Free Amino Acids in the Bleeding Sap and Developing Grain of the Rice Plant[1]

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ABSTRACT

Amino acids in the bleeding sap collected from the culm of the rice plant (*Oryza sativa* L.) and in the ethanolic extract of grain at the midmilky stage (10 days after flowering) were analyzed from seven pairs of lines differing in grain protein content. The concentration of total free amino acids in the developing grain of the high-protein lines was higher than that in the grain of the low-protein lines for all pairs. Only five pairs showed a similar trend for total free amino acids in the sap. The sap and the grain differed in the composition of free amino acids.

Previous studies on the biochemistry of protein accumulation in the developing rice grain showed that lines with a high protein content in the mature grain incorporated amino acids faster than lines low in protein, and had more free amino nitrogen and RNA (1). Physiologists believe that the amino acids of rice grain are derived mainly from the breakdown and translocation of protein already present in the vegetative tissues of the plant at flowering (2).

Plant breeders at the International Rice Research Institute have developed several pairs of lines that are well suited for investigating the biochemical differences between high- and low-protein rice (3). The two members of each pair are genetically similar because they are the progenies of the same cross, but they have different levels of protein in the grain. We analyzed the content and composition of free amino acids in the bleeding sap and developing grain of seven such pairs at the midmilky stage (10 days after flowering) of grain development.

MATERIALS AND METHODS

Seed Source

Seven pairs of dwarf rice lines (*Oryza sativa* L.) from the F5 seeds of IR8 crosses with four high-protein varieties (Rikuto Norin 20, Chow Sung, Omirt 39, and Chok-jye-bi-chal) were selected for their wide differences in grain protein content. Three plants of each line were grown in a pot containing 7.5 kg. soil, 10 g. $(NH_4)_2SO_4$, 4 g. KCl, and 4 g. $Ca(H_2PO_4)_2 \cdot$ The pots were arranged 40 cm. apart in a Mylar house where the plants were watered daily and sprayed regularly with insecticides.

Sap Collection

Cotton balls large enough to hold 0.5 ml. water were extracted for 24 hr. with refluxing 80% (v./v.) methanol. After the excess methanol was squeezed out of the cotton balls they were soaked overnight in 0.33% (v./v.) aqueous thimerosal NFX1

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(merthiolate). Excess merthiolate was squeezed out and the cotton balls were dried at 100°C. in an oven. The dried cotton balls were weighed in groups of ten and individually wrapped with Parafilm (American Can Co.) sheets. The researchers wore gloves when handling the cotton balls.

The sap of the rice plant was collected 10 days after flowering. Ten tillers from each pot were cut off at the internode closest to the panicles. The stems were then individually covered with the cotton balls, which were held in place with Parafilm sheets. The potted plants were enclosed in plastic bags for 12 hr. (from 7 P.M. to 7 A.M.). For uniformity, the panicles of the rest of the tillers were similarly cut off. The gain in weight of the cotton balls was considered the weight of the sap collected for 12 hr. The grains from the selected tillers were collected and kept at 0°C. for analysis.

Chemical Determinations

The developing grains collected 10 days after flowering were assayed for dry weight, protein, and soluble amino nitrogen as described in our previous work (1). In addition, the composition of the free amino acids was determined as described below.

Preparation of Sap for Amino Acid Analysis

The cotton balls were placed in a medium-porosity sintered-glass filter and washed at 25°C. with 20 ml. cold distilled water ten times (complete extraction). The aqueous extracts were evaporated to dryness under reduced pressure at 50°C. The residue was dissolved in 0.5 ml. 0.1N HCl and 2 ml. of sample dilutor (pH 2.2, 0.30N lithium (Li), 0.10M citrate) was then added. The resulting solution was then centrifuged at 500 X g for 10 min. at 25°C. and an appropriate fraction of the clear supernatant liquid was analyzed for amino acids.

Preparation of Free Amino Acids in the Rice Grain

Fifty grains were dehulled and homogenized in 14.15 ml. distilled water, as described by Cruz et al. (1). The homogenate was centrifuged at 500 X g for 15 min. at 4°C. Anhydrous ethanol (40 ml.) was mixed with 10 ml. of the supernatant fluid. The resulting precipitate was removed from the ethanolic solution by filtering the mixture through medium-pore sintered glass and washing it with 80% (v./v.) ethanol. The combined filtrate and washings were evaporated to dryness under reduced pressure at 50°C. The residue was dissolved in 0.5 ml. 0.1N HCl and subsequently diluted with a 2.0-ml. sample dilutor (pH 2.2). This solution was then analyzed for amino acids.

