Different Roles of the Five Alcohol Acyltransferases in Peach Fruit Aroma Development

Bin Peng, Jianlan Xu, Zhixiang Cai, Binbin Zhang, Mingliang Yu, and Ruijuan Ma

Institute of Pomology, Jiangsu Academy of Agricultural Sciences/Jiangsu Key Laboratory for Horticultural Crop Genetic Improvement, No. 50 Zhongling Street, Nanjing, 210014, P.R. China

ADDITIONAL INDEX WORDS. absolute quantification, enzymatic activity, functional survey, oleaginous yeast, *Prunus persica*, sequence analysis, γ -decalactone

ABSTRACT. Peach (*Prunus persica*) fruit emit more than 100 volatile organic compounds. Among these volatiles, γ -decalactone is the key compound that contributes to peach aroma. The final step in lactones biosynthesis is catalyzed by alcohol acyltransferases (AATs). In this study, five AAT genes were isolated in the peach genome, and the ways that these genes contribute toward the peach aroma were studied. The sequence analysis of the five AATs showed PpAAT4 and PpAAT5 are truncated genes, missing important residues such as HXXXD. The expressions of PpAATs were investigated to identify the roles in creating the peach aroma. The results indicated that only PpAAT1 is highly expressed during γ -decalactone formation. A functional survey of the five PpAATs, using the oleaginous yeast expression system, suggested that only PpAAT1 significantly increased the γ -decalactone content, whereas the other four PpAATs did not significantly alter the γ -decalactone content. Enzyme assays on PpAATs heterologously expressed and purified from *Escherichia coli* indicated that only PpAAT1 could catalyze the formation of γ -decalactone. All results indicated that PpAAT1 is a more efficient enzyme than the other four PpAATs during the γ -decalactone biosynthesis process in peach fruit. The results from this study should help improve peach fruit aroma.

Peach (*Prunus persica*) is a high-value stone fruit that is widely cultivated throughout temperate and subtropical zones (Akagi et al., 2016; Crisosto et al., 1999). Over the past few decades, peach cultivars have been mainly selected for enhanced yield, disease resistance, and firmness in a similar way to other fruit crops (Cao et al., 2014; Zhou et al., 2020). As an unintended consequence, peach fruit flavor has significantly declined. Fruit flavor is due to the unique combination of sugars, acids, and volatile compounds, with volatiles being the most essential for good flavor (Goff and Klee, 2006; Pichersky and Gershenzon, 2002). Today, the major complaint from consumers is that many peach cultivars have lost their unique aroma. Therefore, volatile compounds have received considerable attention. Even though more than 100 volatile organic compounds have been identified in peach fruit, only a few of them have high odor active values (Eduardo et al., 2010; Jia et al., 2008). Recent studies have indicated that γ -decalactone is an important volatile that distinguishes peach from other fruits and makes the greatest contribution to peach aroma (Peng et al., 2020; Zhang L. et al., 2017). Apart from its contribution to peach fruit flavor, γ -decalactone is also one of the most

extensively used lactones in the food and fragrance industry because of its “peach-like” aroma (Waché et al., 2003).

In the biotechnology industry, this chemical is mainly obtained from oleaginous yeast (*Yarrowia lipolytica*) by adding a hydroxyl fatty acid (HFA), ricinoleic acid, as the substrate for biotransformation (Mlikova, 2004; Nicaud, 2012). Unfortunately, in plants, the biosynthetic pathway of γ -decalactone has not been fully elucidated. However, some studies have provided enzymes specifically involved in the formation of γ -decalactone in peach fruit, such as epoxide hydrolases, fatty acid desaturase, and acyl-CoA oxidase (Sánchez et al., 2013; Vecchiotti et al., 2009; Zhang L. et al., 2017). In recent years, many studies have shown that alcohol acyltransferases (AATs), which catalyze the transfer of an acyl group from a CoA donor to an alcohol acceptor, are involved in plant aroma biosynthesis (Beekwilder et al., 2004; D’Auria, 2006; Defilipi et al., 2005). For example, in apple (*Malus ×domestica*), a quantitative trait loci (QTLs) analysis indicated that the *alcohol acyltransferase 1* (AAT1) gene is critical during the biosynthesis of fruit aroma (Souleyre et al., 2014). In papaya [*Vasconcellea pubescens* (Moralesquintana et al., 2011)], tomato [*Solanum lycopersicum* (Goulet et al., 2015)], and kiwifruit [*Actinidia chinensis* (Souleyre et al., 2011)], the AATs play very important roles in aroma biosynthesis. The peach fruit storage studies have suggested that AAT is associated with aroma formation (Zhang et al., 2010; Zhou et al., 2018). We previously showed that PpAAT1 are capable of catalyzing internal esterification at the hydroxyl (-OH) and -CoA groups of 4-hydroxydecanoyl-CoA yielding γ -decalactone, and some amino acid substitutions in PpAAT1 are responsible for the low levels of γ -decalactone accumulation in some low-aroma peach cultivars (Peng et al., 2020).

During the analysis of the draft genome of peach, five alcohol acyltransferase genes have been annotated (Verde et al., 2013). Unfortunately, there have been very few studies on how these five AATs affect peach aroma. In this study, the roles of

Received for publication 19 June 2020. Accepted for publication 16 Sept. 2020.
Published online 26 October 2020.

This work was supported by the earmarked fund for the China Agriculture Research System (CARS-30) and grants from the Jiangsu Agriculture Science and Technology Innovation Fund (CX(15)1020).

