Polyamines and ethylene interact in rice grains in response to soil drying during grain filling

Tingting Chen¹, Yunji Xu¹, Jingchao Wang¹, Zhiqin Wang¹, Jianchang Yang¹,* and Jianhua Zhang²,*

¹ Key Laboratory of Crop Genetics and Physiology of Jiangsu Province, College of Agriculture, Yangzhou University, Yangzhou, Jiangsu, China
² School of Life Sciences and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Hong Kong, China

* To whom correspondence should be addressed. E-mail: jcyang@yzu.edu.cn or jhzhang@cuhk.edu.hk

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Abstract

This study tested the hypothesis that the interaction between polyamines and ethylene may mediate the effects of soil drying on grain filling of rice (Oryza sativa L.). Two rice cultivars were pot grown. Three treatments, well-watered, moderate soil drying (MD), and severe soil drying (SD), were imposed from 8 d post-anthesis until maturity. The endosperm cell division rate, grain-filling rate, and grain weight of earlier flowering superior spikelets showed no significant differences among the three treatments. However, those of the later flowering inferior spikelets were significantly increased under MD and significantly reduced under SD when compared with those which were well watered. The two cultivars showed the same tendencies. MD increased the contents of free spermidine (Spd) and free spermine (Spm), the activities of S-adenosyl-L-methionine decarboxylase and Spd synthase, and expression levels of polyamine synthesis genes, and decreased the ethylene evolution rate, the contents of 1-aminocyclopropane-1-carboxylic acid (ACC) and hydrogen peroxide, the activities of ACC synthase, ACC oxidase, and polyamine oxidase, and the expression levels of ethylene synthesis genes in inferior spikelets. SD exhibited the opposite effects. Application of Spd, Spm, or an inhibitor of ethylene synthesis to rice panicles significantly reduced ethylene and ACC levels, but significantly increased Spd and Spm contents, grain-filling rate, and grain weight of inferior spikelets. The results were reversed when ACC or an inhibitor of Spd and Spm synthesis was applied. The results suggest that a potential metabolic interaction between polyamines and ethylene biosynthesis responds to soil drying and mediates the grain filling of inferior spikelets in rice.

Key words: 1-Aminocyclopropane-1-carboxylic acid (ACC), ethylene, grain filling, inferior spikelet, polyamines, rice (Oryza sativa), S-adenosyl-L-methionine decarboxylase, soil drying, superior spikelet.

Introduction

Polyamines (PAs) and ethylene are endogenous plant growth regulators mediating many physiological processes such as cell division, morphogenesis, embryogenesis, fruit set and growth, senescence, and responses to environmental stresses (Alcazar et al., 2006; Yang et al., 2007b, 2008; Jang et al., 2012; Torrigiani et al., 2012). In higher plants, the major PAs are putrescine (Put), spermidine (Spd), and spermine (Spm). Put can be directly synthesized from ornithine via ornithine decarboxylase (ODC; EC 4.1.1.17) or indirectly from arginine via arginine decarboxylase (ADC; EC 4.1.1.19) (Gemperlová et al., 2006). Spd and Spm are synthesized via Spd synthase (EC 2.5.1.16) and Spm synthase (EC 2.5.1.22), respectively, by sequential addition of aminopropyl groups to Put. The aminopropyl groups are generated from S-adenosyl-L-methionine...
expression of PAO genes, and H$_2$O$_2$ content in superior and inferior spikelets (located on proximal secondary branches) which flowered on the first 2 d (located on apical primary branches) within a panicle and those that flowered on the last 2 d within a panicle were separated from the inferior spikelets (which flowered on the last 2 d). The effects of chemical regulators on the levels of PAs and ethylene in the two types of spikelets were observed, and the interaction between the two plant growth regulators affects the grain filling. The purpose of this study was to test the hypothesis that the interaction between PAs and ethylene may be involved in mediating the effects of soil drying on grain filling. The temporal patterns of PAs and ethylene levels, activities of the enzymes involved in PA and ethylene biosynthesis, and expression of genes encoding these enzymes in both superior and inferior spikelets subjected to soil drying during grain filling were investigated, and the effects of chemical regulators on the levels of PAs and ethylene in the two types of spikelets were observed.

In plants, the flavoprotein polyamine oxidase (PAO; EC1.5.3.3) is one of the key enzymes in PA catabolism which oxidizes Spd to $\Delta^1$-pyrroline, 1,3-diminopropane (1,3-DPA) and hydrogen peroxide (H$_2$O$_2$) (Moschou et al., 2008b; Wu et al., 2010). It has been observed that abiotic stress could enhance PAO and increase H$_2$O$_2$ accumulation in tobacco (Nicotiana tabacum L.), leading to programmed cell death (Moschou et al., 2008a). Here, changes in the PAO activity, expression of PAO genes, and H$_2$O$_2$ content in superior and inferior spikelets were also investigated to understand the mechanism by which PAs and ethylene respond to soil drying and regulate grain filling of rice.

### Materials and methods

#### Plant materials and growth conditions

The experiment was conducted at a research farm of Yangzhou University, Jiangsu Province, China (32°30'N, 119°25'E) during the rice-growing season (May–October). Two newly bred japonica 'super' rice (O. sativa L.) cultivars that have numerous spikelets on all panicles and are currently used in local production, Liandao 7 and Huaidao 9, were used. The seeds were sown in the paddy field on 11–12 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 litres in volume) was filled with 20 kg of sandy loam soil [Typic fluvaquents, Entisols (US taxonomy)] that contained 24.2 g kg$^{-1}$ organic matter, 102 mg kg$^{-1}$ alkali-hydrolysable N, 34.5 mg kg$^{-1}$ Olsen-P, and 72.5 mg kg$^{-1}$ exchangeable K. Each pot was planted with three hills, with two seedlings per hill. On the day of transplanting (10–11 June), 1 g of N as urea, 0.3 g of P as single superphosphate, and 0.5 g of 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 29–31 August (50% of plants) and were harvested on 15–16 October. The water level in the pot was kept at 1–2 cm until 8 days post-anthesis (DPA) when soil drying treatments were initiated. The total precipitation during the growing season was 485.5 mm, 69.5% of which was in June and July. The mean solar radiation was 17.6 MJ m$^{-2}$ d$^{-1}$. The temperatures, averaged over a 10 d period from anthesis (30–31 August) to harvest, were 27.1, 24.5, 21.8, 21.3, and 20.4 °C, respectively.

