Response of brassinosteroids to nitrogen rates and their regulation on rice spikelet degeneration during meiosis

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Abstract
The plant steroid hormones brassinosteroids (BRs) play pivotal roles in modulating flower and fruit production. Nitrogen (N) is a key factor affecting rice (Oryza sativa L.) production. We hypothesized that BRs would respond to N application rates at spikelet differentiation (SD) and regulate spikelet degeneration in rice. Three rice cultivars were field-grown and treated with five N rates at SD. Plant N and BRs contents, antioxidant capacity, and energy status were observed during meiosis in young panicles, and their relationships with spikelet degeneration were evaluated. In all the N treatments, the BRs, adenosine triphosphate, and energy charge levels, activities of the enzymes involved in energy metabolism, including cytochrome C oxidase and succinate dehydrogenase, total antioxidant capacity, and grain yield, were the highest, whereas hydrogen peroxide (H2O2) content and vacuolar processing enzymes (VPE) genes expression levels, and spikelet degeneration rate were the lowest when plant N content was 2.6% during meiosis. The rice BRs-deficient mutants showed substantial reduction in BRs levels, antioxidant capacity, and energy status, but great increase in H2O2 content, VPE genes expression levels, and spikelet degeneration rate than the wild type (WT); moreover, these parameters in WT panicles could be regulated, whereas in BRs-deficient mutant panicles, they were not distinctly affected by N application. The results indicated that BRs mediate the effects of N rates on spikelet degeneration, and elevated BRs levels in rice panicles effectively inhibit spikelet degeneration when plant N content is 2.6% during meiosis by elevating antioxidant capacity and energy status.
1 INTRODUCTION

Spikelet degeneration, or spikelet abortion, is very common in cereals such as rice (*Oryza sativa* L.) that is a serious physiological defect and constraint to grain production (Yoshida, Ohmori, Kitano, Taguchi-Shiobara, & Hirano, 2012; Zhang, Sheng, et al., 2019). Reduction or elimination of spikelet degeneration to increase spikelet number is critical for increasing rice grain yield, and great effort has been made to decrease spikelet degeneration in rice using agronomic, genetic, and molecular approaches (Heng et al., 2018; Tang, Sun, Xu, & Yu, 2011; Zhang, Sheng, et al., 2019; Zhang, Zhu, et al., 2019). However, the mechanism underlying spikelet degeneration remains unclear.

Brassinosteroids (BRs) are a recently discovered group of naturally occurring plant steroid hormones comprised of brassinolide (BL), castasterone (CS), and their various derivatives that are widely distributed throughout the plant; BRs produce an array of metabolic changes essential for normal plant growth, development, and stress tolerance (Eremina et al., 2016; Lv et al., 2018; Zhang, Bai, & Chong, 2014; Zhu et al., 2015). Spikelet number per panicle is determined both genetically and environmentally. Among the nutritional factors, nitrogen (N) is one of the most yield-limiting nutrients in crop production, and its proper management is essential to increase spikelet number and consequently increase rice grain yield (Ding et al., 2016; Zhang, Zhu, et al., 2019; Zhang et al., 2013). Our previous work showed that BRs in young panicles mediate the effects of N top-dressing times during panicle development on rice spikelet differentiation and degeneration (Zhang, Zhu, et al., 2019). However, little is known about how BRs respond to treatment with different N rates at spikelet differentiation (SD) or how they regulate spikelet degeneration at pollen mother cell meiosis metaphase (PMCm) in rice.

Like all aerobic organisms, plants have well-developed metabolic pathways that utilize their energetic potential in the presence of oxygen (Calderón, Rotem, Harris, Vela-Corcá, & Levy, 2019; Navrot, Rouhier, Gelhaye, & Jacquot, 2007). One potentially damaging effect of metabolism is the deleterious production of reactive oxygen species (ROS), such as hydrogen peroxide (*H₂O₂*), during normal respiration, photosynthesis, N fixation, and abiotic stress responses (Ahmad, Jaleel, Salem, Nabi, & Sharma, 2010; Chen, Zhang, & Zhang, 2019; Choudhury, Rivero, Blumwald, & Mittler, 2017). Indeed, excessive *H₂O₂* is the main factor that causes programmed cell death (PCD) and rice spikelet degeneration through the destruction of cell membrane integrity (Ali, Xu, Riaz, & Wu, 2019; Heng et al., 2018; Zhang, Sheng, et al., 2019). In addition to ROS levels, cellular energy supply is another important factor that controls growth and development. The enhancement of membrane integrity is associated with the maintenance of a higher energy status, such as adenosine triphosphate (ATP) level (Jin et al., 2013; Liu et al., 2016). Our previous work showed that an appropriate N top-dressing time during rice panicle development could significantly increase BRs levels, which effectively increase spikelet differentiation and decreased spikelet degeneration by stimulating antioxidant activities and energy status in young panicles (Zhang, Zhu, et al., 2019). How BRs mediate effects of various N application rates at SD on rice spikelet degeneration is poorly understood, and the objective of this study was to elucidate their mechanism.

