The role of polyamines in regulating amino acid biosynthesis in rice grains

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Abstract
Polyamines (PAs) are important endogenous plant growth regulators mediating the grain yield and quality in rice. This study was to clarify whether and how PAs regulate amino acid composition and content in milled rice. We grew three categories of rice cultivars differing in protein contents in the field and investigated the relationship of free-PAs concentrations in filling grains with the activities of key enzymes involved in amino acid biosynthesis and with the amino acid contents of milled rice. Cultivars with higher concentrations of free-Spermidine (Spd) and free-Spermine (Spm) exhibited higher amino acid contents than the others. The concentrations of free-Spd and free-Spm were significantly and positively correlated with the contents of essential amino acids (EAAs), non-essential amino acids (NEAAs), and total amino acids (TAAs) in milled rice, whereas the concentration of free-Putrescine (Put) had no effect on these parameters. The concentrations of free-Spd and free-Spm were linearly associated with the activities of glutamine synthetase (GS), glutamate synthetase (GOGAT), aspartate transaminase (AST), aspartate kinase (AK), and alanine transaminase (ALT), but only GOGAT, AST, and ALT were significantly and positively correlated with amino acid content. The application of exogenous Spd or Spm to panicles increased the activities of these key enzymes and amino acid contents, whereas the application of MGBG (an inhibitor of Spd and Spm synthesis) had the opposite effect. Applying Put had no significant effect on these traits. These results suggest that free-Spd and free-Spm enhance amino acid biosynthesis during grain filling in rice, primarily by improving the activities of GOGAT, AST, and ALT.

KEYWORDS
amino acid, biosynthesis, rice (Oryza sativa L.), spermidine, spermine
1 | INTRODUCTION

Rice (Oryza sativa L.) is one of the most important grain crops worldwide and a staple food for over half the human population (Bhullar & Gruissem, 2013; Fitzgerald et al., 2009). In light of recent increases in rice production and greatly improved standards of living, the concept of rice consumption has been gradual shifting toward the need for better nutrition (Bhullar & Gruissem, 2013; Chen et al., 2012; Fitzgerald et al., 2009). Increasing the nutritional quality of rice has become an inevitable trend (Birla et al., 2017) and a popular focus of research (Peng et al., 2014; Yang et al., 2017). The nutritional value of rice mainly depends on its protein content and the abundance of various amino acids, especially essential amino acids (EAs) (Duan & Sun, 2005; Raubenheimer & Simpson, 2016; Wenefrida et al., 2009). Therefore, the nutritional quality of rice could be improved by exploring the factors that influence amino acid composition and content and the underlying mechanisms.

Polymamines (PAs), including putrescine (Put), spermidine (Spd), and spermine (Spm), are important endogenous plant growth regulators that affect the grain yield and quality of cereal crops (Li et al., 2017; Liu, Chang, et al., 2014; Xu, Qiu, et al., 2016; Yang et al., 2014). PAs exist in three states in rice grains: free, soluble-conjugated, and insoluble-conjugated (Chen et al., 2013; Sen et al., 1981). Most PAs in rice grains are free-PAs, accounting for more than 80% of the total PAs (Liang & Lur, 2002; Sen et al., 1981). Free-PAs (especially free-Spd and free-Spm) could enhance grain filling to increase the yields of cereal crops such as rice, wheat, and maize (Liu et al., 2016; Wang, 2013; Xu et al., 2016; Yang et al., 2008). Rice cultivars with better grain filling have higher concentrations of free-Spd and free-Spm in developing grains than cultivars with poor grain filling (Yang et al., 1997). In contrast to superior spikelets of rice, inferior spikelets have relatively lower concentrations of free-Spd and free-Spm and higher concentration of free-Put (Tan et al., 2009; Wang et al., 2012; Yang et al., 2008). Moderate soil drying contributes to grain filling in inferior spikelets of rice by increasing free-Spd and free-Spm levels (Chen et al., 2013). A previous study showed that a rice genotype with better grain milling and appearance qualities had higher concentrations of free-Spd and free-Spm and lower free-Put concentration in grains during the filling period compared with other genotypes (Wang et al., 2007). Moreover, the grain milling and appearance qualities of rice could be improved by increasing free-Spd and free-Spm levels and reducing free-Put level in filling grains (Wang, Zhang, et al., 2007). The free-Spm concentration and the free-Spm/free-Put ratio in roots are significantly and positively correlated with globulin content in milled rice but very significantly and negatively correlated with prolamin content (Liu, Chang, et al., 2014). Similarly, the exogenous application of synthetic Spd increased total protein, albumin, prolamin, and glutelin contents in wheat grains (Li et al., 2017). These findings suggest that free-PAs play crucial roles in determining the nutritional quality of grains. Amino acids, as the components of protein, have been popularly considered as important nutrients for human beings (Duan & Sun, 2005; Raubenheimer & Simpson, 2016). However, little is known about how free-PAs influence amino acid biosynthesis in grains.

The biosynthesis of amino acids in plants is catalyzed by a series of enzymes such as glutamine synthetase (GS), glutamate synthetase (GOGAT), aspartate transaminase (AST), aspartate kinase (AK), and alanine transaminase (ALT) (Chen et al., 2017; Jander & Joshi, 2010; Xiong, 2012; Yang et al., 2020). In general, the first amino acid formed during inorganic nitrogen assimilation in plants is glutamic acid (Glu), which is the ammoniated form of α-ketoglutarate (an intermediate of the tricarboxylic acid cycle in the mitochondria). The Glu is then converted to glutamine via a reaction catalyzed by GS. The glutamine can be again converted to Glu via GOGAT. More than 95% of inorganic nitrogen is assimilated into plants via the GS/GOGAT pathway (Chen et al., 2017; Wang et al., 2007). Subsequently, the Glu can be converted to other amino acids, such as aspartic acid (Asp, the first amino acid in the Asp family) and alanine (Ala, the first amino acid in the Ala family) via reactions catalyzed by AST and ALT, respectively (Azevedo et al., 1997; Curien et al., 2009; Jander & Joshi, 2010). The Asp is converted to β-phosphate aspartic acid via the action of AK. AST and AK are key enzymes in the biosynthesis of Asp family amino acids (Jander & Joshi, 2010; Liang et al., 2013).

In tomato, exogenous Spd treatment promoted the activities of key enzymes of nitrogen metabolism (nitrate reductase [NR], GS, GOGAT, glutamate dehydrogenase [GDH], glutamate-oxaloacetate transaminase [GOT, another name for AST], and glutamic-pyruvic transaminase [GPT, another name for ALT]) and increased the expression of the underlying genes in seedlings under high temperature stress (Shan, 2016). In maize, exogenous Spd treatment effectively alleviated drought stress-induced ammonia toxicity and nitrogen metabolic disorder by enhancing the activities of GS, GOGAT, GDH, GOT, and GPT in seedling leaves (Li, 2019). These findings imply that PAs might regulate the activities of enzymes involved in amino acid biosynthesis in rice grains.

In the current study, we investigated whether PAs play roles in regulating amino acid composition and content in milled rice. The changing patterns of the concentrations of free-PAs and the activities of key enzymes involved in amino acid biosynthesis were measured, and their relationship with amino acid contents in milled rice was examined. To verify the roles of free-PAs in regulating amino acid contents, we studied the effects of chemical regulators on the concentrations of free-PAs in filling grains and the contents of amino acids in milled rice.
2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

The experiment was carried out at the research farm of Yangzhou University, Jiangsu Province, China (32°30′N, 119°25′E), during the rice-growing season in 2019 and 2020. Six rice cultivars were used in this study: Yangfuxian 2 (YFX2), Yangfujing 7 (YFJ7), Yangdao 6 (YD6), Wuyujing 3 (WYJ3), Peiai 64 (PA64), and Nipponbare. The cultivars were divided into three categories according to the protein content in the milled rice: cultivars with low-protein content (YFX2 and YFJ7), medium-protein content (YD6 and WYJ3), and high-protein content (PA64 and Nipponbare) (Table 1). The seeds were obtained from Jiangsu Academy of Agricultural Sciences (Nanjing, Jiangsu, China).