#### Soil-drying treatments

The experiment was a two by three (two cultivars and three levels of soil moisture) factorial design with six treatments. Each treatment had 80 pots as replicates. From 8 DPA till maturity, three levels of soil water potential were imposed by controlling water application: well-watered (WW) treatment was flooded with 1–2 cm water depth in the pot by manually supplying tap water, a moderate soil drying (MD) treatment was maintained at a soil water potential of $-25 \pm 5$ kiloPascal (kPa), and a severe soil drying (SD) treatment was maintained at $-50 \pm 5$ kPa. Soil water potential in the soil drying treatments was monitored in the 15–20 cm soil depth. A tension meter (Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China) consisting of a sensor of 5 cm length was installed in each pot to monitor soil water potential. Tension meter readings were recorded every 3 h from 0600 h to 1800 h. When the readings dropped to the desired value, 0.3 litres and 0.15 litres 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 and determination of endosperm cell division/grain filling rate

Six hundred panicles that headed on the same day were chosen and tagged from 60 pots of each treatment. Thirty tagged panicles from each treatment were sampled at 4 d intervals from anthesis to maturity for the measurement of PA and ethylene levels, their biosynthetic activities, and grain weight. The sampled panicles were divided into three groups (10 panicles each) as subsamples. Fifteen tagged panicles (five panicles formed a subsample) from each treatment were sampled at 2 d intervals from anthesis to 24 DPA for the observation of endosperm cell number. Superior spikelets which flowered on the first 2 d (located on apical primary branches) within a panicle and inferior spikelets (located on proximal secondary branches) which flowered on the last 2 d within a panicle were separated from the sampled panicles. The difference in flowering date between superior and inferior spikelets within a panicle was 3 d for both cultivars. Among one half of the sampled superior or inferior spikelets was...
frozen in liquid nitrogen for 2 min and then stored at −80 °C for determination of the contents of PAs, ACC, and H2O2, the activities of PAO and the enzymes involved in PA and ethylene biosynthesis, and the expression of genes encoding these enzymes. Sixty to eighty sampled grains (spikelets) from each subsample were used for measurement of ethylene production. Another 60–80 sampled grains were dried at 70 °C to constant weight, dehulled, and weighed. Ten to twelve sampled grains, with a small hole cut on the edge of a hull, were fixed in Carnoy’s solution (absolute ethanol:glacial acetic acid:chloroform = 9:3:1, v/v/v) for 48 h, and then kept in 70% (v/v) ethanol pending examination of endosperm cell number. The method for isolation and counting of endosperm cells and the calculation of the total cell number per endosperm was described previously (Yang et al., 2008). The division processes of endosperm cells as well as the processes of grain filling were fitted by the Richards’ (1959) growth equation as described by Zhu et al. (1988):

\[
M(W) = \frac{A}{1 + B e^{-kt}} N
\]

(1)

The endosperm cell division rate or grain-filling rate (G) was calculated as the derivative of Equation 1:

\[
G = \frac{AkBe^{-kt}}{N(1 + Be^{-kt})^{(N-1)/N}}
\]

(2)

where M is the cell number and W is the grain weight, A is the maximum cell number/grain weight, t is the time after anthesis (d), and B, k, and N as defined as that when M or W was from 5% (t1) to 95% (t2) of A. The average cell division/grain-filling rate during this period was therefore calculated from t1 to t2.

**Measurement of leaf water potential**

Leaf water potential of the flag leaves was measured at pre-dawn (0600 h) and midday (1130 h) on 0, 3, 5, 8, 12, 16, 20, 25, and 30 d after withholding water when the sky was clear. Well-illuminated flag leaves were chosen randomly for such measurements. A pressure chamber (Model 3000, Soil Moisture Equipment Corp., Santa Barbara, CA, USA) was used for leaf water potential measurement with six leaves for each treatment.

**Ethylene and ACC analysis**

Ethylene production by the grains was determined according to Beltrano et al. (1994) with modifications. Briefly, 60–80 sampled grains from each subsample were placed between two sheets of moist paper for 1 h at 27 °C in darkness to allow wound ethylene to be evolved. Grains from each subsample were placed between two sheets of moist filter paper and immediately sealed with airtight subaseal stoppers, and incubated in the dark for 12 h at 27 °C. A 1 ml gas sample was withdrawn through the stopper with a gas-tight syringe, and ethylene was measured using a gas chromatograph (HP5890 Series II, Hewlett Packard Com, Palo Alto, CA, USA) equipped with a Porapak Q column (0.3 cm × 200 cm, 50–80 mesh) and a flame ionization detector (FID). Temperatures for the injection port, column, and detector were kept constant at 140, 100, and 200 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 ml min−1, and hydrogen and air were used for FID at the rate of 30 ml min−1 and 300 ml min−1, respectively. The rate of ethylene production was calculated as nmol g−1 dry weight (DW) h−1.

ACC levels in grains were determined according to Cheng and Lur (1996). Ethylene evolved from ACC was assayed by using gas chromatography as described above. The transformation rate as a percentage from ACC to ethylene was 88 ± 4.5, 93 ± 5.6 and 81 ± 4.4, respectively, at early, mid, and late grain-filling stages. ACC content was expressed as nmol g−1 DW.

**Extraction and quantification of PAs**

Free PA fractions were estimated following the method of Flores and Galston (1982), while the soluble conjugated and insoluble conjugated fractions were analysed following the method as described by Liu et al. (2004) with modifications. Briefly, the sampled grains (0.5–1.0 g) were homogenized in a pre-chilled mortar and pestle in 3–5 ml of 5% (v/v) perchloric acid (PCA). The homogenates were incubated at 5 °C for 1 h and then centrifuged at 25 000 g for 20 min. After centrifugation, the supernatant and pellet were collected separately. To extract soluble conjugated PAs, aliquots (2 ml) of the supernatant were mixed with 2 ml of 12 N HCl and heated at 110 °C for 18 h in flame-sealed glass ampoules. After acid hydrolysis, HCl was evaporated from the tubes by further heating at 80 °C, and the residues were resuspended in 0.5 ml of 5% (v/v) PCA. To extract insoluble conjugated PAs, the pellet was rinsed four times with 5% PCA to remove any trace of soluble PA and then dissolved by vigorous vortexing in 2 ml of 1 M NaOH. Ethylene from ACC was assayed by using gas chromatography as described by Xue et al. (2009) with slight modifications. Sampled grains (0.3–0.5 g) were homogenized

**Determination of PA and ethylene biosynthetic enzyme activity**

To determine the activities of the enzymes involved in PA biosynthesis, sampled grains (0.5–1.0 g) were ground to a fine powder and homogenized with 3 ml of extraction buffer (pH 8.0) containing 25 mM potassium phosphate, 50 μM EDTA, 100 μM phenylmethylsulphonyl fluoride, 1 mM 2-mercaptoethanol, and 25 mM ascorbic acid. After centrifugation at 25 000 g at 4 °C for 20 min, the supernatant was dialysed overnight against the extraction buffer. The activities of ADC, ODC, and SAMDC were determined by measuring CO2 evolution as described by Lee et al. (1997). Spd synthase activity was assayed according to Kasukabe et al. (2004). An aliquot of the supernatant was incubated at 37 °C for 30 min in a reaction mixture consisting of 0.1 M TRIS-HCl (pH 8.0), 30 μM Put, 25 μM decarboxylated SAM, and 20 μM adenosine. The reaction product (5'-deoxy-5'-methylthioadenosine) was quantified via HPLC (Waters 2695 Separations Module) equipped with a fluorescence detector (Waters 2475 Multi λ, USA) and a reverse-phase (C18) column (Waters). 1,7-Hexanediane was used as an internal standard. The PA levels were the average of three replicates for each independent sample and are expressed as μmol g−1 DW.

