Exogenous γ-aminobutyric acid (GABA) application at different growth stages regulates 2-acetyl-1-pyrroline, yield, quality and antioxidant attributes in fragrant rice

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ABSTRACT
The aim of this study was to investigate the optimum time for γ-aminobutyric acid (GABA) application to improve the yield and quality in fragrant rice. Pot and field experiments were conducted during 2016–17 with two fragrant rice cultivars (for pot experiment), and five fragrant rice cultivars (for field experiment) which were applied with five GABA levels i.e. no GABA application (CK), application of GABA at 250 mg l−1 with 25 ml pot−1 at tillering stage (S1), panicle initiation stage (S2), heading stage (S3), and at tillering, panicle initiation and heading stages (S4) in the pot experiment. Similarly, the same treatments with 100 ml m−2 were applied to all rice cultivars in the field experiment. The S3 treatment significantly increased the 2-acetyl-1-pyrroline (2AP) contents in Meixiangzhan2 (14.76%) and Yuxiangyouzhan (20.19%) in pot experiment, Meixiangzhan2 (27.27%), Yuxiangyouzhan (40.24%), Basmati (43.07%) and Yungengyou14 (13.66%) in field experiment owing to regulations in the contents of proline, D1-pyrroline-5-carboxylic acid (P5C), GABA and the activities of enzymes involved in 2AP formation. The GABA treatments improved yield and modulated the antioxidant enzyme activities. This study provides a reference for the GABA application to improve yield and quality in fragrant rice.

1. Introduction
Rice is an important cereal crop that feeds billions of people around the world (Mahajan et al. 2010). Fragrant rice is a unique rice type and world-famous due to its special aroma, appearance, and taste, thus regarded as a superfine grain (Giraud 2013). In recent years, it has become more popular and its demand is increasing in international markets worldwide (Hashemi et al. 2013). However, the grain yield was generally lower in fragrant rice than non-fragrant rice cultivars. Thus, some effective strategies are needed and have also been previously employed to improve grain yield production in fragrant rice (Pan et al. 2013; Mo et al. 2015, 2017).

Rice aroma was regarded as one of the most critical quality characters (Das et al. 2018), aroma intensities are closely related to the mixture of aroma compounds in rice (Sansenya et al. 2018). The 2-acetyl-1-pyrroline (2AP) was reported as a crucial volatile compound involved in the intricate volatile chemistry of aroma in fragrant rice (Ren et al. 2017; Boontakham et al. 2019), which can be determined in all parts of fragrant rice excluding root (Maraval et al. 2010; Mo et al. 2015). In recent times, some studies focusing on the formation of aroma have revealed that the betaine-aldehyde dehydrogenase (BADH)-related gene is responsible for the biosynthesis and up–down regulation of 2AP (Bradbury et al. 2005; Chen et al. 2008). Previous studies reported that catalytic activities of proline dehydrogenase (PDH), D1-pyrroline-5-carboxylic acid synthetase (P5CS) and ornithine aminotransferase (OAT) convert the proline, glutamate, and ornithine into a common metabolite i.e. pyrroline-5-carboxylic acid (P5C) and thus act as precursors for several related physiological processes involved in the formation of 2AP in fragrant rice (Mo et al. 2016a; Ghosh and Roychoudhury 2018). Moreover, diamine oxidase (DAO) and soluble protein have also been found to be associated with grain 2AP production in fragrant rice (Li et al. 2016b; Deng et al. 2018).

Indeed, the biosynthesis and regulations of 2AP in fragrant rice are genetic-controlled mechanisms, however, agronomic measures and crop management practices can also affect the yield, enzyme activities, and genes involved in 2AP biosynthesis to a great extent (Bao et al. 2018). For example, Mo et al. (2019a, 2019b) showed that the regulations of 2AP were linked to the dynamics in water and nitrogen application in fragrant rice. Additionally, it appears that the production and accumulation of 2AP can also be linked to the external environmental conditions and abiotic stress factors, e.g. an increment of 2AP contents in rice was detected in response to salt stress (Poonaphredea et al. 2012). Similarly, Mo et al. (2015) reported that the accumulation of GABA and 2AP were enhanced simultaneously by shading or low light
intensity during the grain filling stage in fragrant rice. Besides, Bradbury et al. (2008) indicated that both the biosynthesis of γ-aminobutyric acid (GABA) and the accumulation of Δ1-pyrroline were largely affected by Bdh2 gene. Furthermore, it was reported that GABA was closely associated with the plant growth and grain yield (Li et al. 2017; Li et al. 2019b). Consequently, it is quite feasible to regulate 2AP biosynthesis and grain yield by the exogenous application of GABA in fragrant rice.

GABA is recognized as a signaling molecule that is involved in various physio-biochemical processes to regulate plant stress responses (Ramesh et al. 2017; Routray and Rayaguru 2018). In general, the endogenous concentration of GABA in plants is relatively low but its levels may be enhanced in response to the various stress conditions (Li et al. 2017). Previous studies confirmed that GABA is endowed with the ability to regulate physio-biochemical metabolism in different plants (Yu and Sun 2007; Song et al. 2010). Li et al. (2016a) suggested that exogenous GABA improved the salt stress tolerance in growing wheat plants due to the enhancement of photosynthesis and antioxidant enzyme activities. Exogenous GABA application improved the chlorophyll contents and other photosynthetic attributes in rice seedlings (Li et al. 2017). Xie et al. (2019) revealed that GABA application improved the nutrient uptake in rice. Shang et al. (2011) showed that GABA application played an important role in alleviating chilling injury in cold-stored peach fruit. Li et al. (2019b) demonstrated that GABA application could regulate rice yield and yield-related traits under different nitrogen levels. Hence, exogenous GABA applications can regulate either aroma biosynthesis or plant growth and even the grain yield by affecting the physiological metabolism. Previous studies have well documented the GABA-induced regulations in different plant species, however, a little is known about the effects of GABA application during the different growth stages of fragrant rice to find out the suitable application period or growth stage in fragrant rice. Therefore, the present study was conducted to investigate the effects of exogenous GABA application at different growth stages of fragrant rice on the 2AP contents and the physiological and biochemical attributes involved in 2AP biosynthesis, grain yield formation, grain quality as well as antioxidant defense system in fragrant rice.

