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Changes with Aging of Endogenous Abscisic Acid and Zeatin Riboside in the Root System of Rice

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Abstract : Plant roots produce abscisic acid (ABA) and zeatin riboside (ZR) which are known to counteract the aging of plant organs. Changes in ABA and ZR levels were determined by an enzyme immunoassay method in rice roots, in order to evaluate their roles in root system development, especially of seminal root axis (SRA) and lateral roots (LR). Rice plants were grown for 35 days after sowing (DAS) under submerged soil conditions in root boxes. In the seminal root system, ABA and ZR levels reached the highest peaks at 10 and 21 DAS, respectively. The ABA peak corresponded with the times when the nitrogen concentrations in LR decreased to the lowest level and the ZR peak coincided with the 2nd peak of the nitrogen level, as indicated by our previous finding. A drastic increment in ZR level in the seminal root tip at 6 DAS coincided with rooting of 2nd order LR and closely related to emergence of 4th leaf and 1st node nodal roots, which indicated the significant role of ZR in the early development of rice seedlings. Comparison of LR and SRA revealed that SRA showed a much higher ZR level and much lower content ratio of ABA to ZR than those of LR. Furthermore, the ratio in the seminal root tip was very similar to that of SRA. This indicates that the hormonal characteristics of LR and SRA would be far different.
Key words : Abscisic acid, Aging, Enzyme immunoassay, Lateral roots, *Oryza sativa* L., Seminal root system, Senescence, Zeatin riboside.

水稲根系のエイジングに伴う内生アブシジン酸及びゼアチンリボシドの変化 : 郭　康洙・飯嶋盛雄・山内章・河野恭廣（名古屋大学農学部）

要　旨 : 根系で生産される内生アブシジン酸（ABA）とゼアチンリボシド（ZR）は，エイジングに対して対照的な働きを示すことが知られている．エイジングに伴う根系各器官・部位における，これらの内生ホルモンの濃度変化を酸素免疫測定法で調べた．供試材料は湛水条件下で根箱栽培した水稲品種・愛知旭を用い，その根系の中から主として種子根系を対象に，根軸，その根端，及び側根における両ホルモンの動態を経時的に追跡した．種子根系のABA濃度は播種後10日に，ZR濃度は同21日に最大値に達した．これらのピークのパターンは，既報で示した窒素の根系内の濃度変化と関連性をもつことを示唆した．すなわち，ABA濃度のピークは根軸上の側根の窒素含有率が最低に達する時期に，ZR濃度のピークは同側根の窒素濃度が再び増加のピークに達する時期に，それぞれ一致した．播種後6日の根端のZR濃度の急激な上昇は，側根における2次分枝の急速な開始，第4葉の抽出・展開および第1節根の発根開始時期とほぼ時間的に一致し，根端のZR濃度の動態が，めばえの初期発育の出葉・発根過程に重要な役割を果していることを示すものと推論した．根軸と側根に分けて比較した結果，根軸は側根に比べて明らかに高いZR濃度と低いABA/ZR濃度比をもつことを認めた．なお，根端では，ABA/ZR濃度比は根軸と基本的に同様であった．以上のように，ABA/ZR濃度比，並びにABAとZR濃度の変化を基準として比較した時，側根と根軸の器官としての特徴は大きく異なることを強く示唆した．

キーワード : アブシジン酸，エイジング，酸素免疫測定法，種子根系，水稲，ゼアチンリボシド，側根，老化．

Plant hormones play a principal role in regulating growth and development of crop plants. Abscisic acid (ABA) and cytokinins have been regarded as senescence-promoting and retarding plant hormones respectively[2,19]. However, the role of these hormones in the root system is yet to be examined.

The root system of crop plants consists of several component roots. In rice, lateral roots (LR) develop on the seminal and nodal root axes and account for most of the length and surface area of the entire root system[7]. Our previous studies[10,11] indicated that the physiological acitivities of LR were much higher than those of the seminal root axis (SRA), and it was concluded that the characteristics of the LR are much different from those of the SRA. Hsia and Kao[4] suggested that the LR of soybean plant may play a predominant role in regulating primary leaf senescence by means of cytokinin production. Drew and Saker[1] also claimed the importance of the LR in the

growth of barley root system in terms of plant hormones. These possible roles of LR, however, remain to be confirmed by the quantification of ABA and/or cytokinins in LR. At the moment, LR have never directly been studied in relation to their plant hormonal natures. As a result, only limited information is available for the role of plant hormones in the development of the root system. It is, therefore, of great interest to estimate the amount of ABA and cytokinins in root axes and LR in relation to aging of the root system.

