



## Research article

## Molecular basis for increased 2-acetyl-1-pyrroline contents under alternate wetting and drying (AWD) conditions in fragrant rice

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## ABSTRACT

Factors affecting rice aroma biosynthesis have been well documented previously, however the molecular mechanism lies behind the regulations in grain 2-acetyl-1-pyrroline (2AP) biosynthesis under alternate wetting and drying (AWD) remained largely unexplored. Present study investigated the effects of three irrigation regimes i.e., conventional irrigation (CI), alternate wetting and moderate drying (WMD), and alternate wetting and severe drying (WSD) on the yield, quality traits, intermediates, enzyme activities and genes involved in 2-acetyl-1-pyrroline biosynthesis in two fragrant rice cultivars viz, Meixiangzhan2 and Xiangyaxiangzhan. Results revealed that the levels of intermediates such as  $\Delta 1$ -pyrroline-5-carboxylate (P5C) and  $\Delta 1$ -pyrroline, and the activity of enzymes such as proline dehydrogenase (PRODH),  $\Delta 1$ -pyrroline-5-carboxylate synthetase (P5CS), diamine oxidase (DAO), and gene expressions of *PRODH*, *P5CS2* and *DAO* were comparatively higher under AWD than CI in both aromatic rice cultivars. The levels of gamma-aminobutyric acid (GABA), betaine aldehyde dehydrogenase 2 (*BADH2*) and *BADH2* gene were lower that together led to enhanced 2-AP contents in rice grains. Moreover, WMD irrigation improved yield and yield characters, while WSD irrigation reduced yield and quality traits of rice. Overall, up-regulation of P5C and  $\Delta 1$ -pyrroline and down-regulation of GABA under AWD treatments resulted in enhanced 2AP biosynthesis in both rice cultivars. Evaluation and adoption of AWD (within safe limits) at field level could be an alternative option to conventional flooded rice to get better yield and quality.

## 1. Introduction

Fragrant rice is well-known for its pleasant aroma, fine grain quality and high nutritional value (Wakte et al., 2017; Ashraf et al., 2018). The 'pop-corn' like smell and textural composition of fragrant rice is well appraised by the consumers (Baradi and Martinez, 2015; Ashraf et al., 2017; Ashraf and Tang, 2017) and thus fetching premium prices in international markets (Sakthivel et al., 2009).

Among the fragrant rices, the 'Basmati' rice of India and Pakistan and the 'Jasmine' rice of Thailand are the most popular in Asia (Mo et al., 2015). In recent era, the globalization has exposed the western countries to Asian cuisines, whereas the immigration of Asians to the western world resulted in increased consumption of fragrant rice in Europe, America and Australia (FAO, 2009). Hence, cultivation of fragrant rice could be more profitable to the rice growers than other rice

types.

From the last three decades, the aroma biosynthesis and its regulatory mechanisms in rice were studied in various aspects, however, the chemical nature of the aromatic compounds was firstly identified by Buttery and Ling (1982) and regarded the 2-acetyl-1-pyrroline (2AP) as the main aromatic compounds in fragrant rice which was later confirmed by many researchers (Yoshihashi et al., 2002; Chen et al., 2008; Niu et al., 2008; Mo et al., 2017). Recently, Hashemi et al. (2015) reported that about more than 100 volatile compounds are involved in aroma production in rice whilst more than 300 volatile compounds were identified in both aromatic and non-aromatic rice cultivars by Widjaja et al. (1996).

Previously attempts have been made to understand the mechanism of 2AP biosynthesis (Li et al., 2016; Wakte et al., 2017) and the proposed 2AP biosynthesis pathway has been presented in Fig. 1. Firstly,

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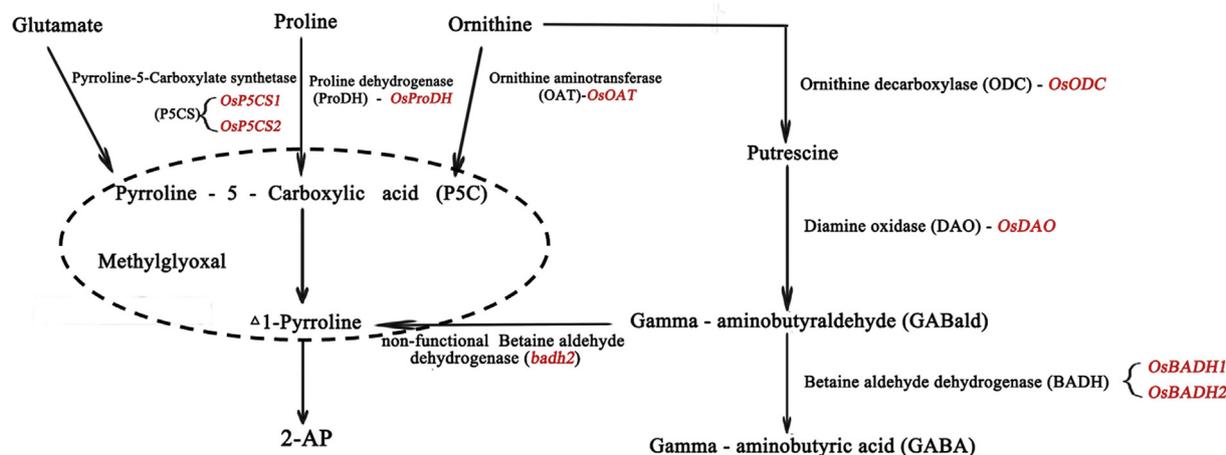


Fig. 1. The schematic representation of 2AP biosynthesis pathway in fragrant rice.

glutamate, proline, and ornithine are converted to  $\Delta 1$ -pyrroline-5-carboxylate (P5C) by the activities of proline dehydrogenase (PRODH),  $\Delta 1$ -pyrroline-5-carboxylate synthetase (P5CS), and ornithine aminotransferase (OAT), P5C/ $\Delta 1$ -pyrroline which non-enzymatically reacts with methylglyoxal to form 2AP (Bradbury et al., 2008; Chen et al., 2008; Kaikavoosi et al., 2015). Secondly, ornithine was catalyzed by ornithine decarboxylase (ODC) to form putrescine (Put), where diamine oxidase (DAO) is the sole enzyme responsible for the conversion of Put to GABald, which cyclizes to either  $\Delta 1$ -pyrroline or produces GABA, respectively, depending upon the functionality of betaine-aldehyde dehydrogenase2 (BADH2) (Ghosh and Roychoudhury, 2018). BADH2 transformed GABald into GABA to inhibit aroma production in non-aromatic rice, whereas non-functional BADH2 enzymes promoted the accumulation of  $\Delta 1$ -pyrroline resulting in accumulated 2AP in aromatic rice (Chen et al., 2008).

