Studies on Nitrogen Metabolism in Crop Plants

XIV. Changes in cytokinins in rice grains during ripening*

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INTRODUCTION

In developing rice grains^{5,13)}, fundamental structure of the endosperm is almost completed on the 5th day after flowering and continuously starch grain begins to accumulate in endosperm cells, while protein appears somewhat lately on the 10th day after flowering. These accumulation processes succeed rapidly to the 20th day, and last slowly until the 30th day after flowering.

There are few reports dealing with the relations between the movement of photosynthate and growth regulators in seeds. WEAVER *et al.*¹²⁾ reported that the application of benzyladenine induced the movement of ¹⁴C-assimilate into fruit clusters of grapevins. Recent evidence indicated that cytokinin in barley grain⁸⁾ affected to the accumulation process and the duration of the filling period.

In this experiment, we have investigated on the qualitative and quantitative changes in cytokinins in rice grains during ripening, and results are discussed in relation to the filling period and the accumulation process.

MATERIALS AND METHODS

Plant cultivation

The rice plant (*Oryza sativa* L.) varieties, Honenwase, Hatsukaori and Moritawase, were used. The rice seedlings grown in nursery bed until the 6th leaf stage were transplanted on sandy loam soil in 1/5000 a Wagner pot with an application of N, P₂O₅ and K₂O fertilizer at the rate of 1, 1 and 1 g on May 15, respectively, and each pot with two seedlings was arranged at the distance of 25×20 cm under natural condition. The rice grains were

collected at some intervals after heading time, and deepfreezed until extraction for cytokinins.

Purification and quantitative estimation of cytokinins

Cytokinins in the materials were partially purified by ion exchange chromatography and the activity was determined chlorophyll preservation and carrot callus growth tests¹⁵⁾. The quantitative estimation of cytokinins were performed with a Shimadzu gas chromatograph Model GC-4A fitted with a hydrogen flame ion detector as trimethylsilyl derivatives.

RESULTS

Cytokinins distribution during ripening of rice grains

Carrot callus bioassay results (fig. 1) show that in the rice grains, there were three zones with cytokinins activities at Rf's 0.0~0.2, 0.3~0.5 and 0.6~0.9. The overall activities of three zones became much higher at the milk ripe stage and then gradually decreased toward the yellow ripe stage. In our early work¹⁴⁾, it has been shown that the cytokinin activity at Rf 0.3~0.5 corresponded to zeatin glucoside and the activity at Rf 0.6~0.9 consisted with zeatin and its riboside, but the active zone at Rf 0.0~0.3 composed of unknown cytokinin except for zeatin ribotide.

Among these cytokinins, the level of free cytokinin being extractable with ethyl acetate such as zeatin and its riboside was much greater in the early ripe stage, whereas bound cytokinin which remained in the aqueous phase increased slightly at the yellow ripe stage (fig. 2).

The amounts of zeatin and its riboside were determined by gas chromatography after purification with TLC, and bound cytokinin was also

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Fig. 1 Changes in cytokinins activities of rice grains during ripening (var. Honenwase). Twenty five g equivalent of rice grains was chromatographed by Toyo No 51 filter paper with n-butanol-acetic acid-water (12:3:5) solvent. A; heading stage (6/V111). B and C; milk ripe stage (11/V111 to 16/V111). D; yellow ripe stage (21/V111)

Fig. 2 Activity levels in free cytokinin which was extractable with ethylacetate (solid line) and bound cytokinin which remained in the aqueous phase (dotted line) of rice grains at the two ripening periods (var. Honenwase). Bound cytokinin was bioassayed as zeatin liberated by hydrolyzing with 1 N HCl. A; heading stage (1/V111). B; yellow ripe stage (20/V111)

estimated as zeatin liberated by hydrolyzing with 1 N hydrochloric acid. Fig. 3 shows the quantitative changes in zeatin contents in rice grains during ripening. Zeatin content per 100 g fresh weight reached the highest value shortly after heading time and declined progressively with maturing. In contrast, when 1000-grain was based, zeatin content increased sharply to the 3 days after heading time and thereafter remained nearly constant for 9 days, during which time the intensive increment in 1000 grains weight occurred, and after which time the level of zeatin decreased rapidly.