We thank Professor C. Madzak (Centre de Biotechnologies Agro-Industrielles, Institut National Agronomique Paris-Grignon, France) for providing the oleaginous yeast expression system: mutant *Po1g* strain (Leu⁻, Δ AEF, Δ AXP, *Suc*⁺ pBR platform) and the pINA1296 expression vector and technical assistance. B.P., J.X., and R.M. conceived the original screening and research plans; B.P., J.X., Z.C., B.Z., M.Y., and R.M. performed the experiments; B.P., J.X., and R.M. wrote the article with contributions from all of us; M.Y. and R.M. supervised and improved the writing.

R.M. is the corresponding author. E-mail: marj311@163.com.

This is an open access article distributed under the CC BY-NC-ND license (<https://creativecommons.org/licenses/by-nc-nd/4.0/>).

the *AATs* involved in γ -decalactone biosynthesis were verified by isolating the *AATs* from an heirloom cultivar Fenghuayulu in the National Peach Germplasm Repository (Nanjing, China). The expression analysis of the five *AATs* during peach fruit ripening was conducted using real-time quantitative polymerase chain reaction (qPCR). Then, the *AATs* were heterologously expressed and functionally characterized using the oleaginous yeast system. Furthermore, we also conducted enzyme assays on *AATs*, which were heterologously expressed and purified from *Escherichia coli*.

Materials and Methods

PLANT MATERIAL. The peach fruit (cultivar Fenghuayulu) were harvested from the National Peach Germplasm Repository. Fenghuayulu is an heirloom Chinese peach cultivar that has been found to have a strong aroma according to numerous consumer panels (Yu et al., 2010; Shen et al., 2013). Peach fruit were selected at 130 d after full bloom (DAFB) and were matched to ensure similar ripeness based on the index of absorbance difference (I_{AD}) (Zhang B. et al., 2017). The fruit were immediately taken back to the experimental laboratory at Jiangsu Academy of Agricultural Sciences within 30 min of collecting. Following this, the samples were mechanically peeled, cored, sliced, frozen in liquid nitrogen, and stored at -80°C for use in further experiments.

SEQUENCE ISOLATION OF FIVE *AATs*. We have previously isolated *PpAAT1* from the cDNA of 'Fenghuayulu' fruit (Peng et al., 2020). Here, we conducted a BLAST search using *PpAAT1* coding sequence (CDS) against PEACH genome [*Prunus persica* Genome version 2.0 (Verde et al., 2013)] to identify alcohol acyltransferase genes. Gene-specific primers for the full-length *AATs* (*PpAAT1*–*PpAAT5*) (Table 1) were designed using their homologous gene sequences (Prupe.5G018100, Prupe.5G018200, Prupe.5G017800, Prupe.5G017900, Prupe.5G018000) in the GDR database (Genome Database for Rosaceae). RNA isolation was performed from peach fruit tissue (130 DAFB) using MiniBEST Plant RNA

Extraction kits (Takara, Dalian, China). Recombinant DNase I (Takara) was used to eliminate DNA. The purity of RNA was estimated by spectrophotometric measurements using an ultraviolet-Vis spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA). The first-strand cDNA was synthesized using a PrimeScript II First Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. All cDNA samples were stored at -80°C . The *PpAAT* genes were amplified using Mastercycler pro (Eppendorf, Hamburg, Germany), and the cDNAs were used as the template. The PCR was carried out with Golden Star T6 Super PCR Mix (Tsingke Co., Nanjing, China), PCR primers (amplification occurred most effectively at $0.4\ \mu\text{M}$ final primer concentration for *PpAAT1* and *PpAAT3*, $0.3\ \mu\text{M}$ for *PpAAT2*, *PpAAT4*, and *PpAAT5*) and cDNA (20 ng) in a final volume of $25\ \mu\text{L}$. Cycling conditions were: initial denaturation 2 min at 98°C followed by 35 cycles of 10 s at 98°C , 60°C for 15 s, 72°C for 2 min, with a final extension step of 72°C for 5 min. The PCR product of each gene is cloned into PMD 18-T (Takara), respectively, according to the manufacturer's instructions and sequenced by Tsingke Co.

SEQUENCE ANALYSIS. Intron–exon structures of the *AAT* genes (Prupe.5G018100, Prupe.5G018200, Prupe.5G017800, Prupe.5G017900, Prupe.5G018000) were performed following the methods of Xue et al. (2012). The amino acid sequences produced were translated using MEGA 6.06 (Tamura et al., 2013). Sequence alignments were performed using MEGA 6.06. Then, the alcohol acyltransferases annotated in other plants were searched in GenBank (National Center for Biotechnology Information, Bethesda, MD). The amino acid sequences for *AATs*, along with other known *AATs*, were phylogenetically analyzed with the Maximum Likelihood method with 1000 bootstrap replicates using MEGA 6.06.

REAL-TIME QUANTITATIVE PCR. In this study, absolute quantification was generated using a standard curve. The above positive recombinant *AAT*-PMD 18-T plasmids were extracted respectively using plasmid miniprep kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Spectrophotometric measurements were carried out using a spectrophotometer

Table 1. Primer pairs for coding sequence (CDS) amplification, heterologous expression, and real-time quantitative PCR (qPCR).

