

Physiological Processes Contributing to the Difference in Grain Amino Acid Content between Two Hybrid Rice (*Oryza sativa* L.) Cultivars

Min Huang*, Hengdong Zhang, Chunrong Zhao, Guanghui Chen and Yingbin Zou

Crop and Environment Research Center for Human Health, College of Agronomy, Hunan Agricultural University, Changsha, 410128, China.

*Corresponding Author: Min Huang. Email: mhuang@hunau.edu.cn.

Abstract: Improving grain amino acid content of rice (*Oryza sativa* L.) is essential for the health of consumers. This study was conducted to identify the physiological processes that contribute to the higher grain amino acid content in hybrid rice cultivar Lingliangyou 268 compared to Luliangyou 996. The results showed that total amino acid content in grains was 9% higher in Lingliangyou 268 than in Luliangyou 996. There was no significant difference in grain nitrogen (N) content between Lingliangyou 268 and Luliangyou 996, while ratio of amino acid to N was 6% higher in Lingliangyou 268 compared to Luliangyou 996. A total of 16 differentially expressed proteins related to amino acid metabolism (e.g., erythronate-4-phosphate dehydrogenase domain containing protein) were identified in grains between Lingliangyou 268 and Luliangyou 996. The identified proteins were involved in 10 molecular functions. Six of the 10 defined functions were related to binding (heterocyclic compound binding, nucleoside phosphate binding, nucleotide binding, organic cyclic compound binding, protein binding, and small molecule binding) and the other 4 defined functions were catalytic activity, enzyme regulator activity, hydrolase activity, and transferase activity. These results indicate that the higher grain amino acid content in Lingliangyou 268 compared to Luliangyou 996 is attributed to increased efficiency of converting N to amino acid that results from altered expression of proteins related to amino acid metabolism.

Keywords: Amino acid content; grain quality; protein expression; protein function; hybrid rice

1 Introduction

Rice (*Oryza sativa* L.) is the staple food for more than 65% of the population in China [1]. In recent years, the demand for high-quality rice production has increased in China as people's living standards are improving [2,3]. Provision of more nutritious foods is ever more demanded in the market place as people's health becomes a priority for the food industry and consumers alike [4]. However, rice is deficient in many nutrients [5]. Therefore, it is important to enhance nutritional value of rice to improve the health of consumers.

Grain amino acid content is an important nutritional component in rice [6]. It has been well-documented that amino acid content in rice grains varies with cultivar, management practice, and environmental condition [4,7-10]. However, the physiological mechanisms for the variation in amino acid content in rice grains are still controversial. Mossé et al. [7] observed that amino acid content increases linearly with nitrogen (N) content in the grain of eight rice cultivars. However, Huang et al. [4] found that increased grain amino acid content in rice with application of earthworm casts was not related to change in grain N content but to improved amino acid metabolism. Consistently, Liang et al. [9] and Liu et al. [10] reported that increased amino acid content in rice grains induced by high temperature during ripening was attributable to changes in

activities of enzymes, including aspartate aminotransferase, aspartokinase, glutamine synthetase, glutamic oxalo-acetic transaminase, and glutamate pyruvate transaminase.

Proteomic analysis is a powerful approach that can provide qualitative and quantitative information of protein expression, which is invaluable in understanding the mechanisms involved in physiological processes [11]. This approach has been successfully used to provide new insights into the mechanisms underlying grain quality traits in cereals [12-14]. However, no such report is available for the grain amino acid content in rice.

In this study, N content, ratio of amino acid to N, and expression and functions of proteins related to amino acid metabolism in grains were compared between two hybrid rice cultivars differing in grain amino acid content. The objective of this study was to identify the physiological processes that contribute to the higher grain amino acid content in hybrid rice.