The temporal patterns of N accumulation in plants, BRs contents, changes in energy status, activities of enzymes involved in energy metabolism, total antioxidant capacity (T-AOC), *H₂O₂* content, and the expression of vacuolar processing enzyme (VPE) genes in young rice panicles at PMCm under different N application rates at SD, as well as their relationships with spikelet degeneration rate, were examined. Transgenic rice plants and chemical regulators were used to further investigate the molecular mechanism underlying how BRs respond to various N application rates at SD and how BRs regulate spikelet degeneration in rice.

2 MATERIALS AND METHODS

2.1 Materials and culture conditions

The experiment was conducted at a research farm at Yangzhou University, Jiangsu Province, China (32°30’N, 119°25’E), during the rice growing season (May-October) in 2016 and 2017. The soil was a sandy loam (Typic Fluvaquent, Entisol, US classification) that contained 24.2 g/kg organic matter, 101.5 mg/kg alkali-hydrolysable N, 34.2 mg/kg Olsen phosphorus, and 68.1 mg/kg exchangeable potassium. The field capacity soil moisture content was 0.187 g/g when measured gravimetrically at a constant drainage rate, with a soil bulk density of 1.34 g/cm³.

Three rice cultivars: *japonica* inbred Wuyunjing 24 (WYJ-24), *indica* inbred Yangdao 6 (YD-6), and *japonica* *indica* hybrid Yongyou 2640 (YY-2640), have different panicle sizes (number of spikelets per panicle) and are currently used in local rice production. These cultivars were grown in the paddy field; the number of spikelets per
panicle is usually less than 150 for WYJ-24, 160–190 for YD-6, and more than 250 for YY-2640 when N application rate was 240–300 kg/ha according to local practice. Across the two experimental years, the seeds were sown in the paddy field on 11–12 May. Thirty-day-old seedlings were then transplanted into a field with a hill spacing of 0.25 m × 0.16 m, with two seedlings per hill. Phosphorus (30 kg/ha as single superphosphate) and potassium (40 kg/ha KCl) were applied and incorporated before transplanting. Weeds, insects, and diseases were controlled as required to avoid yield loss. The heading date (50% plants) of the cultivars was 102–107 days from germination, and plants were harvested 157–158 days after germination.

2.2 | N Treatments

The experiments were conducted in a completely randomized block design with three replicates. The plot size was 5 m × 6 m, and the plots were separated by a 1-m wide alley with plastic film inserted into the soil to a depth of 50 cm to form a barrier between plots. Five N rates were applied at the SD stage as 0, 30, 60, 90, and 120 kg/ha N fertilizer (as urea) (N0, N1, N2, N3, and N4). For all N treatments, N as urea was also applied at pretransplanting (one day before transplanting), early tillering (seven days after transplanting), and panicle initiation at 60, 30, and 60 kg/ha N, respectively. The developmental stages were observed by frequent inspection of the meristems using a microscope and leaf remainder (LR) through peeling and examining leaves as described previously (Zhang et al., 2013). The panicle initiation was defined as the first appearance of a differentiated apex (LR: 4.0–3.5) and the SD as the appearance of glumous flower primordia at the tips of elongating primary rachis branches (the length of the young panicles was approximately 1.0–1.5 mm, LR: 2.0–1.6).

2.3 | Sampling

Sampling and measurements were mainly made at PMCm when the ligule of the flag leaf was 0 cm below that of the penultimate leaf because rice spikelet degeneration mainly occurs at this stage (Wang, Zhang, & Yang, 2018; Zhang, Sheng, et al., 2019; Zhang, Zhu, et al., 2019). One hundred main stems were tagged in each plot during tillering, and young panicles were sampled from the tagged stems at PMCm for the measurement of BRs contents, cytochrome C oxidase (CCO) and succinate dehydrogenase (SDH) activities, levels of ATP and energy charge (EC), T-AOC, H$_2$O$_2$ content, and expression levels of the VPE genes (OsVPE2 and OsVPE3). Aboveground plants sampled at PMCm were oven-dried at 70°C until they reached a constant weight. Tissue N content was determined by micro Kjeldahl digestion, distillation, and titration to calculate aboveground plants N accumulation and content as previously described (Zhang et al., 2013).

At the heading stage, young panicles that emerged by two-thirds from the flag leaf sheath were tagged from 2 m$^2$ plants in each plot to determine the number of differentiated and degenerated spikelets per panicle. Plants on the boarder of the plots were omitted to control for boarder effects. A white and withered spikelet on a panicle was defined as a degenerated spikelet. The number of differentiated spikelets was defined as the sum of the number of developed and degenerated spikelets. The spikelet degeneration rate was defined as the ratio of degenerated to differentiated spikelets. Grain yield was determined from a harvest area of 6 m$^2$ in each plot and adjusted to 14% moisture. Yield components including the total spikelet number per square meter (m$^2$), percentage of fully filled grains, and 1000-grain weight were determined from plants within 2 m$^2$ that were sampled randomly from each plot, excluding those on the borders. The method used for these observations was described previously (Zhang, Chen, Wang, & Yang, 2017).

