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# Transcriptomic Analysis Provides Insights into Foliar Zinc Application Induced Upregulation in 2-Acetyl-1-pyrroline and Related Transcriptional Regulatory Mechanism in Fragrant Rice

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**ABSTRACT:** The involvement of zinc (Zn) in terms of aroma formation has been rarely investigated. This study shows that the regulation of 2-acetyl-1-pyrroline (2AP) biosynthesis was evaluated in two different rice cultivars under foliar Zn application. The results showed that the 2AP and Zn contents in leaves and grains were improved substantially under foliar Zn application. The 2AP content was positively related to the expression *P5CS2* gene, contents of proline, 1-pyrroline, and Δ1-pyrroline-5-carboxylate (P5C), and the activity of pyrroline-5-carboxylate synthase (P5CS) under Zn application in fragrant rice. Multiple transcription factors (TFs) were differently expressed, such as *bZIPs*, *NACs*, and *MYBs*, to play a role under Zn treatments in fragrant rice, suggesting the crucial role of 46 differently expressed TFs in 2AP improvements in fragrant rice. Furthermore, this study showed that the optimal foliar Zn application at a concentration of 30 mg $L^{-1}$ could increase the 2AP content of aromatic rice and keep the yield stable or increase the yield. TFs were involved in regulating to promote the 2AP formation in aromatic rice under the foliar Zn application. However, the relationship between 2AP biosynthesis pathway genes and TFs in fragrant rice remains to be further studied.

**KEYWORDS:** *fragrant rice, 2-acetyl-1-pyrroline, foliar zinc application, transcription factors, gene*

## INTRODUCTION

Fragrant rice is famous for its high-quality taste and rich grain nutrients.[1] Aroma is the most important economic indicator that determines the quality of fragrant rice.[2] Among the fragrant rice types, the Basmati from Indo-Pak regions and the Jasmine from the fields of Thailand are the most popular worldwide.[3] Cultivation fragrant rice often brings greater benefits to growers due to its increasing demands in international markets.[4] Considering the needs of consumers and growers for scented rice, it makes sense to understand the ways and mechanisms of flavoring in rice for further improvements in grain quality characters. Although more than 100 volatile compounds have been found to contribute to the aroma of rice.[5] However, many studies have shown that the most important aroma compound in fragrant rice was 2-acetyl-1-pyrroline (2AP).[6] The 2AP was also biosynthesized in nonaromatic rice, but the 2AP concentration in nonaromatic rice was also significantly reduced compared with that in aromatic rice cultivars.[2]

Generally, external environmental and agronomic measures such as light, temperature, and nutrition significantly affect the contents of 2AP in fragrant rice.[2] Internal genetic factors are also indispensable factors in the biosynthesis factors affecting the rice fragrance.[7] It had been shown that the single recessive gene (fgr) on chromosome 8 regulates rice aroma by encoding betaine aldehyde dehydrogenase 2 (badh2).[3] Thus the recessive *badh2* gene promoted 2AP generation, the dominant *BADH2 gene* inhibited 2AP biosynthesis.[3] Kaikavoosi et al.[8] was the first report of enhancement in 2AP content through overexpression of using the *P5CS* gene. The 2AP biosynthesis pathways had been explored in scented rice.[9] Generally, glutamate, proline, and ornithine were catalyzed by the enzyme proline dehydrogenase (ProDH), 1-pyrroline-5-carboxylate synthetase (P5CS), and ornithine aminotransferase (OAT) to form 1-pyrroline-5-carboxylate (P5C), P5C/Δ1-pyrroline was further formed by nonenzymatic reaction of methylglyoxal with 2AP.[8] Ornithine was catalyzed in a two-step reaction of ornithine decarboxylase (ODC) and diamine oxidase (DAO) to form the GABald.[4] BADH2-catalyzed GABald to form GABA, inhibiting the 2AP biosynthesis in nonfragrant rice, while the inactive BADH2 promoted the conversion of GABald to Δ1-pyrroline, leading to the accumulation of 2AP biosynthesis in fragrant rice[4] (Figure 1).

Zinc (Zn) was an essential micronutrient and involved in various biochemical processes in plants, including protein, nucleic acid, carbohydrate, lipid metabolism, and enzyme activation.[10] It was one of the seven essential microelements in plants and contains, binds, or transports more than 1200 proteins.[11] These proteins included Zn finger structural proteins, transcription factors (TFs), redox enzymes, and hydrolases, which were involved in regulating many metabolic processes such as plant protein synthesis and degradation,

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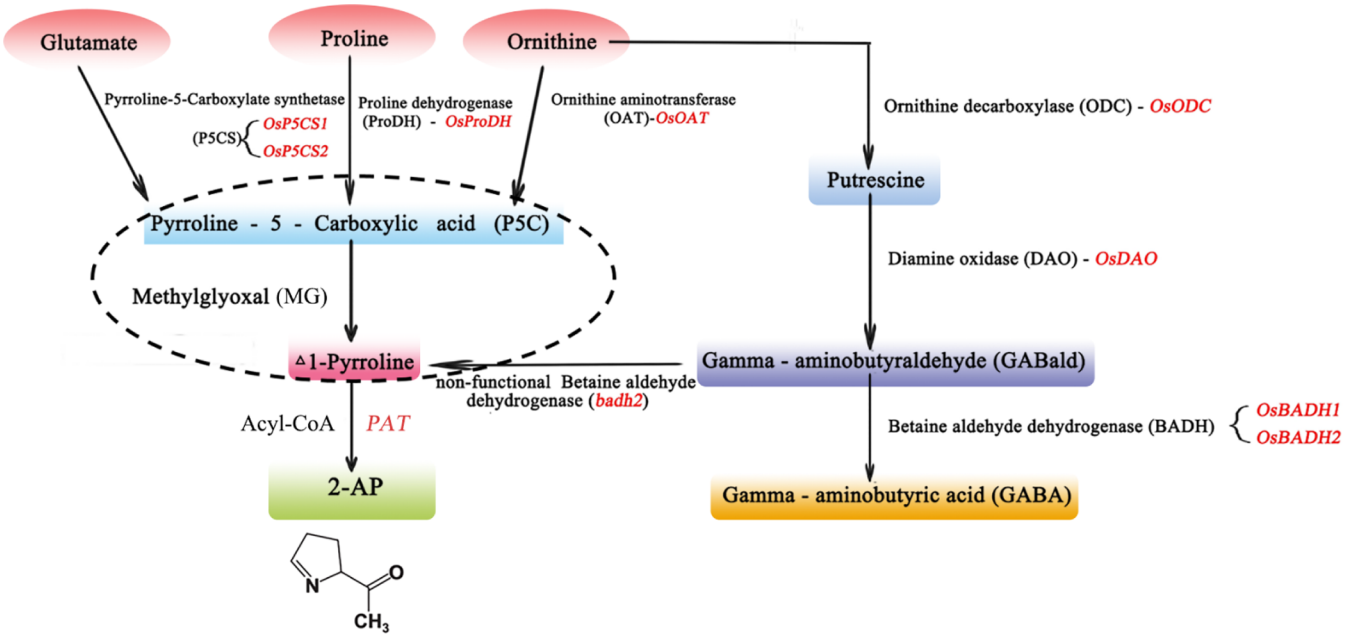