2 Materials and Methods

A field experiment was conducted in Yongan (28°09' N, 113°37'E, 43 m asl), Hunan Province, China in the early rice-growing season in 2016. The average daily mean temperature during the period from heading to maturity was 27.3°C. The soil of the experimental field was a clay with the following properties at the upper 20 cm layer: pH = 6.22, organic matter = 41.8 g kg⁻¹, total N = 1.23 g kg⁻¹, total P = 0.71 g kg⁻¹, total K = 6.56 g kg⁻¹, available N = 132 mg kg⁻¹, available P = 26.8 mg kg⁻¹, and available K = 155 mg kg⁻¹.

Two hybrid rice cultivars, Lingliangyou 268 and Luliangyou 996, were used in this study. The cultivars were chosen based on a preliminary study which showed that Lingliangyou 268 had about 10% higher total amino acid content in grains than Luliangyou 996.

Lingliangyou 268 and Luliangyou 996 were arranged in a randomized complete-block design with three replications with a plot size of 40 m². Seedlings were raised in trays (length × width × height = 58 cm × 25 cm × 2 cm) for machine transplanting. Twenty-day-old seedlings were transplanted with a high-speed rice transplanter (PZ80-25, Dongfeng Iseki Agricultural Machinery Co., Ltd., Xiangyang, China) on 15 April. Transplanting was done at a hill spacing of 25 cm × 11 cm with two seedlings per hill. Missing rice plants were manually replanted within 1 week after transplanting to obtain a uniform plant population. Fertilizers were applied according to the local recommendations. N fertilizer (150 kg N ha⁻¹) was applied in three splits: 50% as basal (1 day before transplanting), 20% at early-tillering (7 days after transplanting), and 30% at panicle initiation. P fertilizer (75 kg P₂O₅ ha⁻¹) was applied as basal. K fertilizer (150 kg K₂O ha⁻¹) was split equally as basal and at panicle initiation. The experimental field was flooded (5-10 cm) from transplanting until 7 days before maturity, when plots were drained. Insects, diseases, and weeds were intensively controlled by chemicals.

Six panicles that headed on the same day were sampled from each plot at 9 days after heading. The samples were placed in plastic bags and kept cool on ice during transport to the laboratory, after which they were hulled and stored at -80 °C until proteins were extracted. Total proteins were extracted using the cold acetone method [15], and then measured via protein DC assay (Bio-Rad) and calculated according to a bovine serum albumin protein standard curve. A proteomic analysis was carried out using iTRAQ (isobaric tags for relative and absolute quantitation) coupled LC-MS/MS (liquid chromatography-mass spectrometry/mass spectrometry). The differentially expressed proteins related to amino acid metabolism pathways in rice grains were identified based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg>), and the functions of the identified proteins were defined according to the Gene Ontology (GO) database (<http://www.geneontology.org>).

Ten hills of rice plants were sampled from each plot at maturity. The sampled plants were hand threshed. About 10 g of grains were hulled and oven-dried at 70°C to a constant weight. The dried grains were ground and sieved (100 mesh) to determine amino acid and N contents. Approximately 1.0 g of the sieved sample was hydrolyzed in HCl (5 M, 5 ml) for 22 h at 110°C and derivatized using the AccQ-Tag reagent kit (Waters, Milford, MA, USA) for 15 min at 55°C. The contents of 17 amino acids, including alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine,

phenylalanine, proline, serine, threonine, tyrosine, and valine, were measured using a high-performance liquid chromatography system (Waters 2695 Separations Module, Waters) equipped with an AccQ-Tag column (3.9×150 mm, $4 \mu\text{m}$ film thickness) and linked simultaneously to a photodiode array detector (Model 2996, Waters). Total amino acid content was the summation of the contents of the 17 individual amino acids. About 0.5 g of the sieved sample was digested with $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$ to determine the N content using a Skalar SAN Plus segmented flow analyzer (Skalar Inc., Breda, the Netherlands). Ratio of amino acid to N was calculated by dividing the total amino acid content by the N content.

Data of the amino acid and N contents and the ratio of amino acid to N were analyzed using analysis of variance (Statistix 8, Analytical software, Tallahassee, FL, USA). Statistical significance was set at the 0.05 probability level.

