Poster Reprint

ASMS 2019
MP610

Dissection of Flag Leaf Metabolic Shift and Their Relationship with Those Occurring Simultaneously in Developing Seed by Application of Non-targeted Metabolomics

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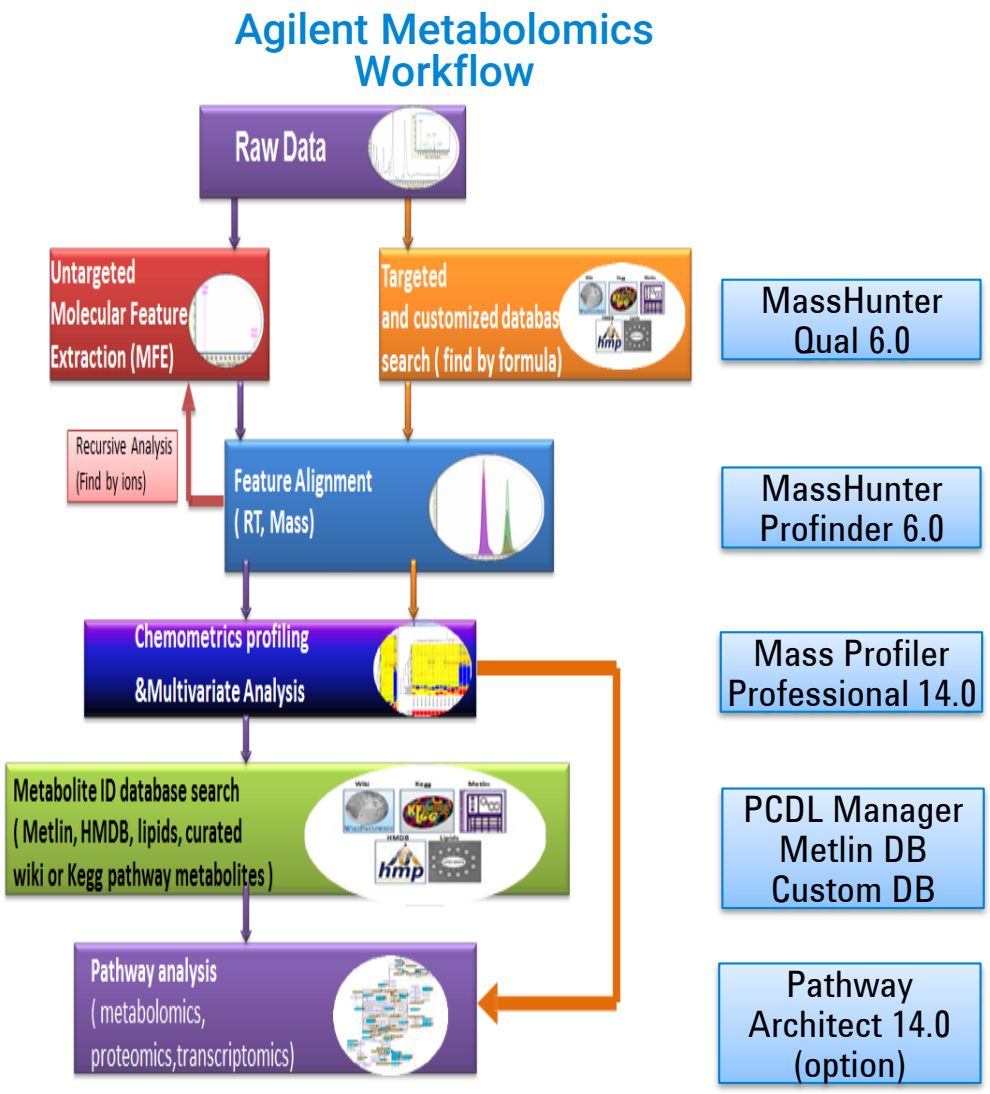
Introduction

The metabolic kinetics of flag leaves in two *japonica* and two *indica* rice cultivars were investigated using a non-targeted metabolomics approach and compared with those in developing seeds. Principle component analysis revealed that the detected flag leaf metabolomes differed significantly depending on both species and developmental stage, with only a few of the metabolites in flag leaves showing the same pattern of change in the four tested cultivars. Further association analysis found that the levels of 45 metabolites in seeds that are associated with human nutrition and health correlated significantly with their levels in flag leaves. Given the different functional and physio-logical states of these two organs, this study revealed not only the function of the tissue-specific metabolites but also provide important insight into the nature of the metabolic flux from source to sink organs in rice.

