Priming Effect of Exogenous ABA on Heat Stress Tolerance in Rice Seedlings is Associated with the Upregulation of Antioxidative Defense Capability and Heat Shock-Related Genes

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Research Article

Keywords: Abscisic acid (ABA), Antioxidative defense, Heat stress, Priming, Rice (Oryza sativa L.)

Posted Date: December 9th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1098035/v1

Priming effect of exogenous ABA on heat stress tolerance in rice seedlings is associated with the upregulation of antioxidative defense capability and heat shock-related genes

Running title: ABA improved ROS-scavenging capability

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Abstract

Heat stress is a major restrictive factor that suppresses rice production. In this study, we investigated the potential priming effect of exogenous abscisic acid (ABA) on heat tolerance in rice seedlings. Seedlings were pretreated with 10 μM ABA by root drenching for 24 h and then subjected to heat stress conditions of 40 °C day/35 °C night. ABA pretreatment significantly decreased leaf withering by 2.5–28.5% and chlorophyll loss by 12.8–35.1% induced by heat stress in rice seedlings. ABA application also mitigated cell injury, as shown by lower malondialdehyde (MDA) content, membrane injury and expression of cell death-related genes *OsKOD1*, *OsCP1* and *OsNAC4*, while expression of *OsBI1*, a cell death-suppressor gene, was upregulated by ABA pretreatment. Moreover, ABA pretreatment improved antioxidant defense capacity, as shown by an obvious upregulation of ROS-scavenging genes and a decrease in ROS content (O_2^- and H_2O_2) and downregulation of the *OsRbohs* gene. The application of fluridone, an ABA biosynthesis inhibitor, increased membrane injury and the accumulation of ROS under heat stress. Exogenous potent antioxidants (proanthocyanidins, PC) significantly alleviated leaf withering by decreasing ROS overaccumulation and membrane injury induced by heat stress. In addition, ABA pretreatment significantly superinduced the expression of ABA-responsive genes *SalT* and *OsWsi18*, the ABA biosynthesis genes *OsNCED3* and *OsNCED4*, and the heat shock-related genes *OsHSP23.7*, *OsHSP17.7*, *OsHSF7* and *OsHsfA2a*. Taken together, these results suggest that exogenous ABA has a potential priming effect for enhancing heat stress tolerance of rice seedlings mainly by improving antioxidant defense capacity and heat shock-related genes.

Key words Abscisic acid (ABA); Antioxidative defense; Heat stress; Priming; Rice (*Oryza sativa* L.)

Abbreviations

ABA	Abscisic acid
H_2O_2	Hydrogen peroxide
HS	Heat stress
HSP	Heat-shock proteins
HSF	Heat-shock factor
MDA	Malondialdehyde
MI	Membrane injury
O_2^-	Superoxide anions
PC	Proantho Cyanidins
qRT-PCR	Quantitative real-time PCR
ROS	Reactive oxygen species
RBOH	Respiratory Burst Oxidase Homolog

Introduction

Global warming has become a severe ecological and environmental problem that has been observed due to the development of industry and population growth. Heat stress is caused by an extremely high temperature or by a lasting threshold of high-temperature weather, which has resulted from global warming (Quint et al. 2016; Xu et al. 2018). Heat stress results in serious threat to crop production worldwide, and the yields of wheat, rice, and maize were decreased by 6.0%, 3.2% and 7.4%, respectively, due to a 1℃ increase in the mean temperature (Zhao et al. 2017; Janni et al. 2020). Rice (*Oryza sativa* L.) is a staple food for most of the world's population. China is a largest rice production country, and the main growing temperature in the growing season of rice occurs during the annual long hot summer season (Shi et al. 2015). Rice production during this time suffers from heat stress, which directly inhibits the survival and transplanting of late rice, as well as the heading, flowering and grain-filling of early rice (Yang et al. 2020). Previous research has shown that widespread extreme high-temperature events of consecutive 40 °C days caused a 30% yield loss in the rice planting area of Southeast China (Tao et al. 2007). Furthermore, heat stress results in a significant decrease in grain yield, total milled rice yield, head rice yield, and total milling revenue with the increasing average growing temperature (Lyman et al. 2013). Thus, the demand of an expanding population under the current situation of global warming remains a huge challenge for food production.

The effect of heat stress on rice production occurs during the entire growth stage. The germination of rice seeds was suppressed by growth temperatures ≥ 35 ℃, as shown by a significant decrease in the germination rate and growth in buds (Yang et al. 2021), and the suppressive effect was evidenced by the overaccumulation of ROS in buds (Yang et al. 2021). The optimal temperature of rice seedlings ranges from 22 ℃ to 28 ℃, while the growth of rice seedlings is severely inhibited under growth conditions of ≥ 35 ℃ (Soda et al. 2018). Heat stress results in withering, browning, abnormalities and water loss of rice leaves, suppresses the growth of seedlings and roots, and causes complete wilting of rice plants (Liu et al. 2016; Kilasi et al. 2018; Li et al. 2020). Heat stress suppresses photosynthetic efficiency by decreasing chlorophyll content, disturbing the combination of chlorophyll-protein complexes and damaging the photosystem structure (Yang et al. 2013; Fan et al. 2017). In addition, heat stress causes excess accumulation of reactive oxygen species (ROS), which results in severe damage to the membrane and cell death (Zhao et al. 2017). Rice plants are more

sensitive to heat stress at the reproductive and grain-filling stages. Heat stress results in the degradation of flowering florets, abortion of pollen and devitalization of pollen viability of rice at the booting stage, which leads to a reduction in filled spikelets and a decrease in the maturation rate (Coast et al. 2016; Fu et al. 2016; Zhang et al. 2018). Heat stress suppresses the grain-filling rate in rice at the grain-filling stage, as shown by the decrease in activity of the starch branching enzyme, which impedes the assimilation of photosynthate into starch in rice grains (Kaneko et al. 2016; Zhang et al. 2016, 2018; Wang et al. 2019). Thus, it is indeed necessary to explore an effective pathway for the improvement of heat tolerance in rice in the key growth period.

Plants generate and accumulate ROS such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) under various environmental stress conditions, such as drought, salinity, alkalinity and extreme temperature (Choudhury et al. 2017). These ROS molecules play an important role in the regulation of development and adaptation to the environment (Mittler 2017). However, excess accumulation of ROS causes oxidative stress in plants, which results in severe damage to the plant cellular membrane, RNA, DNA and proteins (Sewelam et al. 2016). We previously reported that overaccumulation of ROS in rice roots is a main limiting factor in cell damage and plant growth inhibition in rice seedlings under alkaline stress conditions (Zhang et al. 2017). ROS accumulation is an important harmful pathway in the physiological effects of heat stress on plants (Xu et al. 2021). Heat stress causes a remarkable upregulation of *respiratory burst oxidase homolog* (RBOH) genes and an accumulation of ROS in rice seedlings, and excessive ROS levels disturb the balance of ROS production and scavenging, which further results in membrane lipid peroxidation, cell damage, changes in a series of antioxidant enzymes, and even plant death (Liu et al. 2019). Previous studies have shown that excessive accumulation of ROS induced by heat stress causes the decline of pollen viability, spikelet fertility and grain chalkiness in rice plants at reproductive and grain-filling stages, indicating that ROS level is involved in the regulation of yield formation of rice under heat stress condition (Suriyasak et al. 2017; Zhao et al. 2017), and enhancing the antioxidative defense capacity promotes anther development and yield formation under heat stress conditions (Dwivedi et al. 2019). Therefore, studies on the improvement of rice tolerance to various stress factors by reducing oxidative stress induced by overaccumulation of ROS are still required for further insight and will provide a potential useful pathway in rice field production in the future (Kerchev et al. 2015).