3 Results

Total amino acid content in the grain was 9% higher in Lingliangyou 268 than in Luliangyou 996 (Fig. 1(A)). Grain contents of arginine, aspartate, glutamate, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, and valine were 6-15% higher in Lingliangyou 268 than in Luliangyou 996 (Fig. 1(B)). The differences in grain contents of alanine, cysteine, methionine, threonine, and tyrosine were not significant between Lingliangyou 268 and Luliangyou 996.

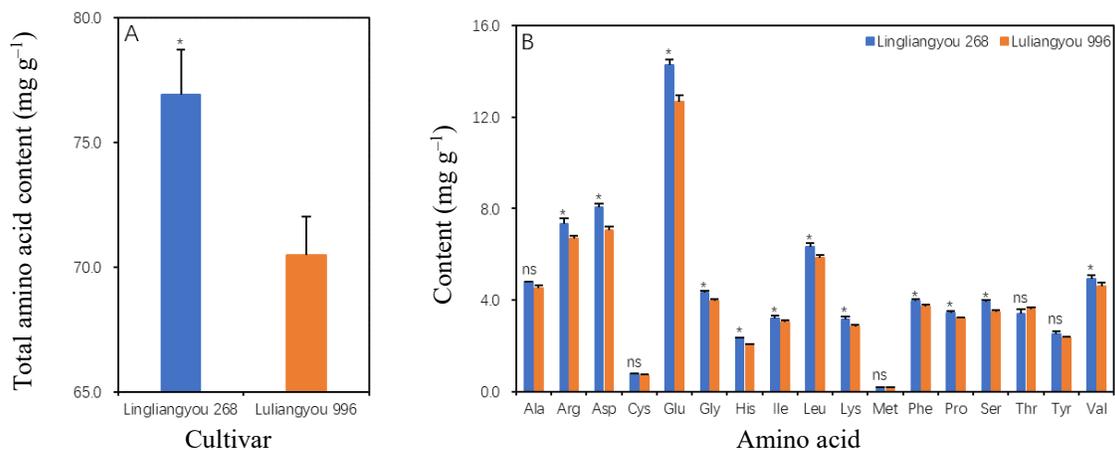


Figure 1: Total (A) and individual amino acid content (B) in grains of two hybrid rice cultivars Lingliangyou 268 and Luliangyou 996. Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, and Val represent alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine, respectively. Error bars represent standard errors ($n = 3$) * and ns denote significant and non-significant differences, respectively, between the two cultivars at the 0.05 probability level

There was no significant difference in grain N content between Lingliangyou 268 and Luliangyou 996 (Fig. 2(A)). Lingliangyou 268 had a ratio of amino acid to N of 5.39, which was 6% higher than that in Luliangyou 996 (Fig. 2(B)).

A total of 16 differentially expressed proteins related to KEGG amino acid metabolism pathways were identified in grains between Lingliangyou 268 and Luliangyou 996 (Tab. 1). Among them, 11 proteins were up-regulated in Lingliangyou 268 compared to Luliangyou 996 with fold changes of 1.20 to 1.33, respectively, and 5 proteins were down-regulated with fold changes of 0.83 to 0.29 in Lingliangyou 268 compared to Luliangyou 996, respectively.

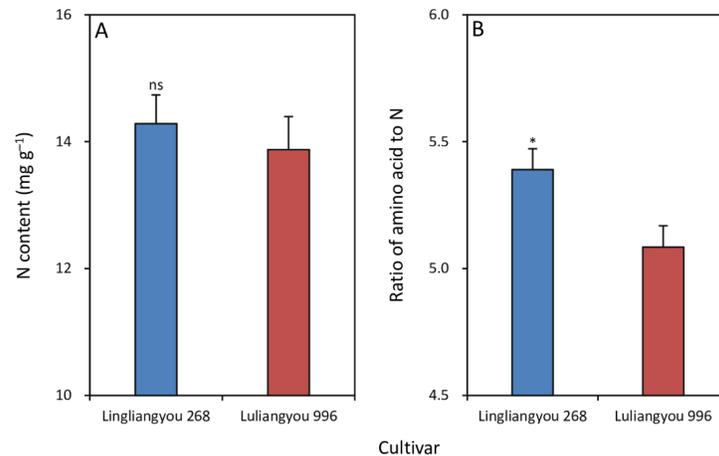


Figure 2: N content (A) and ratio of amino acid to N (C) in grains of two hybrid rice cultivars Lingliangyou 268 and Luliangyou 996. Error bars represent standard errors ($n = 3$) * and ns denote significant and non-significant differences, respectively, between the two cultivars at the 0.05 probability level

