Effect of Brassinolide Applied at the Meiosis and Flowering Stages on the Levels of Endogenous Plant Hormones during Grain-Filling in Rice Plant (*Oryza sativa* L.)

Hitoshi Saka, Seiichi Fujii*, Angela Maria Imakawa, Naoki Kato, Shin-ichiro Watanabe**, Tetsuro Nishizawa and Satoshi Yonekawa

(Graduate School of Agricultural and Life Sciences, The University of Tokyo, Nishitokyo, Tokyo 188-0002, Japan; *Shiraoka Research Station of Biological Sciences, Nissan Chemical Ind. Ltd., Shiraoka, Saitama 349-0294, Japan; **National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan)

Abstract: Brassinolide (BL), a brassinosteroid, applied to rice plants in pots promotes panicle ripening. In this study, we examined the effect of BL applied at the meiosis and flowering stages on endogenous levels of various plant hormones in the panicles of the rice plant (cv. Nipponbare) grown in a field-temperature (F-temp; 25°C on average ranging from 22 to 33°C during ripening periods) condition and low-temperature (L-temp; in phytotron kept at 22°C/17°C) condition in rice cultivation season in Japan. The content of either free- or bound-IAA in the rice spikelet at the milk-ripe stage (10 - 15 days after heading) was higher in the F-temp condition than in the L-temp condition. BL applied twice, 10 days before and on the day of heading, slightly increased the free-IAA content and greatly increased the bound-IAA content at the milk-ripe stage in both condition. BL slightly decreased the ABA content of the spikelet at the milk-ripe stage in the F-temp condition, and slightly increased it in the L-temp condition. The rate of ethylene production was measured only in the F-temp condition. It was markedly high at the milk-ripe stage and low at the dough-ripe stage (21 days after heading). BL treatment clearly increased the rate of ethylene production from the panicles under both light and dark conditions at the milk-ripe stage. These results suggest that BL, which promotes rice ripening, influences in the levels of endogenous plant hormones to play an important role in controlling the sink function during grain-filling.

Key words: Abscisic acid (ABA), Brassinolide (BL), Ethylene, Indoleacetic acid (IAA), Low temperature (L-temp), Milk-ripe stage, Panicle, Rice.

Brassinolide (BL) is known to increase the grain weight and ripening rate of rice (Hirai et al. 1991). It increases both the translocation of assimilates and the accumulation of starch in the panicle (Fujii and Saka 1992, 2001a). BL is assumed to increase the sink capacity of panicles after heading by promoting the accumulation of starch in the panicle, resulting in promotion of panicle ripening (Fujii and Saka 2001a). Cytokinin and gibberellins also are known to promote panicle ripening (Biswas and Choudhuri 1986). A rapid increase of grain weight after flowering and fertilization in rice plants is closely related to the level of endogenous plant hormones. The level of endogenous hormones rapidly increases after heading and flowering. There is a rapid increase in grain weight after fertilization reaching a peak within two weeks followed by a rapid decrease, suggesting that endogenous plant hormones play an important role in increasing sink capacity (Suzuki et al. 1981, Kobayashi et al. 1988, 1989a, 1989b, Michiyama and Saka 1991, Saka et al. 1992, Takagi et al. 1985, 1989). ABA has also been suggested to be involved in assimilate partitioning among spikelets in rice panicles (Tsukaguchi et al. 1999) and in soybean racemes (Kokubun and Honda, 2000). Elucidation of the kinetics of endogenous plant hormones during the ripening process after flowering and fertilization is important for understanding the mechanism of the accumulation of starch and other macromolecular substances in rice panicle. Not only the quantitative performance of each plant hormone, but also the qualitative relationship among plant hormones should be clarified to elucidate the role of plant hormones during grain-filling as a whole.

In this study, we examined the changes in the levels of endogenous plant hormones (IAA, ABA and ethylene) after heading in a field temperature condition (F-temp) and in a low-temperature (L-temp) condition in the rice cultivation season in Japan. We also examined the effect of BL applied twice, at meiosis and flowering stages, on the levels of endogenous plant hormones during the grain-filling process.

Materials and Methods

1. Culture of rice in pots and BL treatment

Received 11 June 2000. Accepted 9 August 2002. Corresponding author: H. Saka (sahito@fm.a.u-tokyo.ac.jp, fax +81-424-64-4391).

Abbreviations: ABA, abscisic acid; BL, brassinolide; F-temp, field temperature; GA, gibberellin; IAA, indoleacetic acid; L-temp, Low-temperature.
Germinated rice seeds (cv. Nipponbare) were sown in 1/5,000a Wagner pots filled with paddy soil in a circle, 20 seeds per pot, and cultivated customarily under the field condition in rice culture season in Japan. All tillers that developed during the growth were cut off with scissors, and single-stem rice plants with only the main shoot were cultivated in pots (Satake 1972). Four-grams of chemical fertilizer (N : P₂O₅ : K₂O = 15 : 15 : 10) were applied as basal dressing and 0.6g ammonium sulfate as top dressing at the early panicle formation stage, 20 days before heading.