**Figure 1.** 2AP biosynthesis pathway.

energy production, auxin synthesis, biofilm stability, and cell division.[12] Although rice needed Zn in far fewer quantities than nitrogen, phosphorus, and potassium. Zn, as a necessary element for rice growth and development, played an important physiological role in enhancing root stem disease resistance, improving yield and quality, participating in metabolism, and improving resistance.[13] Zn-lysine could reduce Cr toxicity in rapeseed (*Brassica napus* L.) by increasing plant growth and reducing Cr absorption.[14,15] Foliar supplementation of Zn- and Fe-lysine could reduce the Cr content and increase the absorption of micronutrients by the soil in *Spinacia oleracea* L.[16,17] On the other hand, Zn deficiency was the main limiting factor for rice quality, and it was also a human nutrition issue of global concern.[18,19] Foliar Zn application is a simple, cost-effective, and effective way of improving the grain Zn concentration.[20] Hu et al.[21] pointed out that the two fragrant rice cultivars (Jiangyong and 80-66) were planted in origin and nonorigin, respectively. The Zn contents of the two fragrant rice cultivars grown in origin were higher than that in nonorigin fragrant rice cultivars, and the Zn content in the same region was also higher than that in the nonfragrant rice cultivars, indicating that the Zn content was positively related to the fragrance of fragrant rice.[21] It had been shown that foliar Zn application could significantly improve the 2AP content in fragrant rice grains at the booting stage.[22] Determination of proline content and PRODH activity after exogenous Zn treatment analyzed the reason for the improvement of 2AP content in fragrant rice.[22] However, the molecular mechanism of foliar Zn application promoting 2AP accumulation of fragrant rice is unclear.

TFs could activate or inhibit transcription by identifying cis-acting elements in the promoter region of the target gene.[23] TFs could respond to external signals in physiological and biochemical aspects. Therefore, the importance of transcriptional regulation in agricultural production could not be ignored.[24] For example, the transcription regulation mechanism of anthocyanin synthesis (research is relatively mature) and the overexpression or deletion of a single TF gene led to the upregulation or downregulation of a large number of downstream genes.[25]

Foliar Zn application can be used as a cultivation technique in fragrant rice production to increase the 2AP concentration and yield in scented rice. However, the lack of study on the molecular mechanism makes it difficult to breed new aromatic rice cultivars with rich aroma through genetic engineering, limiting the promotion of light, fragrant, and straightforward cultivation techniques. To solve this bottleneck, TFs are the most important regulatory factors in the regulation of gene expression in eukaryotes as the inspiration point. Therefore, the key genes of the 2AP biosynthetic pathway were identified in fragrant rice under the foliar Zn application. The TFs involved in the regulation of promotion of the 2AP formation in aromatic rice under the foliar Zn application were selected. It was a necessary precursor to clarify the molecular mechanism of regulating the 2AP formation in aromatic rice under foliar Zn application. This study provides the theoretical rationale for the mechanism of transcriptional regulation of the 2AP biosynthesis in fragrant rice.

## MATERIALS AND METHODS

**Plant Material.** Two fragrant rice cultivars, i.e., "Meixiangzhan2" (M) and "Xiangyaxiangzhan" (X) in the Department of Crop Science and Technology, College of Agriculture, South China Agricultural University, Guangzhou, China, were obtained. Meixiangzhan2 and Xiangyaxiangzhan are all national primary fragrant rice cultivars and also agricultural leading aromatic rice cultivars in Guangdong Province.

**Experimental Details.** The pot experiment was carried in the greenhouse house of the College of Agriculture, South China Agricultural University (Guangzhou, Guangdong Province, China) (23090 N, 113220 E) from March to July 2017. Guangzhou, Guangdong Province, has a subtropical monsoon climate. Each pot was filled with 10 kg of soil. The soil characteristics are listed in Table S1. The sample collection time was from 6:00 to 7:00 am at 7, 15, and 25 days after the heading stage (d AHS). Leaves were collected at 7 d AHS and 15 d AHS. Leaves and grains were collected at 25 d AHS because the grains had matured at 25 d AHS. The rapid clipping leaves (second sheet) and grains were put into a sealed bag with labels prepared in advance and returned to the laboratory in a liquid nitrogen refrigerator. Each part of the fragrant rice organs was divided into four parts. The first fresh sample was reserved at −20 °C for the


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determination of 2AP and Zn contents. The second fresh sample was refrigerated with liquid nitrogen and reserved at −80 °C for the determination of 2AP relation parameters and enzyme activities. The third fresh sample was chilled with liquid nitrogen and reserved at −80 °C for the determination of the gene expression and transcriptomics. All were mixed for three duplications. Grain yield was measured at maturity. The measurement of yield and yield-related traits were based on the published article by Bao et al.[2]

**Treatments.** The experimental treatments consisted of three Zn concentrations: Zn0: 0 mg $L^{-1}$, Zn1: 14 mg $L^{-1}$, and Zn2: 30 mg $L^{-1}$. These Zn concentrations were based on the previously published studies by Mo et al.[22] Zinc chloride solution was sprayed on the leaf surface using a manual sprayer at the heading stage. The 1 L volume of zinc solution was sprayed for each treatment to ensure that each leaf was sprayed. Each pot of fertilizer contained urea (1.45 g N46%), calcium perphosphate (2.08 g P12%), and potassium chloride (1.11 g K60%). Base fertilizer and tillering fertilizer ratio were in the ratio of 7:3 in each pot. Other cultivation measurements were the same.

**2-Acetyl-1-pyrroline (2AP) Concentration.** A fresh sample (1.5 g) was placed in 10 mL of dichloromethane and processed for 4 h at 200 oscillations per minute in the oscillation instrument (HZS-H, China). 2AP concentration was determined by a Shimadzu GCMS-2010 Plus gas-mass spectrometer produced in Japan and 2,4,6-trimethylpyrimidine (TMP) internal standard method.[1] The chromatographic column was RESTEK Rxi-5MS (30 m × 0.32 mm × 0.25 $\mu$m, Shimadzu, Japan). The GCMS-QP 2010 Plus setup program was used: the starting temperature of the GC oven was set to 40 °C for 1 min, then raised at 2 °C $min^{-1}$ to reach 65 °C, 1 min, at 65 °C and then raised at 10 °C $min^{-1}$ to reach 220 °C and 10 min, the peak time of 2AP was 7.5 min. The carrier gas was high-purity helium (purity >99.999%).

**Determination of Zn Concentration.** Determination of Zn concentration in the sample using an atomic absorption spectrophotometer (AA6300C, Shimadzu, Japan) was described by Rao et al.[26] Each sample (0.2 g) was digested with a diacidic mixture of $HClO_4$:$HNO_3$ (1:4 v/v) for 4 h, and the volume was adjusted to 25 mL by filtration.