Extraction methods have a vital impact on non-targeted metabolomics. In this study, we evaluated the effect of three extraction methods on rice seed metabolic profiling and provided a selection strategy for future rice metabolomics studies. The three extraction buffers and two rice cultivars were subjected to metabolomics analysis using an Agilent 1290 UHPLC coupled to an Agilent 6550 iFunnel Q-TOF.



Experimental

Agilent 1290 Infinity UHPLC/ 6550 iFunnel Q-TOF system

LC analysis was performed using an Agilent 1290 Infinity II LC system. MS detection was performed on an Agilent 6550 iFunnel Q-TOF mass spectrometer with an Agilent Jet Stream ion source. The detailed information concerning UHPLC/MS data acquisition can be found in our previous study[1]. Metabolites were annotated by searching a custom "Nature Product" Personal Compound Database and Library (PCD/PCDL), and Massbank and METLIN databases, based on two criteria: (1) the difference between the observed mass and theoretical mass was less than 5 ppm; (2) the main feature of the observed MS/MS spectrum was the same as that in literatures or database. Data acquisition, review and peak area extraction were performed with the MassHunter Acquisition 6.0, MassHunter Qualitative 6.0 and Mass Profinder 6.0 software, respectively.

Sample preparation

Rice plants were planted in a paddy field in Minghang (31.03°N, 121.45°E), Shanghai, during the summer season in 2013. The tillers were marked at the heading dates. Four biological replications of flag leaves at 0, 7, 14 and 28 days after flowering (DAF) were collected, immediately frozen with liquid nitrogen, lyophilized for 48 hours and stored at -80°C until metabolomics analysis. Two flag leaves from two individual plant were pooled as one biological replicate. Samples were ground into fine powder, and methanol extracts from 10 mg of sample were then analyzed by GC/MS and UHPLC/MS in positive and negative mode.

Data processing

For data normalization, peak areas were divided by the sample weight and the median value of each metabolite. The missing values of a given metabolite were imputed with the detected minimum value of the same metabolite for statistical analysis, assuming that they were below the limits of instrument detection. The final statistics matrix with normalized data for the following statistical analysis are available in Table S1. Principle component analysis was performed with SIMCA-P version 11.0. Two-way ANOVA and ASCA were performed using the tool embedded in the MetaboAnalyst website The metabolite-metabolite correlations between grain and flag leaf were analyzed by using Pearson's product-moment correlation method with mean values in R software package. The heatmaps of metabolite ratios were visualized with MultiExperiment Viewer (MeV) version 4.8.

Separation and Investigation of Identified Compound List

The flag leaf samples were collected at 0, 7, 14 and 28 DAF and the methanol extracts of these samples were subjected to non-targeted metabolomics analysis by employing gas chromatography mass spectrometry (GC/MS) and ultra-high performance liquid chromatography-quadrupole time of flight-tandem mass spectrometry (UHPLC/Q-TOF) as previously described. The Agilent ZORBAX Eclipse Plus C18, 3.0x150 mm, 1.8 µm column shows excellent chromatographic performance. Six isomers with very similar chemical structures can be completely separated. Under these conditions, the leucine and isoleucine were also well separated (data not shown).

In our studies, the metabolic profiles of developing seeds (at 7,14 and 28 DAF) of the same four rice cultivars used in this study were characterized. The greatest metabolic differences between the flag leaves and developing seeds as observed by LC/MS was the accumulation of flavonoids in the flag leaves but lipids in seeds (Figure 1A). The PCA score plot clearly demonstrated that the flag leaf metabolome differed significantly from that of seeds (Figure 1B). The results were further confirmed by the identification and quantification of those metabolites.

