Involvement of cytokinins in the grain filling of rice under alternate wetting and drying irrigation

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Abstract

Cytokinins may reflect soil water status and regulate rice (Oryza sativa L.) grain filling. This study investigated the changes in cytokinin levels in rice plants and their relations with grain filling under alternate wetting and drying irrigation. Two ‘super’ rice cultivars were field grown. Three irrigation regimes, alternate wetting and moderate soil drying (WMD), alternate wetting and severe soil drying (WSD), and conventional irrigation (CI, continuously flooded), were imposed after flowering. No significant differences in grain-filling rate, grain weight, and cytokinin content were observed for the earlier-flowering superior spikelets among the three irrigation regimes. For the later-flowering inferior spikelets, however, their grain-filling rate and grain weight were significantly increased in the WMD and significantly reduced in the WSD when compared with those in the CI. Cytokinin contents in shoots (inferior spikelets and the flag leaves) in the WMD at the soil drying time were comparable with those in the CI, but they were significantly increased when plants were rewatered. The WSD significantly reduced cytokinin contents in the shoot either during soil drying or during the rewatering period. Cytokinin contents in roots showed no significant difference between the WMD and CI regimes. The WSD increased \textit{trans}-zeatin-type cytokinins, whereas it reduced isopentenyladenine-type cytokinins, in roots. Grain-filling rate and grain weight of inferior spikelets were very significantly correlated with cytokinin contents in these spikelets. The results suggest that a post-anthesis WMD holds great promise to improve grain filling of inferior spikelets through elevating cytokinin levels in the rice shoot.

Key words: Cytokinins, grain filling, inferior spikelets, soil drying, superior spikelets, super rice.

Introduction

Conventional flooded rice (Oryza sativa L.) cultivation provides more than 75% of the world rice supply for half of the earth’s main staple food, and also consumes about 80% of the total irrigated fresh water resources in Asia (Bouman and Tuong, 2001; Bouman, 2007). To meet the major challenge that rice production needs to increase to feed a growing population under increasing scarcity of water resources, alternate wetting and drying (AWD) irrigation has been developed as a novel water-saving technique and adopted in many countries such as China, Bangladesh, India, and Vietnam (Tuong et al., 2005; Bouman, 2007; Yang et al., 2007; Zhang et al., 2008, 2009b). This technique, characterized by its alternation of periods of soil submergence with periods of non-submergence during the growing season, could substantially reduce irrigation water and lead to an improvement in water use efficiency (WUE) (Bouman and Tuong, 2001; Belder et al., 2004, 2005; Zhang et al., 2008). It has been reported that, when compared with continuously submerged conditions, AWD irrigation can maintain or even increase grain yield (Tuong et al., 2005; Yang et al., 2007; Zhang et al., 2008, 2009b). On the other hand, there are reports that AWD irrigation often reduces, rather than increases, grain yield (Tabbal et al., 2002; Belder et al., 2004). The biochemical mechanism underlying the
increase or reduction in grain yield under AWD irrigation has not yet been understood.

Cytokinins are a class of plant hormones known as key regulators of plant growth and development, including cell division, chloroplast biogenesis, bud and root differentiation, shoot meristem initiation and growth, stress tolerance, and organ senescence (Argueso et al., 2009; Kuroha et al., 2009). It is reported that water stress significantly reduces cytokinin levels in the root exudates and leaves of rice and wheat (Triticum aestivum L.), which are closely associated with plant senescence (Yang et al., 2002b, 2003b). However, very little is known about changes in cytokinins in rice plants and their relationships with yield formation under AWD irrigation.

Grain filling is the final growth stage in cereals when fertilized ovaries develop into caryopses. The degree and rate of grain filling in rice spikelets differ largely with their positions on a panicle. In general, earlier-flowering superior spikelets, usually located on apical primary branches, fill fast and produce larger and heavier grains. However, later-flowering inferior spikelets, usually located on proximal secondary branches, are either sterile or fill slowly and poorly to produce grains unsuitable for human consumption (Mohapatra et al., 1993; Yang et al., 2000). The slow grain-filling problem in inferior spikelets is more aggravated in the newly bred ‘super’ rice cultivars (Zhang et al., 2009c; Yang and Zhang, 2010a). Several studies have shown that variations in grain filling between the superior and inferior spikelets are mediated by plant hormones (Yang et al., 2003a, 2006) and cytokinin levels in rice spikelets are significantly correlated with seed development (Yang et al., 2002a; Zhang et al., 2009a). It is important to know if and how AWD irrigation alters cytokinin levels in rice plants and, consequently, regulates grain filling of inferior spikelets.

The objective of this study was to investigate the changes in cytokinin levels in rice plants and their relationships with grain filling of superior and inferior spikelets under AWD irrigation during the grain-filling period. As biological important cytokinins in most higher plants are isopentenyladenine (iP)-type and trans-zeanin (Z)-type cytokinins (Matsumoto-Kitano et al., 2008), iP, isopentenyladenosine (iPR), Z, and trans-zeanin riboside (ZR) were determined. Another plant hormone, indole-3-acetic acid (IAA), was also measured because auxins and cytokinins interact in the regulation of many physiological processes, including those likely to be involved in the recovery from stress (Havlová et al., 2008).

