



Article

Volatiles and Transcriptome Profiling Revealed the Formation of ‘Taro-like’ Aroma in the Leaf of Pumpkin (*Cucurbita moschata*)

Siying Zhao ^{1,2,†}, Yuehan Qiu ^{1,2,†}, Jianning Luo ^{1,3}, Wenwen Wang ⁴, Haibin Wu ^{1,3}, Xiaoxi Liu ^{1,3}, Gangjun Zhao ^{1,3} , Hao Gong ^{1,3}, Xiaoming Zheng ^{1,3}, Yujuan Zhong ¹, Xian Yang ^{2,*} and Junxing Li ^{1,3,*}

¹ Guangdong Key Laboratory for New Technology Research of Vegetables, Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China

² College of Horticulture, South China Agricultural University, Guangzhou 510640, China

³ Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou 510640, China

⁴ Agilent Technologies (China) Co., Ltd., Beijing 100102, China

* Correspondence: yangxian@scau.edu.cn (X.Y.); lijunxing@gdaas.cn (J.L.)

† These authors contributed equally to this work.

Abstract: ‘Taro-like’ odor is an important economic trait of pumpkin species. The metabolic and molecular bases of this aromatic trait remain largely unexplored. Therefore, in this study, gas chromatography-mass spectrometry, GC-Olfactometry, and RNA-seq technology were used to illuminate the differential volatile compounds, the key volatile compounds, and differentially expressed genes (DEGs) in leaves from two pumpkin samples. Eight volatile compounds, including (E)-2-nonenal, 3-octanol, 2-ethyl-1-hexanol, 1-nonanol, α -terpineol, 2,3-pentanedione, caryophyllene, and 2-acetyl-1-pyrroline, were only detected in the sample with ‘taro-like’ aroma. Moreover, the variable importance in projection scores of all the above eight volatile compounds were >1.0 using PLS-DA analysis. The compounds 2-acetyl-1-pyrroline, 3-octanol, 1-nonanol, and (E)-3,7-dimethyl-2,6-octadienal were identified as the key contributors using GC-Olfactometry analysis. It was determined that 2-acetyl-1-pyrroline might play a significant role in ‘taro-like’ aroma. Furthermore, most of the differential volatile compounds were derived from fatty acids, and the DEGs were also involved in the pathways related to degradation, metabolism, and biosynthesis of fatty acids. Moreover, five genes involved in the accumulation of 2-acetyl-1-pyrroline showed differential expression, and their expression trends were consistent with 2-acetyl-1-pyrroline. This study offers the basis for further studies on the mechanism of ‘taro-like’ aroma in pumpkins.

Keywords: pumpkin; ‘taro-like’ aroma; volatiles; transcriptome



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1. Introduction

Flavor is the core quality trait for horticultural crops, while aroma is an important factor of flavor [1]. In addition, aromatic traits play important roles in life activities, such as pollination, fertilization, seed dispersal, and resistance [2–4]. Therefore, aromatic traits need to be well investigated. Pumpkin is a vital cucurbitaceous vegetable that is widely cultivated and consumed all over the world. However, studies on the aromatic traits of pumpkin are limited, which is mainly due to the complexity of aroma and lack of resources pertaining to unique aroma. In recent years, the pumpkin germplasm resource (*Cucurbita moschata*) with ‘taro-like’ aroma has gained attention because its leaves and fruit have a pleasant aroma [5,6]. In this case, the germplasm resource with a ‘taro-like’ aroma has become an ideal model to study the aromatic characteristics of pumpkin.

Aroma trait is very complex and is determined by the composition, content, threshold, and interaction of volatile compounds [7]. Headspace-solid phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS) has been commonly used to identify and analyze volatile compounds, due to its speediness, good selectivity, and high sensitivity [8]. Until now, there have been few studies conducted on the aroma quality

of pumpkin fruit and their products, rather than leaf [9,10]. To the best of our knowledge, the data available on ‘taro-like’ aromatic traits is limited. In a previous study, we found that 2-acetyl-1-pyrroline, 2-acetylpyrrole, 1-undecanol, and methyl salicylate were four unique compounds in the leaves of pumpkin with ‘taro-like’ aroma [5]. Additionally, 2-acetyl-1-pyrroline made the greatest contribution to the ‘taro-like’ aroma of pumpkin fruit from sample No. 44 [6]. According to a previous report, volatiles will differ in both fruit and leaf [11]. Additionally, judgment of the aromatic traits of leaves can be carried out in the seedling stage, which plays a critical role in the subsequent screening and identification of germplasm resources. Therefore, the volatile compounds and the key contributors should be analyzed to reveal the ‘taro-like’ aroma of pumpkin leaf.

In addition to the analysis of metabolites, the screening of genes related to the anabolism of volatile compounds is also urgently needed to analyze aromatic traits. A previous study reported that the comprehensive analysis of metabolites and genes might be helpful for the screening and identification of aroma-associated genes [12]. Zheng et al. [2] identified that multiple genes played important roles in the tea volatile heterosis using RNA-Seq technology and GC-MS. In recent years, the transcriptome profiles of different pumpkin species have been evaluated using RNA-Seq technology, such as *Cucurbita pepo* [13] and *C. moschata* [14,15]. However, previous studies on pumpkins have mainly focused on growth and development [16], nutrient composition [17], stress response [18], and so on, and the volatile compounds basis and transcriptomic analysis of aromatic traits in pumpkins have not been studied. Therefore, it is necessary to identify the genes associated with aromatic traits in pumpkins using high-throughput sequencing technology, which might provide a reference by which to study the complex metabolic process of volatile compounds production in pumpkins.

To comprehensively analyze the ‘taro-like’ aroma, it is essential to investigate the volatile compounds and genes for metabolic and molecular bases. Thus, in this study, the leaves from two kinds of *C. moschata* germplasms, including No. 312, with ‘taro-like’ aroma, and No. 18292, without ‘taro-like’ aroma, were used to perform the volatiles and transcriptome analyses, which will provide the information for the in-depth research. First, GC-MS was used to assess the composition and content of volatile components, and then the key volatile compounds were identified using the differential volatile compounds and GC-Olfactometry (GC-O) analysis. Second, comparative transcriptomic analysis was conducted on the leaves to detect the differentially expressed genes. At last, correlations among the volatile compounds and genes were analyzed to explore the possible molecular mechanisms of the production of ‘taro-like’ aromas. This study provides a thorough understanding of how pumpkin leaves produce the ‘taro-like’ aroma.