Single-Column Amino Acid Analysis

The analysis was conducted in the Beckman/Spinco Model 120C amino acid analyzer, using only a 69 X 0.9-cm. column of UR-30 resin. Lithium citrate buffers were used. The run was started for the acidic and neutral amino acids strictly following the procedures recommended by Beckman Instruments (4), and was continued for the basic amino acids. The temperature was shifted from 39° to 62.5°C. and the buffer was shifted from pH 4.16 (0.30N Li) to pH 5.36 (0.38N Li) after the appearance of the phenylalanine peak corresponding to 260 min. running time. When the ornithine peak appeared (at 320 min.), the buffer was again changed to pH 6.72 (0.35N Li). An analysis was completed in 10.5 hr.

RESULTS

Amino Acid Content and Composition of the Bleeding Sap

The amount of culm bleeding sap collected 10 days after flowering ranged from 2.10 to 13.1 g. per 10 tillers for the 12-hr. collection period (Table I). It varied widely between lines from the same cross and between crosses. Five high-protein (HP) lines yielded more sap than their low-protein (LP) counterparts for the seven pairs of samples, whereas the remaining two pairs showed the opposite trend.

The total free amino nitrogen in the collected sap was higher in the five HP than in the LP lines. These were the five HP lines which yielded more sap. In four of these five pairs, the free amino nitrogen per gram of sap was higher in the HP lines. The opposite trend was found in the remaining two pairs, where the sap from the HP line had less amino nitrogen and lower weight than the corresponding sap from the LP line. In contrast, the corresponding level of free amino nitrogen of the developing grain was consistently higher in the HP samples for all the seven pairs.

Amino acid analyses showed 32 acids, eight of which were present in minor amounts and which were not positively identified. Most amino acids showed a wide range of values among the 14 samples. The proportion of asparagine, glutamic acid, glutamine, histidine, serine, and threonine tended to be much higher in the free amino acids of the sap from HP lines than in the free amino acids from LP samples in 4 to 6 of the 7 pairs. The asparagine level tended to be higher than that of glutamine. Histidine was the principal amino acid, particularly in the HP samples.

Similar analyses were also obtained from bleeding sap collected 6 and 14 days after flowering. Presumably, the amino acid composition in the sap translocated into the grain remains fairly constant during grain development. Some of these amino acids have been previously identified in rice sap by other workers (5,6).

Free Amino Acid of the Developing Rice Grain

The mean dry weight of the dehulled grain gathered 10 days after flowering ranged from 8.8 to 13.2 mg. for the 14 samples. In the 7 pairs of lines, the differences in mean grain weight within each pair were not significant. Six days after flowering, the mean dry weight of dehulled grain ranged from 2.5 to 3.3 mg. Fourteen days after flowering, the grain weighed from 10.8 to 14.6 mg.

The level of free amino nitrogen in the 10-day-old grain was positively correlated ($r = 0.84^{**}$) with the protein accumulated by the same grain. This relationship confirms our previous findings with varieties and lines differing in protein content in the mature grain (1).

In the two pairs of lines with comparable grain yield and maturity, the composition of the free amino acids of the sap entering the panicle and of the developing grain 10 days after flowering were compared (Table II). The same 32 amino acids were detected in the grain. The free amino acids differed widely in composition from those of protein of the developing IR8 grains found by Palmiano et al. (7). The major free amino acids included alanine, aspartic acid and asparagine, valine, glutamic acid, histidine, and ornithine, whereas the major amino acids of rice protein are alanine, arginine, aspartic acid, glutamic acid, leucine, and valine. Some of these acids have previously been identified by other workers studying the developing rice grain (8,9,10).

The HP grain tended to have more asparagine, γ-aminobutyric acid, glutamine,

histidine, homoserine, and hydroxyproline in its free amino acids than the free amino acids of the LP grain. However, the higher level of free amino nitrogen in the grain, the higher the proportion of the two amides (aspargine and glutamine) relative to the acid forms (aspartic and glutamic acids). Higher levels of free amino acids were found in the HP grain than in the LP counterpart.