When instrumental analyses are employed, a relatively large amount of sample is needed to detect endogenous plant hormones. Since we also aimed to use a limited seminal root portion besides the whole root system in this study, it was not easy to collect a large amount of the sample in a short period of time. Therefore, we employed the enzyme immunoassay (EIA) method in order to detemine endogenous ABA and cytokinin in a small amount of the root sample.

Among different kinds of naturally-occurring cytokinins, we determined zeatin riboside (ZR), because riboside-type cytokinin is one of the most abundant naturally-occurring cytokinins in rice. Furthermore, the main cytokinin in rice root system is zeatin-type and the root system contains a much larger amount of ZR compared to zeatin[5,18].

Thus, we determined endogenous levels of ABA and ZR in the SRA and the concomitant LR by EIA method to find the roles of these hormones in the developing pattern of rice root system.

Materials and Methods

Plant growth and Sampling

Experiment 1. Rice (*Oryza sativa* L. cv. Aichiasahi) plants were sown in root boxes filled with loamy sand soil on July 20, 1995. Five to 10 plants were grown in each root box under submerged conditions for 35 days after sowing (DAS) in a vinyl-house. Fertilizer was not applied to all plants. The size of root boxes was 25 cm in length × 2 cm in width × 40 cm in depth. Plants from 5 root boxes were sampled every day from 2 to 7 DAS, and at 10, 14, 21, 28 and 35 DAS. At each sampling, the shoot was cut just on the soil surface and the root system was sampled by the root box pin-board method to ensure washing of the root system with a minimum loss[9]. Each sampling was completed within 1 h. Immediately after sampling, these root systems were separated into seminal root and nodal root systems. The seminal root systems were then divided into three root portions as we reported previously[10,11]; the basal 4.5 cm-root portion that elongated during 2 DAS (4.5 cm-portion), 1 cm-root tip which was excluded after 14 DAS and the remaining portion of the seminal root system.

Experiment 2. To compare changes of ABA and ZR levels between the SRA and LR, additional plants were sown on August 27 and grown under the same conditions for 14 DAS. Plants from 5 root boxes were sampled at 3, 4, 5, 7, 10 and 14 DAS in the same manner mentioned above. In this experiment, we focused on the basal 5.1 cm-root portion of the seminal root system that elongated during 2 DAS (5.1 cm-portion). In this portion, LR were detached from the SRA using fine forceps.

All dividing procedures of the root system in Exps. 1 and 2 were conducted on the ice within 20 min. Each divided root sample was frozen immediately in liquid nitrogen and stored at −80°C until extracted.

Extraction and Purification of ABA and ZR Samples

We followed the extraction procedures developed by R. Yoshida (Toyama Prefec. Univ., College of Tech.). Each root sample (20 ~200 mg of fresh weight) was homogenized by mortar and pestle with 3 mL of 80% methanol containing 100 mg/L 2,6-*di-tert*-butyl-4-methyl-phenol (Aldrich Chem. Co.) as an antioxidant. The samples were placed in the dark at 4°C for 24 h and centrifuged (10,000 g, 10 min), and the supernatant was decanted. The remained cell debris was re-extracted twice. The combined extract was dried under 4°C *in vacuo* using a centrifugal concentrator (CC-105, Tomy Tech., Inc.). The dried samples were resuspended in 5 ml of 5% methanol containing 0.05 N acetic acid and loaded onto a C_{18} Seppak cartridge (Waters Associates, Millipore Corp.) pre-equilibrated with the elution solvent. The cartridges were then eluted with 5 mL of 55% methanol and the eluate containing both ABA and ZR was dried. The dried samples were resuspended in 3 ml of

distilled water (pH 2.7) and equal volume of ethyl acetate added. The solution was completely partitioned for 10 min and the ethyl acetate layer was decanted. Another 3 mL of ethyl acetate was then added and the procedure was repeated twice. After drying the water fraction and combined ethyl acetate layers separately, the samples were resuspended in 0.4 mL of Tris-buffered saline (25 mM Tris, 1 mM $MgCl_2$ and 10 mM NaCl, pH 7.5). The amount of free ABA and ZR in ethyl acetate and water layers was determined respectively by phytodetek-ABA and ZR test kits (Idetek inc., 1995). Data represent means of two (Exp. 1) and three (Exp. 2) replicate measurements from the same root samples respectively. In Exp. 1, the two replicate values in each datum were very similar, and LSD value was calculated by ANOVA table.