The phytohormone abscisic acid (ABA) plays an important role in the regulation of plant growth and adaptive to various stress factors (Ye et al. 2012; Sah et al. 2016; Dar et al. 2017). One important pathway of ABA action is exerting the priming effect to plants in stress tolerance (Savvides et al. 2016; Vishwakarma et al. 2017), which confer the potential enhanced ability to mount defense responses to impending stress factors (Pastor et al. 2013; Aranega-Bou et al. 2014). The priming effect of ABA has been demonstrated as shown by rice seeds or seedlings pretreated with ABA improved plants survival, growth and grain yield in saline stress conditions (Li et al. 2010; Gurmani et al. 2011, 2013). Wei previously reported that exogenous ABA primes rice seedlings for enhanced tolerance to alkali stress and improved plant growth and grain yield in saline-alkaline paddy field (Wei et al. 2015. 2017). Further analysis on the underlying mechanism of ABA priming showed that ABA priming potentiated to improve the downstream antioxidant defense capacity and stress tolerance-related gene expression for an increased adaptive response to alkaline stress (Liu et al. 2019).

ABA also functions in crops response to heat stress and plays a vital role in crop production under climate warming condition (Suzuki et al. 2016; Li et al. 2021). Application of exogenous ABA improves plant heat tolerance as shown by keeping water balance, regulating stomatal conductance and the regulation of gene expression, like the heat-shock proteins (HSP) (An et al. 2014; Liu et al. 2014; Lei et al. 2015). Additional, ABA also functions in the regulation of rice in the reproductive and grain-filling stage (Islam et al. 2018; Li et al. 2021). The ROS signal pathway plays an important role in plant response to environmental stress and excess accumulation induced by various stress factors result in membrane injury, root damage and even the death of plants (Zhang et al. 2017; Qiu et al. 2019). ABA confers to regulate ROS levels in plants response to environmental stress factors (Wang et al. 2013; Liu et al. 2019), as well as under high temperature condition (Hu et al. 2010). And the ABA-deficient mutants exhibited more sensitive to heat stress (Larkindale et al. 2005). These studies demonstrate a strong correlation between ABA application and regulation of ROS levels in plants response to stress condition (Suzuki et al. 2016; Liu et al. 2019). Current studies on the relationship between ABA and heat stress in rice were mainly focused on the ABA-dependent signal pathway and spraying of exogenous ABA (Wang et al. 2013; Zhang et al. 2019; Li et al. 2020). Research of ABA priming effect on stress tolerance in rice were mainly validated in the response to salt or alkaline stress (Gurmani et al. 2013; Wei et al. 2015, 2017; Liu et al. 2019).

This study aimed to gain insights into the effect and mechanism of ABA priming on heat tolerance in rice by focusing on the effect of ABA priming on ROS-formation or ROS-scavenging pathway. Our results strongly suggest that priming effect of exogenous ABA on heat stress tolerance in rice seedlings is associated with improvement of antioxidative defense capacity and expression of heat stress tolerant genes.

Materials and methods

Plant material and growth conditions

Rice cultivar Huanghuazhan, a elite cultivar suitable to be spreaded in eastern China, was used in this study. It was bred by crossing 'Huangxinzhan' with 'Fenghuazhan' and was resistant to high-temperature stress (China Rice Data Center). Rice seeds were surface-sterilized with 75% (v/v) alcohol for 5 min, and rinsed with deionized water five times. After that, seeds were immersed in water for 2 days, and then were sprinkled onto a petri dish with wet filter paper for pregermination in an incubator under dark condition at 28 °C for 24 h. Eighteen uniformly germinated seeds were transplanted onto a multi-well plate floating on a 320-mL cup containing deionized water for 7 days, and then grown in half-strength Kimura B nutrient solution (Miyake and Takahashi 1983) for another 7 days. All rice seedlings were grown in a controlled growth chamber under the following conditions: 28℃ day/22℃ night, 12 h photoperiod, and 350 mmol photons m^{-2} s^{-1} light intensity.

ABA pretreatment and high-temperature treatment

Abscisic acid (ABA) (Sigma, Inc., St, Louis, MO, USA) was dissolved in a small amount of absolute ethanol and then diluted with deionized water to the desired concentrations (Wei et al. 2015; Liu et al. 2019). Rice seedlings at approximately three-leaf stage were pretreated with 10 μM ABA or deionized water by root-drench for 24 h, respectively. Then these two sets of rice seedlings were transferred to the control or high-temperature stress conditions after rinsed with deionized water, respectively. Thus, four treatments were set: root-drench with deionized water in unstressed condition (CK); root-drench with 10 μM ABA in unstressed condition (ABA); root-drench with deionized water in heat stress (HS), root-drench with 10 μM ABA in high-temperature stress condition (ABA+HS). We used grown temperature at 40℃ to simulate high-

temperature stress. The growth temperature of unstressed condition was set as following: 28℃ day/22℃ night and temperature of high-temperature stress was set as 40℃ day/35℃ night. This growth temperature was set according to the description of rice response to heat stress in South China by Shi et al. (2015) and Huang et al. (2017).

Treatment of rice seedlings with exogenous Fluridone and Proantho Cyanidins (PC)

In this study, exogenous fluridone and proantho cyanidins (PC) were used to examine the mechanism of ABA priming effect. Fluridone is an ABA biosynthesis inhibitor and inhibited rice seedlings growth under alkaline stress (Wei et al. 2015). Proantho cyanidins is an antioxidant that effectively scavenged superoxide anion radicals and hydroxyl radicals, and alleviated alkalinity-induced suppression of rice seedling growth by inhibiting ROS overaccumulation (Rue et al. 2017; Zhang et al. 2017). Two-week-old rice seedlings were pretreated in the solution with deionized water, 10 μM fluridone and 1% PC, by root-drench for 24 h, respectively; and then transferred to control or heat stress conditions aforementioned. The treatments were set as follows: CK, Fluridone, PC, HS, Fluridone+HS, PC+HS, respectively.

Measurement of seedling growth

Photograph of the growth condition of rice seedlings was taken at the indicated treatment hours. The withered leaf rate was investigated at 48, 72 and 96 h of heat stress, respectively. The withered leaf rate was recorded as 1 if the whole leaf was dry and brown, while it was recorded as 0.5 if half of the leaf was dry and brown, respectively (Liu et al. 2020). The withered leaf rate of each treatment was represented by the proportion of withered leaves of whole leaves one cup.