2. Materials and methods

2.1. Experimental description

The pot and field experiments were performed in the greenhouse and under field conditions at Experimental Research Farm, College of Agriculture, South China Agricultural University (SCAU), Guangzhou, P.R. China from 2016 to 2017. The experimental region has a humid subtropical-monsoon type climate with an average air temperature of 24.4°C and average humidity of 79.3% in the growing season of rice. The seeds were collected from the College of Agriculture, South China Agricultural University, Guangzhou, China. The soil of pot experiment was sandy loam with 21.65 g kg⁻¹ organic matter, 152 g kg⁻¹ total N, 1.02 g kg⁻¹ total P, 19.34 g kg⁻¹ total K, and 6.40 soil pH whereas the soil of the field experiment was sandy loam containing 21.74 g kg⁻¹ organic matter, 1.70 g kg⁻¹ total N, 1.30 g kg⁻¹ total P, 20.10 g kg⁻¹ total K, and 6.34 soil pH.

2.2. Experimental treatments and design

The experiments were conducted with two fragrant rice cultivars (Meixiangzhan2 and Xuyiangyouzhan) for pot experiment, and with five fragrant rice cultivars (Meixiangzhan2, Xuyiangyouzhan, Basmati, Xiangyuxiangzhan and Yungengyou14) for field experiment. Those cultivars were globally /regionally popular due to their special aroma and better cooking qualities. The five GABA levels i.e. no GABA application (CK), application of GABA at 250 mg l⁻¹ with 25 ml pot⁻¹ at tillering stage (S1), panicle initiation stage (S2), heading stage (S3), and at tillering, panicle initiation and heading stages (S4) were applied in the pot experiment. Similarly, the same treatments with 100 ml m⁻² were applied to all rice cultivars in the field experiment. The crop management practices and pest control were carried out according to standard cultural practices.

The pot experiment was arranged in randomized complete block design (RCBD) with seven replications for each treatment. The commercial compound fertilizer (N: P₂O₅: K₂O = 15:15:15) was applied in all the pots with 3.5 g pot⁻¹ as basal fertilizer. Each pot (32 cm in diameter and 24 cm in height) was filled with 12 kg of sun-dried soil and transplanted with 30 days old rice seedlings with 3 seedlings per hill and five hills per pot at two days after application of basal fertilizer. An additional dose of fertilizer was applied with 3.5 g per pot at the tillering stage.

The field experiment was arranged in split-plot design with three replications, with rice cultivars were assigned to the main plot and GABA treatments were kept in the subplot. The net plot size was 5 × 3 m. The NPK at 90–150–180 kg ha⁻¹ was applied as basal fertilizer and no additional fertilizer was applied later on. The rice seedlings were transplanted by using rice transplanter (2Z-8A2-PZ80-HDRT25) at 30 × 14 cm planting distance.

2.3. Sampling and measurement

Plant sampling was done during 9:00–11:00 am at the heading stage (HS), 15 days after heading stage (15 d AH), and maturity stage (MS). At HS, 15 d AH and MS thirty flag leaves of the main stem, and at 15 d AH and MS fifteen panicles were sampled randomly. The panicles were divided into two parts: one was stored at −20°C for determination of 2AP content and the other together with all the flag leaves were frozen by liquid nitrogen and stored at −80°C for physio-biochemical assays.

2.3.1. Determination of grain 2AP contents

The grain 2AP contents were determined by the synchroniza- tion distillation and extraction method (SDE) combined with GCMS-QP 2010 Plus (Shimadzu Corporation) as described by Huang et al. (2012). The chromatographic condition was described as following: gas chromatograph equipped with a RESTEK Rxi-5 ms (Shimadzu, Japan) silica capillary column (30 m × 0.32 mm × 0. 25 μm). The temperature of the GC oven was kept at 45°C (1 min), increased at the rate of 2°C per min to 65°C and kept at 65°C for 1 min, and then increased to 220°C at the rate of 10°C per min, and kept at 220°C for 10 min. The auto-injector was AOC-20i, SPL1. High purity helium gas (99.999%, Guangzhou Gases Co., LTD., China) was the carrier gas at a constant flow rate of 2 ml per min. The temperature of the ion source was 200°C. Under these conditions, the retention time of 2AP was 7.5 min. The contents of 2AP in grains were defined as μg g⁻¹.
2.3.2. Determination of proline, PSC, GABA and soluble protein contents in leaves and grains

The proline contents in leaves and grains were determined according to Bates et al. (1973) and the contents of proline were expressed as μg g\(^{-1}\) FW, whereas the PSC contents were determined by Miller et al. (2009) and were defined as μmol g\(^{-1}\) FW. The GABA contents in leaves and grains were determined according to Zhao et al. (2009) and expressed as mg g\(^{-1}\) FW, whereas the soluble protein contents were detected by using the method as described by Kong et al. (2017) and expressed as μg g\(^{-1}\) FW.

2.3.3. Determination of PDH, PSCS, OAT and DAO activities in leaves and grains

Crude enzymes were extracted according to the method represented previously (Tripathi et al. 2013; Naliwajski and Skłodowska 2014). The PDH activity was assayed by the method devised by Ncube et al. (2013) and was expressed as U g\(^{-1}\) min\(^{-1}\) FW, while the PSCS activity was determined according to Sánchez et al. (2002) and was expressed as U g\(^{-1}\) min\(^{-1}\) FW. The OAT activity was measured by using the method of Umair et al. (2011) and was expressed as U g\(^{-1}\) min\(^{-1}\) FW whereas the DAO activity was estimated according to the method of Su et al. (2005) and was expressed as U g\(^{-1}\) min\(^{-1}\) FW.

2.3.4. Determination of antioxidant enzyme activities and malondialdehyde (MDA) contents

The antioxidant enzyme activities i.e. superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), as well as MDA contents, were assayed according to the procedures of Li et al. (2019a). In brief, fresh leaves (0.3 g) were homogenized with 5 ml of 50 mM sodium phosphate buffer (pH 7.8) and centrifuged at 8000 rpm at 4°C for 15 min and the supernatant was the crude enzyme extract. The SOD activity was defined as U g\(^{-1}\) FW, while the POD and CAT activities were defined as U g\(^{-1}\) min\(^{-1}\) FW, as well as the MDA contents were expressed as μmol g\(^{-1}\) FW.