**Measurement of PAO activity and H2O2 content**

PAO activity was determined according to Xue et al. (2009) with slight modifications. Sampled grains (0.3–0.5 g) were homogenized

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in 0.3–0.5 ml of 0.2 M phosphate buffer (pH 6.5) and centrifuged at 8000 g for 15 min at 4 °C. Reaction solution (3.3 ml) contained 2.5 ml of 0.1 M phosphate buffer (pH 6.5), 0.2 ml of crude enzyme extracts, 0.2 ml of peroxidase (250 U ml⁻¹), and 0.2 ml of 4-aminoantipyrine/N,N-dimethylaniline. The reaction was initiated by the addition of 0.2 ml of 20 mM Spd for the determination of PAO activity. A 0.01 change in the absorbance value at 555 nm was regarded as one enzyme activity unit. The content of H₂O₂ in grains was measured by using the method of Brennan and Frenkel (1977), and was expressed as μmol g⁻¹ DW.

Determination of transcript levels of genes

Genes encoding enzymes involved in PA biosynthesis, ADCl, ADC2, ODC, SAMDC, and Spd synthase1, and in ethylene biosynthesis, ACC synthase2, ACC synthase6, ACC oxidase1, ACC oxidase3, and ACC oxidase5, were analysed at the transcript level. These genes were chosen because they were observed to be closely associated with grain filling of rice (Zhu et al., 2011; Supplementary Tables S1, S2 available at JXB online). It is observed that the rice genome contains seven PAO isoforms that are termed OsPAO1 to OsPAO7, but only OsPAO3, OsPAO4, and OsPAO5 are more abundantly expressed in the plants (Ono et al., 2012). Therefore, these three PAO genes were chosen for analysis in the current study. RNA extraction, cDNA preparation, and real-time fluorescence quantification PCR (qRT-PCR) were carried out using the method described by Wei et al. (2012) with minor modifications. Total RNA in grains was extracted with the RNeasy plant mini kit (Qiagen, Germany) following the manufacturer’s protocols, and was isolated and transcribed with oligo(dT) primers using a SuperScript first-strand synthesis system according to the manufacturer’s instructions (Invitrogen, USA). Transcript levels of the genes were measured by qRT-PCR using an iCycler (Bio-Rad, USA) with iQ SYBR Green Supermix (Bio-Rad, USA). The gene-specific primer pairs used for qRT-PCR are listed in Supplementary Table S1. To verify the specificity of each primer set and optimize the PCR annealing temperature and PCR efficiency, the fluorescence signal specificity of PCR amplification was detected for each primer pair and their melting curve (from 55 °C to 95 °C) was examined prior to the experimental measurements. The amplification of the Actin gene was performed as a control, and a standard curve was checked. Three replications were conducted for each sample.

Final harvest

Plants from five pots in each treatment were harvested at maturity for the determination of the number of superior and inferior spikelets per pot and the percentages of sterile spikelets and unfilled grains of the two types of spikelets. The method used for determination followed that of Yoshida et al. (1976). The percentage of sterile spikelets was defined as the number of unfertilized spikelets as a percentage of the total number of spikelets per pot and the percentages of sterile spikelets and unfilled grains was defined as the unfilled fertilized grains (specific gravity <1.0 g cm⁻³) as a percentage of the total number of superior or inferior spikelets.

Chemical applications

Both cultivars were pot grown as described above and plants were well watered during the whole growing season. Each cultivar had 120 pots. Starting at 6 d after full heading, 2 mM Put, 1 mM Spd, 1 mM Spm, 5 mM methylglyoxal-bis (guanylylhydrazone) (MGBG; an inhibitor of Spd and Spm synthesis by inhibiting SAMC), 1 mM Spd+5 mM MGBG, 50 mM ethephon (an ethylene-releasing agent), 50 μM ACC, or 50 μM aminoethoxyvinylglycine (AVG; an inhibitor of ethylene synthesis by inhibiting ACC synthesis) (all from Sigma Chemical Co., St Louis, MO, USA) were applied to panicles using a paint brush that had been dipped in the solutions. The chemicals were applied daily for 4 d at the rate of 4 ml per panicle at each application, with 0.5% (v/v) Teepol (Fluka, Riedel-de-Haen, Germany) as surfactant. The same volume of deionized water containing the same concentrations of Teepol was applied to the control plants. Each chemical treatment had 60 panicles with three replications.

For all the chemical treatments, spikelets on a panicle were divided into two groups, namely the superior and the inferior spikelets. Levels of ethylene, ACC, free PAs (Put, Spd, and Spm), and H₂O₂ in grains were determined at 12 and 20 DPA, respectively. The grain-filling rate was determined at 6 d intervals from anthesis to maturity. Methods for the measurement of free PAs, ethylene, ACC, H₂O₂, and grain-filling rate were the same as described above. Thirty panicles from each treatment were harvested at maturity for the determination of final grain weight.

Statistical analysis

The results were analysed for variance using the SAS/STAT statistical analysis package (version 6.12; SAS Institute, Cary, NC, USA). Data from each sampling date were analysed separately. Means were tested by least significant difference at the P ≤0.05 level (LSD₀.₀₅). Regressions were used to evaluate the relationships of the levels of PAs, ethylene, and ACC, activities of enzymes involved in PA and ethylene biosynthesis, and PAO activities in the grains to the endosperm cell division rate and grain-filling rate. Since the two cultivars showed the same tendencies, the data are presented as an average between the two cultivars. This experiment was also conducted under tank-grown conditions at the same time as the pot-grown experiments, and results from the tank experiment were very similar. Only the pot experiment is reported herein due to space limitations.

Results

Changes in leaf water potentials

Figure 1 illustrates the changes in leaf water potentials during the first 30 d after withholding water. For plants grown under the WW treatment, midday (1130 h) leaf water potentials gradually decreased during grain filling, from −0.53 MPa at the beginning of measurements to −0.98 MPa on 30 d after withholding water (Fig. 1). Both soil drying treatments reduced midday leaf water potentials, and ranged from −0.52 MPa to −1.26 MPa under MD and from −0.54 MPa to −1.75 MPa under SD. As midday leaf water potential lower than −1.5 MPa during grain filling could not inhibit rice growth (Yang et al., 2007a), the results indicate that moderate soil drying during the grain-filling period would not seriously affect the plant water status.

Figure 1 also shows that the pre-dawn leaf water potential under MD was not significantly different from that under the WW treatment, but it was significantly decreased under SD, suggesting that plants under the MD treatment could rehydrate overnight whereas plants under SD could not.

Rates of endosperm cell division and grain filling

Because the MD and SD treatments were imposed from 8 DPA when spikelets completed fertilization, no significant differences among the WW, MD, and SD treatments were observed in the number of spikelets per pot and percentages of sterile spikelets for both superior and inferior carpyopes (Table 1). Both soil drying treatments also had no significant
Effects on the unfilled grain percentage of superior spikelets. However, MD significantly decreased, while SD significantly increased, the unfilled grain percentage of inferior spikelets when compared with the WW treatment (Table 1).