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Used for
<i>PpAAT1</i>	ATGGGTTCATTGTGCCCTCT aggccgttctggccaATGGGTTCATTGT GCCCTCT	TTATTAGATATCTCCTCGACATGC ggggtaccccTTATTAGATATCTCCTC GACATGC	CDS of sequence analysis Heterologous expression
<i>PpAAT2</i>	CATAAACCACGCAATGTGT ATGATTTCCCCATCCGCCC aggccgttctggccaATGATTTCCCCAT CCGCCC	GCATTGAGGAGCTCTCGTGCC TTACATCCTTGACACGATCTTAGC ggggtaccccTTACATCCTTGACACGAT CTTAGC	qPCR CDS of sequence analysis Heterologous expression
<i>PpAAT3</i>	TCAGGGAAGGACCTAAT ATGATTTCCCCATCCGCCCTCC aggccgttctggccaATGATTTCCCCATCC GCCCTCC	CACATTGTGTGATTTAG TTACATCCTTGACACGATCTTAGCTGA ggggtaccccTTACATCCTTGACACGATC TTAGCTGA	qPCR CDS of sequence analysis Heterologous expression
<i>PpAAT4</i>	TTGGGGAGATGGCACAAGGT ATGGGTCCACTGTG aggccgttctggccaATGGGTCCACTGTG TCTACTGTTTCCGGTGAACCGGT	ATGCACACACGTAATTCGTGGCT TCATCTAGTAGTAGCTA ggggtaccccTCATCTAGTAGTAGCTA TCAATGCTTCCCTTGATCACC	qPCR CDS of sequence analysis Heterologous expression
<i>PpAAT5</i>	ATGGCAGATTTTATC aggccgttctggccaATGGCAGATTTTATC TTTATCGCGGTTGAGCTA	TTATTAGATACATCATC ggggtaccccTTATTAGATACATCATC GGCCATCTCCCCAATTGCGTTC	qPCR CDS of sequence analysis Heterologous expression qPCR

(NanoDrop 2000), and the number of copies of individual genes was calculated (Whelan et al., 2003). Each of the recombinant plasmids was diluted to eight gradients (10^0 to 10^{-7}) and used as a template to perform quantitative PCR to establish a standard curve. The qPCR was performed on a Real-Time PCR System (Applied Biosystems ABI-7500, Thermo Fisher Scientific) using TB Green Premix Ex Taq II (Takara). The reactions were performed in three biological replicates and two technical replicates, each containing TB Green Premix Ex Taq II mix (Takara) 10 μ L, 0.4- μ M primer pairs (primers used for the qPCR are listed in Table 1, and the lengths of RT-PCR products of *PpAAT1*–5 were 123, 253, 101, 194, and 162 bp, respectively), 2- μ L template (either cDNA or diluted plasmid DNA), and 0.4- μ L ROX Reference Dye (Takara) in a final volume of 20 μ L. Cycling conditions were as follows: 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s, and 60 or 65 °C for 34 s. For *PpAAT2*, annealing was at 65 °C; for other *PpAATs*, the annealing was at 60 °C. Each qPCR run was followed by a dissociation curve analysis using the Dissociation Curves option of SDS software (Applied Biosystems) at 65 to 99 °C.

HETEROLOGOUS EXPRESSION IN OLEAGINOUS YEAST. The oleaginous yeast mutant Polg strain and the pINA1296 expression vector, which were kindly provided by C. Madzak (Institut National Agronomique, Paris-Grignon, France), were used to conduct heterologous expression of PpAATs. The transformation, fermentation, and γ -decalactone detection steps were performed as described in Peng et al. (2020). Briefly, the *PpAAT1*–5 genes were flanked with the *SfiI/KpnI* (Table 1) restriction site, respectively. Then, both the PCR fragment and the pINA1296 plasmid were digested using *SfiI* and *KpnI* restriction enzymes (NEB, Beijing, China). The linear plasmid and the modified PCR fragment were ligated using T4 DNA ligase (NEB). The constructs were amplified in *E. coli* DH5 α Electro-Cells (Takara) and extracted by a TIANprep Mini Plasmid Kit (Tiangen) according to the manufacturer's instructions. Finally, the pINA1296-PpAAT cassettes were transformed into Polg according to Blazeck et al. (2014). The fermentation of the Polg transformant producing γ -decalactone was conducted according to the protocol described by Braga and Belo (2013), and each construct was tested using eight Polg transformants.

ANALYSIS OF γ -DECALACTONE CONTENT. Lactones were extracted from 2 mL broth with n-hexane (Sigma-Aldrich, St. Louis, MO). The organic phase was analyzed by gas chromatography (GC). The analysis was performed on a GC (7890 A; Agilent Technologies, Santa Clara, CA) equipped with a DB-WAX column (0.32 mm, 30 m, 0.25 μ m, Agilent Technologies). Conditions for the GC analysis were conducted as described in Peng et al. (2020). Briefly, γ -decalactone was extracted by adsorption to a fiber coated with 65 μ m of polydimethylsiloxane and divinylbenzene [PDMS/DVB (Supelco, Bellefonte, PA)]. Conditions for the GC analysis were as follows: an initial oven

temperature of 40 °C was held for 2 min, then increased by 5 °C $\cdot$ min $^{-1}$ to 240 °C, and held for 2 min. Nitrogen was used as a carrier gas at 1.0 mL $\cdot$ min $^{-1}$. A standard curve for γ -decalactone was generated by analyzing a standard concentration series ranging from 1.00 μ g $\cdot$ mL $^{-1}$ to 10.00 mg $\cdot$ mL $^{-1}$.

PREPARATION OF RECOMBINANT PROTEINS AND ENZYME ASSAY. The PpAATs coding sequences with a C-terminal six histidine tag, which were optimized for expression in *E. coli*, were synthesized by GenScript Co. Ltd. (Nanjing, China). The PET-30a (+) was used as expression vector. The heterologous expression in *E. coli* [BL21(DE3) Strain], purification, and confirmation of the identity of the recombinant proteins were conducted by GenScript.

The alcohol acyltransferase activity of recombinant proteins was assayed using 4-hydroxydecanoyl-CoA (Accela ChemBio, Shanghai, China) as a substrate as described by Peng et al. (2020). Briefly, a total reaction volume of 1 mL included 5.1 mg of protein, 10 mM hydroxydecanoyl-CoA, and 50 mM Tris-HCl (pH 8.2). Reactions were performed at 31 °C for 40 min. Boiled protein (nonfunctional) was used as the control. Then the product was collected and analyzed by GC as described above. All the assays were performed in sextuplicate.