2. Materials and Methods

2.1. Plant Materials

Two kinds of *C. moschata* germplasms, including No. 312, with ‘taro-like’ aroma, and No. 18292, without ‘taro-like’ aroma, were used. These two samples (NO.312 and NO.18292) were from the same parents. The female parent and male parent absented and presented ‘taro-like’ aroma, and they were hybridized, separated and bred. These two samples and their parents were obtained from the Vegetable Research Institute, Guangdong Academy of Agricultural Sciences. The two samples (NO.312 and NO.18292) were inbred lines with stable traits and exhibited different aromas. The samples were planted on 9 August 2018 in fields of the Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China. The same cultivation procedures were used for all the plants. The spacing between plants was 60 cm between rows and 100 cm between columns. Fresh leaves from both the samples at their top five nodes were selected on 20 September 2018. Three biological replicates of each experimental group were used, and each biological replicate came from an individual plant. All the samples were immediately frozen in liquid nitrogen, and then stored at -80°C . Moreover, the specific samples used for the different analyses were derived from the same sample pool.

2.2. Standards

C₇–C₄₀ saturated alkane mixture, 1-hexanol ($\geq 99.9\%$), linalool ($\geq 97.5\%$), hexanal ($\geq 95.0\%$), (*E*)-2-hexenal ($\geq 97.0\%$), (*E,E*)-2,4-heptadienal (90%), (*E*)-2-nonenal ($\geq 95.0\%$), decanal ($\geq 95.0\%$), nonanal ($\geq 95.0\%$), benzeneacetaldehyde ($\geq 90.0\%$), α -ionone ($\geq 96.0\%$), β -ionone ($\geq 97.0\%$), methyl salicylate ($\geq 99.0\%$), β -myrcene ($\geq 90.0\%$), and limonene ($\geq 99.0\%$) were bought from Sigma-Aldrich (St. Louis, MO, USA). Benzaldehyde ($\geq 99.5\%$) and benzyl alcohol ($\geq 99.5\%$) were purchased from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China); 2-acetyl-1-pyrroline (10% *w/w* in Toluene) was obtained from TRC (Canada).

2.3. Extraction of Volatile Compounds

Samples were freeze-dried in a vacuum and ground into powder using a tube mill control (IKA, Staufen, Germany). The volatile compounds were extracted via HS-SPME technique. The powdered sample (one gram) was accurately introduced into a 150-mL vial. The vial was sealed and equilibrated at 70 °C for 5 min. The SPME fiber with 2 cm long and extraction conditions were the same as that used in the previous study [5].

2.4. GC-MS Analysis

The desorption and GC-MS conditions were performed analogous to the previous study [5]. Agilent 7890B gas chromatography with an Agilent 5977A mass spectrometer detector (Agilent Technologies, Santa Clara, CA, USA) was used for the detection of volatile compounds.

2.5. Data Preprocessing and Statistical Analyses

The identification of volatile compounds was carried out according to previous reports [5,6]. Mass spectra library NIST 17 was used to provide the mass spectra and retention index (RI) values of volatile compounds. Lastly, 17 available standard substances were also employed for additional verification and were examined in the same capillary column. Additionally, all the entities were filtered using the following parameters: minimum absolute abundance (5000 counts); frequency values (over 60.0% of the replicates in one sample group); and coefficient of variation (no more than 25%), which could ensure the validity of volatile compounds. The unsupervised principal component analysis (PCA) was conducted based on the all identified volatile compounds using MetaboAnalyst (version 4.0, <https://www.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml>, accessed on 24 November 2019). Then, the significant differential volatile compounds were further sorted based on one-way analysis of variance (ANOVA) ($p < 0.01$) and fold change ($FC \geq 1.5$) using SPSS v17 (SPSS Inc., Chicago, IL, USA). Finally, depending on the differential volatile compounds, MetaboAnalyst was used to perform partial least square-discrimination analysis (PLS-DA), PLS-DA variable importance in projection (VIP) scores, and hierarchical clustering analysis (HCA).

2.6. GC-Olfactometry Analysis

The GC-O was carried out and the pivotal volatile compounds were determined according to the previously disclosed method, using the leaf of pumpkin germplasm No. 312 [6]. The HS-SPME and GC-MS conditions were identical to those mentioned above. One experienced assessor was employed for this analysis (repeated twice). First, all odor impressions were discovered by analyzing the undiluted volatile compounds. Then, utilizing split mode, the four stepwise dilution series 1:3, 1:9, 1:27, and 1:81 were applied to analyze the odor impressions. The odor intensities were represented by different numbers (0–3).

2.7. cDNA Library Construction and RNA-Sequencing

Total RNA was extracted from each sample utilizing Trizol reagent following the manufacturer's instructions (Invitrogen, Waltham, MA, USA) and quantified using NanoDrop 2000 (Thermo, Waltham, MA, USA). The RNA integrity was assessed using a RNA Nano 6000 Assay Kit, Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Biomarker Technologies carried out the library establishment and RNA-seq (Beijing, China). A total amount of 1 µg RNA per sample was used. An NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) was utilized for the construction of the sequencing library. After PCR amplification, Agilent Bioanalyzer 2100 system was used to evaluate the library quality. All the libraries were sequenced on Illumina HiSeq X-ten platform. The data were uploaded to the CNGB Sequence Archive (CNSA) of China National Genbank DataBase (CNGBdb) (accession number CNP0002555).

2.8. Mapping of Reads to Reference Genome and Analysis of the Genes

The clean reads were mapped to the reference genome sequence (*Cucurbita moschata*) (http://cucurbitgenomics.org/ftp/genome/Cucurbita_moschata/v1/, accessed on 25 March 2019). On the basis of the reference genome, reads that had a perfect match or a single mismatch were further annotated and examined. The gene functions were annotated using the following databases: nr (NCBI nonredundant protein sequences); Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of Proteins); Swiss-Prot (a manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); eggNOG (Evolutionary Genealogy of Genes); and GO (Gene Ontology). Then, the gene expression levels were calculated using fragments per kilobase of transcript per million fragments mapped. Finally, the significantly differentially expressed genes (DEGs) were identified using the false discovery rate (FDR) < 0.01 and Fold Change ≥ 2. The DEGs were further analyzed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis to investigate their potential functions.

2.9. Validation of DEGs by Quantitative Real-Time PCR Analysis

In order to confirm the accuracy of transcriptomic data, 18 DEGs (9 up-regulated and 9 down-regulated) were selected for qRT-PCR analysis. The total RNA was extracted using UNIQ-10 Trizol Total RNA Extraction Kit (Sangon Biotech, Shanghai, China) and converted into cDNA for qRT-PCR using Maxima Reverse Transcriptase (Thermo Scientific). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The selected genes and their primer sequences are provided in Supplementary Table S1. qRT-PCR was performed using StepOne PLUS fluorescence quantitative PCR instrument (ABI, Foster, CA, USA) and 20-µL reaction volume using 2X SG Fast qPCR Master Mix (High Rox, B639273, BBI, ABI). Three biological and three technical replicates of each sample were conducted for analysis, and the results were calculated using $2^{-\Delta\Delta CT}$. The relative expression was shown as log₂ fold change (No. 312 vs. No. 18292).