2.3.5. Determination of grain yield and yield related traits

For the pot experiment, six pots from each treatment at maturity stage were sampled to determine grain yield and yield-related traits. The panicle number per pot was measured and averaged from the six pots. The filled grain number per pot and total grain number per pot were recorded from the panicles threshed manually, the 1000-grain weight was recorded by counting and weighing five random samples from filled grains (Mo et al. 2017).

For the field experiment, at maturity stage, the panicle number from the fifty representative plants was investigated in each plot. Four hills from different locations of each plot were sampled to determine the total grain number, the filled grain number, grain-filling percentage, and the 1000-grain weight (Mo et al. 2015, 2017).

2.3.6. Determination of grain quality

Grains were stored at room temperature for three months to measure grain quality attributes after sun drying. About 1.5 kg grains from each treatment were weighed and brown rice rate was estimated by using FC2 K rice huller (Jiangsu, China), whereas milled and head milled rice rates were assayed by JN MJ6 rice polishing machine (Zhejiang, China). The percentage of chalky grain and chalkiness degree were analyzed by using SDE-A lightbox (Guangzhou, China). The Infratec1241 grain analyzer (FOSS-TECATOR) was used to determine amylose content, protein content, and alkali of grains (Mo et al. 2015).

2.3.7. Statistical analysis

Microsoft office 2013 was used as a tool to log, dispose, and calculate data. The data analysis and the evaluation of relationships among the indexes were conducted using Statistix version 8 (Analytical Software, Tallahassee, Florida, USA). The significance of differences amongst means of different treatments was determined by the least significant difference (LSD) test at the 0.05 probability level.

3. Results

3.1. 2AP contents

For pot experiment, compared with CK, the GABA application improved the grain 2AP contents in both cultivars. The S3 treatment increased the grain 2AP contents of MX2 and YX by 14.76% and 20.19% as compared to CK whereas the highest 2AP contents were recorded under the S3 and S4 treatments for MX2 and YX, respectively.

For field experiment, the application of GABA led to substantial improvements in grain 2AP contents of YX, XY, and YG14. Compared to CK, the grain 2AP contents of MX2, YX, BS, and YG14 were increased by 27.27%, 40.24%, 43.07%, and 13.66% under the S3 treatment. Moreover, the highest grain 2AP contents were recorded under S3 for MX2 and YX, under S4 for BS and XY as well as under S2 for YG14 (Table 1).

3.2. The proline, PSC, GABA and soluble protein contents in leaves

For pot experiment, the proline contents were significantly affected by the GABA application, all the GABA treatments substantially increased the proline contents for MX2 at HS and MS. The S1 and S3, S2 and S4 treatments significantly improved the proline contents for YX at HS and MS, respectively. However, the GABA application marginally affected the PSC contents for MX2 and YX, for MX2, the S1, S3 and S4 treatments for MX2 and YX, respectively.
while the S1, S2 and S4 treatments significantly improved the P5C contents at 15 d AH. Furthermore, for MX2, the S2, S3 and S4 treatments significantly increased the GABA contents, while for YX, the GABA contents were found higher under the S1 and S4 treatments at MS. Likewise, the S1 and S4 treatments significantly increased the soluble protein contents for YX at HS, however, the S3 and S4 treatments significantly reduced the soluble protein contents for MX2 at HS and 15 d AH, respectively (Table 2).

For field experiment, all the GABA treatments significantly reduced the proline contents for YX at MS, BS at 15 d AH and MS as well as YG14 at HS and MS, and the S1 and S3 treatments reduced the proline contents for MX2 and XY at HS as well as YX at HS, respectively. Additionally, the S3 and S4 treatments significantly increased the soluble protein contents for YX at HS and 15 d AH. Furthermore, all the GABA treatments, S2 and S3 treatments significantly increased the P5C contents for BS at MS and YG14 at HS, YX and YG14 at 15 d AH, respectively. Additionally, the S4 treatment significantly improved the P5C contents for MX2 at MS, BS at HS and YG14 at 15 d AH. Likewise, all the GABA treatments, as well as S2 and S3, S3 and S4 significantly increased the GABA contents for BS at HS, YG14 at 15 d AH and MS, YX and BS at 15 d AH and MS, respectively. Additionally, the S1 treatment significantly improved the GABA contents for YX at MS, YX at HS and 15 d AH as well as YG14 at HS and MS. For the soluble protein contents, the S2 and S3 treatments significantly increased the soluble protein contents for MX2 at MS and YG14 at 15 d AH, while the S1 and S3 treatments significantly increased the soluble protein contents for YX and BS at 15 d AH as well as YG14 at MS, YX and XY at 15 d AH and MS, respectively (Table 2).

### 3.3. The proline, P5C, GABA and soluble protein contents in grains

Significant effects of GABA application on the proline contents were detected. All the GABA treatments significantly increased the proline contents for YX2 and XY at 15 d AH, YG14 at MS, whereas the S1, S2 and S3 treatments significantly improved the proline contents for BS at 15 d AH. Additionally, the S2 and S4 treatments significantly improved the proline contents for MX2 at MS and XY at 15 d AH, YX at 15 d AH and BS at MS, respectively. However, all the GABA treatments significantly decreased the P5C contents for YX at 15 d AH, while the S1, S2 and S4 treatments significantly increased the P5C contents for MX2 and XY at MS, MX2 at MS, BS at 15 d AH and MS as well as MX2 at MS. Furthermore, significant effects were noted on the GABA contents for MX2, YX, BS and YG14. All the GABA treatments, S3 and S4 treatments significantly increased the GABA contents for MX2 at MS as well as BS and YG14 at 15 d AH, YX at 15 d AH and BS at MS, respectively. Likewise, significant effects were recorded regarding the soluble protein contents for YX, BS, XY and YG14. The S1, S2 and S4 treatments improved the soluble protein contents for BS at 15 d AH and MS as well as YG14 at 15 d AH, BS and XY at 15 d AH, YX and BS at 15 d AH as well as XY and YG14 at 15 d AH, respectively, while the S3 treatment significantly decreased the soluble protein contents for YX at MS and YG14 (Table 3).

