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Research article

UPLC-QTOF analysis reveals metabolomic changes in the flag leaf of wheat (*Triticum aestivum* L.) under low-nitrogen stress

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ABSTRACT

Wheat is one of the most important grain crop plants worldwide. Nitrogen (N) is an essential macronutrient for the growth and development of wheat and exerts a marked influence on its metabolites. To investigate the influence of low nitrogen stress on various metabolites of the flag leaf of wheat (*Triticum aestivum* L.), a metabolomic analysis of two wheat cultivars under different induced nitrogen levels was conducted during two important growth periods based on large-scale untargeted metabolomic analysis using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF).

Multivariate analyses—such as principle components analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA)—were used for data analysis. PCA yielded distinctive clustering information among the samples, classifying the wheat flag samples into two categories: those under normal N treatment and low N treatment. By processing OPLS-DA, eleven secondary metabolites were shown to be responsible for classifying the two groups. The secondary metabolites may be considered potential biomarkers of low nitrogen stress. Chemical analyses showed that most of the identified secondary metabolites were flavonoids and their related derivatives, such as iso-vitexin, iso-orientin and methylisoorientin-2″-O-rhamnoside, etc.

This study confirmed the effect of low nitrogen stress on the metabolism of wheat, and revealed that the accumulation of secondary metabolites is a response to abiotic stresses. Meanwhile, we aimed to identify markers which could be used to monitor the nitrogen status of wheat crops, presumably to guide appropriate fertilization regimens. Furthermore, the UPLC-QTOF metabolic platform technology can be used to study metabolomic variations of wheat under abiotic stresses.

1. Introduction

Environmental factors play an important role in the growth and development of plants. Plant metabolites and related pathways can be influenced by exposure of plants to abiotic stresses such as adverse environmental conditions (Dixon and Paiva, 1995; H D et al., 2010). However, during evolution, plants have adapted to survive under harsh conditions, such as by using their enormous metabolic capacity to produce a large variety of secondary metabolites.

Among the environmental factors that affect plants, the soil nitrogen content is one of the most important. Nitrogen (N) is an essential element for plants and is considered the most important mineral nutrient (Robinson, 2005). It plays a key role in many aspects of plant metabolism as a constituent of cell components such as proteins, phytohormones, co-enzymes, chlorophyll, and nucleic acids (Hawkesford et al., 2012). However, farmland in most countries has poor nutrient composition or low nitrogen content. Therefore, N is often one of the limiting factors of crop growth (Diaz et al., 2006; Stevenson, 2008), which could hinder plant metabolism and affect the yield of crops (Hermans et al., 2006; Makino, 2011). Nitrogen limitation of plant growth and development

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functions as a type of abiotic stress. To adapt to nitrogen deficiency and other abiotic stress, thousands of metabolites (more than 200,000 to date) are synthesized during the long-term evolution of plants. The synthesized metabolites—such as phenylpropanoids, quinones, flavonoids, terpenes, glycosides, alkaloids, *etc*.—include both the primary metabolites required by plants and the secondary species-specific metabolites (Sumner et al., 2003).

Wheat is one of the most important grain crop plants worldwide, and its growth is restricted by environmental factors. Currently, few research methods are available to explore the influence of growth factors on wheat, but metabolomics may be applicable. Metabolomics has largely focused on the study of all small-molecule compounds (metabolites) and the related dynamic changes found in or produced by organisms and their tissues and cells (Fiehn, 2002; Hillenmeyer et al., 2010; Oldiges et al., 2007). Plant metabolomics is now considered a widespread and valuable biotechnology. Metabolomics has been used to explore the resistance mechanisms of plants in adverse environments. Cook et al., 2004 demonstrated that the metabolomics of *Arabidopsis thaliana* could change distinctly under low temperature stress based on a comparison of the metabolic fingerprint spectra of *Arabidopsis thaliana* strains with different cold hardiness levels. Previous reports explored the metabolites of fruit and seeds from wine grapes and found that among the selected 32 small-molecule metabolites, the accumulation in fruits of 7 was influenced by drought stress (Grimplet et al., 2009).