3. Results

3.1. Identification of Volatile Compounds and Chemometric Analysis

The GC-MS was used to detect the compositions and contents of volatile compounds. A total of 59 volatile compounds were identified in sample No. 312, including 21 aldehydes, 13 alcohols, 9 ketones, 11 hydrocarbons, 2 esters, and 3 heterocyclic compounds (Table 1), and 51 volatile compounds were identified in sample No. 18292, including 20 aldehydes, 9 alcohols, 8 ketones, 10 hydrocarbons, 2 esters, and 2 heterocyclic compounds (Table 1).

Table 1. Volatiles identified in the leaves of pumpkin sample No. 312 with ‘taro-like’ aroma and sample No. 18292 without ‘taro-like’ aroma.

No.	Volatiles	Retention Index (RI)			No. 18292:No. 312
		RI-Practical	RI-NIST 17 Library ^a	RI-STD ^b	Ratio of Peak Area
Aldehydes					
A1	(E)-2-pentenal	745	754	-	1.00:0.98
A2	hexanal	795	800	797	1.00:0.95
A3	2-hexenal	850	851	852	1.00:0.96
A4	(E)-4-heptenal	894	900	-	1.00:1.13
A5	heptanal	897	901	-	1.00:2.11
A6	methional	903	907	-	1.00:2.67
A7	(E,E)-2,4-hexadienal	907	911	-	1.00:0.81
A8	benzaldehyde	961	962	966	1.00:1.00
A9	octanal	999	1003	-	1.00:1.88
A10	(E,E)-2,4-heptadienal	1008	1012	1014	1.00:1.44
A11	benzeneacetaldehyde	1043	1045	1048	1.00:2.27
A12	nonanal	1100	1104	1100	1.00:1.60
A13	(E,E)-2,4-octadienal	1107	1115	-	1.00:1.26
A14	(E,Z)-2,6-nonadienal	1150	1155	-	1.00:2.61
A15	(E)-2-nonenal	1157	1162	1162	0:1.00
A16	decanal	1202	1206	1204	1.00:2.96
A17	2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde	1221	1220	-	1.00:1.17
A18	2,2,6-trimethyl-1-cyclohexene-1-acetaldehyde	1258	1254	-	1.00:1.39
A19	(E)-3,7-dimethyl-2,6-octadienal	1264	1270	-	1.00:1.85
A20	undecanal	1303	1307	-	1.00:3.43
A21	dodecanal	1405	1409	-	1.00:4.77
Alcohols					
B1	1-penten-3-ol	-	684	-	1.00:0.54
B2	(Z)-2-penten-1-ol	758	767	-	1.00:0.55
B3	1-hexanol	861	868	864	1.00:0.76
B4	cyclohexanol	884	880	-	1.00:0.99
B5	phenol	970	980	-	1.00:2.24
B6	1-octen-3-ol	975	980	-	1.00:0.96
B7	3-octanol	992	994	-	0:1.00
B8	2-ethyl-1-hexanol	1023	1030	-	0:1.00
B9	benzyl alcohol	1032	1036	1041	1.00:0.76
B10	linalool	1096	1099	1103	1.00:1.83
B11	1-nonanol	1166	1173	-	0:1.00
B12	α -terpineol	1196	1189	-	0:1.00
B13	1-decanol	1266	1273	-	1.00:1.35
Ketones					
C1	2,3-pentanedione	-	698	-	0:1.00
C2	3-penten-2-one	728	733	-	1.00:0.54
C3	2,2,6-trimethyl-cyclohexanone	1035	1036	-	1.00:1.30
C4	acetophenone	1065	1065	-	1.00:0.43
C5	(E,E)-3,5-octadien-2-one	1065	1073	-	1.00:1.77
C6	2,6,6-trimethyl-2-cyclohexene-1,4-dione	1143	1144	-	1.00:0.72
C7	(-)-carvone	1245	1242	-	1.00:3.87
C8	α -ionone	1422	1426	1428	1.00:1.10
C9	trans- β -ionone	1479	1486	1486	1.00:1.10
Hydrocarbons					
D1	β -myrcene	985	991	985	1.00:1.77
D2	p-cymene	1024	1022	-	1.00:0.79
D3	limonene	1029	1030	1032	1.00:1.04
D4	γ -terpinene	1057	1060	-	1.00:0.99
D5	naphthalene	1190	1182	-	1.00:0.97
D6	tridecane	1295	1300	-	1.00:1.54
D7	1-tetradecene	1387	1392	-	1.00:1.24
D8	tetradecane	1395	1400	-	1.00:1.40
D9	caryophyllene	1428	1419	-	0:1.00
D10	pentadecane	1495	1500	-	1.00:1.21
D11	α -farnesene	1499	1508	-	1.00:6.55
Esters					
E1	methyl salicylate	1193	1192	1194	1.00:1.25
E2	dimethyl phthalate	1445	1455	-	1.00:1.30
Heterocyclic compounds					
F1	2-ethyl-furan	-	703	-	1.00:0.98
F2	2-acetyl-1-pyrroline	917	922	920	0:1.00
F3	(R)-5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	1537	1532	-	1.00:1.34

^a RI-NIST 17 Library: the published retention index of compounds in NIST 17 library. ^b RI-STD: retention index of standard substance analyzed on the same column was calculated using the homologous series of n-alkanes.

Then, the PCA was employed to gain a preliminary overview of differences/similarities between the two samples based on the compounds shown in Table 1 (Figure 1A). Figure 1A shows the scores scatter plot of the two first principal components (PC1, PC2), displaying the differences between the two samples. The first two principal components explained 85.30% of the total variance, and PC1 and PC2 explained 68.10% and 17.20% of the variances, respectively. In this study, No. 312 and No. 18292 were mainly separated based on PC1. The two samples were not randomly distributed and were divided into two regions. No. 312 was situated on the positive region of PC1 in the scores scatter plot and was isolated from No. 18292, which was mainly located on the negative area of PC1. Moreover, the Supplementary Figure S1 shows the corresponding loadings plot of 59 volatile compounds, demonstrating the relative importance of each volatile and the correlation between the volatile compounds and samples. The loading plots show the distribution of volatile compounds, which corresponds to the distribution of sample points in the scores diagram. Most of the volatile compounds exhibited a large Euclidean distance from origin and were thought to be crucial for discrimination. The volatile compounds, including (*E,E*)-2,4-hexadienal (A7), 1-penten-3-ol (B1), (*Z*)-2-penten-1-ol (B2), 1-hexanol (B3), benzyl alcohol (B9), 3-penten-2-one (C2), acetophenone (C4), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (C6), and *p*-cymene (D2), were associated with No. 18292, and indicted that these volatile compounds had a major effect on the discrimination of No. 18292. Most of the volatile compounds lay in the positive region of PC1, correlated with No. 312, and were considered important for the identification of No. 312.