In both years of the study, seeds were prepared and sown in a seedling bed on 15–16 May. Thirty-day-old seedlings were transplanted into a paddy field on 15–16 June at a hill spacing of 0.16 m × 0.25 m with two seedlings per hill. The field experiment was arranged in a completely randomized block design with three replicates. The plot dimension was 3.50 m × 5.76 m, and plots were separated by a 30-cm-wide gap to prevent water and fertilizer leakage. The field soil was a sandy loam containing 104.72 and 105.23 mg kg⁻¹ alkali-hydrolyzable N, 25.22 and 24.80 mg kg⁻¹ Olsen P, 84.08 and 82.98 mg kg⁻¹ exchangeable K, and 21.83 and 22.05 g kg⁻¹ organic matter in 2019 and 2020, respectively. Wheat (Triticum aestivum L.) was the preceding crop at the study site. For fertilization, 240 kg ha⁻¹ N (actual use of urea) was applied at a ratio of 5:2:3, in which 50% was applied as basal fertilizer before transplanting (1 day before transplanting), 20% as tillering fertilizer at the early tillering stage (7 days after transplanting), and 30% as panicle fertilizer at the panicle initiation stage (30 days before heading; leaf remainder of 4.0–3.5). The day before transplanting, calcium superphosphate (P₂O₅ 13.5%) and potassium chloride (K₂O 62%) were applied at a rate of 445 and 150 kg ha⁻¹, respectively. The plants were irrigated in the field using alternate wetting and moderate drying irrigation (Yang et al. 2009).

Weather conditions (mean air temperature, precipitation, and sunshine hours during the rice-growing period), obtained from a weather station located at the experimental site in two years, are shown in Table S1. Diseases, insects, and weeds were strictly controlled during both rice-growing seasons, and the dates of the major growth and development stages were recorded in detail.

2.2 | Sampling

Four hundred panicles that headed on the same day were chosen and tagged from plants in each plot. Forty tagged rice panicles from each plot were sampled at 6-d intervals from anthesis to maturity and separated into upper, middle, and lower portions relative to the primary branches within each panicle. Grains/spikelets collected from the branches located in the middle portion were frozen in liquid nitrogen for 10 min, stored at −70°C, and used to measure the concentrations of free-PAs (free-Put, free-Spd, and free-Spm) and the activities of key enzymes (GS, GOGAT, AST, AK, and ALT) involved in amino acid biosynthesis.

2.3 | Extraction and quantification of free-PAs

Free-PAs fractions (Put, Spd and Spm) were extracted as described by Flores and Galston (1982) with some modification. Hulled grains (0.5–1.0 g) were homogenized in a pre-chilled mortar and pestle in 3–5 ml of 5% (v/v) perchloric acid (PCA). The homogenate was incubated at 4°C for 2 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min. After centrifugation, the supernatant was collected. A 1 ml aliquot of the supernatant and standard solutions of Put, Spd, and Spm were derivatized at 37°C for 1 h and centrifuged at 15,000 × g for 20 min.
2.4 Measurement of the activities of key enzymes involved in amino acid biosynthesis

Frozen grains were hulled prior to enzyme activity analysis. GS and GOGAT activities were determined following the methods of Liu et al. (2013) and Liu, Yang, et al. (2014). AST and ALT activities were assayed according to Wu et al. (1998). AK activity was determined as described by Brennecke et al. (1996). Soluble protein content was determined using bovine serum albumin (BSA) as the standard (Bradford, 1976). Enzymatic activities were expressed on a soluble protein basis. All chemicals and enzymes used for enzymatic measurements were purchased from Sigma Chemical Company.

2.5 Final harvesting

Rice plants were harvested on 14 October and 16 October in 2019 and 2020, respectively. Plants in a 6 m² area (except border plants) were harvested to determine grain yield with three replicates. Mature grains that had been stored for 3 months were hulled, milled, and used to determine hydrolyzed amino acid contents.

2.6 Determination of hydrolyzed amino acid contents

The milled rice was ground into rice flour and used to measure hydrolyzed amino acid contents according to the method of Mossé et al. (1988) with some modifications. Each rice flour sample (0.1 g) was placed into a 10-ml ampere bottle. After adding 5 ml 6 M HCl, the bottle was sealed and fixed with rubberized fabric to prevent the bottle from breaking, and the sample was digested for 24 h in an oven at 110°C. The sample was allowed to cool prior to filtration in an eliminating digestion liquid. The filtrate (2 ml) was transferred to a cuvette and placed in a freeze drier to remove HCl by vaporizing under a vacuum. The remaining mixture was filtered (0.22 μm) and dissolved in 2 ml sodium buffer (80-2037-67, Biochrom). An automatic amino acid analyzer (Biochrom 30, Biochrom) was used to assay the hydrolyzed amino acid content. Seventeen amino acids were determined in our study, including lysine (Lys), valine (Val), methionine (Met), threonine (Thr), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), aspartic acid (Asp), serine (Ser), glutamine (Glu), glycine (Gly), alanine ( Ala), proline (Pro), cysteine (Cys), tyrosine (Tyr), histidine (His), and arginine (Arg). The total amino acids (TAA) determined were classified into essential amino acids (EAA, including Lys, Val, Met, Thr, Ile, Leu, and Phe) and non-essential amino acids (NEAAs, including Asp, Ser, Glu, Gly, Ala, Pro, Cys, Tyr, His, and Arg) based on whether they can be synthesized in the human body (Wu, 2009).

2.7 Chemical treatment

Two rice cultivars with medium-protein content, YD6 (Indica rice) and WYJ3 (Japonica rice), were subjected to chemical treatment in both years. Plants were grown in a paddy field under the same conditions described above. The chemical treatment was conducted based on the previous reports (Chen et al., 2013; Wang et al., 2012; Yang et al., 2008, 2014) with minor modifications. Beginning at 4 days after full heading, 2 mM Put, 1 mM Spd, 1 mM Spm, 5 mM methylglyoxal-bis (guanylhydrazone) (MGBG, an inhibitor of Spd and Spm synthesis that inhibits S-adenosylmethionine decarboxylase activity), or 1 mM Spd + 5 mM MGBG were applied to panicles using a writing brush that had been dipped in the solution. The synthetic chemicals were applied daily after 6:00 pm for consecutive 5 days at a rate of 4 ml per panicle at each application, with 0.5% (v/v) Teepol (Fluka, Riedel-de-Haen) used as surfactant. The same volume of deionized water containing the same concentration of Teepol was applied to the control plants. Each chemical treatment was applied to an area of 5 m² with three replications. All synthetic chemicals used for treatment were purchased from Sigma Chemical Company.

For all chemical treatments, the grains/spikelets located in the middle portion of a panicle were sampled at 12 days post-anthesis (DPA) and 24 DPA to determine the concentrations of free-PAs and the activities of the five key enzymes. At maturation, grains from 50 treated panicles were collected with three replications, stored for 3 months, and used to evaluate hydrolyzed amino acid content in milled rice. All methods used for measurement were as described above.

2.8 Statistical analysis

Analysis of variance was performed using the SAS/STAT statistical analysis package (version 9.2, SAS Institute). Means were tested by the least significant difference at p = 0.05 (LSD0.05). R software (Corrplot, version 3.5.1, https://cran-project.org) was used to calculate the Pearson correlation coefficient and to graph the data. Regression analysis was used to evaluate the relationships between the concentrations of
free-PAs and the activities of key enzymes involved in amino acid biosynthesis in filling grains. The differences in the data across the two study years and in the interaction between the years and cultivars were not significant.