Plant metabolomic studies of *Arabidopsis thaliana*, potato, tomato, tobacco, peas, barley, and lettuce, have focused only on their associations with water stress, temperature stress, *etc*. (Charlton et al., 2004; Flamini et al., 2013; Gholami et al., 2014; Roldan et al., 2014; Sobolev et al., 2005). Limited studies have focused on metabolite variation of wheat and the identification of biomarkers under mineral nutrient stress, particularly low nitrogen stress. Therefore, the purpose of this study was to increase our understanding of the metabolism of the flag leaf of wheat under low N stress using a metabolomics method.

2. Materials and methods

2.1. Chemicals and reagents

Extraction solvents: Ultrapure water (18 kΩ, Millipore, Solna, Sweden), Methanol and Formic acid (Sigma-Aldrich, Stockholm, Sweden).

Chromatography solvents: Ultrapure water (18 kΩ, Millipore, Solna, Sweden), Acetonitrile (ACN) and Formic acid (Sigma-Aldrich, Stockholm, Sweden).

2.2. Plant material, growth conditions, and experimental design

The samples were selected during 2014 and 2015 in a technology park of Nanyang, Henan Province, China (112°54′15.14″E, 33°15′43.83″). The soil in the park is mortar black soil, with a pH of 7.16. The other nutrient substances in the soil are 12.78 g kg^{-1} organic matter, 0.83 g kg^{-1} total nitrogen, 56.0 mg kg^{-1} available nitrogen, 17.0 mg kg^{-1} rapidly available phosphorus (P_2O_5), and 126 mg kg^{-1} rapidly available kalium (K_2O). Potassium chloride fertilizer (containing 60% K_2O) was present at 175.00 kg hm^{-2} and superphosphate fertilizer (containing 16% P_2O_5) at 843.75 kg hm^{-2}. Phosphorus and kalium fertilizers were used as base manure at a ratio of 1:1. All base manures were applied in the jointing stage of wheat, while other cultivation managements and measures were identical to those of high-yield wheat.

Wheat in the study area was subjected to the following two treatments: one treatment (low-nitrogen) involved fertilization with N (120 kg hm^{-2}), which is referred to as N0, while the other treatment (normal-nitrogen) involved fertilization with N (225 kg hm^{-2}), which is referred to as N1. The study wheat cultivars, Zheng Mai 366 and Ai Kang58, were the dominant species in Henan Province. The wheat was sown on October 13, 2014. There were 2.3×10^6 plants/ha in the study area. The two cultivars were seeded in half of each experimental area. The wheat samples were collected during the jointing and flowering periods of wheat, respectively. Each sample involved eight replicates, each of which was blended from three individual leaves (Table 1). The samples were then placed in liquid nitrogen with silver paper and finally stored in an ultra-low temperature refrigerator at −80 °C prior to analysis.

2.3. Sample preparation

After being ground into powder in an agate mortar with the additional liquid nitrogen, 50 mg of powder sample were weighed into 4 ml vials and configured into solution (V_{solute}: $V_{Solution} = 1:30$) with 75% methanol containing 1‰ formic acid. After being ultrasonic extracted for 15 min and centrifuged for 10 min at 12,000 rpm, the supernatant was collected and filtered through 0.22 μm PTFE membranes (Sigma, America). Filtrate (20 μl) was then prepared for analysis. All samples were randomized to eliminate instrument errors.

2.4. Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry analysis

Compounds in all wheat samples were measured using 2 μl of methanol filtrate and an ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometer (UPLC-QTOF MS) (XEVO G2, Waters Corporation, Milford, MA, USA) equipped with an Acquity BEH C_{18} column (1.7 μm, 2.1 mm × 100 mm) (Waters Corporation, Milford, MA, USA) using optimized mobile phases. The binary solvent system consisted of 0.1% formic acid in deionized water (Mobile phase A) and 0.1% formic acid in acetonitrile (Mobile phase B), with a linear gradient. The linear mobile phase gradient started at 5% B (0.0–2.0 min), increased to 95% B (2.0–22.0 min), maintained at 95% B (22.0–25.0 min), ramped back down to 5% B (25.0–27.0 min), and was held at 5% B (27–30 min). The flow rate was 400 μL/min (shown in Table 1). The column temperature was set at 35 °C and the temperature of the automatic sampler was maintained at 4 °C. The separated analytes were detected using Electro-Spray Ionisation Mass Spectrometry (ESIMS) monitors. For MS detection, the optimum ESI conditions were as follows: source temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 700 L/h; cone gas flow, 50 L/h; capillary voltage, 2.5 kV; sample cone voltage, 21 V; extraction cone, 4 eV; collision energy, 15–60 V; scan range, 100–1000 m/z. The MS detector was set to collect negative (ESI-) and positive (ESI+) ions. The results obtained using

Table 1
List of sampling information of wheat the flag leaf in NanYang.

