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Exogenous application of abscisic acid enhanced 2-acetyl-1-pyrroline biosynthesis, accumulation, and antioxidant activities in aromatic rice

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Abstract

Background Abscisic acid (ABA) is a crucial endogenous hormone in plants, regulating a myriad of processes integral to plant growth and development. However, there has been no reported impact on the production of aromatic rice following the application of exogenous ABA. Aromatic rice not only possesses intense aroma but also boasts higher nutritional value. 2-Acetyl-1-pyrroline (2-AP) is the primary compound responsible for the distinctive aroma of aromatic rice. A two-year field experiment was conducted to explore the impact of exogenous ABA application on the biosynthesis and accumulation of 2-AP, as well as the physiological characteristics, yield, and quality of aromatic rice varieties.

Results The two aromatic rice varieties, Meixiangzhan-2 (MXZ2) and Nanjingxiangzhan (NJXZ), underwent five applications of 20 mg/L abscisic acid (ABA) from the breakthrough stage, whereas the control group (CK) received deionized water sprays.The results indicated that the application of exogenous ABA significantly boosted the 2-AP content by 20.7%, primarily by enhancing the levels of its precursors and the activities of enzymes involved in 2-AP biosynthesis. Exogenous ABA also upregulated the transcription levels of *ProDH*, *P5CS2*, *OAT*, and *DAO4* while downregulating *BADH2* transcription. Furthermore, exogenous ABA strengthened the antioxidant activites (superoxide dismutase, peroxidase, and catalase) of aromatic rice, although it led to increased malondialdehyde content and slight decreases in yield and quality. Notably, compared with superior grains, exogenous ABA application had a more pronounced effect on enhancing the aroma of inferior grains in aromatic rice with fewer negative effects.

Conclusions The exogenous application of ABA from the breakthrough stage notably elevated the biosynthesis and accumulation of 2-AP in aromatic rice production. This was achieved by augmenting the content of its precursors and

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the activities of related synthetic enzymes, along with an enhancement in antioxidant activities. However, the impact on the yield and quality of aromatic rice remained relatively modest.

Keywords Aromatic rice, Abscisic acid, 2-acetyl-1-pyrroline, Antioxidant activities

Background

Rice (*Oryza sativa* L.) is a vital global food crop. As the global economy and population expand rapidly, and arable land and water resources dwindle, maintaining and enhancing rice production is crucial for China's food security [1]. Aromatic rice, as a special subspecies of rice, is a treasure among rice varieties [2]. It not only possesses a unique and intense aroma but also has higher nutritional value than common rice, making it highly favored by consumers [3]. Consequently, the market value of aromatic rice is more than twice as high as that of common rice, leading to higher profits for farmers who cultivate it [4]. However, aromatic rice is vulnerable to various abiotic stresses, including drought, high temperatures, and soil salinization, which frequently lead to decreased yield and quality [5]. In recent years, countries such as India, Pakistan and the United States have been intensifying the breeding and selection process of aromatic rice varieties in order to seize a share of the international aromatic rice market [4]. Therefore, enhancing the stress resistance and the competitiveness of domestic aromatic rice is an urgent task for China's rice research [6].

The unique aroma profile of aromatic rice is attributed to a complex blend of more than 300 organic volatile compounds [7]. Within this mixture, 2-acetyl-1-pyrroline (2-AP) stands out as the key compound responsible for the signature scent of aromatic rice [8]. The biosynthetic mechanism of the aroma compound 2-AP is highly complex (Fig. 1). Thus far, two synthetic pathways in aromatic rice have been reported [9]. Specifically, in the first pathway, amino acids including proline (Pro), glutamate (Glu), and ornithine (Orn) function as nitrogen donors and are sequentially converted into 1-pyrroline-5-carboxylic acid (P5C) by the enzymes proline dehydrogenase (PDH), 1-pyrroline-5-carboxylate synthetase (P5CS), and ornithine aminotransferase (OAT), respectively. Subsequently, P5C undergoes metabolism to produce 1-pyrroline and is ultimately converted into 2-AP [10, 11]. In the alternative pathway, polyamines such as putrescine are converted into γ-aminobutyl aldehyde (GABald) and cyclized to form 1-pyrroline. This process can also promote the production of 2-AP. However, this alternative pathway is suppressed by betaine aldehyde dehydrogenase (BADH), which is encoded by the dominant *BADH2* allele and functions to regulate the conversion of GABald into γ-aminobutyric acid (GABA). The presence of GABA subsequently inhibits the conversion of GABald to 1-pyrroline, ultimately resulting in a decreased production of 2-AP [12]. Conversely, the occurrence of a non-functional badh2 enzyme disrupts the conversion of GABA, thereby facilitating the accumulation of 2-AP [13]. Additionally, besides converting into 1-pyrroline, P5C may also undergo non-enzymatic reactions with methylglyoxal, ultimately producing a certain amount of 2-AP. However, compared with the enzymatic pathway, this pathway may not generate sufficient 2-AP, making the enzymatic pathway the primary focus of aroma research [9, 14].

As one of the five widely existing endogenous hormones in plants, abscisic acid (ABA) participates in multiple plant growth and development processes, including the induction of seed dormancy, the inhibition of seed germination, and the acceleration of maturation [15]. ABA also acts as a "stress hormone" to stimulate the induced resistance of plants themselves (priming), thereby enhancing their resistance to both biotic and abiotic stresses [16–18]. When encountering stress, ABA concentration increases and induces alterations in specific metabolic processes within the plants, contributing to stress resistance. Salt stress induces the accumulation of ABA in plant roots. This ABA is then transported to the aerial parts of the plant via the xylem, resulting in a continuous accumulation of ABA in the leaves. Consequently, the reduction in leaf expansion rate and the promotion of stomatal closure result in decreased transpiration water loss and reduced salt transport in the roots, alleviating salt stress damage to plants [19, 20]. In addition, ABA enhances plant stress resistance by promoting the accumulation of osmolytes. Previous studies have demonstrated that ABA regulates the expression of the *P5CS* gene under salt stress conditions. This regulation leads to the accumulation of osmolytes, such as Pro, betaine, and soluble sugars, which collectively enhance the plant's resistance to salt stress [21]. Islam et al. [22] reported that treatment with ABA significantly elevated the soluble sugar content in plants. This increase helped maintain the stability and integrity of the plasma membrane under stress conditions, thereby preventing the disruption of the membrane's phase structure. Studies have shown that ABA assists plants in resisting adverse conditions such as low temperature, drought, and salinity by regulating their antioxidant defense systems [23]. In terms of low-temperature stress, ABA application enhanced the activity of superoxide dismutase (SOD) in rice seedlings subjected to low-temperature stress, while reducing the increases in peroxidase (POD) activity and malondialdehyde (MDA) content, thereby improving their cold tolerance [24]. Under drought stress, the

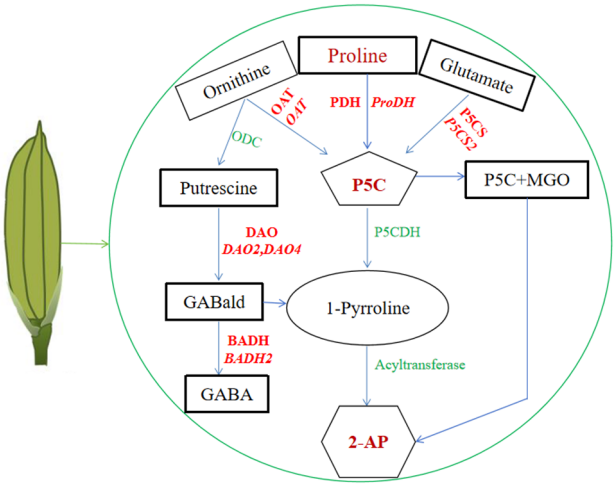

Fig. 1 Biosynthetic pathway of 2-AP. ODC: ornithine decarboxylase; OAT: ornithine aminotransferase; P5CS: 1-pyrroline-5-carboxylate synthetase; PDH: proline dehydrogenase; P5C: 1-pyrroline-5-carboxylic acid; DAO: diamine oxidase; P5CDH: pyrroline-5-carboxylase dehydrogenase; MGO: methylglyoxal; GABald: γ-aminobutyl aldehyde; BADH: betaine aldehyde dehydrogenase; GABA: γ-aminobutyric acid

enhancement of ABA in rice increased SOD activity in leaves, reduced MDA accumulation, alleviated membrane lipid peroxidation, induced stomatal closure, decreased transpiration rate, and reduced excessive water consumption, providing significant protection to rice under drought stress and enhancing its drought resistance [25, 26]. ABA treatment also resulted in a significant enhancement of antioxidant enzyme activities in Platycladus orientalis plants exposed to salt stress conditions. This enhancement was accompanied by an increase in the expression of genes related to reactive oxygen species (ROS) scavenging, an elevated glutathione (GSH) content, and a reduction in the accumulation of H_2O_2 and MDA [27].