STATISTICAL ANALYSES. The comparison tests were analyzed using the Fisher's least significant difference test at $P \leq 0.05$. Significant differences were assumed by IBM SPSS Statistics (version 23; IBM Corp., Armonk, NY). Histograms were prepared with Origin software (version 2019; OriginLab Corp., Northampton, MA).

Results

PEACH AAT SEQUENCES ISOLATION. The coding sequences of the five alcohol acyltransferase genes (*PpAAT1*, *PpAAT2*, *PpAAT3*, *PpAAT4*, and *PpAAT5*) from the 'Fenghuayulu' fruit were cloned and sequenced. We obtained 1353, 1383, 1383, 495, and 966 nucleotide (nt) sequences from the five *AATs*, respectively. The CDS of all five genes is the same as the sequence in the peach genome. The coding sequences for PpAAT1 and PpAAT2 were 76% identical. The coding sequences for PpAAT3 was most closely related (99%) to PpAAT2, and the sequences from PpAAT4 and PpAAT5 were closely related to PpAAT1 with 89% identity. The structural

Fig. 1. Positions and phases of introns in *PpAAT* genes. The term "phase" indicates the position of an intron within a codon. For example, the phases 0, 1, and 2 correspond to the first, second, and third bases of the codon, respectively.

analysis of the five genes in the peach genome revealed that they have different intron–exon structures (Fig. 1). The amino acid sequences analysis of the PpAATs showed that PpAAT1, PpAAT2, and PpAAT3 contained an HXXXD-type ACYL-TRANSFERASE-like motif (HAMCD) (Fig. 2), which is like other known plant acyltransferases. There were no similar motifs in PpAAT4 and PpAAT5. PpAAT1 and PpAAT5 contain another conserved region of other plant AATs, D(N)F(V) GWG (Souleyre et al., 2005; Wang et al., 2014), whereas the Val is substituted by the Phe in PpAAT2 and PpAAT3.

Phylogenetic analysis of the amino acids showed that all the PpAATs were clustered together (Fig. 3). When using *Arabidopsis thaliana* AAT as the outgroup, PpAAT1 was placed near the root of the phylogenetic tree. PpAAT4 had a closer genetic relationship with PpAAT1. PpAAT2 and PpAAT3 clustered together and formed a separate clade. PpAAT5 seemed to have a distant genetic relationship with PpAAT1.

QUANTITATIVE PCR ANALYSIS AT THE RIPE STAGE. The transcript levels of peach *PpAAT* genes associated with the formation of γ -decalactone were investigated by performing an absolute quantitative PCR analysis on the five *PpAAT* genes in peach fruit. At the ripe stage (130 DAFB), the results of absolute quantification showed that only PpAAT1 was highly expressed in the fruit (Fig. 4), and its average expression level was about 800 copies/ng cDNA. The expressions level of *PpAAT2*–*PpAAT5* were less than 10% of *PpAAT1*, and there were no significant changes among those genes.

FUNCTIONAL TESTS ON THE AATs CONTRIBUTING TO γ -DECALACTONE IN OLEAGINOUS YEAST. The oleaginous yeast *Polg* strain and the pINA1296 expression system were used for the functional expressions analysis in this study. The five types of recombinant oleaginous yeast, containing the five *PpAATs* genes and the *Polg* strain with empty vector (treated as the wild type control), showed different γ -decalactone production abilities. The results showed the amount of γ -decalactone produced by the “wild” *Polg* transformant was about 0.3 g·L⁻¹ on average, and the transformants

containing the *PpAAT2*, *PpAAT3*, *PpAAT4*, and *PpAAT5* genes were not significantly different from the “wild” *Polg* transformant. In contrast, the transformant with the type *PpAAT1* gene significantly increased γ -decalactone levels and reached an average of 2.2 g·L⁻¹ (Fig. 5).