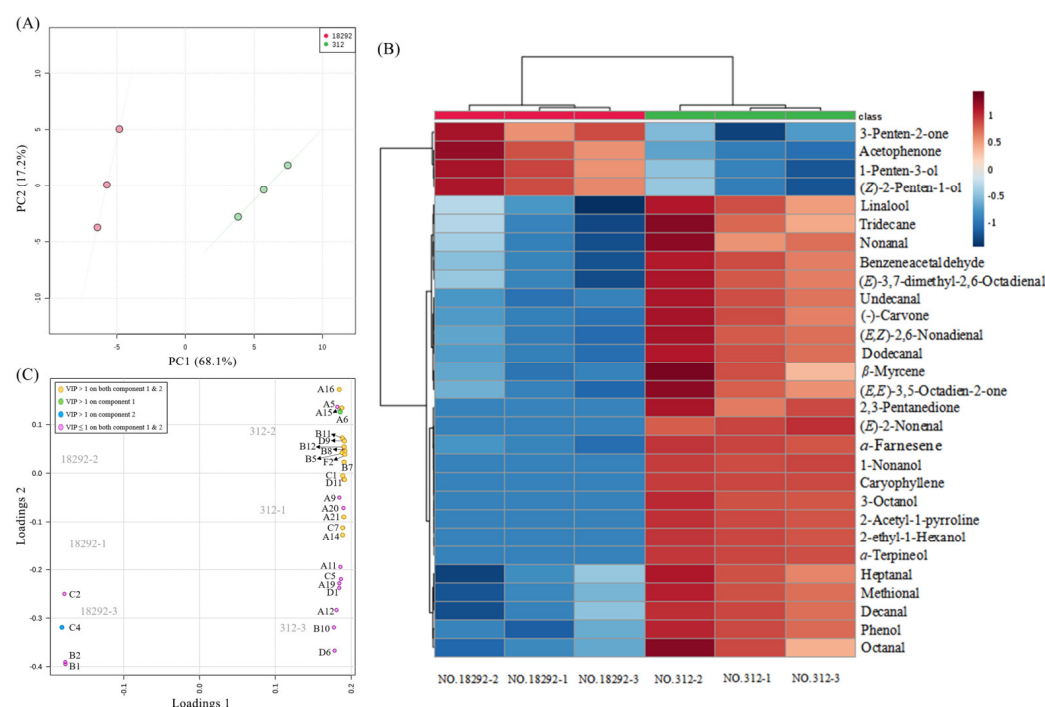


Figure 1. Chemometric analyses. (A) PCA scatter plots of the first two principal components using all the identified volatiles from pumpkin sample No. 312 with ‘taro-like’ aroma and sample No. 18292 without ‘taro-like’ aroma. (B) The heat map and feature-wise HCA dendrogram of the significantly-varied volatiles in two samples. Each sample was tested in triplicate. Red indicating high content, and blue representing low content. (C) Compounds with VIP score are highlighted by different colors using PLS-DA based on the differential volatiles between the samples No. 312 and No. 18292.

3.2. Identification and Analysis of Differential Volatile Compounds

Subsequently, the different volatile compounds between the two samples were identified and analyzed to identify the volatile compounds associated with ‘taro-like’ aroma. The differences in the contents of each compound between the two samples in terms of peak area ratio, as described in a previous study, are also listed in Table 1 [19–21]. A total

of 29 volatile compounds with significant differences were identified, and the volatile compounds, aroma description, *p*-value, and derivative way are listed in Table 2. Eight differential volatile compounds, including (*E*)-2-nonenal (A15), 3-octanol (B7), 2-ethyl-1-hexanol (B8), 1-nonanol (B11), α -terpineol (B12), 2,3-pentanedione (C1), caryophyllene (D9), and 2-acetyl-1-pyrroline (F2), were the only specific volatile compounds detected in No. 312. Moreover, the different volatile compounds were divided into five categories: fatty acids; amino acids; carotenoids; terpenoids; and phenylpropanoid derivative aroma. A total of 20 fatty acid-derived aromatic compounds, such as methional, heptanal, (*E*)-2-nonenal, octanal, undecanal, and (*E,Z*)-2,6-nonadienal, accounted for the largest proportion (68.97%) (Table 2). Notably, the five specific volatile compounds, including (*E*)-2-nonenal, 3-octanol, 2-ethyl-1-hexanol, 1-nonanol, and 2,3-pentanedione, which were only detected in No. 312, belonged to fatty acid-derived volatile compounds. There were four terpenoid-derived aromatic compounds (13.79%), including linalool, α -terpineol, β -myrcene, and caryophyllene, among which the volatile compounds α -terpineol and caryophyllene were only detected in No. 312. Benzeneacetaldehyde, acetophenone, and phenol were the only three phenylpropanoid-derived volatile compounds (10.34%). Both the amino acid-derived volatile and carotenoid-derived volatile accounted for the minimum proportion (3.45%) and contained 2-acetyl-1-pyrroline and α -farnesene, respectively.

Table 2. Differential volatile compounds in the leaves of pumpkin sample No. 312 with ‘taro-like’ aroma and sample No. 18292 without ‘taro-like’ aroma.