However, as shown in table 1, the amounts of zeatin ribotide increased obviously with maturing of grains although zeatin glucoside did not change so much during ripening.

These results suggest that levels in zeatin and its related compounds were closely associated with the accumulation process of the photosynthate into the grains from the other parts of plant.

Table 2 shows the changes in cytokinins content in the developing rice grains of a recently-established variety Hatsukaori and a long-established Moritawase. Generally, it seems that Hatsukaori produces a higher grain yield than Moritawase, because the former has a longer filling period than the latter.

Fig. 3 Changes in zeatin content of rice grains and 1000 grains weight during ripening (var. Honenwase). ●—●; Zeatin content per 100 g fresh weight. ■—■; Zeatin content per 1000 grains. ▲—▲; 1000 grains weight (g)

Table 1 Quantitative distribution of cytokinins in rice grains at the heading and yellow ripe stages (var. Honenwase)

Growth stages	Cytokinins content, $\mu\text{g}/100\text{ g FW}$			
	Ethyl acetate fraction		Aqueous fraction ¹⁾	
	Zeatin	Zeatin riboside	Zeatin ribotide	Zeatin glucoside
Heading stage (5/VIII)	830	trace	245	460
Yellow ripe stage (25/VIII)	430	trace	415	370

Note: 1), Aqueous phase contained bound zeatin such as zeatin glucoside and zeatin ribotide. Zeatin ribotide was precipitated with barium hydroxide, while zeatin glucoside remained in the supernatant fraction. Zeatin contents in the precipitated and supernatant fractions were determined after they were treated with 1 N HCl at 100°C

Table 2 Changes in cytokinins content in rice grains of Hatsukaori and Moritawase during ripening

Date after heading	Hatsukaori			Moritawase		
	Cytokinins content, $\mu\text{g}/100\text{ g FW}$			Cytokinins content, $\mu\text{g}/100\text{ g FW}$		
	Zeatin	Zeatin riboside	Bound zeatin ¹⁾	Zeatin	Zeatin riboside	Bound zeatin ¹⁾
8/VIII (heading time)	—	—	—	765	trace	225
14/VIII	3,620	trace	270	1,885	trace	300
19/VIII	2,979	trace	200	2,920	trace	265
30/VIII	1,800	trace	130	960	trace	330
6/IX	1,180	trace	290	—	—	—

Note: 1) Bound zeatin content was determined after it was treated with 1 N HCl at 100°C

Fig. 4 Distribution of UV absorption (left) and cytokinin activity (right) in the elution of Dowex 50 W column adsorbed with the methanolic extract of rice grains. Elution was carried out with 1 N HCl

It should be also noted that the amounts of zeatin increased remarkably in the grains of Hatsukaori at the 6 days after heading time and thereafter decreased slowly toward the full ripe stage, whereas in the grains of Moritawase, the level of zeatin was lower and that of bound cytokinin was relatively higher than Hatsukaori.

Identification of new bound cytokinin

Some chemical and biological properties of new cytokinin from the slow-moving active zone (R_f 0.0~0.2) were also investigated. 2 Kg of rice grains (Hatsukaori) were collected at the milk ripe stage and extracted with 80% methanol. The methanolic extract was evaporated in vacuo at 35°C. The residue was dissolved in pH 2.5 water and fractionated on Dowex 50 W column. The column was eluted with 1 N hydrochloric acid at the rate of 10 ml per tube, and then assayed for cytokinin activity.