2.4 | Extraction and quantification of BRs

Endogenous BRs in young rice panicles were extracted and purified using a previously described method (Ding, Mao, Yuan, & Feng, 2013) with modifications. Briefly, a 4–6 g sample of fresh young panicles was ground in a tissue crusher (MM400, Retsch Corp) and 0.8 g of the produced power was transferred into a 10-ml centrifuge tube, followed by extraction with 4 ml acetonitrile overnight at 20°C. Extraction, dehydration, and double-layered solid phase extraction (DL/SPE) were performed as previously described (Chen, Lu, Ma, Guo, & Feng, 2009). The quantification of BRs was performed using high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC-ESI-MS/MS) according to the protocol (Ding et al., 2013). The BRs were quantified using a calibration curve of known amounts of standards and based on the ratios of the summed area of the multiple reaction monitoring (MRM) transitions for BRs. Data acquisition and analysis were performed using the Xcalibur Data System (Thermo Fisher Scientific). In this study, 24-epicastasterone (24-epiCS) and 28-homobrassinolide (28-homoBL) were analyzed because of their high biological activity in rice (Bajguz & Tretyn, 2003; Zhang, Sheng, et al., 2019; Zhang, Zhu, et al., 2019). The 24-epiCS and 28-homoBL contents were expressed as pmol/g dry weight (DW).
2.5 | Measurement of CCO and SDH activities

The CCO activity in young rice panicles was measured as previously described (Jin et al., 2013). The assay mixture contained 0.05 mol/L phosphate buffer (pH of 7.5), 0.3 mmol/L reduced cytochrome C, and 0.02 mol/L dimethyl phenylenediamine. The reaction was initiated by adding 0.5 ml crude mitochondrial extract. One unit of CCO activity was defined as an increase of 0.01 in absorbance at 510 nm per minute.

The SDH activity in young rice panicles was measured as previously described (Acevedo et al., 2013). The substrate solution contained 0.05 mol/L potassium phosphate buffer (pH 7.8), 4 mmol/L sodium azide, 1 mmol/L phenazine methosulfate, 0.08 mmol/L dichlorophenolindophenol, and 0.1 mol/L sodium succinate. The mixture was incubated at 30°C for 10 min. The activity was determined by adding 0.5 ml crude mitochondrial extract. One unit of SDH activity was defined as an increase of 0.01 in absorbance at 600 nm per minute. The activities of both CCO and SDH were expressed in unit/g DW.

2.6 | Measurement of ATP content and EC

The extraction and assays of ATP, adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in young rice
panicles were described previously (Zhou et al., 2014) and performed with some modifications. Briefly, approximately 2 g ground sample was extracted with 5 ml 0.6 mol/L perchloric acid. The homogenate was centrifuged at 15,000 g for 10 min at 4°C. A 3 ml aliquot of the supernatant was quickly neutralized to pH 6.5 with 1 mol/L KOH, diluted to a final volume of 5 ml, and passed through a 0.45-mm filter. Measurements of ATP, ADP, and AMP were taken with an HPLC system (Waters 2,695 separation module, Waters Corp) as previously described (Liu et al., 2016). The EC was defined as the ratio of the sum of ATP and 1/2 ADP concentration to the concentrations of ATP, ADP, and AMP, which was calculated with the following formula (Liu et al., 2016):

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EC = \frac{ATP + 0.5 \times ADP}{ATP + ADP + AMP}
\]

### 2.7 Measurement of T-AOC and H₂O₂ content

The T-AOC in young rice panicles was evaluated using a T-AOC kit (Suzhou Comin Biotechnology Co., Ltd.) following the manufacturer instructions. T-AOC was expressed in μmol/g DW. The H₂O₂ content in rice young panicles was measured using a method previously reported (Rao, Lee, Creelman, Mullet, & Davis, 2000) and was expressed in μmol/g DW.

### 2.8 Determination of genes expression levels

The expression levels of two representative VPE genes (OsVPE2 and OsVPE3) associated with PCD in young rice panicles (Deng et al., 2011) were analyzed. Transcript levels of the genes were measured by fluorescent real-time quantitative PCR (qRT-PCR) using an iCycler (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). The gene accession numbers and gene-specific primer pairs used for qRT-PCR are listed in Table S1. Rice Actin 1 was used as an internal reference for all analyses (Table S1). Three replicates were performed for each sample.