A comprehensive two-year field experiment was conducted to assess the effects of abscisic acid (ABA) application, via spraying, on the physiological characteristics, yield, and aroma of aromatic rice. The underlying hypothesis was that ABA treatment could promote the biosynthesis and accumulation of 2-acetylpyridine (2-AP), ultimately enhancing the antioxidant activities in aromatic rice.

A two-year field experiment was conducted to assess the effects of ABA, via spraying, on the physiological characteristics, yield, and aroma of aromatic rice, based on the hypothesis that spraying ABA can promote the biosynthesis and accumulation of 2-AP with enhanced antioxidant activities in aromatic rice. This study aims to offer novel insights into the research domain of aromatic rice and the agricultural application of ABA.

Results

Effects of exogenous ABA on 2-AP biosynthesis and accumulation in aromatic rice

Glu, Orn, and Pro serve as indispensable substrates for the biosynthesis of 2-AP. Specifically, these substrates can be sequentially converted into P5C, a pivotal precursor for the synthesis of 2-AP, through the catalytic actions of P5CS, OAT, and PDH, respectively. The exogenous application of ABA exerted a notable influence on the biosynthesis and accumulation of 2-AP in the grains of aromatic rice, with statistical significance at $p < 0.05$ (Figs. 2, 3, 4, 5, 6 and 7). During the grain-filling stage of both aromatic rice varieties, the trends observed in both superior and inferior grains revealed that the contents of Pro and P5C, as well as the activities of P5CS, OAT, and PDH, followed

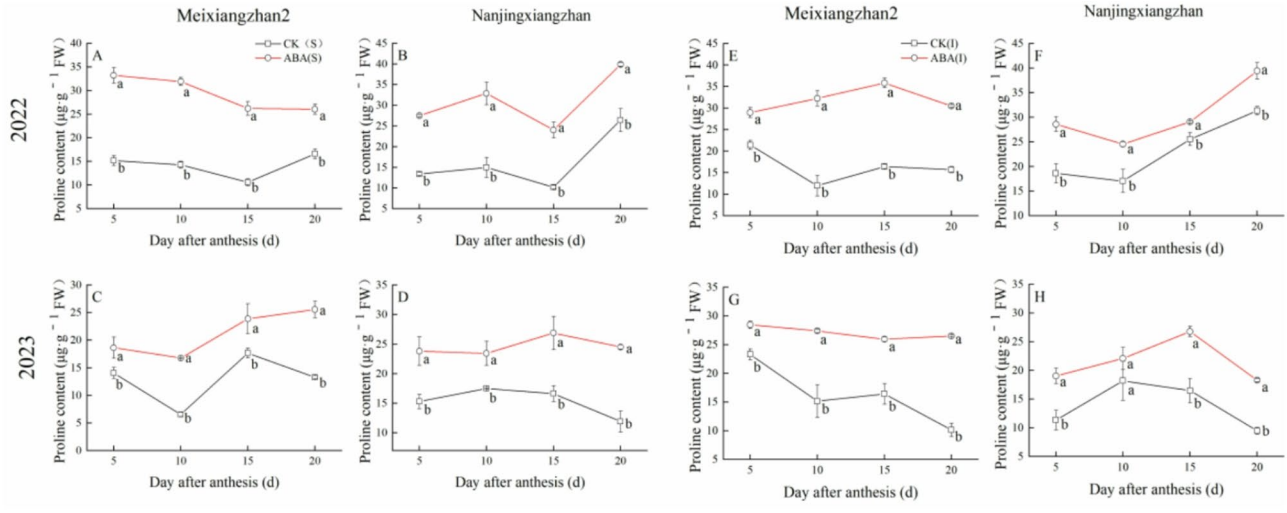

Fig. 2 Proline content under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively. S and I indicate superior grains and inferior grains, respectively

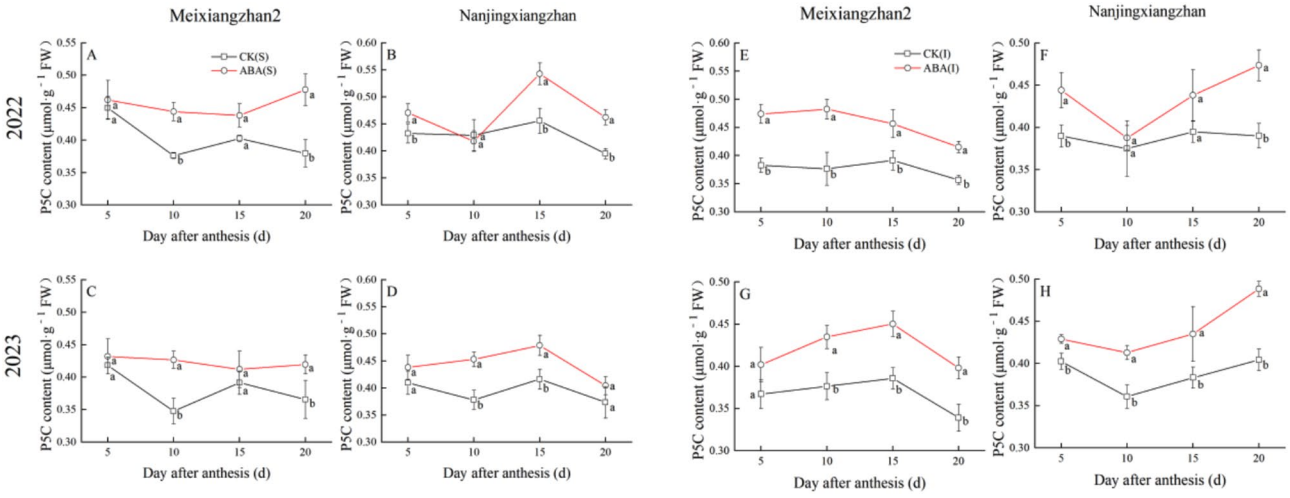

Fig. 3 1-pyrroline-5-carboxylic acid (P5C) content under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively. S and I indicate superior grains and inferior grains, respectively

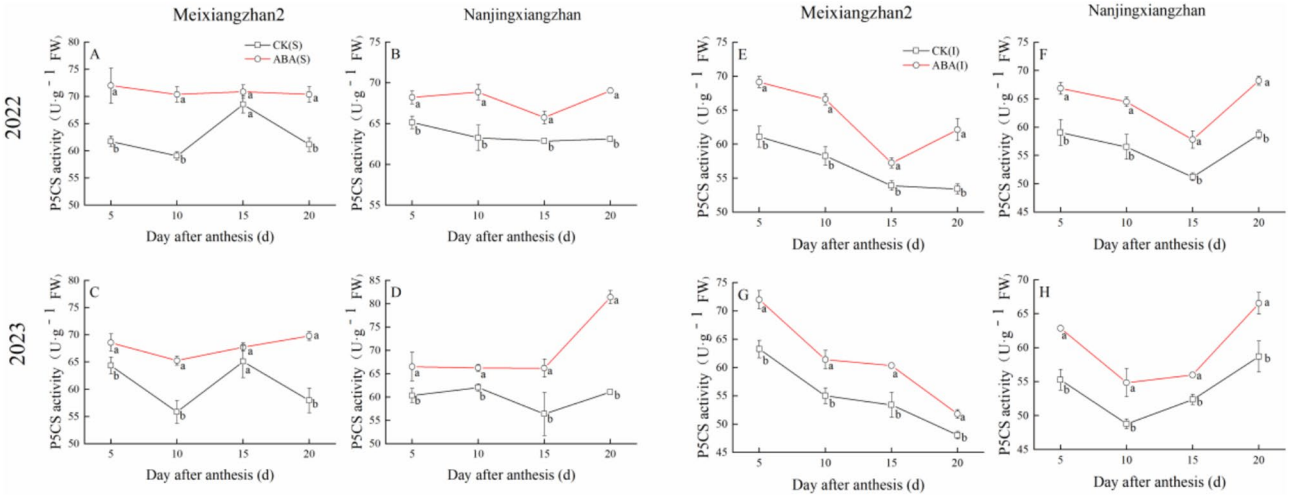

Fig. 4 1-pyrroline-5-carboxylate synthetase (P5CS) activity under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively. S and I indicate superior grains and inferior grains, respectively

the pattern: ABA > CK. Consequently, the 2-AP content in the mature grains of both aromatic rice varieties subjected to ABA treatment was significantly elevated compared with the CK.