No. ^A	Volatile Compound	Aroma Description [8,22] ^B	<i>p</i> Value ^C	Derivative Way [23]
Aldehydes				
A5	heptanal	grass, fresh, citrus, fat	0.003	fatty acid
A6	methional	cooked potato	0.001	fatty acid
A9	octanal	citrus, fat, green, lemon	0.008	fatty acid
A11	benzeneacetaldehyde	green, sweet, flower	0.002	phenylpropanoid
A12	nonanal	grass, citrus, floral	0.008	fatty acid
A14	(<i>E,Z</i>)-2,6-nonadienal	green, metal	0.001	fatty acid
A15	(<i>E</i>)-2-nonenal	cucumber, fat, green	0	fatty acid
A16	decanal	fat, citrus, flower	0.001	fatty acid
A19	(<i>E</i>)-3,7-dimethyl-2,6-octadienal	lemon	0.003	fatty acid
A20	undecanal	oil, sweet	0.001	fatty acid
A21	dodecanal	fresh, citrus	0.001	fatty acid
Alcohols				
B1	1-penten-3-ol	earth	0.003	fatty acid
B2	(<i>Z</i>)-2-penten-1-ol	green, rubber	0.003	fatty acid
B5	phenol	must	0	phenylpropanoid
B7	3-octanol	fat, metal	0	fatty acid
B8	2-ethyl-1-hexanol	heavy, earth	0	fatty acid
B10	linalool	sweet, floral	0.008	terpenoid
B11	1-nonanol	cucumber, fat	0	fatty acid
B12	α -terpineol	mint, oil, anise	0	terpenoid
ketones				
C1	2,3-pentanedione	caramel, fruit	0	fatty acid
C2	3-penten-2-one	sweet	0.004	fatty acid
C4	acetophenone	almond, sweet, flower	0.004	phenylpropanoid
C5	(<i>E,E</i>)-3,5-octadien-2-one	fruit, mushroom, fat	0.004	fatty acid
C7	(-)-carvone	mint	0.003	fatty acid
Hydrocarbons				
D1	β -myrcene	spice	0.01	terpenoid
D6	ttridecane	alkane	0.013	fatty acid
D9	caryophyllene	wood, earth	0	terpenoid
D11	α -farnesene	wood, sweet	0	carotenoid
Heterocyclic compounds				
F2	2-acetyl-1-pyrroline	nut, roast, popcorn-like, sweet	0	amino acid

^A The No. refer to the order in Table 1. ^B The aroma description also refers to http://www.flavornet.org/d_odors.html (accessed on 15 January 2021). ^C The *p* value was calculated using ‘SPSS 17’ software.

The dendrogram of HCA was utilized to visualize the diversity in volatile compounds between the two samples based on the differential volatile compounds. The expression of differential volatile compounds in the two samples was significantly different, and the two main groups were successfully clustered (Figure 1B). In addition, the trend of expression was divided into two categories; the first category had four compounds, including 3-penten-2-one (C2), acetophenone (C4), 1-penten-3-ol (B1), and (Z)-2-penten-1-ol (B2), which were present in high amounts in No. 18292, while the second category contained 25 compounds, which were present in high amounts in No. 312. Crucially, the majority of the volatile compounds in No. 18292 occurred at a lower level than those in No. 312.

Furthermore, the PLS-DA analysis was performed on the 29 differential volatile compounds. The first two components in Supplementary Figure S2 explain 98.20% of the total variance (94.90% and 3.30%, respectively). As shown in Supplementary Figure S2, the two samples were significantly different, where No. 18292 was placed in the negative area of component 1 and No. 312 was located in the positive region of component 1. PLS-DA loading plot is shown in Figure 1C. All the differential volatile compounds with a large Euclidean distance from the origin were considered important for discrimination. The differential volatile compounds, including 3-penten-2-one (C2), acetophenone (C4), 1-penten-3-ol (B1), and (Z)-2-penten-1-ol (B2), lying in the negative region of component 1 and component 2, were associated with NO. 18292. In addition, the rest of the differential volatile compounds were located in the positive region of component 1 and were considered important for the identification of No. 312. The VIP scores for each compound are provided in Supplementary Table S2. As shown in Figure 1C and Table S2, there were 15, 15, and 14 volatile compounds with VIP scores > 1.0 in component 1, component 2, and both components, respectively. Moreover, those volatile compounds with VIP score >1 were situated along the direction of separation on the outermost regions. Furthermore, the 14 differential volatile compounds with VIP >1.0 in component 1 and component 2 showed high levels in No. 312. The VIP scores for the eight differential volatile compounds only identified in No. 312 all exceeded 1.0 in component 1 and component 2.

3.3. The Analysis of Key Volatile Compound Related to ‘Taro-like’ Aroma Using GC-O

Subsequently, in order to find the key volatile compounds for ‘taro-like’ aroma, a four stepwise dilution series (1:3; 1:9; 1:27; 1:81) was used to gradually increase the dilution. A total of 28 odors were detected in the undiluted condition. As the dilution increased, less odor was detected (Table S3). Finally, four distinct odors were detected at the highest dilution (1:81), namely taro, rust, floral, and mint flavor, respectively (Table 3). Moreover, the taro odor displayed the highest intensity with the scale of 3, followed by rust and mint odors with the scale of 2. The floral odor presented the lowest intensity with the scale of 1. Based on the retention time and MS results, the volatile compound for taro, rust, floral, and mint flavor were 2-acetyl-1-pyrroline, 3-octanol, 1-nonanol, and (E)-3,7-dimethyl-2,6-octadienal.

Table 3. The most potent odorants in pumpkin leaf (sample NO.312) with ‘taro-like’ aroma via GC-O.

Retention Time	Odor Quality	Odor Intensity	Volatile Compounds	Identification
12.61–12.91	taro	3	2-acetyl-1-pyrroline	RT, MS
15.11–15.31	rust	2	3-octanol	RT, MS
21.92–22.05	floral	1	1-nonanol	RT, MS
25.96–26.15	mint	2	(E)-3,7-dimethyl-2,6-octadienal	RT, MS

3.4. Identification and Analysis of DEGs

The cDNA library was established using the RNA extracted from the young leaves of the two samples in triplicates. After excluding the low-quality reads, adaptor sequences, and rRNA reads, 63,409,143 and 71,530,686 clean reads were obtained from the No. 18292 and No. 312, respectively (Table S4). As a result, 29,720 genes were identified using the different reference databases (Table S5).

In order to investigate the DEGs related to the variations in aromatic compounds, gene expression levels were compared between No. 312 and No. 18292. A total of 543 DEGs were detected with FDR values of <0.01 and fold change of ≥ 2 (Table S6). Among the 543 DEGs, 302 genes displayed a higher expression level in No. 312 than those in No. 18292. Moreover, in order to better comprehend the function of DEGs in the production of ‘taro-like’ aroma, KEGG enrichment analyses were conducted. As shown in Figure 2A, the results indicated that the 543 DEGs were predominantly enriched in 20 pathways. The top five KEGG pathways included photosynthesis-antenna proteins (KO00196), phenylpropanoid biosynthesis (KO00940), fatty acid degradation (KO00071), ascorbate and aldarate metabolism (KO00053), and fatty acid metabolism (KO01212). Additionally, the same change trend was obviously presented for the same gene using qRT-PCR and RNA-seq (Figure 2B). The qRT-PCR results indicated that the transcriptomic data and the analysis results were reliable and accurate.