**Measurement of Proline, P5C, Δ1-Pyrroline, Methylglyoxal, and GABA Contents.** Proline content was determined according to the published article by Bates et al.[27] A 3% sulfosalicylic acid was used as the extraction solution at 100 °C for 10 min. Two milliliters of glacial acetic and 2 mL of acid ninhydrin were mixed with 2 mL of supernatant in a 15 mL centrifugation tube at 100 °C for 30 min and then in an ice bath for 20 min. It was finally extracted with 4 mL of toluene at 4000 rpm for 5 min. The absorbance was read at 520 nm and represented as $\mu g\ g^{-1}$. The P5C content was determined according to the method of Mezl and Knox.[28] The extraction solution was composed of 10% glycerol, 1% Triton X-100, 50 mM Tris−HCl (pH 8.0), and 1% $\beta$ mercaptoethanol at 4 °C, 12,000 rpm, for 30 min. The supernatant was added to 40 mM $\gamma$-amino-benzaldehyde and 10% trichloroacetic acid (TCA). The absorbance was measured at 440 nm. The extinction coefficient of the calculated P5C content was 2.58 $mM^{-1}\ cm^{-1}$. The Δ1-pyrroline content was measured following the protocols of Holmstedt et al.[29] A 0.2 mM phosphate buffer (pH 7.0) and 0.01 mM $\gamma$-amino-benzaldehyde were mixed in the supernatant at room temperature for 30 min. The absorbance was recorded at 430 nm. The calculated extinction coefficient was 1860 $cm^{-1}$. The methylglyoxal content was estimated following the protocol of Yadav et al.[30] and Banu et al.[31] The sample (0.3 g) was homogenized in 3 mL of extraction solution (PBS) at 4 °C, 8000 rpm, for 5 min. A 5 M perchloric acid and 7.2 mM 1,2-diaminobenzene were added to the supernatant. The absorbance was read at 336 nm. The GABA content was determined by the protocols described by Zhao et al.[32] and Yao et al.[33] The extraction solution consisted of 60% ethanol, 1 M KOH, and 60 mM lanthanum chloride. Sodium hypochlorite (available chlorine, 10%), 0.2 M borate buffer (pH 10.0), and 6% phenol solution were added to the supernatant while shaking. The absorbance was recorded at 645 nm.

**Measurement of the PRODH, P5CS, OAT, DAO, and BADH2 Activity.** The PRODH and BADH2 activities were measured using a Plant Elisa kit (Mlbio, Shanghai, China). The kit was used a double-antibody one-step sandwich method. The sample, standard product, and HRP labeled detection antibody were added to the microhole coated with the antibody and then cultured and thoroughly washed. The absorbance was measured at 450 nm wavelength using BioTek Spectrophotometer EPOCH (BioTek, Vermont) and calculated as U $L^{-1}$. The extraction solutions of P5CS, OAT, and DAO activity were all the same. Units were all expressed as $\mu mol\ g^{-1}$ FW. Fresh samples (0.3 g) were homogenized in 5 mL of 50 mM Tris−HCl buffer (pH 7.5) (containing 7.0 mM $MgCl_2$, 1% glycerol, 1.0 mM KCl, 3.0 mM EDTA-$Na_2$, 1.0 mM DTT, and 5% PVP) at 4 °C, 8000 rpm, for 20 min. The P5CS activity was measured according to the published article by Zhang et al.[34] The reaction mixture contained 50 mmol $L^{-1}$ Tris−HCl buffer, pH 7.0, including 20 mmol $L^{-1}$ $MgCl_2$, 10 mmol $L^{-1}$ ATP, 100 mM hydroxamate-HCl, 50 mM sodium glutamate. The enzyme extraction solution (0.5 mL) was added to initiate the reaction at 37 °C, for 5 min. A 2.5% $FeCl_3$ and 6% TCA (dissolved in 2.5 M HCl) were added to stop the reaction. The absorbance was read at 340 nm. The OAT activity was measured by following the protocol of Chen et al.[35] The enzyme extraction solution was composed of 100 mM potassium phosphate buffer (pH 8.0), 50 mM ornithine, 1 mM pyridoxal-5-phosphate, and 20 mM $\alpha$-ketoglutarate. The enzyme extraction solution (0.05 mL) was added to initiate the reaction. Ten percent TCA was added to stop the reaction. The absorbance was measured at 440 nm. The DAO activity was determined by the protocol described by Su et al.[36] Phosphate buffer (0.1 M), pH 6.5, 0.1 M putrescine, 0.25 U $L^{-1}$ peroxidase, 0.82 mM 4-aminoantipyrine, and enzyme extract were added to the reaction solutions. Twenty millimolar putrescine was added to initiate the reaction. The absorbance was read at 550 nm.

**Real-Time Quantitative RT-PCR (qRT-PCR).** RNA extraction and cDNA synthesis used HiPure Plant RNA Mini Kit and Hiscript II QRT SuperMix for qPCR Kit (+gDNA wiper) (Magen, Guangzhou, China), respectively. Real-time quantitative qRT-PCR used an Option Real-Time PCR System CFX96 instrument (Bio-Rad, CA). The reaction system was 10 $\mu$L. The PCR reaction conditions were 95 °C for 30 s, 95 °C for 5 s, 57 °C for 30 s, and 40 cycles. Actin was used as an internal reference gene. SYBR Premix Ex Taq (Takara Bio Inc.) was used to conduct qRT-PCR. All primers were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA). Primers are listed in Table S2.

**Raw Sequencing Data.** The 2AP contents in leaves reached the highest at 15 d AHS in both fragrant rice cultivars. Transcriptomics analysis was performed on 15 d AHS leaves. Raw sequencing data have been uploaded in the NCBI Gene Expression Omnibus under the accession number PRJNA673016 (http://www.ncbi.nlm.nih.gov/geo). The cDNA library was established for sequencing. Filteration, quality assessment, and comparative analysis were performed on raw sequencing data to obtain gene expression and calculate the fragments per kb per million fragments (FPKM). There were two biological duplications in the mixed transcriptome samples of both fragrant rice cultivars.

**Enrichment Analysis.** Gene expression with a false discovery rate (FDR) <0.05 and fold change ≥2 in the comparative analysis was identified as a significantly differentially expressed gene (DEG).[37] The DEGs were analyzed through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.[38]

**Experimental Design and Statistical Analyses.** Data input and processing were performed on Office 2013 (Microsoft). Analysis of variance was performed on SPSS 25 (Analytical Software, Chicago). Significantly differences of 2AP content, Zn content, with 2AP related indicators, and yield were distinguished by $P < 0.05$ using the least significant difference (LSD). Analysis of transcriptomic sequence was performed on Omicshare platform (https://www.omicshare.com/). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed on MetaboAnalyst platform (http://www.metaboanalyst.ca).

