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Abscisic acid, ethylene and antioxidative systems in rice grains in relation with grain filling subjected to postanthesis soil-drying

Hao Zhang · Kai Liu · Zhiqin Wang ·
Lijun Liu · Jianchang Yang

Abstract This study tested the hypothesis that abscisic acid (ABA) and ethylene may mediate the effect of post-anthesis soil drying on grain filling of rice (*Oryza sativa* L.) through regulating its anti-oxidative systems. Two rice cultivars were pot-grown and exposed, from 9 days post anthesis until maturity, to three soil drying treatments: well-watered (WW), moderate drying (MD), and severe drying (SD). The MD treatment, compared with the WW treatment, increased grain filling rate and the activities of CAT, POD, and SOD in superior and inferior spikelets, whereas the SD treatment decreased them. Both MD and SD treatments enhanced the grain contents of ABA, superoxide anion, and MDA, with a higher rise in the SD than in the MD. The MD treatment decreased ethylene evolution rate and 1-aminocycopropane-1-carboxylic acid (ACC) content, whereas the SD treatment increased them. The ratio of ABA–ACC (ABA/ACC) was the highest under the MD and the lowest under the SD treatment. There was a significant positive correlation between the ABA content, the activities of CAT, POD, SOD, and ABA/ACC with the grain filling rate, while the levels of ethylene and ACC and the contents of superoxide anion and MDA correlated negatively with the parameter. Exogenous chemical application verified the roles of ABA and ethylene in grain filling. The results suggest that an increase in ABA and decrease in ethylene in the grains under post-anthesis moderate soil drying could enhance the anti-oxidative enzyme activities, thus reducing free radicals in grains and accelerating grain filling rate of rice.

Abbreviations

ABA	Abscisic acid
AES	Anti-oxidative enzymes system
ACC	1-Aminocycopropane-1-carboxylic acid
CAT	Catalase
MD	Moderate soil-drying
DPA	Days post anthesis
O_2^-	Superoxide anion
POD	Peroxidase
SD	Severe soil-drying
ROS	Reactive oxygen species
SOD	Superoxide dismutase
WW	Well-watered

H. Zhang (✉) · K. Liu · Z. Wang · L. Liu · J. Yang
Jiangsu Key Laboratory of Crop Genetics and Physiology/Co-Innovation Center for Modern Production Technology of Grain Crops, Yangzhou University, Yangzhou, China
e-mail: haozhang@yzu.edu.cn

Introduction

Grain filling is the final growth stage in cereals when fertilized ovaries develop into caryopses. The filling degree and rate of rice (*Oryza sativa* L.) spikelets differ largely with their positions on a panicle. In general, the earlier-flowering superior spikelets, usually located on apical primary branches, fill fast and produce larger and heavier grains. Conversely, the later-flowering inferior spikelets, commonly located on proximal secondary branches, are either sterile or fill slowly and poorly (Mohapatra et al.

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1993; Yang et al. 2000a). There are many explanations for the physiological mechanism differences between the superior and inferior spikelets attributed mainly to the assimilate supply (Murty and Murty 1982; Fu et al. 2011), hormone balance (Naik and Mohapatra, 1999; Yang et al. 2006a; Zhang et al. 2009), enzyme activities (Nakamura et al. 1989; Kato et al. 2007), and gene expressions (Dai et al. 2009; Jiang et al. 2003; Ishimaru et al. 2003). Grain filling is generally believed to be a process, regulated either genetically or environmentally (Yoshida 1972; Saini and Westgate 2000). Water or drought stress is one of the most important factors limiting crop yields worldwide (Passioura 1996, 2007; Tian et al. 2013). Water stress during the early grain development curtails the kernel sink potential by reducing the number of endosperm cells and amyloplasts formed, thus diminishing grain weight as a result of the decrease in the capacity of endosperm to accumulate starch, both in rate and duration (Bouman and Tuong 2001; Samejima et al. 2004). However, there are reports showing that a moderate soil drying during the grain filling stage could markedly enhance grain growth in both rice and wheat (*Triticum aestivum*), and consequently rise grain weight, especially in the inferior spikelets (Yang and Zhang 2006; Zhang et al. 2012; Chen et al. 2013). The mechanism underlying the decrease or increase in grain filling under soil drying is yet to be well understood.

Abscisic acid (ABA) and ethylene are the two major phytohormones generally suggested to be involved in linking plant responses to stress (Cheng and Lur 1996; Santner and Estelle 2009). A significant ABA accumulation was observed in aborted maize (*Zea mays* L.) kernels under drought stress (Ober et al. 1991), and a reduction in the grain set and kernel weight under water stress in wheat was associated with the elevated levels of ABA (Morgan 1980; Ahmadi and Baker 1999). However, there are reports that the inferior spikelets have a lower ABA content than superior spikelets at the early grain filling stage, and the cell division and grain filling rates are significantly positively correlated with ABA contents in grains in both wheat and rice (Yang and Zhang 2006; Yang et al. 2006a). A high ethylene evolution rate, induced by drought, has frequently been related to the abortion in cotton (*Gossypium hirsutum* L.) and maize (Guinn 1976; Cheng and Lur 1996) and the reduction in wheat grain weight (Beltrano et al. 1999).

It has been reported that many adversities could induce membrane damage, lipid permeability increase, and biological accumulation of free radicals, such as malondialdehyde (MDA), one of the lipid peroxidation products (Rajinder et al. 1981; Huang et al. 2013). Moreover, drought, like other abiotic stress factors, results in oxidative stress through an increase in the reactive oxygen species (ROS), such as the superoxide radical (O_2^-). ROS could destroy the normal cellular metabolism through the oxidative damage to lipids, proteins, and nucleic acids, and cause growth impairment in plants. To eliminate these ROS, plants have developed a complex anti-oxidative enzymes system (AES), including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) (Nunez et al. 2003). It has been reported that the drought damage degree was negatively correlated with CAT, POD, and SOD activities, but corresponded positively with MDA accumulation (Liu et al. 2011). Although the AES and ROS in rice under water stress are well documented, little is known about the relationships of ABA, ethylene, AES, and ROS with the grain filling of the superior and inferior spikelets of rice under water stress.