BL stored in 99.5% ethanol at a concentration of 2.1 × 10⁻⁴ M was diluted to 2.1 × 10⁻⁵ M and 2.1 × 10⁻⁶ M. We applied a BL solution containing a 2,000-time diluted surfactant (Nippol, Nissan Chem. Ind. Ltd.) to the whole rice plants (30 ml per pot) by spraying twice, 10 days before heading and on the day of heading, and their plants were grown in the field. After heading, the plants were divided into two groups. One group was grown in a phytotron under natural daylight (22°C day/17°C night: 12-hour light from 6:00 to 18:00, called L-temp condition hereafter), and the other group in the field condition (25°C on average ranging from 22 to 33°C during the ripening period, called F-temp condition hereafter). For each BL treatment, two pots were used with three replications.

2. Quantification of IAA content
Ten and 15 days after heading, 5 – 7 panicles each with 8 primary rachis-branches were sampled from each pot at 11:00 – 12:00 in the field and phytotron. Ripened spikelets were separated from the panicles and after removing the husks, 1 g each of the grain sample was immersed in 99.5% ethanol. IAA was extracted following the method of Watanabe et al. (1989) based on the report of Kobayashi et al. (1989). The grains immersed in ethanol were homogenized after keeping at 4°C overnight, the extract was filtered by suction and the residue was extracted with ethanol three times. Indole propionic acid (IPA) was added to total extract (filtrate) as an internal standard, and the extract was evaporated under a reduced pressure at room temperature. Saturated sodium bicarbonate (pH 9) was added to the aquatic residue, and it was extracted with diethyl ether (2,6-Di-t butyl-p-cresol at 4.5 × 10⁻⁴ M was added as an antioxidant) six times to remove the neutral diethyl-ether-soluble fraction. The remaining aquatic fraction was adjusted to lower than pH 2 using 2.5 M HCl, and again extracted with diethyl ether. After dehydrating the organic fraction, ether was evaporated under a reduced pressure.

Acidic diethyl-ether fraction thus obtained was dissolved in a small amount of methanol, fractionated by high performance liquid chromatography (HPLC) and quantified. First, the methanol fraction was injected into a Nucleosil 5N(CH₃)₂ column (6 mm × 100 mm). It was eluted with 0.2% acetic acid-methanol at a flow rate of 1.5 ml/min. The eluate was monitored by using a fluorescence spectrophotometer, and the fraction containing IAA and IPA (5.2 – 10.2 min) was collected. The collected fraction was concentrated under a reduced pressure, re-dissolved in 50% methanol, and injected into Inertsil ODS column (5 μm, 4.6 mm × 250 mm, eluted with 0.1 M acetic acid – 50% methanol – water at a flow rate of 0.8 ml/min). The eluate was monitored with a fluorescence spectrophotometer, and the amount of free-IAA was determined from the ratio of the peak area of IAA to that of IPA. The aquatic fraction was neutralized with 3M NaOH, then dissolved in 1M NaOH, and bound-IAA was extracted with diethyl-ether three times. Bound-IAA was quantified by the same method as that for free-IAA. For fluorescence analysis, a Hitachi Fluorescent Spectrophotometer F-1150 was used; wavelength 280nm, L-6200 Intelligent Pump (Hitachi) and oven at room temperature.

3. Quantification of ABA
Acidic diethyl-ether fraction obtained as mentioned above was concentrated under a reduced pressure, dissolved in 0.5 ml ethanol, and subjected to thin layer chromatography (Silica gel HF254, 0.5 mm, Merck Co. Ltd.) to isolate. The spot with Rf value corresponding to ¯-ABA was collected, eluted with methanol, and methanol was evaporated under a reduced pressure. The residue was resolved in 0.5 ml methanol and ABA was quantified using PHYTODETEK-kit (Phytodetek Co. Ltd.) following the protocol. The solvent used for TLC was benzene : butanol : acetic acid = 87.5 : 7.5 : 5.0.

4. Quantification of ethylene
From the rice plants cultivated in the F-temp condition, we cut the panicles at the rachis node, 14 and 21 days after heading. The panicle was weighed and put in a test tube (22.5 mm internal dia., 292.5 mm length) with 5 ml distilled water. After closing with a double rubber stopper, the test tubes were placed in the light at about 5,000 lux or in the dark under 25°C condition. After a 24-hour incubation, air gas in the test tube was collected with a 1-ml injector and ethylene was quantified by FID gas chromatography (Saka et al. 1992). Gas chromatograph used was Shimadzu GC-8 type (Shimadzu Co. Ltd) with 3 mm × 3 m stainless column packed with 50/80 mesh Porapak Q. Carrier gas was N₂ at a flow rate of 50 ml/min and oven temperature was 80°C.