## RESULTS

**2AP Contents, Zn Accumulation, and Gene Expressions Involved in 2AP Biosynthesis.** The 2AP contents were strongly impacted under the foliar Zn application and initially


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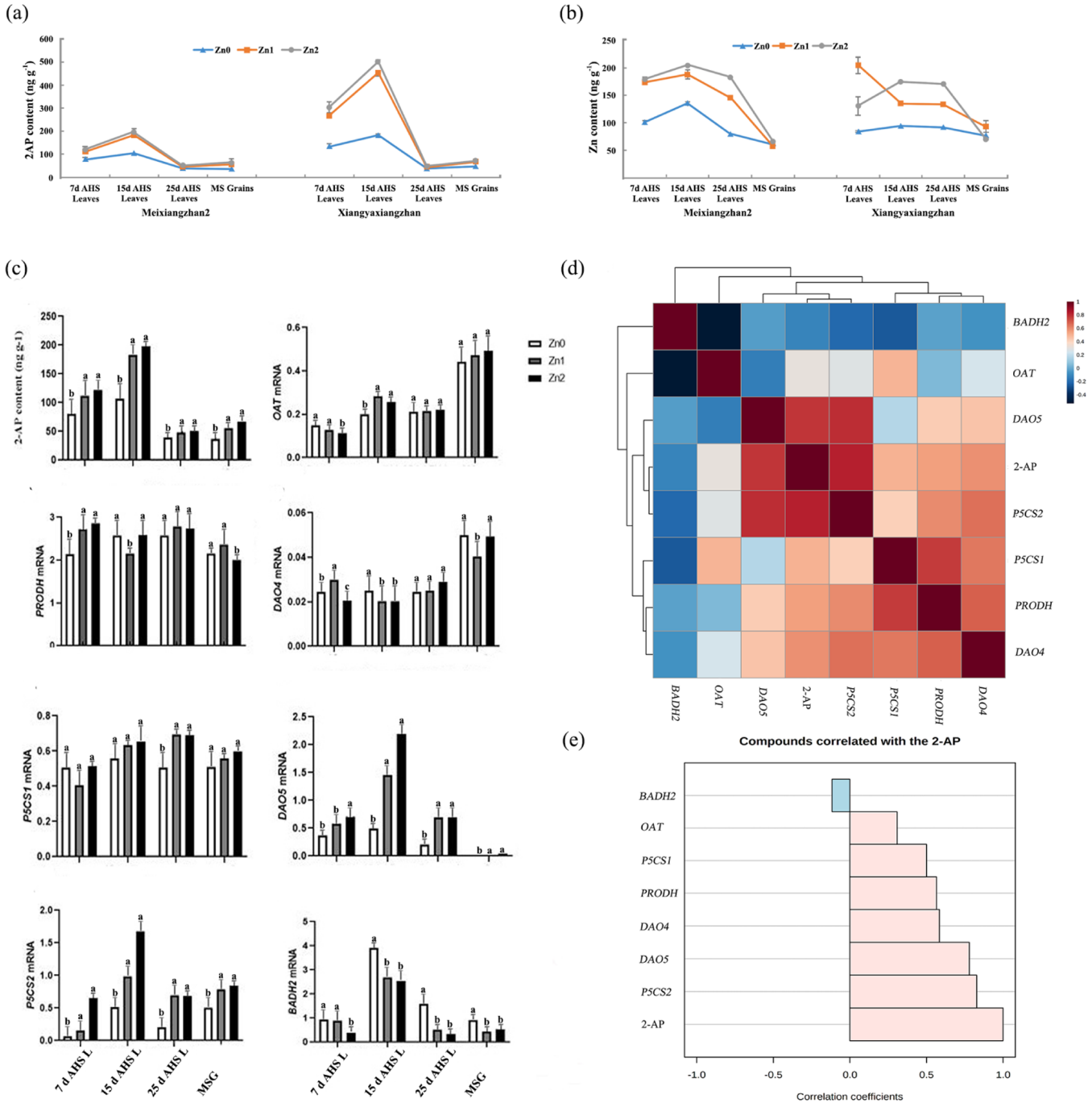


**Figure 2.** Effects of foliar Zn application on (a) 2AP and (b) Zn contents in leaves and grains in fragrant rice. (c) Analysis of transcript levels of 2AP biosynthesis pathway genes. d AHS L, day after heading stage in leaves; MS G, maturity stage in grains. (d) Pearson correlation analysis heatmap. (e) Pattern hunter. Marking the same letters means $P \geq 0.05$ (LSD). There is no significant difference. The difference between different letters means $P < 0.05$ (LSD). The difference is significant.

increased and then decreased from 7 d AHS to 25 d AHS in the leaves of both rice cultivars under different Zn treatments; the contents increased to the highest level in the 15 d AHS leaves. The 2AP contents were significantly increased in 7 d AHS and 15 d AHS leaves and at maturity stage in grains (MS G) under the Zn1 treatment and the Zn2 treatment than Zn0 for both rice cultivars. The 2AP contents were increased by 39.13−85.52 and 45.77−121.68% in both rice cultivars under the Zn treatments (Figure 2a).

The Zn contents in leaves increased till 7 d AHS then decreased to 25 d AHS in Meixiangzhan2 under Zn treatments, reaching the highest level at 15 d AHS. For Xiangyaxiangzhan, the Zn contents were substantially improved to the highest at 7 d AHS in leaves under the Zn1 treatment. The Zn contents in leaves increased till 7 d AHS then decreased to 25 d AHS under the Zn2 treatment, which increased to the highest level at 15 d AHS. The Zn contents in the leaves and grains were increased by 10.4−128.51 and 21−142.71% for Meixiangzhan2 and Xiangyaxiangzhan under Zn1 and Zn2 treatments, respectively, than that under Zn0. The 2AP and Zn contents were the highest at 15 d AHS in leaves for both fragrant rice cultivars (Figure 2b).


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The expression levels of *RODH*, *P5CS1*, *P5CS2*, *OAT*, *DAO4*, *DAO5*, and *BADH2* genes at 7 d AHS, 15 d AHS, 25 d AHS in leaves, and MS in grains were determined by qRT-PCR. The gene expression levels of *P5CS2* and *DAO5* in Zn1 and Zn2 were higher than that under Zn0 for Meixiangzhan2 and Xiangyaxiangzhan at 15 d AHS and 25 d AHS in leaves and MS in grains. The expression levels of *BADH2* were lower in Zn1 and Zn2 than that in Zn0 for Meixiangzhan2 and Xiangyaxiangzhan at 15 d AHS and 25 d AHS in leaves and MS in grains (Figure 2c). Pearson correlation analysis further showed that the *P5CS2* transcript had the strongest positive correlation with the 2AP contents (Figure 2d,e).