The endosperm cell division rate, grain-filling rate, endosperm cell number, and grain weight of superior spikelets were much greater than those of inferior spikelets, and showed no significant differences for the superior spikelets among the three soil moisture treatments (Fig. 2A–D). For the inferior spikelets, however, their endosperm cell division rate, grain-filling rate, endosperm cell number, and grain weight were greatly increased under MD and markedly reduced under SD. As a result, grain yield was increased by 11.1% under MD, and was reduced by 19.6% under SD, relative to that under the WW treatment (data not shown).

Changes in levels of PAs, ethylene, and ACC in grains

Similar to the changes in endosperm cell division and grain-filling rates, the contents of free Spd and free Spm were increased in the grains as grain filling proceeded, reached a peak at 16 DPA for the superior and 24 DPA for inferior spikelets, and decreased sharply thereafter (Fig. 3B, C). Superior spikelets had higher peak values of free Spd and free Spm at the early and mid grain-filling stages (4–20 DPA) and lower contents thereafter when compared with inferior spikelets. The MD treatment increased, while SD decreased, free Spd and free Spm contents in the inferior spikelets when compared with the WW treatment. In contrast to free Spm and free Spm contents, free Put contents were high in both superior and inferior spikelets at the early grain-filling stage, and decreased as grain filling proceeded (Fig. 3A). Inferior spikelets had higher free Put contents than superior spikelets during the grain-filling period. Both soil drying treatments remarkably enhanced free Put accumulation in inferior spikelets, which was enhanced more under SD than under MD. The MD or SD treatment had no significant effects on free PA contents in superior spikelets (Fig. 3A–C).

Generally, contents of both soluble conjugated and insoluble conjugated PAs (Put, Spd, and Spm) were high in superior and inferior spikelets at the early grain-filling stage, and decreased as grain filling proceeded (Supplementary Fig. S1A–F at JXB online). They were much lower than those of the free form of PAs, and showed no significant differences in superior or inferior spikelets among the three soil moisture treatments.

In a sharp contrast to free Spd and free Spm, ethylene evolution from grains was high at the early grain-filling stage, rapidly decreased until 24 DPA for superior spikelets and 36 DPA for inferior spikelets, then increased till 32 DPA for superior and 40 DPA for inferior spikelets, and decreased thereafter (Fig. 4A). The MD treatment reduced, whereas SD substantially increased, ethylene production in inferior spikelets. Both soil drying treatments showed no significant

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**Table 1.** The number of spikelets and the percentages of sterile spikelets and unfilled grains of superior and inferior spikelets of rice. WW, MD, and SD represent well-watered, moderate soil drying, and severe soil drying treatments, respectively, during grain filling (8–45 d post-anthesis). The number of superior and inferior spikelets per pot and the percentages of sterile spikelets and unfilled grains of superior and inferior spikelets were determined from plants of five pots. The percentage of sterile spikelets was defined as the number of unfertilized spikelets as a percentage of the total number of spikelets, and the percentage of unfilled grains was defined as the unfilled fertilized grains (specific gravity <1.0 g cm$^{-3}$) as a percentage of the total number of superior or inferior spikelets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of spikelets per pot</th>
<th>Sterile spikelets (%)</th>
<th>Unfilled grains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superior</td>
<td>Inferior</td>
<td>Superior</td>
</tr>
<tr>
<td>WW</td>
<td>1265±54 a</td>
<td>1183±45 a</td>
<td>2.45±0.16 a</td>
</tr>
<tr>
<td>MD</td>
<td>1273±62 a</td>
<td>1171±38 a</td>
<td>2.31±0.13 a</td>
</tr>
<tr>
<td>SD</td>
<td>1258±46 a</td>
<td>1196±57 a</td>
<td>2.54±0.18 a</td>
</tr>
</tbody>
</table>

The data are presented as an average between the two japonica cultivars Liandao 7 and Huaidao 9 because they showed the same tendencies. Different letters indicate a statistical difference at the $P$=0.05 level ($n$=10) within the column.
effects on ethylene evolution from superior spikelets. A similar pattern was observed for ACC contents in the grains (Fig. 4B). They were increased in inferior spikelets under SD, but decreased under MD. The ACC content was significantly correlated with the ethylene evolution rate ($r=0.989$, $P=0.001$), suggesting that an increase in ethylene production in inferior spikelets under SD is attributed to an enhanced ACC level there.

Changes in enzymatic activities and expression levels of PA biosynthesis genes

Figure 5A–D illustrates the changes in activities of the enzymes involved in PA biosynthesis in grains during filling. Changes in activities of SAMDC and Spd synthase were consistent with the changes in contents of free Spd and free Spm (Fig. 5C, D), and were closely associated with the grain-filling rate (see Fig. 2D). MD increased, whereas SD decreased, SAMDC and Spd synthase activities in inferior spikelets. The changing pattern of ADC activity was similar to that of Put contents, and both MD and SD treatments enhanced ADC activities in inferior spikelets, being more enhanced under SD than under MD (Fig. 5A). Compared with the ADC activity, the ODC activity was much lower, and showed no significant difference between superior and inferior spikelets (Fig. 5B). Differences in the activities of PA biosynthetic enzymes in superior spikelets were not significant among the three soil moisture treatments.

Changes in the expression levels of PA synthesis genes, $ADC1$, $SAMDC$, and $Spd synthase1$, were consistent with those in the activities of ADC, SAMDC, and Spd synthase in superior and inferior spikelets (Fig. 5E, G, H), indicating that these genes regulate PA synthesis and respond to soil drying at the transcriptional level. The expression levels of $ODC$ and $ADC2$ were high at the early grain-filling stage, and decreased as grain filling proceeded (Fig. 5F; Supplementary Fig. S2A at JXB online). They were not significantly different in superior or inferior spikelets among the three soil moisture treatments, suggesting that these genes play a minor role in response to soil drying at the transcriptional level.

Changes in enzymatic activities and expression levels of ethylene biosynthesis genes

Changes in activities of enzymes in ethylene biosynthesis, ACC synthase and ACC oxidase, were very similar to those in ethylene and ACC levels in grains (Fig. 6A, B). The activities of both enzymes in inferior spikelets were increased under
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SD, and were decreased under MD when compared with those under the WW treatment. They showed no significant differences for superior spikelets among the three treatments.

A very similar changing pattern was observed for expression of genes of ethylene biosynthesis, ACC synthase2 and ACC oxidase3, in both superior and inferior spikelets under the WW, MD, or SD treatments (Fig. 6C, D). The expression levels of ACC synthase6, ACC oxidase1, and ACC oxidase7 were high at the early grain-filling stage, and decreased as grain filling proceeded (Supplementary Fig. S2B–D) at JXB online. They showed no significant differences in superior or inferior spikelets among the three soil moisture treatments.