### 2.9 Construction of rice BRs-deficient mutants and N treatments

To further verify the roles of BRs in response to various N application rates at SD and in regulating spikelet degeneration in rice during meiosis, we used two rice BRs-deficient mutants (osd11#1 and osd11#2) in which the OsD11 (LOC_Os04g39430) gene encoding a cytochrome P450 enzyme (CYP724B1) involved in BRs biosynthesis is knocked out. The rice BRs-deficient mutants (osd11#1 and osd11#2) were generated by CRISPR/Cas9 system (Baige) and reported in our previous study (Zhang, Sheng, et al., 2019). The wild type (WT) used to assess the osd11#1 and osd11#2 mutants was the inbred japonica rice cultivar Zhonghua 11 (ZH11). The transgenic osd11#1 and osd11#2 plants displayed significant reduction in BRs contents in young panicles and a sharp increase in spikelet degeneration rate (47.2% and 46.4%) compared to WT plants (14.2%) under normal growth conditions (Zhang, Sheng, et al., 2019).

Seeds of the WT (ZH11) and BRs-deficient mutants (osd11#1 and osd11#2) were sown in the field, and thirty-day-old seedlings were then transplanted into a field with a hill spacing of 0.25 m × 0.16 m, with two seedlings per hill during the rice growing season. The plot size and soil composition were the same as those described above. Two N rates were applied at the SD stage: no N fertilization (N0) and 60 kg/ha (N2). The treatment details and management were the same as those described above. One hundred main stems were tagged in each plot during the tillering period, and young panicles were sampled from the tagged stems at PMCm for measurement of BRs (24-epiCS and 28-homoBL) and H₂O₂ contents, CCO and SDH activities, T-AOC level, and OsVPE2 and OsVPE3 expression levels, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), and intensity of the fluorescence emissions of ROS in young panicles/spikelets. The number of differentiated or degenerated spikelets in a panicle was determined from 2 m² plants sampled from each treatment plot at the heading stage and determined as described above.

### 2.10 TUNEL assay

The spikelet hulls of WT (ZH11) and BRs-deficient mutants (osd11#1 and osd11#2) treated with N0 and N2 conditions were collected at PMCm and fixed in FAA fixation solution (18:1:1 v/v mixture formalin, 70% ethanol, and acetic acid) for 24 hr. The spikelet hull samples were embedded in paraffin and sectioned as previously described (Heng et al., 2018). The TUNEL assay as a direct indicator for PCD was performed with a Dead End Fluorometric TUNEL Kit (Roche) according to the manufacturer’s instructions. The fluorescein isothiocyanate TUNEL (green) and 4’,6-diamidino-2-phenylindole (blue) fluorescent signals were excited at 465–495 nm or 330–380 nm, and detected at 515–555 nm or 420 nm (detection), respectively, using fluorescent microscopy (NIKON ECLIPSE C1).

### 2.11 Intensity of the fluorescence emissions of ROS

The intensity of the fluorescent emissions of ROS in live spikelet hulls cells from WT (ZH11) and mutants (osd11#1

\[\text{Intensity of the fluorescence emissions of ROS} = \frac{\text{Integral of fluorescence} \times \text{Sample area}}{\text{Integral of BSA} \times \text{Sample area}}\]
and osd11#2 treated with N0 and N2 conditions were quantified with the LIVE Green ROS Detection Kit (Invitrogen Molecular Probes) following the manufacturer protocol with some modifications. Briefly, the young spikelets at PMCm were collected and immediately placed in an Eppendorf tube and transported to the laboratory in ice. The young spikelets were washed in warm (30°C) phosphate-buffered solution (PBS) and cut into small pieces before being immediately immersed in 25 mmol/L 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) working solution. The young spikelets were incubated for 30 min at 37°C in the dark. Spikelets stained using the LIVE Green ROS Detection Kit and observed with a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss). The stained spikelets were analyzed with 495 nm and 529 nm excitation and emission wavelengths, respectively.

### 2.12 Chemical treatments

The WT (ZH11) and osd11#1 plants were field-grown under the no N fertilization (N0) treatment at SD as described above. At the onset of PMC meiosis when the ligule of the flag leaf was 10 cm below that of the penultimate leaf, 10 nmol/L exogenous BRs (24-epiCS + 28-homoBL) (eBRs) was applied to the WT and osd11#1 panicles, and 5 mmol/L exogenous H2O2 (eH2O2) was applied to the WT panicles by carefully injecting the solution from the top into the boot of the flag leaf with a 1-mL syringe (Ultra-Fine Needle Insulin Syringe, Becton, Dickinson and Company). Physical damage caused by the injection was not observed when using a very fine 0.33-mm needle. The chemicals were applied daily for 2 days as 1.0 ml solution per panicle at each application (all from Sigma Chemical Co.). Control plants (CK) received the same volume of deionized water as the experimental treatments. Each chemical treatment included 160 young panicles as replicates, and these panicles were tagged.