Results of the elution profile and bioassay are shown in fig. 4. Most of the cytokinin activity was observed in the peak A. It was very effective in promoting the carrot callus growth, but the

peak B and C were less active at the concentration of 300 or 600 g equivalent weight.

The peak A was further resolved by paper chromatography with n-butanol-acetic acid-water (12:3:5) solvent into the three cytokinin substances, whose R_f values were 0.0~0.2, 0.2~0.3 and 0.4~0.5 (fig. 5). The active substances at R_f 0.2~0.3 and 0.4~0.5 corresponded to the R_f value of zeatin ribotide and zeatin glucoside, respectively.

Moreover, the unknown substance at 0.0~0.2 was purified with barium hydroxide because its substance reacted with a phosphate colour reagent, ammonium molybdate solution.

An aliquot solution of the substance was adjusted at pH 8.0 with saturated barium hydroxide and then two volumes of absolute ethanol was added. The obtained supernatant and precipitate were bioassayed respectively. The cytokinin activity was only observed in the supernatant fraction. The precipitate has been identified as phytic acid which was less effective in both the carrot callus growth and rice chlorophyll preservation tests.

The active material in the supernatant was further

Fig. 5 Activities in bound cytokinins of rice grains at the milk ripe stage. Bound cytokinin in the peak A obtained from the Dowex 50 W column was chromatographed by Toyo No 51 filter paper with *n*-butanol-acetic acid-water (12:3:5) solvent

resolved by TLC with *n*-propanol-ammonium hydroxide-water (40:60:5) solvent into the two bands, whose *R_f* values were 0.68 and 0.79. The cytokinin activity was much higher in the latter than in the former. The latter substance reacted with aniline hydrogen phthalate and ninhydrin reagents, but the detail of the chemical nature could not be revealed.

DISCUSSION

Several investigations have demonstrated that endogenous cytokinins can regulate cell division and accumulating process in the various higher plants. A positive correlation between cytokinin level and rate of cell division was found in plum and apple fruits^{6,7)}. In developing cotton fruit, rice, avocado and pumpkin seeds^{1,3,9,11)}, the level of measurable cytokinin activity was much higher during the early stages of development, and as the tissue matured the cytokinin level also decreased.

The fact that the cytokinin activity was correlated with the fruit or seed growth rate may indicate that also in the rice grains cytokinins play a major

role in the accumulation process and influence on the duration of ripening period.

In the present experiment, we showed that levels of zeatin and its related compounds in rice grains changed dramatically with maturation. Among those cytokinins, the highest content of zeatin occurred in several days after heading time, whereas the content of zeatin ribotide increased toward the yellow ripe stage (table 1). However, when 1000-grain was based, the highest content of zeatin remained nearly constant during the intensive increment in 1000 grains weight (fig. 3).

These results suggest that there is a positive correlation between zeatin level and increment rate of rice grains weight. Recently, MICHAEL *et al.*⁸⁾ reported that cytokinins participated in the regulation of barley grain size, possibly by influencing accumulation process and the duration of the filling period. They found that cytokinin activity per grains was significantly lower in the small-grain varieties than in the large-grain ones.

The data of table 2 indicate that grains of Hatsu-kaori having a long-filling period were distinctly higher in zeatin content than those of Moritawase.

Probably, free zeatin is an "active form" as cytokinins for prolongation of the filling period and its ribotide seems to be a "storage form".

In senescent leaves of *Populus X robusta*, HEWITT and WAREING pointed out that fraction Z, which corresponded to zeatin and its riboside, decreased and fraction N, which was tentatively identified as zeatin glucoside, increased to predominate. Contrastively, in the seeds of *Leucadendron daphnoides*, high level of water soluble cytokinin which was found in the unchilled seed, decreased accompanied by a concomitant increase in butanol soluble cytokinin under low temperature treatments.

These and authors results suggest that there is an interconversion mechanism between free and bound cytokinins in the ripening rice grains.