Changes in PAO activity, PAO gene expression, and H₂O₂ content

The PAO activity and expression levels of OsPAO5 were high at the early grain-filling stage, decreased as grain filling proceeded, and were higher in inferior spikelets than in superior spikelets (Fig. 7A, D). They were increased under SD and were decreased under MD for inferior spikelets, and showed no significant differences for superior spikelets among the treatments. The expression of OsPAO3 in inferior spikelets was markedly up-regulated by the SD treatment, but showed no significant difference between the WW and MD treatments (Fig. 7B).
Differences in expression levels of OsPAO3 in superior spikelets and OsPAO4 in either superior or inferior spikelets were not significant among the three treatments (Fig. 7B, C).

Changes in H2O2 contents in both superior and inferior spikelets were very similar to those of PAO activities (Fig. 8). Contents of H2O2 were much higher in inferior spikelets than in superior spikelets. They were decreased under MD and were increased under SD for inferior spikelets, and showed no significant differences in superior spikelets among the soil moisture treatments.

**Relationships of PA and ethylene biosynthesis to grain filling**

Regression analysis demonstrated that, during the active endosperm cell division period (2–10 DPA for superior...
spikelets and 6–18 DPA for inferior spikelets) and during the active grain-filling period (4–24 DPA for superior spikelets and 8–36 DPA for inferior spikelets), the mean endosperm cell division rate and grain-filling rate were very significantly and positively correlated with the mean contents of free Spd and free Spm, the activities of SAMDC and Spd synthase, and the ratio of Spd content to ACC content (Spd/ACC) and Spm content to ACC content (Spm/ACC) in grains ($r=0.925–0.998, P=0.01$; Table 2), whereas it was significantly and inversely correlated with the mean ethylene evolution rate, ACC and H$_2$O$_2$ contents, and activities of ACC synthase, ACC oxidase, and PAO ($r=-0.858$ to $-0.984, P=0.05$ and 0.01). The correlations of free Put contents, activities of ADC and ODC, and the ratio of free Put content to ACC content (Put/ACC) with the rates of endosperm cell division and grain filling were not significant ($r=-0.763$ to 0.712, $P > 0.05$).

**Effects of chemical regulators**

To verify the role of PAs and ethylene in the regulation of grain filling, chemicals involved in promoting or inhibiting PA and ethylene synthesis were applied to the panicles of WW plants at the early grain-filling stage (6–9 d after full heading). When compared with control (treated with deionized water), application of Put significantly increased free Put contents, and had no significant effects on ACC, ethylene, and H$_2$O$_2$ levels, the active grain-filling period, mean grain-filling rate, and grain weight of inferior spikelets (Tables 3–5). Application of Spd or Spm significantly enhanced free Spd or free Spm accumulation, decreased ACC and H$_2$O$_2$ contents and ethylene evolution rate, and increased the grain-filling rate and grain weight of inferior spikelets (Tables 3–5). When MGBG (an inhibitor of SAM decarboxylase), ethephon (an ethylene-releasing agent), or ACC was applied to panicles, contents of free Put, ACC, and H$_2$O$_2$, and the ethylene evolution rate were significantly increased, whereas free Spd, and free Spm contents, the grain-filling rate, and grain weight of inferior spikelets were significantly reduced. Application of AVG, an inhibitor of ACC synthase, showed the opposite effects (Tables 3–5). Except for MGBG which significantly reduced the grain-filling rate and grain weight, the other chemical regulators had no significant effects on PA and ethylene levels, grain-filling rate, and grain weight of superior spikelets (Supplementary Tables S2–S4 at JXB online).
Previous work done by Yang and Zhang (2006) has shown that a controlled soil drying or a moderate soil drying imposed during grain filling can improve grain filling in wheat and rice where plant senescence is unfavourably delayed such as by heavy use of nitrogen. The present data demonstrated that such a practice could also significantly reduce unfilled grains, accelerate endosperm cell division and grain-filling rates, and increase grain weight of inferior spikelets in ‘super’ rice cultivars (Table 1, Fig. 2A–D). This finding would have great significance to improve grain filling of modern rice cultivars, especially for the newly bred ‘super’ rice showing poor grain filling, such as many unfilled inferior caryopses (Yang and Zhang, 2010).

It is generally believed that both PAs and ethylene are endogenous plant growth regulators and play important roles in plant growth and development and responses to environmental stresses (Alcazar et al., 2006; Yang et al., 2007; Jang et al., 2012; Torrigiani et al., 2012). Prior to this study, however, little information was available describing the synchronous changes of ethylene and PA levels, activities of enzymes involved in PA and ethylene synthesis, and expression levels of key genes encoding these enzymes in rice grains, and their responses to soil drying and relationships to grain filling. The results herein showed that the temporal profile of ethylene evolution, ACC contents, ACC synthesis, and ACC oxidase activities, and expression levels of \( ACC \text{ synthase}_2 \) and \( ACC \text{ oxidase}_2 \) in inferior spikelets were rather different from that of the grain-filling rate (Figs 2D, 4A, B, 6A–C). Ethylene evolution rates, ACC contents, and activities of \( ACC \text{ synthase} \) and \( ACC \text{ oxidase} \) were significantly and negatively correlated with the endosperm cell division and grain-filling rates (Table 2). Application of an inhibitor of ethylene synthesis (AVG) increased, while applying ACC or an ethylene-releasing agent (ethephon) significantly reduced, the grain-filling rate and grain weight of inferior spikelets (Table 5). The data suggest that ethylene plays a role in inhibiting grain filling.
The mechanism underlying the effect of ethylene on the rice grain filling is little understood. It is suggested that ethylene may enhance the breakdown of cytokinins (Bollmark and Eliasson, 1990) which play an important role in maintaining cell division in the endosperm (Yang et al., 2002; Davies, 2004). High levels of ethylene and ACC in grains can inhibit endosperm cell division, leading to a low grain-filling rate and grain weight (Yang et al., 2006b). Ethylene has been observed to promote H$_2$O$_2$ generation and thereby inhibits plant growth and development (Kendrick and Chang, 2008; Hou et al., 2013). In this study, the increase in H$_2$O$_2$ content was closely associated with the decrease in the grain-filling rate and grain weight of inferior spikelets when ACC or ethephon was applied (Tables 4, 5). There is a report that ethylene can act as a signal to induce the expression of α-amylase genes and thereby reduce starch accumulation in the storage organs (Rook et al., 2001). Wang et al. (2012) observed that the activities of three enzymes involved in the sucrose to starch conversion in rice grains, sucrose synthase, ADP glucose pyrophosphorylase, and soluble starch synthase, were significantly reduced by application of ACC or ethephon, but substantially enhanced by application of AVG, to the panicles at the early grain-filling stage. These results suggest that ethylene reduces the grain-filling rate through inhibition of the key enzymes involved in sucrose to starch conversion in rice grains.

The effect of water deficit stress on ethylene production has remained a matter of debate (Morgan and Drew, 1997). The present results showed that the ethylene and ACC levels and ethylene biosynthetic activities in inferior spikelets were reduced under MD and greatly increased under SD (Figs 4A, B, 6A, B), indicating that the production of ethylene in rice grains may depend on the severity and duration of soil drying. Presumably, the decrease in ethylene production and its biosynthetic activity contributed to the increase in the grain-filling rate and grain weight for inferior spikelets under MD. On the other hand, the increase in ethylene production induced by SD inhibited the grain filling of these spikelets.