### 2.13 Statistical analysis

Analysis of variance (ANOVA) was performed using the SAS/STAT statistical analysis package (version 9.2, SAS Institute). The statistical model included sources of variation due to replication, year, cultivar, N treatment and the

### TABLE 1 Grain yield and yield components of rice under various nitrogen treatments

| Year/item | WYJ-24 | YD-6 | YY-2640 |
|-----------|--------|------|---------|
|           | N0     | N1   | N2     | N3     | N4     | N0     | N1   | N2     | N3     | N4     | N0     | N1   | N2     | N3     | N4     |
| 2016 Total spikelets (x10^4 m^-2) | 2.50c | 2.86b | 3.11a | 2.92ab | 2.80b | 3.05d | 3.42c | 3.65b | 3.90a | 3.57bc | 4.55c | 5.13c | 5.47b | 5.80b | 6.01a |
| 2017 Fully filled grains (%) | 92.3a | 92.1a | 91.6a | 88.3b | 87.1b | 90.2a | 90.0a | 89.8a | 88.5a | 84.6b | 80.7a | 79.9a | 79.1a | 78.9a | 78.2a |
| 1000-grain weight (g) | 27.8a | 27.6a | 27.5a | 27.3a | 27.0a | 28.6a | 28.5ab | 28.2ab | 28.2ab | 27.1b | 24.9a | 24.6ab | 24.3bc | 24.2bc | 23.7c |
| Grain yield (g/m^2) | 635c | 716b | 782a | 722b | 660c | 792c | 880b | 938a | 985a | 826c | 933d | 988cd | 1041bc | 1103ab | 1142a |

Note: N0, N1, N2, N3, and N4 indicate the N rate applied at spikelet differentiation at 0, 30, 60, 90, and 120 kg/ha, respectively. Values of grain yield are means of plants of 6 m^2 harvested from each plot and adjusted to 14% moisture. Values of total spikelets per m^2, fully filled grain percentage, and 1000-grain weight are means of plants harvested from 2 m^2 from each plot in each treatment.
interaction of year × cultivar, year × N treatment, cultivar × N treatment, and year × cultivar × N treatment. Data from each sampling date were analyzed separately. Means were tested by a least significant difference (LSD) test at \( p = .05 \) within the same cultivar.

3 | RESULTS

3.1 | Plant N accumulation and content, spikelet differentiation and degeneration, and grain yield

The computed \( F \)-values for differences between or among years, cultivars, and treatments are presented in Table S2. Most of the measurements showed significant difference \( (p < .05) \) among the cultivars and treatments; however, the variations due to years and interactions of year × N treatment, and year × cultivar × N treatment were not significant \( (p \geq .05) \) for most of the measurements recorded (Table S2).

The N accumulation in plants and plant N content increased in a dose-dependent manner with N application rate (Figure 1a–d), and no significant differences among the N treatments were observed with respect to the number of differentiated spikelets (Figure 1e,f). Compared with the no N fertilization treatment (N0) at SD, the application of N at SD significantly decreased spikelet degeneration rate, which was the lowest in N2, N3, and N4 treatments for WYJ-24, YD-6, and YY-2640, respectively. In contrast to the spikelet degeneration rate, the total spikelets and grain yield were the highest at N2, N3, and N4 treatments for WYJ-24, YD-6, and YY-2640, respectively, even though the fully filled grain percentage and 1000-grain weight showed a slight decrease when more N was applied at SD (Table 1).

3.2 | Change in BRs contents and the correlation among plant N content, BRs contents, spikelet degeneration rate, and grain yield

Changes in the BRs contents (24-epiCS and 28-homoBL) in young panicles varied with N application rates and cultivars (Figure 2a–d). Both 24-epiCS and 28-homoBL contents initially increased in young panicles with N application rate, peaked in the N2 and N3 treatments in young panicles of WYJ-24 and YD-6 cultivars, respectively; these values then decreased sharply when higher N rate was applied. For YY-2640, 24-epiCS and 28-homoBL contents in young panicles significantly increased with N application rate and were the highest at the N4 treatment (Figure 2a–d).

The 24-epiCS and 28-homoBL contents in young panicles correlated with plant N content with inverted-U polynomial equations \( E = -40.7x^3 + 229x^2 - 356x + 207 \) and \( H = -97.6x^3 + 573x^2 - 964x + 583 \), respectively (where \( E \) is the 24-epiCS contents, \( H \) is the 28-homoBL content, and \( x \) is the plant N content; \( R^2 = 0.911 \) to 0.916, \( p = .01 \)) (Figure 3a,b). The spikelet degeneration rate correlated with the plant N content with a polynomial equation \( S = 2.84x^3 - 9.61x^2 - 8.96x + 46.1 \) (where \( S \) is the spikelet degeneration rate and \( x \) is plant N content; \( R^2 = 0.902, p = .01 \)) (Figure 3c), and correlated with the 24-epiCS and 28-homoBL contents in young panicles with exponential decay equations \( S = 1.22 + 48.8e^{-0.017E} \) and \( S = 4.57 + 55.9e^{-0.012H} \), respectively, where \( E \) is the 24-epiCS content and \( H \) is the 28-homoBL content.
28-homoBL; \( R^2 = 0.968 \) to 0.958, \( p = .01 \) (Figure 3d,e). The grain yield was significantly and positively correlated with 24-epiCS and 28-homoBL contents (0.611 to 0.972, \( p = .05 \) or 0.01), and negatively correlated with spikelet degeneration rate (−0.739 to −0.980, \( p = .05 \) or 0.01) (Table S3).