**Correlation Analyses between 2AP Contents in Leaf and Grain and Related Parameters.** The P5C contents (at 7 and 15 d AHS in leaves and MS in grains), proline contents (at 15 d AHS in leaves and MS in grains), 1-pyrroline contents (25 d AHS in leaves and MS in grains), methylglyoxal contents (at 15 d AHS in leaves), PRODH activity (at 7 d AHS in leaves, 15 d AHS in leaves, and MS in grains), P5CS activity (at 15 d AHS in leaves), DAO activity (at 15 d AHS in leaves), and OAT activity (at 15 d AHS in leaves) were significantly increased for both fragrant rice cultivars under Zn1 and Zn2 treatments than those under Zn0. The 1-pyrroline contents (at 7 d AHS in leaves), GABA contents (at 7 d AHS in leaves), DAO activity (at 7 d AHS in leaves and MS in grains), and P5CS activity at (MS in grains) were significantly increased for Meixiangzhan2 under Zn1 and Zn2 treatments than those under Z0. Moreover, the methylglyoxal contents, P5CS activity, and DAO activity were significantly increased for Xiangyaxiangzhan in grains at MS under Zn1 and Zn2 treatments than that under Zn0. The OAT activity was improved for the four periods (at 7, 15 d AHS, and 25 d AHS in leaves, and MS in grains) under Zn1 and Zn2 treatments than that in Zn0. Compared with Zn0, the Zn1 and Zn2 treatments had no significant effect on the BADH2 activity of the four periods (Table S3). The associations of grain 2AP contents at MS with other investigated indices are exhibited in Table 1. The 2AP contents at 15 d AHS and 25 d AHS in leaves showed a significant positive correlation with 2AP contents in grains at MS. The proline content in leaves at 15 d AHS and in grains at MS showed a strong positive correlation with 2AP contents at MS in grains. Moreover, there was a positive correlation between 2AP contents at MS in grains and P5C and 1-pyrroline. 2AP contents at MS in grains showed a strong positive correlation with the P5CS activity at 15 d AHS in leaves. Therefore, the 2AP contents in grains at MS were positively related to proline, P5C, and 1-pyrroline contents and P5CS activity.

**Table 1. Correlation Analyses of the Investigated Parameters between 2AP Content in Leaf and Grain Rice[a]**

| index | 7 d AHS leaves | 15 d AHS leaves | 25 d AHS leaves | MS grains |
|---|---|---|---|---|
| 2AP | 0.777 | 0.908* | 0.849* | 1 |
| proline | −0.407 | 0.943** | −0.494 | 0.913* |
| P5C | 0.811 | 0.811 | −0.192 | 0.858* |
| 1-pyrroline | 0.375 | −0.195 | 0.553 | 0.894* |
| methylglyoxal | −0.003 | 0.061 | 0.159 | 0.29 |
| GABA | 0.405 | 0.657 | −0.106 | 0.164 |
| ProDH | 0.704 | 0.618 | −0.473 | 0.683 |
| P5CS | 0.45 | 0.868* | 0.583 | 0.723 |
| DAO | 0.563 | 0.513 | −0.131 | 0.662 |
| OAT | 0.495 | 0.107 | −0.167 | 0.322 |
| BADH2 | −0.073 | −0.374 | 0.387 | −0.374 |

[a]7 d AHS, 7 d after heading stage; 15 d AHS, 15 d after heading stage; 25 d AHS, 25 d after heading stage; MS, maturity stage. ** and * represent significance at the 0.05 and 0.01 probability levels, respectively.

**Sequencing Statistics.** Raw reads of each sample were ranged from 46.02, 46.05, 53.41, 47.33, 49.87, to 48.09 million (Table S4). More than 96.9% of high-quality reads (HQ clean reads) were obtained. The Reads on ribosomal RNA were removed by HQ clean reads, called total reads used for subsequent analyses (Table S4). In sum, the mapping ratio of six data sets ranged from 85.81 to 86.77%. Among these, 84.96−85.95% were uniquely mapped reads and 0.75−0.87% were multiple mapped reads (Table S4). The averages of Q20, Q30, and GC contents were 98.08, 94.14, and 55.01%, respectively (Table S5). In total, 57 185 genes and 361 new genes were identified in all of the samples (Table S6). Thus, the quality of sequencing was qualified and can be used as raw data for subsequent analysis.

**Differentially Expressed Genes (DEGs).** A total of 308, 824, 306, and 507 DEGs were revealed in M-Zn0 vs M-Zn1, M-Zn0 vs M-Zn2, X-Zn0 vs X-Zn1, and X-Zn0 vs X-Zn2, respectively (Figure 3a). It was obvious that more DEGs appeared in the Zn2 treatment in both aromatic rice cultivars. PCA (Figure 3b) and sample-to-sample clustering analysis (Figure 3c) indicated that the same cultivar of control and Zn treatment were clustered together.

**GO Enrichment Analysis.** Functional annotation of 17 321 genes was conducted using the GO enrichment analysis, which was most frequently related to biological processes (6507), followed by molecular function (6746), and cellular components (4068). Seventeen thousand three hundred twenty-one DEGs were significantly enriched in 29 GO terms ($q$-value $\leq$ 0.05). The two most highly enriched GO terms in the biological process were "cellular process" (GO: 0009987), "single-organism process" (GO: 0044699), "metabolic process" (GO: 0008152), and "biological regulation" (GO: 0065007) and "catalytic activity" (GO: 0003824) and "Binding" (GO: 0005488) were the two most highly enriched terms in the molecular functions. The most highly represented cellular component was "cell" (GO: 0005623) and "cell part" (GO: 0044464), followed by "organelle" (GO: 0043226) and "membrane" (GO: 0016020). There were more strongly enriched in M-Zn0 vs M-Zn2 and X-Zn0 vs X-Zn2 than in M-Zn0 vs M-Zn1 and X-Zn0 vs X-Zn1. Moreover, more genes with diverse functional categories (GO terms) were involved in the response of Zn treatment in M-Zn0 vs M-Zn2 and X-Zn0 vs X-Zn2, compared with M-Zn0 vs M-Zn1 and X-Zn0 vs X-Zn1 (Table S7).

**KEGG Pathway.** The KEGG pathway database was used to further explore the metabolic pathways in which DEGs were significantly enriched (Table S8). Among the 33 DEGs that were M-Zn0 vs M-Zn1, the significantly enriched KEGG pathways were "Plant-pathogen interaction" and "Plant hormone signal transduction". Among the 113 DEGs that were M-Zn0 vs M-Zn2, the significantly enriched KEGG pathways were "Starch and sucrose metabolism", "Pentose and glucuronate interconversions", "Plant-pathogen interaction", "Nitrogen metabolism", "Cyanoamino acid metabolism", "Phenylpropanoid biosynthesis", and "Plant hormone signal transduction". Among the 37 DEGs that were X-Zn0 vs X-Zn1, "Phenylpropanoid biosynthesis" was most highly enriched. Among the 70 DEGs that were X-Zn0 vs X-Zn2, the significantly