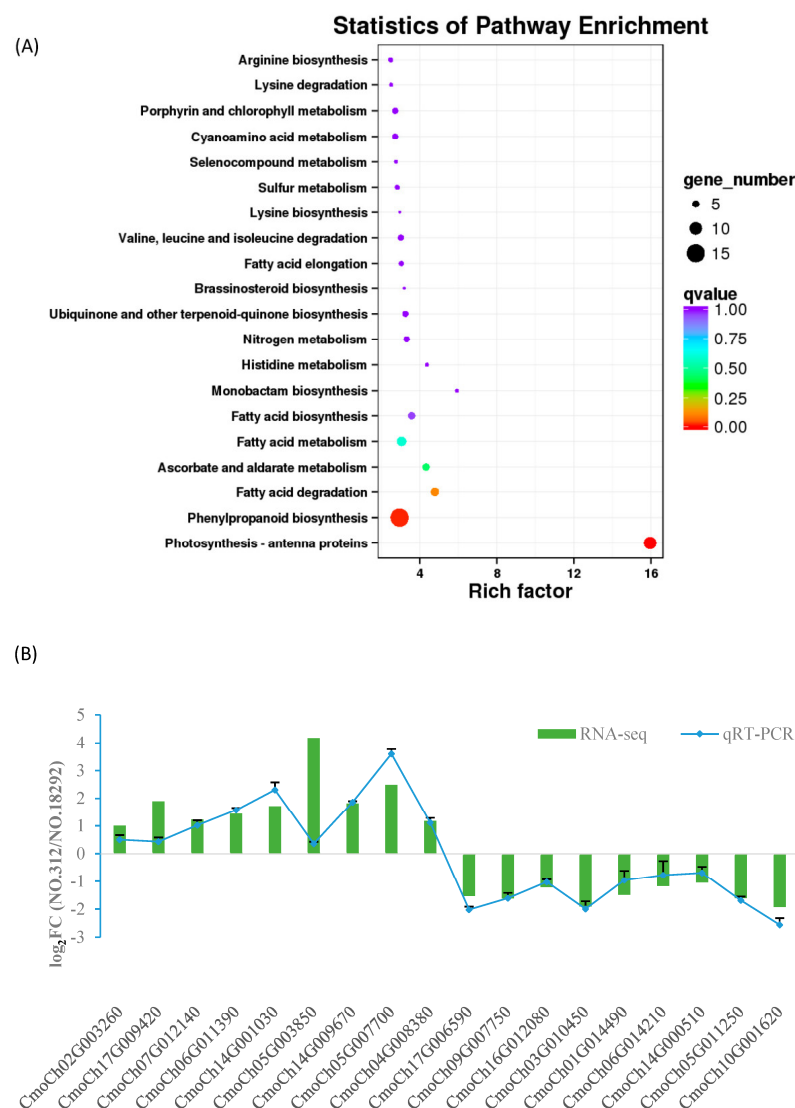


Figure 2. The pathways analysis and the validation of RNA-seq data. (A) KEGG pathway enrichment for differentially expressed genes; (B) the validation of RNA-seq data using qRT-PCR in the selected DEGs. Relative expression ratio of each DEG is presented in a log₂ Fold Change (No. 312 with ‘taro-like’ aroma vs. No. 18292 without ‘taro-like’ aroma). The values are mean $\pm$ SD and error bars represent standard deviations from three independent replicates.

4. Discussion

The metabolic and molecular mechanisms of ‘taro-like’ aromatic traits have not been systematically and comprehensively studied. Consequently, in order to reveal these mechanisms, the combined volatile compounds and transcriptomic analyses were performed using leaves from two pumpkin-inbred lines with/without ‘taro-like’ aroma.

4.1. Analyses of Key Volatile Compounds Associated with the ‘Taro-like’ Aroma in Pumpkin

A comprehensive study of volatile compounds is a key for studying the ‘taro-like’ aromatic trait of pumpkin leaf. In this study, a total of 59 volatile compounds were identified, in which the aldehydes and alcohols were the most important chemical groups isolated from the *C. moschata* leaves (Table 1). Similar results were found in *C. moschata* puree using SPME-GC-MS technique [24]. In our previous study, only 28 volatile compounds were identified in the leaf of pumpkin with ‘taro-like’ aroma, and alcohols and aldehydes were the main compounds [5]. Moreover, 42 volatile compounds were identified in the pumpkin fruit with ‘taro-like’ aroma, and hydrocarbons, aldehydes, and ketones were the main volatile compounds [6]. These various results might be caused by the different tested samples (different germplasm resources, different plant parts) and a GC-MS instrument. In addition, 28 and 42 major volatile compounds were reported in the leaf and fruit of pumpkin with ‘taro-like’ aroma [5,6]; 20 and 20 of which were also found, respectively, in this study. In general, the PCs replace the original dataset when they have >85% cumulative reliability of the original data. According to the PCA results, PC1 and PC2 could explain 85.30% of the total variance, and an excellent separation of the two germplasms was observed in Figure 1A, indicating that the distinct variances in composition and contents of volatile compounds allowed for a clear differentiation of the volatiles between two samples.

The identification of the essential volatile compounds related to a particular fragrance from a huge number of volatile compounds is the key to understanding aromatic traits. In this study, the key candidate volatile compounds were screened and obtained using differential analysis between the two samples. The PLS-DA analysis based on the 29 differential volatile compounds showed a clear difference between the two samples (Figure S2), indicating that the 29 differential volatile compounds could well reflect the different aroma between the two samples, and might also be associated with the differences in aromatic trait. In order to identify biomarkers to screen the pumpkin samples, VIP scores were calculated to identify the key differential volatile compounds. According to a previous study, the VIP score, when exceeding 1.0, has a significant impact on the PLS-DA discriminant process [25]. There were 14 differential volatile compounds, including caryophyllene, 1-nonanol, α -terpineol, 2-ethyl-1-hexanol, 2-acetyl-1-pyrroline, (*E*)-2-nonenal, 3-octanol, α -farnesene, 2,3-pentanedione, dodecanal, undecanal, (-)-carvone, phenol, and (*E,Z*)-2,6-nonadienal, with VIP scores greater than 1.0 in both component 1 and component 2 (Figure 1C). This was defined as the most influential data points in the VIP scores plots, suggesting that all these compounds might be main aroma contributors to the pumpkin ‘taro-like’ aromatic traits. In addition, eight volatile compounds with VIP scores >1, including (*E*)-2-Nonenal, 3-octanol, 2-ethyl-1-hexanol, 1-nonanol, α -terpineol, 2,3-pentanedione, 2-acetyl-1-pyrroline, and caryophyllene, were detected only in No. 312. These eight volatile compounds might play important roles for the ‘taro-like’ aroma. According to the results of GC-O analyses, four volatile compounds, covering 2-acetyl-1-pyrroline, 3-octanol, 1-nonanol, and (*E*)-3,7-dimethyl-2,6-octadienal, presented taro, rust, floral, and mint odor, respectively. Thus, it was determined that 2-Acetyl-1-pyrroline might be a key volatile compound associated with the ‘taro-like’ aroma of pumpkin leaf. Furthermore, 2-acetyl-1-pyrroline is the characteristic aromatic compound of rice [22] and is also found in coconut [26], mung bean [27], muskmelon [28], etc. It has a pleasing scent and is referred to as being nut-, roast-, popcorn-like, and sweet to taste. More significantly, Lin et al. [29] observed that a rice mutant, SA0420, had a pleasant “taro-like” aroma in both its leaves and grains due to the higher 2-acetyl-1-pyrroline content. Furthermore, 2-acetyl-1-pyrroline is the key contributor to the ‘taro-like’ aroma of pumpkin fruit [6].