Alternate wetting and drying (AWD) is a water-saving technique which improves water use efficiency in rice (Chu et al., 2014). Studies have shown that AWD can substantially affect the rice growth, development, yield, and grain quality attributes, however, some controversies still exist among scientists. For example, AWD lead to substantial improvements in growth and yield of rice with better water use efficiencies (Carrizo et al., 2017; Sandhu et al., 2017; Li et al., 2018), whereas others reported that although AWD improves growth and water use efficiency but no significant affects were observed on rice yield (Ye et al., 2013). Nonetheless, AWD under safe limits may lead to substantial improvements in growth, yield and quality attributes of rice (Yang and Zhang, 2010). In addition, AWD at tillering, booting and filling stage could enhance grain 2AP contents of fragrant rice (Ren et al., 2017).

Hence, water regulations could alter the enzyme activities and aroma biosynthesis in rice but the molecular mechanism of the overall biochemical pathways related to aroma biosynthesis and its related genes expression analysis was rarely studied. Present study investigated the precursors and enzyme activities related to 2AP biosynthesis as well as the expression of 2AP related genes in rice under AWD conditions. This study could further strengthen the understandings about the molecular mechanisms of the aroma biosynthesis in fragrant rice.

## 2. Materials and methods

### 2.1. Plant materials and treatment conditions

A pot experiment was conducted in the green house of the Experimental Research Farm, College of Agriculture, South China Agricultural University, Guangzhou, China (23090 N, 113220 E and 11 m above the sea level) during March–July, 2017. Rice grown in this

season is called ‘early rice’ in double rice cropping system of South China. This region is characterized by subtropical monsoon type of climate with 21–29 °C mean annual temperatures and > 75% average humidity (Ashraf et al., 2017; Li et al., 2016; Mo et al., 2017). The seeds of two fragrant rice cultivars i.e., Meixiangzhan2 (Lemont  $\times$  Fengaozhan) and Xiangyaxiangzhan (Xiangsimiao126  $\times$  Xiangyuanzhan) were obtained from College of Agriculture, South China Agricultural University, Guangzhou, China. The seeds of both rice cultivars were surface sterilized with 5% sodium hypochlorite solution and then immersed in water for 12 h and allowed to germinate in dark thermostatic incubator at 38 °C for 12 h. The germinated seeds were sown in soil containing plastic trays for nursery growing. The experimental soil was collected from paddy fields, air dried, passed through 4 mm sieve, thoroughly mixed. Each pot was filled with 10 kg of soil. The experimental soil was sandy loam containing 23.34 g kg<sup>-1</sup> organic matter, 1.139 g kg<sup>-1</sup> total nitrogen, 1.136 g kg<sup>-1</sup> total phosphorus, 24.41 g kg<sup>-1</sup> total potassium, 127.03 mg kg<sup>-1</sup> available phosphate, 114.27 mg kg<sup>-1</sup> alkali-hydrolyzable nitrogen, 61.34 mg kg<sup>-1</sup> available potassium, 6.14 pH. Thirty-day-old seedlings were transplanted with five hills per pot and four seedlings per hill. From heading stage to maturity, three irrigation regimes viz., (i) conventional irrigation (CI), (ii) alternate wetting and moderate soil drying (WMD), when the soil water potential reached  $-15 \pm 5$  kPa, and then was flooded with 2–3 cm water depth, and (iii) alternate wetting and severe soil drying irrigation (WSD), the soil water potential reached  $-25 \pm 5$  kPa, and then was flooded with 2–3 cm water depth. Soil moisture meter hermetically sealed with vacuum gauge was used to measure the soil water potential with the depth of 10–15 cm below the soil surface.

Each pot was applied with 181 mg kg<sup>-1</sup> urea (46% N), 333 mg kg<sup>-1</sup> calcium superphosphate (12% P<sub>2</sub>O<sub>5</sub>) and 117 mg kg<sup>-1</sup> potassium chloride (60% K<sub>2</sub>O) with 70% at basal and 30% at tillering stage. Crop was managed by following the recommendations governed by the province. Plants were harvested on July 4th, 2017. The fresh panicles were harvested at maturity and stored at  $-80$  °C for bio-chemical and molecular analysis.

### 2.2. Determination of grain 2AP contents, P5C content, $\Delta 1$ -Pyrroline and GABA content

The 2AP contents in grains was evaluated by synchronization distillation and extraction method (SDE) combined with GCMS-QP 2010 Plus (Shimadzu Corporation, Japan) as described by Mo et al. (2015).

The  $\Delta 1$ -pyrroline-5-carboxylate (P5C) contents were determined by following the protocols devised of Mezl and Knox (1976). Fresh grains (0.1 g) were ground into powder with liquid nitrogen. The extraction solution contained 50 mM Tris-HCl (pH 8.0), 10% glycerol, 1% tritonX-

100, and 1%  $\beta$ -mercaptoethanol. The supernatant was added to a mixture containing 10% trichloroacetic acid (TCA) and 40 mM  $\gamma$ -aminobenzaldehyde. The absorbance was noted at 440 nm. The concentration of P5C was determined using the extinction coefficient  $2.58 \text{ mM}^{-1} \text{ cm}^{-1}$ .

The amount of  $\Delta 1$ -pyrroline was determined by the method of Holmstedt et al. (1961). The reaction mixtures were mixed with 0.01 mM  $\gamma$ -aminobenzaldehyde (in 0.02 mM phosphate buffer, pH 7.0) and 0.2 mM phosphate buffer (pH 7.0). The mixtures were kept at room temperature for 30 min, the absorbance of the reaction solution was measured at 430 nm using the extinction coefficient  $1860 \text{ cm}^{-1}$ .