Compared with CK, in MXZ2, ABA treatment increased Pro content, P5C content, activities of P5CS, OAT, and PDH, and 2-AP content in superior grains by 111.6%, 13.9%, 13.6%, 13.5%, 27.1%, and 102.0%, respectively, and in inferior grains by 104.3%, 21.3%, 12.5%, 16.2%, 24.4%, and 38.3%, respectively. In NJXZ, ABA treatment enhanced the Pro content, P5C content, P5CS, OAT, and PDH activities, and 2-AP content in superior grains by 102.7%, 12.5%, 17.0%, 25.0%, 48.9%, and 21.9%, respectively, and in inferior grains by 61.3%, 13.9%, 14.1%, 22.3%, 47.4%, and 20.7%, respectively. Furthermore, the results also revealed that the Pro and P5C contents, P5CS, OAT, PDH activities, and 2-AP content in the inferior grains were slightly higher than those in the superior grains. Overall, exogenous ABA application significantly elevated the Pro and P5C contents, P5CS, OAT, and PDH activities throughout the grain filling process in aromatic rice. Consequently, this led to an increase in the 2-AP content in both superior and inferior grains at maturity.

Effects of exogenous ABA on genes related to 2-AP biosynthesis in aromatic rice

The exogenous application of ABA significantly modulated the expression levels of certain genes associated with 2-AP biosynthesis in the grains of both aromatic rice varieties, with statistical significance at $p < 0.05$

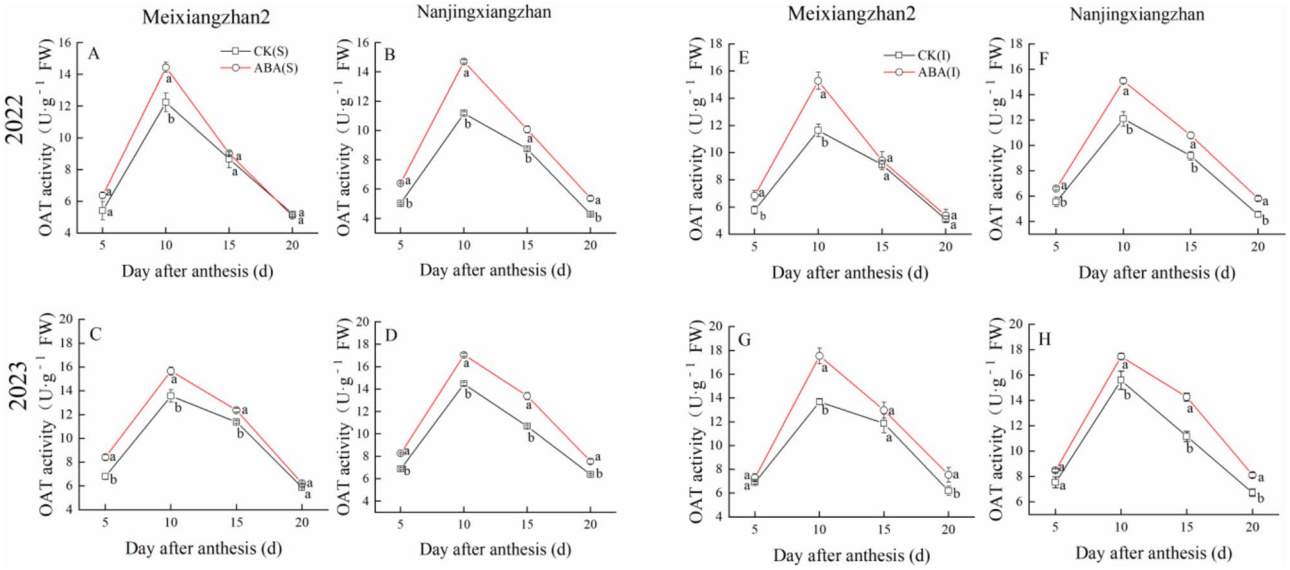

Fig. 5 Ornithine aminotransferase (OAT) activity under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively. S and I indicate superior grains and inferior grains, respectively

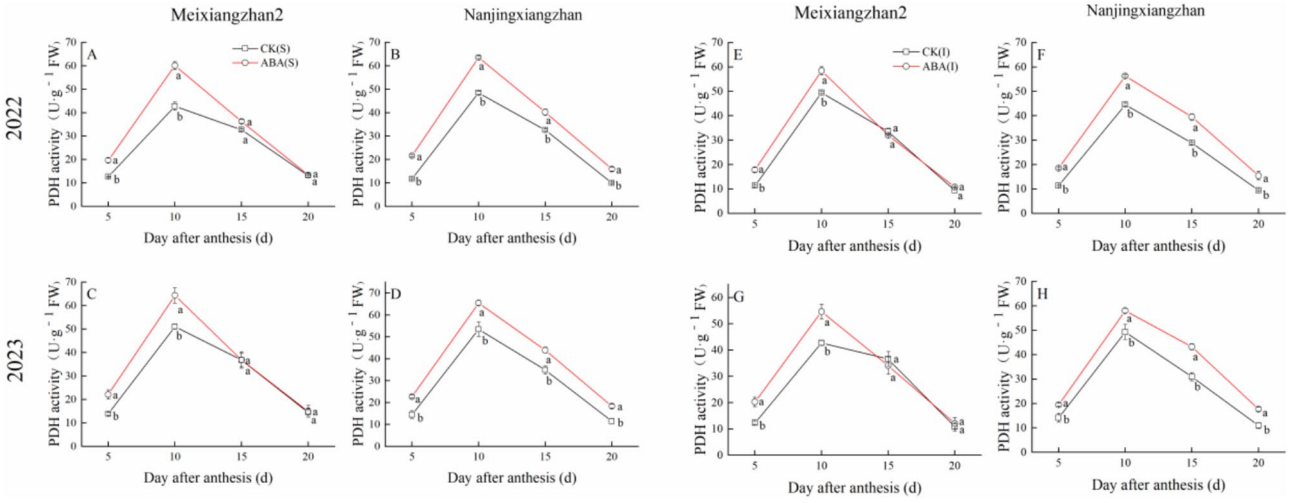

Fig. 6 Proline dehydrogenase (PDH) activity under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively. S and I indicate superior grains and inferior grains, respectively

(Fig. 8). Compared with CK, in MXZ2, ABA treatment significantly upregulated the transcription levels of *ProDH*, *P5CS2*, *OAT*, and *DAO4* by 43.5%, 58.4%, 75.56%, and 15.19%, respectively, while downregulating the transcription level of *BADH2* by 46.43%. In NJXZ, ABA treatment significantly upregulated the transcription levels of *ProDH*, *P5CS2*, *OAT*, and *DAO4* by 119.33%, 92.3%, 1.12%, and 17.18%, respectively, while downregulating the transcription level of *BADH2* by 14.21%. The results also indicated that ABA treatment exerted a suppressive effect on the transcription level of *DAO2*. Overall, exogenous application of ABA facilitated the expression of genes involved in 2-AP biosynthesis, thereby laying a favorable molecular foundation for the biosynthesis and accumulation of 2-AP in the grains of aromatic rice.

Effects of exogenous ABA on antioxidant activities in flag leaves of aromatic rice

Exogenous ABA significantly enhanced the resistance of flag leaves in aromatic rice ($p < 0.05$) (Figs. 9 and 10). Throughout the grain filling process of both rice varieties, the MDA content and the activities of SOD, POD, and CAT generally followed a trend of ABA > CK. Specifically, in MXZ2, ABA treatment elevated the MDA content