### Table 2. Correlations of the mean endosperm division rate and grain-filling rate with the mean contents of free polyamines, 1-aminocyclopropane-1-carboxylic acid (ACC), and hydrogen peroxide (H$_2$O$_2$), the ethylene evolution rate, and activities of the enzymes involved in polyamine and ethylene biosynthesis/catabolism in rice spikelets during the endosperm division and grain-filling periods. Data are from Figs 2B and D, 3 A–C, 4A and B, 5A–D, 6A and B, 7A, and 8.

<table>
<thead>
<tr>
<th>Correlations with</th>
<th>Mean endosperm cell division rate</th>
<th>Mean grain-filling rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine (Put)</td>
<td>-0.763</td>
<td>-0.571</td>
</tr>
<tr>
<td>Spermidine (Spd)</td>
<td>0.925**</td>
<td>0.995**</td>
</tr>
<tr>
<td>Spermine (Spm)</td>
<td>0.923**</td>
<td>0.963**</td>
</tr>
<tr>
<td>ACC content</td>
<td>-0.981**</td>
<td>-0.948**</td>
</tr>
<tr>
<td>Ethylene evolution rate</td>
<td>-0.984**</td>
<td>-0.956**</td>
</tr>
<tr>
<td>Activity of enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine decarboxylase</td>
<td>-0.691</td>
<td>-0.558</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>-0.478</td>
<td>0.548</td>
</tr>
<tr>
<td>S-Adenosyl-l-methionine</td>
<td>0.988**</td>
<td>0.998**</td>
</tr>
<tr>
<td>decarboxylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spd synthase</td>
<td>0.994**</td>
<td>0.997**</td>
</tr>
<tr>
<td>ACC synthase</td>
<td>-0.955**</td>
<td>-0.940**</td>
</tr>
<tr>
<td>ACC oxidase</td>
<td>-0.957**</td>
<td>-0.939**</td>
</tr>
<tr>
<td>Polyamine oxidase</td>
<td>-0.953**</td>
<td>-0.858*</td>
</tr>
<tr>
<td>Put/ACC</td>
<td>0.712</td>
<td>0.639</td>
</tr>
<tr>
<td>Spd/ACC</td>
<td>0.972**</td>
<td>0.996**</td>
</tr>
<tr>
<td>Spm/ACC</td>
<td>0.981**</td>
<td>0.981**</td>
</tr>
<tr>
<td>H$_2$O$_2$ content</td>
<td>-0.957**</td>
<td>-0.979**</td>
</tr>
</tbody>
</table>

*, ** Correlation significance at $P$=0.05 and $P$=0.01 levels, respectively ($n=6$).

The data are presented as an average between the two japonica cultivars Liandao 7 and Huaidao 9 because they showed the same tendencies. Different letters indicate a statistical difference at $P$=0.05 ($n=8$) within the column.

### Table 3. Effects of applied chemical regulators on contents of free putrescine (Put), free spermidine (Spd), and free spermine (Spm) in inferior spikelets of rice. Plants were pot grown and were well watered. The panicles were treated with 2 mM Put, 1 mM Spd, 1 mM Spm, 5 mM methylglyoxal-bis (guanylhydrazone) (MGBG), 1 mM Spd + 5 mM MGBG, 50 mM ethephon, 50 μM 1-aminocyclopropane-1-carboxylic acid (ACC), or 50 μM aminoethoxyvinylglycine (AVG) daily for 4 d starting at 6 d post-anthesis. Control plants were treated with deionized water. The content (μmol g$^{-1}$ DW) of free Put, Spd and Spm in the spikelets was determined at 12 and 20 d post-anthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>12 d post-anthesis</th>
<th>20 d post-anthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free Put</td>
<td>Free Spd</td>
</tr>
<tr>
<td>Control</td>
<td>9.26±0.43 b</td>
<td>1.54±0.08 b</td>
</tr>
<tr>
<td>2 mM Put</td>
<td>11.7±0.63 a</td>
<td>1.51±0.05 b</td>
</tr>
<tr>
<td>1 mM Spd</td>
<td>9.43±0.45 b</td>
<td>2.74±0.16 a</td>
</tr>
<tr>
<td>1 mM Spm</td>
<td>9.42±0.54 b</td>
<td>1.63±0.14 b</td>
</tr>
<tr>
<td>5 mM MGBG</td>
<td>12.2±0.71 a</td>
<td>0.65±0.04 d</td>
</tr>
<tr>
<td>1 mM Spd + 5 mM MGBG</td>
<td>9.42±0.62 b</td>
<td>1.49±0.10 b</td>
</tr>
<tr>
<td>50 μM ethephon</td>
<td>11.7±0.62 a</td>
<td>0.97±0.07 c</td>
</tr>
<tr>
<td>50 μM ACC</td>
<td>11.4±0.62 a</td>
<td>1.01±0.09 c</td>
</tr>
<tr>
<td>50 μM AVG</td>
<td>7.12±0.47 c</td>
<td>2.86±0.15 a</td>
</tr>
</tbody>
</table>
The present results demonstrated that, in contrast to those in ethylene production, changes in free Spd and free Spm contents in grains followed a similar pattern to the endosperm cell division and grain-filling rates (Figs 2B, D, 3B, C). The endosperm cell division and grain-filling rates were significantly and positively correlated with free Spd and free Spm contents and the activities of SAMDC and Spd synthase (Table 2). The increases or decreases in the contents of higher free PAs (Spd and Spm), activities of SAMDC and Spd synthase1, and expression levels of SAMDC and Spd synthase1 in inferior spikelets were observed to be closely associated with the increase or decrease in the grain-filling rate under MD or under SD treatment (Figs 2B, D, 3B, C, 5C, D, G, F). Application of Spd or Spm to panicles at the early grain-filling stage significantly increased, whereas application of MGBG, an inhibitor of SAMDC, significantly reduced, the grain-filling rate and grain weight of inferior spikelets (Table 5). These results demonstrate that free Spd and free Spm and their biosynthetic activity play an important role in responding to soil drying and regulating grain filling of rice.