Materials and methods

Plant materials and cultivation

Field experiments were conducted at a research farm of Yangzhou University, Jiangsu Province, China (32°30’ N, 119°25’ E, 21 m altitude) during the rice growing season (May–October) of 2007 and 2008. The soil was a sandy loam (Typic fluvaquents, Entisols, US classification) that contained 24.5 g kg⁻¹ organic matter, 103 mg kg⁻¹ alkali hydrolysable N, 34.1 mg kg⁻¹ Olsen-P, and 68.2 mg kg⁻¹ exchangeable K. The field capacity soil moisture content, measured after constant drainage rate and made gravimetrically, was 0.189 g g⁻¹, and bulk density of the soil was 1.34 g cm⁻³. The total precipitation during the grain-filling period was 40.5 mm in 2007 and 44.6 mm in 2008. The mean solar radiation during grain filling was approximately 18.2 MJ m⁻² d⁻¹ in 2007 and 17.8 MJ m⁻² d⁻¹ in 2008.

Two ‘super’ rice (Oryza sativa L.) cultivars currently used in local production, HD-9 (Huaidao 9, japonica) and LY-9 (Lian-goupeijiu, an indica F1 hybrid), were grown in the field. Seedlings were raised in the field with a sowing date of 10 May and transplanted on 10 June at a hill spacing of 0.2×0.2 m with two seedlings per hill. N (60 kg ha⁻¹ as urea, P (30 kg ha⁻¹ as single superphosphate), and K (40 kg ha⁻¹ as KCl) were applied and incorporated before transplanting. N as urea was also applied at mid-tillering (40 kg ha⁻¹) and at panicle initiation (25 kg ha⁻¹). Both cultivars (50% of plants) headed on 20–22 August, and were harvested on 15 October. Except for drainage at the end of tillering (11–15 July), the water level in the field was kept at 1–2 cm until 6 d after heading, when AWD irrigation treatments were initiated.

Treatments

The experiments were laid out in a completely randomized block design with three replicates. Plot dimensions were 5×4 m and plots were separated by an alley 1 m wide with plastic film inserted into the soil to a depth of 50 cm to form a barrier. Treatments consisted of three irrigation regimes including alternate wetting and moderate soil drying (WMD), alternate wetting and severe soil drying (WSD), and conventional irrigation (CI), and were applied from 6 d after heading to maturity. In the WMD regime, fields were not irrigated until the soil water potential had reached –25 kilopascal (kPa) (soil moisture content 0.161 g g⁻¹) at 15–20 cm depth. While in the WSD regime, water was withheld until the soil water potential reached –50 kPa (soil moisture content 0.136 g g⁻¹) at 15–20 cm depth. The CI regime maintained plots with a continuous flood of 2–3 cm water depth until one week before harvest as are recommended farming practices. Soil water potentials of –25 kPa in the WMD regime and –50 kPa in the WSD regime were chosen as previous studies have shown that a mild soil-drying (a soil water potential of –25 kPa at 15–20 cm depth) during the grain-filling period did not seriously reduce grain yield, whereas a severe soil-drying (a soil water potential of –50 kPa) reduced yield by as much as 30% when compared with the CI regime (Yang et al., 2007; Zhang et al., 2008). Four tensionmeters were installed in each plot, and readings were recorded at 12:00 h each day. When soil water potential reached the threshold, a flood with 2.0–2.5 cm water depth was applied to the plots. The amount of irrigation water was monitored with a flow meter (LXSQ-50 Flow meter, Shanghai Water Meter Manufacturing Factory, Shanghai, China) installed in the irrigation pipelines. A rain shelter consisting of a steel-frame covered with a plastic sheet was used in each block to minimize the effect of rainfall precipitation on the treatments, and was moved off after the rain.

Determination of grain-filling rate

Two hundred panicles that headed on the same day were chosen and tagged for each plot. The flowering date and the position of each spikelet on the tagged panicles were recorded. Ten to twelve tagged panicles from each plot were sampled at 4 d intervals from anthesis to maturity. Superior spikelets that flowered on the first 2 d within a panicle and inferior ones that flowered on the last 2-d within a panicle were separated from the sampled panicles, which consisted of 120–150 spikelets (grains) at each sampling time. The difference in flowering date between superior and inferior spikelets was 3 d within a panicle. Sampled spikelets were dried at 70 °C to constant weight, dehulled, and weighed. The processes of grain filling were fitted by Richards’ growth equation (Richards, 1959) as described by Zhu et al. (1988):
Grain-filling rate \((R)\) was calculated as the derivative of the equation (1):

\[
R = \frac{AkBe^{-kt}}{N(1+Be^{-kt})^2}
\]

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

Leaf water potential and photosynthetic rate measurement

For leaf water potential measurement, six flag leaves were sampled from each treatment at 0 (the initial of the treatments) and 11, 22, and 34 d after treatments (DAT) when soil water potential was approximately –25 kPa in the WMD regime and –50 kPa in the WSD regime (Fig. 1). Leaf water potentials were measured at midday (11.30 h). A pressure chamber (Model 3000, Soil Moisture Equipment Corp., Santa Barbara, CA, USA) was used for leaf water potential measurement.

The photosynthetic rate of the flag leaves was determined at 11 (D1) and 22 (D2) DAT when soil water potential was approximately –25 kPa in the WMD regime and –50 kPa in the WSD regime. Photosynthesis was measured as the rate of carbon dioxide uptake by the leaves above the canopy using a gas exchange analyser (Li-Cor 6400 portable photosynthesis measurement system, Li-Cor, Lincoln, NE, USA) from 09.00 to 11.00 h when photosynthetic active radiation above the canopy was 1300–1500 \(\mu\)mol m\(^{-2}\) s\(^{-1}\). The measurement was made on the upper surface of each flag leaf, using six leaves from each treatment.

