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The role of volatile compounds and genes that involved in ester biosynthesisduring strawberry fruit (*Fragaria* × *ananassa* Duchesne) development

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Abstract: Strawberry (*Fragaria* × *ananassa* Duchesne) is one of the most crucial berry fruits because of its nutrients and pleasant taste. The present research is to identify volatile compounds, study the biosynthesis pathway during three developmental stages, and *insilico* analysis of lipoxygenase (LOX), alcohol dehydrogenase (ADH), and alcohol acyltransferase (AAT) genes in strawberries. The results indicated that 68 volatile compounds were identified in different developmental stages. The gas chromatography/mass spectrometry showed that the amounts of esters increased during the development of strawberry fruit, while aldehydes and alcohol components decreased during the red stage. The results showed LOX gene expression decreased during fruit development, while ADH and AAT gene expression increased in ripe fruit. It seems that alcohols have a minor contribution to producing the aroma of fruits due to early consumption. Furthermore, esters in the red stage play a significant role in the aroma of ripe fruit. The knowledge of the phytochemical profile of strawberries in the growing stages could be used in different applications of these materials in various fields, including food, medical, and pharmaceutical industries, and production of food essences and natural flavorings, as well as fragrance design.

Key words: alcohols, aldehydes, esters, lipoxygenase pathway, strawberry fruit.

Vloga hlapnih spojin in genov vključenih v biosintezo estrov pri razvoju plodov jagodnjaka (*Fragaria* × *ananassa* Duchesne)

Izvleček: Žlahtni jagodnjak (*Fragaria* × *ananassa* Duchesne) je ena izmed najpomembnejših vrst jagodičevja zaradi vsebnosti hranil v plodovih in dobrega okusa. Namen raziskave je bil *insilico* določiti hlapne spojine in njihovo biosintezo med tremi obdobji razvoja plodov in sicer delovanje genov za lipoksigenazo (LOX), alkohol dehidrogenazp (ADH) in alkohol aciltransferazo (AAT). V različnih razvojnih stopnjah je bilo identificiranih 68 hlapnih spojin. Analiza s plinsko kromatografijo in masno spektrometrijo je pokazala, da se med razvojem plodov jagodnjaka povečuje količina estrov medtem, ko se količina aldehidov in alkoholov zmanjšuje v rdečem obdobju razvoja plodov. Rezultati so pokazali, da se je delovanje genov za LOX zmanjševalo med razvojem plodov, medtem, ko se je delovanje genov za za ADH in AAT povečalo v zrelih plodovih. Izgleda, da imajo alkoholi manjši delež pri tvorbi arome plodov zaradi njihove hitre porabe, imajo pa estri v rdečem stadiju razvoja pommebmo vlogo pri aromi zrelih plodov. Vedenje o fitokemkičnem profile plodov jagodnjaka v rastnih obdobjih bi lahko bilo uporabljeno za različne namene in področja kot so prehrana, medicinska in farmacevtska uporaba, pri pripravi dodatkov hrani, naravnih barvilih kot pri načrtovanju vonjav.

Ključne besede: alkoholi, aldehidi, estri, cikel lipoksigenaze, plod jagodnjaka.

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1 INTRODUCTION

Strawberry is one of the most popular fruit crops cultivated worldwide, valued for its economic importance and consumer demand. In recent years, its production has significantly contributed to national economies, including over three billion dollars annually in the United States (Lu et al., 2020). Plants produce a wide range of volatile compounds (secondary metabolites), including alcohols, aldehydes, esters, ketones, lactones, terpenoids, and apocartenoids, which are not always essential for plant reproduction and survival. Moreover, volatile compounds play a role in protecting the plant against environmental stresses (Effah et al., 2019). Volatile compounds are an indicator of fruit ripening, which affect the aroma of the fruit and play a vital role in the acceptability and choice of fruit by consumers (Christensen et al., 2023). Esters are the most abundant volatile compounds of strawberry fruit (Yan et al., 2018).

The most significant step in the biosynthesis of volatile compounds is the availability of their precursor substrate, the amount and composition of which are strictly controlled during fruit development (Song et al., 2003). The activity of lipoxygenase enzyme (LOX) is one of the fundamental processes during fruit ripening, and its products have essential functions in the biosynthesis of volatile compounds (Li et al., 2014). Esters, alcohols, acids, and carbonyls in fruits are produced from the oxidative reduction of linolenic acid and linoleic acid by the LOX pathway, which mainly forms C6 volatile compounds (Ei Hadi et al., 2013). C6 volatiles are normally produced after chewing herbivore attack. In continuing the lipoxygenase pathway, aldehyde compounds can either be converted into their isomers by isomerases or reduced to alcohol by the alcohol dehydrogenase (ADH) enzyme. ADH can use aldehydes as a substrate, which quickly converts the C6 aldehydes of the ripening fruit into alcohol. ADH enzyme is dependent on NAD and NADP and is responsible for providing precursors that determine the production of the type of ester in strawberries (Yan et al., 2018). Alcohols produced through the lipoxygenase pathway are used as substrates for the enzyme alcohol acyltransferase (AAT) to produce esters (Cumplido-Laso et al., 2012). Furthermore, alcohols act as signaling molecules in stress conditions (biotic and abiotic) and induce the expression of defense genes (Weihua et al., 2020). AAT enzyme catalyzes the biosynthesis of esters, and in fruits with more aroma, AAT enzyme is more active (Beekwilder et al., 2004). AAT enzyme catalyzes the biosynthesis of esters and is responsible for the final stage of ester production, which shows a multifold increase in the gene expression of the Rosaceae family in the middle stages until the ripe fruit (Song et al., 2008).