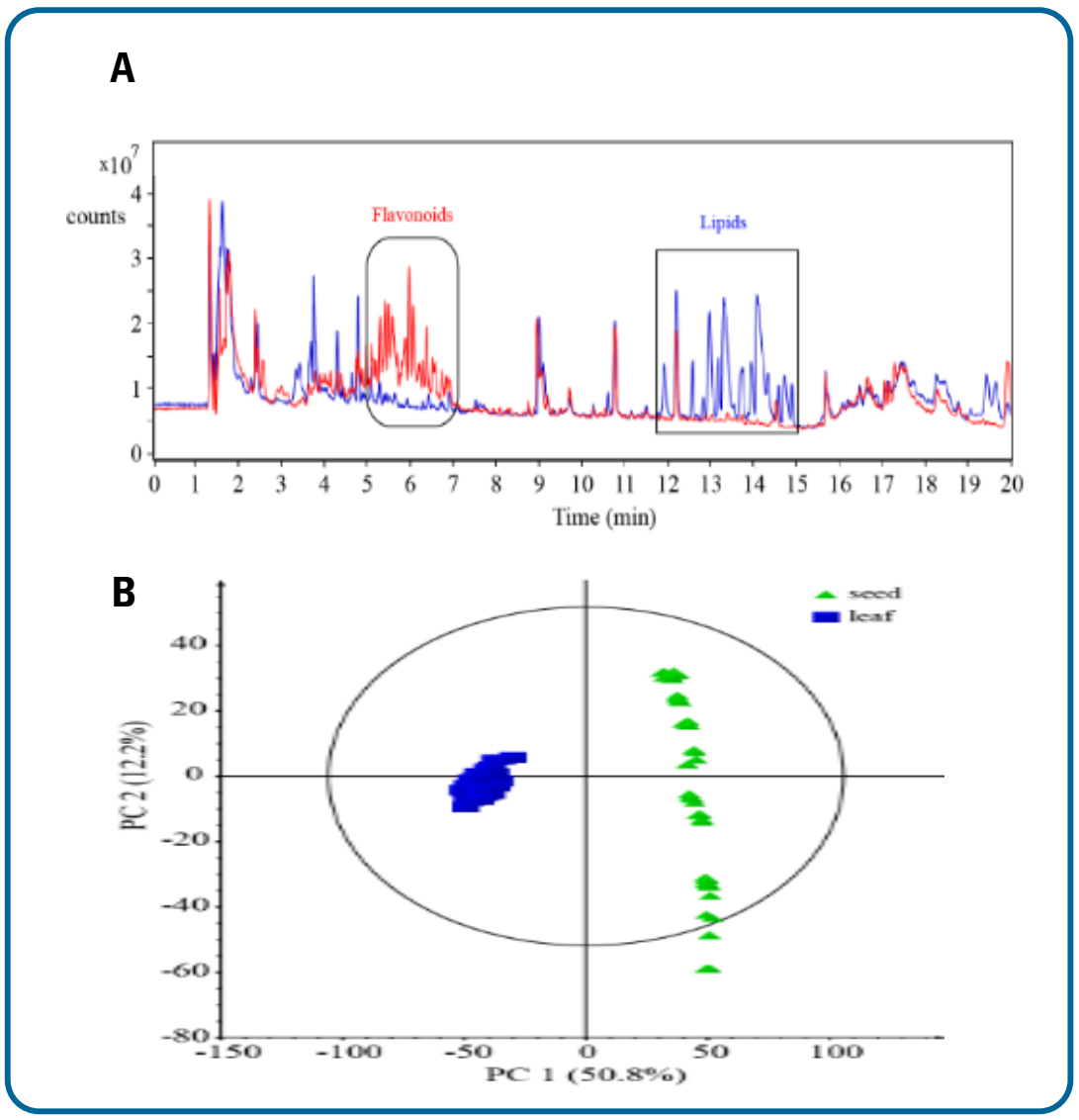


Figure 1. Difference between metabolomes of rice flag leaf and developing seed.

A total of 207 metabolites were identified by retention time and MS/MS spectra, including 38 amino acids and dipeptides, 37 carbohydrates and organic acids, 25 lipids, eight nucleotides, 10 cofactors, five benzene derivatives, 63 flavonoids, 12 hydroxycinnamate derivatives, three terpenoids and six miscellaneous metabolites.(Table 1)