The objective of this study was to test the hypothesis that the interaction between ABA and ethylene is involved in grain filling through mediating AES and ROS. The changes of ABA, ethylene, AES, and ROS levels in the superior and inferior spikelets of rice, subjected to post-anthesis soil drying and their relationships with grain filling, were investigated. The effects of the chemical regulators in the grains were also studied to verify the role of the two hormones, ABA and ethylene.

Materials and methods

Plant materials and growth conditions

The experiment was conducted at a farm of Yangzhou University, Yangzhou, China (32° 30′N, 119° 25′E) during the rice growing season (May–October) of 2010, and repeated in 2011. Two rice cultivars, Wuyunjing 8 (*japonica*) and Yangdao 6 (*indica*) were grown in pots. The seeds were sown in the paddy field on 8–10 May. Thirty-day-old seedlings were then transplanted to porcelain pots. Each porcelain pot (30 cm in height and 25 cm in diameter, 14.72 l in volume) was filled with 20 kg of sandy loam soil (Typic fluvaquents, Entisols, US classification) that contained 2.42 g kg^{-1} organic matter and 110 mg kg^{-1} alkali-hydrolysable N, 34.6 mg kg^{-1} Olsen-P, and 66.6 mg kg^{-1} exchangeable K. Each pot was planted with three hills with two seedlings per hill. On the day of transplanting (8–10 June), 1.0 g N as urea, 0.3 g P as single superphosphate, and 0.5 g K as KCl were mixed into the soil in each pot. N as urea was also applied at mid-tillering (0.5 g per pot) and panicle initiation (0.8 g per pot) stages. Both cultivars headed on 24–26 August (50 % of plants), flowered on 26–28 August, and were harvested on 19–20 October. The water level in the pot was kept at 1–2 cm until 9 days post anthesis (DPA) when soil drying treatments were initiated. The total precipitation during the growing season was 496.4 mm, 67.2 % of which was in June and July. The mean solar radiation was

17.8 MJ m^{-2} day^{-1}. The temperatures, averaged over the 10-day period from anthesis to harvest, were 27.8, 27.2, 24.4, 21.6, 21.1, and 20.2 °C, respectively.

Soil drying treatments

The experiment was a 2 × 3 (two cultivars and three levels of soil moisture) factorial design with six treatments. Each treatment consisted of 60 pots as replicates. From 9 DPA until maturity, three levels of soil water (ψ_{soil}) potentials were imposed by controlling the water application. The well-watered (WW) treatment was flooded with 1–2 cm water depth in the pot ($\psi_{soil} = 0$ kPa) by manually applying tap water; the moderate soil drying (MD) treatment was maintained at -20 ± 5 kPa; the severe soil drying (SD) treatment was maintained at -40 ± 5 kPa. The soil water potentials in the soil drying treatments were monitored in the 15–20 cm soil depth. A tension meter (Soil Science Research Institute, Nanjing, China) consisting of a sensor of 5 cm length was installed in each pot to monitor the water potential. The tension meter readings were recorded every 3 h from 0600 to 1800 h. When the readings dropped to the desired value, 0.4 and 0.2 l of tap water per pot was added to the MD and SD treatments, respectively. The pots were placed in a field and sheltered from rain by a removable polyethylene shelter, which was placed over them during rain.

Sampling

Six hundred panicles that headed on the same day were chosen and tagged from each treatment. Thirty tagged panicles from each treatment were sampled at 3 days intervals from anthesis to maturity. The sampled panicles were divided into three groups (10 panicles each) as subsamples. Both superior and inferior grains that developed from the fertilized spikelets were removed. One half of the sampled grains were used for measurements of ABA, 1-aminocyclopropane-1-carboxylic acid (ACC), O_2^-, MDA contents, and the activities of the anti-oxidative enzymes. Sixty to eighty sampled grains were used for the measurement of ethylene production. About 80-100 grains were dried at 70 °C to constant weight, dehulled, and weighed. The superior spikelets that flowered on the first 2 days (located on apical primary branches) within a panicle and the inferior ones (located on the proximal secondary branches) that flowered on the last 2 days within a panicle were separated from the sampled panicles. The difference in the flowering date between the superior and inferior spikelets within a panicle was 3–4 days. The grain filling processes of both superior and inferior spikelets were fitted by the Richards's growth equation (Richards 1959), as described by Zhu et al. (1988):

$$W = \frac{A}{(1 + Be^{-kt})^{1/N}} \qquad (1)$$

Grain filling rate (R) was calculated as the derivative of the Eq. (1)

$$R = \frac{AkBe^{-kt}}{N(1 + Be^{-kt})^{(N+1)/N}} \qquad (2)$$

where W is the grain weight, A is the maximum grain weight, t is the time after anthesis (d), and B, k, and N are coefficients determined by regression. The active grain filling period was defined as that the time interval when W was from 5 % (t_1) to 95 % (t_2) of A. The average grain filling rate during this period was therefore calculated from t_1 to t_2.

ABA extraction, purification, and quantification

The methods for extraction and purification of ABA were modified from those described by Bollmark et al. (1988) and He (1993). Samples of 0.8–1.0 g spikelets were ground in a mortar (at 0 °C) in 10 ml 80 % (v/v) methanol extraction medium containing 1 mM butylated hydroxytoluene as an antioxidant. The extract was incubated at 4 °C for 4 h and centrifuged at 4,800×g for 15 min at the same temperature. The supernatants were passed through Chromosep C$_{18}$ columns (C$_{18}$ Sep-Pak Cartridge, Waters Corp, Millford, MA, USA), prewashed with 10 ml 100 % and 5 ml 80 % methanol, respectively. The hormone fractions were dried under N$_2$ and dissolved in 2 ml phosphate buffered saline (PBS) containing 0.1 % (v/v) Tween 20 and 0.1 % (w/v) gelatin (pH 7.5) for analysis by enzyme-linked immunosorbent assay (ELISA).