### 3.4. The PDH, P5CS, OAT and DAO activities in leaves

For pot experiment, all the GABA treatments significantly increased the PDH activity for MX2 at HS and BS whereas the S2 and S3 treatments significantly decreased the PDH activity for YX at 15 d AH. For the P5CS activity, the S1 treatment significantly increased the P5CS activity for MX2 at MS as well as YX at HS and MS whereas the S3 and S4 treatments significantly increased the P5CS activity for MX2 at HS, YX at MS and MX2 at HS, respectively. Furthermore, the S3, both the S2 and S4 treatments significantly increased the OAT activity for YX at HS, MX2 at 15 d AH, respectively. Likewise, the GABA treatments showed a significant effect on the DAO activity. All the GABA treatments significantly increased the DAO activity for MX2 at MS, while the S3 and S4 treatments significantly increased the DAO activity for YX at 15 d AH. Additionally, the S1, S2 and S4 treatments substantially improved the DAO activity for YX at HS and MS, MX2 at HS and YX at MS, YX at 15 d AH and MS, respectively (Table 4).

For field experiment, all the GABA treatments significantly decreased PDH activity for XY at HS and the S1 and S4 treatments significantly reduced the PDH activity for BS at HS, YX at 15 d AH and XY at MS, respectively, while the S3 and S4 treatments significantly increased the PDH activity for XY at 15 d AH and YG14 at HS. Moreover, GABA treatments significantly affected the P5CS activity for MX2, YX, BS and XY. All the GABA treatments significantly increased the P5CS activity for MX2 at HS, and the S1 and S3 treatments significantly increased the P5CS activity for XY at HS and 15 d AH as well as YG14 at HS, YX at HS and 15 d AH as well as XY at HS, respectively, while the S2, S3 and S4 treatments significantly decreased P5CS activity for BS at HS, and the S2 and S4 treatments significantly reduced the P5CS activity for MX2 at MS, YX and XY at MS. Furthermore, the S3 and S4 treatments significantly increased the OAT activity for MX2 at HS and MS as well as XY at HS, while the S2 treatment significantly decreased the OAT activity for YX at 15 d AH and MS. However, all the GABA treatments significantly decreased the DAO activity for XY at HS whereas the S1, S2, S3 and S4 treatments significantly increased the DAO activity for YG14 at HS and BS at MS, MX2 at 15 d AH, YX at 15 d AH and BS at MS, MX2 and YX at 15 d AH, respectively (Table 4).

### 3.5. The PDH, P5CS, OAT and DAO activities in grains

The GABA application marginally affected the PDH and DAO activities, however, the S1 and S2 treatments substantially improved the PDH activity for MX2 at MS whereas the S2 and S3, S3 and S4 treatments decreased the PDH activity significantly for BS at MS and YG14 at 15 d AH, respectively. All the GABA treatments significantly decreased the DAO activity for BS at 15 d AH. Furthermore, all the GABA treatments significantly increased the P5CS activity for BS and YG14 at 15 d AH and XY at MS whereas the S1, S2 and S4 treatments significantly decreased the P5CS activity for YX at MS. The S2 and S3, S3 and S4 treatments led to decline in the P5CS activity for MX2 at 15 d AH, MX2 at MS and XY at 15 d AH, respectively. Moreover, the S1 treatment reduced the P5CS activity significantly for MX2 at 15 d AH and MS. Likewise, the OAT activities were significantly affected by the GABA treatments. All the GABA treatments significantly...
decreased the OAT activity for MX2 at 15 d AH and YG14 at MS whereas the S1 and S4, S3 and S4 treatments significantly increased the OAT activity for BS at 15 d AH and MS, XY at 15 d AH, respectively, and the S2 and S3 treatments significantly increased the OAT activity for YX at 15 d AH and BS at MS, MX2 at MS and BS at 15 d AH, respectively (Table 5).

3.6. Grain yield and yield related traits

Significant variations were noted in both yield and yield traits under the different GABA treatments. For MX2 and YX, the S4 treatment enhanced the grains per panicle by 14.40% and 25.06%, respectively, whereas for BS, the 1000-grain weight under the S4 treatment was significantly higher as compared with CK. The grain yield was found higher in YX under the S4 treatment than CK. Moreover, both the S2 and S3 treatments produced 16.70% and 24.77% higher grains per panicle for XY whereas the S3 and S4 treatments improved the filled grain percentage for YG14 by 16.87% and 11.05%, respectively. In addition, the S2 treatment significantly increased the grain yield for MX2 and YX by 16.90% and 22.93% as compared with CK (Table 6).

3.7. The SOD, POD, CAT activities and MDA contents

Significant effects of the GABA treatments on the antioxidant enzyme activities and the MDA contents were noted. All the GABA treatments improved the SOD activity for MX2 and XY at HS whereas the S1 and S3 treatments significantly improved the SOD activity for XY and YG14. The S1 and S4, S2 and S3 treatments substantially enhanced the SOD activity for MX2 and YX at 15 d AH, XY at HS, respectively, while the S2 and S4 treatments decreased the SOD activity for MX2 and BS, BS, respectively. Likewise, all the GABA treatments resulted in higher POD activities for XY and BS at MS and MX2 at HS. The S1 and S2, S3 and S4 treatments significantly increased the POD activity for XY at HS and BS at 15 d AH, as well as YG14 at MS, XY at HS, respectively, nevertheless, the S1 and S2 treatments reduced the POD activity for MX2 at 15 d AH and MS as well as XY at MS. Additionally, the S4 treatment significantly increased the POD activity for XY and BS at HS and 15 d AH. Furthermore, the CAT activity was substantially increased under GABA application for XY, BS and YG14 at MS as well as MX2 at 15 d AH, whereas the S3 and S4, S2 and S4 treatments significantly increased the CAT activity for XY at 15 d AH and MS, MS, MS at BS and 15 d AH, respectively. The S1 and S3 treatments significantly reduced the CAT activity for MX2 at MS and XY at HS. Moreover, the S2, S3 and S4 treatments significantly reduced the CAT activity for XY and YG14 at 15 d AH as well as XY, YG14 and XY at HS and YG14 at MS, respectively. Moreover, the higher contents of MDA were detected for BS and YG14 at HS under the GABA treatments and the S3 and S4 treatments

