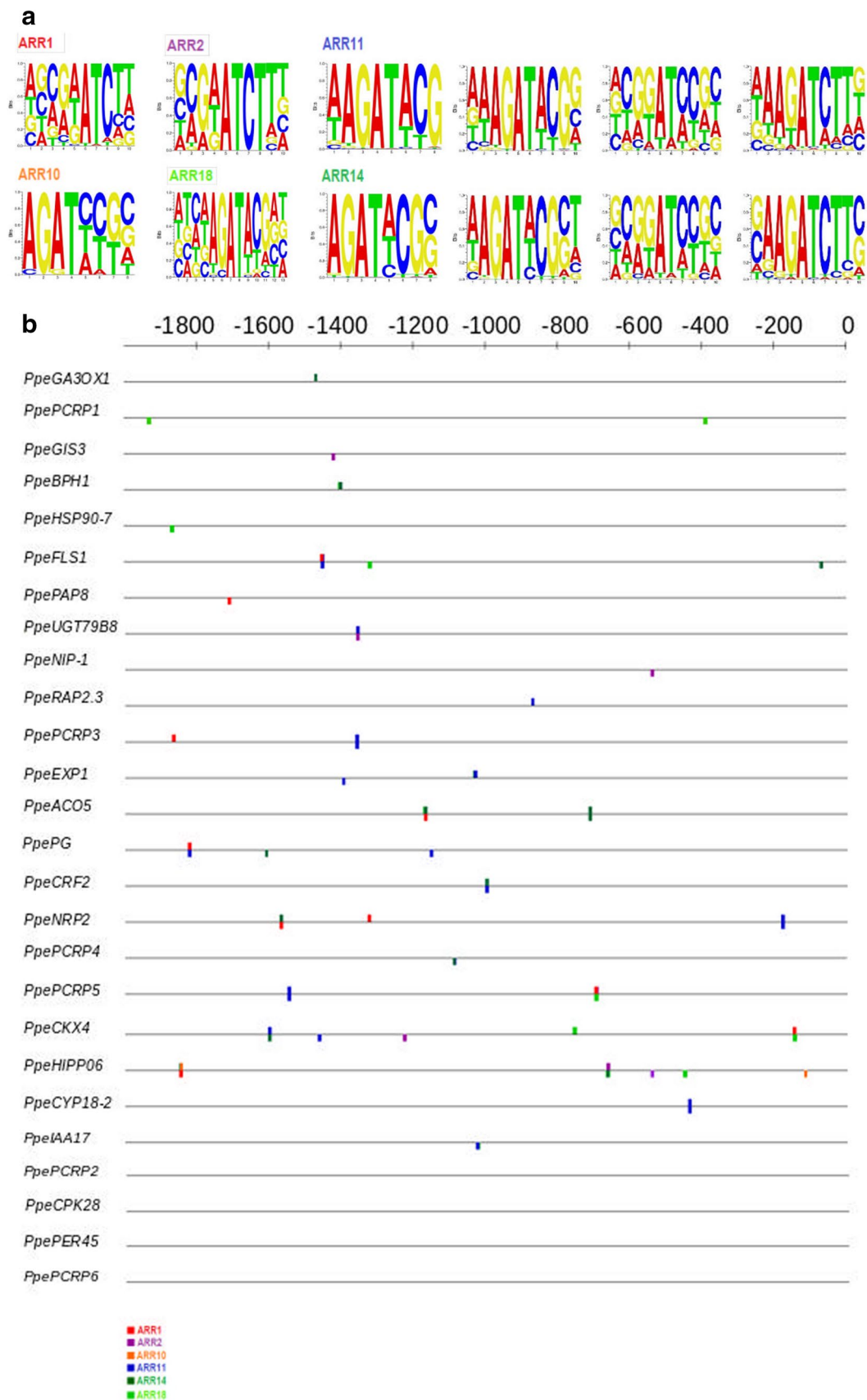
◄**Fig. 4** Type-B responsive regulatory elements in the upstream region of "Putative *Prunus persica* orthologs of Arabidopsis cytokinin-regulated genes." **a** Position weight matrix (PWM) of the six Type-B Response Regulators (ARR1, ARR2, ARR10, ARR11, ARR14, and ARR18) known in Arabidopsis in response to cytokinin (from JASPAR and Arabidopsis PBM databases). **b** Localization of the ARR1 (red square), ARR2 (purple square), ARR10 (orange square), ARR11 (blue square), ARR14 (green square) and ARR18 (light green square) motifs found in the upstream region of 26 putative cytokinin responsive genes of *Prunus persica*. (Color figure online)