Additionally, a previous study showed that aromatic compounds can be derived from diverse biosynthetic pathways, such as isoprenoid/terpenoid, fatty acid, and alkaloid pathways [23]. In this study, fatty acid-derived volatile compounds accounted for the largest proportion (Table 2). Generally, fatty acids are the major precursors of volatile compounds in fruits and vegetables, as well as in tomatoes [4]. A variety of plant flavor compounds are biosynthesized and accumulated in specialized anatomical structures [23]. More importantly, these tissues contain many enzymes and highly expressed genes, which are involved in the production of metabolites, such as volatile compounds. Therefore, understanding the key biosynthetic pathways and regulation of volatile compounds is crucial.

4.2. Genes' Expression Patterns and Correlations among Volatile Compounds and Genes

In order to clarify the main contributions of aroma, it is necessary to study the molecular mechanisms and metabolic pathways for more effectively breeding new varieties with aroma traits. It was necessary to identify the potential genes that were related to the 'taro-like' aromatic trait. A total of 543 DEGs were identified, on which KEGG enrichment pathway analysis was performed. In this study, it was found that most of the differential volatile compounds were derived from fatty acids. The DEGs were involved in fatty acid degradation, fatty acid metabolism, and fatty acid biosynthesis (Figure 2A), thereby affecting the fatty acid levels. Therefore, it was speculated that the enrichment of DEGs in fatty acid pathways might result in the production of differential volatile compounds. The aromatic-related derivatives of fatty acids are formed by α - and β -oxidation and lipoxygenase pathways, in which the degradation of straight-chain fatty acids by α - and β -oxidation is the main process for the formation of flavor compounds [23]. Additionally, the relationship between the differential volatile compounds derived from fatty acids and the genes requires further studies.

According to the results of metabolite profiling, the 2-acetyl-1-pyrroline has been determined to be the key volatile for 'taro-like' aroma. Therefore, the biosynthesis pathway of 2-acetyl-1-pyrroline was focused further (Figure 3) and the intermediates of its main metabolic processes, as well as the key enzymes and genes involved in its biosynthesis, were further studied. The biosynthesis pathway of 2-acetyl-1-pyrroline is very complex, involving many genes [30]. Uridine diphosphate (UDP)-glucose pyrophosphorylase 2 (*UGP2*, CmoCh14G008490) is a positive regulated gene for Uridine diphosphate glucose (UDP-glucose) in different metabolic pathways, such as starch and sucrose metabolism (KO00500), galactose metabolism (KO00052), and other sugar metabolism pathways. In this study, the expression level of *UGP2* in No. 312 with 'taro-like' aroma was significantly higher than that in No. 18292 without 'taro-like' aroma (Table 4). UDP-glucose is used as a glucose donor and the fleshy corollas of *B. Latifolia* have a high content of 2-acetyl-1-pyrroline due to its high glucose content [30,31]. Moreover, glucose is a precursor of methylglyoxal, which is a direct substrate for the synthesis of 2-acetyl-1-pyrroline [31]. Therefore, the 'taro-like' aroma associated with 2-acetyl-1-pyrroline in pumpkin might be caused by the high glucose content due to the high expression level of *UGP2*. In addition, glyceraldehyde-3-phosphate (G3P) has been identified as a precursor for the synthesis and accumulation of methylglyoxal [22]. Triosephosphate isomerase (*TPI*, CmoCh02G003260) gene was found to be highly expressed in No. 312 (Table 4) and involved in the biosynthesis of amino acids (KO01230), fructose and mannose metabolism (KO00051), and other metabolic pathways. *TPI* can positively regulate G3P, resulting in the accumulation of methylglyoxal and promoting the synthesis of 2-acetyl-1-pyrroline. Additionally, 1-deoxy-D-xylose-5-phosphate synthase (*DXS*, CmoCh16G008020) gene has been reported to be a key gene for the synthesis of terpenoid from prickly ash [32] and can catalyze the decomposition of G3P. *DXS* had low expression levels in No. 312 (Table 4) and resulted in the accumulation of G3P for methylglyoxal. Furthermore, ornithine acts as a nitrogen source for 2-acetyl-1-pyrroline by γ -aminobutyraldehyde (GABald), which is spontaneously cycled to Δ 1-pyrroline and then acylated at the C-2 position of acyl-CoA to synthesize 2-acetyl-1-pyrroline [33]. Palmitoyl-protein thioesterase (*PPT*, CmoCh06G008220) gene

can catalyze acetyl-CoA to form fatty acids. However, this gene had a significantly low expression level in No. 312 (Table 4) and resulted in the accumulation of acyl-CoA, which might lead to the synthesis of 2-acetyl-1-pyrroline. The previous studies have reported that a single recessive gene *fgr* present on chromosome 8 of rice is related to rice aroma, and can determine the content of 2-acetyl-1-pyrroline in rice [34]. The *fgr* gene encodes a defective betaine-aldehyde dehydrogenase (BADH2), which leads to the accumulation of 2-acetyl-1-pyrroline [35]. However, researchers have also found aroma mutants with functional BADH2 [29], suggesting that the deficiency of BADH2 might not be the only cause of rice aroma phenotype with 2-acetyl-1-pyrroline. In this study, the *BADH* gene (CmoCh10G001620) was detected, but its expression level did not show significant difference based on the strict filtering parameters (FDR < 0.01) in transcriptomic analysis. The expression level of *BADH* gene in No. 312 was lower than that in NO. 18292 using qRT-PCR, which indicated that *BADH* gene might be significantly involved in the changed content of 2-acetyl-1-pyrroline associated with ‘taro-like’ aroma. Subsequent studies should concentrate on the identification of candidate genes and the development of molecular markers of 2-acetyl-1-pyrroline for ‘taro-like’ aromatic trait, which might rapidly and accurately judge the ‘taro-like’ aroma by the genotype and establish a molecular-assisted selective breeding technology system for the ‘taro-like’ aromatic trait in pumpkinkin.

