Reviewer 2 #

Comments and Suggestions for Authors

Nitrogen use efficiency (NUE) is important topic in reality and scientific interest. The present study has great results on the new insights into the regulatory mechanisms of nitrogen fertilization in the crop that is very important to identify the potential molecular protein. However, the manuscript should be improved for publication in IJMS. The authors have to address the following points:

**Response:** Thank you for your comments and hard work. We appreciate your helpful suggestions on our paper. As for the questions, our thoughts are listed below:

Abstract should be avoid the suggested results.

**Response**: Thank you very much for your hark work and valuable comments. According to your suggestion, we have removed the suggested results in the abstract.

In the introduction:

- The Nitrogen fertilizer should be taken into account to Ammonia (NH3).

The author should refer to the paper: Environmental Chemistry 2017, 14 (5), 327-337

**Response**: Thank you very much for your comments. According to your suggestion, we have added this reference in the manuscript.

Pham, T. D., Do, T.T., Doan, T.H.Y., Nguyen, T.A.H., Mai, T.D., Kobayashi, M., Adachi, Y. Adsorptive removal of ammonium ion from aqueous solution using surfactant-modified alumina. Environ. Chem. 2017, 14, 327-337.

- The novelty of this research needs emphasize

**Response:** Thank you very much for your valuable suggestion. According to your suggestion, we have rewritten the discussion part, and emphasized the novelty of this research. **Please see Line 298-309.**

3. Results

- Capture of Figure 1 should be in one page.

**Response:** Thank you very much for your comments. We have revised it in our manuscript.

- The umbers in Fig. 3 A and B are too small to see anything.

**Response:** Thank you very much for your comments. We apologize for this mistake. According to your suggestion, we have reworked the figure, and make it more clearly to reflect the result.

- How to evaluate Molecular Function. The comparison with other previously published data is needed.

**Response:** Thank you very much for your comments. The enrichment of **molecular function** that belongs to GO analysis was performed according to the method described in the previous studies (Hu et al., 2017; Zhou et al., 2018). The details were as follows:

Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (www. http://www.ebi.ac.uk/GOA/). **Firstly**, Converting identified protein ID to UniProt ID and then mapping to GO IDs by protein ID. If some identified proteins were not annotated by UniProt-GOA database, the InterProScan soft would be used to annotated protein's GO functional based on protein sequence alignment method. **Then** proteins were classified by Gene Ontology annotation based on three categories: biological process, cellular component and **molecular function**. For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the differentially expressed protein against all identified proteins. The GO with a corrected p-value < 0.05 is considered significant.

In addition, according to your comments, we have added the references in our manuscript.

**References:**

Hu, Z.; Liu, A.; Bi, A.; Amombo, E.; Gitau, M. M.; Huang, X.; Liang, Chen.; Fu, J. Identification of differentially expressed proteins in bermudagrass response to cold stress in the presence of ethylene. Environ. Exp. Bot. 2017, 139, 67-78.

Zhou, H.; Finkemeier, I.; Guan, W.; Tossounian, M. A.; Wei, B.; Young, D.; Huang, J.; Messens, J.; Yang, X.; Zhu, J.; Wilson, M.H.; Shen, W.; Xie, Y.; Foyer, C.H. Oxidative stress‐triggered interactions between the succinyl‐and acetyl‐proteomes of rice leaves. Plant Cell Environ. 2018, 41, 1139-1153.

- So many data in Table 2 that is difficult to follow. What is the differences between the highlights of data.

**Response:** Thank you very much for your valuable comments. As you said, it is difficult to follow what we want to highlight in the Table 2 that contains lots of data. Therefore, we have reworked the tables and rewritten the results and discussion sections in our manuscript. In fact, what we want to highlight is the differentially expressed proteins

regulated by nitrogen fertilizer in the two rice cultivars. Thus, we emphatically analyzed the proteins in the comparisons of H_N15 vs H_CK and 681_N15 vs 681_CK, in our new manuscript. We believe the new version of the manuscript is easier to understand, and has a more fluent scientific discourse. Please see **new Table 2-4 and supplementary Table S3-S5.**

4. Discussion

- What are enough amounts of GS to incorporate NH4+.

**Response:** Thank you very much for your comments. We are sorry for this illogical deduction. According to your helpful comments, we have changed it into 'the relative higher level of GS expression in Quanliangyou 681 could enhance the incorporation of $NH4^+$ into organic compounds, which is beneficial to increase grain production'.

- How to evaluate electron transfer and transmembrane transport.

**Response:** Thank you very much for your comments. We apologize for the mistake. In fact, what we want to express is 'energy metabolism and material transport' in cell, and we have revised it in the manuscript. In the study, we considered that the higher the expression level of these proteins involved in energy metabolism and material transport, the stronger the enhancement degree of these two processes by nitrogen fertilizer.

Respiration is the process of organic matter oxygenolysis and **energy production**. Most oxidative degradation of respiratory substrates occurs through dehydrogenation reactions. Hydrogen acceptors, such as NAD+, FAD or NADP+, will be transferred into NADH, FADH2 or NADPH after accepting hydrogen ion and electron, which promotes synthesis of ATP to provide energy for organism (Buchanan, 2015).