Measurement of chlorophyll content

The chlorophyll content was measured according to the theory as described by Wellburn and Lichtenthaler (1984), with some modifications as described by Liu et al. (2019). Leaf samples (0.1 g) were extracted using a 10 mL mixture of ethanol (5 mL) and acetone (5 mL) under dark condition. The absorbance of the supernatant was determined at 645 and 663 nm using a spectrophotometer (UV-2700, Shimadzu, Kyoto, Japan) until the whole leaves whitening. The total chlorophyll content unit fresh weight was calculated using the following formula: $(20.29\times A_{645} + 8.05\times A_{663})\ V/(1000\times W)$.

Measurement of malondialdehyde (MDA) content and membrane injury (MI)

The MDA content was determined by the thiobarbituric acid reaction as described by Heath and Packer (1968). Leaf samples were homogenized in 1 mL of 50 mM phosphate buffer (pH 7.8) after smashed at refrigerated condition with liquid nitrogen, and centrifuged at 12,000 × g for 15 min. Subsequently, 400 μL of supernatant was mixed with 1 ml of 0.5% thiobarbituric acid, and the mixture was boiled for 20 min. The absorbance of the resulting supernatant after cooled and centrifuged was measured at 532, 600, and 450 nm using a spectrophotometer (UV-2700, Shimadzu, Kyoto, Japan). The MDA concentration was calculated using the following formula: $6.45\times(A_{532} - A_{600}) - 0.56 \times A_{450}$. Finally, the MDA content in leaf was calculated according to the fresh weight of leaf of each treatment.

Membrane injury (MI) was represented by measured by relative electrolytic leakage (Tantau and Dörffling 1991). Rice leaves (2 g fresh weight) were randomly selected from each treatment group, washed with deionized water to remove surface-adhered electrolytes. Leaf samples were submerged in 15 ml of deionized water in 50 mL conical tubes and kept at 20°C for 1 h. The electrical conduction of the effusion was then measured with a DDS-12 conductivity meter (Lida Inc., Shanghai, China) and recorded as R1. The tissue samples were killed by heating tubes in a boiling bath for 40 min, cooled to 20°C, and the electrical conduction of the effusion was measured again which recorded as R2. The MI was evaluated using the formula MI (%) =R1/R2 ×100%.

Measurement of ROS levels

The O_2^- contents were measured as described by Elstner and Heupel (1976) by monitoring nitrite formation from hydroxylamine in the presence of O_2^-, with some modifications as described by Jiang and Zhang (2001). Absorbance values at 530 nm were calibrated to calculate the contents of O_2^- from the chemical reaction of O_2^- and hydroxylamine.

The H_2O_2 contents were measured as described by monitoring the A_{415} of the titanium-peroxide complex (Brennan and Frenkel 1977). Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2. The analytical reagent used to measure the H_2O_2 and O_2^- contents were acquired from the determination kit, according to the manufacturer's instructions (Comin Biotechnology Co., Ltd. Suzhou, China) (Zhang et al. 2017; Liu et al. 2019).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Rice leaves were sampled in liquid nitrogen and ground using a bench-top ball-mill (Scientz-48, Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China) at 50 Hz for 30 s. Total RNA was extracted with TRIzol reagent (TaKaRa Bio Tokyo, Japan) and first-strand cDNA was synthesized using M-MLV reverse transcriptase (Thermo, Carlsbad, CA, USA) according to the manufacturer's protocols. Quantitative real-time PCR (qRT-PCR) was performed to determine the transcriptional expression of genes, including nine *OsRboh* family genes (*OsRboh1-OsRboh9*) (Liu et al. 2012), two ABA-response genes, *SalT* and *OsWsil18*; two ABA-biosynthesis genes, *OsNCED3* and *OsNCED4*; four heat shock-related genes, *OsHSP23.7* (Zou et al. 2012), *OsHSP17.7* (Sato and Yokoya 2008), *OsHSF7* (Liu et al. 2009) and *OsHsfA2a* (Wang et al. 2013); and 20 ROS-scavenging genes (R1-R20) (Fang et al. 2015; Liu et al. 2019). Gene-specific primers were designed using Primer 5.0 software (Table S1; Table S2 and Table S3).

The housekeeping gene β-actin (GenBank ID: X15865.1) was used as an internal standard. PCR was conducted in a 20 μL reaction mixture containing 1.6 μL of cDNA template (50 ng), 0.4 μL of 10 mM specific forward primer, 0.4 μL of 10 mM specific reverse primer, 10 μL of 2× SYBR® *Premix Ex Taq*™ (TaKaRa, Bio Inc.), and 7.6 μL of double-distilled H_2O in a PCR machine (qTOWER2.2. Analytic Jena. GER). The procedure was performed as follows: 1 cycle for 30 s at 95°C, 40 cycles for 5 s at 95°C, and 20 s at 60°C, and 1 cycle for 60 s at 95°C, 30 s at 55°C, and 30 s at 95°C for melting curve analysis. The level of relative expression was computed using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen 2001).

Experimental design and statistical analyses

All of the experiments were conducted in a controlled growth chamber with five biological replicates, each consisting of 3 cups of rice seedlings, with eighteen seedlings each cup. Statistical analyses were performed using the statistical software SPSS 21.0 (IBM Corp., Armonk, NY). Based on one-way analysis of variance (ANOVA), Duncan's multiple range test (DMRT) was used to compare differences in the means among treatments. The significance level was $P < 0.05$.

Results

ABA priming rescued rice seedling from wilting and death under heat stress

There was no significant difference of leaf withering with or without ABA pretreatment at 24 and 48 h of high-temperature stress (Fig. 1A, B). While pretreatment with exogenous ABA significantly rescued rice seedling from wilting and death as shown by the lower withered leaf rates of seedlings pretreated with ABA at 72 and 96 h of high-temperature stress (Fig. 1C-E), withered leaf rate of rice seedlings was decreased by 28.5% and 15.8% by ABA pretreated under heat stress condition (Fig. 1F). Rice seedlings pretreatment with ABA significantly increased chlorophyll content by 11.3%, 25.9% and 13.8%, compared with without ABA pretreatment under heat stress condition (Fig. 1G).

ABA priming mitigated membrane injury induced by heat stress

Exogenous ABA pretreatment significantly mitigated cell injury as shown by a lower accumulation of MDA and MI (Fig. 2A, B). Compared to HS treatment, MDA content was decreased by 22.4%, 22.1% and 10.8%, and MI was decreased by 14.1%, 13.2% and 7.9%, at 48, 72 and 96 h of heat stress, respectively (Fig. 2A, B). In addition, a cell death suppressor, *OsBI1*, was significantly downregulated and the cell death-related genes, *OsKOD1*, *OsCP1*, *OsNAC4*, were significantly upregulated by ABA pretreated under heat stress condition (Fig. 2C-F). The relative expression level was increased by 37.1%, 47.2% and 50.2% with ABA pretreatment at 48, 72 and 96 h of heat stress condition, respectively (Fig. 2C).

ABA priming decreased ROS accumulation and improved ROS-savaging capacity under heat stress

Pretreatment with exogenous ABA significantly inhibited ROS accumulation as shown by lower O_2^- and H_2O_2 content in rice leaves under heat stress condition (Fig. 3). Compared to HS treatment, content of O_2^- was decreased by 5.9%, 19.6% and 22.2% (Fig. 3A), and content of H_2O_2 was decreased by 8.3%, 16.5% and 16.1% with ABA pretreatment at 48, 72 and 96 h, respectively (Fig. 3B).