Recoveries of ABA and ZR

Synthetic compounds of (±) −ABA and *trans*-ZR (Sigma Chem. Co.) were used for the determination of the recoveries and standards. Recoveries of both ABA and ZR after purification were 68±3% (n=3) and 81±8% (n =3) respectively.

Polyclonal antibody

Before using the EIA test kits, preliminary determination of ABA and ZR was carried out for testing the sampling technique, especially separation of the LR from the SRA, and also for estimating the minimum amounts of the LR and SRA for the EIA method. In order to produce the polyclonal antibodies for these preliminary experiments, authentic (±) − ABA and *trans*-ZR were conjugated to bovine serum albumin for the antigens by the procedures of Hansen et al.[3] and Philosoph-Hadas et al.[12] respectively. Immunization of rabbits (Japanese white species) was performed to raise anti-(±)-ABA and anti-*trans*-ZR antibodies. Then, we conducted the preliminary experiments several times using the polyclonal antibodies.

Morphological analysis

At each sampling in Exp. 1, seminal root systems from five replicate plants were fixed with FAA (Formalin, Acetic acid, 70% EtOH ; 1 : 1 : 18 parts by volume), and the length and number of the LR that emerged on the 4.5 cm-portion of the root axis were measured. The LR were classified into two components. L-type LR have long and thick root axis and produce further higher order LR on it, S-type LR have short and thin root axis and do not produce higher LR[8]. In addition, developmental patterns of the shoot and nodal root systems were observed.

Results

Experiment 1. ABA levels in the whole root system showed rapid increase at 5 and 10 DAS when the highest value was recorded, thereafter decreased significantly at 14 DAS and then maintained almost constant levels (Fig. 1). The trend in the seminal root system was just the same as that in the whole root system. In the basal 4.5 cm-seminal root portion, the profile was similar to the seminal and whole root systems, although the small peak at 5 DAS was not appeared. In the case of the seminal root tip, a small peak at 5 DAS was noticed but the highest peak was not apparent at 10 DAS. Instead, a significant increment was observed at 14 DAS. The total content per sample at 14 DAS was very small, however, because the root tips had become extremely thin. Therefore, the high value would be caused by the small fresh weight.

ZR levels in the whole root system showed a sudden increment at 14 DAS when the highest level was recorded, and then decreased gradually (Fig. 2). The profile of the seminal root system was similar to that of the basal 4.5 cm-seminal root portion, except for the value at 10 DAS. The levels in seminal roots increased gradually and the highest levels were observed at 21 DAS and then gradually decreased. The peak at 14 DAS in the whole root system would be caused by the increment in the number of nodal roots, since nodal roots emerged from the 2nd node (2nd node nodal roots) should start to emerge at around 14 DAS. This suggested that the changes of the ZR level in the whole root system might be related to the rooting paterns of the nodal roots. Seminal root tip showed a drastic increment at 6 DAS when the content was 9.1 times that of previous day, and it decreased to about half in the next day followed by a constant level for 7 days. The time in the peak at 6 DAS coincided with the time when the numerous 2nd order LR started to emerge on the basal 4.5 cm-portion of the seminal root system (Fig. 3). The number increased 10.1 times from 5 to 6 DAS. Furthermore, it was just the previous