Gama amino butyric acid (GABA) contents in grains were measured by the method of Zhao et al. (2009) and Yao et al. (2008). The extraction solution contained 60% ethanol, 60 mM lanthanum chlorid and 1 M KOH. The supernatant was added to 0.2 M borate buffer (pH 10.0) and 6% phenol solution and sodium hypochloride (available chlorine, 10%) was added while shaking. The absorbance of the reaction mixture was measured at 645 nm.

### 2.3. Determination of proline dehydrogenase (PRODH, EC 1.5.99.8) activity in grains

The proline dehydrogenase (PRODH) activity in grains was measured using Plant PRODH Elisa Kit (Mlbio, Shanghai, China) according to the manufacturer's protocol. The absorbance was measured by using BioTek Spectrophotometer EPOCH (BioTek, Vermont, USA) at 450 nm. PRODH activity was expressed as  $\text{U L}^{-1}$ .

### 2.4. Determination of $\Delta 1$ -pyrroline-5-carboxylate synthetase (P5CS, EC 1.5.1.12) activity in grains

The  $\Delta 1$ -pyrroline-5-carboxylate synthetase (P5CS) activity was measured by the method devised by Zhang and Lu (1995). Fresh samples (0.3 g) were extracted with 50 mM Tris-HCL buffer (pH 7.5) containing 7.0 mM  $\text{MgCl}_2$ , 1.0 mM KCl, 3.0 mM ethylenediamine tetraacetic acid (EDTA), 1.0 mM DL-Dithiothreitol (DTT) and 5% insoluble polyvinyl polypyrrolidone (PVP). The reaction mixture contained 50 mM Trise-HCL buffer (pH 7.0), 20 mM  $\text{MgCl}_2$ , 50 mM sodium glutamate, 10 mM ATP, 100 mM hydroxamate-HCL and enzyme extract. The reaction was stopped by adding 2.5%  $\text{FeCl}_3$  and 6% trichloroacetic acid (TCA), dissolved in 2.5 M HCl. The absorbance was read at 340 nm. P5CS activity was expressed as  $\mu\text{mol g}^{-1} \text{ FW}$ .

### 2.5. Determination of ornithine aminotransferase (OAT, EC 4.1.1.17) activity in grains

The ornithine aminotransferase (OAT) activity was recorded according Chen et al. (2001). Fresh samples (0.3 g) were extracted with 50 mM Tris-HCL buffer (pH 7.5) containing 7.0 mM  $\text{MgCl}_2$ , 1.0 mM KCl, 3.0 mM EDTA, 1.0 mM DTT and 5% PVP. The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 50 mM ornithine, 20 mM  $\alpha$ -ketoglutarate, 1 mM pridoxal-5-phosphate and the enzyme extract. The reaction was stopped by adding 10% TCA. The absorbance was read at 440 nm. OAT activity was expressed as  $\mu\text{mol g}^{-1} \text{ FW}$ .

### 2.6. Determination of diamine oxidase (DAO, EC 1.4.3.6) activity in grains

The diamine oxidase (DAO) activity was determined according to Su et al. (2005). Fresh samples (0.3 g) were extracted with 50 mM Tris-HCL buffer (pH 7.5) containing 7.0 mM  $\text{MgCl}_2$ , 1.0 mM KCl, 3.0 mM EDTA, 1.0 mM DTT and 5% PVP. The reaction solutions contained 0.1 M phosphate buffer, pH 6.5, 0.1 M putrescine (Put), 0.25  $\text{U L}^{-1}$  peroxidase, 0.82 mM 4-aminoantipyrine and enzyme extract. The absorbance was measured at 550 nm and DAO activity was expressed as  $\mu\text{mol g}^{-1} \text{ FW}$ .

### 2.7. Determination of betaine aldehyde dehydrogenase2 (BADH2, EC 1.2.1.8) activity in grains

The betaine aldehyde dehydrogenase2 (BADH2) enzyme activity was measured using Plant PRODH Elisa Kit (Mlbio, Shanghai, China) according to the manufacturer's protocol. The absorbance was measured by using BioTek Spectrophotometer EPOCH (BioTek, Vermont, USA) at 450 nm. BADH2 activity was expressed as  $\text{U L}^{-1}$ .

### 2.8. Real-time quantitative RT-PCR

Fresh grains (0.03 g) were collected for total RNA extraction. Total RNA was extracted using HiPure Plant RNA Mini Kit (Magen, Guangzhou, China). The quality and quantity of RNA was assessed by Nanodrop 2000. The Hiscript II QRT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) was used to synthesize cDNA from 500 ng of total RNA. The following mixtures were prepared in qPCR tubes: 4.4  $\mu\text{l}$  cDNA, 0.2  $\mu\text{l}$  each for forward and reverse primers, 5  $\mu\text{l}$  2\*chamQ SYBR qPCR Master MiX and 0.2  $\mu\text{l}$  ROX reference Dye 1, ddH<sub>2</sub>O to 20  $\mu\text{l}$  (Vazyme, Nanjing, China). Real-time quantitative RT-PCR (qRT-PCR) was conducted in CFX96 real-time PCR System (Bio-Rad, Hercules, CA, USA). Each RNA sample was performed in triplicate. A negative control without cDNA template was always included. Primers used for qRT-PCR were listed in Table 1. All primers were designed using the software tool Primer 5.

### 2.9. Determination of yield and yield related traits

Five pots were randomly harvested from each treatment at maturity, threshed manually and sun dried (adjusted to ~14% moisture contents) to get grain yield. Panicle number per pot was measured by counting the panicle numbers of each hill in five different pots in each treatment and averaged. Grains were threshed manually from each panicle to count total number of grains and number of filled grains per panicle. To record 1000-grain weight, five random samples from filled grains were counted, weighed and averaged.

### 2.10. Determination of grain quality traits

After sun drying, the grains were stored at room temperature for at least three months to determine grain quality. Thereafter, about 500 g rice grain from each treatment was taken from storage and brown rice rate was estimated using a rice huller (Jiangsu, China) while milled rice and head rice rates were estimated by using a Jingmi testing rice grader (Zhejiang, China). Grains with chalkiness and chalkiness degree were assessed by using an SDE-A light box (Guangzhou, China). Grain amylose and protein contents were determined through an Infratec-1241 grain analyzer (FOSS-TECATOR).