The mouse monoclonal antigen and antibody against ABA and immunoglobulin G-horse radish peroxidase (IgG-HRP), used in ELISA, were produced at the Phytohormones Research Institute, China Agricultural University, China (see He 1993). The methods for quantification of ABA by ELISA and the recovery test were described previously (Yang et al. 2001b). The recovery percentage of ABA in spikelets was 85.0 ± 4.9. The specificity of the monoclonal antibody and other possible nonspecific immunoreactive interferences were checked previously and proved reliable (Xie et al. 2003).

Ethylene and ACC determination

The ethylene that evolved from spikelets was determined according to Beltrano et al. (1994) with modifications. Briefly, the sampled spikelets or sheaths were placed between two sheets of moist paper for 1 h at 27 °C in darkness to allow wound ethylene to subside. Each sample contained 0.5–0.8 g spikelets or 1–2 g sheaths. Spikelet/

sheaths were then transferred into 10 ml glass vials containing moist filter paper and immediately sealed with airtight stoppers and incubated in the dark for 24 h at 27 °C. A 1 ml gas sample was withdrawn through the airtight stoppers with a gas-tight syringe, and ethylene was assayed by a gas chromatograph (HP5890 Series II, Hewlett Packard, Palo Alto, CA, USA) equipped with a Porapak Q column (0.3 cm × 200 cm, 50–80 mesh) and flame ionization detector (FID). The temperatures of the injection port, column, and detector were kept constant at 140, 100, and 200 °C, respectively. Nitrogen was used as the carrier gas at a flow rate of 30 ml min^{-1}, and hydrogen and air were utilized for FID at the rate of 30 and 300 ml min^{-1}, respectively.

To examine the time-course changes in ethylene production by the spikelets after sampling, the spikelets sampled at 12 day before heading were incubated for 6, 12, 18, 24, and 30 h, respectively, for ethylene measurement through the same method described above.

ACC in the spikelets and sheaths was determined according to the method suggested by Cheng and Lur (1996). The ethylene evolved from ACC was assayed by using gas chromatography, as described above. The transformation rate as a percentage from ACC to ethylene was 90.3 ± 5.42 for spikelets and 88.5 ± 6.4 for sheaths, on average. ABA and ACC concentrations and the rate of ethylene evolution were expressed on a dry weight basis.

Measurements of CAT, POD, and SOD activities

CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm for 3 min (Aebi 1984). POD activity was assayed by the method described by Cakmak and Marschner (1992). SOD activity was determined through measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT), according to the method of Giannopolitis and Ries (1977). The protein content was determined according to the method of Bradford (1976).

Production rate of O_2^- and MDA content determination

The production rate of O_2^- was determined according to Elstner and Heupel (1976) by monitoring the nitrite formation from hydroxylamine in the presence of O_2^-. The absorbance was read at 530 nm. MDA content was assayed via the thiobarbituric acid (TBA) reaction (Heath and Packer 1968).

Chemical applications

The cultivar Yangdao 6 was used for the chemical application. The experimental plants were grown in porcelain

pots under the same conditions as described above. From 9 DPA till maturity, either WW or SD treatments were imposed to the plants. The treatment details, water control, and rain prevention were described previously. Synthetic ABA, ethephon (an ethylene-releasing agent), cobaltous nitrate [$Co(NO_3)_2$, an inhibitor of ethylene synthesis] (all from Sigma, St Louis, MO, USA), and fluridone (Fluka, Riedel-de Haën, Germany), an inhibitor of ABA synthesis, were applied to the plants for both WW and SD treatments. The preparation of the chemical solutions was described elsewhere (Ober and Sharp 1994; Cheng and Lur 1996; Sharp et al. 2000). Starting at 9 DPA, either 20×10^{-6} M ABA, or 20×10^{-6} M fluridone, or 50×10^{-3} M ethephon, or 5×10^{-5} M $Co(NO_3)_2$, were sprayed at the rate of 100 ml per pot on the top of the plants (panicles) daily, for 5 day. The time of application of the exogenous hormones was from 16.00 to 18.00 h. The relative humidity (%RH) during the every day spraying, was 68.6, 73.9, 73.2, 61.9, and 65.9 %, respectively. All the solutions contained ethanol and Tween 20 at final concentrations of 0.1 % (v/v) and 0.01 % (v/v), respectively. The control plants were sprayed with the same volume of deionized water, containing the same concentrations of ethanol and Tween 20. Each chemical treatment had 20 pots. The concentrations of ABA, ethylene evolution rate, and CAT, POD, and SOD activities as well as the O_2^- production rate and MDA contents in the grains were determined 3 and 7 day after the chemical treatments (16 and 20 DPA). Twenty panicles for each treatment were harvested at maturity for the determination of yield and its components.

Statistical analysis

The results were analyzed for variance by using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA). The data from each sampling date were analyzed separately and the means were tested by the least significant difference at the $P_{0.05}$ level ($LSD_{0.05}$). Linear regression was used to evaluate the relationships of grain filling rate, levels of ABA, ethylene, ACC, and the ratio of ABA to ACC with AES and ROS. The differences in data across the two study years and in the interaction between the year and cultivar were not significant ($F < 1$). Therefore, the data from both years were averaged.