Lastly, the physiological significance of a new bound cytokinin separated from the slow-moving active zone (*R_f* 0.0~0.2) could not be revealed in this experiment, but it wants yet to be confirmed.

SUMMARY

Qualitative and quantitative changes of cytokinins in rice grains were investigated during ripening. Three rice varieties, Honenwase, Hatsu-kaori and Moritawase were used. The results obtained are as follows.

1. Three zones of cytokinins activities in rice grains were present at Rf 0.0~0.2, 0.3~0.5 and 0.6~0.9 by paper chromatography with n-butanol-acetic acid-water (12:3:5) solvent, and these activities became much higher at the milk ripe stage and then gradually decreased toward the yellow ripe stage. However, among these cytokinins, the activity level of free cytokinin being extractable with ethyl acetate such as zeatin and its riboside was much greater in the early ripe stage, whereas bound cytokinin in the aqueous phase increased slightly at the yellow ripe stage.
2. Quantitative determination of cytokinins in rice grains showed that the highest content of zeatin occurred in 3 days after heading time and declined progressively with maturing, but the content of zeatin ribotide increased toward the yellow ripe stage. However, when 1000-grain was based, the highest content of zeatin remained nearly constant for 9 days, during which time the intensive increment in 1000 grains weight occurred, and after which time the level of zeatin decreased rapidly.
3. The amounts of zeatin increased considerably in the grain of Hatsu-kaori having a longer filling period, whereas in the grains of Moritawase the level of zeatin was lower and that of bound cytokinin was relatively higher than Hatsu-kaori.
4. A new bound cytokinin was separated from the slow-moving active zone (Rf 0.0~0.2) but the chemical properties were not revealed.

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〔和 文 摘 要〕

作物の窒素代謝に関する研究

第14報 水稻穀実の登熟過程におけるサイトカイニン
の変化について折 谷 隆 志・葭 田 隆 治
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本研究は水稻穀実の登熟過程において、穀実のサイトカイニンが量的、質的にどのように変化するか、また、これらのサイトカイニンが穀実における同化産物の蓄積過程においてどのような役割を果たしているかを解明しようと試みたもので得られた結果は次の通りである。

1. 水稻の穀実には遊離サイトカイニンとしてゼアチン、ゼアチンリボシド (Rf 0.6~0.9) を含むバンド、結合型サイトカイニンとしてゼアチンリボシドとゼアチングルコシドを含むバンド (Rf 0.3~0.5), と新しい活性バンド (Rf 0.0~0.2) の3つの活性区分が認められた。一般にこれらのサイトカイニン活性は乳熟期ごろに最も高く、黄熟期には著しく低下した。
2. 穀実の遊離型と結合型サイトカイニンの比をみると、登熟初期にはゼアチンを主体とした遊離型が多いが後期にはゼアチンリボシドを主体とした結合型が増加した。
3. 穀実の遊離型サイトカイニンの主体をなすゼアチンの推移をみると、これは単位生体重当たりとして計算された場合には、出穂後約3日目に著しく増加し、以降、穀実の発育に伴って減少している。しかし、これを1,000粒当たりとして計算してみると、ゼアチン含量は出穂後3日目に最高に達し、それ以降登熟の最も著しい約9日間はほぼ一定の値を保ち以後、減少した。
4. 最近育成された登熟期間の長い〈はつかおり〉では登熟期間の短い〈森田早生〉に比べて穀実のゼアチン含量は全登熟期間を通じて著しく高かった。これに対して森田早生ではむしろ結合型サイトカイニンがやや高い傾向を示していた。これらの結果から、穀実の機能維持のためにゼアチンは活性型として重要な役割を果たしており、ゼアチンリボシドはむしろ貯蔵型とも考えられる。
5. 新しい活性物質 (Rf 0.0~0.2) は活性の低いフィチン酸と未知の活性物質から成り立っているが、この未知の物質の化学的特性は明らかではなかった。