Hormonal extraction, purification, and quantification

Hormones in roots, the flag leaves, and both superior and inferior spikelets were determined at D1, D2, W1, and W2. The roots in soil were dug out by a spade (the soil volume around roots was 20×20×20 cm). The roots were carefully rinsed and detached from their nodal bases. Both the fresh weight and dry weight (dried in an oven at 70 °C) of roots were calculated.

The methods for extraction and purification of Z, ZR, iP, iPR, and IAA were modified from those described by Bollmark et al. (1988) and Yang et al. (2001, 2002a). Samples consisting of 1–2 g dehulled and frozen grains with their embryos removed, or 2–3 g frozen leaves/roots, were ground in an ice-cold mortar in 5–10 ml 80\% (v/v) methanol extraction medium containing 1 mM butylated hydroxytoluene (BHT) as an antioxidant. The extract was incubated at 4 °C for 4 h and centrifuged at 5000 g for 15 min at the same temperature. The supernatants were passed through Chromosep C18 columns (C18 Sep-Pak Cartridge, Waters Corp., Millford, MA, USA), prewashed with 10 ml 100\% (v/v) 80\% (v/v) methanol, and 5 ml 80\% (v/v) methanol, respectively. About 5 ml of the purified fraction containing cytokinins and auxins was collected and dried under 

Cytokinin oxidase/dehydrogenase activity assay

To measure cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) activity, leaves and roots were cut into small pieces. Samples consisting of 0.8–1.2 g dehulled grains or 1.5–2.0 g leaves/roots were powdered with liquid nitrogen using a pestle and mortar and extracted with a 1.5-fold excess (v/w) of 0.2 M TRIS-HCl buffer, pH 8.0, containing 1 mM phenylmethylsulphonyl fluoride and 3\% Triton X-100. Tissue debris was removed by centrifugation at 12 000 g for 10 min. The extract was loaded onto a Sephadex G-25 (50×2.5 cm) column equilibrated with 0.1 M TRIS-HCl, pH 8.0, to remove the low molecular mass fraction. The protein fraction was then used to assay CKX activity. The assay was performed according to the method described by Frébort et al. (2002), with some modifications. Samples were incubated in a reaction mixture (total volume 0.6 ml in an Eppendorf tube) of 100 mM McIlvaine.
buffer, pH 6.0, 0.5 mM electron acceptor 2,3-dimethoxy-5-methyl-1,4-benzoquinone, and 0.25 mM substrate iPR for 2–10 h at 37 °C. For determination of specific activities, protein contents in the samples were assayed according to Bradford (1976) with bovine serum albumin as a standard.

Final harvesting
All plants were harvested on 14–15 October. Grain yield was determined from all plants from a 5 m² site (except border plants) in each plot and adjusted to a moisture content of 0.14 g H₂O g⁻¹ fresh weight. Above-ground biomass and yield components, i.e. total number of spikelets m⁻², the percentage of filled grains, and grain weight, were determined from plants of a 1 m² site (excluding the border ones) sampled randomly from each plot. The percentage of filled grains was defined as the filled grains (specific gravity ≃1.06 g cm⁻³) as a percentage of the total number of spikelets. The harvest index was expressed as the grain yield over total above-ground biomass. WUE for irrigation was defined as the ratio of grain yield over the amount of irrigation water during the whole rice growing season.

Exogenous cytokinin application
Both the cultivars were used for exogenous cytokinin application. Plants were grown in eight cement tanks in open field conditions. Each tank (0.3 cm height, 1.6 m width, and 8.8 m length) was filled with sandy loam soil with the same nutrient contents as the field experiment. Thirty-day-old seedlings raised in the field were transplanted on 12 June into the tanks at a hill spacing of 0.20×0.20 m with two seedlings per hill. N (6 g m⁻² as urea), P (3 g m⁻² as single superphosphate), and K (3 g m⁻² as KCl) were applied and incorporated before transplanting. N as urea was also applied at mid-tillering (3 g m⁻²) and at panicle initiation (3 g m⁻²). The water level in the tank was kept at 1–2 cm until 6 d after heading, when irrigation treatments were initiated. Two irrigation regimes, CI and WSD, were conducted from 6 d after heading to maturity. The treatment methods for both CI and WSD regimes were the same as those in the field.

Starting at the initial of irrigation treatments, 50 μM kinetin (6-β-furfurylaminopurine, from Sigma, St Louis, MO, USA) was sprayed on the leaves and panicles in both the CI and WSD regimes. The chemical was applied daily for 8 d at the rate of 100 ml m⁻² at each application. Preparation of the chemical solution was described previously by Zhang et al. (2009a). The solution contained ethanol and Tween 20 at final concentrations of 0.1% (v/v) and 0.01% (v/v), respectively. Plants sprayed with the same volume of deionized water containing the same concentrations of ethanol and Tween 20 were taken as a control. The chemical treatment was an area of 3 m² with four replications.

For each treatment, spikelets on a panicle were divided into two groups, i.e. the superior and the inferior. Superior spikelets were those that flowered on the first two days and inferior ones were those flowered on the last two days on a panicle. Grain weight was determined at 6 d intervals from anthesis to maturity. The measurement method was the same as described above. Twenty plants (172–176 panicles) for 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. Measurements were made at midday (11.30 h) when soil water potentials were –25 kPa in the WMD regime and –50 kPa in the WSD regime. Data are averages observed for the two study years because they behaved the same. Vertical bars represent ± standard error of the mean (n=12) where these exceed the size of the symbol.