3 | RESULTS

3.1 | Free-PAs concentrations in filling grains

The free-Put concentration in grains was very high during the early grain filling stage and sharply decreased as grain filling progressed in the six rice cultivars examined (Figure 1a,b). The concentrations of free-Spd and free-Spm increased during grain filling, reached a peak at 18 DPA, and decreased sharply thereafter (Figure 1c–f). Among the rice cultivars, the concentrations of free-Spd and free-Spm from highest to lowest were high-protein cultivars (PA64 and Nipponbare) > medium-protein cultivars (YD6 and WYJ3) > low-protein cultivars (YFX2 and YFJ7) (Figure 1c–f). However, the free-Put concentration showed no obvious trend among the different cultivars (Figure 1a,b). Similar results were obtained in both years of the study (Figure 1).

3.2 | Activities of key enzymes involved in amino acid biosynthesis

The activities of five key enzymes involved in amino acid biosynthesis displayed a typical unimodal curve, increasing first and then decreasing (Figure 2). For all the six cultivars, activities of GS (Figure 2a,b), AK (Figure 2g,h), and ALT (Figure 2i,j) in grains uniformly reached a peak at 18 DPA, while activities of GOGAT (Figure 2c,d) and AST (Figure 2e,f) reached a peak at 12 DPA. The mean activities of GOGAT, AST, and ALT in grains also showed the trend of high-protein cultivars (PA64 and Nipponbare) > medium-protein cultivars (YD6 and WYJ3) > low-protein cultivars (YFX2 and YFJ7) (Figure 2).

3.3 | Amino acid composition and content in milled rice

The 17 amino acids were detected in milled rice, but the contents of individual amino acids varied with rice varieties (Tables 2 and 3). The variations in the contents of various amino acids in milled rice were similar among the six rice cultivars, with higher Glu (11.44–14.05 mg g⁻¹), Asp
(6.36–7.53 mg g⁻¹), Leu (5.64–6.45 mg g⁻¹), and Arg (5.00–5.71 mg g⁻¹) contents and lower Cys (0.42–0.66 mg g⁻¹), Met (1.54–2.03 mg g⁻¹), His (1.58–2.27 mg g⁻¹), and Lys (2.08–2.83 mg g⁻¹) contents (Tables 2 and 3).

The contents of EAAs, NEAAs, and TAAs were the highest in high-protein cultivars (PA64 and Nipponbare), intermediate in medium-protein cultivars (YD6 and WYJ3), and the lowest in low-protein cultivars (YFX2 and YFJ7) (Table 4). The ratio of EAA to TAA tended to be higher in Indica rice than in Japonica rice, whereas the opposite trend was detected for the NEAA to TAA ratio (Table 4).

**FIGURE 2** Changes in the activities of five key enzymes (GS, GOGAT, AST, AK, and ALT) involved in amino acid biosynthesis in filling grains of six different rice cultivars. Vertical bars represent ± SE of the mean (n = 6) where these exceed the size of the symbol. GS, glutamine synthetase; GOGAT, glutamate synthetase; AST, aspartate transaminase; AK, aspartate kinase; ALT, alanine transaminase.
TABLE 2  Contents of hydrolyzed essential amino acids (EAAs) in milled rice of six different rice cultivars

| Year | Cultivar | Lys (mg g⁻¹) | Val (mg g⁻¹) | Met (mg g⁻¹) | Thr (mg g⁻¹) | Ile (mg g⁻¹) | Leu (mg g⁻¹) | Phe (mg g⁻¹) |
|------|----------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| 2019 | YFX2     | 2.08 ± 0.03 f| 4.14 ± 0.07 a| 1.66 ± 0.07 efg| 3.00 ± 0.08 cd| 3.33 ± 0.08 a| 6.18 ± 0.07 cd| 3.24 ± 0.09 e|
|      | YFJ7     | 2.49 ± 0.04 cde| 3.82 ± 0.06 ef| 1.78 ± 0.06 cdef| 2.90 ± 0.10 de| 3.09 ± 0.06 cd| 5.77 ± 0.06 g| 3.66 ± 0.08 d|
|      | YD6      | 2.62 ± 0.06 bc| 3.88 ± 0.08 def| 1.98 ± 0.12 ab| 2.78 ± 0.07ef| 2.89 ± 0.05 e| 5.64 ± 0.10 g| 4.48 ± 0.04 b|
|      | WYJ3     | 2.47 ± 0.08 de| 3.57 ± 0.09 g| 1.54 ± 0.07 g| 2.82 ± 0.09 ef| 2.92 ± 0.10 e| 5.93 ± 0.12 f| 4.81 ± 0.09 a|
|      | PA64     | 2.83 ± 0.06 a | 4.05 ± 0.06 abc| 1.82 ± 0.10 bcde| 3.15 ± 0.10 bc| 3.25 ± 0.07 ab| 6.45 ± 0.04 a| 4.14 ± 0.08 c|
|      | Nipponbare| 2.69 ± 0.09 ab| 3.99 ± 0.08 bcd| 1.98 ± 0.08 ab| 3.44 ± 0.10 a| 3.09 ± 0.06 cd| 6.38 ± 0.08 ab| 4.46 ± 0.08 b|
| 2020 | YFX2     | 2.13 ± 0.05 f | 4.10 ± 0.04 ab| 1.70 ± 0.07 defg| 3.05 ± 0.10 cd| 3.26 ± 0.07 ab| 6.12 ± 0.08 de| 3.31 ± 0.09 e|
|      | YFJ7     | 2.41 ± 0.07 e | 3.80 ± 0.10 f | 1.84 ± 0.13 bcd| 2.93 ± 0.09 de| 2.97 ± 0.10 de| 5.71 ± 0.08 g| 3.72 ± 0.07 d|
|      | YD6      | 2.70 ± 0.11 ab| 3.94 ± 0.05 cde| 2.03 ± 0.06 a| 2.72 ± 0.07 f| 2.97 ± 0.10 de| 5.73 ± 0.08 g| 4.40 ± 0.07 b|
|      | WYJ3     | 2.40 ± 0.15 e | 3.63 ± 0.08 g | 1.62 ± 0.08 fg | 2.95 ± 0.09 de| 2.86 ± 0.10 e| 6.02 ± 0.08 ef| 4.74 ± 0.07 a|
|      | PA64     | 2.74 ± 0.07 ab| 4.12 ± 0.06 ab| 1.75 ± 0.10 cdef| 3.23 ± 0.11 b| 3.18 ± 0.06 bc| 6.39 ± 0.09 ab| 4.20 ± 0.09 c|
|      | Nipponbare| 2.60 ± 0.08 bcd| 3.85 ± 0.10 def| 1.90 ± 0.11 abc| 3.53 ± 0.10 a| 3.16 ± 0.10 bc| 6.29 ± 0.09 bc| 4.54 ± 0.10 b|

Analysis of variance

|          | Year   | Cultivar | Year × Cultivar |
|----------|--------|----------|-----------------|
|          | NS     | NS       | 55.80**         |
| Year     | NS     | NS       | 39.24**         |
| Cultivar | NS     | NS       | 17.91**         |
| Year × Cultivar | NS | NS | 45.73** |
|          | NS     | NS       | 23.26**         |
|          | NS     | NS       | 79.01**         |
|          | NS     | NS       | 295.24**        |