ABA pretreatment also suppressed the transcriptional expression of *OsRboh* genes. As shown in Fig. 4, an obvious upregulation was showed in the *OsRbohs* family genes by heat stress, and the relative expression level of *OsRboh1*, *OsRboh4*, *OsRboh5*, *OsRboh6* and *OsRboh7* was reached to a higher level. Among these *OsRbohs* family genes, *OsRboh2*, *OsRboh3*, *OsRboh5* and *OsRboh7* was significantly suppressed by ABA pretreatment (Fig. 4).

We further analyzed the relative expression levels of 20 ROS-scavenging genes, as shown in Fig. 5, almost the all ROS-scavenging genes were upregulated by heat stress. Furthermore, ABA pretreatment significantly super-upregulated the expression level of 16 ROS-scavenging genes except for R5, R7, R13 and R20 (Fig. 5).

Exogenous ABA biosynthesis inhibitor (Fluridone) suppressed rice seedlings growth under heat stress

As shown in Fig.6A, growth condition of rice seedlings with application of fluridone (treatment of Fluridone+HS), an ABA biosynthesis inhibitor, was similar to the HS treatment. The withered leaf rate and chlorophyll content were not statistically significant between Fluridone+HS and HS treatment (Fig.6B, C), as well as the accumulation of O_2^- and H_2O_2 (Fig. 6F, G). Accumulation of MDA and MI with application of fluridone was significantly higher than that of HS treatment at 48, 72 h of heat stress condition, respectively (Fig.6D, E).

Application of exogenous antioxidant (Proantho cyanidins, PC) rescued rice seedlings from leaf withering induced by heat stress

In this study, application of exogenous PC significantly rescued rice seedlings from leaf withering as shown by a decrease of 47.8%, 44.7% and 33.5% of leaf withered rate at 48, 72 and 96 h, compared to HS treatment (Fig. 6A, B). Content of chlorophyll was increased by 13.6%, 31.3% and 34.8% with application of PC under heat stress condition, compared to HS treatment (Fig. 6C). In addition, membrane injury was significantly mitigated by PC as shown by a lower accumulation of MDA content and MI in rice seedlings of PC+HS treatment (Fig. 6D, E). Consistently, accumulation of O_2^- and H_2O_2 was decreased by 25.3%-41.1% and 39.8%-45.6% with application of PC, compared to HS treatment (Fig. 6F, G).

ABA pretreatment upregulated stress tolerance-related genes under heat stress

ABA signaling pathway was exactly activated by heat stress and ABA pretreatment as shown by an upregulation of two ABA-responsive genes, *Salt* and *OsWsi18* (Fig.7A, B). While the expression levels of *Salt* and *OsWsi18* was significantly superinduced by 34.2%-47.8% and 25.9%-26.9% with ABA pretreatment, compared to HS treatment (Fig.7A, B). The expression levels of two ABA biosynthesis genes, *OsNCED3* and *OsNCED4*, were increased by 40.8%-71.3% and 32.5%-54.0% by ABA pretreatment,

compared to HS treatment (Fig.7C, D).

To gain further insights into the mechanism of ABA pretreatment for heat stress, two heat shock protein (HSP) genes, *OsHSP23.7* and *OsHSP17.7*, and two heat shock transcription factors (HSF), *OsHSF7* and *OsHsfA2a*, were analyzed in this study. All these stress tolerance-related genes were significantly upregulated by ABA, HS and ABA+HS treatment, while the relative expression levels of these four genes were significantly super-upregulated by ABA pretreatment under heat stress condition (Fig.7 E-H). The gene relative expression levels of *OsHSP23.7*, *OsHSP17.7*, *OsHSF7* and *OsHsfA2a* was increased by 28.4%-36.9%, 32.9-49.7%, 31.0%-42.6% and 33.3%-50.5% with ABA pretreatment under heat stress condition, respectively (Fig.7E-H).

Discussion

Heat stress is characterized by extreme or lasting high-temperature climate for a long time, which has become an enormous meteorological disaster for crop production (Xu et al. 2021). Heat stress results in severe inhibition in crop growth and yield formation as shown by increasing leaves withering and death (Wei et al. 2012; Kilasi et al. 2016; Liu et al. 2018), damaging cell membrane and photosynthetic structure (Essemine et al. 2017; Soda et al. 2018), impairing pollen swelling (Endo et al. 2009; Das et al. 2014; Wang et al. 2019), reducing spikelets (Fu et al. 2016; Zhang et al. 2018) and the final grain filling (Chen et al. 2017; Suriyasak et al. 2017). Recently, it was shown that application of exogenous phytohormones alleviated heat-induced damage in plants and enhanced plant heat tolerance (Li et al. 2021). ABA plays an important role in crops response to environmental stress. Previous studies have reported the priming effect of exogenous ABA on tolerance to alkaline stress in rice seedlings (Wei et al. 2015, 2017; Liu et al. 2019). And we previous showed rice seeds soaked with exogenous ABA significantly improved seed growth under lasting high temperature stress condition (Yang et al. 2021). In the present study, we have firstly shown that rice seedlings pretreated with exogenous ABA significantly mitigated the heat-induced leaf withering (Fig. 1), membrane injury (Fig. 2) and overaccumulation of ROS (Fig. 3, 4), and improved ROS-scavenging capability (Fig. 5). In addition, there was some evidence showed that application of the ABA biosynthesis inhibitor, fluridone, compromised tolerance to heat stress in rice seedlings (Fig. 6), while application of the antioxidant, PC, improved tolerance of the seedlings to heat stress (Fig. 6). Pretreatment with ABA

also upregulated gene expression levels related to ABA signal and heat shock and transcription factor (Fig. 7). These data collectively suggest that improving ROS-scavenging capability and upregulating heat shock related genes are important mechanism of exogenous ABA priming for heat tolerance in rice seedlings (Fig. 8).

ABA is an important "stress phytohormone" in plants, which has been evidenced by the action in various stress conditions as drought, salt, alkali, cold and heat temperature (Dar et al. 2017; Vishwakarma et al. 2017). Exogenous ABA plays a vital in the improvement of stress tolerance by multiple methods, such as foliage spray, adding into solution or seed soaking (Gurmani et al. 2011, 2013; Wang et al. 2013). Another important mechanism of ABA for enhancing stress tolerance in plants is the priming effect, which help plants to acquire a potential capacity to enhance defense response to subsequent stress factors (Pastor et al. 2013; Aranega-Bou et al. 2014). This priming effect has recently been validated in rice response to salt or alkali stress as shown by seed presoaking or root drenching with exogenous ABA significantly improved the survival rate, plant growth and grain yield of rice (Gurmani et al. 2011; Wei et al. 2015, 2017). Application of exogenous ABA plays active effect in plants response heat stress (Islam et al. 2018). However, few studies have reported on the priming effect of ABA in the heat stress responses. We previously reported that ABA primes rice seeds for enhanced heat stress tolerance as shown by ABA presoaking improved ROS-scavenging capacity, inhibiting ROS overaccumulation and mitigating membrane injury (Yang et al. 2021). Results of the present study showed that the ABA responsive genes, *Salt* and *OsWsi18* (Fig. 7A-7B), and ABA-biosynthesis genes, *OsNCED3* and *OsNECD4* (Fig. 7C-7D), were significantly upregulated by heat stress, and application of the ABA inhibitor suppressed rice seedlings growth (Fig. 6), which indicated that the ABA signal was indeed participated in the response to heat stress in rice seedlings. However, rice seedlings were withered or eventually dead induced by heat stress for more than 4 days (Fig. 1), indicating that this activation levels of ABA induced by heat stress may not be sufficient to effectively cope with the heat stress factor. Nevertheless, ABA pretreatment upregulated the expression of ABA-responsive and ABA-biosynthesis genes to a higher degree (Fig. 7), as well as a great increasing of ROS-scavenging genes (Fig. 5), heat shock-related genes (Fig. 7), and a remarkable decrease of ROS accumulation (Fig. 3) and cell death in rice seedlings under heat stress condition (Fig. 2). In addition, we previously reported that ABA soaking on rice seeds efficiently improved seed viability and bud growth under continuous heat stress