Results

Soil and leaf water potentials

It took 5–9 d to reach a soil water potential of –25 kPa in the WMD regime and 11–13 d to reach a soil water potential of –50 kPa in the WSD regime (Fig. 1A, B). For plants grown in the CI regime, midday (11.30 h) leaf water potentials decreased gradually during grain filling, from approximately –0.76 MPa at the beginning of the measurements to about –1.26 MPa on 35 DAT (Fig. 2A, B). Leaf water potentials were reduced as soil water potential decreased, and ranged from –1.09 to –1.49 MPa when the soil water potential was –25 kPa in the WMD regime and from –1.38 to –2.07 MPa when the soil water potential was –50 kPa in the WSD regime. The two cultivars showed similar trends in leaf water potential as a function of soil water potential (Fig. 2A, B). As midday leaf water potential lower than –1.5 MPa during grain filling could not inhibit

Fig. 2. Changes in leaf water potentials the flag leaf of rice. Both japonica cultivar HD-9 (A) and indica hybrid LY-9 (B) were field-grown. CI, WMD, and WSD indicate conventional irrigation, alternate wetting and moderate soil drying, and alternate wetting and severe soil drying, respectively, during grain filling (6–48 d after heading). Measurements were made at midday (11.30 h) when soil water potentials were –25 kPa in the WMD regime and –50 kPa in the WSD regime. Data are averages observed for the two study years because they behaved the same. Vertical bars represent ± standard error of the mean (n=12) where these exceed the size of the symbol.
rice growth (Cai et al., 2002; Yang et al., 2007), the results indicate that a moderate soil-drying during the grain-filling period would not seriously affect plant water status.

**Leaf photosynthetic rate and grain-filling rate**

The photosynthetic rate of the flag leaf showed no significant difference between the CI and WMD regimes when soil water potential was −25 kPa (D1, D2), but it was greater in the WMD regime than in the CI regime when plants were rewatered (W1, W2) (Fig. 3A, B). Compared with the CI regime, the WSD regime significantly reduced photosynthetic rates either during soil drying (D1, D2) or during the rewatering period (W1, W2), with a greater reduction at a soil water potential of −50 kPa. The two cultivars behaved the same.

The grain-filling process, mean grain-filling rate, active grain-filling period, and final grain weight of superior spikelets showed no significant difference among the three irrigation regimes (Fig. 4A, B; Table 1). However, The WMD regime markedly accelerated, whereas the WSD regime significantly decreased, grain-filling rate of inferior spikelets when compared with the CI regime. The WSD regime significantly shortened, while the WMD regime little affected, the active grain-filling period of inferior spikelets. As a result, the WMD regime significantly increased the grain weight of inferior spikelets, and the WSD regime exhibited an opposite effect. In the same irrigation regime and for the same type of spikelets, the cultivar HD-9 showed a greater grain-filling rate and grain weight than the cultivar LY-9 (Table 1).

![Fig. 3](image-url). The photosynthetic rate of the flag leaf of rice. Both japonica cultivar HD-9 (A) and indica hybrid LY-9 (B) were field-grown. CI, WMD, and WSD indicate conventional irrigation, alternate wetting and moderate soil drying, and alternate wetting and severe soil drying, respectively, during grain filling (6–48 d after heading). D1 and D2 are the measurement time at 11 d and 22 d after treatment, respectively, when soil water potentials were −25 kPa in the WMD regime and −50 kPa in the WSD regime, and W1 and W2 are the measurement time at 13 d and 24 d after treatment, respectively, when plants were rewatered. Data are averages observed for the two study years because they behaved the same. The asterisk (*) represents significant difference (P=0.05) between the WMD regime and the CI regime or between the WSD regime and the CI regime. Vertical bars represent ± standard error of the mean (n=6) where these exceed the size of the symbol.

![Fig. 4](image-url). Grain-filling processes of the superior and inferior spikelets of rice. Both japonica cultivar HD-9 (A) and indica hybrid LY-9 (B) were field-grown. CI, WMD, and WSD indicate conventional irrigation, alternate wetting and moderate soil drying, and alternate wetting and severe soil drying, respectively, during grain filling (6–48 d after heading). Data are averages observed for the two study years because they behaved the same. Vertical bars represent ± standard error of the mean (n=6) where these exceed the size of the symbol.
Grain yield and WUE

As the irrigation treatments started after heading, neither the panicle number m⁻² nor the spikelets per panicle were influenced by the treatments (data not shown), and the differences in sink size (total number of spikelets m⁻²) among the three treatments were insignificant (Table 2). However, grain yield varied significantly with the treatments. Compared with those in the CI regime, the percentage of filled grains, grain weight (mean weight of all the filled grains on a panicle), and grain yield in the WMD regime were significantly increased. However, they were all markedly reduced in the WSD regime. The harvest index was significantly increased in both WMD and WSD regimes, with the latter increased more than the former (Table 2), indicating that soil drying during grain filling enhances the partitioning of assimilates from vegetative tissues to grains.

Water application after heading was 104–113 mm to the WMD regime and 58–74 mm to the WSD regime, which is 36–44% more ZR than Z and 16–22% more iPR than iP. Contents of Z and ZR, or iP and iPR showed a similar pattern of change during grain filling (data not shown). At the same measurement time, there was no significant difference in Z+ZR contents in superior spikelets among the irrigation treatments (Fig. 5A, C). Z+ZR contents in inferior spikelets, however, differed largely with irrigation treatments and the measurement time. In the WMD regime and when soil water potential was ~25 kPa (D1, D2), Z+ZR contents in inferior spikelets was comparable with those in the CI regime. The WMD regime significantly increased Z+ZR contents in inferior spikelets at the rewatering time (W1, W2). While the WSD regime regime significantly increased WUE (grain yield over the amount of irrigation water during the whole rice growing season), while WUE in the WSD regime showed no significant difference when compared with that in the CI regime (Table 3).