Notes: Data are presented as mean ± standard error of the mean (n = 3). Different letters indicate statistical significance at the p = 0.05 level within the same column. Abbreviations: Lys, lysine; Val, valine; Met, methionine; Thr, threonine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine.
**. F values significant at the p = 0.01 level. NS, non-significant at the p = 0.05 level.
| Year  | Cultivar | Asp       | Ser       | Glu       | Gly       | Ala       | Pro       | Cys       | Tyr       | His       | Arg       |
|-------|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
|       |          | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  | (mg·g⁻¹)  |
| 2019  | YFX2     | 6.70 ± 0.10 e | 3.03 ± 0.08 e | 12.20 ± 0.10 d | 3.26 ± 0.07 c | 4.22 ± 0.10 def | 4.02 ± 0.10 a | 0.57 ± 0.05 abc | 3.84 ± 0.12 bc | 1.58 ± 0.13 e | 5.05 ± 0.12 c |
|       | YFJ7     | 7.05 ± 0.08 d | 3.08 ± 0.07 c | 13.00 ± 0.08 c | 3.40 ± 0.10 b | 4.12 ± 0.04 cd | 3.37 ± 0.14 b | 0.66 ± 0.04 a | 3.62 ± 0.09 def | 1.70 ± 0.07 de | 5.22 ± 0.12 bc |
|       | YD6      | 6.59 ± 0.07 ef | 3.61 ± 0.06 d | 11.44 ± 0.11 e | 3.60 ± 0.09 a | 4.31 ± 0.11 cd | 4.09 ± 0.07 a | 0.62 ± 0.08 ab | 4.02 ± 0.11 ab | 1.93 ± 0.08 bc | 5.66 ± 0.11 a |
|       | WYJ3     | 6.46 ± 0.06 fg | 3.94 ± 0.11 c | 14.05 ± 0.10 a | 3.18 ± 0.06 cde | 3.99 ± 0.12 g | 2.77 ± 0.08 f | 0.63 ± 0.06 ab | 3.52 ± 0.08 f | 2.19 ± 0.09 a | 5.71 ± 0.15 a |
|       | PA64     | 7.53 ± 0.06 a | 5.42 ± 0.09 a | 12.24 ± 0.08 d | 3.02 ± 0.09 f | 5.44 ± 0.10 a | 3.08 ± 0.10 cd | 0.57 ± 0.04 abc | 3.55 ± 0.06 ef | 1.78 ± 0.14 cd | 5.05 ± 0.12 c |
|       | Nipponbare | 7.20 ± 0.08 bc | 5.02 ± 0.04 b | 13.90 ± 0.08 ab | 3.11 ± 0.08 def | 5.18 ± 0.09 b | 3.23 ± 0.13 bc | 0.42 ± 0.07 d | 3.74 ± 0.12 cde | 1.64 ± 0.11 de | 5.21 ± 0.13 bc |
| 2020  | YFX2     | 6.65 ± 0.09 e | 3.14 ± 0.05 e | 12.12 ± 0.05 d | 3.20 ± 0.10 cd | 4.29 ± 0.09 cde | 4.11 ± 0.06 a | 0.52 ± 0.06 bcde | 3.78 ± 0.12 cde | 1.64 ± 0.12 de | 5.14 ± 0.05 bc |
|       | YFJ7     | 7.12 ± 0.11 cd | 3.00 ± 0.07 e | 13.09 ± 0.07 c | 3.32 ± 0.12 bc | 4.20 ± 0.11 def | 3.30 ± 0.13 b | 0.60 ± 0.09 abc | 3.60 ± 0.08 de | 1.60 ± 0.10 de | 5.30 ± 0.10 b |
|       | YD6      | 6.50 ± 0.11 fg | 3.55 ± 0.08 d | 11.52 ± 0.08 e | 3.56 ± 0.10 a | 4.42 ± 0.03 c | 4.13 ± 0.11 a | 0.61 ± 0.08 abc | 4.14 ± 0.10 a | 2.02 ± 0.10 b | 5.8 ± 0.12 a |
|       | WYJ3     | 6.36 ± 0.07 g | 3.88 ± 0.10 c | 14.02 ± 0.09 a | 3.23 ± 0.10 cd | 4.04 ± 0.09 ef | 2.82 ± 0.12 ef | 0.60 ± 0.10 a | 3.29 ± 0.08 f | 1.64 ± 0.12 a | 5.62 ± 0.10 a |
|       | PA64     | 7.46 ± 0.10 a | 5.49 ± 0.09 a | 12.18 ± 0.10 d | 3.11 ± 0.07 def | 5.39 ± 0.11 a | 3.09 ± 0.13 de | 0.55 ± 0.09 abc | 3.50 ± 0.12 f | 1.71 ± 0.14 de | 5.00 ± 0.13 c |
|       | Nipponbare | 7.31 ± 0.08 b | 5.11 ± 0.08 b | 13.85 ± 0.07 b | 3.04 ± 0.09 ef | 5.11 ± 0.11 b | 3.31 ± 0.14 b | 0.49 ± 0.03 cd | 3.82 ± 0.11 c | 1.62 ± 0.12 de | 5.30 ± 0.12 b |

Analysis of variance

| Year  | Cultivar | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |
|-------|----------|----|----|----|----|----|----|----|----|----|----|
| Cultivar | 174.70** | 967.59** | 798.18** | 37.60** | 204.78** | 137.72** | 6.70** | 22.96** | 40.45** | 29.70** |

Notes: Data are presented as mean ± standard error of the mean (n = 3). Different letters indicate statistical significance at the p = 0.05 level within the same column. Abbreviations: Asp, aspartic acid; Ser, serine; Glu, glutamic acid; Gly, glycine; Ala, alanine; Pro, proline; Cys, cysteine; Tyr, tyrosine; His, histidine; Arg, arginine. **, F values significant at the p = 0.01 level. NS, non-significant at the p = 0.05 level.
### 3.4 Relationship of free-PAs concentration and amino acid content in rice

The correlations between free-PAs (Put, Spd and Spm) concentrations in filling grains and amino acid contents in milled rice varied, depending on the grain filling stage (Figure 3). Both free-Spd and free-Spm were significantly and positively correlated with Ser, Ala, Thr, and Leu contents at the early or middle grain filling stages. The free-Spd concentration was also positively correlated with Lys and Asp contents, while the free-Spm concentration was positively correlated with Phe content (Figure 3). However, the correlations between free-Put concentration and the contents of individual amino acids largely differed with grain filling stages, especially for Lys and Phe (Figure 3). In general, the free-Put concentration at the middle grain filling stage was negatively correlated with contents of EAAs, NEAAs, and TAAs in milled rice (Figure 3). Both free-Spd and free-Spm concentrations at the early and middle grain filling stages were very significantly or significantly and positively correlated with the contents of EAAs, NEAAs, and TAAs in milled rice and positively correlated with the EAAs/TAAs ratio (Figure 3).

Regression analysis showed that both free-Spd (Figure 4f-j) and free-Spm (Figure 4k-o) concentrations were significantly and positively correlated with the activities of five key enzymes (GS, GOGAT, AST, AK, and ALT) \(r = 0.52^* (p = 0.05)\) to \(0.91^{**} (p = 0.01), n = 42\), fitting a linear polynomial. The free-Put concentration was highly significantly and positively correlated with the activities of GS (Figure 4a), AST (Figure 4c), AK (Figure 4d), and ALT (Figure 4e) \(R^2 = 0.60^{**} \text{ to } 0.84^{**}, p = 0.01, n = 42\), fitting a quadratic polynomial, and was also highly significantly and positively correlated with GOGAT activity (Figure 4b) \(R^2 = 0.79^{**}, p = 0.01, n = 42\), fitting a cubic polynomial. The regression equations are shown in Table S2. Based on the simulated regression equations, the activities of GS, GOGAT, AST, AK, and ALT would be reduced when the free-Put concentration was greater than 5.29, 6.81, 6.51, 4.91, and 5.70 \(\mu\text{mol g}^{-1} \text{ DW}\), respectively.