### 3.3 | Changes in enzyme activities involved in energy metabolism, ATP content, and EC, and their correlations with BRs contents and spikelet degeneration rate

The CCO and SDH activities, ATP and EC levels in young panicles were significantly and positively correlated with BRs (24-epiCS and 28-homoBL) contents \( (r = .917 \) to 0.980, \( p = .01 \)) (Table S3). Compared with no N fertilization at SD (N0), the application of N fertilizer at SD greatly increased CCO and SDH activities, ATP and EC levels in young rice panicles at PMCm; these values were the highest in the N2, N3, and N4 treatments for WYJ-24, YD-6, and YY-2640, respectively, in all the N treatments (Figure 4a–h). The activities of CCO and SDH, levels of ATP and EC were significantly and negatively correlated with spikelet degeneration rate \( (r = −.901 \) to −0.957, \( p = .01 \)) (Table S3).

### 3.4 | Changes in T-AOC and \( \text{H}_2\text{O}_2 \) content, OsVPE2 and OsVPE3 expression levels, and their correlations with BRs and spikelet degeneration rate

Similar to the CCO and SDH activities, and ATP and EC levels in response to N treatments, the T-AOC level in young panicles was significantly positively correlated with BRs contents
ZHANG et Al. (r = .974 to 0.979, p = .01) (Table S3). Compared with N0 treatment, the T-AOC level significantly increased at PMCm when N was applied at SD. Among all the N treatments, T-AOC level was the highest in N2, N3, and N4 treatment groups for WYJ-24, YD-6, and YY-2640, respectively (Figure 5a,b). In contrast to T-AOC level, the H2O2 content and OsVPE2 and OsVPE3 relative expression levels in young panicles at PMCm were significantly negatively correlated with BRs contents (r = −.839 to −0.905, p = .01) (Table S3) and were the lowest in the N2, N3, and N4 treatment groups for WYJ-24, YD-6, and YY-2640, respectively, in all the N treatments (Figure 5c–h). The T-AOC level was significantly negatively correlated with spikelet degeneration rate (r = −.937, p = .01), whereas the H2O2 content and OsVPE2 and OsVPE3 expression levels were significantly positively correlated with the spikelet degeneration rate (r = .907 to .939, p = .01) (Table S3).

3.5 | Effects of N treatments on WT and rice BRs-deficient mutants

The BRs contents, CCO and SDH activities, and ATP, EC, and T-AOC levels were significant lower in the BRs-deficient mutants (osd11#1 and osd11#2) panicles than
in WT panicles, whereas H₂O₂ content, intensity of the fluorescence emissions of ROS, OsVPE2, and OsVPE3 expression levels, and TUNEL signal in young panicles/spikelets, as well as the spikelet degeneration rate of WT plants. However, the N2 treatment had no significant effects on these factors in osd11#1 and osd11#2 plants (Figures 6a–c; 7a–d; 9a–f; 10a–l).

### 3.6 Effects of chemical regulators on WT and rice BRs-deficient mutant

Compared with no chemical application (CK), the application of exogenous BRs (24-epiCS + 28-homoBL) to WT and osd11#1 panicles significantly increased BRs (24-epiCS and 28-homoBL) contents, CCO and SDH activities, and ATP, EC, and T-AOC levels, but significantly decreased the H₂O₂ content, intensity of the fluorescence emissions of ROS, OsVPE2, and OsVPE3 expression levels, and TUNEL signal in young panicles/spikelets, as well as the spikelet degeneration rate of WT plants. However, the N2 treatment had no significant effects on these factors in osd11#1 and osd11#2 plants (Figures 6a–c; 7a–d; 9a–f; 10a–l).
levels of OsVPE2 and OsVPE3 in young panicles, and the spikelet degeneration rate compared with CK (Figure S2a–d).

4 | DISCUSSION

It is believed that N application at SD can prevent differentiated spikelets from degeneration and increase grain yield in rice (Kamiji, Yoshida, Palta, Sakuratani, & Shiraiwa, 2011; Zhang, Zhu, et al., 2019; Zhang et al., 2013). Therefore, a higher N rate applied at panicle development is strongly recommended in rice production to increase number of spikelets per panicle and grain yield (Ghaley, 2012; Kamiji et al., 2011). Our previous work also found that different N top-dressing times during panicle differentiation could regulate spikelet differentiation and degeneration in rice (Zhang, Zhu, et al., 2019). The present study further found that the effectiveness of N application rate at SD in decreasing spikelet degeneration varied largely with the rice cultivars differing in panicle size. With increased N rates, the spikelet degeneration rate decreased, while total spikelet number and grain yield increased in the cultivar YY-2640 with more differentiated spikelets on a panicle. On the other hand, spikelet degeneration rate increased and total spikelet number and grain yield decreased at N3 and/ or N4 treatment when compared with N2 and/ or N3 treatment in the WYJ-24 and YD-6 cultivars, which had less differentiated spikelets per panicle (Figure 1a–h; Table 1). These results suggest that a rice cultivar with more differentiated spikelets per panicle needs a higher N application rate at SD or more N accumulation at meiosis to prevent spikelet degeneration than a cultivar with less differentiated spikelets per panicle. However, too high N rate applied at SD or too more plant N accumulation at meiosis may not beneficial to inhibit spikelet degeneration and enhance grain yield in rice, especially in cultivars with more differentiated spikelets.