Results

Grain weight and grain filling rate

The grain weight and filling rate varied with the grain type and soil drying treatment (Fig. 1). The grain filling

Fig. 1 Effect of soil drying on grain weight (**a**, **c**) and grain filling rate (**b**, **d**) of the superior and inferior spikelets of rice. Both *japonica* cultivar Wuyunjing 8 (**a**, **b**) and *indica* cultivar Yangdao 6 (**c**, **d**) were grown in pots. WW, MD, and SD indicate well-watered, moderate soil drying, and severe soil drying, respectively, during the grain filling period (from 9 DPA until maturity). S and I represent the superior grain and inferior grain, respectively. *Vertical bars* represent ± SE of the mean (n = 6) where these exceed the size of the symbol
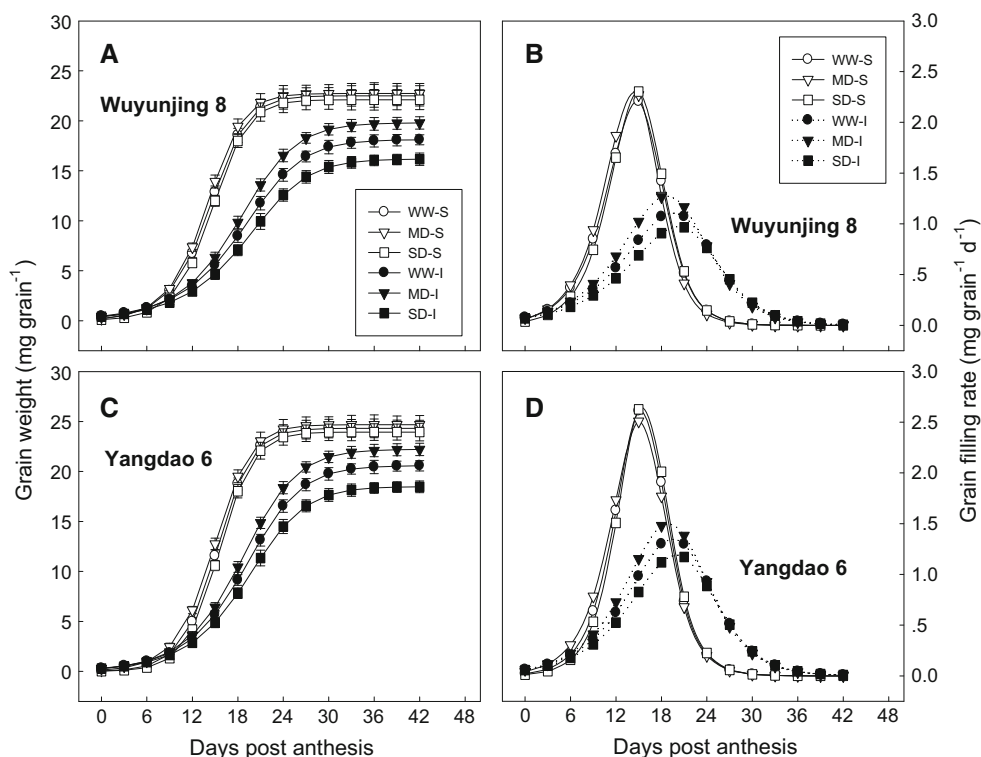
procession of the superior spikelets showed no significant difference among the three soil drying treatments. However, the grain weight and filling rate of the inferior spikelets markedly increased in the MD treatment, whereas the values of these indices significantly decreased in the SD treatment, when compared with the WW treatment. There was no difference in these parameters caused by the cultivars.

Activities of CAT, SOD and POD in grains

The activity levels of CAT, SOD, and POD were increased continuously after the anthesis, reached a maximum, and declined thereafter (Fig. 2). Very similar to the grain filling rate, the highest value of CAT, POD, and SOD activities appeared at the maximum grain filling rate stage. At the early grain filling stage, these activities were with lower magnitude in the inferior than in the superior spikelets, whereas the results were reversed at the middle and late grain filling stages. The activities of the enzymes reaching a maximum appeared later and were affected by the soil drying treatments to a higher degree in the inferior spikelets than in the superior ones (Fig. 2). The MD treatment stimulated the actions of the three enzymes. Under the SD treatment, the enzymatic operations were significantly increased at the early stage of the treatment and decreased at its late stage when compared with those under the WW (Fig. 2).

Production rate of O_2^- and MDA content in grains

The production rate of O_2^- showed no considerable difference between the superior and inferior spikelets at the early grain filling stage, and at the end of grain filling it was greater in the superior than in the inferior ones (Fig. 3). There was no substantial variation in the production rate of O_2^- between WW and MD. The SD treatment significantly increased the production rate of O_2^- in the spikelets. The effect of soil drying on O_2^- production was more pronounced in the inferior than in the superior spikelets (Fig. 3a, b). The tendency of MDA content was very similar to that of the production rate of O_2^- (Fig. 3c, d).