Similar to free-PAs, the relationship between the activities of the five enzymes and the amino acid contents in milled rice varied with grain filling stages (Figure 5). The activities of GOGAT, AST, and ALT at the early and middle grain filling stages were positively correlated with the contents of EAAs, NEAAs, and TAAs and the EAAs/TAAs ratio in milled rice, while the activities of GS and AK during grain filling period were not significantly correlated with the contents of EAAs, NEAAs, TAAs, or their ratios in milled rice (Figure 5).

| Year | Cultivar | EAAs content\(^a\) (mg g\(^{-1}\)) | NEAAs content\(^b\) (mg g\(^{-1}\)) | TAAs content\(^c\) (mg g\(^{-1}\)) | EAAs/TAAs | NEAAs/TAAs |
|------|----------|-----------------------------------|-----------------------------------|-----------------------------------|-----------|-----------|
| 2019 | YFX2     | 23.63 ± 0.14 e                    | 44.47 ± 0.31 f                    | 68.10 ± 0.18 e                    | 0.35 ± 0.00 abc | 0.65 ± 0.00 def |
|      | YFJ7     | 23.51 ± 0.19 e                    | 45.22 ± 0.05 e                    | 68.73 ± 0.17 d                    | 0.34 ± 0.00 ef  | 0.66 ± 0.00 ab  |
|      | YD6      | 24.27 ± 0.11 cd                   | 45.87 ± 0.20 d                    | 70.14 ± 0.19 c                    | 0.35 ± 0.00 bcde | 0.65 ± 0.00 bcde |
|      | WYJ3     | 24.06 ± 0.16 d                    | 46.44 ± 0.30 c                    | 70.50 ± 0.44 c                    | 0.34 ± 0.00 f   | 0.66 ± 0.00 a   |
|      | PA64     | 25.69 ± 0.12 ab                   | 47.68 ± 0.26 b                    | 73.37 ± 0.17 b                    | 0.35 ± 0.00 ab  | 0.65 ± 0.00 ef  |
|      | Nipponbare | 26.03 ± 0.34 a                  | 48.65 ± 0.42 a                    | 74.68 ± 0.47 a                    | 0.35 ± 0.00 abc | 0.65 ± 0.00 def |
| 2020 | YFX2     | 23.67 ± 0.11 e                    | 44.59 ± 0.13 f                    | 68.26 ± 0.24 de                   | 0.35 ± 0.00 abcd | 0.65 ± 0.00 cdef |
|      | YFJ7     | 23.38 ± 0.09 e                    | 45.13 ± 0.29 e                    | 68.51 ± 0.33 de                   | 0.34 ± 0.00 f   | 0.66 ± 0.00 a   |
|      | YD6      | 24.49 ± 0.24 c                    | 46.03 ± 0.37 cd                   | 70.52 ± 0.62 c                    | 0.35 ± 0.00 abc | 0.65 ± 0.00 def |
|      | WYJ3     | 24.22 ± 0.23 cd                   | 46.42 ± 0.06 c                    | 70.64 ± 0.25 c                    | 0.34 ± 0.00 def | 0.66 ± 0.00 abc |
|      | PA64     | 25.61 ± 0.20 b                    | 47.39 ± 0.20 b                    | 73.00 ± 0.29 b                    | 0.35 ± 0.00 a   | 0.65 ± 0.00 f   |
|      | Nipponbare | 25.87 ± 0.28 ab                 | 48.96 ± 0.35 a                    | 74.83 ± 0.18 a                    | 0.35 ± 0.00 cde | 0.65 ± 0.00 bcd |

**Notes:** EAAs/TAAs, the ration of EAAs to TAAs; NEAAs/TAAs, the ration of NEAAs to TAAs.  
Data are presented as mean ± standard error of the mean \((n = 3)\). Different letters indicate statistical significance at the \(p = 0.05\) level within the same column.  
\(**, F values significant at the \(p = 0.01\) level. NS, non-significant at the \(p = 0.05\) level.  
\(a\)The sum of all the individual essential amino acid content.  
\(b\)The sum of all the individual non-essential amino acid content.  
\(c\)The sum of contents of EAAs and NEAAs.
Effects of chemical regulators

To verify the roles of PAs in regulating the activities of key enzymes involved in amino acid biosynthesis in filling grains and the amino acid contents in milled rice, we treated panicles with synthetic Put, Spd, Spm, or MGBG (which inhibits Spd and Spm synthesis by inhibiting S-adenosylmethionine decarboxylase activity) at the early grain filling stage (4–8 days after full heading). Compared to the control, the application of Put increased free-Put concentration, while application of Spd or Spm increased free-Spd or free-Spm concentrations, in the grains of the two cultivars examined (Table 5). The application of MGBG increased free-Put concentration and reduced free-Spd and free-Spm concentrations in grains, but its effect on free-PAs was eliminated by concomitant treatment with Spd (Table 5). The application of Spd or Spm to panicles enhanced the activities of the five key enzymes, whereas the application of MGBG had the opposite effect (Table 5). The effects of Put application differed between the two measurement times (12 and 24 DPA). Put treatment significantly decreased the activities of the five enzymes in grains at 12 DPA, whereas it had no significant impact on these enzymes at 24 DPA (Table 5).

The application of Spd or Spm increased the contents of EAAs, NEAAs, and TAAs in milled rice, and the application of MGBG decreased the contents of EAAs, NEAAs, and TAAs, whereas the effects of MGBG treatment were eliminated by concomitant treatment with Spd (Table 6). The application of Put did not affect the contents of EAAs, NEAAs, or TAAs in milled rice (Table 6). Finally, the application of Spd or Spm tended to increase the proportion of EAAs in TAAs, especially in rice cultivar YD6 (Table 6).

4 | DISCUSSION

The PAs can regulate protein content in milled rice (Liu, Chang, et al., 2014) and in wheat grains (Li et al., 2017). Accordingly, it is possible that PAs could play crucial roles in amino acid biosynthesis in rice grains, as various amino acids are the building blocks of protein. It is of great significance to investigate how PAs regulate amino acid biosynthesis for improving rice nutritional quality.

A previous report indicated that treatment of exogenous PAs could elevate the protein-filling duration, protein percentage per grain, protein yield per unit area, and some EAAs contents in the grains of mung bean, contributing to the improvement in the quality of mung bean protein (Farhangi-Abriz et al., 2017). In our study, rice cultivars with higher protein content exhibited higher contents of EAAs, NEAAs, and TAAs in milled rice (Table 4) and higher concentrations of endogenous free-Spd and free-Spm in filling grains (Figure 1c–f). The concentrations of free-Spd and free-Spm at the early and middle grain filling stages were highly significantly or significantly and positively correlated with the contents of EAAs, NEAAs, and TAAs in milled rice and positively correlated with the EAAs/TAAs ratio (Figure 3). When exogenous Spd or Spm was applied to panicles at the initial filling stage, the contents of EAAs, NEAAs, and TAAs in milled rice were increased, whereas MGBG had the opposite effects (Table 6). Additionally, exogenous Spd or Spm increased the EAAs/TAAs ratio in milled rice of cultivar WYJ3. However, endogenous free-Put concentration in filling grains did not show obvious regularity due to rice cultivars differing in protein contents (Figure 1a,b). The free-Put concentration at the middle grain filling stage was negatively correlated with the proteins of EAAs, NEAAs, and TAAs in milled rice (Figure 3), and exogenous Put treatment had no effects on these contents in milled rice (Table 6). Taken together, these results indicate that free-Spd and free-Spm in filling grains are beneficial to the production of TAAs, especially EAAs.
FIGURE 4  Relationships between the concentrations of free-PAs [free-Put (a–e), free-Spd (f–j), free-Spm (k–o)] and the activities of five key enzymes [GS (a, f, k), GOGAT (b, g, l), AST (c, h, m), AK (d, i, n), and ALT (e, j, o)] involved in amino acid biosynthesis in filling grains of six different rice cultivars. Data used for the calculations are from Figures 1 and 2. Determination coefficients ($R^2$ or $r$) are shown, and asterisks * and ** represent statistical significance at $p = 0.05$ and $p = 0.01$ levels, respectively ($n = 84$)
increased the concentrations of free-Spd and free-Spm in TAAs ratio (Figure 5). Exogenous Spd or Spm application and ALT were also positively correlated with the EAAs/NEAAs, and TAAs in milled rice, and the activities of AST significantly and positively correlated with the contents of EAAs, NEAAs, and TAAs in milled rice (Table 6). Moreover, exogenous Put had few effects on the contents of EAAs, NEAAs, and TAAs in milled rice (Table 6). In summary, PAs might affect amino acid contents in milled rice by regulating the activities of key enzymes involved in amino acid biosynthesis. Specifically, free-Spd and free-Spm might increase the contents of EAAs, NEAAs, and TAAs in milled rice primarily by activating GOGAT, AST, and ALT during the grain filling period.