studied in numerous fruit species (McAtee et al. 2013). However, much less is known about the molecular mechanisms by which cytokinins affect fruit growth and development. The sequencing of the peach genome (Verde et al. 2013, 2017) and subsequent in silico analyses have led to the identification of the cytokinin signaling and homeostasis gene families (Immanen et al. 2013), including 23 type-B authentic response regulators (Zeng et al. 2017). However, the molecular basis underlying the physiological effect of cytokinin, cytokinin signaling, or cytokinin homeostasis on fruit set, growth and ripening are still poorly understood. Recently, comparative transcriptomic analysis between flat shape and the traditional round fruit revealed nineteen putative genes involved in fruit shape and development; however no cytokinin-related genes were identified (Guo et al. 2018). By identifying cytokinin-regulate genes that are expressed in peach fruits, the role that cytokinin and cytokinin-regulated genes may play in fruit set and growth can be explored.

Peach cytokinin responsive genes

A total of 26 putative cytokinin-regulated genes were detected in the peach genome (Table 1). The distribution of these genes within the peach genome is not random; rather, eight cytokinin-regulated genes are located on Pp01 (four of which are within a 3 Mb region), five on Pp02 (three of which are within an area less than 1 Mb) and four on Pp05 (two of which are within less than 1 Mb of each other) (Fig. 1). Based upon nearest neighboring molecular markers that are located on the TxE map as well as the peach genome; five of the cytokinin-response genes (*PpeCKX4*, *PpeFLS1*, *PpeNRP2*, *PpePCRP2*, *PpePCRP4*) map within QTL regions for blooming date, chilling requirement or ripening on the TxE map (Table 1) (Olukolu and Kole 2012). These cytokinin-response genes include an ACC oxidase (*PpeCKX4*), a flavonol synthase (*PpeFLS1*), a NAP1-related protein (*PpeNRP2*), as well as two genes with unknown functions (*PpePCRP2* and *PpePCRP4*). Four of these genes are expressed in peach fruit mesocarp, as determined by RT-qPCR analyses (*PpeCKX4*, *PpeNRP2,* and *PpePCRP2*) or transcripts detected in a peach fruit mesocarp transcriptomic database (the genes determined by RT-qPCR as well as *PpePCRP4*); suggesting that although *PpCRP4* is expressed

in peach fruit mesocarp, the levels of expression are low. We were unable to detect transcript accumulation of *PpeFLS1* in peach fruit mesocarp, nor were transcripts identified in the peach fruit mesocarp transcriptomic database, suggesting that this gene may not express in peach fruit mesocarp, but may have a tissue-specific expression in other organs. Future analysis of these genes and their expression in segregating populations for these QTLs may reveal whether these cytokinin-response genes are segregating with these QTLs.

PpeCKX4, a putative ortholog of the Arabidopsis cytokinin oxidase 4 (*AtCKX4*), is expressed in the peach fruit mesocarp and the transcript levels increased six-fold after one-hour incubation in trans-zeatin. CKX4 catalyzes the degradation of cytokinins by degrading unsaturated carbon chains through an oxidation mechanism (Werner et al. 2006). *PpeCKX4* promoter-region contains several cis-elements that are potential binding sites for type-B ARR transcription factors (Powell et al. 2019), suggesting that this gene is regulated by one or several of the peach type-B ARR DNA-binding transcriptional regulators. There have been 23 type-B ARR genes identified in the peach genome, twice as many as the Arabidopsis genome (Zeng et al. 2017). Future studies towards further characterizing this large type-B ARR gene family may provide further insight into the transcriptional regulation of the *PpeCKX4* and the conservation among other Prunus fruit species. In the case of *PpeCKX4,* this is interesting since it maps between RosCos_2343 and RosCos_1223, a region of less than one cM where there is a QTL for blooming date in sweet cherry (Dirlewanger et al. 1999).