Index	Nitrogen treatments	Cultivars	Sampling period
A	Low nitrogen level (LN)	Zheng Mai 366 (LNZ)	Jointing stage
B	Low nitrogen level (LN)	Zheng Mai 366 (LNZ)	Flowering stage
C	Low nitrogen level (LN)	Ai Kang 58 (LNA)	Jointing stage
D	Low nitrogen level (LN)	Ai Kang 58 (LNA)	Flowering stage
E	Normal nitrogen level (NN)	Zheng Mai 366(NNZ)	Jointing stage
F	Normal nitrogen level (NN)	Zheng Mai 366(NNZ)	Flowering stage
G	Normal nitrogen level (NN)	Ai Kang 58(NNA)	Jointing stage
H	Normal nitrogen level (NN)	Ai Kang 58(NNA)	Flowering stage

positive ionization mode did not show additional distinct variances within the data.

2.5. Data analysis

Data preprocessing—including alignment, peak detecting, peak integration, and retention time (Rt) correction—was performed using Markerlynx XS™ software (Waters Corporation, Milford, USA). The optimized parameters were: Rt range of 1–24 min, mass range of 100–1000 Da, mass tolerance of 0.02 D, and Rt window of 0.2 min. Data were normalized to total intensity (area) using Markerlynx. Principal component analysis (PAC) was then applied for unsupervised multivariate analysis using SIMCA-P software (version 12.0, Umetric, Umea, Sweden). Additionally, orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was applied for supervised multivariate analysis. Moreover, Kruskal–Wallis ANOVA and univariate statistical analysis of the detected data were conducted using SPSS 20.0 and Origin 8.0 with a significance level of 0.01.

3. Results and discussion

3.1. Principal component analysis of metabolites profiles

To provide an overview of the similarities and differences among the samples, data from the UPLC-QTOF were analyzed based on principle components analysis (PCA). The analysis method was conducted using detailed MS information, including retention time, ion peak intensities, ion peak area, and MS (*m/z*) ions. Preliminary PCA with mean centering was performed on all samples to inspect the clustering and omit outliers. PCA was considered an unsupervised model to operate without any anthropogenic factors, and may