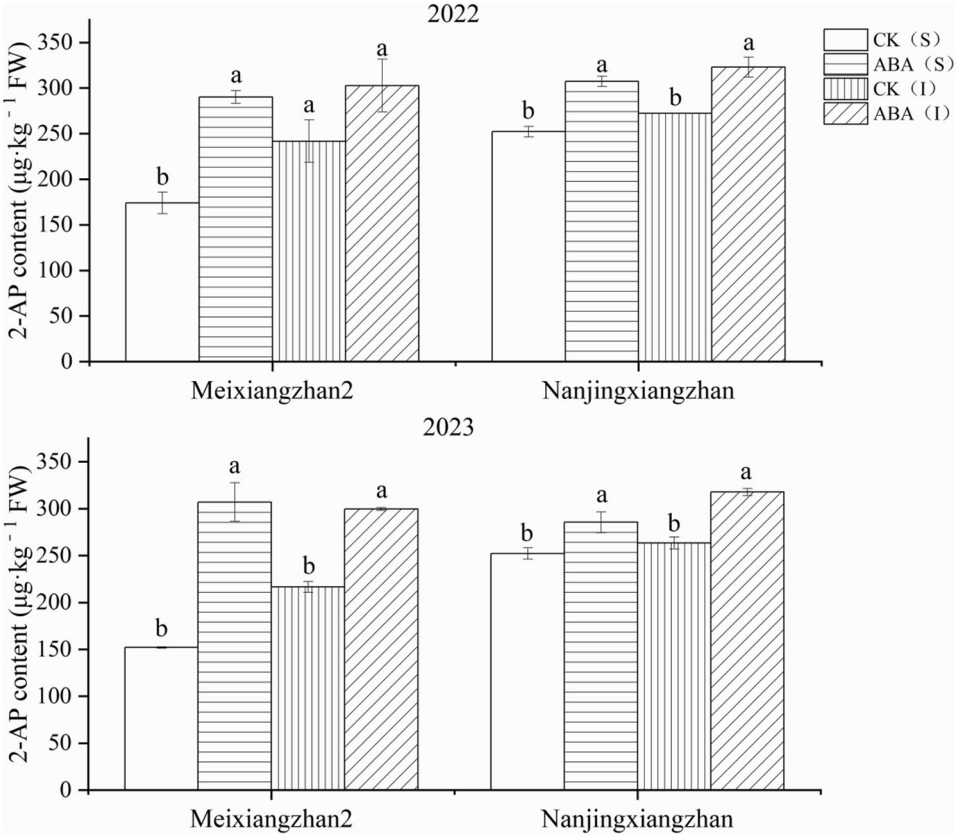

Fig. 7 2-acetyl-1-pyrroline (2-AP) content under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively. S and I indicate superior grains and inferior grains, respectively

by an average of 43.1% and enhanced the activities of SOD, POD, and CAT by 30.2%, 12.3%, and 3.8%, respectively, relative to CK. Similarly, in NJXZ, ABA treatment increased the MDA content by an average of 36.2% and augmented the activities of SOD, POD, and CAT by 20.3%, 16.7%, and 2.6%, respectively, relative to CK. In summary, the exogenous application of ABA notably augmented the antioxidant activities in the flag leaves of aromatic rice, albeit accompanied by an increase in MDA content.

Effects of exogenous ABA on yield and yield components of aromatic rice

The exogenous application of ABA exerted certain adverse effects on the yield and yield components of two aromatic rice varieties (Table 1). In general, ABA spraying slightly reduced the yield, panicle number, grain number per panicle, 1000-grain weight of superior grains and medium grains, and seed setting rate of both aromatic rice varieties. Specifically, in the year 2022, ABA spraying significantly ($p < 0.05$) decreased the 1000-grain weight of medium grains in NJXZ and the seed setting rate of both varieties. However, notably, exogenous ABA application also led to a slight increase in the 1000-grain weight of inferior grains. When compared with the CK, ABA treatment increased the 1000-grain weight of inferior grains by 2.1% in MXZ2 and 4.6% in NJXZ, respectively. In conclusion, the results of this experiment indicated that ABA treatment was not favorable for enhancing the yield of aromatic rice but was beneficial for increasing the 1000-grain weight of inferior grains.

Effects of exogenous ABA on aromatic rice quality

Furthermore, ABA spraying significantly elevated protein and amylose content by 2.7% and 1.4%, respectively ($p < 0.05$). In conclusion, while the overall effect of exogenous ABA spraying on aromatic rice quality was limited, it did exhibit a beneficial effect on enhancing the milled rice rate, protein content, and amylose content of the rice.

The effect of exogenous ABA spraying on the quality of aromatic rice was limited (Table 2). When compared with the CK, no statistically significant differences were observed in the brown rice rate, head rice rate, chalkiness rate, or chalkiness degree of the two aromatic rice varieties subjected to ABA treatment. Notably, exogenous ABA spraying led to a significant increase in the milled rice rate of MXZ2 in both years, with increases of

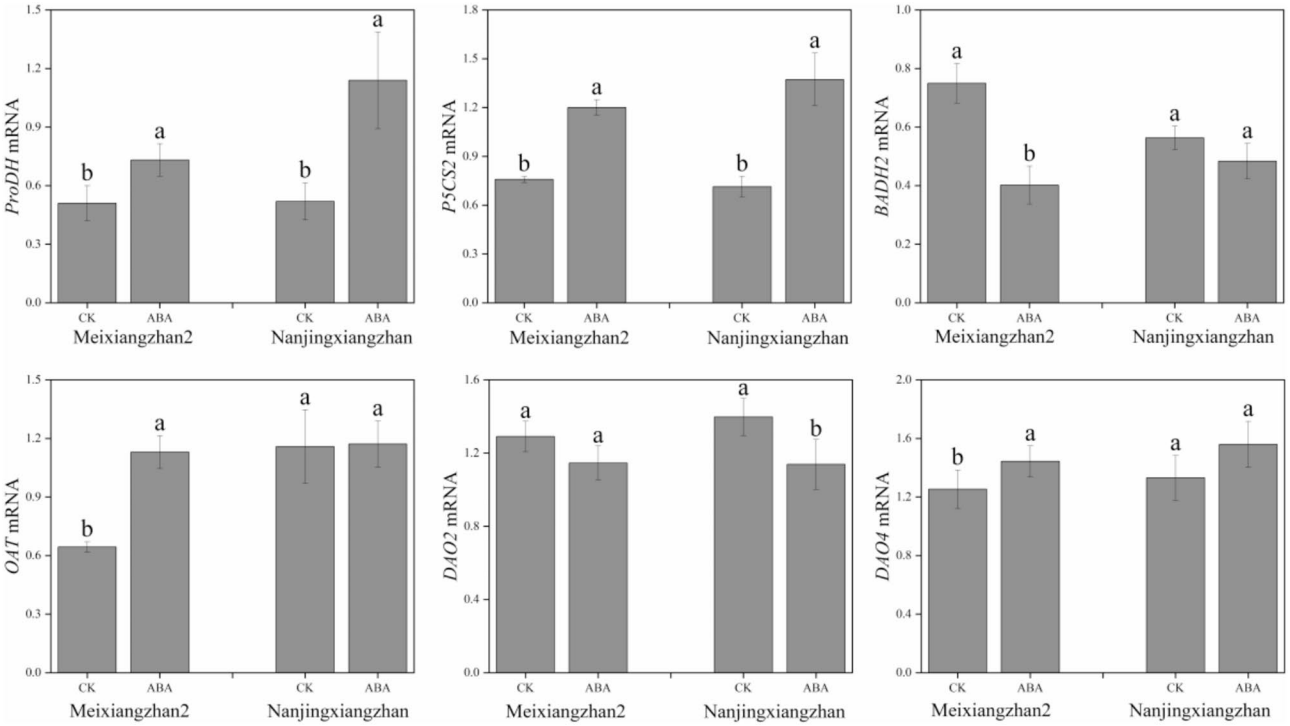

Fig. 8 Expression of 2-acetyl-1-pyrroline (2-AP) biosynthesis related genes under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively

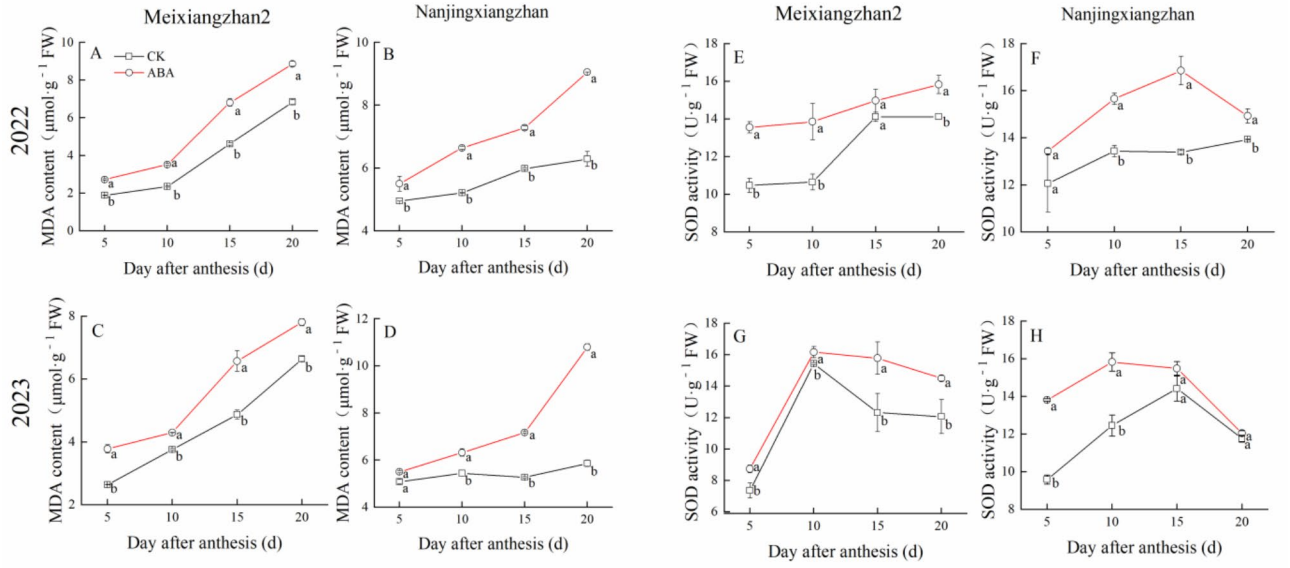

Fig. 9 Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively

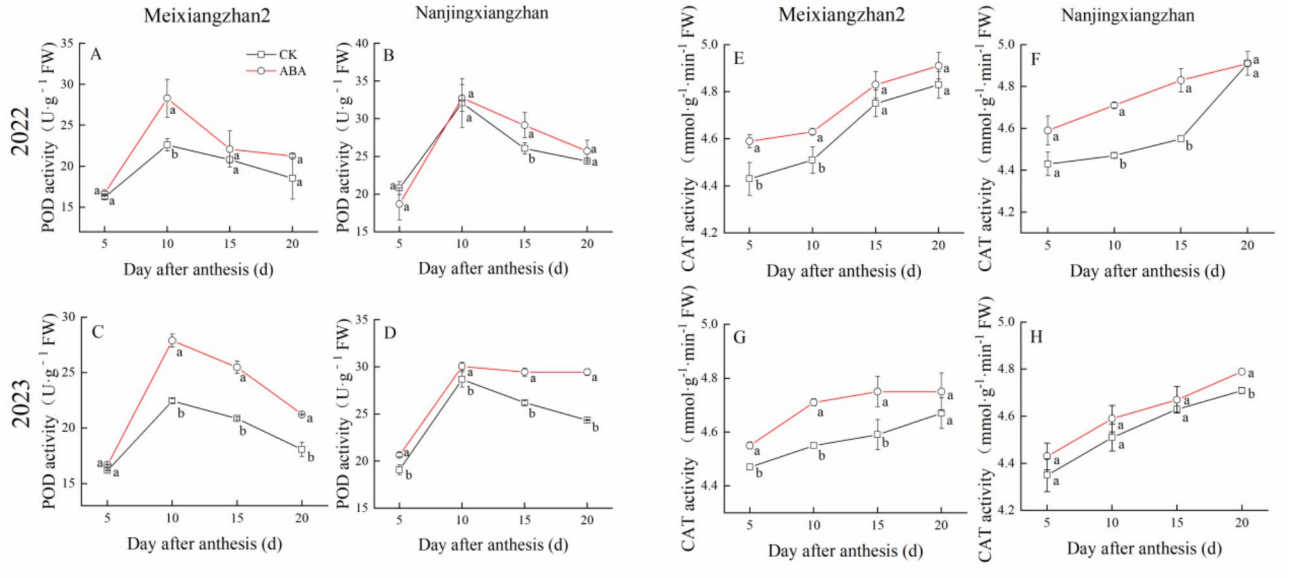

Fig. 10 Activities of peroxidase (POD) and catalase (CAT) under different treatments. Distinct lowercase letters are used to indicate statistically significant differences between the treatments ($p < 0.05$). CK and ABA indicate foliar application of deionized water and abscisic acid, respectively

Table 1 Yield and yield components of aromatic rice under different treatments

Year (Y)	Variety (V)	Treatment (T)	Panicle number per hill	1000-grain weight (g)			Grain number per panicle	Seed setting rate (%)	Yield ($t \cdot ha^{-1}$)
				S	M	I			
2022	MXZ2	CK	20.00 ± 3.46a	20.68 ± 0.83a	19.95 ± 0.03a	15.62 ± 0.88a	74.36 ± 4.56a	87.57 ± 1.14a	5.18 ± 0.41a
		ABA	17.00 ± 5.57a	20.61 ± 0.09a	19.60 ± 0.23a	15.95 ± 0.91a	74.22 ± 1.39a	84.40 ± 1.23b	4.72 ± 0.09a
	NJXZ	CK	21.33 ± 5.13a	25.69 ± 1.10a	22.74 ± 0.17a	15.91 ± 0.58a	74.88 ± 3.76a	88.55 ± 0.86a	6.35 ± 0.48a
		ABA	17.67 ± 2.08a	24.22 ± 0.82a	20.01 ± 0.67b	16.27 ± 1.41a	72.24 ± 5.51a	84.50 ± 1.04b	5.13 ± 0.31a
2023	MXZ2	CK	19.67 ± 2.08a	21.70 ± 1.66a	19.71 ± 0.13a	15.19 ± 0.74a	81.41 ± 4.18a	84.98 ± 0.68a	4.89 ± 0.05a
		ABA	17.67 ± 1.53a	21.66 ± 1.49a	19.44 ± 0.07a	15.26 ± 0.97a	80.06 ± 4.16a	84.61 ± 1.04a	4.72 ± 0.12a
	NJXZ	CK	21.00 ± 2.65a	23.32 ± 0.05a	22.34 ± 0.26a	15.30 ± 1.01a	76.11 ± 8.18a	86.51 ± 0.74a	5.76 ± 0.14a
		ABA	18.67 ± 3.06a	23.15 ± 1.08a	20.85 ± 1.78a	15.92 ± 1.33a	66.31 ± 8.73a	86.48 ± 1.09a	5.66 ± 0.23a
ANOVA		Y	ns	0.95 **	0.45 **	ns	ns	2.22 **	0.61 **
		V	ns	ns	ns	ns	ns	ns	ns
		T	ns	1.55 **	1.97 **	ns	2.46 *	21.82 **	18.91 **
		Y×V	ns	ns	ns	ns	ns	1.98 **	0.26 **
		Y×T	ns	0.91 **	ns	ns	ns	ns	ns
		V×T	ns	ns	8.97 **	ns	ns	0.12 **	2.35 **
		Y×V×T	ns	ns	37.24 **	ns	ns	ns	ns

Different lowercase letters indicate significant differences among treatments of a variety in the same year ($p < 0.05$); * and ** indicate the effect is significant at 0.05 and 0.01 levels, and ns indicates no significant effect. CK and ABA indicate foliar application of deionized water and abscisic acid, respectively. S, M and I indicate superior grains, medium grains and inferior grains, respectively

4.4% and 8.4%, respectively, in comparison with the CK. Meanwhile, the influence of ABA on the milled rice rate of NJXZ was not apparent. ABA spraying significantly increased protein and amylose content by 2.7% and 1.4% respectively ($p < 0.05$). In summary, the impact of exogenous ABA spraying on the quality of aromatic rice was limited, but it did exhibit a beneficial effect on improving the milled rice rate, protein, and amylose content of aromatic rice.

Discussion

Effects of exogenous ABA on the biosynthesis and accumulation of 2-AP in aromatic rice

2-AP is the most significant component among various aroma-contributing factors in aromatic rice. Our study represents the inaugural report on the ability of exogenous ABA application in field experiment to elevate 2-AP synthesis in aromatic rice, while elucidating the fundamental mechanisms involved. The results indicated a marked increase in 2-AP content following ABA treatment. Furthermore, by analyzing the biosynthetic

Table 2 Aromatic rice quality under different treatments

Year (Y)	Variety (V)	Treatment (T)	Brown rice rate %	Milled rice rate %	Head rice rate %	Chalky rice rate %	Chalkiness degree %	Protein content %	Amylose content %
2022	MXZ2	CK	75.54 ± 0.14a	61.76 ± 0.17b	56.13 ± 0.13a	3.65 ± 0.58a	0.56 ± 0.01a	6.87 ± 0.06b	17.67 ± 0.02b
		ABA	76.30 ± 0.31a	64.47 ± 0.13a	52.12 ± 1.13b	3.67 ± 0.56a	0.55 ± 0.01a	7.17 ± 0.06a	18.09 ± 0.05a
	NJXZ	CK	76.32 ± 0.20a	59.48 ± 0.32a	46.13 ± 0.55a	2.68 ± 0.58a	0.45 ± 0.02a	7.92 ± 0.05b	19.35 ± 0.04a
		ABA	76.18 ± 0.44a	54.56 ± 1.59b	46.86 ± 0.41a	3.00 ± 1.00a	0.47 ± 0.01a	8.10 ± 0.03a	19.40 ± 0.03a
2023	MXZ2	CK	72.48 ± 0.23a	59.12 ± 0.51b	49.94 ± 0.37b	3.68 ± 0.58a	0.55 ± 0.02a	7.07 ± 0.07b	17.84 ± 0.03b
		ABA	72.63 ± 0.21a	64.14 ± 0.13a	55.72 ± 0.41a	4.33 ± 0.58a	0.55 ± 0.01a	7.31 ± 0.04a	18.15 ± 0.04a
	NJXZ	CK	73.13 ± 0.18a	61.94 ± 0.13a	54.28 ± 1.78a	3.33 ± 0.56a	0.47 ± 0.01a	8.17 ± 0.06a	19.94 ± 0.06b
		ABA	72.12 ± 0.15b	62.44 ± 0.26a	53.24 ± 1.41a	3.33 ± 0.55a	0.46 ± 0.02a	8.25 ± 0.01a	20.25 ± 0.05a
ANOVA		Y	703.74 **	35.85 **	39.55 **	ns	0.02 **	51.51 **	413.17 **
		V	2.25 **	81.02 **	49.60 **	ns	ns	1496.08 **	7658.68 **
		T	ns	7.18 **	0.59 **	0.08 **	0.02 **	60.14 **	176.01 **
		Y×V	ns	117.00 **	ns	ns	ns	0.28 **	213.33 **
		Y×T	ns	39.50 **	17.82 **	ns	ns	ns	2.90 **
		V×T	15.51 **	97.30 **	1.19 **	ns	ns	ns	ns
		Y×V×T	0.24 **	6.40 **	ns	ns	ns	0.19 *	20.28 **