### 2.11. Experimental design and statistical analyses

The experiment was arranged in completely randomized design

**Table 1**  
Primer sequences of genes encoding enzymes involved in 2AP synthesis in rice grains.

Gene name	Accession No.	Primer sequences
PRODH	AK121010	F 5'-TCATCAGACGAGCAGAGGAGAACAGG-3' R 5'-CCCAGCAITGACGCTTGAACC-3'
P5CS2	AK101230	F 5'-GAGGTTGGCATAAGCAGACAG-3' R 5'-CTCCCTTGTGCGCCGTTC-3'
DAO	AK099435	F 5'-TGGCAAGATAGAAGCAGAAGT-3' R 5'-GTCCATACGGGCAACAAA-3'
BADH2	AB096083	F 5'-GGTGGTCTTCCTCAGGTGTGC-3' R 5'-CATCAACATCATCAACACCACTAT-3'

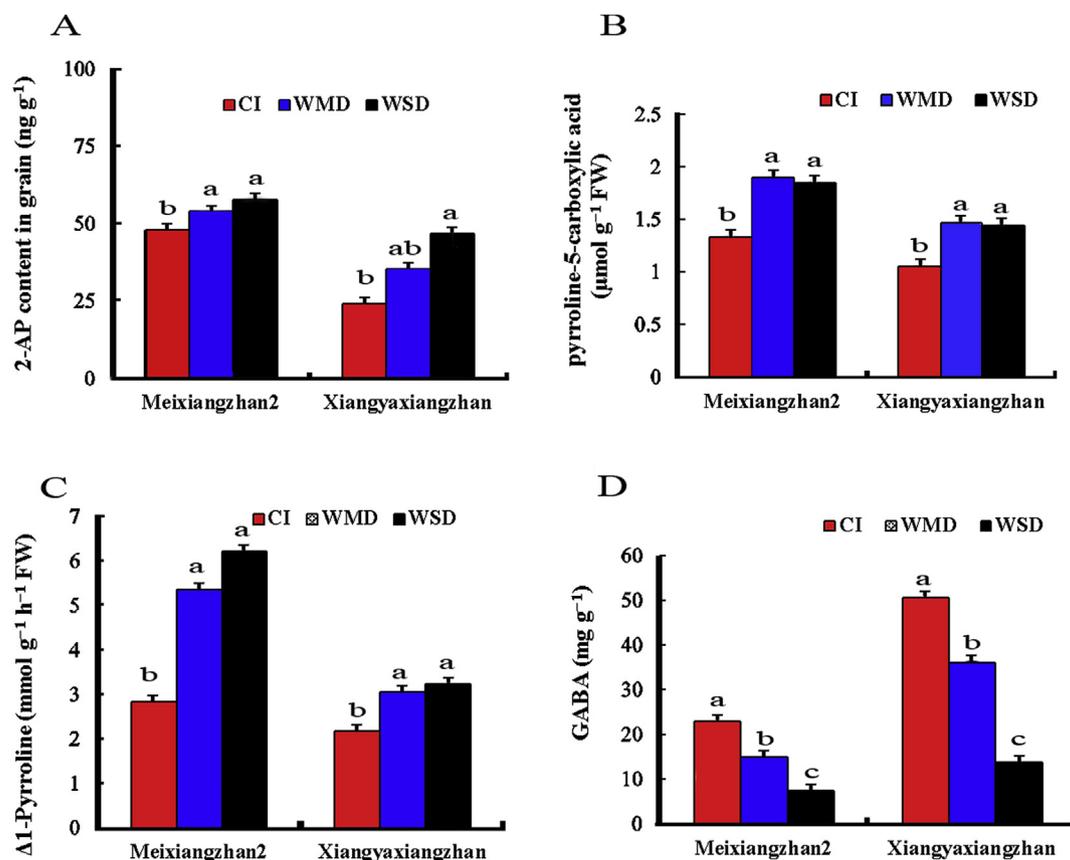


Fig. 2. The effect of AWD treatments on the (A) 2AP, (B) P5C, (C)  $\Delta 1$ -Pyrroline and (D) GABA contents in Meixiangzhan2 and Xiangyaxiangzhan. CI: conventional irrigation; WMD: alternate wetting and moderate soil drying; WSD: alternate wetting and severe soil drying irrigation. The different letter above the column indicates difference at  $P < 0.05$  by LSD tests. Capped bars represent standard errors.

(CRD) with three replications. All data were subjected to analysis of variances (ANOVA) according to the module for completely randomized design (CRD) using Statistix 8 (Analytical software, Tallahassee, Florida, USA). The data were analyzed by one-way analysis of variance and the differences amongst means were separated by using least significant difference (LSD) test at 5% significance level.

### 3. Results

#### 3.1. Effect of AWD on the 2AP, P5C, $\Delta 1$ -Pyrroline and GABA levels in grains of fragrant rice

AWD treatments substantially improved the grain 2AP contents in both rice cultivars. Compared with CI, the 2AP contents were increased by 10.61%–20.29% in Meixiangzhan2 and 45.86%–93.87% in Xiangyaxiangzhan under MWD and WSD, respectively. Comparatively, Meixiangzhan2 accumulated higher 2AP contents than Xiangyaxiangzhan (Fig. 2A). The P5C contents were noticeably higher in WMD ( $1.9 \pm 0.18$  and  $1.47 \pm 0.17 \mu\text{mol g}^{-1}$ ) and WSD ( $1.85 \pm 0.08$  and  $1.44 \pm 0.06 \mu\text{mol g}^{-1}$ ) than in CI ( $1.33 \pm 0.08$  and  $1.05 \pm 0.05 \mu\text{mol g}^{-1}$ ) in Meixiangzhan2 and Xiangyaxiangzhan, respectively (Fig. 2B).  $\Delta 1$ -Pyrroline contents were also followed the same trend as P5C i.e.,  $\text{WMD} > \text{WSD} > \text{CI}$  in both rice cultivars (Fig. 2C). The GABA contents were decreased by 34.56–67.97% and 28.67–73.03% for Meixiangzhan2 and Xiangyaxiangzhan, respectively under AWD treatments than CI (Fig. 2D).