| Cultivar | Treatment | HS | 15 d AH | MS | HS | 15 d AH | MS | HS | 15 d AH | MS | HS | 15 d AH | MS |
|----------|-----------|----|---------|----|----|---------|----|----|---------|----|----|---------|----|
| MX2      | CK        | 12.43 | 9.98b | 8.79c | 0.57a | 0.63c | 1.19a | 0.05c | 0.06bc | 0.10c | 13.42a | 12.94a | 9.64a |
|          | S1        | 19.23c | 12.39a | 11.61b | 0.46b | 0.92ab | 1.32a | 0.05c | 0.05c | 0.05d | 13.88a | 13.71a | 8.99a |
|          | S2        | 21.03b | 10.03b | 11.62b | 0.62a | 0.85b | 1.45a | 0.10b | 0.08b | 0.25a | 13.26ab | 13.15a | 8.66a |
|          | S3        | 17.52c | 10.30b | 12.89b | 0.44b | 0.76bc | 1.47a | 0.10b | 0.06bc | 0.15b | 12.23b | 13.32a | 10.19a |
|          | S4        | 22.67a | 11.08ab | 20.42a | 0.47b | 0.31a | 1.11a | 0.51a | 0.31a | 0.05d | 14.31b | 10.17b | 10.46a |
| YX       | CK        | 14.89b | 13.27b | 16.26b | 0.71b | 0.88a | 0.93bc | 0.11b | 0.11b | 0.09d | 11.19c | 11.92a | 11.56a |
|          | S1        | 17.62d | 22.89a | 16.19b | 0.78b | 0.62b | 1.13a | 0.17b | 0.10bc | 0.16b | 12.25a | 11.56a | 11.22a |
|          | S2        | 14.85b | 13.02b | 22.47b | 0.75b | 0.74ab | 0.98abc | 0.10d | 0.08c | 0.10c | 11.29abc | 10.11a | 9.86b |
|          | S3        | 17.72a | 13.31b | 16.09b | 0.94a | 0.75ab | 0.91c | 0.20a | 0.13ab | 0.13b | 12.02abc | 11.50a | 11.64a |
|          | S4        | 14.10b | 13.66b | 19.61b | 0.72b | 0.72ab | 1.16a | 0.14c | 0.15a | 0.16a | 12.09ab | 11.08a | 11.41a |

The means in the same column followed by different lowercase letters for the same cultivar differed significantly at P = 0.05 according to the LSD test. CK: no spraying of GABA; S1: spraying GABA at tillering stage; S2: spraying GABA at panicle initiation stage; S3: spraying GABA at heading stage; S4: spraying GABA at all the three periods above. MX2: Meixiangzhuan; YX: Yuxiangyouhan; BS: Basmati; XY: Xiangyaxianghan; YG14: Yungengyou 14.
significantly increased the MDA contents for MX2 at MS, YX at HS and 15 d AH as well as XY at 15 d AH, but the S1 and S2 and S3 treatments significantly decreased the MDA contents for YG14 at MS and MX2 at 15 d AH, respectively (Table 7).

3.8. Grain quality

Significant effects were noted on the grain quality traits under the GABA treatments. For instance, the S4 treatment significantly increased the grain protein contents in MX2, YX and BS whereas all the GABA treatments significantly reduced the rate of chalky grains and the degree of chalkiness for MX2 and BS as well as the alkali for YG14. The S2, S3 and S4 treatments substantially reduced the rate of chalky grains and the degree of chalkiness for XY. Moreover, higher amylose contents for BS were found under the S2 and S4 treatments, while for YG14, the amylose contents were lower under the same treatments. Additionally, the S4 treatment significantly improved the rate of chalky grains and the degree of chalkiness for XY. Furthermore, the application of GABA could transiently increase the rate of chalky grains and the degree of chalkiness for three aromatic rice cultivars (Xie et al. 2019). The positive effects of GABA on different plants under adverse conditions have also been widely studied. For instance, the application of GABA could significantly improve the growth and productivity of black cumin under water deficit conditions (Rezaei-Chiyaneh et al. 2018). Exogenous application of GABA could transiently increase the tolerance to root hypoxia of Prunus rootstock (Salvatierra et al. 2016). In the present study, GABA-induced modulations in 2AP contents and enzymes involved in its biosynthesis as well as grain yield of rice (Tables 1, 4, 5 and 6) were estimated.

4. Discussion

GABA is a non-protein amino acid that acts as a signaling molecule that regulates growth and development, metabolism and stress response in plants (Ramesh et al. 2017). It was found that the GABA concentration tends to be higher in response to environmental stresses such as drought, UV irradiation, mechanical damage, extreme temperature, salinity, and hypoxic conditions (Bouché and Fromm 2004). The positive effects of GABA on different plants under adverse conditions have also been widely studied. For instance, the application of GABA could significantly improve the growth and productivity of black cumin under water deficit conditions (Rezaei-Chiyaneh et al. 2018). Exogenous application of GABA could transiently increase the tolerance to root hypoxia of Prunus rootstock (Salvatierra et al. 2016). In the present study, GABA-induced modulations in 2AP contents and enzymes involved in its biosynthesis as well as grain yield of rice (Tables 1, 4, 5 and 6) were estimated.

GABA application during the different growth stages of fragrant rice could result in regulations in physiological metabolism and growth. For example, the application of GABA at panicle initiation stage could regulate the aroma formation for three aromatic rice cultivars (Xie et al. 2019). Xie et al. (2020) indicated that spraying GABA at the initial heading stage could modulate the grain 2AP contents, grain yield and grain quality in fragrant rice. In the present study, application of GABA at heading stage (S3) or at all the

### Table 3. Effects of different γ-aminobutyric acid application periods on the contents of proline, PSC, GABA and soluble protein in grains in field experiment.