Cytokinin levels in superior and inferior spikelets

Both superior and inferior spikelets, leaves, and roots contained 36–44% more ZR than Z and 16–22% more iPR than iP. Contents of Z and ZR, or iP and iPR showed a similar pattern of change during grain filling (data not shown). At the same measurement time, there was no significant difference in Z+ZR contents in superior spikelets among the irrigation treatments (Fig. 5A, C). Z+ZR contents in inferior spikelets, however, differed largely with irrigation treatments and the measurement time. In the WMD regime and when soil water potential was ~25 kPa (D1, D2), Z+ZR contents in inferior spikelets was comparable with those in the CI regime. The WMD regime significantly increased Z+ZR contents in inferior spikelets at the rewatering time (W1, W2). While the WSD regime

Table 1. Effects of alternate wetting and soil drying during grain filling on the mean grain-filling rate, active grain-filling period, and grain weight of superior and inferior spikelets of rice

Both japonica cultivar HD-9 and indica hybrid LY-9 were field-grown. CI, WMD, and WSD indicate conventional irrigation, alternate wetting and moderate soil drying, and alternate wetting and severe soil drying, respectively, during grain filling (6–48 d after heading). The mean grain-filling rate and active grain-filling period were calculated according to Richards’ growth equation (Richards, 1959). The data are averages observed for the two study years, and are expressed as means ± standard error (n=3). Different letters indicate statistical significance at the P=0.05 level within the same column and within the same cultivar.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Mean grain-filling rate (mg kernel⁻¹ d⁻¹)</th>
<th>Active grain filling period (d)</th>
<th>Grain weight (mg grain⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Superior</td>
<td>Inferior</td>
<td>Superior</td>
</tr>
<tr>
<td>HD-9</td>
<td>CI</td>
<td>1.67±0.12 a</td>
<td>0.64±0.011 b</td>
<td>16.2±0.5 a</td>
</tr>
<tr>
<td></td>
<td>WMD</td>
<td>1.68±0.15 a</td>
<td>0.71±0.014 a</td>
<td>16.0±0.7 a</td>
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<td></td>
<td>WSD</td>
<td>1.68±0.21 a</td>
<td>0.60±0.012 c</td>
<td>15.7±0.8 a</td>
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<tr>
<td>LY-9</td>
<td>CI</td>
<td>1.52±0.11 a</td>
<td>0.59±0.012 b</td>
<td>15.8±0.6 a</td>
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<tr>
<td></td>
<td>WMD</td>
<td>1.56±0.14 a</td>
<td>0.63±0.013 a</td>
<td>15.6±0.5 a</td>
</tr>
<tr>
<td></td>
<td>WSD</td>
<td>1.54±0.17 a</td>
<td>0.53±0.011 c</td>
<td>15.3±0.7 a</td>
</tr>
</tbody>
</table>

Table 2. Effects of alternate wetting and soil drying during grain filling on grain yield, above-ground biomass, and harvest index of rice

Both japonica cultivar HD-9 and indica hybrid LY-9 were field-grown. CI, WMD, and WSD indicate conventional irrigation, alternate wetting and moderate soil drying, and alternate wetting and severe soil drying, respectively, during grain filling (6–48 d after heading). Values of total number of spikelets m⁻², percentage of filled grains, grain weight, and above-ground biomass were means of plants harvested from 6 m² of each treatment. The grain yield was the means from 30 m² of each treatment. The harvest index is calculated as the grain yield over total above-ground biomass. The data are averages observed for the two study years, and are expressed as means ± standard error (n=3). Different letters indicate statistical significance at the P=0.05 level within the same column and within the same cultivar.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Total number of spikelets (×10⁶ m⁻²)</th>
<th>Filled grain (%)</th>
<th>Grain weight (mg grain⁻¹)</th>
<th>Grain yield (g m⁻²)</th>
<th>Above-ground biomass (g m⁻²)</th>
<th>Harvest index (kg kg⁻¹)</th>
</tr>
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<tr>
<td>HD-9</td>
<td>CI</td>
<td>53.8±2.3 a</td>
<td>76.5±1.8 b</td>
<td>27.2±0.5 b</td>
<td>1109±51 b</td>
<td>2249±115 a</td>
<td>0.493±0.004 b</td>
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<td></td>
<td>WMD</td>
<td>53.5±1.8 a</td>
<td>81.4±2.1 a</td>
<td>28.4±0.4 a</td>
<td>1228±64 a</td>
<td>2398±127 a</td>
<td>0.512±0.005 a</td>
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<td>WSD</td>
<td>53.4±2.2 a</td>
<td>72.3±1.6 c</td>
<td>25.1±0.6 c</td>
<td>971±43 c</td>
<td>1878±103 b</td>
<td>0.517±0.003 a</td>
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<tr>
<td>LY-9</td>
<td>CI</td>
<td>55.9±2.3 a</td>
<td>72.9±2.2 b</td>
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<td>989±35 b</td>
<td>2043±89 a</td>
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<td></td>
<td>WMD</td>
<td>55.8±2.8 a</td>
<td>77.6±2.4 a</td>
<td>25.4±0.3 a</td>
<td>1070±48 a</td>
<td>2154±97 a</td>
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<td>WSD</td>
<td>55.3±2.4 a</td>
<td>68.4±1.5 c</td>
<td>22.9±0.5 c</td>
<td>856±37 c</td>
<td>1685±78 b</td>
<td>0.508±0.005 a</td>
</tr>
</tbody>
</table>
significantly reduced Z+ZR contents in inferior spikelets either at a soil water potential of -50 kPa (D1, D2) or at the rewatering time (W1, W2).