#### 3.2. Analysis of 2AP biosynthesis enzyme activities in grains of fragrant rice under alternate wetting and drying irrigation treatments

AWD differentially affected the activities of PRODH, P5CS, OAT,

DAO and BADH2 in both rice cultivars. Compared with CI, the activities of PRODH and DAO were increased 24.56–107.02% and 37.31–82.09% under WMD and WSD treatments in both rice cultivars (Fig. 3A and D). BADH2 activity was found to decrease significantly under WSD treatments in both cultivars, whereas WMD was found statistically similar ( $P > 0.05$ ) with CI (Fig. 3E). P5CS activities were found higher only in WSD treatments than CI in both cultivars (Fig. 3B). No significant difference was noted among CI and AWD treatments for OAT activity in grains (Fig. 3C).

#### 3.3. Effects of AWD on genes related to 2AP biosynthesis in grains

Real-time PCR analyses depicted that levels of *PRODH*, *P5CS2* and *DAO* transcripts were higher in AWD treatments than CI (Fig. 4A, B, C). *BADH2* transcript was present at significantly lower levels in WMD and WSD treatments than CI. AWD treatments decreased the expression of *BADH2* genes by 3–4.8 fold and 2.39–3.56 fold for Meixiangzhan2 and Xiangyaxiangzhan, respectively (Fig. 4D).

#### 3.4. Effect of AWD treatments on the yield and related traits

For Meixiangzhan2, the grain panicle<sup>-1</sup>, filled grain percentage and grain yield were increased by 21.32%, 0.65% and 28.57%, respectively in WMD than CI, however, WSD reduced the grain panicle<sup>-1</sup>, filled grain percentage and 1000 grain weight by 18.44%, 5.59% and 14.92%, respectively. For Xiangyaxiangzhan, compared with CI, no significant improvements were observed regarding panicle number pot<sup>-1</sup>, grain panicle<sup>-1</sup>, and 1000-grain weight under AWD treatments. The filled grain percentage was 3.94% higher in WMD whilst 10.36% lower in WSD than CI. Furthermore, grain yield of Meixiangzhan2 and Xiangyaxiangzhan was decreased by 35.71%, and 14.78%, respectively

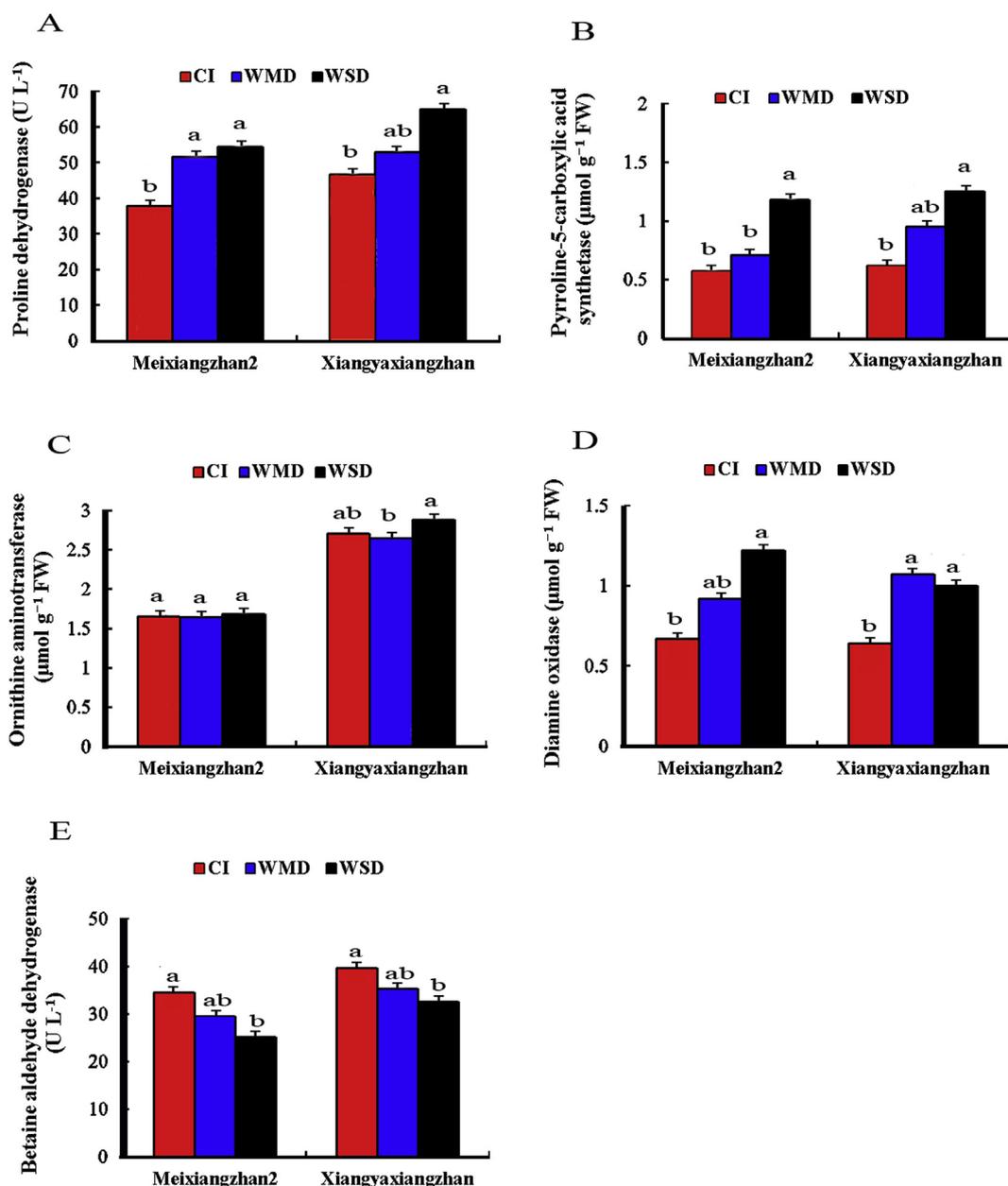


Fig. 3. Effects of AWD on the activities of (A) proline dehydrogenase (PRODH), (B)  $\Delta$ 1-pyrroline-5-carboxylate synthetase (P5CS), (C) ornithine aminotransferase (OAT), (D) diamine oxidase (DAO), and (E) betaine aldehyde dehydrogenase2 (BADH2) in both fragrant rice cultivars. CI: conventional irrigation; WMD: alternate wetting and moderate soil drying; WSD: alternate wetting and severe soil drying irrigation. The different letter above the column indicates difference at  $P < 0.05$  by LSD tests. Capped bars represent standard errors.

under WSD than CI (Table 2).