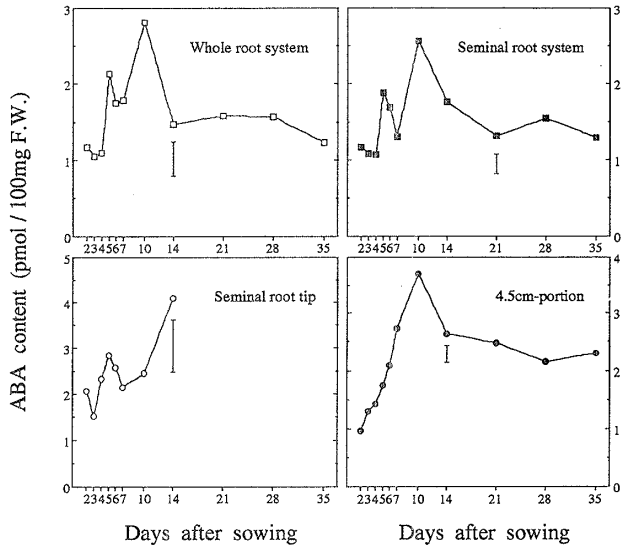

Fig. 1. Changes in the content of abscisic acid (ABA) at each root part in Exp. 1.
Each value represents mean of two point measurements. 4.5 cm-portion means basal 4.5 cm-portion of the seminal root that elongated during 2 days after sowing. Vertical error bars are LSD ($p=0.05$) calculated from ANOVA table.

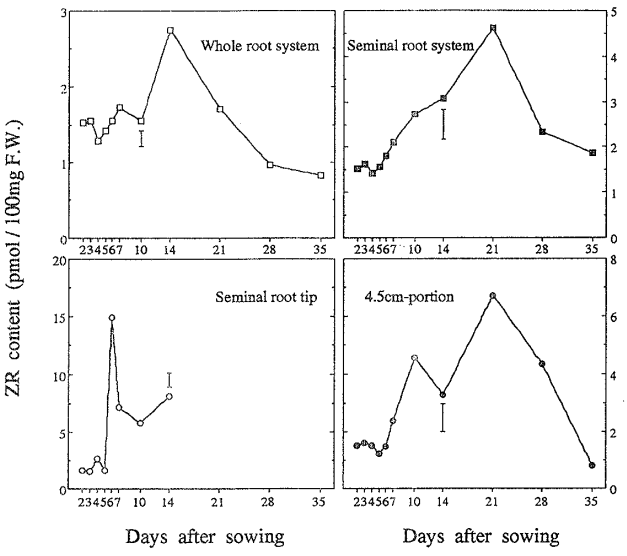

Fig. 2. Changes in the content of zeatin riboside (ZR) at each root part in Exp. 1.
Each value represents mean of two point measurements. Refer to Fig. 1 for the explanation of the 4.5 cm-portion. Vertical error bars are LSD ($p=0.05$) calculated from ANOVA table.

day when the 1st node nodal roots started to emerge (Table 1), and the emergence of 4th leaf (data is not shown). These facts indicated that the increment of the ZR level in the

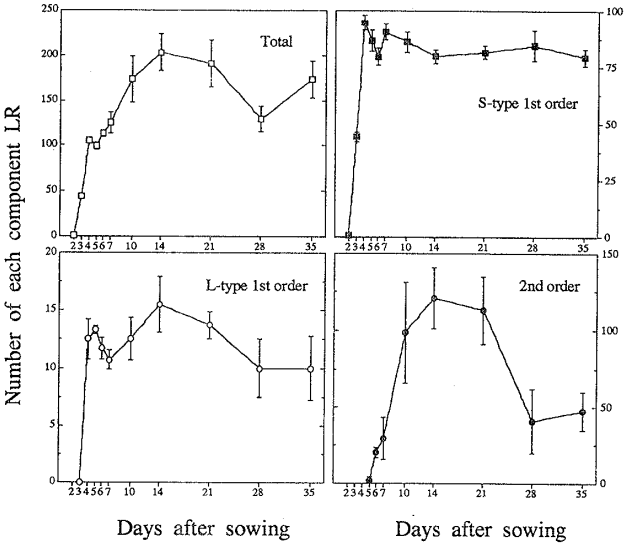

Fig. 3. Changes in the number of each component lateral root (LR) that emerged on the basal 4.5 cm-portion of the seminal root axis in Exp. 1.
Each value represents mean of five replicate measurements ± standard error. Refer to the text for the explanation of each component LR.

seminal root tip would also have close correlation with the emergence of those roots.