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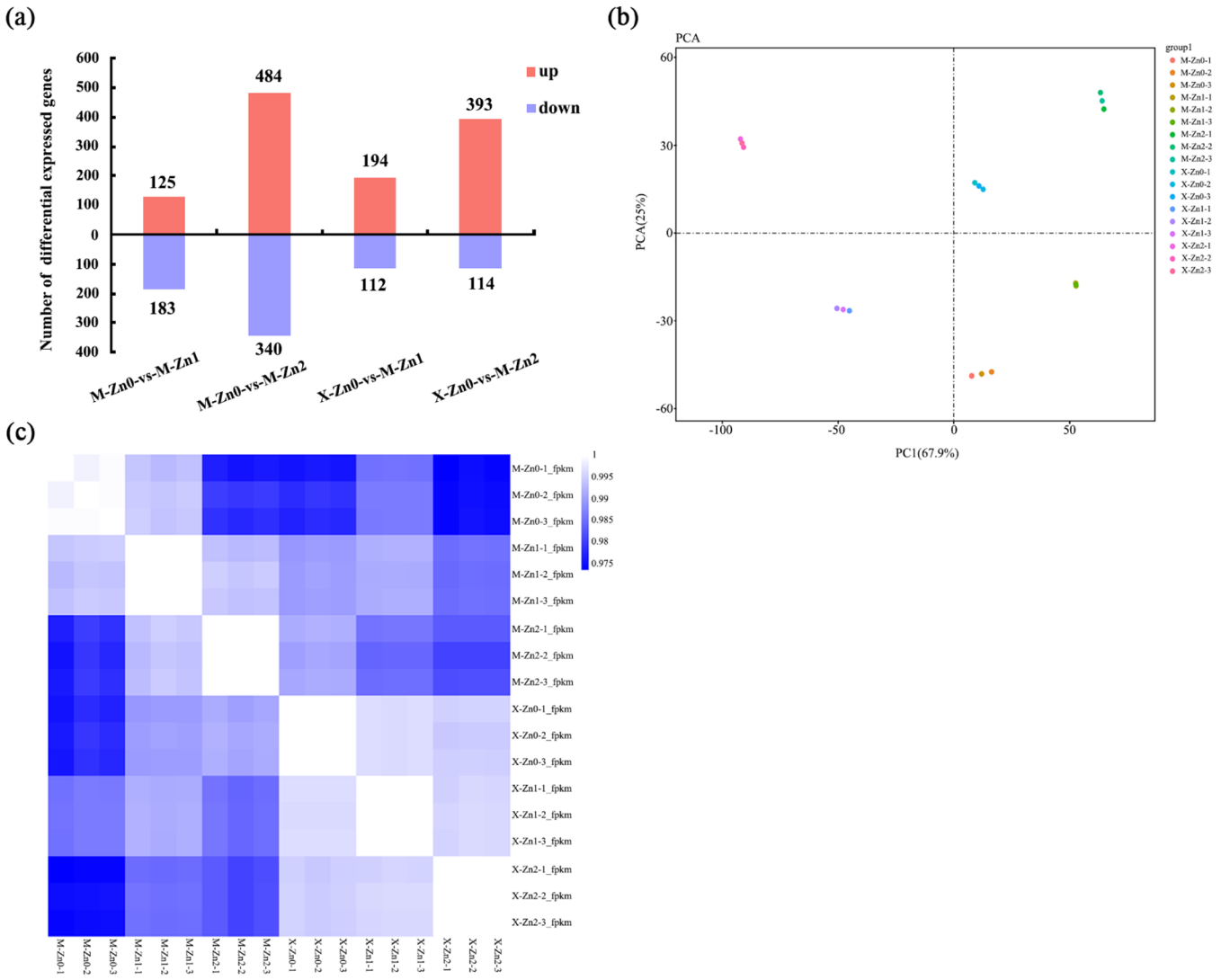


**Figure 3.** Summary of differentially expressed genes (DEGs) and sample clustering and correlation analysis. (a) DEGs. (b) Principal component analysis (PCA). (c) Sample-to-sample clustering analysis. The darker the color, the greater the difference.

enriched KEGG pathways were "starch and sucrose metabolism", "Pentose and glucuronate interconversions", "Amino sugar and nucleotide sugar metabolism", "plant-pathogen interaction", and "plant hormone signal transduction". These results indicate that "Starch and sucrose metabolism" and "plant hormone signal transduction" may have a regulatory effect on 2AP biosynthesis. Therefore, the following pathways caught our attention.

**Starch and Sucrose Metabolism and Plant Hormone Signal Transduction.** The pathways involved in starch and sucrose metabolism and plant hormone signal transduction were found to be highly enriched in the analysis carried out on the basis of DEGs. In the present study, 17 and 11 DEGs emerged in starch and sucrose metabolism and plant hormone signal transduction in Meixiangzhan2 and Xiangyaxiangzhan cultivar, respectively (Figure 4a,b). Thirteen DEGs in starch and sucrose metabolism and plant hormone signal transduction were randomly selected by qRT-PCR analysis. The relative expression levels of all selected genes obtained by the qRT-PCR analysis were consistent with the results calculated by the FPKM value (Figure 4c), indicating that the RNA-seq results were reliable.

**Transcription Factors.** Currently, about 1611 TFs were detected in the rice transcriptome, about 2.6% of the rice genome.[39] The key TFs associated with zinc in rice are exhibited in Figure 5. Among them, bZIP (6), NAC (15), IRO (1), MYB (8), bHLH (2), OFP (1), AP2-ERE (1), TiFy (3), TA2 (1), and WRKY (8) were differently expressed in different treatments and different cultivars (Figure 5a). PCA and PLS-DA showed that IROs had the greatest regulatory effect on the 2AP biosynthesis under foliar Zn application, followed by bZIP, MYB, bHLH, and WRKY (Figure 5b,c).

**Effect of Zn Treatments on the Yield and Related Traits.** For Meixiangzhan2, compared with Zn0, the effective grain panicle$^{-1}$ and filled grain percentage were increased by 14.3−18.5 and 8.9−14.88%, which increased the grain yield by 24.3−37.8%, respectively, in Zn1 and Zn2. For Xiangyaxiangzhan, compared with Zn0, the grain yield remained stable under foliar Zn application (Table 2).

## DISCUSSION

2AP has been confirmed to be an important characteristic compound that affects the aroma of aromatic rice.[40] Many studies have shown that fertilizers and micronutrients can