Different lowercase letters indicate significant differences among treatments of a variety in the same year ($p < 0.05$); * and ** indicate the effect is significant at 0.05 and 0.01 levels, and ns indicates no significant effect. CK and ABA indicate foliar application of deionized water and abscisic acid, respectively

pathway of 2-AP, this study assessed the levels of precursor compounds and the activities of associated enzymes. Notably, application of exogenous ABA resulted in significant elevations in Pro and P5C content in aromatic rice, suggesting that the enhanced biosynthesis of 2-AP was attributed to the increased availability of precursors. Previous research has demonstrated that ABA enhances plant stress tolerance by facilitating the accumulation of osmolytes, including Pro, and modulating the expression of the *P5CS* gene [20]. Previous research confirmed that Pro and P5C were crucial precursors for 2-AP formation [11, 28]. In this study, the molecular basis for the increase in P5C was likely the upregulation of *ProDH* and *P5CS2* expression under ABA treatment, leading to enhanced PDH and P5CS activities. Pro was converted into P5C, a precursor of 2-AP, through catalysis by PDH [29]. P5CS could also reduce Glu to glutamate-semialdehyde, which spontaneously converted to P5C [30]. P5CS is crucial for the biosynthesis of 2-AP, and overexpression of *P5CS2* could significantly elevate 2-AP content [31]. Hence, it was reasonable to speculate that increased expression of *ProDH* and *P5CS2* in ABA-treated plants played a crucial role in enhancing 2-AP levels. Additionally, the conversion of Orn to P5C was significant for 2-AP production, and foliar application of Orn was shown to significantly increase 2-AP content [32]. In this study, exogenous ABA application upregulated *OAT* expression and increased OAT activity, indicating that ABA can enhance Orn metabolism in aromatic rice to increase P5C synthesis, thereby promoting 2-AP biosynthesis.

Apart from the aforementioned pathways, there existed alternative pathway for synthesizing 2-AP, which was regulated by BADH and DAO [12, 33]. DAO catalyzed the formation of GABald from putrescine, which then underwent cyclization to produce 1-pyrroline, the immediate precursor of 2-AP. However, the presence of BADH catalyzed the conversion of GABald into GABA, thereby restraining the production of 2-AP in aromatic rice [34]. Our findings indicated that exogenous application of ABA modulated this biosynthetic pathway by upregulating the transcription level of *DAO4* and downregulating those of *DAO2* and *BADH2*. This suggested that ABA not only enhanced DAO activity by increasing *DAO4* expression but also reduced BADH activity by decreasing *BADH2* expression, thereby facilitating the accumulation of GABald and promoting 2-AP biosynthesis. Furthermore, it was reasonable to speculate that the contribution of *DAO2* to DAO activity might not be as significant as that of *DAO4*. Hence, even though ABA treatment restricted *DAO2* expression in this study, the elevated *DAO4* expression was sufficient to compensate for this loss, consistent with previous findings [35]. The possible pathway through which exogenous ABA influenced 2-AP biosynthesis was illustrated in Fig. 11. Overall, this study revealed an increase in 2-AP content after exogenous ABA application during the breakthrough stage in the field experiment and preliminarily explored the associated mechanisms. Notably, in both treatments, the contents of Pro, P5C, as well as the activities of P5CS, OAT, and PDH, along with 2-AP content, were slightly elevated in inferior grains compared with superior grains, indicating that inferior grains significantly contributed to the aroma of aromatic rice. Moreover, exogenous ABA application represented an effective method of enhancing 2-AP content in both superior and inferior grains, particularly in the latter.

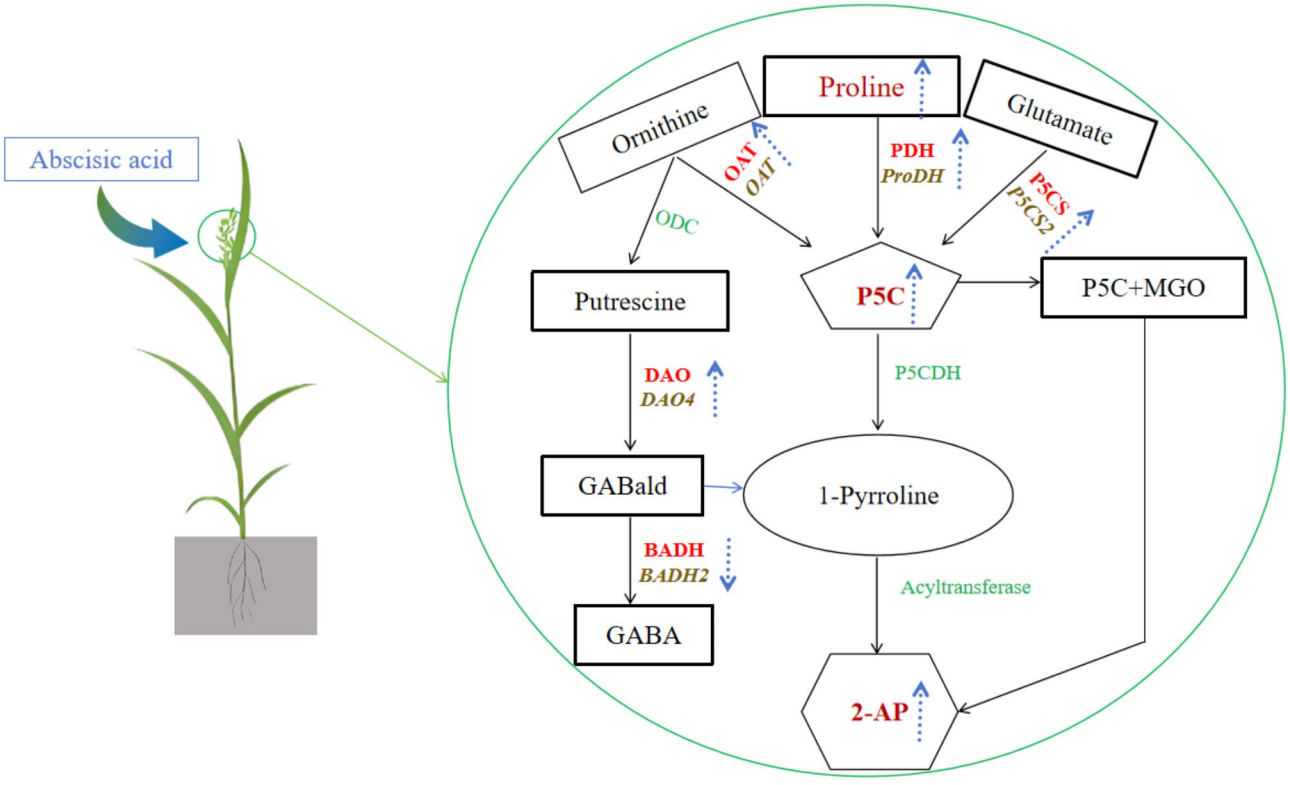

Fig. 11 The potential pathways by which abscisic acid (ABA) influences 2-acetyl-1-pyrroline (2-AP) biosynthesis in aromatic rice. ODC: ornithine decarboxylase; OAT: ornithine aminotransferase; P5CS: 1-pyrroline-5-carboxylate synthetase; PDH: proline dehydrogenase; P5C: 1-pyrroline-5-carboxylic acid; DAO: diamine oxidase; P5CDH: pyrroline-5-carboxylase dehydrogenase; MGO: methylglyoxal; GABald: γ-aminobutyl aldehyde; BADH: betaine aldehyde dehydrogenase; GABA: γ-aminobutyric acid