The biosynthesis of volatile compounds is an important part of the fruit development, and their production during fruit ripening affects its final quality and taste (Li et al., 2021). The economic value of strawberry fruit and the role of its volatile compounds make it indispensable as a valuable fruit in various industries such as food, pharmaceuticals, and cosmetics. This study aims to identify the volatile compounds present at three key developmental stages of strawberry fruit—green, white, and red—using GC-MS. Additionally, it investigates the expression patterns of three key genes involved in volatile biosynthesis, namely LOX, ADH, and AAT, through RT-PCR analysis. Bioinformatic analyses were also conducted to predict the subcellular localization and functional properties of these proteins. We hypothesize that both the composition of volatile compounds and the expression levels of LOX, ADH, and AAT genes vary significantly across fruit developmental stages, correlating with changes in aroma profiles during ripening.

2 MATERIALS AND METHOD

2.1 PLANTS COLLECTION

Strawberry plants (Albion cultivar) were cultivated in a greenhouse (60-75 % humidity) under light and temperature conditions (16 hours of light and 8 hours of darkness at 25-27 °C). Water and nutrient solutions were provided directly to the plant and growth was carried out under controlled conditions (without stress). Then the fruits were harvested in different stages of development (green, white, and red stages). After collection, fruits were frozen at -80 °C for molecular studies.

2.2 ANALYSIS OF VOLATILE COMPOUNDS DURING STRAWBERRY FRUIT DEVELOPMENT

Volatile compounds from strawberry fruits at different developmental stages were analyzed using the Headspace Solid Phase Micro-Extraction (HS-SPME) method, following the protocol described by Kafkaz et al. (2005) with modifications. For each stage (green, white, and red), 10 g of fresh fruit were collected, immediately ground to a fine consistency, and placed into a sealed 20 ml glass vial. A silica fiber coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB) was then inserted into the vial's headspace and exposed to the sample volatiles for 30 minutes at 65 °C to allow adsorption of

compounds onto the fiber. After extraction, the fiber was immediately transferred to the injection port of the Gas Chromatography-Mass Spectrometry (GC-MS) system for thermal desorption.

GC-MS analysis was performed using an HP-5MS capillary column (30 m length × 0.25 mm inner diameter). Helium was used as the carrier gas at a constant flow rate of 1 ml min^{-1}. The oven temperature program began at 50 °C (held for 1 minute), then ramped to 200 °C at 4 °C per minute, followed by a 2-minute hold. The injector and detector temperatures were set at 280 °C. Mass spectra were recorded with an HP 5989A detector, scanning from m/z 40 to 400. Volatile compounds were identified by comparing their mass spectra and retention times with those in the NIST library database.

2.3 THE EXPRESSION OF AAT, ADH, AND LOX GENES DURING STRAWBERRY FRUIT DEVELOPMENT

For molecular investigations, the sequence of AAT KX450225.1, ADH X15588.1, and LOX AJ578035.1 genes in strawberry plants was extracted and then the primer design was done via Oligo Analyzer software (Table 1). Elongation factor 1-alpha gene (EF1) (DAA80492.1) was considered as the reference gene and the stage of receptacle formation was regarded as the control. Extraction of total RNA and reaction of cDNA synthesis of samples were performed using the YektaTajhiz Azma kit. Then the PCR reaction with temperature program 95 °C (10 min), 95 °C (15 S), 60 °C (1min), and 72 °C (15 S) in 40 cycles was performed via CFX96™ Real-Time System Bio-Rad (USA). The ΔΔCt method was used for the statistical analysis of the gene expression obtained in the present research.

2.4 BIOINFORMATICS ANALYSIS OF AAT, ADH, AND LOX PROTEINS OF STRAWBERRY FRUIT

To identify LOX (CAE17327.1), ADH (CAA33613.1), and AAT (AAG13130.1) enzymes in strawberry fruit, protein sequences were extracted from the NCBI database. Sequence alignment was performed by Mega7 software. Protein characteristics and possible location of proteins were predicted by ProtParam and LOCtree3 software. Moreover, the ligand binding site, and second and three-dimensional structures of proteins were predicted using COACH, Phyre2, and I-TASSER software, respectively (Faghani et al., 2022).