Experiment 2. The contents of ABA and ZR were compared between the LR and SRA of the basal 5.1 cm-seminal root portion (Table 2). The ZR content in the SRA was always significantly higher than that in the LR and the difference was much pronounced with the progression of age. In the content of ABA, however, no consistent difference was found between LR and SRA.

Discussion

In this study, endogenous ABA and ZR levels in the rice root system during the early developmental stages were separately measured in different parts or organs with the progression of age. We found several peaks in the ABA and ZR levels in different parts of the root system (Exp. 1), and the different nature of LR and SRA in terms of ABA and ZR levels (Exp. 2).

Firstly, in the seminal root system, the highest levels of the ABA and ZR contents were observed at 10 and 21 DAS respectively (Figs. 1 and 2). When these times are compared with our previous experiment[10], 10 DAS was the time when the introgen concentration in the LR of the seminal root system was de-

Table 1. Changes in the number of the coleoptilar, first node nodal roots and seminal root length in Exp. 1.

Days after sowing	3	4	5	6	7	10
Coleoptilar node nodal root number	2.7±0.9	4.2±0.6	4.7±0.5	5.2±0.6	5.2±0.6	5.2±0.6
First node nodal root number	—	—	—	—	2.4±0.8	5.8±2.0
Whole nodal root number	2.7±0.9	4.2±0.6	4.7±0.5	5.2±0.6	7.6±1.2	11.0±2.3
Seminal root length (cm)	9.7±0.9	14.8±1.3	17.0±1.5	20.3±1.5	21.6±1.5	26.4±2.8

Each value represents mean of twenty replicate measurements ± standard deviation.

Table 2. Changes in the contents of abscisic acid (ABA) and zeatin riboside (ZR) in the lateral roots (LR) and seminal root axis (SRA) of the basal 5.1cm-seminal root portion which was elongated during 2 days after sowing in Exp. 2.

	ABA (pmol/100 mg F.W.)		ZR (pmol/100 mg F.W.)	
DAS	LR	SRA	LR	SRA
3	—	—	0.217±0.064	0.841±0.146
4	1.817±0.004	1.127±0.053	0.291±0.074	0.915±0.189
5	1.873±0.025	1.907±0.077	0.592±0.009	1.214±0.153
7	1.365±0.079	1.621±0.235	0.513±0.046	2.434±0.314
10	1.816±0.050	2.376±0.272	0.665±0.078	3.976±0.669
14	2.583±0.090	2.130±0.023	0.661±0.070	3.437±0.132

Each value represents mean of three point measurements±standard deviation. The values of ABA at 3 days after sowing (DAS) were not presented because of failure in the determinations.

creased to almost the lowest level. On the contrary, 21 DAS was just the time when the concentration in the LR reached the 2nd peak. Samuelson et al.[14,15] documented that ZR levels in barley root system decreased at low nitrogen supply conditions, and concluded that cytokinins might be involved in regulation of nitrate reductase activity. These reports strongly support the present result showing that the sudden increment of ZR level at 21 DAS would be closely related to the nitrogen metabolism in the seminal root system especially in the LR.

Secondly, the drastic increment of ZR content in the seminal root tip at 6 DAS corresponded with the emergence of the 2nd order LR on the SRA and with that of the 1st node nodal roots (Figs. 2 and 3, and Table 1). Moreover, the peak in the whole root system (Fig. 2) would correspond to the emergence of the 2nd node nodal roots. Based on the fact that supraoptimal levels of cytokinins inhibit root growth[19] and LR generally do not emerge nearby the main root tips, Jesko[6] and Wightman et al.[22] pronounced that the cytokinins produced in the main root tips inhibit the development and growth of LR. At the same time, however, cytokinins also play as stimulators of LR initiation under optimal concentration[21]. At 6 DAS, the seminal root length was 20.3 cm (Table 1), and so distance between the site of 2nd order LR emergence in the basal 4.5 cm-portion and seminal root tip was relatively high, and ZR levels around the basal portion might be reduced to the optimal level for LR initiation. In fact, ZR levels in SRA of the basal 5.1 cm-seminal root portion at 7 DAS was twice higher than that at 5 DAS (Table 2). Therefore, results obtained from this study could be interpreted to show that emergence of the 2nd order LR near the root base would be caused by supraoptimal levels of ZR produced in the seminal root tip. Of course, initiation of LR would also be controlled by other factors. On the contrary, the relationship with the 1st order S- and L-type LR could not recognized. This means the hormonal regulation of LR development would be different