condition (Yang et al. 2021). These results suggest that exogenous ABA enhances tolerance to heat stress in rice seedlings by the priming effect which potentiate multiple downstream pathways response to heat stress.

Reactive oxygen species (ROS) plays vital role in the regulation of plants response to various stress factors (Choudhury et al. 2017; Mittler 2017). ROS serve as the signaling messenger in a series of physiological processes required for the growth regulation and stress response at low levels (Sewelam et al. 2016; Mittler 2017). However, environmental stress induces overaccumulation of ROS in cells, which result in oxidative stress and even cell death in plants (Choudhury et al. 2017; Zhang et al. 2017). In rice, excessive accumulation of ROS has been identified as a key causal factor in the inhibition of seed germination and seedlings growth under various stress conditions due to the result of oxidative stress, especially for the severe cellular damage to roots (Guan et al. 2017; Zhang et al. 2017). Heat stress caused multiple physiological effects to rice including membrane and photosynthesis damage, disturbance of ROS accumulation and carbohydrate (Xu et al. 2021). We previously showed that increasing of ROS intracellular was the primary for the inhabitation of seed germination and bud growth under lasting heat stress condition (Yang et al. 2021). In the present study, heat stress caused a remarkable increase of ROS in rice seedlings as shown by a gradually rising accumulation of O_2^- and H_2O_2 in leaves at the indicated time (Fig. 3A, B), as well as the upregulation of a series of *OsRboh* genes (Fig. 4). Meanwhile, rice seedlings presented a significant membrane injury as shown by the increase of MDA and MI (Fig. 2A, B), as well as a number of cell death-related genes, *OsKOD1*, *OsCP1* and *OsNAC4*, (Fig. 2D-F) under heat stress condition. In addition, several ROS-scavenging genes were significantly upregulated by heat stress (Fig. 5). These results indicated that ROS signal pathway was activated in the response to heat stress in rice seedlings; however, the ROS levels was too high in turn leaded to severe injury to cell membrane, and final resulted in withering and even death of rice seedlings (Fig. 1). Application of exogenous antioxidant, PC, significantly rescued rice seedlings from death by decreasing the ROS content and membrane injury (Fig. 6), indicating that overaccumulation of ROS is an important mechanism for inhibiting rice seedlings induced by heat stress. Nevertheless, rice seedlings pretreatment with ABA significantly improved antioxidative defense capacity as shown by a series of ROS-scavenging genes (Fig. 5), and decreased the ROS accumulation (Fig. 3A, B) and membrane injury (Fig. 2), which achieved the similar effect with the PC. On the contrary, application of fluridone was noneffective

to decrease the ROS accumulation and membrane injury (Fig. 6). These data demonstrate that ABA primes for enhanced heat tolerance in rice seedlings mainly by improving of the ROS-scavenging capacity, which is accordant to our previous study in alkaline stress (Liu et al. 2019).

The ROS levels in plants is codetermined by the ROS formation which mainly regulated by the RBOH genes, and scavenging pathway that constituted by antioxidant enzymes (Choudhury et al. 2017). The ROS formation may be induced by various stress factors, as well as ABA, while ROS levels would affect the ABA biosynthesis and catabolism (Ishibashi et al. 2012, 2015; Suriyasak et al. 2017). Thus, the "cross-effect" of ROS and ABA levels play a vital role in plants response to environmental stress conditions (Ye et al. 2011; Liu et al. 2019). In this study, almost all these *OsRboh* genes were upregulated during the heat stress process, indicating that heat stress resulted in accumulation of ROS by inducing the transcriptional expression of *OsRboh* genes in rice seedlings. Among these *OsRboh* genes, *OsRboh1*, *OsRboh4*, *OsRboh6*, *OsRboh8* and *OsRboh9* was induced by ABA priming and heat stress, which indicated that ABA induced the expression of *OsRboh* genes to increasing ROS levels in the regulation of plant growth and response to stress factors (Li et al. 2021). Nevertheless, *OsRboh2*, *OsRboh3*, *OsRboh5* and *OsRboh7* was suppressed by ABA pretreatment under heat stress (Fig. 4), which may demonstrated another potential mechanism in the "cross-effect" of ROS and ABA in plants response to environmental stress, that was ABA priming may inhibit the expression of *OsRboh2*, *OsRboh3*, *OsRboh5* and *OsRboh7* to decrease ROS formation under heat stress condition. In further studies, it would be interesting to gain further insights of the correlation between ROS formation and ABA levels by using the mutants or transgenic plants in ROS or ABA pathways.

Reprograming the gene expression is an important pathway for plants to cope with multiply environmental stress conditions. Recently, a number of genes have been identified for enhancing heat tolerance in plants (Hoang et al. 2019; Su et al. 2019). Heat shock proteins and heat shock transcription factor are known as the vital defense mechanism for plants or animals to resist heat stress condition and numerous studies have demonstrated that overexpression of the HSP or HSF genes contributed to improve stress tolerance in rice (Zou et al. 2009, 2012; Cheng et al. 2015; Liu et al. 2015). ABA has a potential regulation effect in genetic network in plants response to the stresses (Liu et al. 2019). In this study, the ABA-responsive genes and ABA biosynthesis genes were superinduced by ABA pretreatment under heat stress (Fig. 7A-D), indicating the

ABA signal pathway was indeed activated by ABA priming. In addition, two HSP genes, *OsHSP23.7* and *OsHSP17.7*; and two HSF genes, *OsHSP23.7* and *OsHSP17.7* were significantly upregulated by ABA-priming under heat stress (Fig. 7E-H). These data represented another important mechanism of ABA-priming effect, indicating that ABA was involved in the multiple gene transcriptional regulatory network in plant under stress conditions.

In summary, in this study, ABA priming super-increased the ABA signal in rice seedlings under heat stress, so as to greatly upregulate ROS-scavenging capability and expression of heat shock-related genes, for an increasing adaptive response to heat stress.

Acknowledgements

This work is supported by the Science and Technology Project of Education Department of Jiangxi Province (Project Numbers: GJJ190868); the Natural Science Foundation of Jiangxi Province (Project Numbers: 20202BABL213046); the Doctorial Scientific Research Fund of Yichun University (Project Numbers: 210-3360119017) for their financial support.