| Cultivar | Treatment | Proline content (μg g⁻¹ FW) | PSC content (μmol g⁻¹ FW) | GABA content (mg g⁻¹ FW) | Soluble protein content (μg g⁻¹ FW) |
|----------|-----------|-----------------------------|---------------------------|--------------------------|-----------------------------------|
| MX2      | CK        | 3.30a                       | 0.04c                     | 1.08bc                   | 0.52c                             |
|          | S1        | 2.97a                       | 0.61ab                    | 1.28ab                   | 0.64ab                            |
|          | S2        | 3.26a                       | 0.63a                     | 1.21bc                   | 0.67a                             |
|          | S3        | 4.53a                       | 0.10oa                    | 0.86dc                   | 0.81b                             |
|          | S4        | 3.51bc                      | 0.96ab                    | 0.74d                    | 0.82b                             |
| YX       | CK        | 2.14a                       | 0.98bc                    | 0.93a                    | 0.93a                             |
|          | S1        | 2.06a                       | 0.64bc                    | 1.13a                    | 1.13a                             |
|          | S2        | 3.20a                       | 1.02a                     | 1.10ab                   | 1.05ab                            |
|          | S3        | 3.50bc                      | 1.03a                     | 0.87c                    | 0.54b                             |
|          | S4        | 3.26a                       | 0.63b                     | 0.87c                    | 0.54b                             |
| BS       | CK        | 1.64a                       | 0.94d                     | 0.59b                    | 0.56b                             |
|          | S1        | 2.09a                       | 0.98bc                    | 0.93a                    | 0.93a                             |
|          | S2        | 1.30a                       | 0.46b                     | 0.89c                    | 0.80a                             |
|          | S3        | 1.67c                       | 1.13a                     | 0.86a                    | 0.86a                             |
|          | S4        | 1.05a                       | 1.50ab                    | 0.87b                    | 0.92b                             |
| XY       | CK        | 1.49a                       | 1.95a                     | 1.14a                    | 1.56a                             |
|          | S1        | 1.36b                       | 2.05a                     | 1.30a                    | 0.49ab                            |
|          | S2        | 1.80a                       | 0.53b                     | 1.13a                    | 0.56a                             |
|          | S3        | 2.17a                       | 0.40b                     | 1.10a                    | 0.49ab                            |
|          | S4        | 2.01a                       | 0.37b                     | 1.28a                    | 0.43b                             |
| YG14     | CK        | 3.39ab                      | 0.65ab                    | 1.14b                    | 0.92a                             |
|          | S1        | 3.98a                       | 0.72a                     | 2.35a                    | 0.89a                             |
|          | S2        | 2.77b                       | 0.61b                     | 2.24ab                   | 0.74b                             |
|          | S3        | 3.53ab                      | 0.57b                     | 2.05b                    | 0.87ab                            |
|          | S4        | 3.79a                       | 0.61b                     | 2.25a                    | 0.89ab                            |

The means in the same column followed by different lowercase letters for the same cultivar differed significantly at P = 0.05 according to the LSD test. CK: no spraying of GABA; S1: spraying GABA at tillering stage; S2: spraying GABA at panicle initiation stage; S3: spraying GABA at heading stage; S4: spraying GABA at all the three application periods on the contents of proline, P5C, GABA and soluble protein in grains in field experiment.
Table 4. Effects of different γ-aminobutyric acid application periods on the activities of PDH, P5CS, OAT and DAO in leaves.

| Cultivar | Treatment   | Pot experiment | Field experiment |
|----------|-------------|----------------|------------------|
|          | PDH activity (U g⁻¹ min⁻¹ FW) | OAT activity (U g⁻¹ min⁻¹ FW) | DAO activity (U g⁻¹ min⁻¹ FW) |
|          | HS 15 d AH MS | HS 15 d AH MS | HS 15 d AH MS |
| MIX2     | CK           | 4.27c          | 28.80a           |
|          | S1           | 6.00b          | 36.67a           |
|          | S2           | 7.38a          | 43.45a           |
|          | S3           | 5.32b          | 38.06a           |
|          | S4           | 5.30b          | 38.06a           |
| YX       | CK           | 6.61bc         | 72.40b           |
|          | S1           | 7.03b          | 36.67a           |
|          | S2           | 6.25c          | 36.67a           |
|          | S3           | 7.27b          | 5.70b            |
|          | S4           | 9.20a          | 5.44b            |
| BS       | CK           | 37.41a         | 82.61abc         |
|          | S1           | 33.05b         | 74.38b           |
|          | S2           | 26.76a         | 38.06a           |
|          | S3           | 27.10a         | 34.59a           |
|          | S4           | 30.48a         | 34.59a           |
| XY       | CK           | 27.68a         | 72.40b           |
|          | S1           | 27.77a         | 75.37ab          |
|          | S2           | 25.56a         | 73.63a           |
|          | S3           | 25.84a         | 86.66a           |
|          | S4           | 26.44a         | 49.91c           |
| BS       | CK           | 39.45c         | 89.55a           |
|          | S1           | 35.05b         | 86.16a           |
|          | S2           | 38.95a         | 89.23a           |
|          | S3           | 28.65c         | 96.11a           |
|          | S4           | 25.90c         | 67.24c           |
| XY       | CK           | 31.23a         | 34.17b           |
|          | S1           | 26.53b         | 34.12a           |
|          | S2           | 24.68bc        | 35.43a           |
|          | S3           | 22.28c         | 25.3a             |
|          | S4           | 26.18bc        | 38.81b           |
| YG14     | CK           | 23.47c         | 37.86a           |
|          | S1           | 30.51a         | 38.78a           |
|          | S2           | 24.29c         | 37.81a           |
|          | S3           | 28.02b         | 32.89a           |
|          | S4           | 28.48ab        | 35.98a           |

The means in the same column followed by different lowercase letters for the same cultivar differ significantly at P = 0.05 according to the LSD test. CK: no spraying of GABA; S1: spraying GABA at tillering stage; S2: spraying GABA at panicle initiation stage; S3: spraying GABA at heading stage; S4: spraying GABA at all the three periods above. MIX2: Meixiangzhan2; YX: Yuxiangyouzhan; BS: Basmati; XY: Xiangyaxiangzhan; YG14: Yungengyou 14.
three stages (i.e. tillering stage, panicle initiation stage and heading stage) (S4) promoted the grain 2AP contents whereas application of GABA at panicle initiation stage (S2) improved heading stage (S4) promoted the grain 2AP contents whereas three stages (i.e. tillering stage, panicle initiation stage and heading stage) (S4) promoted the grain 2AP contents whereas application of GABA at panicle initiation stage (S2) improved grain yield of rice (Tables 1 and 6).