### 3.5. Effect of AWD treatments on grain quality attributes

The quality traits of both rice cultivars had not been affected under WMD treatment than CI. For Meixiangzhan2, WSD reduced brown rice, milled rice and head milled rice by 6.94%, 10.65% and 16.69% than CI, respectively. For Xiangyaxiangzhan, under WSD, the head milled rice percentage was reduced by 24.18% while grain chalkiness degree was enhanced by 71.23 and 234.95%, in Meixiangzhan2 and Xiangyaxiangzhan, respectively (Table 3).

### 3.6. Relationships among 2AP, enzymes and intermediates of 2AP biosynthesis

2AP was significantly and positively correlated with P5C and  $\Delta$ 1-

Pyrroline contents, whilst significantly and negatively with BADH2 activity and GABA contents. BADH2 activity was significantly and positively correlated with GABA, while significantly and negatively with P5C and  $\Delta$ 1-Pyrroline contents. Moreover, P5CS activity has positive relations with PRODH and DAO activity as well as  $\Delta$ 1-Pyrroline with P5C contents (Table 4).

### 3.7. Relationships among 2AP and genes of 2AP biosynthesis

Significant correlations were detected between 2AP and P5CS2 in response to AWD treatments whereas no significant relations were found between other genes and 2AP (Table 5).

## 4. Discussion

Rice aroma biosynthesis is affected by several agronomic and

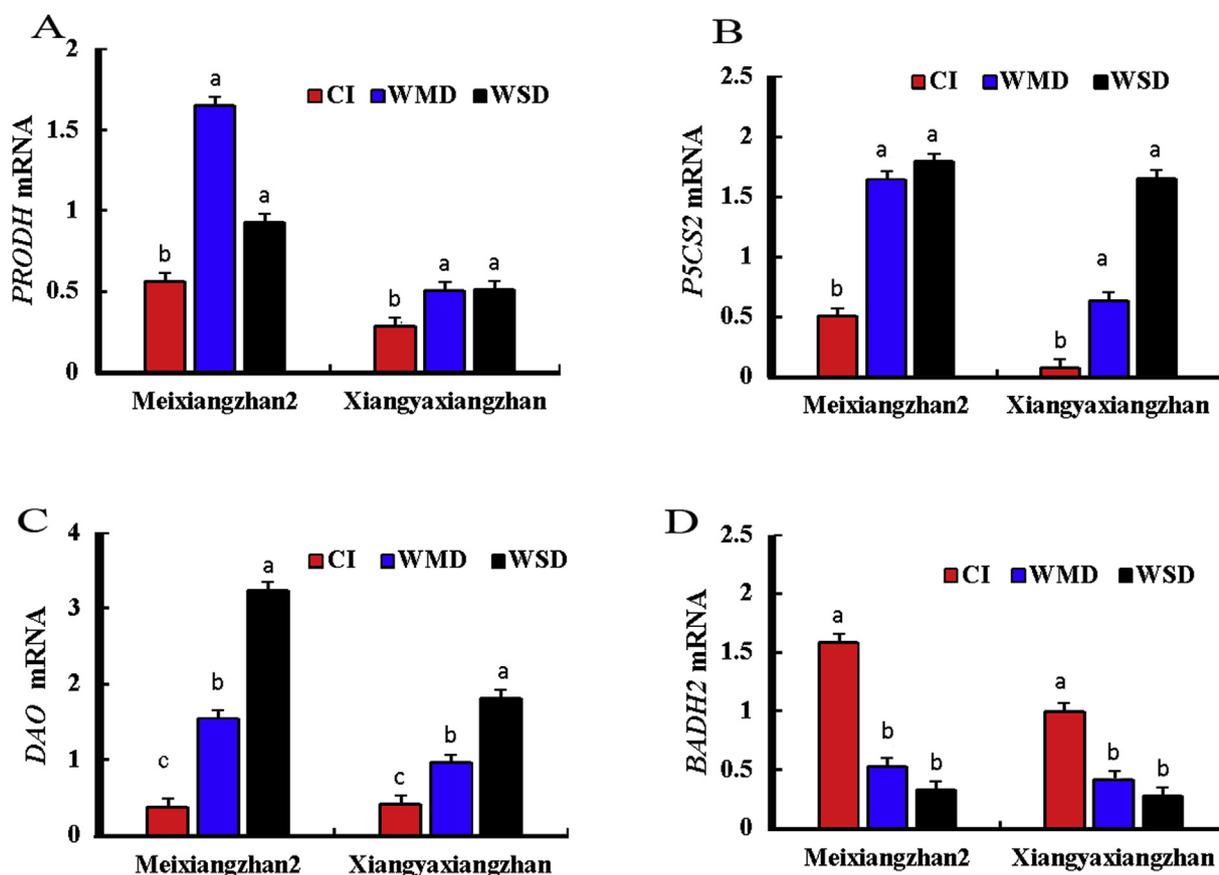


Fig. 4. Analysis of transcript levels of (A) *PRODH*, (B) *P5CS2*, (C) *DAO*, and (D) *BADH2* in the grains of both fragrant rice cultivars under AWD treatments. CI: conventional irrigation; WMD: alternate wetting and moderate soil drying; WSD: alternate wetting and severe soil drying irrigation. The different letter above the column indicates difference at  $P < 0.05$  by LSD tests. Capped bars represent standard errors.

Table 2

Effect of alternate wetting and drying irrigation treatments on yield and yield related traits.