3 RESULTS AND DISCUSSION

3.1 IDENTIFICATION OF VOLATILE COMPOUNDS DURING STRAWBERRY FRUIT DEVELOPMENT

GC-MS results showed that 68 volatile compounds were identified in different stages of strawberry development. The number of identified compounds gradually increased during fruit development, which was more in the red stage than in other stages. The 13 compounds identified in the green stage included esters (1 compound), aldehydes (3 compounds), alcohols (3 compounds), terpenoids (2 compounds), alkanes (2 compounds), and other compounds (2 compounds). The most significant compounds in the green stage included trans, 2-hexenal (18.27 %), and myrtenol (1.64 %), which in this stage 68 % of the compounds belonging to aldehydes (Figure 1a). The number of volatile compounds detected in the white stage (Figure 1b) was significantly increased compared to the green stage (30 compounds). Volatile compounds

Table 1: The sequence of primers in Real-Time PCR evaluation.

Target Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
LOX	AGTGTGCTTCACCCGATACA	GTCTTCTCAAGTACCCCACCA
ADH	AGGAGGGATTGTGGAGAGTG	TTATCCTGAGCAGGTCACACA
AAT	ATGAGCGTTACCCCTTGCTT	GCACCCCAGGACTTGAGAAA
EF1	TGAGATGCACCACGAAGCTC	CCAACATTGTCACCAGGAAGT

in the white stage contained esters (6 compounds), aldehydes (6 compounds), alcohols (6 compounds), terpenoids (4 compounds), alkanes (4 compounds), and other compounds (4 compounds) (Supplementary table 1) (70 % alcohols). The most crucial compounds included 3-hexen-1-ol (15.07 %), linalool (4.04 %), myrtenol (9.21 %), and methyl salicylate (2.63 %).

The most important red phase compounds included ethyl hexanoate (24.98 %), gamma-decalactone (12.8 %), linalool (9.10 %), gammado-decalactone (5.28 %), hexanoic acid, hexyl ester (3.33 %) and trans-2-hexenal (2.89 %) (Supplementary table 1). At this stage, 79 % of the identified compounds belonged to esters (Figure 1c). It should be noted that numerous and diverse volatile compounds were observed in different developmental stages of strawberry fruit. Aldehydes, alcohols, and esters were the most abundant volatile compounds in the green, white, and red stages, respectively.

Volatile compounds result from several chemical changes, including hydroxylation, methylation, oxidation/reduction, and acetylation, which are produced through various biological pathways (El Hadi et al., 2013). It has been reported that the volatile compounds of strawberry fruit contain about 350 compounds (Yan et al., 2018), but the HS-SPME GC-MS results of the present study identified 68 compounds, which gradually increased during fruit development (13 compounds in the green stage, 30 compounds in the white stage and 37 compounds in the red stage). The investigation showed that gamma-decalactone and ethyl hexanoate are the dominant volatile compounds in the ripe fruit of strawberry. Esters often play a role in fruit maturation and ripening, and their presence is difficult to detect in the initial stages of fruit development, but the amount of these compounds increases in ripe fruit, which can be different depending on the species and cultivars (Padilla-Jimenez et al., 2019).

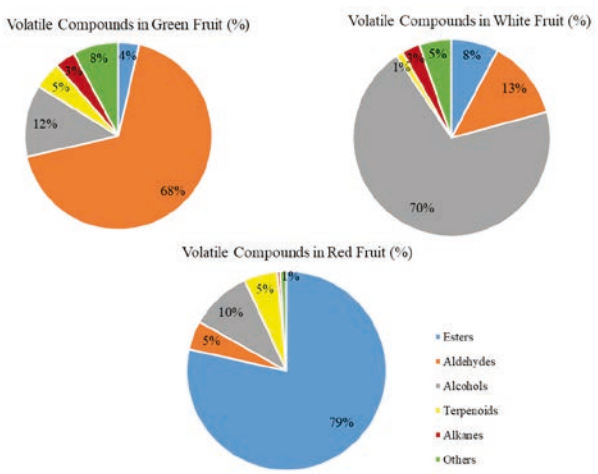

Figure 1: Abundant of volatile compounds identified in three stages of strawberry fruit development. a green stage, b white stage, c red stage

In this study, we observed a clear increase in the number and diversity of volatile compounds as strawberry fruit progressed from the green to the red (ripe) stage, with 13, 30, and 37 compounds identified in the green, white, and red stages, respectively. This trend indicates that fruit ripening is associated with enhanced metabolic activity, particularly in pathways responsible for aroma compound biosynthesis. One of the most notable findings was the significant increase in ester production during ripening, with the highest levels detected in the red stage. This is consistent with previous reports that identified esters such as ethyl butanoate, ethyl hexanoate, and 2-methyl butanoate as major contributors to strawberry aroma, accounting for 20 % to 90 % of total volatiles (Yan et al., 2018). Our results also support the findings of Forney et al. (2000), who emphasized the importance of methyl and ethyl esters in defining the characteristic strawberry scent. Interestingly, we found that aldehyde levels were the highest in the green stage, while alcohol content peaked during the white stage. This pattern may reflect the sequential activation of the LOX and ADH pathways during early fruit development.

