The Changes of Endogenous Absciscic Acid in Developing Grain of Two Rice Cultivars with Different Grain Size*

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Abstract : Two cultivars of rice (*Oryza sativa* L.) with different grain size were investigated to measure the free ABA contents during grain development. In both cultivars, the content and concentration of endogenous ABA in developing grain increased after heading, and attained a maximum value at about the middle of the linearly increasing period of grain dry weight. The ABA content and concentration subsequently decreased and maintained a low level after the termination of increase in grain dry weight. Higher ABA content was found in cultivar BG 1, which showed a larger grain size and a higher rate of grain filling, than in the other cultivar, Koshihikari, throughout all stages except at heading. The ABA concentration in both cultivars, on the other hand, showed similar values. These results suggest that ABA seems to play some role in assimilate accumulation into rice grain and to cause genetic difference in this process.

Key words : Absciscic acid, Endogenous content, Grain-filling process, Grain size, Rice.

粒大の異なるイネ二品種における内生アブシジン酸含量の粒発育に伴う変化 : 加藤恒雄・桜井直樹*・倉石 晋**
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要 旨 : 粒大の異なるイネ二品種, BG 1 とコシヒカリを用いて, 出穂開花後の発育中の粒における遊離型の内生アブシジン酸 (ABA) 含量の変化を観察した. 両品種とも一粒当たり内生 ABA 含量および ABA 濃度は, 出穂後増加し粒重増加期の中間において最大値に達し, その後減少して粒重増加が完了した時期以降は低いレベルにとどまった. 粒重増加速度および最終粒重の高い品種 BG 1 はどの時期においても高い一粒当たり ABA 含量を示した. 一方, ABA 濃度に関しては, 両品種に差は認められなかった. これらの結果は, 発育中の米粒内の内生 ABA が同化産物の粒への蓄積に対して何らかの役割を果たし, かつ粒重増加速度の品種間差の一因となりうることを示唆する.

キーワード : アブシジン酸, イネ, 内生含量, 粒重増加過程, 粒大.

Grain filling is one of the most important developmental processes in cereals, since the process directly determines final grain weight and grain yield. It has been well known that the rate of grain filling varies widely with the genotype^{4,6,10,14}. However, we have no evidence of the factors causing the genetic variation in the rate of grain filling or the mechanism regulating this process.

The rate of grain filling can be regarded as the rate of assimilate accumulation from source organ to grain, the most active sink organ after anthesis. Recent studies suggest abscisic acid (ABA) plays some role in assimilate accumulation in a number of sink organs^{2,8} by promoting unloading of assimilate from sieve tube to sink apoplast^{3,13,15} and/or uptake of the unloaded assimilate by stor-

age cell^{12,13}. Thus we analyzed the ABA content in developing rice grain as one of the internal factors causing the genetic variation in the rate of assimilate accumulation.

Oritani and Yoshida⁹ found higher activity of ABA-like substance only at an earlier stage of rice grain development. However they did not analyze the level of the endogenous ABA in detail. In the present paper, we quantified the endogenous ABA level during grain development of two rice cultivars, 'BG 1' with larger grain size, and 'Koshihikari' with a smaller size, since a close and positive correlation generally exists between final grain weight and rate of grain filling⁶.

Materials and Methods

1. Plant materials

Two cultivars of rice (*Oryza sativa* L.), BG 1 and Koshihikari, were used in this experiment. In 1989, 120 plants each were cultivated

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in the paddy field of Hiroshima Agricultural College at a space of 30 cm × 15 cm with single planting. A randomized block design with two replications was employed. Total amounts of nitrogen, phosphorous and potassium applied as fertilizer were 9.5 and 9 g m⁻², respectively.

2. Characterization of grain-filling process

The heading date of a panicle was individually recorded. From each cultivar, five panicles were sampled every five days from five to 50 days after their respective heading dates. Fertile unhulled grains on the panicles were collected, dried and measured for their average one-grain weight.