In the mung bean study of Farhangi-Abriz et al. (2017), PAs-treated plants produced more these amino acids in grains including Thr, Phe, Asp, Glu, Tyr, Met, His, and Arg. In the present study, the concentrations of free-Spd and free-Spm in filling grains were significantly and positively correlated with the contents of certain amino acids in milled rice, including four EAAs (Thr, Leu, Lys, and Phe) and three NEAAs (Ser, Ala, and Asp) (Figure 3). It is easily found that Thr, Phe, and Asp are three amino acids which could be commonly enhanced by PAs in grains of rice and mung bean. Other amino acids were differently influenced by PAs, which may be attributed to the difference between the two crops. Based on the origin of their carbon skeletons, Asp, Lys, and Thr are important members of the Asp family, while Ala and Leu are members of the Ala family (Chen et al., 2017). AST and ALT were the key rate-limiting enzymes in the biosynthesis of amino acids in the Asp family and Ala family, respectively (Wang, Zhu, et al., 2007; Xiong, 2012; Zhang, 2012). These observations indicate that free-Spd and free-Spm in filling grains regulate amino acid contents in milled rice by enhancing the activities of AST and ALT during the grain filling period. In the future, more attention should be paid to the relationship between free-Spd/free-Spm and the metabolism of these certain amino acids (Thr, Leu, Lys, Phe, Ser, Ala, and Asp).

Free-Put in grains had some impact on the contents of amino acids, including Phe, Lys, His, Arg, Met, and Ile, but the correlations between free-Put content and the levels of these amino acids were inconsistent or even opposite at different grain filling stages (Figure 3). Perhaps this result reflects the special relationship between free-Put concentration and the activities of the five key enzymes involved in amino acid biosynthesis. Here, based on simulated regression equations, the activities of the five key enzymes (GS, GOGAT, AST, AK, and ALT) were predicted to be inhibited by endogenous free-Put in grains when its concentration exceeds 5.29, 6.81, 6.51, 4.91, and 5.70 μmol g⁻¹ DW, respectively (Table S2). The results of the chemical treatment confirmed this hypothesis. Exogenous Put treatment caused endogenous

The mechanism underlying the role of PAs in regulating amino acid contents in milled rice is poorly understood. There are documentations showing that the application of exogenous Spd activated nitrogen metabolizing enzymes (NR, GS, GOGAT, GDH, GOT, and GPT) in the seedlings of tomato (Shan, 2016) and maize (Li, 2019) exposed to abiotic stress. Du et al., (2017) further observed that leaf-applied Spd (1 mM) treatment could increase gene expressions and activities of nitrogen metabolizing enzymes and enhance total amino acids in cucumber root under Ca(NO₃)₂ stress. These initially hinted that PAs might influence amino acid biosynthesis in plants by regulating the enzymes related to nitrogen metabolism. In our work, the concentrations of free-Spd and free-Spm in filling grains were linearly correlated with the activities of five key enzymes (GS, GOGAT, AST, AK, and ALT) involved in amino acid biosynthesis at different grain filling stages (Figure 3). Perhaps this result reflects the special relationship between free-Spd/free-Spm and the activities of the five certain amino acids (Thr, Phe, Asp, Glu, Tyr, Met, His, and Arg). In the future, more attention should be paid to the relationship between free-Spd/free-Spm and the metabolism of these certain amino acids (Thr, Leu, Lys, Phe, Ser, Ala, and Asp).

![FIGURE 5 Correlation of the activities of five key enzymes (GS, GOGAT, AST, AK, and ALT) involved in amino acid biosynthesis at different grain filling stages with amino acid contents in milled rice.](Image)

E-GFS, early grain filling stage; M-GFS, middle grain filling stage; L-GFS, late grain filling stage. The activity of key enzyme at the E-GFS, M-GFS, and L-GFS was the mean of values examined at 6 and 12 days post-anthesis, 18, 24, and 30 days post-anthesis, and 36 and 42 days post-anthesis, respectively. Data used for the calculations are from Table 4 and Figure 2. The blue and red circles indicate positive and negative correlations between parameters, respectively. ***, **, and * indicate significance at p = 0.001, p = 0.01, and p = 0.05 levels, respectively.

### Table 4

| Parameter | 6 DPA | 12 DPA | 18 DPA | 24 DPA | 30 DPA | 36 DPA | 42 DPA |
|-----------|-------|--------|--------|--------|--------|--------|--------|
| TAAs (μmol g⁻¹ DW) | 5.29 | 5.70 | 4.91 | 4.54 | 3.81 | 3.51 | 3.01 |
| NEAAs (μmol g⁻¹ DW) | 5.29 | 5.70 | 4.91 | 4.54 | 3.81 | 3.51 | 3.01 |
| EAAs (μmol g⁻¹ DW) | 5.29 | 5.70 | 4.91 | 4.54 | 3.81 | 3.51 | 3.01 |

Specifically, free-Spd and free-Spm might increase the concentrations of free-Put but then declined as free-Put concentration increased further (Figure 4a–e), implying that the activities of these enzymes are inhibited when free-Put exceeds a certain concentration. When exogenous Spd was applied to panicles, excessive free-Put (8.67 μmol g⁻¹ DW) significantly reduced the activities of the five enzymes in grains at 12 DPA, while the activities of these enzymes at 24 DPA were not affected by a low concentration of free-Put (4.45 μmol g⁻¹ DW) (Table 5, Table S2). Moreover, exogenous Put had few effects on the contents of EAAs, NEAAs, and TAAs in milled rice (Table 6). In summary, PAs might affect amino acid contents in milled rice by regulating the activities of key enzymes involved in amino acid biosynthesis.
## TABLE 5
Effects of applied chemical regulators on the contents of free-put, free-spd, and free-spm and the activities of key enzymes involved in amino acid biosynthesis in filling grains of two rice cultivars

| Cultivar | Treatment | 12 DPA | 24 DPA |
|----------|-----------|--------|--------|
|          | Free-Put  | Free-Spd | Free-Spm | GS activity | GOGAT activity | AST activity | AK activity | ALT activity |
|          | (μmol g⁻¹ DW) | (μmol g⁻¹ DW) | (μmol g⁻¹ DW) | (U g⁻¹ h⁻¹) | (U g⁻¹ h⁻¹) | (μmol g⁻¹ min⁻¹) | (U g⁻¹ h⁻¹) | (μmol g⁻¹ min⁻¹) |
| YD6      | Control   | 7.73 ± 0.11 b | 6.84 ± 0.13 b | 4.06 ± 0.12 b | 3.64 ± 0.13 b | 6.80 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 2 mM Put  | 8.67 ± 0.14 b | 4.55 ± 0.14 b | 3.88 ± 0.14 b | 3.12 ± 0.14 b | 6.90 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 1 mM Spd  | 7.85 ± 0.12 b | 5.02 ± 0.12 b | 4.10 ± 0.12 b | 3.31 ± 0.12 b | 6.90 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 1 mM Spm  | 7.62 ± 0.12 b | 5.80 ± 0.12 b | 4.37 ± 0.12 b | 3.57 ± 0.12 b | 6.90 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 5 mM MGBG | 8.72 ± 0.12 b | 3.80 ± 0.12 b | 1.95 ± 0.12 b | 5.23 ± 0.12 b | 6.90 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 1 mM Spd + 5 mM MGBG | 7.72 ± 0.14 b | 4.25 ± 0.12 b | 3.90 ± 0.14 b | 3.20 ± 0.14 b | 6.90 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
| WYJ3     | Control   | 6.73 ± 0.14 b | 4.13 ± 0.13 b | 2.63 ± 0.13 b | 3.64 ± 0.13 b | 6.80 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 2 mM Put  | 7.81 ± 0.16 a | 6.06 ± 0.16 a | 3.11 ± 0.16 a | 3.20 ± 0.16 a | 6.80 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 1 mM Spd  | 6.73 ± 0.14 b | 5.05 ± 0.14 b | 4.10 ± 0.14 b | 3.31 ± 0.14 b | 6.80 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 1 mM Spm  | 6.65 ± 0.14 b | 5.80 ± 0.14 b | 4.37 ± 0.14 b | 3.57 ± 0.14 b | 6.80 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 5 mM MGBG | 7.90 ± 0.14 a | 3.80 ± 0.14 a | 1.95 ± 0.14 a | 5.23 ± 0.14 a | 6.80 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |
|          | 1 mM Spd + 5 mM MGBG | 6.73 ± 0.16 b | 4.15 ± 0.16 b | 3.20 ± 0.16 b | 3.20 ± 0.16 b | 6.80 ± 0.11 b | 5.45 ± 0.15 b | 3.97 ± 0.10 b |