Changes of iP+iPR contents in both superior and inferior spikelets under different irrigation treatments were very similar to those of Z+ZR contents (Fig. 5B, D). However,
iP+iPR contents were much lower than Z+ZR contents, and were about 50% of the latter. In the same irrigation regime, at the same measurement time, and for the same type of spikelets, the cultivar HD-9 contained more Z+ZR and iP+iPR than the cultivar LY-9 (Fig. 5A–D), which was consistent with a greater grain-filling rate and grain weight for HD-9 than for LY-9 (Tables 1, 2; Fig. 4A, B).

Cytokinin levels in the flag leaf and roots

At each measurement time and for both cultivars, the WSD regime significantly reduced Z+ZR or iP+PR contents in the flag leaf, with a greater reduction at a soil water potential of –50 kPa, when compared with the CI regime (Fig. 6A–D). Both Z+ZR and iP+iPR contents in the leaves showed no significant differences between CI and WMD regimes when soil water potential was –25 kPa (D1, D2), but they were greater when plants were rewatered in the WMD regime. The changes of Z+ZR or iP+iPR contents in the leaves under different treatments were closely associated with those of the photosynthetic rate (refer to Fig. 3). Regression analysis showed that the photosynthetic rate was significantly correlated with both Z+ZR contents (r=0.97**, P=0.01) and iP+iPR contents (r=0.98**, P=0.01).

For both cultivars, the differences in Z+ZR or iP+iPR contents in roots between CI and WMD regimes were not significant either at the soil drying or at the rewatering time. In the WSD regime and at all the measurement time, Z+ZR contents were significantly increased, whereas iP+iPR contents were significantly decreased, in roots when compared with those in the CI regime (Fig. 7A–D).

CKX activity in plant organs

To understand better the responses of cytokinins to various irrigation treatments, the activity of CKX, an enzyme responsible for cytokinin degradation (Kuroha et al., 2009), was determined. As shown in Fig. 8A and B, there were no significant differences in CKX activity in superior spikelets among the treatments at each measurement time for both cultivars. For inferior spikelets and the flag leaf, CKX activity showed a similar changing pattern at the same irrigation regime and at the same measurement time (Fig. 8C–F). With an exception that CKX activities were significantly decreased at the soil drying time in the WSD regime, no significant difference in CKX activities was observed either in inferior spikelets or in the leaves among the three treatments.

When compared with the CI regime, the WSD regime significantly reduced, whereas the WMD regime little affected, CKX activity in roots either at the soil drying or at the rewatering time (Fig. 8G, H).

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Fig. 6. Contents of trans-zeatin+trans-zeatin riboside (Z+ZR) (A, C) and isopentenyladenine+isopentenyladenosine (iP+iPR) (B, D) in the flag leaf of rice. Both japonica cultivar HD-9 (A, B) and indica hybrid LY-9 (C, D) were field-grown. CI, WMD, and WSD indicate conventional irrigation, alternate wetting and moderate soil drying, and alternate wetting and severe soil drying, respectively, during grain filling (6–48 d after heading). D1 and D2 are the measurement time at 11 d and 22 d after treatment, respectively, when soil water potentials were –25 kPa in the WMD regime and –50 kPa in the WSD regime, and W1 and W2 are the measurement time at 13 d and 24 d after treatment, respectively, when plants were rewatered. Data are averages observed for the two study years because they behaved the same. The asterisk (*) represents significant difference (P=0.05) between the WMD regime and the CI regime or between the WSD regime and the CI regime. Vertical bars represent ±standard error of the mean (n=6) where these exceed the size of the symbol.
Changes in IAA contents

Similar to cytokinin levels in superior spikelets, IAA contents in superior spikelets showed no significant difference among the treatments and at each measurement time (Fig. 9A, B). Except at 22 DAT (D2) and 24 DAT (W2) at which IAA contents in both inferior spikelets and leaves were significantly reduced in the WSD regime, no significant difference in IAA contents in both organs was observed among the treatments (Fig. 9C–F). At either the soil drying or the rewatering time, IAA contents in roots were significantly increased in the WSD regime, while they changed little in the WMD regime, when compared with those in the CI regime. The two cultivars behaved the same (Fig. 9G, H).

Correlation between contents of cytokinins/IAA and grain-filling rate

Because no significant differences in grain-filling rate, grain weight, or contents of cytokinins and IAA were observed for superior spikelets among the treatments, the relationship between the hormonal levels and grain filling was only analysed for inferior spikelets. As shown in Table 4, the mean grain-filling rate and the grain weight of inferior spikelets were very significantly and positively correlated with both Z+ZR and iP+iPR contents in these spikelets at each measurement time ($r = 0.90^{**}$ to $0.99^{**}$, $P = 0.01$). The correlations of IAA in inferior spikelets with the mean grain-filling rate and final grain weight were insignificant ($r = 0.52–0.79$, $P > 0.05$) (Table 5).