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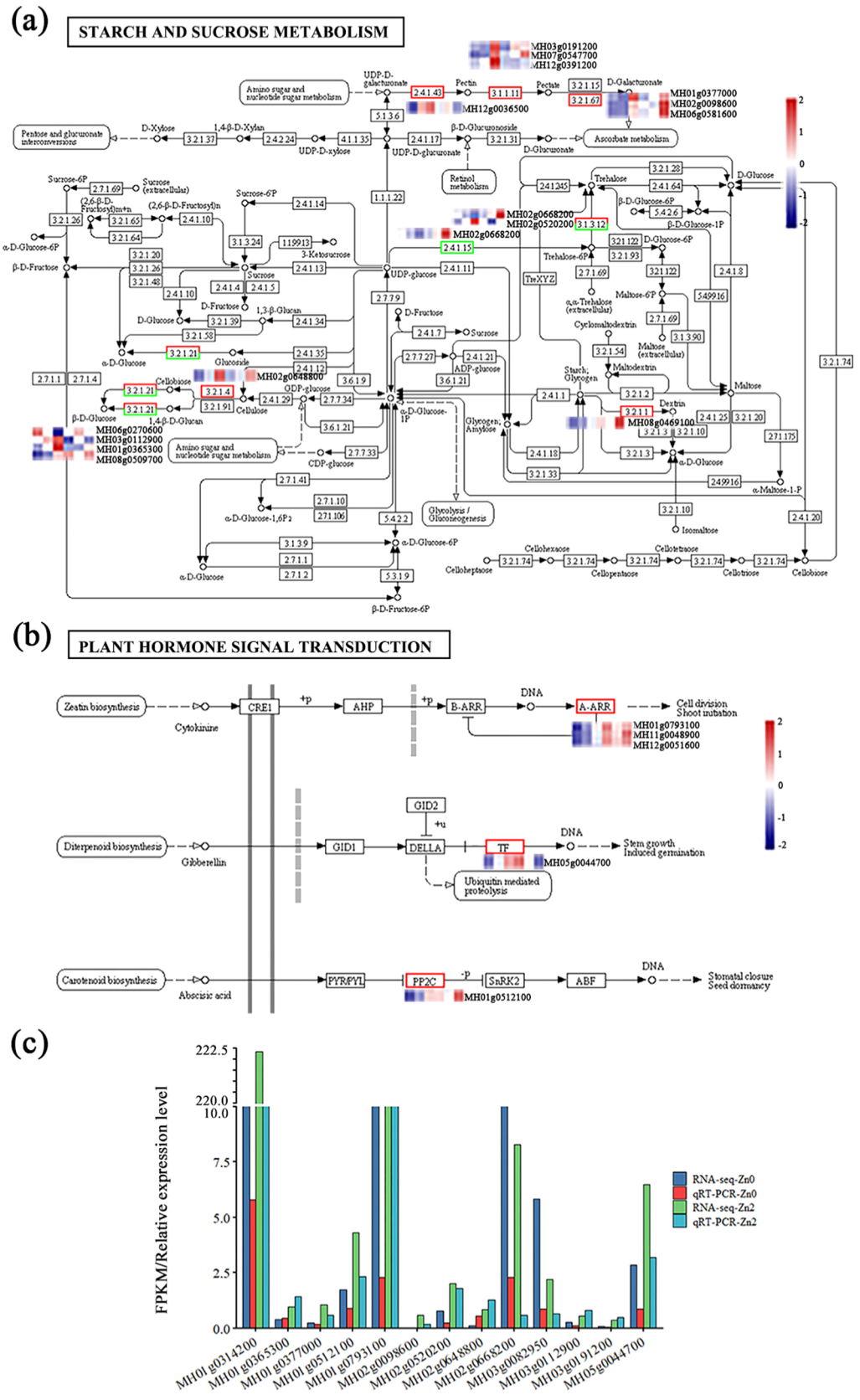


**Figure 4.** Regulations of the KEGG pathway (a) starch and sucrose metabolism and (b) plant hormone signal transduction in comparisons between M-Zn0 and M-Zn1, Zn2 between X-Zn0 and X-Zn1, Zn2. Red box: upregulated. Blue box: downregulated. (c) Comparison of the FPKM value obtained by RNA-seq analysis with the gene expression obtained by qRT-PCR analysis.

promote the accumulation of 2AP in fragrant rice. Our study also depicted significant effects of foliar Zn application on 2AP and Zn contents in fragrant rice (Figure 2a,b). The 2AP and Zn contents were increased to the highest level in 15 d AHS leaves



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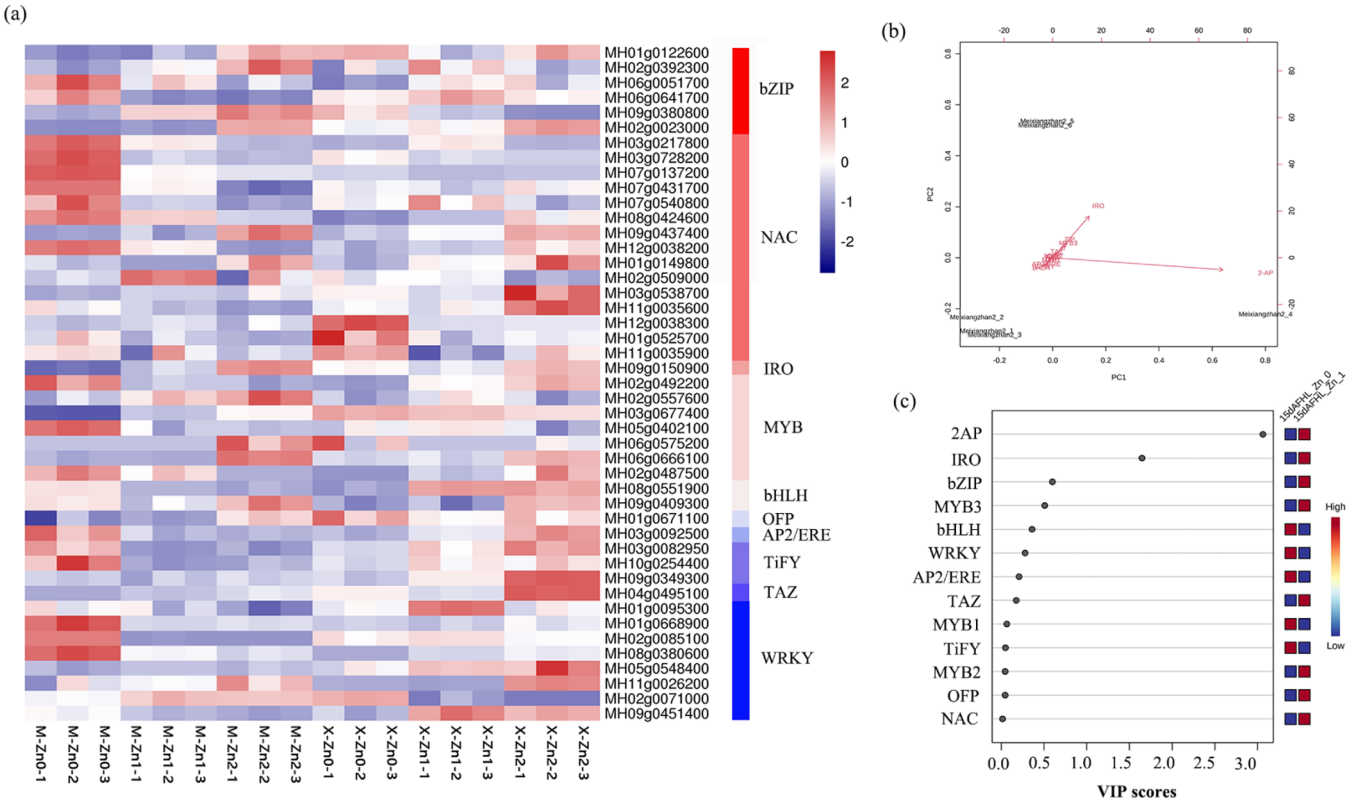


**Figure 5.** (a) Heatmap of known 33 TFs. Red means downregulation. Blue means upregulation. (b) Principal component analysis (PCA). (c) Partial least squares discriminant analysis (PLS-DA).