The sap samples from these two pairs showed some relationships which were not apparent in the combined data for the seven pairs, probably because of the greater differences in the content of free amino nitrogen observed for the paired samples in Table II than in the combined data in Table I. However, the levels of asparagine,

TABLE I. PROPERTIES OF CULM SAP AND GRAIN AND AMINO ACID COMPOSITION OF CULM SAP COLLECTED 10 DAYS AFTER FLOWERING FROM SEVEN PAIRS OF LINES DIFFERING IN PROTEIN CONTENT[a]

Property	Low-Protein Lines		High-Protein Lines	
	Range	Mean	Range	Mean
Properties of culm sap				
Wt. of sap collected (g./10 tillers)	2.10– 13.1	5.53	3.69– 7.25	6.00
Amino N content of sap (γ/10 tillers)	5.91–174	65.0	18.0 –183	90.1
Properties of dehulled grains				
Dry wt. (mg./grain)	8.85– 12.7	10.9	9.30– 13.2	11.6
Protein (mg./grain)	0.53– 1.29	0.94	0.96– 1.51	1.25
Soluble amino N (γ/grain)	4.53– 9.61	6.25	7.86– 10.5	8.67
Amino acid content of collected sap[b] (γ/10 tillers)				
Alanine	1.44– 5.89	3.18	1.90– 7.15	4.65
Arginine	2.00– 75.9	31.7	3.19– 63.6	28.1
Asparagine	0.92– 66.9	19.7	6.89– 88.8	51.1
Aspartic acid	2.47– 15.0	6.22	4.76– 13.7	8.83
Cystine and valine	3.21– 77.1	31.6	9.28– 98.7	41.5
γ-Aminobutyric acid	0.09– 3.10	0.91	0.46– 2.76	1.30
Glutamic acid	2.89– 13.5	6.51	2.61–197	36.7
Glutamine	1.99– 49.3	18.1	2.61– 76.0	33.1
Glycine	0.87– 5.89	2.55	1.34– 7.95	4.37
Histidine	1.45–515	95.8	22.9 –249	91.8
Homoserine	trace– 5.70	1.24	trace– 5.44	3.59
Hydroxyproline	trace– 2.46	0.51	trace– 2.94	1.17
Isoleucine	1.23– 35.4	12.2	0.78– 49.3	21.5
Leucine	1.82– 52.1	15.5	0.91– 50.0	21.5
Lysine	1.50– 25.8	8.87	0.97– 31.3	13.2
Methionine	trace– 5.15	1.83	0.11– 5.89	2.52
Ornithine	1.56– 5.95	3.04	1.92– 6.31	3.85
Phenylalanine	1.41– 30.6	9.87	1.46– 34.6	13.7
Proline	0.82– 7.31	3.26	2.33– 10.8	6.19
Serine	3.25– 15.0	7.69	4.36– 54.1	20.0
Threonine	1.72– 29.0	10.9	2.62– 39.2	19.0
Tryptophan	0.10– 13.6	4.48	3.55– 10.5	8.18
Tyrosine	1.46– 25.5	8.05	1.49– 36.9	16.3
Ammonia	0.03– 1.51	0.47	0.05– 0.85	0.39
Total amino acids	36.8 –683	300	90.4 –910	452

[a]Crude protein content of mature grain (N × 5.95, % dry basis): low-protein lines, 8.90 to 15.0% (mean, 12.4%); high-protein lines, 10.3 to 17.4% (mean, 14.9%).

[b]Collection period of 12 hr. (7 P.M. to 7 A.M.).

TABLE II. MEAN AMINO ACID COMPOSITION OF THE BLEEDING SAP AND DEVELOPING GRAIN COLLECTED FROM TWO PAIRS OF LOW-PROTEIN (LP) AND HIGH-PROTEIN (HP) LINES 10 DAYS AFTER FLOWERING[a]