Effect of exogenous cytokinin application

To verify the role of cytokinins in grain filling, a synthetic cytokinin, kinetin, was applied at the early grain-filling stage (6–13 d after heading) in both CI and WSD regimes. As shown in Table 5, the application of kinetin significantly increased the grain-filling rate and the grain weight of inferior spikelets either in the CI regime or in the WSD regime, with a greater increase in the WSD regime than in the CI regime, when compared with the respective control (plants received deionized water). There were no significant differences in grain-filling rate and grain weight of superior spikelets when kinetin was applied to plants in both the CI and WSD regimes, which may be explained by cytokinin levels in superior spikelets being sufficient during grain filling and these spikelets would be less sensitive/or even not respond exogenous cytokinin application under such a condition.
Fig. 8. Cytokinin oxidase/dehydrogenase (CKX) activity in superior spikelets (A, B), inferior spikelets (C, D), the flag leaf (E, F), and roots (G, H) of rice. Both *japonica* cultivar HD-9 (A, C, E, G) and *indica* hybrid LY-9 (B, D, F, H) were field-grown. CI, WMD, and WSD indicate conventional irrigation, alternate wetting and moderate soil drying, and alternate wetting and severe soil drying, respectively, during grain filling (6–48 d after heading). D1 and D2 are the measurement time at 11 d and 22 d after treatment, respectively, when soil water potentials were −25 kPa in the WMD regime and −50 kPa in the WSD regime, and W1 and W2 are the measurement time at 13 d and 24 d after treatment, respectively, when plants were rewatered. Data are averages observed for the two study years because they behaved the same. The asterisk (*) represents significant difference (P=0.05) between the WMD regime and the CI regime or between the WSD regime and the CI regime. Vertical bars represent ± standard error of the mean (n=6) where these exceed the size of the symbol.
In previous studies on AWD irrigation, the grain yield of rice was increased (Tuong et al., 2005; Yang et al., 2007; Zhang et al., 2008, 2009b), but reduced in others (Tabbal et al., 2002; Belder et al., 2004) when compared with continuously submerged conditions. The discrepancies between the studies are probably attributed to the variations in soil hydrological conditions and the timing of the irrigation method applied (Belder et al., 2004). The results of the present study showed that grain yield significantly decreased when soil water potential was reduced to ~50 kPa

**Discussion**

In previous studies on AWD irrigation, the grain yield of rice was increased (Tuong et al., 2005; Yang et al., 2007; Zhang et al., 2008, 2009b), but reduced in others (Tabbal et al., 2002; Belder et al., 2004) when compared with continuously submerged conditions. The discrepancies between the studies are probably attributed to the variations in soil hydrological conditions and the timing of the irrigation method applied (Belder et al., 2004). The results of the present study showed that grain yield significantly decreased when soil water potential was reduced to ~50 kPa.
when AWD was imposed during the grain-filling period. However, grain yield and WUE were significantly increased when soil water potential was reduced to –25 kPa in AWD (Tables 2, 3). Such results indicate that the drying condition in AWD is the most important factor affecting grain yield, and soil drying to –25 kPa is beneficial to grain growth during the grain-filling period.

Modern rice cultivars, especially the newly bred ‘super’ rice, have numerous spikelets on a panicle and achieve during the grain-filling period.

Table 4. Correlations of the mean grain-filling rate and the final grain weight of superior spikelets with the contents of trans-

<table>
<thead>
<tr>
<th>Sampling time</th>
<th>Mean grain-filling rate</th>
<th>Final grain weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z+ZR iP+iPR IAA</td>
<td>Z+ZR iP+iPR IAA</td>
</tr>
<tr>
<td>D1</td>
<td>0.90* 0.87* 0.52</td>
<td>0.96** 0.98** 0.72</td>
</tr>
<tr>
<td>W1</td>
<td>0.96* 0.92** 0.51</td>
<td>0.98** 0.93** 0.61</td>
</tr>
<tr>
<td>D2</td>
<td>0.91** 0.92** 0.62</td>
<td>0.95** 0.99** 0.71</td>
</tr>
</tbody>
</table>

Table 5. Effects of kinetin application on grain-filling rate and grain weight of superior and inferior spikelets of rice

The plants were grown in cement tanks that were filled with soil. CI indicates conventional irrigation and WSD represents alternate wetting and severe soil drying irrigation during grain filling (6–8 d after heading). The plants were sprayed with 50 μM kinetin daily for 8 d starting at the initial of irrigation treatments. Control plants received deionized water. The grain-filling rate was calculated according to Richards’ (1959) equation. Grain weight was measured from 20 plants in each treatment at maturity. The data are averages observed for the two study years, and are expressed as means ± standard error (n=8). Different letters indicate statistical significance at the P=0.05 level within the same column and within the same cultivar.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Irrigation treatment</th>
<th>Kinetin application</th>
<th>Mean grain-filling rate (mg kernel⁻¹ d⁻¹)</th>
<th>Grain weight (mg grain⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Superior</td>
<td>Inferior</td>
</tr>
<tr>
<td>HD-9</td>
<td>CI</td>
<td>Control</td>
<td>1.65±0.11 a</td>
<td>0.652±0.010 b</td>
</tr>
<tr>
<td></td>
<td>WSD</td>
<td>Control</td>
<td>1.61±0.14 a</td>
<td>0.678±0.012 a</td>
</tr>
<tr>
<td></td>
<td>LY-9</td>
<td>CI</td>
<td>1.53±0.12 a</td>
<td>0.603±0.009 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1.54±0.11 a</td>
<td>0.631±0.012 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 μM kinetin</td>
<td>1.52±0.14 a</td>
<td>0.529±0.018 d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 μM kinetin</td>
<td>1.53±0.16 a</td>
<td>0.578±0.011 c</td>
</tr>
</tbody>
</table>
conductance and, consequently, increase grain weight, harvest index, and WUE (Yang et al., 2001, 2002b; Yang and Zhang, 2010b).