Cultivars	Treatments	Panicle number pot <sup>-1</sup>	Grains panicle <sup>-1</sup>	Filled grain percentage (%)	1000 grain weight (g)	yield pot <sup>-1</sup> (g)
Meixiangzhan2	CI	32.79a	85.5b	63.44b	22.86a	40.6b
	WMD	33.21a	103.73a	67.54a	22.45a	52.2a
	WSD	32.09a	69.73c	59.89c	19.45b	26.1c
Xiangyaxiangzhan	CI	28.99a	119.66a	66.47b	18.78a	43.3b
	WMD	29.11a	125.75a	69.09a	18.66a	47.1ab
	WSD	28.44a	116.75a	61.93c	17.96a	36.9c

Different letter above the table indicates difference at  $P < 0.05$  by LSD tests. Capped bars represent standard errors. CI: conventional irrigation; WMD: alternate wetting and moderate soil drying; WSD: alternate wetting and severe soil drying irrigation.

external climatic factors e.g., fluctuating temperature, salinity, shading, plant nutrition/fertilizer, gamma irradiation, harvesting time and storage conditions (Gay et al., 2010; Goufo et al., 2010; Tananuwong and Lertsiri, 2010; Mo et al., 2015; Prodhan et al., 2017; Ren et al., 2017; Sansenya et al., 2017). This study focuses on the AWD-induced changes

in molecular mechanism of rice aroma biosynthesis. It was found that AWD improved the grain 2AP contents and such increment is dependent on irrigation treatments and rice cultivars (Fig. 2A), which are corroborated with the previous reports of Prodhan et al. (2017). Due to the specificity of the fragrant rice cultivars, the grain 2AP contents were

Table 3

Effect of alternate wetting and drying irrigation treatments on grain quality.

Cultivars	Treatments	Brown rice (%)	Milled rice (%)	Head milled rice (%)	Chalkiness degree (%)	Protein content (%)	Amylose content (%)
Meixiangzhan2	CI	75.32a	56.34a	43.44a	1.85b	10.98a	15.34a
	WMD	74.91a	57.11a	44.23a	1.67b	10.78a	15.65a
	WSD	70.09b	50.34b	36.19b	6.43a	10.65a	15.12a
Xiangyaxiangzhan	CI	75.66a	59.66a	46.07a	1.03b	10.33a	14.24a
	WMD	74.11a	58.45a	47.19a	1.12b	10.89a	14.67a
	WSD	73.88ab	56.75a	34.93b	3.45a	10.12a	14.33a

Different letter above the table indicates difference at  $P < 0.05$  by LSD tests. Capped bars represent standard errors. CI: conventional irrigation; WMD: alternate wetting and moderate soil drying; WSD: alternate wetting and severe soil drying irrigation.

**Table 4**  
Relationships among 2AP, enzymes and precursors of 2AP biosynthesis.

Index	2AP content	BADH2 activity	PRODH activity	OAT activity	DAO activity	P5CS activity	P5C content	$\Delta 1$ -pyrroline content	GABA content
2AP content	1	-0.924**	0.1957	-0.7196	0.5318	0.3706	0.8522*	0.8248*	-0.97038**
BADH2 activity	-0.924**	1	-0.35140	0.6214	-0.7466	-0.5397	-0.9199*	-0.9559**	0.909*
PRODH activity	0.1957	-0.35140	1	0.4901	0.6825	0.8777*	0.329	0.2765	-0.3951
OAT activity	-0.7196	0.6214	0.4901	1	-0.0574	0.2968	-0.6156	-0.6564	0.536
DAO activity	0.5318	-0.7466	0.6825	-0.0574	1	0.8444*	0.7201	0.6953	-0.605
P5CS activity	0.3706	-0.5397	0.8777*	0.2968	0.8444*	1	0.389	0.3967	-0.5517
P5C content	0.8522*	-0.9199*	0.329	-0.6156	0.7201	0.389	1	0.9431**	-0.8018
$\Delta 1$ -pyrroline content	0.8248*	-0.9559**	0.2765	-0.6564	0.6953	0.3967	0.9431**	1	-0.7708
GABA content	-0.97038**	0.909*	-0.3951	0.536	-0.605	-0.5517	-0.8018	-0.7708	1

\*Significant at  $P < 0.05$ ; \*\*Significant at  $P < 0.01$ .

**Table 5**  
Relationships among 2AP and genes of 2AP biosynthesis.

Index	2AP content	<i>BADH2</i>	<i>P5CS2</i>	<i>PRODH</i>	<i>DAO</i>
2AP content	1	-0.247	0.8439*	0.6974	0.7019
<i>BADH2</i>	-0.247	1	-0.6888	-0.2899	-0.7155
<i>P5CS2</i>	0.8439*	-0.6888	1	0.654	0.8645
<i>PRODH</i>	0.6974	-0.2899	0.654	1	0.4237
<i>DAO</i>	0.7019	-0.7155	0.8645	0.4237	1

\*Significant at  $P < 0.05$ .

different under AWD treatments. Under WSD treatment, the content of 2AP in both cultivars was significantly higher than CI, which may be closely related to changes in precursors and intermediates of 2AP. Compared with CI, significant differences were noticed in Meixiangzhan2 whilst no significant difference in Xiangyaxiangzhan for grain 2AP under WMD treatment which may be related to the sensitivity of different cultivars to water stress/levels (Fig. 2A). Proline, P5C,  $\Delta 1$ -pyrroline, GABA and methylglyoxal were recognized as precursors and intermediates for the 2AP biosynthesis (Huang et al., 2008; Poonlaphdecha et al., 2012). Although proline and methylglyoxal had been found to accumulate in response to stresses, yet the accumulation under AWD treatment were remained limited in rice. Li et al. (2016) reported positive correlation of 2AP contents with the activities of PRODH, OAT and P5CS, along with P5C contents in fragrant rice which indicated that the higher 2AP contents are related to the increased enzyme activities involved in its biosynthesis. Ghosh and Roychoudhury (2018) also found that the aromatic rice varieties showed higher P5C levels and increased P5CS, OAT, and PDH activities as compared to non-aromatic varieties. Poonlaphdecha et al. (2016) confirmed that  $\Delta 1$ -pyrroline was a limiting factor for 2AP synthesis in rice. Our results showed that up-regulation of P5C and  $\Delta 1$ -pyrroline and down-regulation of GABA under AWD treatments resulted in enhanced 2AP biosynthesis in both fragrant rice cultivars. However, the exact mechanism related to the changes in enzyme activities and genes expression related to 2AP biosynthesis under AWD is still unclear, which needs to study in future.