An additional four cytokinin-response genes (*PpeACO5, PpeCPK28, PpeCYP18-2, PpePCP1*) have recently been reported as candidate genes that are differentially expressed in a segregating population for fruit softening rate (Carrasco-Valenzuela et al. 2019). By exogenously applying trans-zeatin to ripe peach fruits, we were able to determine significant changes in the transcript accumulation of three of these genes (*PpeCPK28*, *PpeCYP18-2*, and *PpePCRP1*) in response to cytokinin treatment. A significant change in transcript accumulation of *PpeACO5* in response to exogenous application of trans-zeatin was not detected by RT-qPCR analyses, due to variations detected between the biological replicates (Supplementary Fig. 1), suggesting that this gene may also respond to environmental factors. Exogenous application of cytokinin has been shown to decrease the transcript level of *ACO5* in five-day-old Arabidopsis seedlings (Brenner et al. 2005). However, light has also been shown to affect cytokinin signaling via the transcriptional regulation of *CKI1* (Dobisova et al. 2017). Perhaps the variability of *PpeACO5* between biological replicates is associated with light conditions.

PpeCPK28 encodes a calcium-dependent protein kinase. The Arabidopsis ortholog of this gene is associated
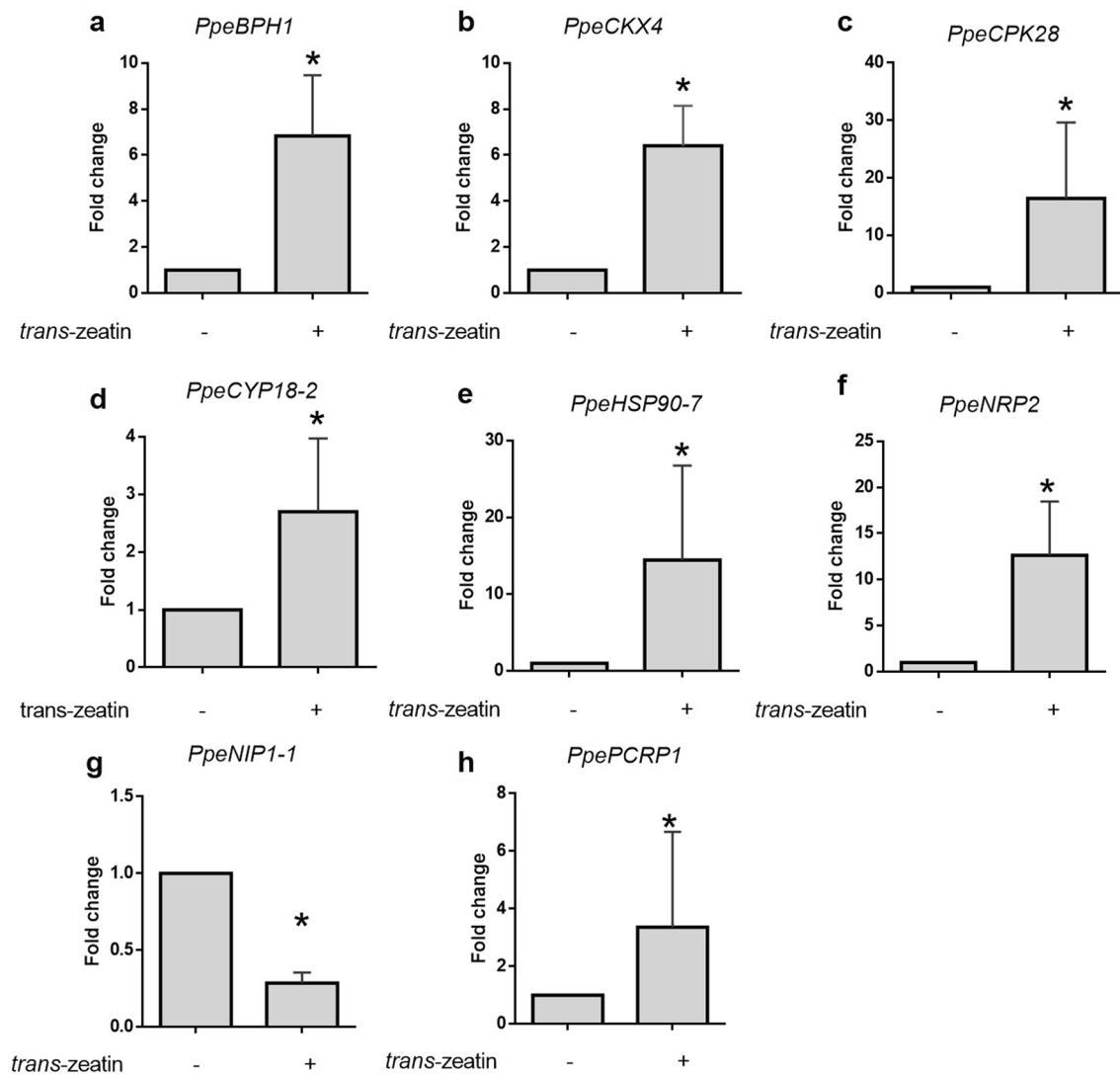
Fig. 5 RT-qPCR analysis of "Putative *Prunus persica* orthologs of Arabidopsis cytokinin-regulated genes" in peach fruits treated exogenously with cytokinin. Expression (transcript accumulation) was quantified in samples treated with *trans*-zeatin cytokinin (+) and in untreated samples (−). Relative transcript abundance was normalized with the reference gene *UBQ10*. The data is shown as "Fold change" for each gene. The genes **a** *PpeBph1,* **b** *PpeCKX4*, **c** *PpeCPK28,* **d** *PpeCYP18-2,* **e** *PpeHSP90-7,* **f** *PpeNRP2,* **g** *PpeNIP1-1,* **h** *PpeP-CRP1* increase their expression in response to exogenous cytokinin and *PpeNIP1-1* decreases its expression. Asterisks (*) indicate a statistically significant difference (non-parametric FRIEDMAN test, n = 3, p value ≤ 0.05)