Metabolite Name	Formula	Class	Tissue
Apigenin-6-C-β-glucoside-8-C-α-arabinoside II	C26H28O14	Flavonoid	Leaf
Chrysoeriol C-hexoside derivant	C25H28O12	Flavonoid	Leaf
Chrysoeriol O-glucoside	C22H22O11	Flavonoid	Leaf
Isoorientin-7,2''-di-O-glucoside	C33H40O21	Flavonoid	Leaf
Isoorientin C-hexoside-C-hexoside II	C36H36O18	Flavonoid	Leaf
Isoorientin	C21H20O11	Flavonoid	Leaf
Isovitexin 2''-O-(6'''-(E)- feruloyl)-glucopyranoside	C37H38O18	Flavonoid	Leaf
Syringetin 3-O-β-D-glucopyranoside	C23H24O13	Flavonoid	Leaf
Tricin derivant I	C36H36O18	Flavonoid	Leaf
Tricin derivant III	C36H36O18	Flavonoid	Leaf
Tricin derivant IV	C36H36O18	Flavonoid	Leaf
Tricin derivant V	C36H36O18	Flavonoid	Leaf
Tricin 4'-O-(erythro-β-guaiacylglyceryl) ether 7-O-β-D-glucopyranoside	C33H36O16	Flavonoid	Leaf
Tricin 4'-O-(syringyl alcohol)ether O-hexoside	C32H34O15	Flavonoid	Leaf
Tricin 4'-O-(threo-β-4-hydroxyphenylglyceryl) ether	C26H24O10	Flavonoid	Leaf
Tricin 4'-O-(threo-β-syringylglyceryl) ether 7''-O-β-D-glucopyranoside	C34H38O17	Flavonoid	Leaf
Tricin 7-O-(2''-O-β-D-glucopyranosyl)-β-D-glucuronopyranoside	C29H34O17	Flavonoid	Leaf
Tricin isomer	C17H14O7	Flavonoid	Leaf
Tricin O-glucoside O-guaiacylglyceryl ether	C33H36O16	Flavonoid	Leaf
Tricin O-guaiacylglyceryl ether'-O-glucopyranoside derivate	C36H36O18	Flavonoid	Leaf
Tricin-O-hexoside derivative	C33H34O15	Flavonoid	Leaf
1-O-Palmitoylhexitol	C27H44O7	Lipid	Leaf
Pregna-5,20-dien-3-ol	C21H32O	Others	Leaf
1-linoleoylglycerol	C21H38O4	Lipid	Seed
2-linoleoylglycerol	C21H38O4	Lipid	Seed
1-myristoylglycerophosphocholine	C22H46NO7P	Lipid	Seed
2-myristoylglycerophosphocholine	C22H46NO7P	Lipid	Seed
1-stearoylglycerophosphoethanolamine	C23H48NO7P	Lipid	Seed
2-stearoylglycerophosphoethanolamine	C23H48NO7P	Lipid	Seed
Cystine	C6H12N2O4S2	Amino acid	Seed
Homovanillic acid	C9H10O4	Monoaromatics	Seed

Table 1. Metabolites detected only in rice flag leaf or seed.

Results and Discussion

Kinetic Patterns of Flag Leaf Metabolomes

To gain a global view of the metabolic differences across all analyzed samples, principle component (PC) analysis on the identified metabolites was subsequently performed. PC 1, accounting for 24.0% of the total variance, separated samples of *japonica* from those of *indica* (Figure 2), indicating different metabolic profiles in flag leaves of these two subspecies.