**Table 2. Effect of Zn Treatments on Yield and Yield-Related Traits**

| cultivars | treatments | panicle number pot$^{-1}$ | grain panicle$^{-1}$ | filled grain percentage (%) | 1000 grain weight (g) | yield (t/hm$^2$) |
|---|---|---|---|---|---|---|
| Meixiangzhan2 | Zn0 | 33.69$^a$ | 99.5$^b$ | 77.44$^b$ | 22.86$^a$ | 5.93$^b$ |
| | Zn1 | 34.21$^a$ | 113.73$^a$ | 84.39$^a$ | 22.45$^a$ | 7.37$^a$ |
| | Zn2 | 34.09$^a$ | 117.86$^a$ | 88.97$^a$ | 22.88$^a$ | 8.17$^a$ |
| Xiangyaxiangzhan | Zn0 | 29.99$^a$ | 119.66$^a$ | 83.47$^a$ | 18.78$^a$ | 5.62$^a$ |
| | Zn1 | 30.11$^a$ | 125.75$^a$ | 82.93$^a$ | 18.66$^a$ | 5.86$^a$ |
| | Zn2 | 29.44$^a$ | 122.78$^a$ | 82.67$^a$ | 19.96$^a$ | 5.96$^a$ |

$^{a,b}$ Different letter above the table indicates difference at $P < 0.05$ by LSD tests. Capped bars represent standard errors.

(Figure 2a,b). Li et al.[41] found that the application of manganese (Mn) had a positive effect on the 2AP formation of aromatic rice and improved the quality characters. Mo et al.[22] demonstrated that 2AP concentrations of detached aromatic rice panicles in vitro were higher when Zn was added to the basic culture medium. Exogenous application of mixed micronutrients significantly improves the quality of aromatic rice, including the aroma of aromatic rice.[42] Furthermore, Mo et al.[5] revealed that Si fertilization affects the 2AP content of aromatic rice by increasing the proline content and the PRODH activity in leaves and grains. Foliar applications of selenium (Se) improved the grain 2AP concentration.[43]

Previously, it has been reported that precursors and intermediates (proline, P5C, Δ1-pyrroline, methylglyoxal, and GABA) and enzymes (ProDH, P5CS, OAT, DAO, and BADH2) were all involved in the 2AP biosynthetic pathway.[44−46] Our results showed that the 2AP contents in aromatic rice were strongly positively correlated with the P5CS activity and proline, P5C, and Δ1-pyrroline levels (Table 1). These findings corroborate previous studies about the 2AP biosynthetic process in fragrant rice.[2] Proline has been confirmed to be a precursor of 2AP in aromatic rice, whereas higher proline content has been shown to result in the rich aroma in fragrant rice.[45] Poonlaphdecha et al.[45] found that P5C/Δ1-pyrroline was the rate-limiting factor of 2AP biosynthesis in fragrant rice. Methylglyoxal was identified as the carbon source for 2AP.[44] Our results showed that the 2AP contents in grains at MS were positively related to proline, P5C, 1-pyrroline, and methylglyoxal (Table S3). As for the relationship between 2AP and GABA content in fragrant rice, previous research results were different. Mo et al.[1] showed that the contents of 2AP and GABA had a positive correlation under all shading treatments. On the contrary, some studies had found that the concentrations of 2AP and GABA in rice were negatively correlated.[46] In addition, studies had shown that changes in the 2AP content had no effect on the GABA content.[45] This result was consistent with the results of our study. That is, the increase in the 2AP concentration had no effect on the change of the GABA content (Table S3).

Zn was normally involved in regulating carbohydrate metabolism, protein synthesis, gene expression, auxin metabolism, pollen formation, and biofilm stability.[47] TFs not only


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regulated plant growth and morphogenesis but also played an important role in plant stress response.[48] Our study also depicted significant effects of foliar Zn application on several TFs in fragrant rice (Figure 5). There were at least two gene expression regulatory pathways in plants, including ABA dependence and ABA nondependence, which involved MYC, MYB, bZIP, and CBF/DREB transcription factor and the corresponding cis-acting element (ABRE, DRE/CRT, LTRE).[49] bZIP functioned by modifying or forming dimers after translation. The promoter sequences of many stress-related genes contained ABA response elements (ABREs) and the activated bZIP were able to bind to the ABREs, thereby regulating the downstream gene expression.[50] OsNAC2 could directly activate chlorophyll degradation gene *OsSGR* and *OsNYC3*, increase abscisic acid (ABA) content, and accelerate leaf senescence.[51] Therefore, we hypothesized that foliar Zn application might indirectly regulate plant hormones by regulating the ABA-dependent TFs. On the other hand, it was also possible that Zn directly regulated plant hormones. Studies in maize show that short-term Zn deficiency can reduce the ABA content of sensitive varieties and increase the ABA content of resistant varieties.[52] Zn was the structural component of aldehyde carboxylase and was involved in sucrose and starch synthesis.[53] ABA was the product of carotenoid cleavage, and carotenoids were precursors of many aroma substances.[54] Therefore, these may be the reason why the pathways involved in starch and sucrose metabolism and plant hormone signaling were significantly enriched in the analysis carried out based on DEGs under Zn treatments (Figure 4).[55]

This study showed that the 2AP contents in leaves reached the highest levels at 15 d AHS in both fragrant rice cultivars (Figure 2a). It was predicted that the transcription level of the regulating 2AP biosynthesis genes would be active at 15 d AHS in leaves in both fragrant rice cultivars. Therefore, transcriptomic analysis and preliminary screening of TFs were carried out (Figure 5). 2AP biosynthesis was not only influenced by the related genes but may also be related to the regulation of TFs. The expression patterns of *P5CS2*, *DAO5*, and *BADH2* genes were consistent with the accumulation pattern of the 2AP contents (Figure 2a,c), indicating that *P5CS2*, *DAO5*, and *BADH2* genes were the node genes of 2AP biosynthesis in fragrant rice. Pearson correlation showed that the expression level of *P5CS2* had the strongest positive correlation with the 2AP content (Figure 2d,e).

The Zn2 (30 mg $L^{-1}$) treatment had a positive effect on the yield and aroma of fragrant rice. The *P5CS2* gene was selected as the key gene regulated by foliar Zn application in the 2AP biosynthesis pathway of aromatic rice. PCA and PLS-DA showed that IROs had the greatest regulatory effect on the 2AP biosynthesis under foliar Zn application, followed by bZIP, MYB, bHLH, and WRKY. However, the regulation of the *P5CS2* gene by TFs required technologies such as Y1H (yeast 1 hybrid), EMSA (electrophoresis mobility shift assay), and transient expression system for further verification.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c03655.

Table S1: properties of the soil characteristics. Table S2: primer sequences of genes. Table S3: effects of foliar spray Zn on the 2AP, precursors, intermediates, and the enzymes in leaves and grains of fragrant rice. Table S4: statistics of raw sequencing data results. Table S5: output statistics of sequencing. Table S6: gene quantity statistics. Table S7: GO enrichment analysis. Table S8: KEGG pathways (PDF)

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## Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

2AP, 2-acetyl-1-pyrroline; P5C, 1-pyrroline-5-carboxylate; PRODH, proline dehydrogenase; P5CS, 1-pyrroline-5-carboxylate synthetase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; DAO, diamine oxidase; BADH2, betaine aldehyde dehydrogenase 2; TFs, transcription factors; d AHS, days after heading stage; MS, maturity stage; MS G, maturity stage in grains; Y1H, yeast 1 hybrid; EMSA, electrophoresis mobility shift assay

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