The mechanism by which effects of N application rates at SD on spikelet degeneration varies with rice cultivars with different panicle size is not well understood. Different N top-dressing times during panicle differentiation can induce the opposite variations in spikelet degeneration rate and BRs levels in young rice panicles (Zhang, Zhu, et al., 2019). Present results showed that different N rates applied at SD could regulate the BRs levels in young rice panicles at PMCm, and these BRs levels correlated with spikelet degeneration rate with exponential decay equations, as well as were significantly positively correlated with grain yield (Figures 2a–d; 3d,e; Table S3). Furthermore, the rice BRs-deficient mutants (osd11#1 and osd11#2) panicles with significant reduction in BRs contents and a higher spikelet degeneration rate compared with WT panicles (Figure S2a–d—WT, ZH11) under various nitrogen (N) treatments. N0 and N2 indicate the N rate applied at spikelet differentiation at 0 and 60 kg/ha, respectively. Vertical bars represent ± standard error of the mean (n = 6) where these exceed the size of the symbol. Different letters above the bars indicate the least significant difference at p = .05.
of WT plants, but had no distinct effects on the BRs contents of *osd11*#1 and *osd11*#2 panicles (Figure 6a–c). When exogenous BRs were applied to WT and *osd11*#1 panicles with no N fertilization at SD (N0), the BRs contents in young panicles at PMCm significantly increased and spikelet degeneration rate significantly decreased, especially in *osd11*#1 panicles (Figure S1a, b and Figure S2d). These results suggest that elevated BRs levels in young panicles play vital roles in reducing spikelet degeneration in rice through mediating the effect of N application rates at SD.

Interestingly, we observed that both spikelet degeneration rate and BRs contents in young panicles correlated with plant N content with polynomial relationship (Figure 3a–c). Such a relationship suggests that the higher BRs levels are in the young panicle, the smaller rate of spikelet degeneration. The maximum levels of BRs and the lowest spikelet degeneration

![Figure 7](image7.jpg)

**FIGURE 7** Changes in activities of cytochrome C oxidase (CCO) (a) and succinate dehydrogenase (SDH) (b), adenosine triphosphate (ATP) content (c), energy charge (d) in young panicles of rice BRs-deficient mutants (*osd11*#1 and *osd11*#2) and wild type (WT, ZH11) under various nitrogen (N) treatments. N0 and N2 indicate the N rate applied at spikelet differentiation at 0 and 60 kg/ha, respectively. Vertical bars represent ± standard error of the mean (n = 6) where these exceed the size of the symbol. Different letters above the bars indicate the least significant difference at p = .05.

![Figure 8](image8.jpg)

**FIGURE 8** Changes in total antioxidant capacity (T-AOC) (a), hydrogen peroxide (H₂O₂) content (b), and relative expression levels of *OsVPE2* and *OsVPE3* (c, d) in young panicles of rice BRs-deficient mutants (*osd11*#1 and *osd11*#2) and wild type (WT, ZH11) under various nitrogen (N) treatments. N0 and N2 indicate the N rate applied at spikelet differentiation at 0 and 60 kg/ha respectively. Vertical bars represent ± standard error of the mean (n = 6) where these exceed the size of the symbol. Different letters above the bars indicate the least significant difference at p = .05.
The rate can be achieved as a plant N content of 2.6% during meiosis. These results indicate that 2.6% N in plant can be used as a threshold for N top-dressing dosage at SD for increasing BRs levels in young panicles and inhibiting spikelet degeneration, while still increasing grain yield in rice.