Notes: The panicles were daily applied with 2 mM Put, 1 mM Spd, 1 mM Spm, 5 mM methylglyoxal bis (guanylhydrazone) (MGBG), 1 mM Spd + 5 mM MGBG at the early grain filling stage (4–8 days after full heading). Control plants were applied with deionized water. Concentrations of free-put, free-spd, free-spm, and activities of GS, GOGAT, AST, AK, and ALT in grains were determined at 12 and 24 days post-anthesis (DPA). Data are presented as mean ± standard error of the mean (n = 6). The mean is the average of replicates observed for the two study years as there was no significant difference between two years. Different letters indicate statistical significance at the p = 0.05 level within the same cultivar.
Put level to increase to 8.67 μmol g\(^{-1}\) DW and 7.81 μmol g\(^{-1}\) DW in the grains of YD6 and WYJ3, respectively, leading to corresponding decreases in enzymatic activities (Table 5). Therefore, it is quite possible that free-Put would promote the activities of the five key enzymes assayed at its concentration of lower than 4.91 μmol g\(^{-1}\) DW in grains, thereby positively regulating amino acid biosynthesis in milled rice.

### CONCLUSIONS

The free-Spd and free-Spm could enhance amino acid biosynthesis in rice grains by improving the activities of GOGAT, AST, and ALT, and especially tend to increase the ratio of EAAs to TAAs in milled rice by enhancing AST and ALT activities. Increases in levels of free-Spd and free-Spm in filling grains through genetic improvement, chemical regulations, or cultivation techniques would benefit amino acid biosynthesis. Further researches are needed to elucidate the mechanism underlying free-Spd and free-Spm regulating the amino acid contents in milled rice.

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### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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### REFERENCES

Azevedo, R. A., Arruda, P., Turner, W. L., & Lea, P. J. (1997). The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. *Phytochemistry, 46*(3), 395–419. [https://doi.org/10.1016/S0031-9422(97)00319-1]

Bhullar, N. K., & Gruissem, W. (2013). Nutritional enhancement of rice for human health: The contribution of biotechnology. *Biotechnology Advances, 31*(1), 50–57. [https://doi.org/10.1016/j.biotechadv.2012.02.001]

Birla, D. S., Malik, K., Sainger, M., Chaudhary, D., Jaival, R., & Jaiwal, P. K. (2017). Progress and challenges in improving the nutritional quality of rice (*Oryza sativa* L.). *Critical Reviews in Food Science and Nutrition, 57*(11), 2455–2481. [https://doi.org/10.1080/10408398.2015.1084992]

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254. [https://doi.org/10.1016/0003-2697(76)90527-3]

Brennecke, K., Neto, A. J. S., Lugli, J., Lea, P. J., & Azevedo, R. A. (1996). Aspartate kinase in the maize mutants ASK1-LT19 and OPAQUE-2. *Phytochemistry, 41*(3), 707–712. [https://doi.org/10.1016/0031-9422(95)00634-6]

Chen, T. T., Xu, Y. J., Wang, J. C., Wang, Z. Q., Yang, J. C., & Zhang, J. H. (2013). Polyamines and ethylene interact in rice grains in response
to soil drying during grain filling. *Journal of Experimental Botany*, 64(8), 2523–2538. https://doi.org/10.1093/jxb/erl115

Chen, Y. H., Jia, L., & Li, J. (2017). *Plant biochemistry*. Higher Education Press.

Chen, Y., Wang, M., & Ouwerkerk, P. B. F. (2012). Molecular and environmental factors determining grain quality in rice. *Food and Energy Security*, 1(2), 111–132. https://doi.org/10.1002/fe3.111

Curien, G., Bästlein, O., Robert-Genthon, M., Cornish-Bowden, A., Cardenas, M. L., & Dumas, R. (2009). Understanding the regulation of aspartate metabolism using a model based on measured kinetic parameters. *Molecular Systems Biology*, 5, 271. https://doi.org/10.1038/msb.2009.29

Ditomasso, J. M., Shaff, J. E., & Kochian, L. V. (1989). Putrescine-induced wounding and its effects on membrane integrity and ion transport processes in roots of intact corn seedlings. *Plant Physiology*, 90(3), 988–995. https://doi.org/10.1104/pp.90.3.988

Du, J., Shu, S., An, Y. H., Zhou, H., Guo, S. R., & Sun, J. (2017). Influence of exogenous spermidine on carbon–nitrogen metabolism under Ca(NO₃)₂ stress in cucumber root. *Plant Growth Regulation*, 81(1), 103–115. https://doi.org/10.1007/s10725-016-0193-8

Duan, M. J., & Sun, S. S. M. (2005). Profiling the expression of genes controlling rice grain quality. *Plant Molecular Biology*, 59(1), 165–178. https://doi.org/10.1007/s11103-004-7507-3

Farhangi Abriz, S., Faegi Analou, R., & Nikpour Rashidabad, N. (2017). Polyamines, affected the nitrogen partitioning, protein accumulation and amino acid composition of mung bean under water stress. *Journal of Crop Science & Biotechnology*, 20(4), 279–285. https://doi.org/10.1007/s12892-017-0079-0

Fitzgerald, M. A., Mccouch, S. R., & Hall, R. D. (2009). Not just a grain of rice: the quest for quality. *Trends in Plant Science*, 14(3), 133–139. https://doi.org/10.1016/j.plants.2008.12.004

Flores, H. E., & Galston, A. W. (1982). Analysis of polyamine in higher plants by high performance liquid chromatography. *Plant Physiology*, 69(3), 701–706. https://doi.org/10.1104/pp.69.3.701

Jander, G., & Joshi, V. (2010). Recent progress in deciphering the biosynthesis of aspartate-derived amino acids in plants. *Molecular Plant*, 3(1), 54–65. https://doi.org/10.1093/mp/spp104

Li, J., Chen, Q., Lv, X. K., Liu, Y., & Liao, Y. C. (2017). Effects of polyamines and ethylene on grain quality of wheat. *Acta Agriculturae Boreali-occidentalis Sinica*, 26(8), 1156–1164. https://doi.org/10.7606/j.issn.1004-1389.2017.08.008

Li, L. J. (2019). *Mitigation effect and regulation mechanism of exogenous spermidine (Spd) on maize under drought stress* (Doctoral Dissertation, Northeast Agricultural University, Haerbing, China).