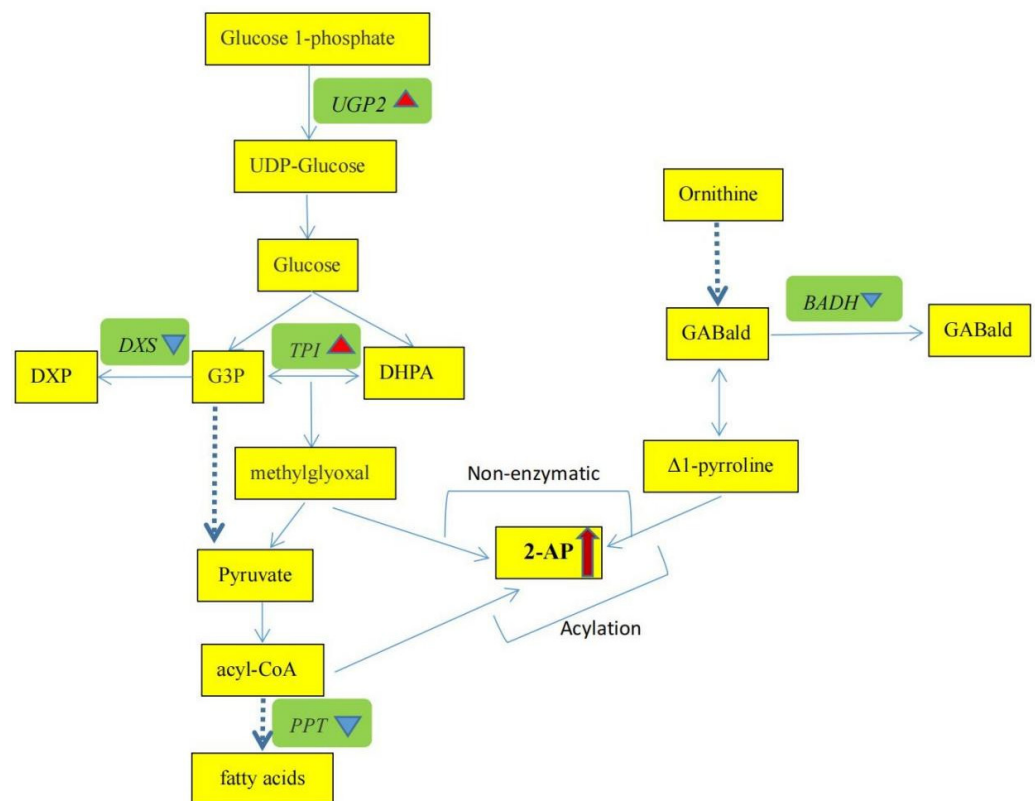


Figure 3. Expression patterns of the 2-acetyl-1-pyrroline-related genes. The green boxes represent genes and the yellow boxes represent metabolites. The red triangle represents the higher level in No. 312, the blue triangle represents the lower level in No. 312, and red arrow represents the higher content of 2-acetyl-1-pyrroline in No. 312.

Table 4. The expression levels from RNA-seq of four genes related to 2-acetyl-1-pyrroline in the leaves of pumpkin sample NO.312 with ‘taro-like’ aroma and sample NO.18292 without ‘taro-like’ aroma.

Gene Name	Gene ID	Expression Level-FPKM in No. 18292	Expression Level-FPKM in No. 312	Regulated (No. 312/No. 18292)
UGP2	CmoCh14G008490	5.65	11.88	up
TPI	CmoCh02G003260	9.20	21.08	up
DXS	CmoCh16G008020	11.43	2.96	down
PPT	CmoCh06G008220	49.06	15.29	down

5. Conclusions

In this study, the analysis of volatile compounds in the leaves of pumpkin with and without ‘taro-like’ aroma showed obvious differences in terms of composition and relative content of volatile compounds. A total of twenty-nine differential volatile compounds were identified, and only eight volatile compounds, including (E)-2-nonenal, 3-octanol, 2-ethyl-1-hexanol, 1-nonanol, α -terpineol, 2,3-pentanedione, caryophyllene, and 2-acetyl-1-pyrroline, were detected in the pumpkin with ‘taro-like’ aroma, among which 2-acetyl-1-pyrroline was identified as the key volatile compound for ‘taro-like’ aroma in pumpkin leaf using differential analysis and GC-O analysis. In addition, a total of 543 DEGs were identified using transcriptomic analysis, and were enriched in metabolic and biosynthetic pathways. Most of the differential volatile compounds belonged to fatty acid-derived volatile compounds, and DEGs were enriched in fatty acid pathways. Moreover, it was found that five genes associated with the biosynthesis of 2-acetyl-1-pyrroline, such as *BADH*, *TPI*, *UGP2*, *DXS*, and *PPT*, showed significantly altered levels. Overall, these findings offer new insights into the identification of ‘taro-like’ aroma in pumpkin and molecular mechanism of metabolism, and offer a scientific basis for the cultivation of high-quality pumpkin with ‘taro-like’ aroma using a molecular-assisted selective breeding technology. In the future, 2-acetyl-1-pyrroline can be used as the phenotype marker of ‘taro-like’ aroma to develop molecular markers based on the gene mapping, which can improve breeding efficiency.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12112641/s1>, Figure S1: The loading diagram of the all identified volatiles in two materials based on the PCA analysis; Figure S2: PLS-DA of the differential volatiles in two materials; Table S1: Primer sequences used for qRT-PCR; Table S2: The variable importance projection (VIP) score of 29 differential volatiles between two pumpkin materials; Table S3: The presentation of odor using GC-O analysis with different dilution; Table S4: Statistical tables of sequencing data from two pumpkin materials; Table S5: All annotated genes based on different databases; Table S6: The differentially expressed genes between two pumpkin materials.

Author Contributions: J.L. (Junxing Li) and X.Y. designed and planned the experiments. S.Z. and Y.Q. performed the GC-MS and qRT-PCR experiment. J.L. (Junxing Li) performed the GC-O and reference standards experiments. J.L. (Jianning Luo), H.G., X.Z. and Y.Z. prepared the samples. H.W., X.L. and G.Z. performed transcriptome analysis. W.W. provided the GC-MS instrument. S.Z., Y.Q. and J.L. (Junxing Li) analyzed the GC-MS data. S.Z., Y.Q. and J.L. (Junxing Li) wrote the article. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data have been deposited in the CNGB Sequence Archive (CNSA) of China National Genebank DataBase (CNGBdb) (accession number CNP0002555).

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