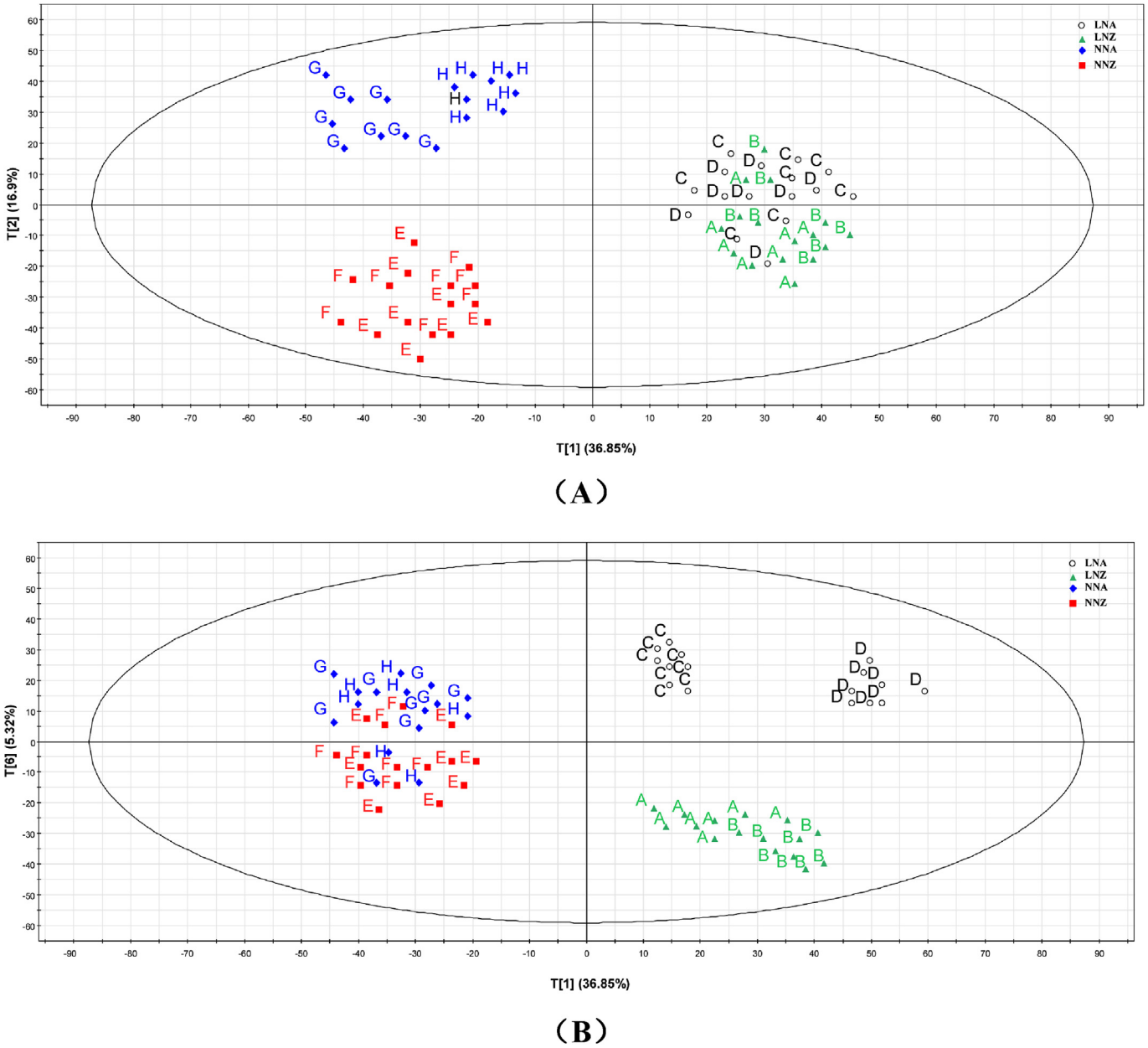
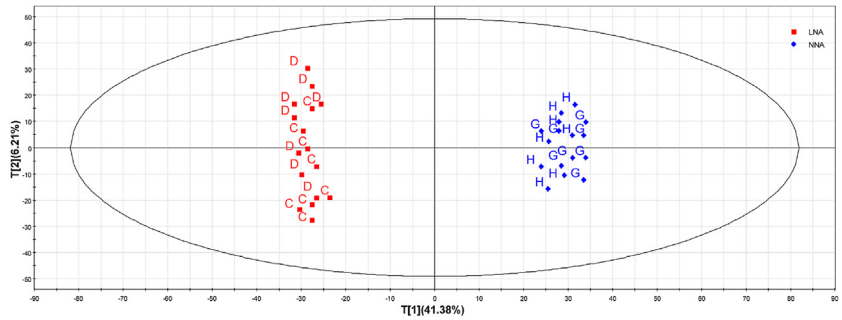
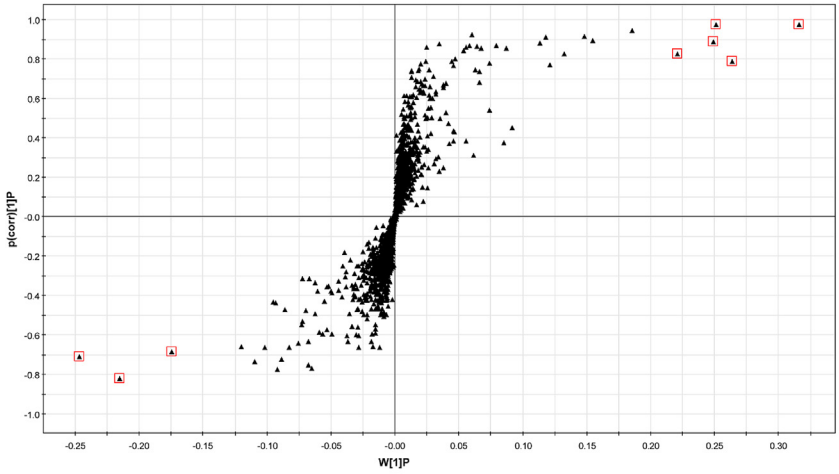