Levels of ABA, ethylene, ACC, and ABA/ACC in grains

The ABA concentration in both the superior and inferior spikelets was exceedingly low at the early grain filling stage, but increased quickly and reached a peak at 12 DPA in the superior spikelets, and at 18 DPA in the inferior spikelets, and then sharply decreased (Fig. 4a, b). At the early and middle grain filling stage, the superior spikelets had a much higher ABA concentration than the inferior, whereas the trend was reversed at the late grain filling stage. There was no significant difference in the ABA content of the superior spikelets among the three soil moisture treatments. However, both the MD and SD treatments markedly enhanced the ABA
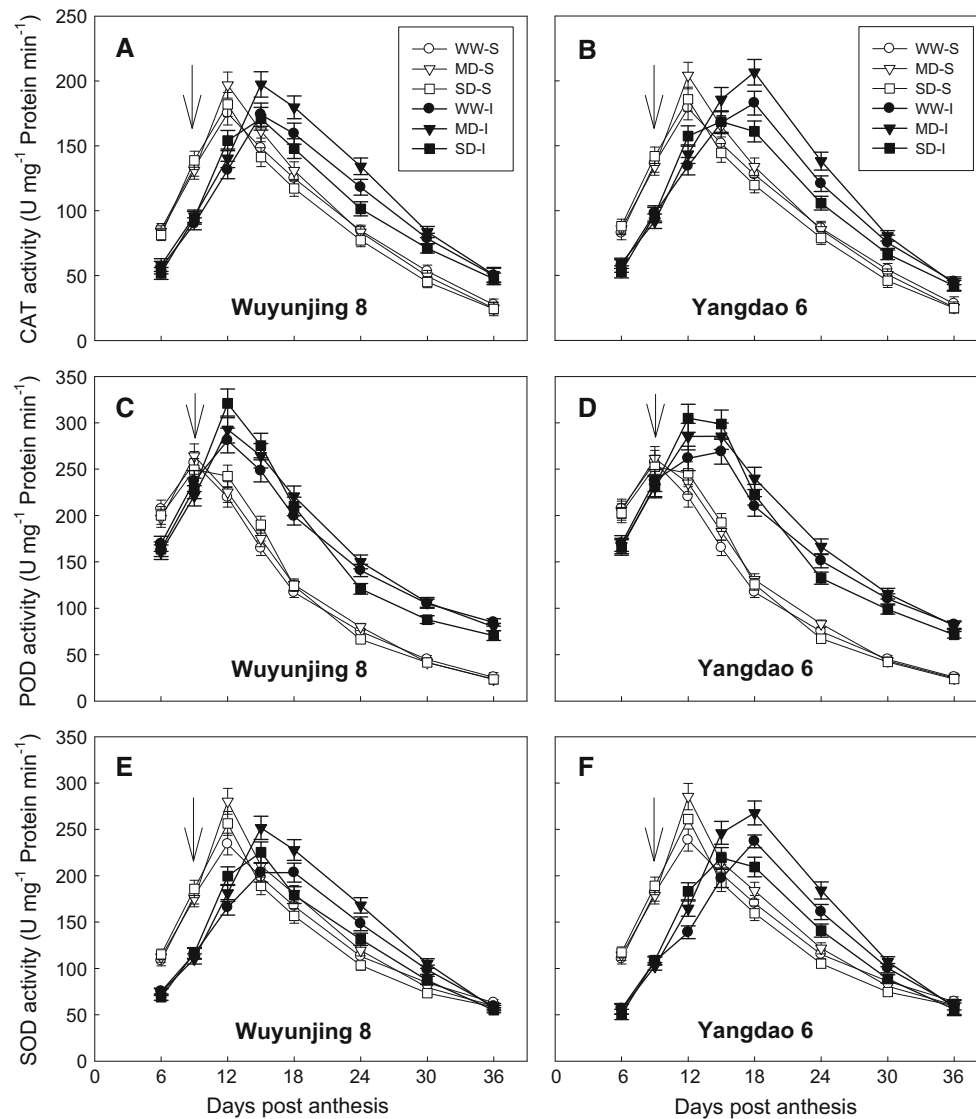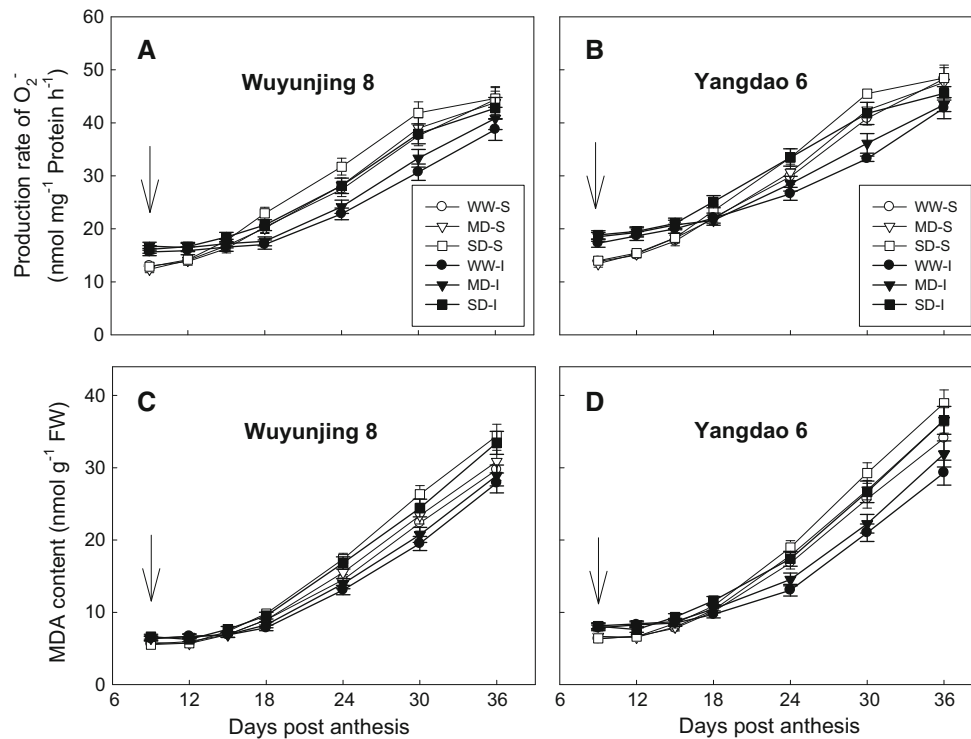
Fig. 2 Effect of soil drying on CAT (**a**, **b**), POD (**c**, **d**), and SOD (**e**, **f**) activity in the superior and inferior spikelets of rice. Both *japonica* cultivar Wuyunjing 8 (**a**, **c**, **e**) and *indica* cultivar Yangdao 6 (**b**, **d**, **f**) were grown in pots. WW, MD, and SD indicate well-watered, moderate soil drying, and severe soil drying, respectively, during the grain filling period (from 9 DPA until maturity). S and I represent the superior grains and inferior grains, respectively. Arrows indicate the start of the period in which water was withheld. *Vertical bars* represent ± SE of the mean (n = 6) where these exceed the size of the symbol

concentration of the inferior spikelets when compared to WW, and this rise was more apparent in the SD than in the MD spikelets (Fig. 4a, b).

In sharp contrast to the instance of ABA content, the ethylene evolution from both the superior and the inferior spikelets was very high at the early grain filling stage, followed by a slight reduction until 9 DPA; then it declined quickly from 9 DPA until 24 DPA, increased thereafter, and decreased again from 30 DPA (Fig. 4c, d). During the grain filling period, the inferior spikelets showed a much higher ethylene evolution rate than the superior spikelets among the three soil moisture treatments. The ethylene release from both superior and inferior spikelets was

reduced in the MD treatment, whereas it was substantially increased in the SD treatment, A similar change pattern was observed in the ACC concentration in both the superior and the inferior spikelets (Fig. 4e, f). The ACC concentration was significantly correlated with the ethylene evolution rate (r = 0.99**, P < 0.01).