Carboxylate esters, such as ethyl acetate, are lipophilic molecules that usually have a low odor threshold. They are widely present in many beverages and food products that provide a pleasant aroma and affect its desirable properties. Ethyl esters, including ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, are different in terms of sensory properties and have been identified in apples and pears (Saerens et al., 2010).

The present research showed that aldehydes and alcohols are the second most volatile compounds in strawberry fruit. Aldehydes compounds such as hexanal, decanal, benzaldehyde, benzeneacetaldehyde, nonanal and beta-cyclocitral, trans-2-hexanal were identified in different stages of strawberry development, and the concentration of aldehydes gradually decreased during fruit ripening. Hexenal, trans-2-hexenal, and cis-3-hexenal-1-ol are important volatile compounds in green stages and unripe fruits, which produces a unique aroma (Xu et al., 2017). Investigations showed that E, Z-2,6-nonadienal and E-2-nonanal are considerable volatile compounds in cucumber, which contributing to the taste of cucumber along with ketones and esters (Chen et al., 2015). Moreover, hexanal and trans-2-hexanal aldehydes are the main components of kiwifruit in the unripe stage (Carcia et al., 2013). The amount of aldehydes depends on the cultivar and the degree of immaturity of the fruit, which gradually decreases during the fruit ripening (Kafkaz et

al., 2017). The produced aldehydes are converted into alcohols to improve the stability of compounds, and finally, alcohols are consumed as substrates for the production of esters (Lu et al., 2021). Therefore, there is a specific coordination between metabolic pathways during fruit development, so the product of one metabolic reaction can be used as a substrate in another pathway (Preeti et al., 2019).

The alcohols produced in strawberry fruit are benzene methanol, myrtenol, methyl chavicol, 3-hexen-1-ol, benzyl alcohol, linalool, and eugenol. Furthermore, alcohol compounds act as signaling molecules in stress conditions (Aguero et al., 2015). Studies showed that the amount of alcohol was constant during the development of the strawberry-Portola cultivar, while the amount of alcohol decreased significantly during fruit ripening in the Cigaline cultivar (Lu et al., 2020). Moreover, the investigations of blackberry (*Rubus ulmifolius* Schitt) showed that aliphatic alcohols increased during fruit development. Thus, the most abundant volatile compounds in ripe fruit include aldehydes, alcohols, ketones, and terpenoids, which indicates the activation of the biosynthetic pathway of these compounds in the final stages of fruit development (Castro et al., 2023). The current research showed different terpenoid compounds such as 1, 8-cineole, neophytadienen, delta-cadiene, hexadecane epoxide, pulegone, and limonene in different stages of strawberry fruit development. 8 alkanes, 3 ketones, 2 lactones, 1 furan, and 1 phenol were observed in this study, which showed different concentrations in different stages of fruit development. These compounds have a significant effect on characteristics, such as the aroma of strawberries (Kafkas et al., 2017).

3.2 THE EXPRESSION OF LOX, ADH AND AAT GENES DURING STRAWBERRY FRUIT DEVELOPMENT

RT-PCR results showed different relative expression patterns for LOX, ADH, and AAT genes in different developmental stages of strawberries (Figure 2). The results indicated that the LOX gene had the highest expression in the green fruit, while the LOX gene expression in the red stage had a significant decrease compared to the previous stages. It appears that as the fruit gets closer to the final stages of its development, the expression of the LOX gene decreases ($p \leq 0.05$) (Figure 2d). ADH and AAT genes showed a significant increase in the relative expression level from the green to the red stage. In other words, the expression levels of ADH and AAT genes increased in the white stage compared to the green stage. The results showed that the expression of ADH and AAT genes gradually increased during fruit development and reached the highest level in the red stage ($p \leq 0.05$) (Figure 2. e, f).

Evaluation of LOX pathway gene expression in different developmental stages of strawberry showed that the LOX gene expression level in the green stage was significantly higher than the other two genes, but expression of LOX gene decreased during fruit development. The findings revealed that the ADH gene had a very low expression level in the green stage, while it reached the highest expression level in the red fruit. Moreover, AAT gene expression increased significantly during strawberry fruit development.

The study of LOX gene expression in apples (Schiller et al., 2015), peaches (Zhang et al., 2010), and kiwifruit (Zhang et al., 2006) showed that the expression of the LOX gene decreased during fruit development, so that the highest expression was observed in the unripe fruit. The analysis of LOX gene expression in pears showed that the expression level was low in the early stages of development, then the expression increased in the later stages. At the stage of fruit ripening, the expression of LOX reached the lowest level according to the changes in aldehydes (Li et al., 2014). Studies have shown that LOX gene suppression completely blocks the biosynthesis pathway in transgenic *Zea ma*ys (Christensen et al., 2023). In addition, LOX gene expression is regulated according to tissue type, developmental stage, phytohormones such as abscisic acid, jasminic acid, salicylic acid, and nitric oxide, and environmental stimuli (injury, water deficit, and pathogen attack) (Chen et al., 2015).