As shown in Fig. 2, a two-intersecting-line was fitted on the data of one-grain weight throughout 10 samplings according to the method described by Kato⁶⁾. The rate and duration of grain filling and the final grain weight were determined from this line (Fig. 2 and Table 1).

3. Sampling for ABA quantification

ABA in the developing grains, only for the free ABA, was extracted and analyzed mainly according to the method of Kuraishi et al.⁷⁾. About 20 g fr wt of grains, including hulls, was sampled by hand-threshing from intact panicles to quantify the endogenous ABA level. After the measurement of fresh weight, the grains were immediately fixed in 80% cold ethanol, and stored at -80°C until use. Such sampling was carried out every seven days from 0 to 35 days after heading.

4. Extraction of endogenous ABA

The grains were homogenized in 80% ethanol with an ultrasonic-homogenizer (Ultratullax TP 18/10 S2, Ika-Werk, Breisgau, Germany) after adding [¹⁴C] 2, *cis*-(R, S)-ABA ([¹⁴C] c-ABA, Amersham, Buckinghamshire, U. K., 204 MBq mmol⁻¹) as an internal standard. The homogenate was filtrated through a glass-filter (GF3, Nippon Rikagaku Kikai Co., Tokyo, Japan), and concentrated in vacuo. After adjusting the pH of the solution below 2.8 with 0.1 M of phosphoric acid, the solution was filtrated successively with 3.0 μm and 1.2 μm of millipore filters (Nihon Millipore Kogyo Co., Yonezawa, Japan) and 0.2 μm of a nylon filter (Sartorius, Goettingen, Germany). The filtrate was partitioned three times with the same volume of dichloromethane. The fractions were combined and dried in

vacuo. The residue was resuspended with 25% acetonitrile (20 mM acetic acid).

5. Fractionation with high-performance liquid chromatography (HPLC)

After passing through a disposable filter (EKICRODISK, Gelman Sciences Japan, Tokyo, Japan), the solution was injected into HPLC system (LC-6A Gradient System G-I, Shimadzu Co., Kyoto, Japan) with HPLC column of Radial Pak μ-Bondapak C₁₈ (Waters Associates, Milford, MA) to collect ABA fraction. The injected solution was isocratically eluted with 25% acetonitrile (20 mM acetic acid) at a flow rate of 2 mL min⁻¹. After monitoring the absorbance at 254 nm, the eluates corresponding to the respective retention times for 2, *cis*- and 2, *trans*-(R, S)-ABA (c-ABA and t-ABA) were fractionated. Both eluates of ABA fraction were combined and dried in vacuo, resuspended with methanol, and then completely dried again and stored in a desiccator at reduced pressure.

6. Quantification with gas-liquid chromatography-electron capture detector (GLC-ECD)

The dried sample was methylated with diazomethane using a diazomethane generator (Wheaton Scientific, Millville, NJ). After evaporation, the methylated sample dissolved in methanol was introduced into a GLC system (G-180, Yanagimoto Manufacturing Co., Kyoto, Japan) equipped with a ⁶³Ni electron capture detector (C-ECD, Yanagimoto Manufacturing Co., Kyoto Japan). The temperature of the column was increased from 150 to 210°C at a rate of 5°C min⁻¹. The temperature of the injector and detector were 220°C. The flow rates of nitrogen carrier gas and ionized gas were set at 40 and 80 mL min⁻¹, respectively. The capillary column of 0.53 mm inner diameter × 10 m length, methyl silicone liquid phase (Quadrex Co., New Haven, CT), was used. Fig. 1 shows an example of the GLC-ECD chromatogram obtained from the present experiment. From the ratio of the peak area of c-ABA for a sample to that for an authentic standard, the content of c-ABA (c-ABA methyl ester) involved in the sample was determined. The determination with GLC-ECD was triplicated for the same methylated sample.

A portion of the above methylated sample was injected into the HPLC system to correct

Fig. 1. GLC-ECD chromatogram of a developing rice grain sample.