Results

1. Effect of BL treatment in the spikelet weight during grain-filling of rice plant grown under the F-temp and L-temp conditions
In the control plants without BL treatment, grain weight (Table 1) and panicle weight (Table 2) increased during the ripening process more rapidly in the F-temp condition than in the L-temp condition. In the rice plants treated with BL, the grain weight 10 days
Table 1. Effect of BL on the fresh weight of grains in the panicles of rice plants used for analyzing IAA and ABA under F-temp and L-temp conditions.

| BL (M) | F-temp condition | L-temp condition |
|--------|-----------------|-----------------|
|        | 10° 15°         | 10° 15°         |
| 0      | 14.6±0.7 25.2±0.3 | 11.5±0.5 18.7±0.9 |
| 2.1x10^{-9} | 16.9±0.9 25.5±0.5 | 11.4±0.6 19.9±0.7 |
| 2.1x10^{-8} | 16.3±0.7 25.3±0.4 | 11.5±0.3 20.7±0.6 |

*: Days after heading.

The experiment was performed in the F-temp (See in Fig. 1) and L-temp condition.

3. Change in ABA content and the effect of BL on it during grain-filling process

Fig. 2 shows the change in ABA (cis-type) content in the rice spikelets during the milk-ripe stage. In the F-temp condition, the ABA content of the control plants 15 days after heading was about 1.5 times higher than that 10 days after heading, suggesting that it increased with the progress of ripening. BL treatment decreased the ABA content either 10 or 15 days after heading. In the L-temp condition, ABA content 15 days after heading was not significantly different from that 10 days after heading, but BL treatment tended to increase the content 10 or 15 days after heading. BL was suggested to increase the ABA content in the F-temp condition but to decrease it in the L-temp condition.

4. Effect of BL on ethylene production in rice panicle

Fig. 3 shows the change in ethylene production in rice panicles during the ripening process in the F-temp condition. The rate of ethylene production in the panicle at the milk-ripe stage in the F-temp condition was significantly higher (about 1.5 times) in the light than in the dark as in our previous report (Saka et al. 1992). However, at the dough-ripe stage (21 days after heading), the rate of ethylene production was nearly the same in both light and dark conditions. BL treatment increased the rate of ethylene production 14 days after heading (milk-ripe stage) in both light and dark conditions, but did not affect the ethylene production 21 days after heading (dough-ripe stage). BL treatment was found to promote ethylene production strikingly at the middle of the milk-ripe stage. However, the dose-dependent effect of BL on ethylene production was obscure like other plant hormones examined in this experiment.

Discussion

The levels of endogenous plant hormones (IAA, GA, cytokinin, ethylene) that are low during the vegetative stage increase markedly during the period from flowering to the milk-ripe stage in rice life cycle.

The content of IAA, both free- and bound-IAA, rapidly increase during the early ripening stage after heading; it is particularly high in the spikelet at the milk-ripe stage. In our previous report (Saka et al. 1992), the rate of ethylene production was significantly higher (about 1.5 times) in the light than in the dark. However, at the dough-ripe stage (21 days after heading), the rate of ethylene production was nearly the same in both light and dark conditions. BL treatment increased the rate of ethylene production 14 days after heading (milk-ripe stage) in both light and dark conditions, but did not affect the ethylene production 21 days after heading (dough-ripe stage). BL treatment was found to promote ethylene production strikingly at the middle of the milk-ripe stage. However, the dose-dependent effect of BL on ethylene production was obscure like other plant hormones examined in this experiment.

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-ripe stage. At this stage, the content of bound-IAA is 2 - 2.5 times higher than that of free-IAA, but the contents of both free- and bound-IAA decrease thereafter and there is no difference between the contents of free- and bound-IAA at the dough-ripe stage (Kobayashi et al. 1989b). In the culms and leaves at the vegetative stage, the contents of free- and bound-IAA are nearly the same and about 1/30 of those in the spikelet at the milk-ripe stage. GA is synthesized from geranylgeranyl-2-phosphate via kaurene-related compounds. In the culms and leaves in the vegetative stage, early-13-hydroxylation pathway which produces GA₉ from GA₁₉ via GA₁₉ and GA₂₀, is operating alone, but in reproductive stage, non-13-hydroxylation pathway, by which GA₄ is produced from GA₂₀ aldehyde via GA₂₀, appears additionally in the culms, leaves and spikelets as well as in anthers (Kobayashi et al. 1988, Kobayashi et al. 1989a). The role of GA₄ in reproductive growth is interesting but the mechanism is yet unknown. Endogenous ABA consists of cis- and trans-type but the majority is cis-type. The ABA content increases during the period from flowering to milk-ripe stage like endogenous IAA, and rapidly decreases thereafter (Kato et al. 1993, Tsukaguchi et al. 1999). The content is increased by shading the plants and is higher in superior spikelets than in inferior spikelets and shading exaggerated the inherently different ABA dynamics between superior and inferior spikelets, which suggest that ABA may accelerate the accumulation of assimilates (Tsukaguchi et al. 1999). A small amount of ABA applied at the heading stage has been reported to increase the ABA content of spikelets and promote rice ripening, and the
Fig. 2. ABA contents in panicles of rice plants under F-temp and L-temp conditions with and without BL treatment. Error bars indicate S.D. of 3 replicates. See in Fig.1 for F-temp and L-temp condition.