The 2AP is considered one of the most important volatiles responsible for the fragrance of aromatic rice (Buttery et al. 1983). Previous studies reported proline as the most important precursor of 2AP (Daygon et al. 2017). The grain 2AP contents are directly related to the enhancement in proline contents in grains (Wang et al. 2013). Besides, proline or 2AP is likely to transport from leaves to grains (Buttery et al. 1983; Maraval et al. 2010). In the present study, GABA application significantly reduced the proline contents in leaves at MS and the contents of proline at MS were substantially decreased as compared with proline contents at 15 d AH (Table 2), the result in field experiment showed the transference of proline from leaves to grains during grain filling thus resulted in more proline contents in grains which involved in 2AP formation. The significant and positive correlation between grain proline and 2AP contents

| Cultivar | Treatment | Panicle number | Grains per panicle | Filled grain percentage (%) | 1000-grain weight (g) | Grain yield (g m⁻²) |
|---------|-----------|----------------|--------------------|-----------------------------|-----------------------|---------------------|
| MX2     | CK        | 228.48a        | 147.51bc           | 67.28a                      | 21.48b                | 416.74b             |
| S1      | 254.62a   | 171.08bc       | 77.87a             | 21.72b                      | 44.03a                | 408.24a             |
| S2      | 215.88a   | 153.55bc       | 63.58a             | 20.78c                      | 47.56a                | 399.14a             |
| S3      | 271.98a   | 157.93ab       | 50.99a             | 20.54d                      | 41.24a                | 395.49a             |
| S4      | 212.94a   | 168.76a        | 42.19b             | 20.00e                      | 40.36a                | 390.69a             |
| YX      | CK        | 202.44a        | 187.43ab           | 70.96a                      | 21.72b                | 459.20a             |
| S1      | 209.16a   | 176.99a        | 66.61a             | 20.83c                      | 44.56a                | 456.70a             |
| S2      | 204.96a   | 177.75b        | 68.92a             | 21.19c                      | 46.20a                | 452.20a             |
| S3      | 197.40a   | 197.48a        | 63.58a             | 20.54d                      | 41.24a                | 448.10a             |
| S4      | 200.34a   | 186.96a        | 54.82a             | 20.00e                      | 40.36a                | 443.20a             |
| BS      | CK        | 218.44a        | 173.69a            | 79.90a                      | 24.77b                | 604.22a             |
| S1      | 205.80a   | 133.50a        | 75.21a             | 21.62b                      | 55.73b                | 618.20b             |
| S2      | 210.84a   | 143.30a        | 72.22bc            | 25.66b                      | 696.08a               | 635.16a             |
| S3      | 199.08a   | 142.35a        | 77.87a             | 25.17b                      | 481.21a               | 642.24a             |
| S4      | 206.22a   | 127.11b        | 68.14c             | 27.31a                      | 560.63b               | 647.36a             |
| YG14    | CK        | 213.78a        | 134.88b            | 63.51a                      | 21.79a                | 439.36a             |
| S1      | 206.64a   | 150.43a        | 62.59ab            | 21.52a                      | 422.25a               | 432.56a             |
| S2      | 211.68a   | 157.41a        | 56.46a             | 21.55a                      | 425.78a               | 437.13a             |
| S3      | 204.54a   | 168.28a        | 67.28b             | 21.86a                      | 418.21a               | 432.56a             |
| S4      | 206.22a   | 168.62a        | 62.50a             | 21.38a                      | 471.56a               | 437.36a             |

The means in the same column followed by different lowercase letters for the same cultivar differ significantly at P = 0.05 according to the LSD test. CK: no spraying of GABA; S1: spraying GABA at tillering stage; S2: spraying GABA at panicle initiation stage; S3: spraying GABA at heading stage; S4: spraying GABA at all the three periods above. MX2: Meixiangzhan2; YX: Yuxiangyouzhan; BS: Basmati; YG14: Yungengyou 14.
| Cultivar | Treatment | SOD activity (U g⁻¹ FW) | POD activity (U g⁻¹ min⁻¹ FW) | CAT activity (U g⁻¹ min⁻¹ FW) | MDA content (μmol g⁻¹ FW) |
|----------|-----------|-------------------------|-------------------------------|-------------------------------|---------------------------|
|          |           | HS 15 d AH MS HS 15 d AH MS | HS 15 d AH MS(S4) HS 15 d AH MS |                  |                           |
| MX2      | CK        | 12.29c                  | 154.28b                      | 262.76a                       | 63.46d                    |
|          | S1        | 91.44b                  | 223.60a                      | 245.35a                       | 74.92c                    |
|          | S2        | 182.94a                 | 173.78b                      | 184.61b                       | 79.69c                    |
|          | S3        | 189.66a                 | 229.22a                      | 262.38a                       | 112.31a                   |
|          | S4        | 110.20b                 | 252.76a                      | 273.38a                       | 99.31b                    |
|          |           | MX2 CK                  | 12.29c                       | 154.28b                       | 262.76a                   |
|          | S1        | 91.44b                  | 223.60a                      | 245.35a                       | 74.92c                    |
|          | S2        | 182.94a                 | 173.78b                      | 184.61b                       | 79.69c                    |
|          | S3        | 189.66a                 | 229.22a                      | 262.38a                       | 112.31a                   |
|          | S4        | 110.20b                 | 252.76a                      | 273.38a                       | 99.31b                    |
| YX       | CK        | 56.21c                  | 126.77b                      | 217.93b                       | 69.65c                    |
|          | S1        | 43.09d                  | 163.25a                      | 282.69a                       | 73.98b                    |
|          | S2        | 90.45a                  | 119.21b                      | 173.29b                       | 74.93b                    |
|          | S3        | 79.63b                  | 117.47b                      | 262.70a                       | 71.82bc                   |
|          | S4        | 56.53c                  | 81.35c                       | 132.89d                       | 99.63a                    |
| BS       | CK        | 248.67a                 | 340.64a                      | 352.23a                       | 80.21d                    |
|          | S1        | 223.51b                 | 295.00a                      | 360.96a                       | 81.87d                    |
|          | S2        | 157.25d                 | 337.04a                      | 290.79b                       | 98.64c                    |
|          | S3        | 199.88c                 | 241.17b                      | 351.07a                       | 111.71b                   |
|          | S4        | 118.44e                 | 238.36b                      | 234.36c                       | 126.17a                   |
| XY       | CK        | 48.95e                  | 200.50c                      | 250.12d                       | 82.41c                    |
|          | S1        | 165.84b                 | 264.52b                      | 295.31c                       | 82.85c                    |
|          | S2        | 75.86d                  | 233.57c                      | 250.08d                       | 72.79d                    |
|          | S3        | 113.08c                 | 339.44a                      | 444.88a                       | 109.04a                   |
|          | S4        | 193.19a                 | 357.12a                      | 390.51b                       | 92.99b                    |
| YG14     | CK        | 75.62c                  | 138.78c                      | 159.30c                       | 89.51c                    |
|          | S1        | 98.16b                  | 205.60b                      | 229.72b                       | 107.59c                   |
|          | S2        | 67.12c                  | 137.69c                      | 223.19b                       | 89.21c                    |
|          | S3        | 120.23a                 | 231.62a                      | 294.29a                       | 110.74b                   |
|          | S4        | 41.65d                  | 139.27c                      | 267.70a                       | 130.81a                   |