PpAAT1	MGSLCPLLPVNRFEPELI TPAKPTPI ETQQLSDI DDQGLRFHFVPI I S	50
PpAAT2	MI SPSALLFQVNRKAEI TPAKPTPHETKLLSNI DDQESLRFQVSVI MS	50
PpAAT3	MI SPSALLFQVNRKPELI TPAKPTPHETKLLSNI DDQESLRFQVPI MS	50
PpAAT4	MGPLCPLLPVNRFEPELI TPAKPTPLETKLLSDI DNQVGI RFHYSVI MT	50
PpAAT5	 MADFI AVELEKGRS GNI QQVTR	22
PpAAT1	YKNNPSMKGNDVAVVI REALSRALVYYYPLAGRLREGPNRKLMEVCNNEG	100
PpAAT2	YKNNPSMKGNDAVEVI REALSRALVYYYPLAGRLREGPNRKLMEVCNNEG	100
PpAAT3	YKNNPSMKGNDAVEVI REALSRALVYYYPLAGRLREGPNRKLMEVCNNEG	100
PpAAT4	YKNNPSMKGNDVAVVI KEALSALVFFYYPLAGRLREGPNRKLMDVCNNEG	100
PpAAT5	LTCGGFI LGLRVNHTI CECDVGLAQFLNAI GEMAKGADS.	62
PpAAT1	VLFI EANADVLTLEQLGDI LPPCPVLEEFSLNPPGSDGI LGCPLLLVQVT	150
PpAAT2	VLFI EANADVTLAQLGDI APPSPFLEEFLYNVPDSDGI LGCPLLLI QVT	150
PpAAT3	VLFI EANADVTLAQLGDI APPSPFLEEFLYNVPDSDGI LGCPLLLI QVT	150
PpAAT4	... VEANADVLTLEQLGDI LPPCPVLFVLFNPLGSDGI LDCPLLSCYFR	147
PpAAT5	... PSI PPLWAQELLNAI DPPTI TLPHYEYEQLLDS. ... QGSFI I DQSD	105
PpAAT1	RLTCGGFI FGLRI NHAMCDVGLAKFLNAI GEMAQGADSLSVPPVWAREL	200
PpAAT2	RLSCGGFI LALRLNHTMCDVGLAKFLNAI GEMAQGADSLSVPPVWAREL	200
PpAAT3	RLSCGGFI LALRLNHTMCDVGLAKFLNAI GEMAQGADSLSVPPVWAREL	200
PpAAT4	YWLCI TFFI LGLVATTR.	164
PpAAT5	MAQRSFYFGPQI TALR.	122
PpAAT1	LNARDPPTVTRWHYEYDQLLDSQGSFI AAI DQSNMAQRSFYFGPQI RAL	250
PpAAT2	LGARDPPRI TCAHHEYEEGI DAQGSFPSN. KPNMVQRSFYFGSKEI KAI	249
PpAAT3	LGARDQPRI TCVHHEYEEGI DAQGSFPSN. KPNMVQRSFYFGSKEI KAI	249
PpAAT4		164
PpAAT5		122
PpAAT1	RKHLPPHLSTCSSFELI TACVWRCRTLSLRLNPKDVTIRI SCAVNARGKSI	300
PpAAT2	RNHI PPQLSTCTTFDLI TACLWKRTLALRMNPKQVVRSLCLVSARGK. R	298
PpAAT3	RNHI PPQLSTCTTFDLI TACLWKRTLALRMNPKQVVRSLCLVSARGK. R	298
PpAAT4		164
PpAAT5	. KHLPPHLSTCSSFELI TACI WRCRTLSLRLNPKDVTIRLSICI I NARGKRM	171
PpAAT1	NDLCLPSGFYGNAFSI PTAVSTVELLCASPLGYGVELVRKS KAQMKEYM	350
PpAAT2	HNVRPLPGYGNVAFPAAVSEAKDVCTNPLGYALELVMKAKATVNEEYM	348
PpAAT3	HNVRPLPGYGNVAFPAAVSEAKDVCTNPLGYALELVMKAKATVNEEYM	348
PpAAT4		164
PpAAT5	DDLCLPLGFYGNALGVPTI VSTVELVCASPLGYGVELVRKNAQMKEYM	221
PpAAT1	QSLADFFVI RGRPLPMGWNVFI VSDNRHTGFGEFVDVGWRPLFAGLARA	400
PpAAT2	RSVADLMEI RGRLPKYPLTGNFI VSDTSRAGFGEVSFGWCKPVFGGPAKA	398
PpAAT3	RSVADLMEI RGRLPKYPLTGNFI VSDTSRAGFGEVSFGWCKPVFGGPAKA	398
PpAAT4		164
PpAAT5	QSLAHFLVI RGRPPPMAWNVI VSDNRHTGLGEFVDVGWRPVYAGLARA	271
PpAAT1	FSMI SFYVRDNNQEEFGTLVPI CLPSTSLERFEEELKKMTLEHVEEI SK	450
PpAAT2	LDLI SFYVQHKCNNE. EGVLVPI CLPLSDMERFQELERMTVETVQTMED	447
PpAAT3	LDLI SFYVQHKCNNE. EGVLVPI CLPLSDMERFQELERMTVETVQTMED	447
PpAAT4		164
PpAAT5	VHVI SFYVRENQEEEFRI LVPI CLPRMCLERFEQELKRMILEHVDDVSK	321
PpAAT1		450
PpAAT2	I YDTKSAKI VSR	459
PpAAT3	I YDTKSAKI VSR	459
PpAAT4		164
PpAAT5		321

Fig. 2. The amino acid sequence differences among the PpAATs. The RED frames were the HXXXD-type and D(N)F(V)GWG motif.

Fig. 3. Phylogenetic analysis of PpAATs with other plant AATs. GeneBank accession numbers are as follows: (NP_199955.1) *Arabidopsis thaliana*, ViKAAT1(ART85743.1) *Vitis labrusca* × *Vitis vinifera* 'Kyoho', SLAAT(NP_001234496.1) *Solanum lycopersicum*, PMAAT(BAE48668.1) *Prunus mume*, AEAAT(AIC83789.1) *Actinidia eriantha*, RrAAT(AEZ53172.1) *Rosa rugosa*, FVAAT(AAN07090.1) *Fragaria vesca*, FCAAT(ACT82247.1) *Fragaria chiloensis*, PPAAT(AFW03968.1) *Physalis peruviana*, BanAAT1(AX025506.1) *Musa sapientum*, ADAAT(AIC83790.1) *Actinidia deliciosa*, DKA-AAT1(AKE98481) *Diospyros kaki*, EJAAT1(AHC32224.2) *Eriobotrya japonica*, AAT1-GSa(AGW30203.1) *Malus domestica* 'Granny Smith', AAT1-RGa(AGW30200.1) *M. domestica* 'Royal Gala', PCAAT(AAS48090.1) *Pyrus communis*, PusaAAT(AJD18611) *Pyrus ussuriensis*, CSAAT(NP_001275839.1) *Citrus sinensis*, CmeAAT2(BAC58010.1) *Cucumis melo*, VCAAT1(ACT82248.1) *Vasconcellea cundinamarcensis*.

ENZYMATIC ACTIVITY OF THE FIVE ALCOHOL ACYLTRANSFERASES. In vitro enzyme assays showed the five PpAATs were heterologously expressed and purified from *E. coli*. Enzyme activity analysis was performed by detecting the production of γ -decalactone when using 4-hydroxydecanoyl-CoA as a substrate. The results showed that the γ -decalactone can be detected only in the reaction of PpAAT1 (Fig. 6), and its production of γ -decalactone was about 17 nmol·L⁻¹ on average. Meanwhile, there was no detectable level of γ -decalactone in other PpAAT reactions and control proteins (boiled).