Liang, C. G., Zhang, Q., Li, J., Xiong, D., Xu, G. L., Wang, Y., Liu, Q., Huang, P., & Li, T. (2013). Effects of high temperature on aspartate metabolism enzyme activities and aspartate-family amino acids contents at rice grain-filling stage. *Chinese Journal of Rice Science*, 27(1), 71–76. https://doi.org/10.3969/j.issn.1001-7216.2013.01.010

Liang, Y. L., & Lur, H. S. (2002). Conjugated and free polyamine levels in normal and aborting maize kernels. *Crop Science*, 42(4), 1217–1224. https://doi.org/10.2135/cropsci2002.1217

Liu, C., Yang, Y., Pan, K., Zhu, T., Li, W., & Zhang, L. (2014). Carbon and nitrogen metabolism in leaves and roots of dwarf bamboo (*Fargesia denudata Yi*) subjected to drought for two consecutive years during sprouting period. *Journal of Plant Growth Regulation*, 33, 243–255. https://doi.org/10.1007/s00344-013-9367-z

Liu, L. J., Chang, E. H., Xiong, Y. W., Bian, J. L., Wang, Z. Q., & Yang, J. C. (2014). Relationships of organic acid and polyamines exudated from roots with grain cooking quality and protein components in rice. *Journal of Yangzhou University (Agricultural and Life Sciences Edition)*, 35(3), 48–53. https://doi.org/10.16872/j.cnki.1671-4652.2014.03.011

Liu, Y., Liang, H. Y., Lv, X. K., Liu, D. D., Wen, X. X., & Liao, Y. C. (2016). Effect of polyamines on the grain filling of wheat under drought stress. *Plant Physiology and Biochemistry*, 100, 113–129. https://doi.org/10.1016/j.plaphy.2016.01.003

Liu, Z. L., Li, Y. J., Hou, H. Y., Zhu, X. C., Rai, V., He, X. Y., & Tian, C. J. (2013). Differences in the arbuscular mycorrhizal fungi-improved rice resistance to low temperature at two N levels: Aspects of N and C metabolism on the plant side. *Plant Physiology and Biochemistry*, 71, 87–95. https://doi.org/10.1016/j.plaphy.2013.07.002

Mossé, J., Huet, J. C., & Baudet, J. (1988). The amino acid composition of rice grain as a function of nitrogen content as compared with other cereals: A reappraisal of rice chemical scores. *Journal of Cereal Science*, 8(2), 165–175. https://doi.org/10.1016/S0733-5210(88)80027-4

Peng, B., Kong, H. L., Li, Y. B., Wang, L., Zhong, M., Sun, L., Gao, Z. J., Zhang, Q. L., Luo, L. J., Wang, G. W., Xie, W. B., Chen, J. X., Yao, W., Peng, Y., Lei, L., Lian, X. M., Xiao, J. H., Xu, C. G., Li, X. H., & He, Y. Q. (2014). OsAAP6 functions as an important regulator of grain protein content and nutritional quality in rice. *Nature Communications*, 5, 4847. https://doi.org/10.1038/ncomms5847

Raubenheimer, D., & Simpson, S. J. (2016). Nutritional ecology and human health. *Annual Review of Nutrition*, 36(1), 603–626. https://doi.org/10.1146/annurev-nutri-071715-051118

Sen, K., Choudhuri, M. M., & Ghosh, B. (1981). Changes in polyamine contents during development and germination of rice seeds. *Phytochemistry*, 20(4), 631–633. https://doi.org/10.1016/0031-9422(81)85147-3

Shan, X. (2016). Effects of exogenous spermidine on carbon and nitrogen metabolism mechanism in tomato seedlings under high temperature stress (Master dissertation, Nanjing Agricultural University, Nanjing, China).

Tan, G. L., Zhang, H., Fu, J., Wang, Z. Q., Liu, J. L., & Yang, J. C. (2009). Post-anthesis changes in concentrations of polyamines in superior and inferior spikelets and their relation with grain filling of super rice. *Acta Agronomica Sinica*, 35(12), 2225–2233. https://doi.org/10.3724/SJPP.1006.2009.02225

Wang, J. C. (2013). *Regulation of polyamines and ethylene to the grain filling of rice* (Master dissertation, Yangzhou University, Yangzhou, China).

Wang, J. Y., Zhu, S. G., & Xu, C. Q. (2007). *Biochemistry*. Higher Education Press.

Wang, Z. Q., Xu, Y. J., Wang, J. C., Yang, J. C., & Zhang, J. H. (2012). Polyamine and ethylene interactions in grain filling of superior and inferior spikelets of rice. *Plant Growth Regulation*, 66(3), 215–228. https://doi.org/10.1007/s10725-011-9644-4

Wang, Z. Q., Zhang, H., Wang, X. M., Zhang, Z. C., & Yang, J. C. (2007). Relationship between concentrations of polyamines in filling grains and rice quality. *Acta Agronomica Sinica*, 33(12), 1922–1927.

Wenefrida, I., Utomo, H. S., Blanche, S. B., & Linscombe, S. D. (2009). Enhancing essential amino acids and health benefit
components in grain crops for improved nutritional values. *Recent Patents on Gene and Drug Research, 3*(3), 219–225. https://doi.org/10.2174/187221509789318405

Wu, G. Y. (2009). Amino acids: metabolism, functions, and nutrition. *Amino Acids, 37*(1), 1–17. https://doi.org/10.1007/s00726-009-0269-0.

Wu, L. H., Jiang, S. H., & Tao, Q. N. (1998). Determination of plant transaminase (GOT and GPT) activity by colorimetric method and its application. *Chinese Journal of Soil Science, 29*(3), 136–138. https://doi.org/10.19336/j.cnki.trtb.1998.03.015

Xiong, D. (2012). Effects of low-light on the accumulation of amino acids and related enzyme activities at rice grain-filling stage (Master Dissertation, Sichuan Agricultural University, Chengdu, China).

Xu, Y. J., Qian, X. Y., Li, Y. Y., Wang, Z. Q., & Yang, J. C. (2016). Effect of alternate irrigation in partitioned roots on the kernel-filling and its related physiological characteristics in maize. *Acta Agronomica Sinica, 42*(2), 230–242. https://doi.org/10.3724/SP.J.1006.2016.00230

Xu, Y. J., Qiu, M. T., Li, Y. Y., Qian, X. Y., Gu, J. F., & Yang, J. C. (2016). Polyamines mediate the effect of post-anthesis soil drying on starch granule size distribution in wheat kernels. *The Crop Journal, 4*(6), 444–458. https://doi.org/10.1016/j.cj.2016.05.004

Yang, J. C., Cao, Y. Y., Zhang, H., Liu, L. J., & Zhang, J. H. (2008). Involvement of polyamines in the post-anthesis development of inferior and superior spikelets in rice. *Planta, 228*(1), 137–149. https://doi.org/10.1007/s00425-008-0725-1

Yang, J. C., Zhu, Q. S., Wang, Z. Q., & Cao, X. Z. (1997). Polyamines in rice grains and their relations with grain plumpness and grain weight. *Acta Agronomica Sinica, 23*(4), 385–392.

Yang, Q. Q., Wu, H. Y., Li, Q. F., Duan, R. X., Zhang, C. Q., Sun, S. S., & Liu, Q. Q. (2017). Characterization of agronomy, grain physico-chemical quality, and nutritional property of high-lysin 35R transgenic rice with simultaneous modification of lysine biosynthesis and catabolism. *Journal of Agricultural and Food Chemistry, 65*(21), 4296–4304. https://doi.org/10.1021/acs.jafc.7b00621

Yang, J. C., Huang, D. F., Duan, H., Tan, G. L., & Zhang, J. H. (2009). Alternate wetting and moderate soil drying increases grain yield and reduces cadmium accumulation in rice grains. *Journal of the Science of Food and Agriculture, 89*(10), 1728–1736. https://doi.org/10.1002/jsfa.3648

Yang, Q. Q., Zhao, D. S., & Liu, Q. Q. (2020). Connections between amino acid metabolisms in plants: lysine as an example. *Frontiers in Plant Science, 11*, 928. https://doi.org/10.3389/fpls.2020.00928

Yang, W. B., Yin, Y. P., Li, Y., Cai, T., Ni, Y. L., Peng, D. L., & Wang, Z. L. (2014). Interactions between polyamines and ethylene during grain filling in wheat grown under water deficit conditions. *Plant Growth Regulation, 72*(2), 189–201. https://doi.org/10.1007/s10725-013-9851-2

Zhang, Q. (2012). Effects of high temperature on main amino acids metabolism enzymes activities and nutritional quality at rice grain-filling stage (Master dissertation, Sichuan Agricultural University, Chengdu, China).

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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