with perception and intracellular signal propagation (Matschi et al. 2013), as well as a key regulator of gibberellic acid and jasmonic acid levels (Shi et al. 2018). The *PpeCPK28* transcript levels were twenty times higher in the trans-zeatin treated fruits when compared with the untreated fruits (Fig. 4). However, there are no potential binding sites for type-B ARR transcription factors in the promoter region of *PpeCPK28* (Fig. 3), suggesting that the differential expression of *PpeCPK28* in response to exogenous cytokinin application occurs indirectly in response to type-B ARR transcriptional regulators or is a type-B ARR independent pathway.

PpeCYP18-2 encodes a peptidyl-prolyl cis–trans isomerase. Studies in rice have suggested that this gene plays a role in drought stress. Overexpression of *CYP18-2* improved rice drought tolerance by modulating the pre-mRNAs of drought-responsive genes (Lee et al. 2015). In Arabidopsis, this gene has shown to be a cytokinin-response gene (Bhargava et al. 2013; Brenner and Schmülling 2015). Our results demonstrate that the cytokinin-responsiveness of *CYP18*-2 is conserved in peach. There was a greater than three-fold increase in *PpeCYP18-2* transcripts after one hour of exogenous trans-zeatin treatment on peach fruit mesocarp (Figs. 3, 4). The presence of Type-B ARR 11-type cis-elements in

the promoter region of this gene suggests that the ARR-11 transcriptional regulator may regulate the *PpeCYP18-2* gene.