The **energy-dependent transport** of solutes through vacuolar membrane of plants is driven by two kinds of $H^+$ pumps: vacuolar (V type) $H^+$ -ATPase and $H^+$-translocating (pyrophosphate-energized) inorganic pyrophosphatase ($H^+$-PPase) (Sarafian et al.; 1992). The ATP-binding cassette (ABC) transporter superfamily is the largest transporter gene family. The proteins bind ATP and utilize the energy to drive the transport of diverse molecules, including sugars, amino acids, peptides, proteins, metal ions, and plenty of hydrophobic compounds and metabolites across the plasma membrane and intracellular membranes (Higgins, 1992; Dean and Allikmets, 1995; Dean et al.; 2001).

**References:**

Buchanan, B.B.; Gruissem, W.; Jones, R.L. (Eds.). Biochemistry and molecular biology of plants. John Wiley & Sons, 2015.

Sarafian, V.; Kim, Y.; Poole, R.J.; Rea, P.A. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of Arabidopsis thaliana. P. Natl. Acad. Sci. USA 1992, 89, 1775-1779

Higgins, C.F. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 1992, 8, 67-113.

Dean, M.; Allikmets, R. Evolution of ATP-binding cassette transporter genes. Curr.Opin. Genet. Dev. 1995, 5, 779-785.

Dean, M.; Hamon, Y.; Chimini, G. The human ATP-binding cassette (ABC) transporter superfamily. J. Lipid Res. 2001, 42, 1007-1017.

- Conclusions should separate to other section in which the originality is again emphasized.

**Response:** Thank you very much for your comments. According to your suggestion, we have separated the conclusion part to other sections.

5. Materials and Methods.

- Protein extraction from rice needs the references. No cited papers are not convincing.

Protein concentrations determined by BCA must cite at least one reference.

Also, Trypsin digestion should add the cited paper.

Please see the paper related to standard protein: Colloid and Polymer Science 296 (1), 145-155

**Response:** Thank you very much for your comments. We appreciate your helpful suggestions on our paper, and we have added the references. Besides, according to your recommendation, what we find is the article of Huang et al. (2018), but the content seems to be irrelevant with our study. So, could you please provide the full name of the recommended article? Thank you very much.

**References**

Hu, Z., Liu, A., Bi, A., Amombo, E., Gitau, M. M., Huang, X., Liang, Chen., Fu, J. Identification of differentially expressed proteins in bermudagrass response to cold stress in the presence of ethylene. Environ. Exp. Bot. 2017, 139, 67-78

Zhou, H.; Finkemeier, I.; Guan, W.; Tossounian, M. A.; Wei, B.; Young, D.; Huang, J.; Messens, J.; Yang, X.; Zhu, J.; Wilson, M.H.; Shen, W.; Xie, Y.; Foyer, C.H. Oxidative stress - triggered interactions between the succinyl - and acetyl - proteomes of rice leaves. Plant Cell Environ. 2018, 41, 1139-1153.

Walker, J.M. The bicinchoninic acid (BCA) assay for protein quantitation. In The protein protocols handbook . Humana Press, Totowa, NJ. 2009, pp. 11-15.

Huang, Y.; Yamaguchi, A.; Pham, T.D.; Kobayashi, M. Charging and aggregation behavior of silica particles in the presence of lysozymes. Colloid Polym. Sci. 2018, 296, 145-155. (?)

- Detail of HPLC and LC-MS/MS should be added. The validations of methods are needed.

**Response:** Thank you very much for your comments. According to your helpful suggestion, we have added detail information about the methods of HPLC and LC-MS/MS.

Western blotting is used as the traditional approach to validate the reliability of this method (HPLC and LC-MS/MS). But there are several disadvantages of western blotting approach: **firstly**, it is not guaranteed that each protein can obtain antibodies with good specificity when multiple proteins are verified; **secondly**, western blotting system mainly relies on antibodies; when many antibodies have certain non-specific adsorption, it is easy to cause quantitative errors.

In this study, we adopt a new method, parallel reaction monitoring (PRM), which has emerged as an alternative method of targeted quantification. PRM is an ion monitoring technology based on high resolution and high precision mass spectrometry, which can selectively detect target proteins and target peptides (such as those modified after translation), so as to accurately quantify the target proteins/peptides (Peterson et al.; 2012; Rauniyar, 2015). When the abundance of proteins to be verified is good, 10-100 proteins can be simultaneously and accurately quantified by PRM verification. In addition, we have added the references in our manuscript.

**References:**

Peterson, A.C.; Russell, J.D.; Bailey, D.J.; Westphall, M.S.; Coon, J.J. Parallel reaction monitoring for high resolution and high mass accuracy quantitative, targeted proteomics. Mol. Cell. Proteomics 2012, 11, 1475-1488.

Rauniyar, N. Parallel reaction monitoring: a targeted experiment performed using high resolution and high mass accuracy mass spectrometry. Int. J. Mol. Sci. 2015, 16, 28566-28581.