ATP is the chemical energy for cellular metabolism and is often referred to as the “energy currency” of the cell. Moreover, an ATP deficiency can induce excessive ROS production, thereby leading to lipid membrane peroxidation. By contrast, exogenous ATP treatment can reduce cell membrane permeability and delay senescence in plants (Jin et al., 2013; Liu et al., 2016). SDH catalyzes the oxidation of succinate to fumarate, which is associated with the generation of ATP in mitochondria, as well as CCO, which is the last enzyme in the respiratory electron transport chain in mitochondria and plays a critical role in energy production (Jin et al., 2013; Liu et al., 2016). Our present data showed that the CCO and SDH activities, and ATP and EC levels were significantly positively correlated with the BRs content in panicles, and significantly negatively correlated with spikelet degeneration rates in plants treated with various N rates at SD (Figures 1g,h; 2a–d; 4a–h; Table S2). Moreover, rice BRs-deficient mutants (osd11#1 and osd11#2) plants showed significant higher spikelet degeneration rate and lower BRs contents, and were accompanied by lower CCO and SDH activities, and decreased levels of ATP and EC in young panicles than in WT panicles when treated with either N0 or N2 treatment. These parameters in WT panicles could be regulated, whereas in osd11#1 and osd11#2 panicles, they were not distinctly affected by N application (Figures 6a–c; 7a–d). Applying exogenous BRs to the WT and osd11#1 panicles with no N fertilization at SD (N0) significantly increased...
BRs contents, CCO and SDH activities, ATP and EC levels in young panicles at PMCm and consequently decreased spikelet degeneration rate (Figure S1a-f and Figure S2d). These results indicate that properly N applied at SD and/or when plant N content is 2.6% during meiosis, BRs levels increase and could reduce spikelet degeneration by elevating the energy status in young rice panicles at PMCm.

It has been proposed that spikelet degeneration is mainly triggered by excessive ROS production that can result in PCD in young rice spikelets (Ali et al., 2019; Heng et al., 2018; Zhang, Sheng, et al., 2019). There are reports showing that excessive H2O2 production and accumulation can induce PCD by activating OsVPE2 and OsVPE3 expressions in young rice panicles (Heng et al., 2018; Zhang, Sheng, et al., 2019). Endogenous antioxidant systems, including enzymatic and nonenzymatic groups, can be promoted by exogenous BRs application (Kartal, Temel, Arican, & Gozukirmizi, 2009; Wu, Zhang, Ervin, Yang, & Zhang, 2017; Zhang, Zhu, et al., 2019). Our results show that during meiosis in young rice panicles, the H2O2 content and OsVPE2 and OsVPE3 expression levels were significantly and negatively correlated with BRs contents and significantly and positively correlated with spikelet degeneration rate. Conversely, T-AOC was positively correlated with BRs contents in these plants (Figures 1g,h; 2a–d; 5a–h; Table S3). Further evidences suggested that there were substantial reduction in T-AOC and H2O2 burst in rice BRs-deficient mutants (osd11#1 and osd11#2) panicles, which showed significant increase in the intensity of the fluorescence emissions of ROS, OsVPE2, and OsVPE3 expression levels, TUNEL signal (a direct indicator for PCD) and spikelet degeneration rate compared to WT panicles in both N0 and N2 treatments. Furthermore, these parameters in WT panicles could be regulated by N application, whereas in osd11#1 and osd11#2 panicles, there was no distinct affect observed with N application (Figures 6a–c; 8a–d; 9a–l; 10a–f). Applying BRs to rice panicles during meiosis significantly increased the T-AOC level and decreased H2O2 content, OsVPE2 and OsVPE3 expression levels, as well as the spikelet degeneration rate; however, the opposite effects were observed when H2O2 was applied to young rice panicles (Figure S1g and Figure S2a–d). These results demonstrate that properly N applied at SD and/or a plant N content of 2.6% during meiosis could increase BRs levels and enhance antioxidant capacity in young rice panicles to protect differentiated spikelets from degeneration through mitigating the deleterious effects of ROS during meiosis.

5 | CONCLUSIONS

Increased BRs contents in young rice panicles during meiosis can decrease spikelet degeneration rate. Responses of BRs levels in the young panicles to N application rates at SD vary with the rice cultivars differing in the spikelet number on the panicle. A plant N content of 2.6% can be used as a dosage threshold for N top-dressing at SD that can increase BRs levels in young panicles during meiosis and consequently reduce spikelet degeneration and increase grain yield in rice. BRs mediate the effects of N application rates at SD on the rice spikelet degeneration by regulating the antioxidant capacity and energy status in the panicles during meiosis.

ACKNOWLEDGEMENTS

We are grateful for grants from National Natural Science Foundation of China (31901445, 31771710, 31461143015, 31901444), the National Key Research and Development Program of China (2016YFD0300206-4; 2018YFD0300800; 2016YFD0305020), the Project funded by China Postdoctoral Science Foundation (2018M640528), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Top Talent Supporting Program of Yangzhou University (2015-01), the Council of Hong Kong Baptist University (1806439), the Open Project from Joint International Research Laboratory of Agriculture and Agri-Product Safety of Yangzhou University (JRK2018004) and the Training Programs of Innovation and Entrepreneurship for Undergraduates of Jiangsu Province (201911117010Z).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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**SUPPORTING INFORMATION**

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

**How to cite this article:** Zhang W, Fu L, Men C, et al. Response of brassinosteroids to nitrogen rates and their regulation on rice spikelet degeneration during meiosis. *Food Energy Secur*. 2020;00:e201. https://doi.org/10.1002/fes3.201