The identified differentially expressed proteins were involved in 10 GO-defined functions (Fig. 3). Six of the 10 defined functions were related to binding (heterocyclic compound binding, nucleoside phosphate binding, nucleotide binding, organic cyclic compound binding, protein binding, and small molecule binding), and the number of up-regulated proteins was 2- to 5-fold higher than that of down-regulated proteins for these binding activities. The other 4 defined functions of the identified differentially expressed proteins were catalytic activity, enzyme regulator activity, hydrolase activity, and transferase activity. The catalytic activity involved 15 of the 16 identified differentially expressed proteins, and the number of up-regulated proteins was nearly 2-fold higher than that of down-regulated proteins for this function. The number of up-regulated proteins was also higher than that of down-regulated proteins for the enzyme regulator activity and transferase activity, whereas an opposite trend was observed for the hydrolase activity.

4 Discussion

Consistent with the preliminary study, the present study showed that total amino acid content in grains in Lingliangyou 268 was about 10% higher than that in Luliangyou 996. This cultivar difference in total amino acid content in the grain was mainly due to differences in grain contents of arginine, aspartate, glutamate, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, and valine. On the other hand, the higher total amino acid in grains in Lingliangyou 268 than in Luliangyou 996 was attributable to increased efficiency of converting N to amino acid (i.e., higher ratio of amino acid to N) but not related to grain N content. This finding is not in agreement with that of Mossé et al. [7], who reported that amino acid content increased linearly with N content in rice grains with a correlation coefficient close to one, but consistent with some previous studies [4,9,10], which indicated that changes in amino acid content were related to changes in amino acid metabolism in rice grains.

Although several differentially expressed proteins related to amino acid metabolism pathways were identified in grains between Lingliangyou 268 and Luliangyou 996 in this study, some commonly-recognized key enzymes in amino acid synthesis in the rice grain, including aspartate aminotransferase, aspartokinase, glutamine synthetase, glutamic oxalo-acetic transaminase, and glutamate pyruvate transaminase [9,10], are not found in the differentially expressed proteins. This fact suggests that it should be prudent to choose analysis of these enzymes to investigate the physiological mechanisms governing grain amino acid content in rice in future studies.

Table 1: Identified differentially expressed proteins related to amino acid metabolism pathways in grains in rice cultivar Lingliangyou 268 compared to Luliangyou 996 based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database

Number	Locus ID	Protein name	Fold change
Up-regulated proteins			
1	LOC_Os01g12830.1	Erythronate-4-phosphate dehydrogenase domain containing protein, expressed	1.27
2	LOC_Os01g16714.1	Flavin monooxygenase, putative, expressed	1.28
3	LOC_Os01g46380.1	Ketol-acid reductoisomerase, chloroplast precursor, putative, expressed	1.22
4	LOC_Os03g27230.1	Phospho-2-dehydro-3-deoxyheptonate aldolase, chloroplast precursor, putative, expressed	1.33
5	LOC_Os03g48300.1	Histidine acid phosphatase, putative, expressed	1.21
6	LOC_Os04g28250.1	Cysteine proteinase inhibitor precursor, putative, expressed	1.20
7	LOC_Os05g06750.1	Dihydrolipoyl dehydrogenase, mitochondrial precursor, putative, expressed	1.20
8	LOC_Os05g35290.1	Phenylalanine ammonia-lyase, putative, expressed	1.27
9	LOC_Os06g36770.1	Serine/threonine protein phosphatase 2A 55 kDa regulatory subunit B, putative, expressed	1.21
10	LOC_Os06g36840.1	Cysteine synthase, putative, expressed	1.25
11	LOC_Os12g22030.1	Serine hydroxymethyltransferase, mitochondrial precursor, putative, expressed	1.22
Down-regulated proteins			
12	LOC_Os01g22520.1	Dihydrolipoyl dehydrogenase 1, mitochondrial precursor, putative, expressed	0.83
13	LOC_Os06g04820.1	Leucine carboxyl methyltransferase 1, putative, expressed	0.29
14	LOC_Os07g30170.1	Nitrilase, putative, expressed	0.82
15	LOC_Os11g34710.1	Ser/Thr protein phosphatase family protein, putative, expressed	0.66
16	LOC_Os12g38900.1	Chorismate mutase, chloroplast precursor, putative, expressed	0.42