The data herein showed that the WSD regime significantly reduced cytokinin levels in the shoots (inferior spikelets and the flag leaf) and grain-filling rate and grain weight of inferior spikelets (Figs 5, 6; Table 1). When kinetin, a synthetic cytokinin, was applied to plants in the WSD regime at the early grain-filling stage, the grain-filling rate and grain weight of inferior spikelets were significantly increased (Table 5). These results suggest that the reduction in grain-filling rate and grain weight of inferior spikelets in the WSD regime would be attributed to the decreased cytokinin levels in the shoots.

It is noteworthy that neither cytokinin contents nor grain-filling rate/grain weight of superior spikelets were markedly affected by the irrigation treatments (Table 1; Figs 4, 5). A similar observation was also made by Xu et al. (2007) who reported that non-flooded mulching cultivation in rice remarkably affected hormonal levels and the grain weight of inferior spikelets but little affected those of superior spikelets. The mechanism involved is not clear. A probable explanation is that earlier-flowering superior spikelets dominate later-flowering inferior spikelets in cytokinin levels, endosperm development, and grain filling (Yang et al., 2002a, 2006; Zhang et al., 2009a), and both the WMD and WSD regimes in this study could not markedly alter the hormonal level in superior spikelets and, consequently, significantly affect the filling of these spikelets.

Interestingly, a very different changing pattern between tZ-type cytokinins and iP-type cytokinins in roots was observed in the WSD regime (Fig. 7). Z+ZR contents were significantly increased, whereas iP+iPR contents were significantly decreased in the root under such a condition when compared with those in the CI regime. It is believed that tZ-type cytokinins are mainly transported from the root to the shoot and iP-type cytokinins are mainly transported from source organs to sink organs (Matsumoto-Kitano et al., 2008). The greater accumulation of Z+ZR in roots could be attributed partly to the significant reduction of CKX activities there (Fig. 8G, H) and partly to the reduced transport of tZ-type cytokinins to the shoots (Yang et al., 2002b, 2003b). While decreased iP-type cytokinins in roots in the WSD regime may account for the reduction in biosynthesis and/or the reduced transport of these cytokinins in/from source leaves, it is noteworthy that, in the WMD regime and when plants were rewatered, Z+ZR contents in the leaves and inferior spikelets were significantly increased, while they remained unchanged in roots when compared with those in the CI regime (Figs 5–7). Since the increase in transport of cytokinins from the roots to the shoots was not observed under such a condition (data not shown), the results imply that tZ-type cytokinins might also be produced in the shoot, in agreement with the notion that cytokinin synthesis is localized in discrete sites throughout the plant (Argueso et al., 2009).

Unexpectedly, a significant reduction in CKX activities in inferior spikelets, leaves, and roots were observed in the WSD regime (Fig. 8C–H). A similar result was observed by Havlová et al. (2008) who reported that water stress significantly reduced CKX activities in the leaves and roots of tobacco (Nicotiana tabacum L.). The mechanism underlying the decrease in CKX activity in plants under the WSD regime or under water stress is not clear. A probable explanation is that plants may keep certain levels of cytokinins to maintain growth by down-regulating CKX activity when the synthesis/transport of cytokinins in/into tissues is much decreased under severe soil-drying or water stress.

There are reports that auxins and cytokinins could interact in conferring drought tolerance and regulating organogenesis and seed development (Yang et al., 2003a; Havlová et al., 2008; Pernisová et al., 2009). The data in this study showed that IAA levels in rice plants were only altered in the WSD regime (Fig. 9). Under such a regime, IAA contents in shoots (inferior spikelets and leaves) were significantly reduced at the late grain-filling stage (22 and 24 DAT), whereas they were significantly increased in roots throughout the grain-filling period, in good agreement with a greater accumulation of tZ-type cytokinins there. The reduction in IAA in the shoot would be associated early senescence of both source and sink organs (Noodén, 1980; Davies, 2004; Ghanem et al., 2008). The reason for a greater accumulation of IAA in the roots in the WSD regime remains to be elucidated. It is suggested that cytokinins could act as auxin transport inhibitors to inhibit auxin efflux (Pernisová et al., 2009). It would be possible that a higher cytokinin level contributes to a higher IAA level in the root, leading to the maintenance of root growth under adverse conditions (Havlová et al., 2008). The present results demonstrated that the grain-filling rate and grain weight of inferior spikelets were very significantly correlated with cytokinin content, but not significantly correlated with IAA content, in these spikelets (Table 4), suggesting that the alteration of cytokinin levels in rice plants under WMD and WSD treatments plays a major role in the regulation of grain filling. Further investigation is needed to understand the cross-talk between cytokinins and auxins or between cytokinins and ABA in responses to irrigation regimes and in regulating seed development.

In conclusion, the present results demonstrate that the WMD regime could significantly increase, whereas the WSD regime significantly reduced, grain-filling rate and grain weight of inferior spikelets in ‘super’ rice when compared with the CI regime. The increase in cytokinin (Z+ZR, iP+iPR) levels in the shoot (inferior spikelets and leaves) in the WMD regime during rewatering contributes to a greater grain-filling rate and a heavier grain weight of inferior spikelets. On the other hand, a reduction in grain-filling rate and grain weight of inferior spikelets in the WSD regime is mainly attributed to the decreased cytokinin levels in the shoot. A moderate wetting and drying regime during the grain-filling phase holds great promise to improve grain filling of inferior spikelets in ‘super’ rice through regulating cytokinin levels in the plants.
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References


Bouman BAM. 2007. A conceptual framework for the improvement of crop water productivity at different spatial scales. Agricultural Systems 93, 43–60.