As shown in Fig. 4, the ratio of ABA to ACC in both superior and inferior spikelets was the highest in the MD treatment and the lowest in the SD treatment, suggesting that the increase in the ACC concentration, relative to the increase in ABA concentration, was greater under the SD treatment. The two cultivars have the same changing tendency (Fig. 4g, h).

Fig. 3 Effect of soil drying on production rate of O_2^- (**a**, **b**) and MDA (**c**, **d**) content in the superior and inferior spikelets of rice. Both *japonica* cultivar Wuyunjing 8 (**a**, **c**) and *indica* cultivar Yangdao 6 (**b**, **d**) were grown in pots. WW, MD, and SD indicate well-watered, moderate soil drying, and severe soil drying, respectively, during grain filling (from 9 DPA until maturity). S and I represent the superior grains and inferior grains, respectively. *Arrows* indicate the start of the period in which water was withheld. *Vertical bars* represent ± SE of the mean (n = 6) where these exceed the size of the symbol

Correlations of grain filling rate, levels of ABA, ethylene, ACC, and the ratio of ABA to ACC with the antioxidative enzymes activities, and MDA and O_2^- contents. As shown in Table 1, the ABA content and the ratio ABA to ACC were significantly or very significantly positively correlated with the CAT, POD, and SOD activities, and very significantly negatively correlated with the MDA content and the production rate of O_2^-. The ABA content, ABA/ACC, and the CAT, POD, and SOD activities were correlated very significantly positively with the grain filling rate, while the levels of ethylene and ACC and the contents of superoxide anion and MDA correlated very significantly and negatively with the grain filling intensity.

Effects of chemical applications

The application of ABA or $Co(NO_3)_2$, an inhibitor of ethylene synthesis, considerably increased CAT, POD, and SOD activities, whereas it decreased the production rate of O_2^- and the MDA content in the inferior spikelets under both WW and SD treatments at 16 and 20 DPA. Conversely, the application of ethephon, an ethylene-releasing substance, or fluridone, an inhibitor of ABA biosynthesis, showed an opposite effect. There were no significant differences in the CAT, POD, and SOD activities, and the

production rate of O_2^- and MDA content of the superior spikelets when chemicals were applied to panicles under both WW and SD treatments at 16 and 20 DPA (Supplementary Table 1). When fluridone was applied, the ABA concentration of the superior and inferior spikelets was significantly reduced under both WW and SD treatments. The application of ABA produced the opposite effects in the inferior spikelets under both WW and SD treatments. No influence was observed on ABA contents in the superior spikelets when ABA was employed in the panicles under the SD treatment. When $Co(NO_3)_2$ was utilized, the ethylene evolution rate was substantially diminished. The application of ethephon to WW spikes increased the ethylene evolution level (Supplementary Table 1).

Under the WW and SD treatment, when $Co(NO_3)_2$ or ABA was applied to the panicles, the quantity of the filled grains and grain weight were enhanced, and the grain yield was augmented. The opposite results were observed when ethephon or fluridone was used (Supplementary Table 2).

Discussion

Previous studies have described that soil drying during grain filling decreased grain filling rate, shortened the grain
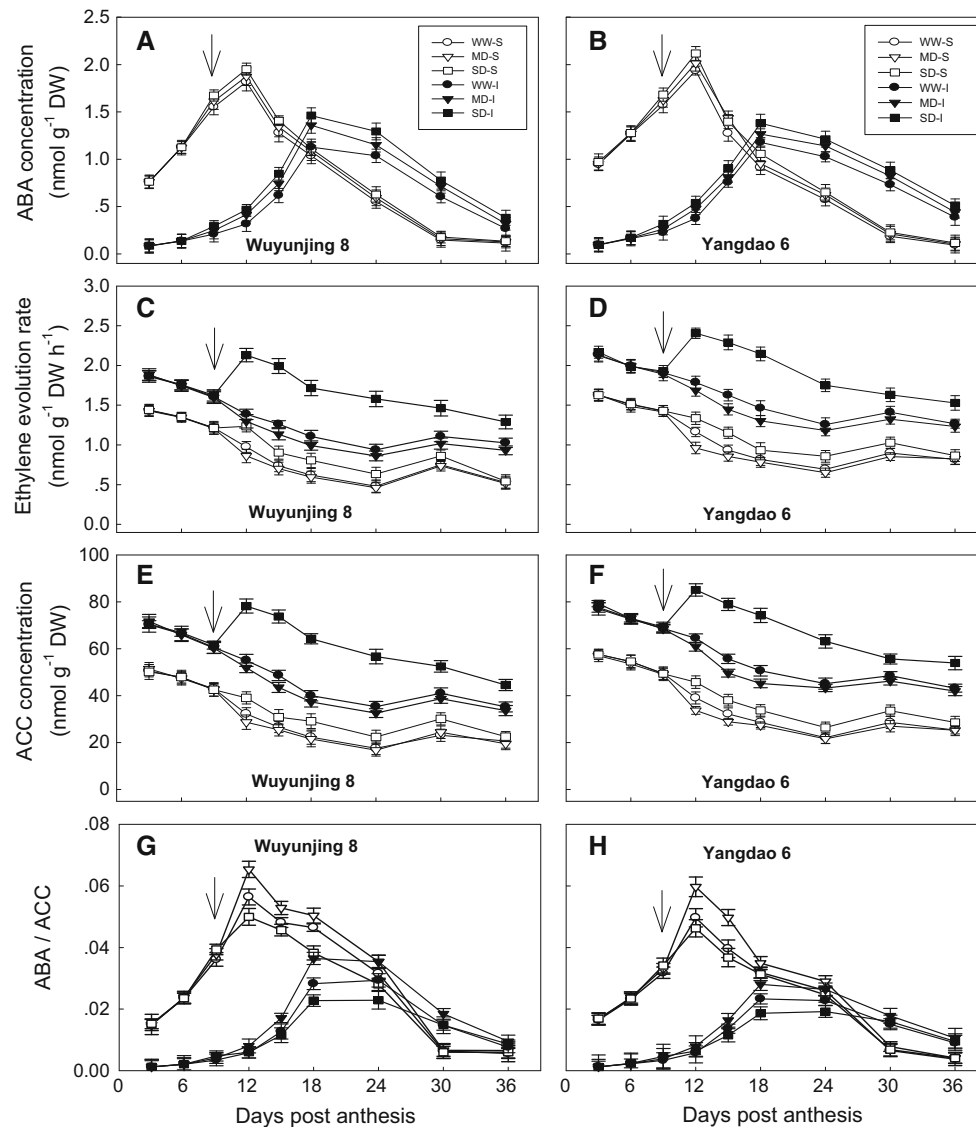
Fig. 4 Effect of soil drying on ABA concentration (**a**, **b**), ethylene evolution rate (**c**, **d**), ACC concentration (**e**, **f**) and the ratio of ABA to ACC (**g**, **h**) in the superior and inferior spikelets of rice. Both *japonica* cultivar Wuyunjing 8 (**a**, **c**, **e**, **g**) and *indica* cultivar Yangdao 6 (**b**, **d**, **f**, **h**) were grown in pots. WW, MD, and SD indicate well-watered, moderate soil drying, and severe soil drying, respectively, during the grain filling period (from 9 DPA until maturity). S and I represent the superior grains and inferior grains, respectively. *Arrows* indicate the start of the period in which water was withheld. *Vertical bars* represent ± SE of the mean (n = 6) where these exceed the size of the symbol