There has been reported that over-accumulation of binding protein in the endosperm significantly can increase amino acid content in rice grains [16]. In this study, six functions related to binding were found for the identified differentially expressed proteins, and the number of up-regulated proteins higher than that of down-regulated proteins in the grain of Lingliangyou 268 compared to Luliangyou 996 for these binding activities. Therefore, increased binding proteins were partly responsible for the increased efficiency of converting N to amino acid and the higher grain amino acid content in Lingliangyou 268

compared to Luliangyou 996. In addition, this study also showed that the number of up-regulated proteins was higher than that of down-regulated proteins in grains of Lingliangyou 268 compared to Luliangyou 996 for the catalytic activity, enzyme regulator activity and transferase activity, whereas an opposite trend was observed for the hydrolase activity. These findings indicate that increases in proteins involved in catalytic, enzyme regulator, and transferase activities and decreases in hydrolase proteins were also responsible for the increased efficiency of converting N to amino acid and the higher grain amino acid content in Lingliangyou 268 than in Luliangyou 996.

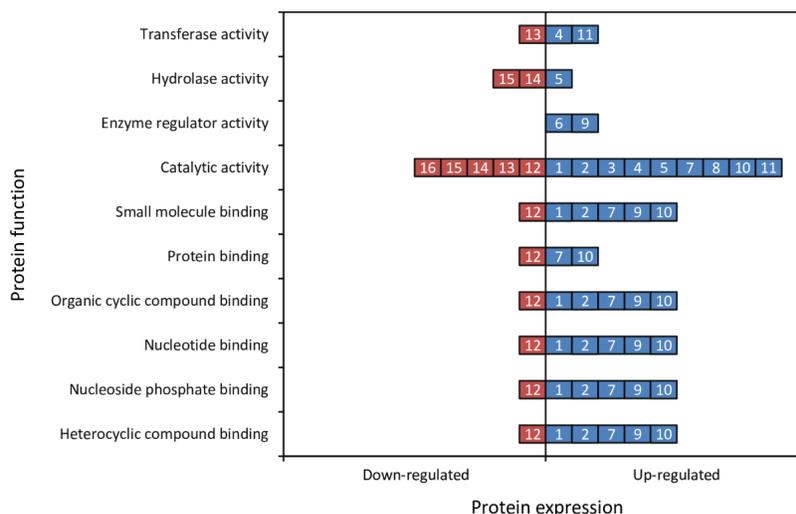


Figure 3: Functions of up-regulated and down-regulated proteins related to amino acid metabolism pathways in hybrid rice grains based on the Gene Ontology (GO) database. Numbers within boxes correspond to protein numbers given in Tab. 1

5 Conclusion

The higher grain amino acid content in hybrid rice cultivar Lingliangyou 268 compared to Luliangyou 996 is attributed to increased efficiency of converting N to amino acid that results from altering expression of proteins related to amino acid metabolism, including increased proteins involved in binding, catalytic, enzyme regulator, and transferase activities and decreased hydrolase proteins.

Acknowledgement: This work was supported by the National Key R&D Program of China (2016YFD0300509) and the Earmarked Fund for China Agriculture Research System (CARS-01).