Effects of exogenous ABA on antioxidant activities in flag leaves, yield and quality of aromatic rice

Previous studies indicated that SOD, POD, and CAT all played pivotal roles in maintaining cellular structure and function in plant tissues as well as quenching reactive oxygen species (ROS) [36]. Functioning as the initial shield against harm from ROS, the antioxidant enzyme SOD promptly eliminated intracellular peroxides [37]. Both POD and CAT were key enzymes for H_2O_2 scavenging. POD decomposed H_2O_2 using phenolic compounds as substrates [38], whereas CAT, as the terminal enzyme in a series of antioxidant enzymes during biological oxidation, converted H_2O_2 molecules into H_2O and O_2 [37]. ABA, functioning as a "stress hormone" in plants, assisted plants in resisting abiotic stresses including low temperature, drought, and salinity by regulating their antioxidant defense systems [8, 15]. In this experiment, the activities of SOD, POD, and CAT in aromatic rice treated with ABA were significantly enhanced, consistent with previous findings. In South China, frequent short-term extreme weather and long-term acid rain significantly impact rice yield [39]. Furthermore, the complex microclimate in paddy fields, which is challenging to measure, influences rice yield formation [40]. Therefore, an enhanced antioxidant system induced by ABA in rice can stabilize production, and higher antioxidant activities can better maintain cellular stability, ensuring the progression of various physiological activities. However, this experiment also observed a significant increase in MDA content in rice treated with ABA. MDA content reflected the degree of cellular damage under stress conditions, and ABA application under stress often effectively reduced MDA accumulation [25]. It was speculated that overly frequent spraying or excessively high ABA concentrations may have adversely affected rice growth in the field, leading to MDA accumulation and a slight decrease in final yield. Although exogenous ABA spraying in this study had some negative effects on the yield, partial yield components, and quality of both aromatic rice varieties, it also improved the 1000-grain weight and milled rice rate of inferior grains [41–43]. Notably, the increases in grain weight and 2-AP content in inferior grains indicated that ABA had a pronounced effect on inferior grains of aromatic rice, and exogenous ABA application was an effective method for optimizing various indicators of inferior grains. Overall, this study confirmed that spraying ABA after the breakthrough stage in field-grown aromatic rice was a favorable approach to improving antioxidant activities, albeit with some adverse effects on yield and quality. Therefore, exploring the optimal

concentration, timing, and frequency of exogenous ABA application in subsequent experiments to achieve simultaneous improvements in aroma and yield of aromatic rice is valuable and essential. Despite the importance of ABA-mediated 2-AP biosynthesis in aromatic rice, the biological functions of certain genes involved in this process remain poorly understood, highlighting the need for additional research to elucidate the regulatory role of ABA in 2-AP biosynthesis.

Conclusions

Exogenous application of ABA can effectively increase the contents of precursors such as Pro and P5C by upregulating the transcription levels of *ProDH*, *P5CS2*, *OAT*, and *DAO4*, while downregulating the transcription level of *BADH2*, ultimately leading to a significant enhancement of 2-AP content in the mature grains. Additionally, exogenous ABA application can effectively enhance the antioxidant activities (SOD, POD, and CAT), conducive to a more stable production of aromatic rice. This research discovered the phenomenon that exogenous ABA application can significantly promote the biosynthesis and accumulation of 2-AP in aromatic rice grown in field conditions without causing notable reduction of yield and rice quality, and preliminarily explored the underlying mechanisms. Future studies should focus on how to more rationally apply exogenous ABA to synergistically increase both the 2-AP content and yield of aromatic rice.

Materials and methods

Experimental site and details

The experiment was conducted at the Experimental Base of South China Agricultural University (113°64′E, 23°24′N) from 2022 to 2023. The soil type was lateritic red soil. Soil samples from the topsoil layer (0–20 cm) were analyzed before the field experiment, revealing total nitrogen concentrations of 0.69 g kg^{-1} in 2022 and 0.71 g g kg^{-1} in 2023, total phosphorus of 0.33 g kg^{-1} in 2022 and 0.30 g g kg^{-1} in 2023, total potassium of 13.19 g kg^{-1} in 2022 and 13.12 g kg^{-1} in 2023, and soil pH values of 5.1 and 5.3, respectively. The climate data is shown in Fig. 12. The experimental cultivars, Meixiangzhan-2 (MXZ2) and Nanjingxiangzhan (NJXZ), were high-quality aromatic rice provided by Guangdong Xianmei Seed Co., Ltd., with growth periods of 130–140 days.

The experiment was conducted using a randomized block design with two treatments (ABA and CK) and three replicates. Seeds were sown on July 15, 2022 and July 10, 2023, and transplanted on August 1, 2022 and August 26, 2023, respectively. Harvesting occurred on November 2, 2022 and October 26, 2023. Each test plot was 5.0 m×5.0 m, with a transplanting density of 16 cm×30 cm and 5 seedlings per hill. A specific aromatic rice fertilizer (N: P_2O_5: K_2O = 15: 4: 6 and 10% organic matter) was applied as basal and top dressing in a 5:2 ratio. The rice fields were continuously flooded with river water throughout the growing season. Field management, including pest and weed control, followed local cultural practices.

During the breakthrough period, ABA was sprayed onto the experimental group, while the control group (CK) received deionized water. The spraying started at

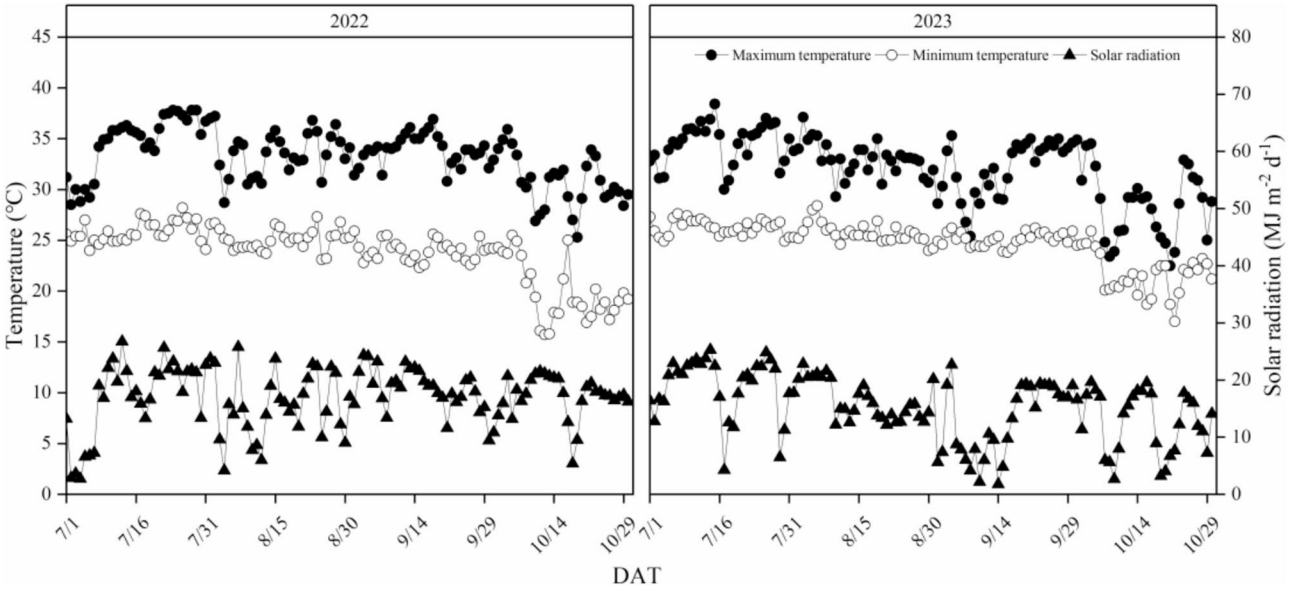

Fig. 12 Climate conditions in the experimental base of South China Agricultural University. DAT, growth date

the breakthrough stage and continued until the flowering stage, occurring once every 3 days for a total of 5 times in each plot. The amount of spraying in each plot was 2 L and the spraying time was controlled at 10: 00–14: 00. Preliminary experiments (data not shown in the manuscript) tested different concentrations of exogenous ABA (10, 20 and 30 mg L^{-1}) on growth of aromatic rice. We found that 20 mg L^{-1} ABA could promote obviously the growth of aromatic rice. Based on the results, 20 mg L^{-1} ABA (produced by Beijing Coolaber Technology Co., Ltd.) was used to study its effects on growth and physiology in aromatic rice in this study.