PpePCRP1 encodes an adenine nucleotide alpha hydrolases-like protein. The Arabidopsis orthologs of this have been characterized not only as a cytokinin-response gene (Bhargava et al. 2013; Brenner and Schmülling 2015) but also a universal stress protein whose transcript levels increase in response to drought stress (Isokpehi et al. 2011). Here, we have shown that the cytokinin-responsiveness of the *PpePCRP1* is conserved in peach, with a two-fold increase in transcript levels being detected after one hour of exogenous trans-zeatin treatment of peach fruit mesocarp tissue. Additionally, this gene is expressed at high levels in the ripe peach fruit mesocarp. The in silico promoter analyses of this gene revealed putative cis-elements for ARR-18, suggesting that a type-B ARR transcription factor may also regulate this gene.

Other cytokinin-response genes that were identified in peach and demonstrated to respond at the transcriptional level to exogenous application of trans-zeatin are *PpeBPH1*, *PpeHSP90-7*, and *PpeNIP1-1*. Whereas the transcript levels of *PpeBPH1* and *PpeHSP90*-7 increased in response to the exogenous application of trans-zeatin in the peach fruit mesocarp, the levels of *PpeNIP1-1* decreased.

In Arabidopsis, *BPH1* is a protein that is hypersensitive to abscisic acid (Woo et al. 2018). The protein encoded by this gene acts as a receptor using the CRL3 complex as a ligand, downregulating cellular responses mediates by ABA (Woo et al. 2018). In Arabidopsis, the loss of function mutant *bph1* has increased drought resistance due to enhanced stomatal closure (Woo et al. 2018). The peach *PpeBPH1* transcript levels increased six-fold in response to exogenous application of trans-zeatin on peach mesocarp. This regulation may occur due to a coordinated response of Type B ARR transcriptional regulators since the in silico analysis of the promoter region of *PpeBPH1* contains *cis*-elements associated with ARR11, ARR14 and ARR18 (Figs. 3, 4).

The *PpeHSP90-7* gene encodes a chaperone protein (90 kDa Heat Shock Protein) that plays an important role in stress and protein folding stability after damage caused by reactive oxygen species (ROS) (Song et al. 2009; Chong et al. 2015). In Arabidopsis, HSP90.70 also participates in pathogen resistance (Lu et al. 2003; Takahashi et al. 2003) and is located primarily in the endoplasmic reticulum and chloroplasts (ER) (Ishiguro et al. 2002). Exogenous application of trans-zeatin on peach fruit mesocarp, increase the *PpeHSP90-7* transcript levels by 15-fold (Fig. 4). Since the promoter region of this gene also has ARR18 type cis-elements, this gene may be regulated downstream of the ARR18 transcriptional regulator in a similar manner to *PpeBPH1* (Fig. 3).

The *PpeNIP1-1* gene encodes an aquaporin (also referred to as a nodulin-like protein). In Arabidopsis, *AtNIP1-1* encodes an arsenic transporter protein As (III), conferring tolerance to this metalloid (Kamiya et al. 2008). Furthermore, the AtNIP1-1 protein interacts with the Calcium-dependent protein kinase, CPK31 (Ji et al. 2017). Type-B transcription factors ARR1 and ARR11 (Taniguchi et al. 2007) modulate the expression of *AtNIP1-1*. In our experiments, the exogenous application of trans-zeatin on peach fruit mesocarp significantly reduced the transcript abundance of *PpeNIP1-1*. In silico analysis of the promoter region of this gene revealed a putative ARR2 cis-element. (Fig. 3), thereby suggesting that an ARR2 transcriptional regulator may regulate the *PpeNIP1-1* gene.

In conclusion, based upon a comparative genomic analysis between Arabidopsis and *Prunus persica*, we have identified cytokinin-regulated genes that are expressed in peach fruits, respond to exogenous application of trans-zeatin, and are target genes that may be regulated by the cytokinin Type-B Response Regulators. Further analyses of these genes and the regulatory factors that regulate the expression of them may provide new insight into the role that cytokinin plays during fruit development as well as the identification of candidate genes associated with QTLs or eQTLs for future peach breeding programs.

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