The means in the same column followed by different lowercase letters for the same cultivar differ significantly at \( P = 0.05 \) according to the LSD test. CK: no spraying of GABA; S1: spraying GABA at tillering stage; S2: spraying GABA at panicle initiation stage; S3: spraying GABA at heading stage; S4: spraying GABA at all the three periods above. MX2: Meixiangzhan2; YX: Yuxiangyouzhan; BS: Basmati; XY: Xiangyaxiangzhan; YG14: Yungengyou 14.
The means in the same column followed by different lowercase letters for the same cultivar differ significantly at \( P = 0.05 \) according to the LSD test. CK: no spraying of GABA; S1: spraying GABA at tillering stage; S2: spraying GABA at panicle initiation stage; S3: spraying GABA at heading stage; S4: spraying GABA at all three periods above. MX2: Meixiangzhan2; YX: Yuxiangyouzhan; BS: Basmatic; XY: Xiangyaxiangzhan; YG14: Yungengyou14.

Table 9. Correlation analyses between 2AP contents in grains at maturity and the investigated parameters in field experiment.

| Index | Heading stage (HS) | 15 days after heading stage (15 d AH) | Maturity stage (MS) |
|-------|-------------------|-------------------------------------|---------------------|
| P5C   |                   |                                     |                     |
| Grain | −0.7699**        | 0.4072**                            | −0.5030ns           |
| Leaf  | 0.2176ns         | 0.7112**                            | 0.8447**            |
| Proline |               | 0.2952ns                            | 0.3991*             |
| Grain | 0.7385**         | 0.6693**                            | 0.5615**            |
| Leaf  | 0.1149ns         | 0.2386ns                            | −0.2709ns           |
| PSCS  |                   |                                     |                     |
| Grain | −0.4417*         | 0.7546**                            | 0.0901ns            |
| DAO   | −0.3162ns        | 0.4224*                             | −0.0220ns           |
| Grain | −0.1082ns        | −0.5615**                           | 0.2150ns            |
| Leaf  | −0.3112ns        | 0.4178**                            | −0.5760**           |

*Significant at \( P < 0.05 \); **Significant at \( P < 0.01 \); ns nonsignificant at \( P > 0.05 \) level.
positive role of GABA application at suitable growth stage in aroma formation in fragrant rice.

Previously, exogenous GABA applications improved the growth, development, and photosynthesis in plants (Luo et al. 2011; Li et al. 2016a). GABA affected grain yield which on the other hand was significantly associated with grain weight (Li et al. 2019b). In the present study, the S2 treatment substantially improved the grain yield owing to an improvement in grains per panicle (Table 6). For example, grains per panicle as well as grain yield were increased under the S2 treatment for YX and BS. Moreover, grain yield was significantly and positively associated with grains per panicle and filled grains percentage (Figure 1). Furthermore, GABA-induced modulations in antioxidant enzyme activities i.e. SOD, POD, and CAT and MDA contents were previously noted (Li et al. 2016a). Malekzadeh et al. (2014) indicated an increase in antioxidant enzyme activities under GABA application which confirms our findings that application of GABA significantly increased the SOD activity at 15 d AH, CAT activity at 15 d AH and MS, as well as POD activity at HS and MS (Table 7). Interestingly, contrary to the reports of Priya et al. (2019), exogenous GABA application enhanced the MDA contents, which indicates that exogenous GABA application might cause stress in fragrant rice. However, AL-Quraan et al. (2015) demonstrated that the significant increase in GABA and MDA accumulation occurs simultaneously under treatment with the three 1,2,3-thiadiazole compounds and discovered GABA molecule may act as a defense mechanism to alleviate oxidative damages. Further, GABA can trap MDA directly and indirectly, as reported by Deng et al. (2010).

Additionally, GABA also plays an important role in ion absorption, nitrate uptake and nitrogen metabolism in plants (Ma et al. 2016). For instance, GABA could regulate the nitrate concentration and metabolism in the leaves of Pakchoi (Brassica campestris ssp. chinensis Makino) (Li et al. 2018). In the present study, different rice cultivars differed in their grain quality attributes, for example, significant influences in grains with chalkiness, chalkiness degree were observed under the GABA treatments, thereinto, down-regulation was noted in MX2, YX, and XY, but in YX and YG14, the two attributes were on the increase, while for protein, a substantial increase was observed in MX2 and BS whereas significant reduction was noted in XY and YG14 (Table 8). The growth and productivity of rice varies among different rice genotype (Li et al. 2019b). These results suggested that the interactive effects of GABA and cultivar influenced grain quality attributes, however, the molecular mechanisms about GABA-cultivar interaction need to be further studied. Overall, exogenous GABA applications during different growth stages of fragrant rice modulated the grain 2AP contents, enzymes involved in its formation and grain yield.
5. Conclusions
In conclusion, GABA application could improve the grain yield and 2AP contents in fragrant rice. Application of GABA at heading stage or at all (tillering, panicle initiation, and heading) stages significantly increased the grain 2AP contents across all cultivars due to the improvement of proline contents in leaves, grain proline and GABA contents. A significant increase in grain yield was noted under the GABA treatments owing to an increment in filled grains percentage, grains per panicle. Besides yield, GABA application also affected the grain quality attributes. Overall, our result suggested that the application of GABA regulate the grain yield formation and 2AP accumulation in fragrant rice.

Authors’ contributions
Z.M. designed the experiments; Z.G., W.X., Y.L., L.M. and R.G. investigated the traits; Z.G., W.X. and U.A. analyzed the data and wrote the manuscript; Z.M., U.A., G.P., H.T., M.D., S.W., and X.T. revised and edited the manuscript. X.T. was the leader of our research team. All authors read and approved the final manuscript.

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