The results demonstrated that the expression of LOX, ADH, and AAT genes varied significantly across strawberry fruit developmental stages, showing a dynamic correlation with the profile of volatile compounds detected by GC-MS. Notably, LOX expression was the highest in the green stage and decreased sharply toward the red stage. This trend aligns with the observed accumulation of aldehydes in the green fruit, suggesting that LOX is actively involved in the early generation of aldehyde volatiles during the initial stages of fruit development. Similar LOX expression patterns have been reported in other fruits, supporting its role in the lipoxygenase pathway for aldehyde biosynthesis (Lu et al., 2018, Iaria et al., 2012). In contrast, ADH and AAT gene expression levels increased progressively during Strawberry fruit development, peaking at the red stage. This pattern closely mirrors the increase in alcohols in the white stage and esters in the red stage, as identified by GC-MS. ADH catalyzes the reduction of aldehydes into alcohols, which serves as a critical step toward ester formation. AAT, which uses alcohol and acyl-CoA substrates to form esters, showed a strong upregulation in the red stage—corresponding

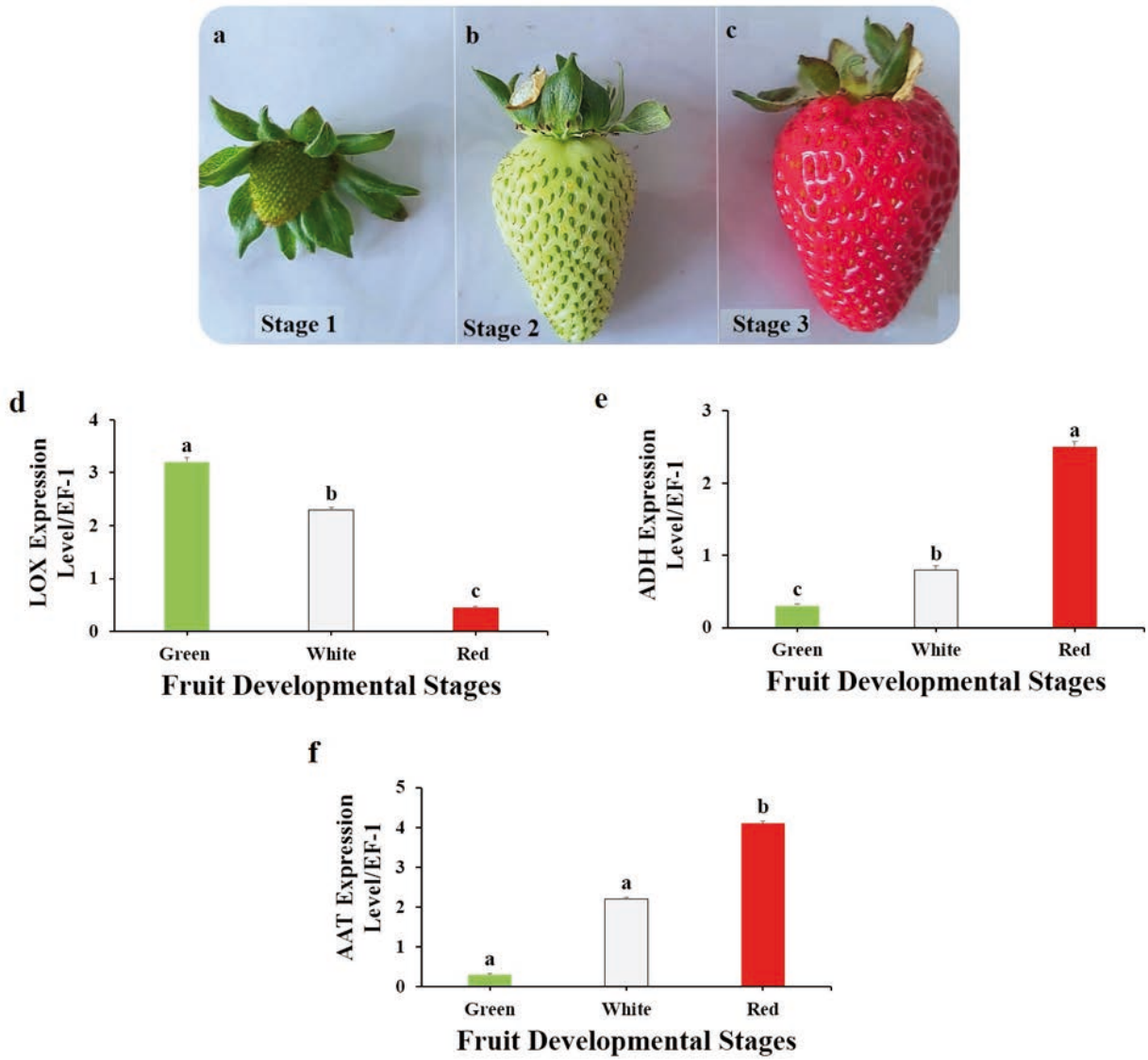

Figure 2: The relative expression pattern of genes involved in the biosynthesis of volatile compounds in three developmental stages of strawberry fruit (a green stage, b white stage, and c red stage). d, e, f is the expression of LOX, ADH, and AAT genes, respectively. Different letters in each column indicate significant differences at the $p \leq 0.05$ level