Discussion

Some fruits, such as tomato, rely on several compounds to form their unique aroma (Buttery et al., 1989; Klee, 2010). However, some fruits use a monocompound in their aroma production process. In strawberry (*Fragaria × ananassa*), 4-hydroxy-2,5-dimethyl-3(2H)-furanone is the key compound contributing to its aroma (Larsen and Poll, 1992; Wein et al., 2002), whereas γ -decalactone makes the most important chemical contribution to peach aroma because it activates human olfactory receptors (Maga and Katz, 1976).

In plants, paralog AATs have been shown to be involved in different physiological processes, such as pollination, seed

spreading, and defense responses (Frost et al., 2008). To determine which PpAAT is responsible for peach aroma, we first conducted a sequences analysis. The results showed that PpAAT4,5 lacked an acyl transferase-like HXXXD motif. This phenomenon may indicate they are truncated genes, missing important residues such as HXXXD and therefore are not active. Substitute in another conserved region (D(N) F(V) GWG) was observed in PpAAT2 and PpAAT3. Whether this substitution affects enzyme activity requires further research. An expression analysis of the five PpAATs was conducted to identify their roles in γ -decalactone formation. However, only PpAAT1 was highly expressed during γ -decalactone formation. The results indicated that only PpAAT1 is likely responsible for peach aroma. Interestingly, a similar phenomenon has been observed in apple and tomato. For example, there are five AAT paralogs in tomato, but only AAT1 is correlated with ripe fruit aroma (Goulet et al., 2015). In apple, a structural characterization and substrate specificity analysis suggested that only AAT1 was related to fruit aroma biosynthesis (Souleyre et al., 2014).

In this study, the oleaginous yeast mutant Polg strain and the pINA1296 expression system were used to conduct a heterologous expression analysis that examined the catalytic activities of the alcohol acyltransferases encoded by the five PpAAT genes. The results showed that only the recombinant strain harboring PpAAT1 obviously increased γ -decalactone contents. The results confirmed that PpAAT1 was more efficient than the other four alcohol acyltransferases in γ -decalactone formation. This strain Polg was obtained by deleting two genes, which encoded for the two secreted proteases (alkaline extracellular protease: AEP and acid extracellular protease: AXP), from the wild-type strain W29 through genetic modification (Madzak et al., 2004). The purpose of knocking-out them was to avoid the presence of proteases in the culture supernatant, because they could be able to degrade the heterologous proteins of interest (Madzak et al., 2000). Thus all the secretory pathway of the strain remains fully functional with the wild-type strain, and the function of the exogenous proteins can also be verified (Madzak et al., 2000). The aim of this study was to compare the activities of the five alcohol acyltransferases, which catalyze the transfer of the acyl group (from coenzyme A) donor to the alcohol acceptor (D'Auria, 2006). In oleaginous yeast, 4-hydroxy-decanoyl-CoA is the direct precursor substance of γ -decalactone (Braga and Belo, 2016). Unfortunately, in oleaginous yeast, whether the precursor 4-hydroxy-decanoyl-CoA is catalyzed by an

Fig. 4. Gene expression of the five *PpAATs* in the mesocarp of ripe fruit (130 d after full bloom). Data are presented as mean $\pm$ SE (n = 6). Letters indicate significant different gene expression levels ($P \leq 0.05$).

Fig. 6. Enzymatic activity of the five alcohol acyltransferase in peach fruit. Data are presented as mean $\pm$ SE (n = 6), and letters represent significant differences at $P \leq 0.05$.

Fig. 5. GC analysis of γ -decalactone production from oleaginous yeast strain Polg containing different *PpAATs*. Data are presented as mean $\pm$ SE (n = 8), and letters represent significant differences at $P \leq 0.05$.

enzyme like an alcohol acyltransferase is not proven so far. Nevertheless, our results showed only PpAAT1 significantly increased the γ -decalactone content in oleaginous yeast, and this finding seemed to indicate that PpAAT1 was more efficient than other AATs in the process of catalyzing the 4-hydroxy-decanoyl-CoA to γ -decalactone. The oleaginous yeast system in this study might be an effective tool to study the activity of different alcohol acyltransferases in peach.

Enzymatic activity of PpAATs also revealed that only PpAAT1 can use 4-hydroxydecanoyl-CoA to form γ -decalactone. This means that among these five alcohol acyltransferases, only PpAAT1 might be the most important enzyme

that forms the peach-like aroma. In future research, we will investigate whether several other alcohol acyltransferases in peach fruit are involved in different physiological processes.

Conclusions

In this study, five AAT genes were identified in the peach genome. Gene expression experiments and heterologous expression experiments indicated that PpAAT1 is a more efficient enzyme than the other four alcohol acyltransferases during the γ -decalactone biosynthesis process in peach fruits.