- o: Untreated control, •: 2.1 X 10^-9 M, : 2.1 X 10^-11 M

Fig. 3. Amounts of ethylene produced in panicles of rice under F-temp conditions with and without BL treatment. Measurement was for 24 h in light or dark condition. Error bars indicate S.D. of 3 replicates.

- o: Untreated control, •: 2.1 X 10^-9 M, : 2.1 X 10^-8 M

Effect of ABA is intensified by shading (Nakamura and Nakajima 1997). In the culms, leaves, roots and panicles of rice plant, zeatin, ribosylzeatin or isopentenyl-adenosine is detectable. At the flowering stage, the zeatin content increases, and at the milk-ripe stage, zeatin glucoside such as cis-ribosylzeatin-o-glucoside predominate. Thereafter, the contents of these cytokinins decrease rapidly (Takagi et al. 1985, 1989). The production of ethylene, which is known as a ripening and senescence hormone in various plant species does not vary so much with the growth stage such as vegetative and reproductive stages in rice leaves. However, the ethylene production in the panicle rapidly increases at the early milk-ripe stages showing a change similar to that in other plant hormones (Michiyama and Saka 1991, Saka et al. 1992). Interestingly, the rate of ethylene production in the panicle at the milk-ripe stage is extremely higher in the light than in the dark, but that in the leaf blade of the flag leaf is higher in the dark than in the light (Lee et al. 1981, Saka et al. 1992).

In the present study, the changes in the endogenous contents of IAA and ABA and ethylene production in the spikelets during the ripening stage were quantitatively similar to those described before, although dynamic
and qualitative fluctuation like GA and cytokinin after heading in rice were not observed. Treatment with BL twice (10 days before heading and the day of heading) increased the IAA (both free- and bound-) content and ethylene production in the F-temp condition and clearly increased the content of IAA (both free- and bound-) and ABA (Fig. 1, 2 and 3) in the L-temp condition. We obtained significant information on the exogenous BL-dependent fluctuations of endogenous contents of IAA and ABA and ethylene production during the grain-filling in rice not only in a normal temperature condition, but also in a low temperature condition. BL treatment also increased panicle weight and grain weight (Tables 1 and 2) as in previous reports (Hirai et al. 1991, Fujii & Saka 1992). Since BL promotes assimilate translocation and accumulation of carbohydrates in the panicles (Fujii & Saka 1992, 2001a) even in the L-temp condition, it may promote ripening by regulating the amounts of endogenous hormones such as IAA and ABA, not only in the field condition but also in the L-temp condition. Especially, the promotive activity of BL on the levels of endogenous plant hormones in the L-temp condition is interesting because the additive effect of BL and IAA on lamina joint-cell elongation has been reported (Fujii and Saka, 2001b). Under low temperature condition, BL may maintain or rescue the sites of action of the other endogenous plant hormones mentioned such as IAA and ABA to promote grain-filling after anthesis in rice plant. However, BL applied at two different concentrations showed similar effects on rice ripening and on the contents of endogenous plant hormones as in our previous study (Fujii and Saka 1992, 2001a). This is probably because BL is poorly absorbed and hardly translocated in the plants (Nishikawa et al. 1994).

Exogenous BL application at much lower concentrations than other plant hormones had significant effects on the growth and development in rice plant (Fujii and Saka 1992, 2001a, 2001b). Reproductive plant organs like pollen grains and immature seeds contain a large amount of brassinosteroids molecules (Fujikawa 1999). These brassinosteroids performance strongly suggest that they directly participate in the physiological function of reproductive plant growth and development. However, to our knowledge, there are no reports on the kinetics of endogenous brassinosteroids in rice plant. Further detailed research on the kinetics of endogenous brassinosteroids in the life cycle of rice is essential to analyze and elucidate the physiological interrelationship between endogenous brassinosteroids and other endogenous plant hormones in rice.

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