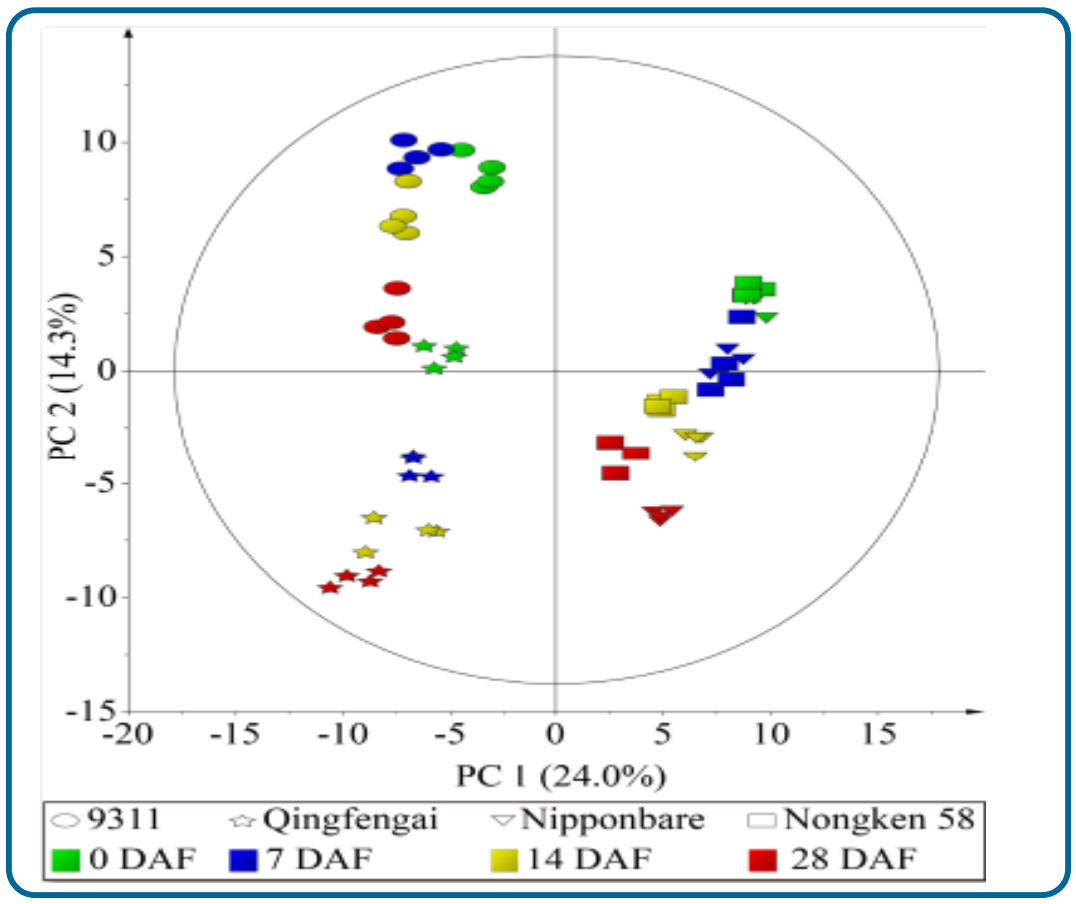


Figure 2. Principal component (PC) analysis of the metabolomes of flag leaves.

Metabolites with high leverage and low SPE (Leverage/squared prediction error) were picked out as well-modeled metabolites that contributed significantly to the model described. The part results of twenty well-modeled metabolites, including eight amino acids, two carbohydrates, five cofactors, two lipids, two nucleotides and one terpenoids, stood out based on the pattern of time. (Figure 3)

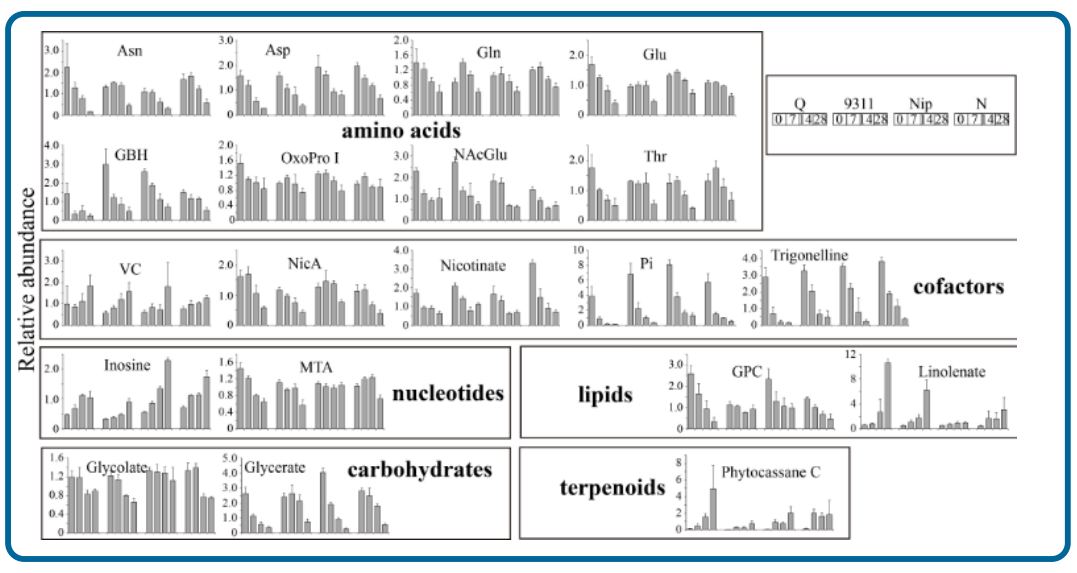


Figure 3. Changes of the well-modeled following the major pattern of time.