References

1. Peng, S., Tang, Q., Zou, Y. (2009). Current status and challenges of rice production in China. *Plant Production Science*, 12(1), 3-8.
2. Liao, X., Chen, Q., Pan, G. (2002). Status and strategies for improving high-quality rice production in China. *Journal of Agrotechnical Economics*, (5), 32-34.
3. Peng, S. (2016). Dilemma and way-out of hybrid rice during the transition period in China. *Acta Agronomica Sinica*, 42(3), 313-319.
4. Huang, M., Zhao, C., Zhou, X., Chen, G., Zou, Y. et al. (2018). Earthworm responses to cropping rotation with oilseed rape in no-tillage rice fields and the effects of earthworm casts on human-essential amino acid content in rice grains. *Applied Soil Ecology*, 127, 58-63.
5. Birla, D. S., Malik, K., Sainger, M., Chaudhary, D., Jaiwal, R. et al. (2017). Progress and challenges in improving nutritional quality of rice (*Oryza sativa* L.). *Critical Reviews in Food Science and Nutrition*, 57(11), 2455-2481.

6. Wang, L., Zhong, M., Li, X., Yuan, D., Xu, Y. et al. (2008). The QTL controlling amino acid content in grains of rice (*Oryza sativa*) are co-localized with regions involved in the amino acid metabolism pathway. *Molecular Breeding*, 21(1), 127-137.
7. Mossé, J., Huet, J. C., Baudet, J. (1988). The amino acid composition of rice grain as a function of nitrogen content as compared with other cereals: a reappraisal of rice chemical scores. *Journal of Cereal Science*, 8(2), 165-175.
8. Cheng, Z., Huang, X., Zhang, Y., Qian, J., Yang, M. et al. (2005). Diversity in the content of some nutritional components in husked seeds of three wild rice species and rice varieties in Yunnan Province of China. *Journal of Integrative Plant Biology*, 47(10), 1260-1270.
9. Liang, C., Zhang, Q., Xu, G., Wang, Y., Ryu, Q. et al. (2013). High temperature during grain filling enhances aspartate metabolism in grains and results in accumulation of aspartate-family amino acids and protein components. *Rice Science*, 20(5), 343-348.
10. Liu, Q., Wu, X., Ma, J., Li, T., Zhou, X. et al. (2013). Effects of high air temperature on rice grain quality and yield under field condition. *Agronomy Journal*, 105(2), 46-454.
11. Dutt, M. J., Lee, K. H. (2000). Proteomic analysis. *Current Opinion in Biotechnology*, 11(2), 176-179.
12. Lin, Z., Zhang, X., Yang, X., Li, G., Tang, S. et al. (2014). Proteomic analysis of proteins related to rice grain chalkiness using iTRAQ and a novel comparison system based on a notched-belly mutant with white-belly. *BMC Plant Biology*, 14, 163.
13. Chen, L., Huang, Y., Xu, M., Cheng, Z., Zhang, D. et al. (2016). iTRAQ-based quantitative proteomics analysis of black rice grain development reveals metabolic pathways associated with anthocyanin biosynthesis. *PLoS One*, 11(7), e0159238.
14. Guo, B., Luan, H., Lin, S., Lv, C., Zhang, X. et al. (2016). Comparative proteomic analysis of two barley cultivars (*Hordeum vulgare* L.) with contrasting grain protein content. *Frontiers in Plant Science*, 7, 542.
15. Wu, X. L., Xiong, E. H., Wang, W., Scali, M., Cresti, M. (2014). Universal sample preparation method integrating trichloroacetic acid/acetone precipitation with phenol extraction for crop proteomic analysis. *Nature Protocols*, 9, 362-374.
16. Kawakatsu, T., Wang, S., Wakasa, Y., Takaiwa, F. (2010). Increased lysine content in rice grains by over-accumulation of BiP in the endosperm. *Bioscience, Biotechnology, and Biochemistry*, 74(12), 2529-2531.