The sample was extracted from BG 1's grains collected at 14 days after heading. c-ABA : 2, *cis*-(R, S)-ABA, t-ABA : 2, *trans*-(R, S)-ABA.

the ABA methyl ester. In this case, the concentration of solvent, acetonitrile (20 mM acetic acid), was increased linearly from 25 to 50% over 8 min, then held at 50%. The eluate of ABA methyl ester fraction was mixed with scintillation cocktail (ACS-II, Amersham, Buckinghamshire, U. K.), measured the radioactivity of the recovered [^{14}C] c-ABA with a scintillation counter (LSC-701, Aloka Co., Tokyo, Japan) to estimate the recovery rate. All data for c-ABA level were collected based on the corresponding recovery rate.

The above procedure of ABA quantification was duplicated.

7. Qualitative analysis with gas chromatography-mass spectrometry (GC-MS)

To verify whether the substance detected with the GLC-ECD is the ABA methyl ester, a qualitative GC-MS was conducted. Except for the addition of the internal standard, the same procedure as mentioned above was carried out from about 60 g fr wt of BG 1's grains sampled at 14 days after heading. The ABA methyl ester fraction corrected from the above HPLC system was injected into GC-MS system (GCMS-QP 1000, Shimadzu Co., Kyoto, Japan). The sample was separated with the

Table 1. Grain-filling characteristics of the two rice cultivars.

Trait	Cultivar	
	BG 1	Koshihikari
Days from sowing to heading	99.3	95.7
Rate of grain filling (mg day ⁻¹ grain ⁻¹)	1.68	0.78
Duration of grain filling (days)	24.7	27.1
Final grain weight (mg grain ⁻¹)	41.4	21.2

column of CBS (Shimadzu Co., Kyoto, Japan). The temperature of the column was set at 100°C during the first 2.5 min, and then increased up to 250°C at a rate of 20°C min⁻¹, and held at 250°C. Full mass spectrum of the ABA methyl ester fraction was obtained after the fragmentation with electron impact (70eV).

Results

1. Grain-filling process

Fig. 2 and Table 1 clearly demonstrate that BG 1 showed about twice as high rate of grain filling as that of Koshihikari. On the other hand, the two cultivars exhibited nearly the same days-to-heading and the same duration of grain filling. These results concurred well with those of the previous paper⁶⁾.

2. Verification of ABA in the sample

The full mass spectrum of an authentic ABA methyl ester standard showed four major peaks in mass charge ratio of 190 (base peak), 164, 134 and 125, which characterize a typical ABA methyl ester. The sample also showed these four peaks at the same retention time in GC-MS as the authentic standard. The authentic standard and the sample resembled well to each other in the relative ion intensities of the four major peaks (Table 2). Thus, the existence of ABA in the present sample was verified.

3. Changes in endogenous free ABA level

Fig. 3 shows the changes in endogenous ABA (c-ABA) content (per grain) after heading. BG 1 and Koshihikari contained a

Fig. 2. The changes of grain dry weight after heading in two rice cultivars.
Closed circle and solid line : BG 1, Open circle and broken line : Koshihikari.

Table 2. Relative intensity of major ions in full mass spectrometry of an authentic ABA and a sample prepared from rice developing grain.

Mass charge ratio	Relative intensity(%) ¹⁾	
	Authentic ABA	Sample
190	100.0	100.0
162	39.4	36.2
134	40.2	28.7
125	40.4	28.7

1) Percentage to the base peak (mass charge ratio=190).

similar amount of ABA in their grains at heading. During the following 14 days, the contents of ABA drastically increased in both cultivars, and attained maximum values. BG 1 showed an apparently higher maximum content, 3.6 ng grain⁻¹, than Koshihikari, 2.3 ng grain⁻¹. From 21 to 28 days after heading, both cultivars showed a sharp decrease in their ABA contents. A large difference between the ABA contents of the two cultivars persisted after the maximum value. In both cultivars, the time when the endogenous ABA attained the maximum content (14 days after heading) coincided with the middle of linearly increasing period of grain dry weight.