with the highest ester accumulation at fruit ripening. The dependence of ADH expression pattern on developmental stages has also been observed in many fruits, including apricot (Gonzalez-Aguero et al., 2009), pears (Gai-hua et al., 2017), and melons (Jin et al., 2016). The increased accumulation of ADH expression can probably be due to changes in cytoplasmic pH and cytoplasmic ion concentration caused by membrane leakage (Speirs et al., 1998). Investigations indicated that overexpression and silencing of the ADH gene in tomatoes cause a significant change in the content of alcohols (especially hexanol and 3-z-hexanol), respectively (Manriquez et al., 2006). ADH gene expression is induced by the abscisic acid hormone and various environmental stresses such as low temperature, drought, salinity, and mechanical damage (Davik et al., 2013). The transcript level of this gene in corn increased rapidly under oxygen deficiency conditions and the production of alcohols occurred through fermentation and then decreased under anaerobic conditions (Zeng et al., 2020).

The metabolism of esters is controlled by environmental factors (light and temperature), transcription fac-

tors, and AAT gene expression during fruit development (Zhou et al., 2021). Studies in bananas (Beekwilder et al., 2004), apples (Li et al., 2006), apricot (Gonzalez-Aguero et al., 2009), papaya (Balboltin et al., 2010), peach (Zhang et al., al., 2010) and pear (Chen et al., 2020) showed that the expression of AAT gene in these fruits starts from the initial stages of fruit development (low expression) and the maximum expression of this gene is observed in the final developmental stages of fruit. Therefore, it can be concluded that the lack of esters production in the initial stages is due to the lack of enzyme activity (Chen et al., 2020). Fruits produce various types of esters, the variety of which depends on the substrate specificity of the relevant enzymes (Liu et al., 2019). AAT enzymes use a variety of alcohol and acyl substrates available to form esters (Perez et al., 2002). Overexpression of AAT in transgenic tobacco plants leads to a significant increase in methyl benzoate concentration, which indicates that methanol and benzoyl-CoA were used as substrates (Li et al., 2008).

The observed increase in AAT gene expression during fruit ripening, particularly in the red stage, suggests that its regulation is tightly linked to developmental and hormonal signals. This upregulation coincides with the highest levels of ester production detected by GC-MS, highlighting AAT's central role in determining the final aroma profile of ripe strawberry fruit. Our findings are consistent with previous reports in apricot and other fruits, where suppression of AAT expression significantly reduced ester biosynthesis (Zhou et al., 2021). The accumulation of esters in the ripening stages may also be influenced by physiological processes such as cell wall degradation, which releases methanol and other alcohols serving as substrates for AAT activity (Beekwilder et al., 2004). This aligns with our observation that alcohol content increases before esters, suggesting a stepwise activation of the volatile biosynthetic pathway.

In addition to substrate availability, the regulation of AAT expression itself appears to be controlled by hormonal signaling and transcription factors. Ethylene and abscisic acid—both known to increase during ripening—are likely contributors to the induction of AAT in the red stage (Ortiz et al., 2010; Cumplido-Laso et al., 2012). Transcription factors such as ERFs and MYBs have also been shown to regulate AAT and other aroma-related genes (Wang et al., 2023). The activation of AAT by ERF overexpression in apples (Li et al., 2020) and the influence of MYBs on aldehyde biosynthesis (Lu et al., 2020) suggest that transcriptional regulation is a critical mechanism behind the coordinated rise of volatiles during ripening. Furthermore, recent evidence suggests that small RNAs such as miRNAs also participate in post-transcriptional control of aroma biosynthetic genes (Singh et al., 2021). While not directly assessed in our work, this layer of regulation may contribute to the fine-tuning of gene expression during fruit development.

3.3 BIOINFORMATICS INVESTIGATION OF GENES INVOLVED IN THE BIOSYNTHESIS OF STRAWBERRY FRUIT VOLATILE COMPOUNDS

The coding region of the LOX sequence in the strawberry plant encodes 844 amino acids with a molecular weight of 100,477 Da. The alignment of the LOX protein sequence in the Rosaceae family indicates the similarity and high conservation of the amino acids. The Pfam software has predicted the sequence of this protein as belonging to the Lipoxygenase family, which consists of two domains (PLAT/LH2 and Lipoxygenase). The second structure of strawberry LOX protein consists of 27 % alpha helix and 29 % beta sheets (98 % accuracy). The results of COACH software revealed that LOX protein can bind to fatty acids. The protein binding site with higher C-Score includes amino acids at position 293, 294, 297, 394, 532, 535, 536, 540, 541, 545, 579, 582, 587, 594, 598, 738, 742, 748, 795 and it is 844 (Table 2). The prediction of the intracellular location of LOX protein showed that this protein is located in the chloroplast.