Sampling

At heading stage, approximately 300 panicles were chosen and labeled within each plot on a specified day. Following flowering, samples were gathered every 5 days at 10:00 a.m., comprising 20 flag leaves and 30 labeled panicles per plot. These samples were swiftly plunged into liquid nitrogen for 3 min before being stored at −80 °C for detailed analysis. Specifically, 20 flag leaves were employed to evaluate the MDA content and the enzymatic activities of SOD, POD, and CAT. Among the tagged panicles, 10 were allocated for measuring the contents of Pro and P5C, another 10 for enzymatic assessments, and the final 10 for RNA extraction. At the maturity stage, an extra 10 panicles from each plot were sampled and preserved at −80 °C for the measurement of 2-AP content. Additionally, the grains were categorized into three groups: superior grains from the top three primary branches (excluding the second grain), inferior grains from the three secondary branches at the base (excluding the first grain), and medium grains, which encompassed the rest [44].

Measurement of 2-AP content and synthesis related enzymes activities

The 2-AP content was measured using the method outlined by Luo et al. [45], with mature grain samples ground to powder and weighed (5 g per replicate, $n = 3$). Extraction involved sonication in dichloromethane at 4 °C for 4 h, followed by the addition of sodium sulfite anhydrous. The supernatant was analyzed using GCMS-QP 2010 Plus (Shimadzu, Japan), with conditions identical to Okpala et al. [46]. Results were reported in μg kg^{-1} FW. Pro content was determined according to Luo et al. [47], involving homogenization in 3% sulfosalicylic acid and boiling with ninhydrin reagent. Absorbance was measured at 520 nm, with results in μg g^{-1} FW. P5C content was measured as described by Wu et al. [48], with extracts reacting with 2-aminobenzaldehyde and recorded at 440 nm. The extinction coefficient was 2.58 mmol/L·cm, and results were reported in μmol g^{-1} FW.

For enzyme activity measurements, grains were homogenized in Tris-HCl buffer (pH 7.6) containing $MgCl_2$, KCl, ethylenediamine tetraacetic acid (EDTA), DL-Dithiothreitol (DTT), and polyvinyl polypyrrolidone (PVP). The activity of P5CS (EC 1.5.1.12) was measured using the method of Bao et al. [28]. Enzyme extract was added to a reaction mixture and incubated at 37 ℃ for 5 min. The reaction was stopped with a stop buffer (2.5 mol L^{-1} HCl containing 2.5% $FeCl_3$ and 6% TCA), and absorbance was read at 340 nm. P5CS activity was expressed as U g^{-1} FW. OAT (EC 4.1.1.17) activity was determined following the study of Chen et al. [49]. Enzyme extract was mixed with a reaction solution containing potassium phosphate buffer (pH 8.0), ornithine, α-ketoglutarate and 1 mmol L^{-1} pridoxal-5-phosphate. After incubating at 37 ℃ for 30 min. The reaction was stopped with TCA and o-aminobenzaldehyde, and absorbance was recorded at 440 nm. OAT activity was calculated using an extinction coefficient (2.68 mmol L^{-1} cm^{-1}) and expressed as U g^{-1} FW. Proline dehydrogenase (PDH, EC 1.5.99.8) activity was measured according to the method of Li et al. [50] method. Enzyme extract was added to a reaction mixture containing proline, cytochrome c, phosphate buffer (pH 7.4) and Triton X-100. After incubating at 37 ℃ for 30 min, TCA and 2-aminobenzaldehyde in ethanol were added and the supernatant's absorbance was measured at 440 nm. PDH activity was calculated using a molar extinction coefficient (2.71×10^3 min^{-1} cm^{-1}) and expressed as U g^{-1} FW.

Isolation and quantification of RNA via RT-qPCR

Total RNA samples were extracted from rice grains collected ten days post-flowering using RNA Trizol reagent (RNAiso Plus, Takara Bio Inc., Shiga, Japan), and subsequently reverse-transcribed into cDNA utilizing the Revertase Transcription kit (Perfect Real Time, Takara Bio Inc., Shiga, Japan). Quantification of the cDNA products was carried out using a real-time PCR detection system (Bio-Rad CFX96, CA, USA), in accordance with the manufacturer's guidelines for SYBR Green Master Mix (Vazyme Co., Ltd., Nanjing, China). Each sample was assayed in triplicate, with the specific operational procedures outlined in Luo et al. [51]. The rice Actin gene served as an internal control for normalization. PCR primer design was facilitated by Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA), and the sequences of the primers utilized were provided in Table 3.

Measurement of MDA content and SOD, POD, CAT activities

The MDA content was assessed using the thiobarbituric acid (TBA) oxidation method [36]. Under high temperature and acidic conditions, MDA reacted with TBA, and

Table 3 Primer sequences of genes encoding enzymes involved in 2-acetyl-1-pyrroline (2-AP) biosynthesis

Gene name	Accession no.	Primer sequences
ProDH	AP014966.1	F 5′-TCATCAGACGAGCAGAGGAGAACAGG-3′
		R 5′-CCCAGCATTGCAGCCTTGAACC-3′
P5CS2	AP014957.1	F 5′-GAGGTTGGCATAAGCACAG-3′
		R 5′-CTCCCTTGTCGCCGTTC-3′
BADH2	AB09683	F 5′-GGTTGGTCTTCCTTCAGGTGTGC-3′
		R 5′-CATCAACATCATCAAACACCACTAT-3′
OAT	AP014959.1	F 5′-GCCCTTGGTGCTGGAGTA-3′
		R 5′-AGCCCTTTCAACGAGACCTT-3′
DAO2	AP014960.1	F 5′-TCGTTCGCATCAAGGTTGG-3′
		R 5′-TCAGACAGAAGGGTGCCGTA-3′
DAO4	AP014960.1	F 5′-TGGCAAGATAGAAGCAGAAGT-3′
		R 5′-GTCCATACGGGCAACAAA-3′

the absorbance of the resulting solutions was measured at 532 nm, 600 nm, and 450 nm. The MDA content was expressed in μmol g^{-1} FW. SOD (EC 1.15.1.1) activity was determined by the nitro blue tetrazolium (NBT) photochemical reduction method [52]. One unit of SOD activity (U) was defined as the amount required to inhibit 50% of the NBT photoreduction reaction. The reaction mixture contained sodium phosphate buffer (pH 7.8), methionine buffer, NBT buffer, EDTA-Na_2 buffer, riboflavin solution, and enzyme extract. The color intensity change was measured spectrophotometrically at 560 nm. SOD activity was expressed in U g^{-1} FW. POD (EC 1.11.1.7) activity was determined by measuring guaiacol oxidation in the presence of hydrogen peroxide (H_2O_2) [53]. The reaction mixture comprised enzyme extract, H_2O_2, guaiacol, and sodium phosphate buffer (pH 7.0). The absorbance was measured spectrophotometrically at 470 nm, with an increase of 0.01 in absorbance representing one unit (U) of POD activity. POD activity was expressed in U g^{-1} FW. CAT (EC 1.11.1.6) activity was assessed using the ammonium molybdate method [54]. This method measures the amount of H_2O_2 hydrolyzed by CAT per unit weight of fresh plant tissue over time. After mixing enzyme extract with H_2O_2 and allowing the reaction to proceed for 2 min, sodium chloride solution and ammonium molybdate were added, and the absorbance was recorded at 405 nm to measure residual H_2O_2. The activity unit was mmol g^{-1} min^{-1}.

Measurement of grain yield and quality

At the maturity stage, rice was harvested, and the yield for each treatment was documented after eliminating impurities, with the final yield standardized to a 14% moisture content. Prior to harvest, the panicle number was counted using 30 hills of plants per plot. Subsequently, 10 hills of plants from each plot, selected based on the average number of effective panicles, were used to determine the spikelet number per panicle, 1000-grain weight, and seed setting rate. Additionally, another 10 plants from each plot were randomly sampled and allowed to dry naturally in the sun. Following a 3-month storage period at room temperature, these samples were evaluated for rice milling quality, appearance quality, protein content, and amylose content.

Statistical analysis

The data were analyzed through the application of analysis of variance (ANOVA), with means compared using the least significant difference (LSD) test at a 0.05 probability level, employing SPSS 20.0 software (Statistical Product and Service Solutions Inc., Chicago, IL, USA). Subsequently, Origin 2024b (OriginLab, Northampton, MA, USA) was utilized for the creation of figures.

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Author contributions
SD, PX, LP, JL and XT conceived and designed the experiments; SD, PX, LP, YW and YL performed the experiments; SD, LP and XY wrote the frst version of manuscript; PX, YZ and ZH performed the gene expression analysis and analyzed the RNA-seq data; XT provided the guidance during the experiment and paper writing; all authors have read and approved the final manuscript.

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Data availability
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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