	Amino Acid Content			
	Sap[b]		Grain	
Amino Acid	LP lines	HP lines	LP lines	HP lines
	γ/10 tillers		mg./g. grain dry wt.	
Alanine	1.99	4.74	0.30	0.56
Arginine	2.41	10.9	0.15	0.27
Asparagine	1.27	62.5	0.13	0.38
Aspartic acid	3.46	12.1	0.49	0.56
Cystine and valine	6.01	56.8	0.10	0.43
γ-Aminobutyric acid	0.32	1.72	0.03	0.14
Glutamic acid	3.14	104	0.46	0.70
Glutamine	2.07	39.3	0.07	0.35
Glycine	1.06	5.93	0.11	0.17
Histidine	4.56	94.1	0.41	2.14
Homoserine	0.73	7.00	0.13	0.38
Hydroxyproline	trace	2.27	0.01	0.25
Isoleucine	1.67	26.6	0.06	0.12
Leucine	2.33	27.2	0.11	0.18
Lysine	1.63	18.8	0.08	0.13
Methionine	0.72	3.52	0.02	0.06
Ornithine	2.57	3.93	0.26	0.47
Phenylalanine	1.66	18.4	0.06	0.09
Proline	1.52	4.77	0.13	0.17
Serine	3.62	35.1	0.19	0.42
Threonine	2.04	23.4	0.10	0.23
Tryptophan	0.82	7.02	0.01	0.03
Tyrosine	1.72	16.9	0.07	0.11
Ammonia	0.03	0.36	0.004	0.008
Total	47.4	587	3.48	8.34

[a]Crude protein (N × 5.95) contents (dry basis) of the dehulled mature grain were 9.0 and 10.3% in the LP lines and 12.5 and 15.4% in the HP lines.

[b]Collection period of 12 hr. (7 P.M. to 7 A.M.).

glutamic acid, glutamine, histidine, and isoleucine in the sap of HP lines are more than 15 times higher than in the sap of LP lines. Hence, these acids showed a greater increase than the 12-fold difference in total free amino acids of the sap between LP and HP lines.

The proportion of asparagine relative to aspartic acid in the sap was higher than in the developing grain. The levels of asparagine and glutamine in the free amino acids of sap and grain, however, were comparable. The asparagine concentration tended to be higher than the glutamine concentration in the sap and grain of HP lines. However, the proportion of aspartic acid to the amide or asparagine form in the sap was greater than the proportion of glutamic acid to the glutamine form.

DISCUSSION

The higher level of free amino acids in the developing grain of HP lines was related to the higher level of free amino acids in the corresponding sap entering the

grain in five of the seven pairs studied. The rate of translocation of the amino acids from the vegetative tissues, and the rate of assimilation of soil nitrogen by roots, may directly affect the level of free amino acids in the sap and in the grain, and ultimately affect the accumulation of protein during grain development in lines differing in protein content. Physiologists, however, believe that the grain nitrogen comes mainly from the nitrogen accumulated in the plant before flowering, because the total nitrogen content of the plant changes little during grain development and because the increase in nitrogen content in the grains is almost matched by the decrease in nitrogen content in the vegetative tissues (2). Presumably, a genetically high protein content in the grain is associated with a more-rapid rate of breakdown of protein in the vegetative tissues, and subsequent translocation of the free amino acids thus formed to the developing grain. Similar findings have been made in wheat (11).

Environmental factors, particularly late application of fertilizer, probably increase the amount of nitrogen assimilated by plant roots, which results in a higher level of free amino acids in the sap. Hence, environmental factors probably increase the protein content of the grain by increasing the level of free amino acids, which are the substrates for protein accumulation. Other biochemical factors, such as the rate of amino acid incorporation by the grain and the RNA level, may be less readily affected by environment. However, Martinović et al. (12) found that levels of RNA are closely related to total protein in wheat. Previous work in our laboratory (13) indicated that an increase in grain protein content in a rice variety involved increases in the quantity of glutelin and prolamin, but not of the soluble proteins.

The differences in free amino acid composition between the sap and the ripening grain, and the composition of rice-grain protein indicate that the developing rice grain has more than one amino acid pool as demonstrated in other plant tissues (14,15).

The higher level of asparagine in the sap relative to glutamine is interesting. Izumi (16) showed that glutamine, and not asparagine, accumulates in the rice plant during photosynthesis. However, our sap collection was done during the night, and Mitsui et al. (17) found that in the dark the rice plant produces asparagine from the breakdown of proteins. Other workers (18,19,20) have found that the asparagine concentration of the leaves is directly related to the nitrogen nutrition of the plant.

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