Little is known about higher PAs (Spd and Spm) regulating grain growth when subjected to soil drying during grain filling. It is reported that a mild water deficit during grain filling could increase the activities of sucrose synthase, ADP glucose pyrophosphorylase, and soluble starch synthase in

**Table 4.** Effects of applied chemical regulators on ethylene evolution rate and contents of 1-aminocyclopropane-1-carboxylic acid (ACC) and hydrogen peroxide (H2O2) in inferior spikelets of rice. Plants were pot grown and were well watered. The panicles were treated with 2 mM putrescine (Put), 1 mM spermidine (Spd), 1 mM spermine (Spm), 5 mM methylglyoxal-bis (guanylhydrazone) (MGBG), 1 mM Spd + 5 mM MGBG, 50 mM ethephon, 50 μM ACC, or 50 μM aminoethoxyvinylglycine (AVG) daily for 4 d starting at 6 d post-anthesis. Control plants were treated with deionized water. The ethylene evolution rate and the contents of ACC and H2O2 were determined at 12 and 20 d post-anthesis, and were expressed as nmol g⁻¹ DW h⁻¹, nmol g⁻¹ DW, and μmol g⁻¹ DW, respectively.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>12 d post-anthesis</th>
<th>Ethylene</th>
<th>ACC</th>
<th>H2O²</th>
<th>20 d post-anthesis</th>
<th>Ethylene</th>
<th>ACC</th>
<th>H2O²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.36 ± 0.12 b</td>
<td>81.7 ± 4.76 b</td>
<td>12.3 ± 0.58 b</td>
<td>1.74 ± 0.07 b</td>
<td>65.7 ± 3.45 b</td>
<td>9.73 ± 0.44 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM Put</td>
<td>2.23 ± 0.10 b</td>
<td>78.6 ± 4.32 b</td>
<td>11.7 ± 0.64 b</td>
<td>1.76 ± 0.06 b</td>
<td>64.4 ± 3.12 b</td>
<td>9.54 ± 0.36 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM Spd</td>
<td>1.37 ± 0.07 c</td>
<td>57.8 ± 3.44 c</td>
<td>8.57 ± 0.26 c</td>
<td>1.14 ± 0.05 c</td>
<td>51.5 ± 2.83 c</td>
<td>6.45 ± 0.39 c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM Spm</td>
<td>1.32 ± 0.08 c</td>
<td>58.4 ± 2.49 c</td>
<td>8.69 ± 0.45 c</td>
<td>1.19 ± 0.06 c</td>
<td>53.7 ± 2.37 c</td>
<td>6.56 ± 0.45 c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 mM MGBG</td>
<td>3.45 ± 0.16 a</td>
<td>97.1 ± 6.33 a</td>
<td>15.4 ± 1.13 a</td>
<td>2.45 ± 0.08 b</td>
<td>76.8 ± 5.48 a</td>
<td>12.9 ± 0.67 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM Spd + 5 mM MGBG</td>
<td>2.42 ± 0.11 b</td>
<td>82.6 ± 5.25 b</td>
<td>11.6 ± 0.74 b</td>
<td>1.82 ± 0.09 b</td>
<td>66.2 ± 3.35 b</td>
<td>9.88 ± 0.52 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 mM ethephon</td>
<td>3.21 ± 0.19 a</td>
<td>96.3 ± 5.54 a</td>
<td>14.5 ± 1.12 a</td>
<td>2.53 ± 0.12 a</td>
<td>74.6 ± 4.67 a</td>
<td>12.6 ± 0.45 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 μM ACC</td>
<td>3.35 ± 0.22 a</td>
<td>98.9 ± 6.57 a</td>
<td>15.2 ± 1.25 a</td>
<td>2.61 ± 0.14 a</td>
<td>75.2 ± 4.29 a</td>
<td>13.1 ± 0.74 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 μM AVG</td>
<td>1.29 ± 0.08 c</td>
<td>55.5 ± 3.83 c</td>
<td>8.35 ± 0.39 c</td>
<td>1.15 ± 0.06 c</td>
<td>50.6 ± 2.46 c</td>
<td>6.32 ± 0.37 c</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data are presented as an average between the two japonica cultivars Liandao 7 and Huaidao 9 because they showed the same tendencies. Different letters indicate a statistical difference at P = 0.05 (n = 8) within the column.

**Table 5.** Effects of applied chemical regulators on the active grain-filling period (AGFP), mean grain-filling rate (MGFR), and grain weight of inferior spikelets of rice. Plants were pot grown and were well watered. The panicles were treated with 2 mM putrescine (Put), 1 mM spermidine (Spd), 1 mM spermine (Spm), 5 mM methylglyoxal-bis (guanylhydrazone) (MGBG), 1 mM Spd + 5 mM MGBG, 50 mM ethephon, 50 μM 1-aminocyclopropane-1-carboxylic acid (ACC), or 50 μM aminoethoxyvinylglycine (AVG) daily for 4 d starting at 6 d post-anthesis. Control plants were treated with deionized water. The active grain-filling period and mean grain-filling rate were calculated according to Richards’ (1959) equation. Grain weight was measured from 30 panicles in each treatment at maturity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AGFP (d)</th>
<th>MGFR (mg grain⁻¹ d⁻¹)</th>
<th>Grain weight (mg grain⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.5 ± 1.2 a</td>
<td>0.584 ± 0.021 b</td>
<td>18.5 ± 0.32 b</td>
</tr>
<tr>
<td>2 mM Put</td>
<td>28.3 ± 0.8 a</td>
<td>0.579 ± 0.017 b</td>
<td>18.2 ± 0.36 b</td>
</tr>
<tr>
<td>1 mM Spd</td>
<td>28.9 ± 0.5 a</td>
<td>0.674 ± 0.025 a</td>
<td>21.7 ± 0.53 a</td>
</tr>
<tr>
<td>1 mM Spm</td>
<td>29.1 ± 1.3 a</td>
<td>0.675 ± 0.023 a</td>
<td>21.8 ± 0.46 a</td>
</tr>
<tr>
<td>5 mM MGBG</td>
<td>25.2 ± 1.1 b</td>
<td>0.478 ± 0.026 c</td>
<td>13.4 ± 0.42 c</td>
</tr>
<tr>
<td>1 mM Spd + 5 mM MGBG</td>
<td>28.3 ± 0.9 a</td>
<td>0.567 ± 0.031 b</td>
<td>17.8 ± 0.37 b</td>
</tr>
<tr>
<td>50 mM ethephon</td>
<td>24.6 ± 0.8 b</td>
<td>0.498 ± 0.022 c</td>
<td>13.6 ± 0.25 c</td>
</tr>
<tr>
<td>50 μM ACC</td>
<td>24.5 ± 0.7 b</td>
<td>0.489 ± 0.027 c</td>
<td>13.3 ± 0.31 c</td>
</tr>
<tr>
<td>50 μM AVG</td>
<td>28.7 ± 1.0 a</td>
<td>0.693 ± 0.034 a</td>
<td>22.1 ± 0.55 a</td>
</tr>
</tbody>
</table>

The data are presented as an average between the two japonica cultivars Liandao 7 and Huaidao 9 because they showed the same tendencies. Different letters indicate a statistical difference at P = 0.05 (n = 8) within the column.
wheat grains (Yang et al., 2004). Application of Spd or Spm to rice panicles at the early filling stage significantly enhanced, while applying MGBG significantly decreased, the activities of these enzymes in grains (Wang et al., 2012). These results suggest that, in contrast to ethylene, PAs may play a role in grain filling through regulating the key enzymes involved in the sucrose to starch conversion there. It is possible that increases in higher PAs (Spd and Spm) and their biosynthetic activity under MD enhanced the key enzymes involved in the sucrose to starch conversion in grains, leading to an increase in the grain-filling rate and grain weight. On the other hand, the decreases in higher PA synthesis under SD inhibited these enzymes in grains and resulted in a slow grain-filling rate and a low grain weight of inferior spikelets.