Author contributions

Xiaolong Liu and Ping Ji designed the study; Ping Ji, Qizhou Chen, Fang Lu, Yunyun Yang, Xin Chen, Xiaobo Zhang and Hongtao Yang performed the laboratory experiments; Xiaolong Liu and Ping Ji performed the data collection, statistical analysis and figure mapping; Xiaolong Liu and Ping Ji wrote the manuscript; Xiaolong Liu, Changjie Jiang and Zhengwei Liang participated in the modification of the manuscript; Xiaolong Liu provided scientific expertise.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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772 Statements and Declarations

773 We declare that we have no financial and personal relationships with other people or
774 organizations that can inappropriately influence our work, there is no professional or
775 other personal interest of any nature or kind in any product, service and/or company
776 that could be construed as influencing the position presented in, or the review of the
777 manuscript.
778
779 The authors declare that they have no competing financial interests.
780

Figure legends

Fig. 1 ABA priming rescued rice seedlings from wilting under high temperature stress. Two-week-old rice seedlings were root-drenched with or without 10 μM ABA for 24 h, and then subjected to unstress or high temperature stress conditions. Photographs of seedling growth (**A, B, C, D**) were taken at 24, 48 h, 72 h, and 96 h, respectively. Photograph of leaf wilting (**E**) was taken at 72 h. Withered leaf rate (**F**) and chlorophyll content (**G**) of leaf in rice seedlings were counted after 48, 72, and 96 h. Values are means ± SD, $n=5$. Different letters on the column represent significant difference ($P<0.05$) between different treatment based on Duncan's test.

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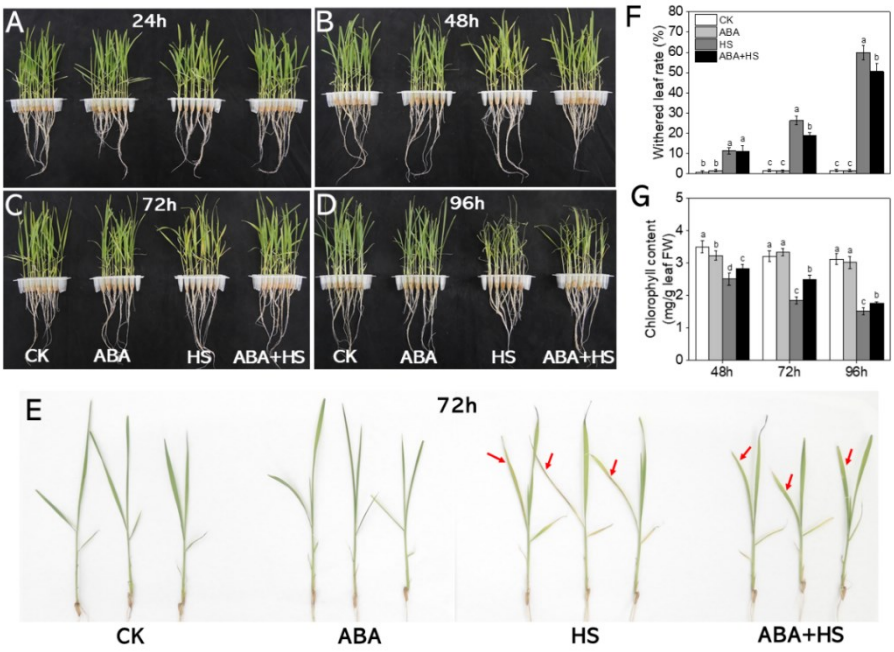
Fig. 8 A schematic mechanism of ABA in response to heat stress in rice seedlings.

Figure 1



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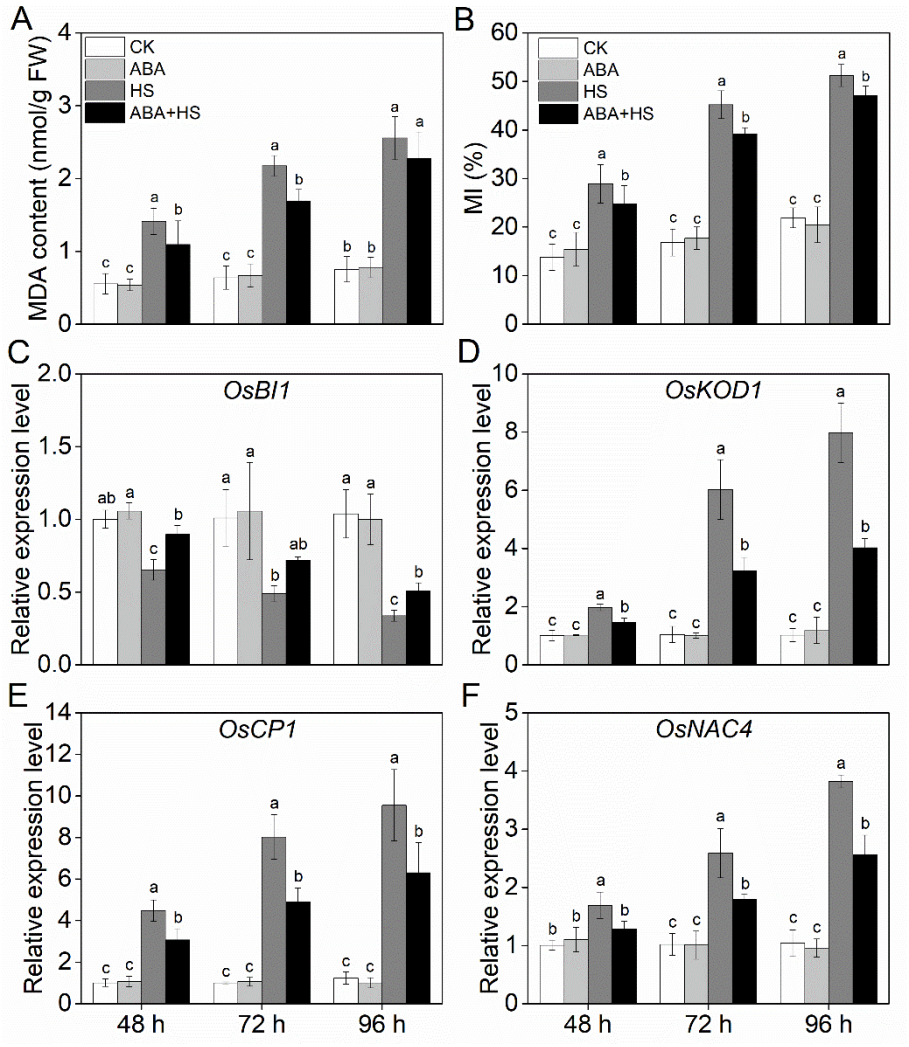