Fig. 4 shows changes in the ABA (c-ABA)

Fig. 3. The changes of ABA (c-ABA) content after heading in developing grain of two rice cultivars.
See Fig. 2 for the symbols.
Vertical line at a circle means S. E. for the average (n=4).

Fig. 4. The changes of ABA (c-ABA) concentration after heading in developing grain of two rice cultivars.
See Fig. 2 for the symbols.
Vertical line at a circle means S. E. for the average (n=4).

concentrations (per g fr wt) after heading. ABA concentrations of both cultivars increased drastically during 14 days after heading. After attaining the maximum concentrations,

they decreased to a similar level at heading. The maximum concentrations of ABA were the same for the two cultivars. Note that the times at the maximum ABA concentration (14 days after heading) and the reduced one (28 days) corresponds to the middle and the end of the linearly increasing period in grain dry weight. In Fig. 4, no remarkable difference was detected between the two cultivars in the ABA concentration.

Discussion

The present data definitely revealed that ABA exists in developing rice grain. Furthermore, in both cultivars, the endogenous free ABA content and concentration increased after heading and attained a maximum value at about the middle of the linearly increasing period of grain dry weight. The content and concentration of ABA subsequently decreased and maintained comparatively lower level after the termination of increase in dry grain weight. ABA content was higher in cultivar BG 1, which showed a larger grain size and a higher rate of grain filling, than in the cultivar Koshihikari, throughout all stages except at heading time. ABA concentration, on the other hand, was similar in both cultivars.

A number of studies^{1,5,11,13)} have examined the changes in endogenous ABA in developing sink organs using two or more genotypes and discuss the genotypic differences.

Schussler et al.¹³⁾ used three soybean cultivars with different seed weight, and demonstrated that a cultivar with the largest grain size contained the highest content and concentration of endogenous ABA in seed coat and cotyledones of a developing seed. This result provided one of the bases for their conclusion that the ABA stimulates unloading and uptake of sucrose in sink organ, and the difference in ABA content causes the difference in seed growth rate among these cultivars. Ackerson¹⁾ also examined three soybean cultivars with different seed weight, and obtained a positive correlation between endogenous ABA content and rate of embryo growth. He suggested, however, a possible role of ABA on embryogenesis rather than on the assimilate accumulation. In tomato seeds Groot et al.⁵⁾ compared endogenous ABA levels between an ABA-deficient mutant and its wild type. They concluded that a reduced

ABA level in the mutant's seeds did not influence the accumulation of dry matter and storage proteins in the seeds. On the other hand, they also described that the fruit, a much more active sink organ than seed in tomato, of the ABA-deficient mutant reduced its size compared to the wild type. Quarrie et al.¹¹⁾ examined the changes in ABA in developing wheat grain using two F_6 lines which differed in the grain growth rate and final grain weight. They could not obtain any causal relationships between the changes in ABA and grain growth rate and final grain weight, and they concluded that the ABA does not affect the assimilate accumulation in wheat grain.

Although there exist some conflicting results in the past, the present experiment of developing rice grain revealed that the cultivar with a higher rate of grain filling included a higher content of endogenous ABA when the grain grew most vigorously. As for the concentration of ABA, the two cultivars did not differ remarkably. When the rate of grain filling is divided by the final grain weight, that is, expressed on a unit weight basis, the two cultivars show little difference. Thus, the present results would not conflict with the consideration that ABA promotes the assimilate accumulation into rice grain and causes the genetic difference in this process. The possibility that the results for ABA were merely a consequence of ABA transport from leaf to grain accompanied with a mass flow of assimilate cannot be denied. Additional information is needed to arrive at a final conclusion for the role of ABA on the regulation of assimilate accumulation in developing rice grain and its genetic variation.

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