The present results showed that MD decreased, while SD increased, PAO activity and OsPAO5 expression in inferior spikelets (Fig. 7A, D). Their increase or decrease was closely associated with the decrease or increase in Spd and Spm contents and with the increase or decrease in H2O2 content in the spikelets (Figs 3B, C, 8). When Spd or Spm was applied, the H2O2 content was significantly reduced in inferior spikelets (Table 4). It has been reported that abiotic stress could enhance PAO and increase H2O2 accumulation in plants and thereby induce programmed cell death (Moschou et al., 2008a). The results indicate that a reduction in PAO biosynthesis under MD would increase Spd and Spm and decrease H2O2 levels in inferior spikelets, leading to increases in endosperm cell division and grain-filling rates and grain weight. On the other hand, the decreased contents of higher PAs and increased H2O2 induced by an enhanced PAO under the SD treatment would inhibit the growth of inferior caryopses.

It is proposed that higher PAs (Spd and Spm) and ethylene share a biosynthetic precursor SAM, and increases in Spd and Spm biosynthesis are likely to affect the rates of ethylene synthesis (Walden et al., 1997; Ravanel et al., 1998; Liang and Lur, 2002). The results presented here demonstrated that the endosperm cell division rate and grain-filling rate correlated not only with levels of free PAs (Spd and Spm), ACC, and ethylene, but also with the ratio of free Spd to ACC (Spd/ACC), and free Spm to ACC (Spm/ACC) (Table 2). The increase in contents of free PAs (Spd and Spm), and their biosynthetic activity under MD were closely associated with the decrease in ethylene production and ethylene biosynthetic activity, and the results were reversed under the SD treatment (Figs 3B, C, 4A, 5C, D, 6A, B). Application of Spd, Spm, or AVG significantly increased free Spd and free Spm contents in inferior spikelets, whereas they significantly reduced the ACC content and ethylene evolution rate, and the opposite effects were observed when MGBG, ethephon, or ACC was applied (Tables 3, 4), implying that higher PAs (Spd and Spm) and ethylene exhibit an antagonistic relationship. Similar observations are also reported in oat (Avena L.) (Fuhrer et al., 1982), chickpea (Cicer L.) (Gallardo et al., 1995), Arabidopsis thaliana (Hu et al., 2006), maize (Feng et al., 2011), and tomato (Solanum lycopersicum) (Nambooben et al., 2012). It is therefore speculated that a potential metabolic interaction or competition between higher free PAs (Spd and Spm) and ethylene biosynthesis may mediate the effects of soil drying on the grain filling of inferior spikelets of rice.

Interestingly, neither PA/ethylene biosynthesis nor endosperm cell division/grain filling rate of superior spikelets were markedly affected by the MD and SD treatments (Table 1, Figs 2–6). Similar observations were also made by Xu et al. (2007) and Zhang et al. (2010) who reported that non-flooded mulching cultivation and alternate wetting and drying irrigation remarkably affected hormonal levels and grain weight of inferior spikelets of rice, but little affected those of superior spikelets. The mechanism involved is not clear. A probable explanation is that earlier flowering superior spikelets dominate over later flowering inferior spikelets in hormonal levels, endosperm development, and grain filling (Yang et al., 2002, 2006b; Zhang et al., 2009), and both MD and SD treatments in this study could not markedly alter the PA and ethylene biosynthesis in superior spikelets and, consequently, significantly affect the endosperm development and grain filling of these spikelets.

It is noteworthy that changes in contents of both soluble conjugated and insoluble conjugated PAs were not related to the grain-filling rate, and were not affected by the soil drying treatments (Supplementary Figs S1, S2 at JXB online), suggesting that these forms of PAs could play a minor role in responding to soil drying and in regulating grain filling.

The results presented here showed that both MD and SD treatments significantly increased free Put accumulation and ADC activity in inferior spikelets (Figs 3A, 5A), but free Put contents were not significantly correlated with the grain-filling rate (Table 2). No significant effects were detected on the grain-filling rate and grain weight when Put was applied to panicles (Table 5). A similar observation was made by Liang and Lur (2002) who reported that Put was abundant in developing maize kernels but there was no difference in the Put levels in the apical endosperm of shaded and control plants. The results infer that the role of free Put in developing seeds could be to respond to soil drying, but Put per se would play a minor role in seed development.

Many studies have suggested that free PAs accumulate via either the ODC or the ADC pathway (Walden et al., 1997; Papadakis and Roubelakis-Angelakis, 2005), although there are reports that a few plant species, including A. thaliana, lack the ODC pathway for PA synthesis (Hanfrey et al., 2001; Kusano et al., 2007). The present results showed that increases in free Put contents were closely associated with increases in ADC activities and ADC1 expression levels under both MD and SD treatments (Figs 3A, 5A, E). The soil drying treatments exhibited no significant effect on ODC activities and ODC expression levels (Fig. 5B, F). The results imply that Put synthesis in rice grains is primarily via ADC, rather than ODC, confirming the operation of an arginine→agmatine→Put pathway in the growth and development of rice seed (Smith, 1970).

In conclusion, moderate soil drying imposed during the grain-filling period of rice can accelerate endosperm cell division and grain-filling rates and increase the grain weight of inferior spikelets. The accelerated seed development is closely associated with an enhanced free Spd and free Spm biosynthesis and a decreased ethylene biosynthesis in these caryopses. Under severe soil drying, an increase in ethylene
biosynthesis and a decrease in free Spd and free Spm biosynthesis contribute to the reduction in the grain-filling rate and grain weight. A potential metabolic interaction or competition between higher free PAs (Spd and Spm) and ethylene biosynthesis mediates the effects of soil drying on grain filling of rice. Further investigation is needed to understand the cross-talk between PAs and ethylene and its response to abiotic stress, and the relationship to grain development in rice by use of mutants or transgenic plants with an attenuated capacity to respond to or synthesize the growth regulators.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Changes in contents of soluble conjugated and insoluble conjugated putrescine, spermidine, and spermine in superior and inferior spikelets of rice.

Figure S2. Changes in expression levels of the polyamine synthesis gene, ADC2, and ethylene synthesis genes, ACC synthase6, ACC oxidase1, and ACC oxidase7, in superior and inferior spikelets of rice.

Table S1. The sequences of primers for genes in the polyamine and ethylene biosynthetic pathway and genes encoding polyamine oxidase in rice grains.

Table S2. Effects of applied chemical regulators on contents of free putrescine, free spermidine, and free spermine in superior spikelets of rice.

Table S3. Effects of applied chemical regulators on ethylene evolution rate and contents of 1-aminocyclopropane-1-carboxylic acid and hydrogen peroxide in superior spikelets of rice.

Table S4. Effects of applied chemical regulators on the active grain-filling period, mean grain-filling rate, and grain weight of superior spikelets of rice.

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