filling stage, and thereafter reduced the number of filled grains and their weight (Rahman and Yoshida 1985; Wardlaw and Willenbrink 2000). Our study also showed similar results in SD (Fig. 1). We observed, however, that MD increased the grain weight and filling rate, especially in the inferior spikelets, when compared to WW. That indicates that if a water deficit was controlled properly during grain filling, similarly to the conditions in the MD treatment, the moderate soil drying maintained at −20 ± 5 kPa after the fertilization sensitive period, it would not seriously affect plant status and could enhance

assimilates remobilization from vegetative tissues to grains, especially to those in the inferior spikelets, thus accelerating grain filling and improving grain weight (Fig. 1). This result is consistent with our previous observations on rice (Zhang et al. 2010, 2012). Yang et al. (2000b, 2001a, 2006b) also reported that moderate soil drying increased the sucrose phosphate synthase and α-/β-amylase activity in stems and sheaths, enhanced the grain sink activity, thus promoted the operation of carbohydrates in stem and sheath to grain. At the same time, the moderate soil drying also improved the key enzymes activity in the

Table 1 Correlations of grain filling rate, levels of ABA, ethylene, ACC, and the ratio of ABA to ACC with CAT, POD and SOD activities, and MDA and O_2^- contents in the rice spikelets

Membrane lipid peroxidation/grain filling rate	Grain filling rate	ABA	Ethylene	ACC	ABA/ACC
CAT	0.768**	0.725**	0.153	0.147	0.626**
POD	0.400**	0.457**	0.613**	0.624**	0.202*
SOD	0.789**	0.767**	0.039	0.026	0.692**
MDA	−0.651**	−0.603**	−0.318**	−0.347**	−0.517**
O_2^-	−0.650**	−0.634**	−0.316**	−0.346**	−0.537**
Grain filling rate	–	0.688**	−0.229*	−0.239*	0.764**

Both *japonica* cultivar Wuyunjing 8 and *indica* cultivar Yangdao 6 were grown in pots. Data are from Fig. 1, 2, 3 and 4. The asterisks (** and *) represent the statistical significance at the $P = 0.01$ and $P = 0.05$ level, respectively

sucrose-to-starch pathway in grains, favored the grain filling rate, thus augmented the quantity of filled grains and enhanced grain weight. This suggests that the gain from the accelerated grain filling rate could outweigh the possible loss of photosynthesis as a result of a shortened grain filling period when subjected to water stress during grain filling.

Hormones play important roles in the adaptation of plant growth and development to water stress (Sharp et al. 2004). We established that both MD and SD treatments markedly increased the ABA concentration of the inferior spikelets when compared to WW, with a higher elevation under the SD than under the MD. There was no significant difference in the ABA concentration of the superior spikelets among the three soil moisture treatments (Fig. 4a, b). Numerous observations exist that a reduction in grain set and kernel growth in wheat (Morgan 1980; Ahmadi and Baker 1999), and a decreased rate of endosperm cell division rate in maize (Ober et al. 1991) under soil drying are associated with elevated levels of ABA. However, our results showed that the change in ABA concentration in the grains followed a pattern similar to that of the grain filling rate (Figs. 1b, d, 4a, b). The latter was very significantly positively correlated with the ABA concentration (Table 1). Under the MD treatment, an enhanced ABA accumulation in the grains was closely associated with an increased grain filling rate. The application of ABA to WW panicles considerably fostered ABA concentration at 16 and 20 DPA, whereas the application of fluridone, an indirect inhibitor of ABA synthesis, had the opposite effect (Supplementary Table 1, 2). These effects indicate that the slow grain filling may be attributed to a low ABA concentration in the grains, and the higher ABA content enhances grain filling rate.