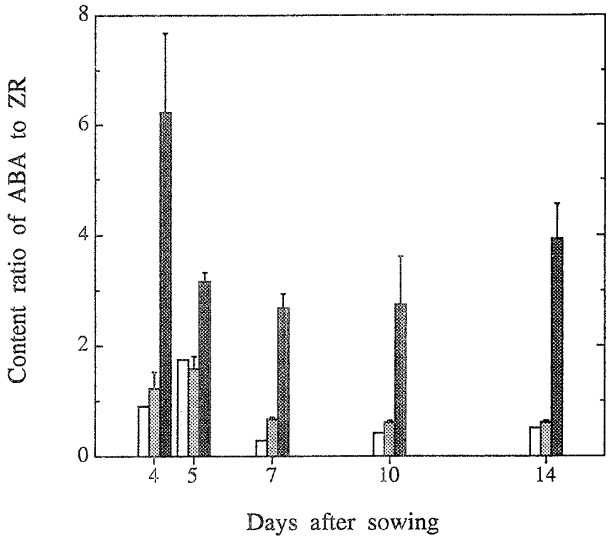

Fig. 4. Changes in the content ratio of abscisic acid (ABA) to zeatin riboside (ZR) in the seminal root tip (□) in Exp. 1, seminal root axis (▨) and lateral roots (■) of the basal 5. 1 cm-seminal root portion in Exp. 2. Vertical error bars are standard deviations of three replicate measurements. The error bars in the seminal root tip are not presented because of two point measurements.

among different types or orders of LR. Detailed study on the optimal levels in ZR is necessary to examine the participation of ZR in the emergence of each component LR.

Thirdly, ABA and ZR levels were separately measured in the LR and SRA in Exp. 2. The ZR levels in SRA were significantly higher than those in LR (Table 2). Root tips are thought to be the main sites of cytokinin production in a root system[16,17,20]. There are hundreds of root tips in LR, whereas the only one root tip exists in SRA. Accordingly, total production of ZR is supposed to be much higher in LR than in SRA. Plant hormones generally move from the synthesized sites to where they should act[6,13]. Indeed, ZR are regarded as the translocating cytokinin type[4,16,20]. Therefore, ZR content in SRA obtained in this experiment would involve the synthesized ZR *in situ* and the translocated one from other parts of the root system. Since we can not evaluate the real production of ZR *in situ* in LR and SRA, a different approach is necessary for this purpose.

When the net amount of ABA and ZR were compared between the basal seminal root portions of Exps. 1 and 2, the contents in the Exp. 2 were found to be much lower than those in the Exp. 1 (data not shown). Although we completed the dividing procedure into LR and SRA within 10 to 20 minutes, one possible explanation for the result is sample loss during the procedure. Since ZR is more water-soluble than ABA, the loss of ZR during the dividing procedure might be more accountable for the low level of ZR in LR. More improved experimental device should be employed to overcome this problem. In spite of the supposed loss, however, the trend of the ZR level in the Exp. 2 corresponded well with that in the Exp. 1. Moreover, evaluation of endogenous plant hormones in LR has never been conducted before, and this study is the first trial.

In order to compare the characteristics of the seminal root tip (Exp. 1), LR and SRA (Exp. 2) in terms of the hormonal distributing patterns, the content ratio of ABA to ZR was calculated (Fig. 4). The ratios in the seminal root tip and SRA were more or less similar, however the ratio in the LR was 1.8 to 6.3 times greater than seen in the other two. This result strongly indicated that characteristics of the LR and SRA in their hormonal works are quite different, and that seminal root tip is very similar to the main root axis in its hormonal nature. However, the reason that ZR tends to accumulate much more in SRA than LR is still not clear.

We conclude from this study that ABA and ZR in the seminal root system of rice may have close relation to the nitrogen metabolism of the LR. Moreover, LR and SRA are quite different in their kinetics of ABA and ZR levels. Further detailed experiments are needed to define the physiological role of LR and SRA in the formation of rice root system.

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