Alignment of the ADH protein sequence in the Rosacea family indicated that this protein is highly conserved among co-family species. This protein belongs to the Zinc-binding dehydrogenase family. The secondary structure of ADH protein consists of 26 % alpha helix, 27 % beta sheets, and 4 % Tm helix. The results have determined that ADH protein can bind to NADH. The protein binding site with a higher C-Score includes amino acids at positions 48, 49, 50, 178, 182, 203, 204, 205, 206, 207, 227, 228, 232, 272, 273, 275, 278, 296, 297, 298, 321, 322, 323 and 373 (Table 2). ADH protein is mainly located in the cytosol and the non-secretory pathway of the cell.

AAT sequence alignment indicated low similarity and conservation in the Rosacea family. The sequence of this protein is predicted from the Transferase family. The secondary structure of the AAT is composed of 31 % alpha helix, 23 % beta sheets, and 4 % TM helix. The 3D structure (Figure 3) is modeled on the most likely organism related to *Sorghum bicolor* (L.) Moench and the transferase family. Moreover, the results showed that AAT interacts with NADH. The binding site with a higher C-Score includes amino acids at positions 36, 37, 38, 157, 305, 376, 402, 406, 407, 408, and 410 (Table 2). Prediction of the intracellular location showed that AAT is located in the cytosol and the non-secretory pathway.

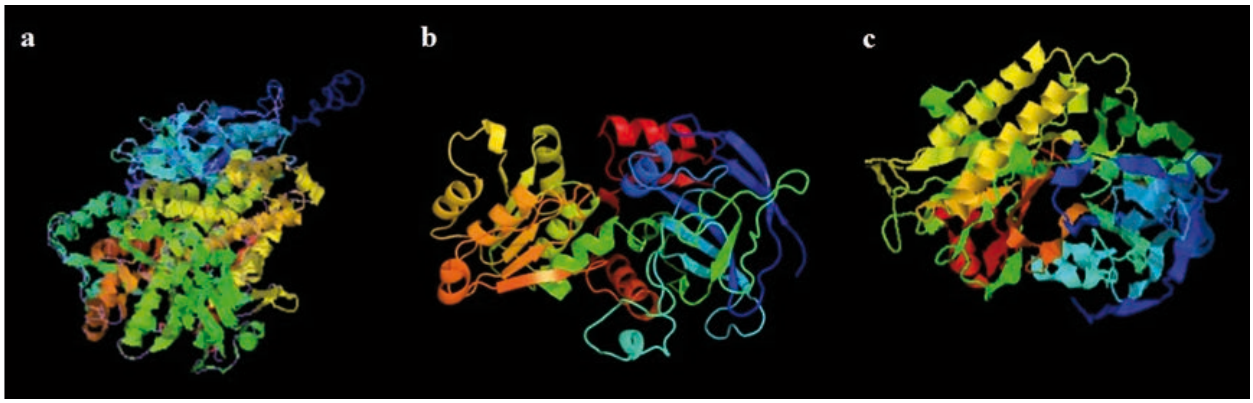

Figure 3: Three-dimensional protein structure predicted by I-TASSER software, a LOX protein, b ADH protein, c AAT protein.

Bioinformatics analysis showed that the LOX enzyme belongs to the family of nonheme iron-containing dioxygenases and are found in plants, animals, and fungi (Viswanath et al., 2020). Plant LOX sequence has a highly conserved catalytic site, lipoxygenase domain (C-terminal), and PLAT/LH2 motif (N-terminal), which cooperating with the lipid bilayer. The catalytic site region has several conserved histidine amino acids that play a role in ligand binding (Wang et al., 2019). The studies of different plants showed that the cellular location of LOX was observed in the chloroplast and cytoplasm, probably due to the chromosomal duplication and evolution of this gene (Guo et al., 2017). Several MYC and MYB motifs were identified in the LOX of plants, and these regulatory elements of the promoter region play an essential role in regulating gene expression in stress conditions (Liu et al., 2020). Investigations presented that LOX is coded by multiple gene families and is active in different cell organelles (Hou et al., 2015). Based on the primary structure and sequence similarity, plant LOX13 is classified into two subfamilies (type I and type II) where type I lacks a temporary peptide (Kang et al., 2021). Exon and intron studies indicated that LOX has 6-9 introns and different exon lengths. This property can probably be due to the loss or gain of introns during evolution, which has created a specific functional role for LOX (Liu et al., 2020).