Ethylene is an endogenous plant growth regulator mediating many physiological processes, such as cell division, embryogenesis, fruit set and growth, senescence, and responses to environmental stress factors (Davies 2004; Yang et al. 2006a, b). Our results showed that MD reduced the ethylene evolution from both superior and

inferior spikelets, whereas the SD treatment stimulated it significantly (Fig. 4c, d). We speculate that the production of ethylene in water-stressed rice grain may depend on the severity and duration of soil drying. A very similar change pattern was observed in the ACC concentration in both superior and inferior spikelets (Fig. 4e, f). Our data demonstrated that the rise in ethylene production is attributed to the enhanced level of ACC, a precursor of ethylene. There are reports indicating that higher levels of ethylene and ACC have frequently been related to abortion in maize (Zea mays) (Cheng and Lur 1996) and a reduction in grain weight of wheat (Triticum aestivum) (Beltrano et al. 1994; Yang et al. 2006a) and rice (Yang et al. 2006b). We observed that the inferior spikelets showed a much higher evolution rate of ethylene and ACC concentrations than the superior spikelets under the three soil moisture treatments during grain filling (Fig. 4c–f). There was a significant negative correlation between the levels of ethylene and ACC with the grain filling rate. The application of ethephon to WW panicles substantially increased the ethylene evolution at 16 and 20 DPA, but decreased the grain filling rate, grain weight, and yield, whereas the application of $Co(NO_3)_2$, an inhibitor of the ethylene synthesis, had the opposite effect (Supplementary Table 1, 2).

The facilitating mechanism of ABA and ethylene on grain filling is not completely understood. The interaction between ABA and ethylene in the superior and inferior spikelets of rice has been described and the relationship between this interaction and the rate of grain filling from the assimilates supply (Mohapatra et al. 1993), the enzymatic activity (Kato et al. 2007; Zhang et al. 2012), and the mechanism at a molecular level (Ishimaru et al. 2005; Wang et al. 2008) has been represented. Plant hormones can act either synergistically or antagonistically, and it is the balance between promoting and inhibiting agents that ultimately determines plant growth and development (Davies 2004). Our results demonstrated that the changes in the ratio of ABA to ACC in the grains were also associated with the grain filling rate (Figs. 1b, d, 4g, h). The

rate of grain filling correlated not only with the levels of ABA and ethylene, but also with the ratio of ABA to ACC (Table 1). These findings suggest that antagonistic interactions between ABA and ethylene may mediate and influence grain filling in rice when subjected to soil drying during meiosis.

Previous studies have described that the lipid permeability of the plant membrane increased under adversities, contributing to the biological accumulation of free radicals, such as O_2^- and MDA (Liu et al. 2011). In order to cope with the harsh environment, plants themselves could generate a complex AES, such as CAT, POD, and SOD to counteract and eliminate these hazardous substances. It was reported that under moderate water stress the CAT, POD, and SOD activities in rice seedlings were stimulated constantly over time, while the SOD and POD effects increased under severe water stress conditions (Jiang et al. 1991). Wang et al. (2007) found that the operation level of CAT, POD, and SOD in the flag leaves of rice grew constantly under moderate water stress, and the activities of the three enzymes decreased over time and along with the aggravation of the stress degree. Our results showed that compared to WW, MD increased the production rate of O_2^- and MDA content in spikelets, but there was no significant difference between WW and MD (Fig. 3). At the same time, the MD treatment increased the activities of the three enzymes, mentioned above (Fig. 2), indicating that AES could eliminate the harmful effects caused by the MD treatment. However, AES could not deal with the accumulation of hazardous substances in the SD treatment (Figs. 2, 3). Meanwhile, we noticed that there were no substantial variations in the production rate of O_2^- and MDA content between the superior and inferior spikelets at the early grain filling stage, while they were considerably greater in the superior than in the inferior spikelets at the end of grain filling (Fig. 3). Moreover, the activity of the three enzymes in the superior spikelets was higher than that in the inferior at the early grain filling stage, whereas the results were reversed at the late grain filling stage (Fig. 2). The evidence infers that the effect of the soil drying on the production rate of O_2^-, MDA content, and AES was remarkably dissimilar because of the variations of the soil drying status, the measurement time, and the differences between the inferior and superior spikelets.

Agarwal et al. (2005) proposed that ABA enhanced the CAT and SOD activities, reduced the H_2O_2 and membrane lipid peroxidation contents, and the responses of SOD to ABA was most obvious. Our results showed that the ABA level was very significantly positively correlated with the CAT, POD, and SOD activities, and very significantly, but negatively correlated with MDA content and the production rate of O_2^- (Table 1). When ABA was applied to the spikes at the early grain filling stage (9–13 DPA), the ABA

concentration in the spikelets was elevated or substantially exceeded that of the control (Supplementary Table 1). The CAT, POD, and SOD activities were greatly intensified, while the O_2^- production rate and MDA content were considerably lower in the inferior spikelets, leading to the improvement of grain filling and the promotion of grain weight and yield (Supplementary Table 1, 2). Our data clearly demonstrated that ABA could enhance the effects of the enzymes protecting the membrane from lipid peroxidation. We also observed that after the application of ethephon to the spikes at the early grain filling stage (9-13 DPA), the activities of CAT, POD, and SOD decreased significantly, and the O_2^- production rate and MDA content increased greatly in the inferior spikelets, causing a decline in the grain filling rate and grain weight (Supplementary Table 1, 2). Basing on the above results, we speculated that ethylene could enhance the active oxygen system and stimulate the free radicals production.

It is noteworthy that the ratio of ABA to ACC level was significantly or very significantly positively correlated with the activities of CAT, POD, and SOD, and very significantly negatively correlated with MDA content and the production rate of O_2^-. The grain filling rate was very significantly positively correlated with CAT, POD, and SOD activities, but very significantly negatively correlated with MDA content and production rate of O_2^- (Table 1). The results indicate that ABA could increase CAT, POD, and SOD activities and enhance the ability of the plant to eliminate free radicals, while ethylene could promote the formation of active oxygen system and increase the content of free radicals. The higher ratio ABA to ACC may enhance the endosperm cell physiological activity, restrain or delay the endosperm cell senescence, enhance the grain filling rate, finally leading to an increase in the grain weight.

In conclusion, the effects of post-anthesis soil drying on grain filling were mediated by the balance between ABA and ethylene through regulating anti-oxidative systems. The moderate soil drying during grain filling could enhance ABA and reduce ethylene levels; moreover, the increase in the ratio of ABA to ethylene could stimulate the activities of the anti-oxidative systems and the ability of the plant to counteract the free radical in grains, and consequently accelerate the grain filling during the grain filling stage of rice.

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