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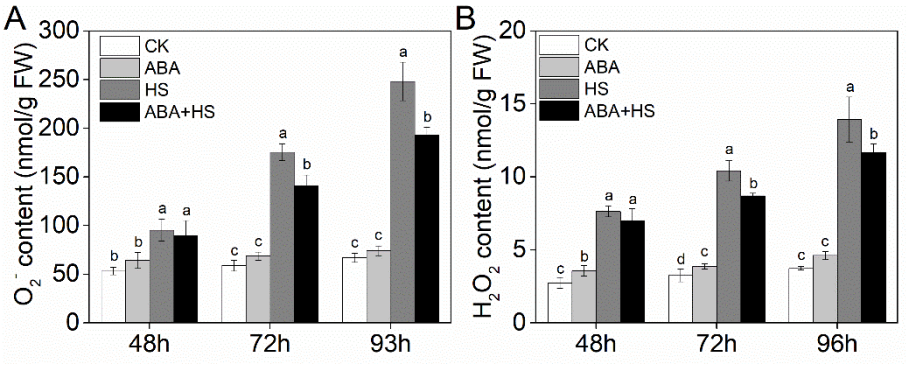

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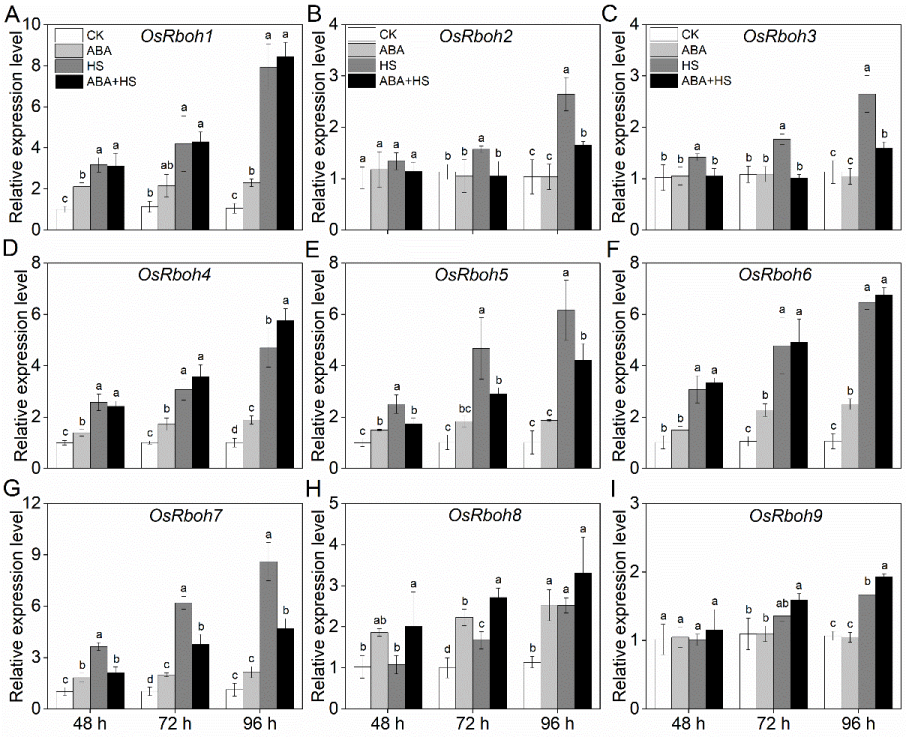

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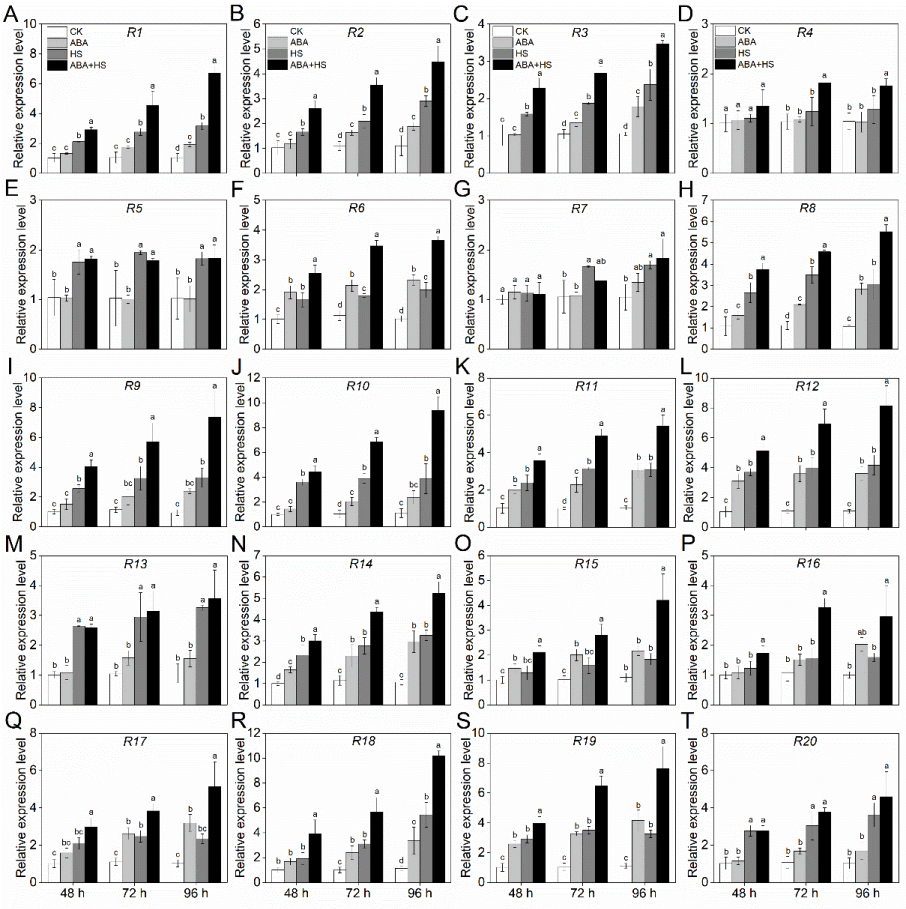

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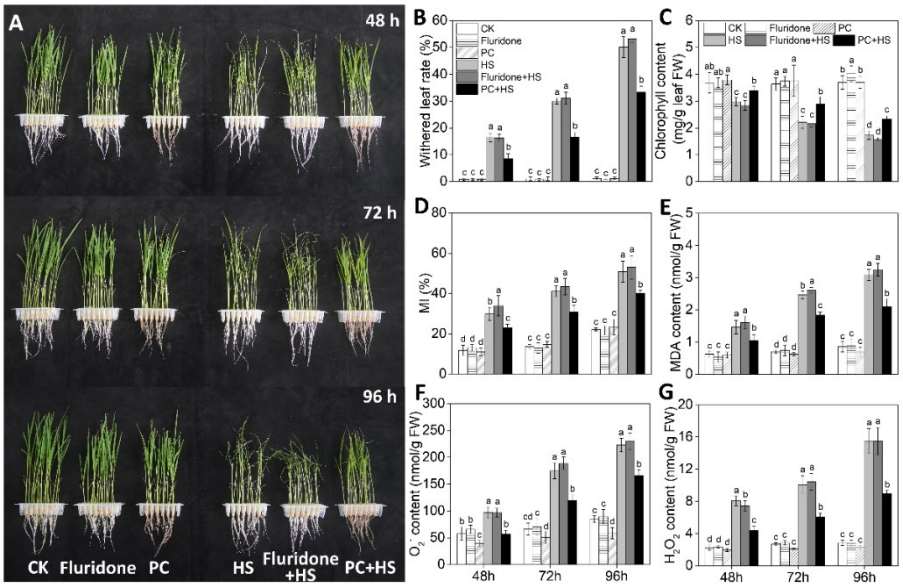