Furthermore, the accumulation of 2AP is associated with transcriptional expression of *P5CS* genes (Hinge et al., 2016; Huang et al., 2008). For example, two functional genes coding *P5CS*, *P5CS1* and *P5CS2*, have been identified in rice related to 2AP biosynthesis (Hien et al., 2003). In rice, expression of *P5CS1* gene was stimulated by the treatment of high salt, dehydration, cold, and ABA (Igarashi et al., 1997), whilst *P5CS2* was induced by salt and cold stress (Hur et al., 2004). Our results reported that the expression levels of *P5CS2* genes was found to be significantly higher in WMD and WSD than CI (Fig. 4B), and significantly and positively correlated with 2AP in response to AWD treatments (Table 5). However, no response expression of *P5CS1* gene to different irrigation treatments was detected (data not shown). *P5CS1* has multiple as well as additional subtypes and is reported to have more than one isoforms (Taverniers et al., 2004). Kaikavoosi et al.

(2015) reported that the overexpression of *P5CS* gene led to enhance 2AP level in transgenic aromatic rice.

The DAO transformed Put into GABald (Struve and Christophersen, 2003) which further cyclizes spontaneously to  $\Delta 1$ -pyrroline or it produces GABA, depending on the absence or presence of functional BADH2 enzyme (Ghosh and Roychoudhury, 2018). BADH2 transformed GABald into GABA to inhibit aroma production in non-aromatic rice, whereas nonfunctional BADH enzymes promoted the accumulation of  $\Delta 1$ -pyrroline resulting in enhanced aroma production in aromatic rice varieties (Chen et al., 2008). In the current study, increased DAO activity and decreased BADH activity were noted in WMD and WSD, compared to CI (Fig. 3D and E). GABA accumulation in rice plants has always been shown to respond to biotic and abiotic stress (Bown and Shelp, 1997; Chung et al., 2009; Kinnersley, 2000). Previous studies found that the some relationship exists between the concentration of 2AP and GABA in fragrant rice. For example, Mo et al. (2015) found that all the shading treatments significantly increased the concentration of 2AP and GABA and reported positive associations between them which suggest the primary pathway for GABA production is the 'GABA shunt' (Fait et al., 2008) and/or there may be another enzyme exists in rice that catalyses the conversion of GABald to GABA. According to Poonlaphdecha et al. (2012) salt stress activates the 2AP contents but does not affect GABA contents. Low GABA content was also noted in isogenic lines of aromatic rice, as compared to non-aromatic lines as well as the negative correlation between the concentration of 2AP and GABA (Chen et al., 2008; Ghosh and Roychoudhury, 2018). This supports our results that increase in the concentration of 2AP with a decrease in GABA level (Fig. 2B), as well as negative correlation of GABA with 2AP concentration under WMD and WSD treatments in both rice cultivars (Table 4).

Two homologous genes encoding *BADH* in rice were *BADH1* and *BADH2*, respectively. The *BADH1* had significant correlation with salt tolerance in rice, whilst *BADH2* was only responsible for fragrance of rice (He et al., 2015). Comparing the two *BADH2* alleles, the deletion of 8 bp in exon 7 and 3 SNP in exon 13 were found responsible for aroma variation (He et al., 2015). The mRNA levels can be controlled co-ordinately by gene transcription efficiency and mRNA decay. The coding sequence mutation would shift open reading frame and result in the introduction of premature stop codons and mRNAs that contain premature stop codons could be degraded by nonsense-mediated decay (Wang et al., 2016). Niu et al. (2008) reported that the down-regulation of *OsBADH2* gene expression significantly increased the accumulation 2AP concentration. Hinge et al. (2016) showed that the *badh2* expression was negatively correlated with 2AP accumulation in fragrant cultivars over non-fragrant cultivars. Prodhhan et al. (2017) found that the down-regulation of the recessive *badh2* allele was responsible for the significant elevation of 2AP concentration under different temperature regimes in rice. In our study, the decrease in the transcription level of *BADH2* gene led to reduce BADH2 activity and thus accounted for higher 2AP concentration under AWD treatments (Figs. 3E and 4D).

In addition, the increase in yield was mainly due to the increase of

filled grain percentage under WMD (Table 2). Besides yield, quality is another important factor in rice. The effects of WMD treatments for all quality parameters were observed non-significant as compared with CI, however, the WSD reduced the head milled rice and induced grain chalkiness in both cultivars (Table 3). These confirm that AWD treatments can have a substantial effect on grain yield and quality of fragrant rice, whereas the WMD not only increased 2AP concentration but also increased yield. Previously, Wopereis et al. (1996) reported that leaf expansion and tillering decreased under the severe water stress condition, which could affect the grain yield in rice. Cao et al. (2017) found that the lower root biomass also decreased rice nutrient uptake and rice yield formation under severe water stress conditions. Hence, WMD can potentially be employed in fragrant rice to have better yields, rice aroma contents and other rice quality attributes. Although WSD increased the 2AP contents in both rice cultivars, the yield loss and grain quality loss were obviously harmful under WSD conditions.

## 5. Conclusion

AWD treatments regulated the precursors' accumulation, enzymes activities and genes expression of the enzymes that regulate the production of 2AP in both rice cultivars. The higher 2AP contents in Meixiangzhan2 than Xiangyaxiangzhan showed the genetic difference regarding production potential of 2AP biosynthesis. The WMD was found better than CI and WSD regarding grain yield, rice quality and rice aroma formation. Up-regulation of P5C and  $\Delta 1$ -pyrroline under AWD treatments results in enhanced 2AP biosynthesis in both rice cultivars through induced genes expression (*PRODH*, *P5CS2*, *DAO*) and enzyme activities (*PRODH*, *P5CS*, *DAO*), and down-regulation of GABA through inhibition of *BADH2*. Furthermore, this study provides the theoretical evidence for high yield, good quality and water saving cultivation of fragrant rice and further suggests that the evaluation and adoption of AWD (within safe limits) at field level could be alternative option to flooded rice.

## Authors' contributions

XT, GB, and ZM designed the experiment. GB, UA, CW, XW, LH performed the experiment, data collection, lab analysis, and data analysis. GB, AZ, and UA contributed in providing chemicals, reagent, analyses, and tools. GB and UA prepare the initial draft. XT, ZM and UA finalized the initial draft. All authors read and approved the final manuscript.

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