Metabolic Relationship between Rice Flag Leaf and Seed

It is interesting to investigate the metabolic association between flag leaves and seeds in order to better understand the metabolic relationship between source and sink organs. Most of the positive correlations were observed between primary metabolites (amino acids, carbohydrates, cofactors, lipids and nucleotides) and hydroxycinnamates in seeds and primary metabolites in flag leaves. (Figure 4)

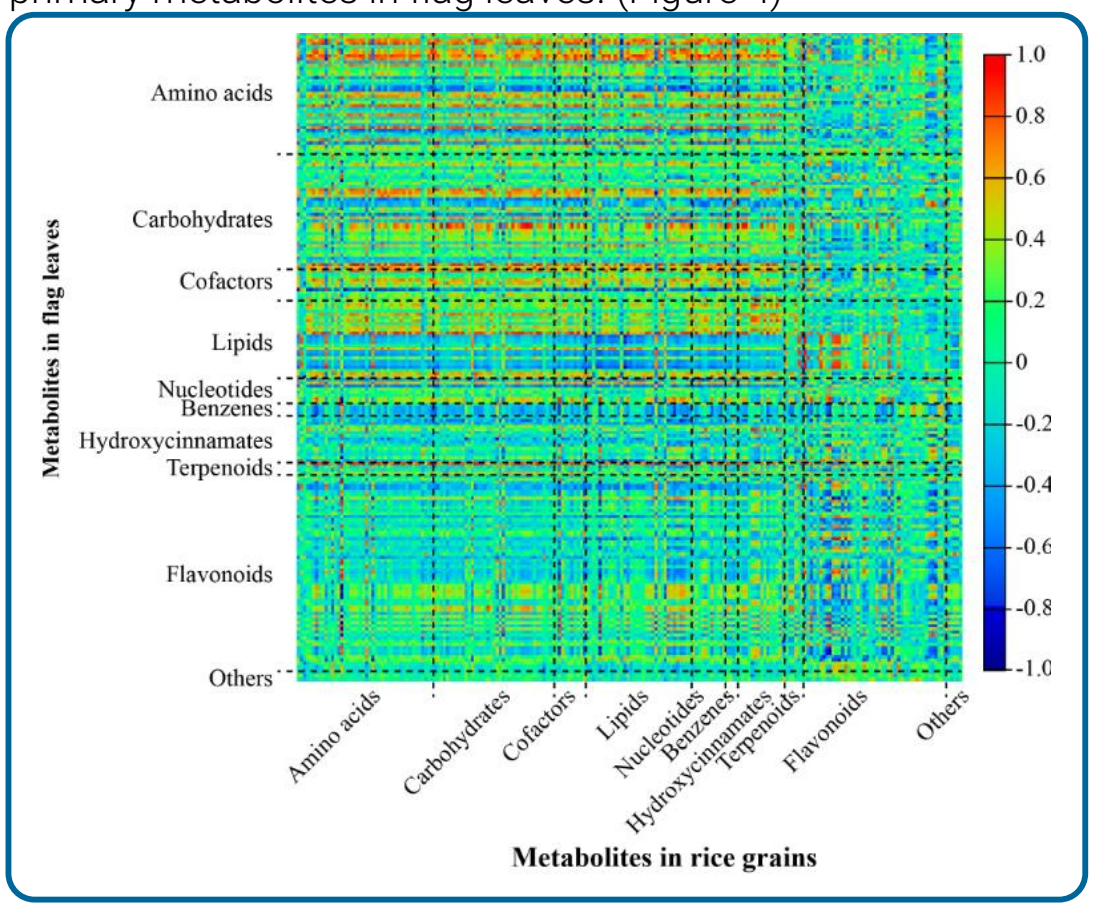


Figure 4. Heatmap of metabolite-metabolite correlation between developing rice grains and flag leaves. Rectangles represent Pearson correlation coefficient (r) values of metabolite pairs (see correlation color key).

Conclusions

This study investigated the metabolite patterns of rice flag leaves together with seed development (from flowering to seed desiccation), and compared it with that in developing seeds reported previously, revealing both cultivar-, tissue- and development-dependent metabolic kinetics in rice. It furthermore allowed association of metabolic changes in flag leaves with those in seeds, providing important hints for us to better understand the metabolic relationship between source and sink tissues of these species. This observation, combined with future works additionally employing transcriptomics and proteomics analysis with the Agilent integrated biology technique, will facilitate both the exploration of fundamental questions regarding the relationship between source and sink as well as their potential applications in rice seed nutrition improvement.

Reference:

1. S. Li et al. Journal of Chinese Agricultural Mechanization **2017** 38(2):108-113

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Published in USA, June 2, 2019

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