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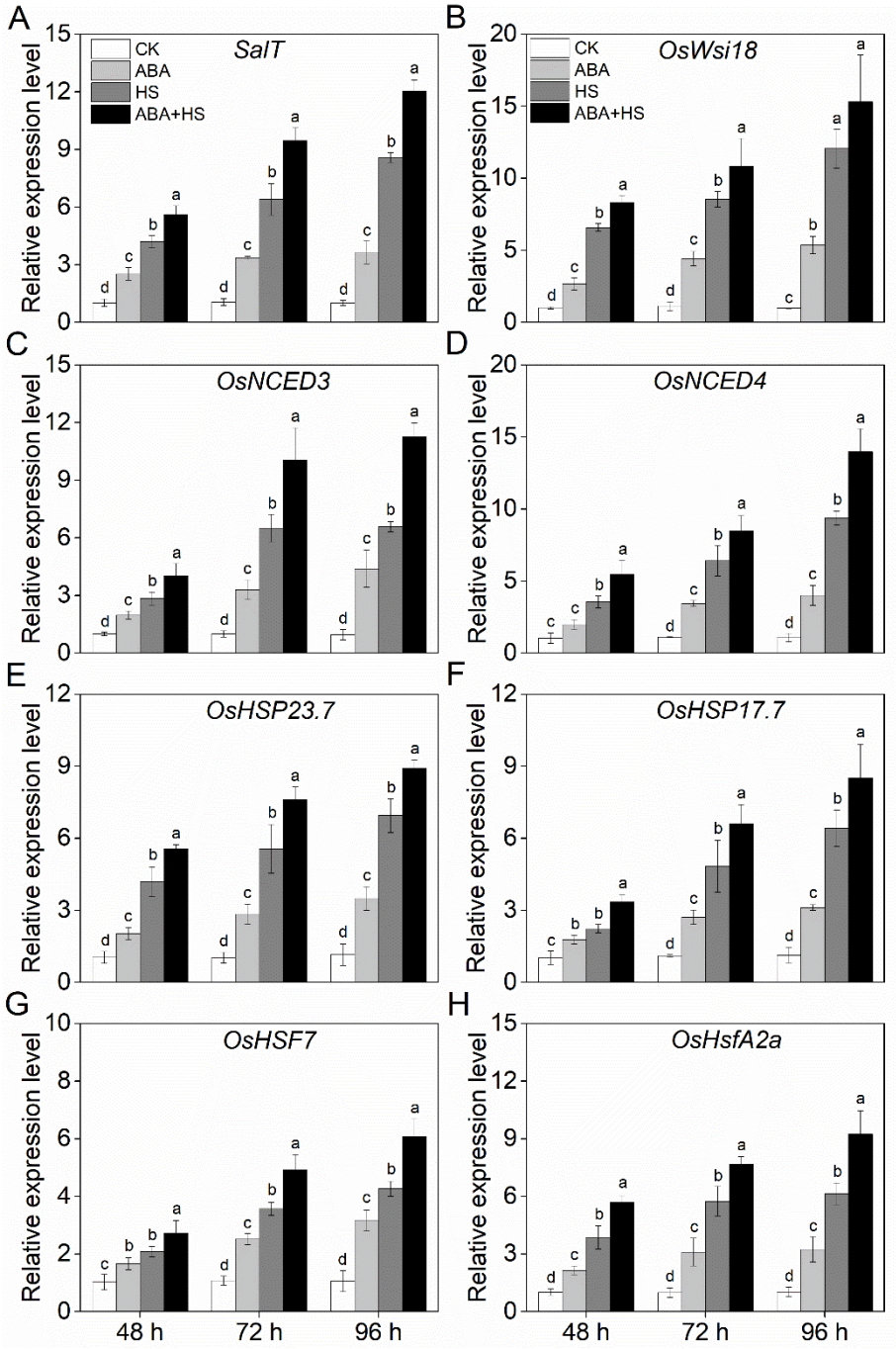

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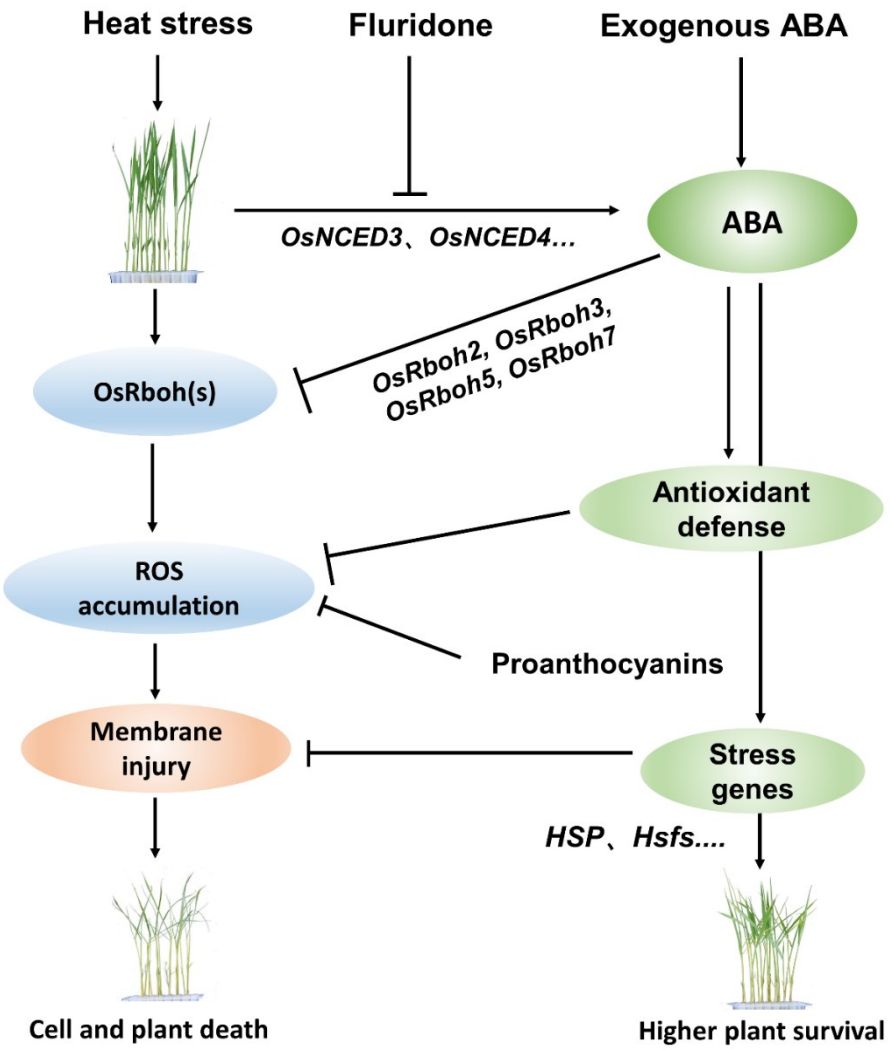

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Supplementary Files

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- SupplementalMaterials.pdf