The ADH enzyme belongs to the large family of dehydrogenases/reductases and plays a crucial role in converting aldehydes to alcohols during fruit ripening. It is a glycoprotein with diverse physiological roles, and

Table 2: Protein characteristics that predicted by I-TASSER and COACH software

protein	PDB Hit	Organism	Ligand	C-score	Z-score	Lig binding site
LOX	1HSS	*Triticum aestivum* L. alpha-amylase	POL	0.9	2.9	31,71,111,153,187,189,190,192,193,214,216,217,233,295,296,303
	3WN6	*Oryza sativa* L. alpha-amylase	POL	0.9	2.6	71, 154,187,189,190,193,214,217,233, 296
	1HSS	*Triticum aestivum* alpha-amylase	POL	0.8	2.5	111,154, 189192,193,214,216,217,233,295
ADH	1YP4	*Solanum lycopersicum* L.	GLC	0.9	2.6	71,111,154,187,189,190,192,193,214,216,217,233,295,296
	1YP4	*Solanum lycopersicum*	GLC	0.8	2.5	71,111,154,187,189,190,192,193,214,216,217,233,295,296
	1YP3	*Solanum lycopersicum*	GLC	0.8	2.5	71,111,154,157,189,190,214,216
AAT	1YP4	*Solanum lycopersicum*	GLC	0.9	3.0	93,94,95,96,110,111,174,184,185,186,187,190,210,211,212,213,248,249,264,265,296,323,345
	1YP4	*Solanum lycopersicum*	GLC	0.9	2. 9	93,94,95,96,110,111,174,184,185,186,187, 190,210,211,212,213
	1YP3	*Solanum lycopersicum*	GLC	0.8	2.8	93,94,95,96,110,111,174,184,185,186,187,210,211,212

it is classified into three major classes—Class I, II, and III—based on differences in structure, function, and cofactor specificity (such as NAD^+ or $NADP^+$) (Jornvall et al., 1995). Class I ADHs are the most common in plants and are primarily involved in fermentative pathways and aroma-related metabolism. Class II and III ADHs are typically associated with detoxification processes or more specialized metabolic functions. Structurally, ADH proteins are composed of four conserved subdomains or cores—A, B, C, and D—which together form the active enzyme. Core A typically contains binding sites for cofactors like NAD^+/$NADP^+$. Core B includes the catalytic zinc-binding site. Core C and D contribute to substrate binding and overall protein stability.

All the amino acids participating in the coreA were placed in the catalytic domain (50 % neutral amino acids and 33 % hydrophobic). Core C is a significant functional unit surrounded by four cysteine amino acids (Goihberg et al., 2007). Studies indicated that this enzyme contains zinc-binding (206-340 amino acids), NADPH-binding, and GroES-like (163-36 amino acids) domains in plants (Hayward 2004). Phylogenetic studies determined that zinc-binding domains in the ADH protein of each plant family have about 80 % similarities, which are known as GHE(X)2G(X)5G(X)2V pattern (Elleuche et al., 2014). ADH enzymes have 8-10 exons and 7-9 introns, which were changed during evolution to adapt to environmental changes. Furthermore, the cellular location of this protein was identified in the cytoplasm of different plants (Borras et al., 2014).

AAT enzyme belongs to acyltransferases family, commonly known as BAHD (Bontpart et al., 2015). The proteins belonging to this family have several common motifs, such as the HXXXD motif, which is highly conserved in higher plants and yeasts and can play a role in the catalytic mechanism (D'Auria, 2006; Molina and Kosma, 2015). The conserved sequence DFGWG is located near the C-terminal and maintains the structural integrity of the enzyme (El-Sharkawy et al., 2005). LXXYYPLAGR is the third conserved motif (less conservation compared to other motifs) which is located at the N-terminal of the sequence and is used in acyltransferases involved in the synthesis of fruits esters (Balbontin et al., 2010). The phylogenetic analysis determined that the AAT enzyme is classified into five clades where species with common motifs, such as the HXXXD domain and DFGWG, are placed in one clade (Tuominen et al., 2011).

4 CONCLUSIONS

This study analyzed volatile compound profiles and gene expression patterns during three developmental stages of strawberry fruit (green, white, and red). GC-MS results revealed a progressive increase in the number of volatile compounds, with esters peaking in the red stage. Aldehyde levels were the highest in green fruit, while alcohols peaked in the white stage. Gene expression analysis showed that **LOX** was highly expressed in the green stage, while **ADH** and **AAT** were significantly upregulated in the red stage. These expression patterns corresponded with changes in volatile compound composition. Bioinformatic predictions suggested that LOX, ADH, and AAT proteins localize to the chloroplast, cytosol, and non-secretory pathways, respectively. Our integrated analysis of gene expression and metabolite profiling provides insight into the temporal coordination of key enzymes involved in flavor development in strawberry. These data not only confirm findings from other fruit systems but also highlight the potential of manipulating LOX, ADH, and AAT expression to enhance fruit aroma quality in breeding programs. This study enhances understanding of aroma biosynthesis during strawberry ripening and provides insights valuable for improving fruit flavor and potential applications in food, pharmaceutical, and fragrance industries.

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