Literature Cited

- Akagi, T., T. Hanada, H. Yaegaki, T.M. Gradziel, and R. Tao. 2016. Genome-wide view of genetic diversity reveals paths of selection and cultivar differentiation in peach domestication. *DNA Res.* 23:271–282, doi: 10.1093/dnares/dsw014.
- Beekwilder, J., M. Alvarez-Huerta, E. Neef, F.W. Verstappen, H.J. Bouwmeester, and A. Aharoni. 2004. Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant Physiol.* 135:1865–1878, doi: 10.2307/4356543.
- Blazeck, J., A. Hill, L. Liu, R. Knight, J. Miller, A. Pan, P. Otoupal, and H. Alper. 2014. Harnessing *Yarrowia lipolytica* lipogenesis to create a platform for lipid and biofuel production. *Nat. Commun.* 5:3131.
- Braga, A. and I. Belo. 2013. Immobilization of *Yarrowia lipolytica* for aroma production from castor oil. *Appl. Biochem. Biotechnol.* 169:2202–2211.
- Braga, A. and I. Belo. 2016. Biotechnological production of gamma-decalactone, a peach like aroma, by *Yarrowia lipolytica*. *World J. Microb. Biot.* 32:169.
- Buttery, R.G., R. Teranishi, R.A. Flath, and L.C. Ling. 1989. Fresh tomato volatiles, p. 213–222. In: R.G. Buttery, R. Teranishi, and F. Shahidi (eds.). *Flavor chemistry: Trends and developments*. Amer. Chem. Soc. Symp. Ser. 388. Amer. Chem. Soc., Washington, DC.

- Cao, K., Z. Zheng, L. Wang, X. Liu, G. Zhu, W. Fang, S. Cheng, P. Zeng, C. Chen, and X. Wang. 2014. Comparative population genomics reveals the domestication history of the peach, *Prunus persica*, and human influences on perennial fruit crops. *Genome Biol.* 15:415, doi: 10.1186/PREACCEPT-1190140987122406.
- Crisosto, C.H., F.G. Mitchell, and Z. Ju. 1999. Susceptibility to chilling injury of peach, nectarine, and plum cultivars grown in California. *HortScience* 34:1116–1118, doi: 10.1016/S0304-4238(99)00028-X.
- D'Auria, J.C. 2006. Acyltransferases in plants: A good time to be BAHD. *Curr. Opin. Plant Biol.* 9:331–340, doi: 10.1016/j.pbi.2006.03.016.
- Defilipi, B.G., A.A. Kader, and A.M. Dandekar. 2005. Apple aroma: Alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. *Plant Sci.* 168:1199–1210, doi: 10.1016/j.plantsci.2004.12.018.
- Eduardo, I., G. Chietera, D. Bassi, L. Rossini, and A. Vecchiatti. 2010. Identification of key odor volatile compounds in the essential oil of nine peach accessions. *J. Sci. Food Agr.* 90:1146–1154, doi: 10.1002/jfsa.3932.
- Frost, C.J., M.C. Mescher, C. Dervinis, J.M. Davis, J.E. Carlson, and C.M. De Moraes. 2008. Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3-hexenyl acetate. *New Phytol.* 180:722–734, doi: 10.1111/j.1469-8137.2008.02599.x.
- Goff, S.A. and H.J. Klee. 2006. Plant volatile compounds: Sensory cues for health and nutritional value? *Science* 311:815–819, doi: 10.1126/science.1112614.
- Goulet, C., Y. Kamiyoshihara, N.B. Lam, T. Richard, M.G. Taylor, D.M. Tieman, and H.J. Klee. 2015. Divergence in the enzymatic activities of a tomato and *Solanum pennellii* alcohol acyltransferase impacts fruit volatile ester composition. *Mol. Plant* 8:153–162, doi: 10.1016/j.molp.2014.11.007.
- Jia, H., K. Hirano, and G. Okamoto. 2008. Effects of fertilizer levels on tree growth and fruit quality of 'Hakuho' peaches (*Prunus persica*). *Engei Gakkai Zasshi* 68:487–493.
- Klee, H.J. 2010. Improving the flavor of fresh fruits: Genomics, biochemistry, and biotechnology. *New Phytol.* 187:44–56, doi: 10.1111/j.1469-8137.2010.03281.x.
- Larsen, M. and L. Poll. 1992. Odour thresholds of some important aroma compounds in strawberries. *Z. Lebensm. Unters. Forsch.* 195:120–123, doi: 10.1007/BF01201770.
- Madzak, C., C. Gaillardin, and J.M. Beckerich. 2004. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: A review. *J. Biotechnol.* 109:63–81, doi: 10.1016/j.jbiotec.2003.10.027.
- Madzak, C., B. Tréton, and S. Blanchinroland. 2000. Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytica*. *J. Mol. Microbiol. Biotechnol.* 2:207–216, doi: 10.1038/sj.jim.2900821.
- Maga, J.A. and I. Katz. 1976. Lactones in foods. *Crit. Rev. Food Sci. Nutr.* 8:1–56.
- Mlikova, K. 2004. Acyl-CoA oxidase, a key step for lipid accumulation in the yeast *Yarrowia lipolytica*. *J. Mol. Catal. B-Enzym.* B. 28:81–85, doi: 10.1016/j.molcatb.2004.01.007.
- Moralesquintana, L., L. Fuentes, C. Gaeteastman, R. Herrera, and M.A. Moyaleón. 2011. Structural characterization and substrate specificity of VpAAT1 protein related to ester biosynthesis in mountain papaya fruit. *J. Mol. Graph. Model.* 29:635–642, doi: 10.1016/j.jmgm.2010.11.011.
- Nicaud, J.M. 2012. *Yarrowia lipolytica*. *Yeast* 29:409–418.
- Peng, B., M. Yu, B. Zhang, J. Xu, and R. Ma. 2020. Differences in PpAAT1 activity in high- and low-aroma peach varieties affect γ -decalactone production. *Plant Physiol.* 182:2065–2080, doi: 10.1104/pp.19.00964.
- Pichersky, E. and J. Gershenzon. 2002. The formation and function of plant volatiles: Perfumes for pollinator attraction and defense. *Curr. Opin. Plant Biol.* 5:237–243, doi: 10.1016/S1369-5266(02)00251-0.
- Sánchez, G., M. Venegas-Calderón, J.J. Salas, A. Monforte, M.L. Badenes, and A. Granell. 2013. An integrative “omics” approach identifies new candidate genes to impact aroma volatiles in peach fruit. *BMC Genomics* 14:1–23, doi: 10.1186/1471-2164-14-343.
- Shen, Z.J., R.-J. Ma, M.-L. Yu, Z.X. Cai, and J.-L. Xu. 2013. Establishment of peach primary core collection based on accessions conserved in National Fruit Germplasm Repository of Nanjing. *Acta Hort. Sinica* 40:125–134.
- Souleyre, E.J., D. Chagne, X. Chen, S. Tomes, R.M. Turner, M.Y. Wang, R. Maddumage, M.B. Hunt, R.A. Winz, C. Wiedow, C. Hamiaux, S.E. Gardiner, D.D. Rowan, and R.G. Atkinson. 2014. The AAT1 locus is critical for the biosynthesis of esters contributing to “ripe apple” flavour in ‘Royal Gala’ and ‘Granny Smith’ apples. *Plant J.* 78:903–915, doi: 10.1111/tpj.12518.
- Souleyre, E.J., D.R. Greenwood, E.N. Friel, S. Karunairetnam, and R.D. Newcomb. 2005. An alcohol acyl transferase from apple (cv. Royal Gala), MpAAT1, produces esters involved in apple fruit flavor. *FEBS J.* 272:3132–3144, doi: 10.1111/j.1742-4658.2005.04732.x.
- Souleyre, E.J.F., C.S. Günther, M.Y. Wang, R.D. Newcomb, and K.B. Marsh. 2011. Ester biosynthesis in kiwifruit—From genes to enzymes to pathways. *Acta Hort.* 127:205–211, doi: 10.1016/j.scienta.2010.10.022.
- Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30:2725–2729, doi: 10.1093/molbev/mst197.
- Vecchiatti, A., B. Lazzari, C. Ortugno, F. Bianchi, R. Malinverni, A. Caprera, I. Mignani, and C. Pozzi. 2009. Comparative analysis of expressed sequence tags from tissues in ripening stages of peach (*Prunus persica* L. Batsch). *Tree Genet. Genomes* 5:377–391, doi: 10.1007/s11295-008-0193-6.
- Verde, I., A.G. Abbott, S. Scalabrin, S. Jung, S. Shu, F. Marroni, T. Zhebentyayeva, M.T. Dettori, J. Grimwood, and F. Cattonaro. 2013. The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. *Nat. Genet.* 45:487–494, doi: 10.1038/ng.2586.
- Waché, Y., M. Aguedo, J.-M. Nicaud, and J.-M. Belin. 2003. Catabolism of hydroxyacids and biotechnological production of lactones by *Yarrowia lipolytica*. *Appl. Microbiol. Biotechnol.* 61:393–404, doi: 10.1007/s00253-002-1207-1.
- Wang, G.Z., C. Xin, T.T. Zhao, L.S. Liang, M.A. Qing-Hua, and G.X. Wang. 2014. Cloning, characterization and expression of alcohol acyltransferase gene which responses to exogenous ethylene in peach fruit. *For. Res.* 27:158–167.
- Wein, M., N. Lavid, S. Lunkenbein, E. Lewinsohn, W. Schwab, and R. Kaldenhoff. 2002. Isolation, cloning and expression of a multifunctional O-methyltransferase capable of forming 2,5-dimethyl-4-methoxy-3(2H)-furanone, one of the key aroma compounds in strawberry fruits. *Plant J.* 31:755–765, doi: 10.1046/j.1365-313X.2002.01396.x.
- Whelan, J.A., N.B. Russell, and M.A. Whelan. 2003. A method for the absolute quantification of cDNA using real-time PCR. *J. Immunol. Methods* 278:261–269, doi: 10.1016/S0022-1759(03)00223-0.
- Xue, J., W. Yue, W. Ping, W. Qiang, Y. Le-Tian, P. Xiao-Han, W. Bin, and C.J.P.O. Jian-Qun. 2012. A primary survey on bryophyte species reveals two novel classes of nucleotide-binding site (NBS) genes. *PLoS One* 7:e36700.
- Yu, M.-L., R.-J. Ma, Z.-J. Shen, and Z.X. Cai. 2010. Research advances in peach germplasm in China. *Jiangsu J. Agr. Sci.* 26:1418–1423.
- Zhang, B., B. Peng, C. Zhang, Z. Song, and R. Ma. 2017a. Determination of fruit maturity and its prediction model based on the pericarp index of absorbance difference (I_{AD}) for peaches. *PLoS One* 12:e0177511.

- Zhang, B., J.Y. Shen, W.W. Wei, W.P. Xi, C.J. Xu, I. Ferguson, and K. Chen. 2010. Expression of genes associated with aroma formation derived from the fatty acid pathway during peach fruit ripening. *J. Agr. Food Chem.* 58:6157–6165, doi: 10.1021/jf100172e.
- Zhang, L., H. Li, L. Gao, Y. Qi, W. Fu, X. Li, X. Zhou, Q. Gao, Z. Gao, and H. Jia. 2017b. Acyl-CoA oxidase 1 is involved in gamma-decalactone release from peach (*Prunus persica*) fruit. *Plant Cell Rep.* 36:829–842, doi: 10.1007/s00299-017-2113-4.
- Zhou, H., Z. Ye, and M. Su. 2018. Effects of MAP treatment on aroma compounds and enzyme activities in flat peach during storage and shelf life. *HortScience* 53:511–523, doi: 10.21273/HORTSCI12631-17.
- Zhou, H., X. Zhang, M. Su, J. Du, X. Li, and Z. Ye. 2020. Effects of ultraviolet-c pretreatment on sugar metabolism in yellow peaches during shelf life. *HortScience* 1:1–8, doi: 10.21273/HORTSCI14554-19.