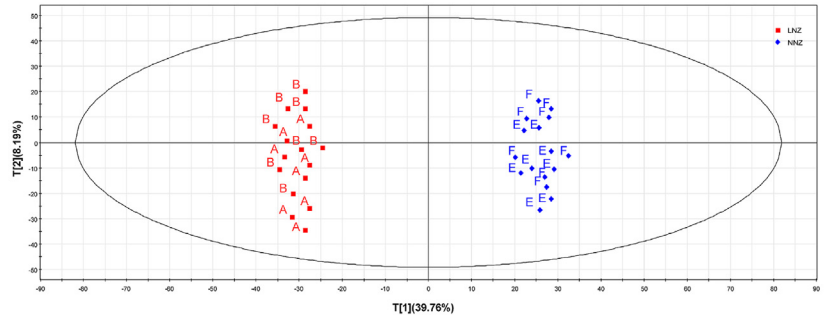
Fig. 1. Scores plots of PCA based on UPLC-QTOF data. (A) PCA plot with the scores of the first two principal components, (B) PCA plot with the scores of the first principal component and sixth principal component. (○) group on LNA samples; (▲) group on LNZ samples; (◆) group on NNA samples; (■) group on NNZ samples.



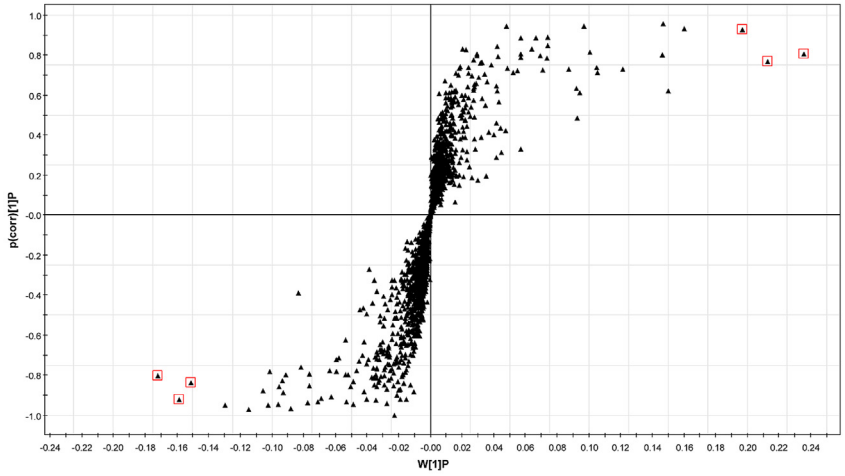
(A)



(B)



(C)



(D)

Fig. 2. Scores plots and S-plot of OPLS-DA model. (A) scores plot of cultivar Ai kang 58 samples under low nitrogen treatment and normal treatment; (B) S-plot of cultivar Ai kang 58 samples under low nitrogen treatment and normal treatment; (■) group on LNA samples; (◆) group on NNA samples. (C) scores plot of cultivar Zheng mai 366 samples under low nitrogen treatment and normal treatment; (D) S-plot of cultivar Zheng mai 366 samples under low nitrogen treatment and normal treatment. (■) group on LNZ samples; (◆) group on NNZ samples.

reflect the primary data, which was conducive to understanding the holistic data and eliminating abnormal samples to improve the accuracy of the PCA model.

The PCA indicated that low nitrogen stress, wheat cultivars, and growth periods had a combinatory effect on metabolite variation of the samples. Among the three factors influencing metabolite variation, the effect of low nitrogen stress was most significant. First, the samples were markedly different and separated based on NO and N1 levels, and respectively gathered in their related areas (Fig. 1). Six principal components (PC) were retained in the final PCA model ($R^2X = 0.676$, $Q^2 = 0.466$).

In the model, PC1 explained 36.85% of the total variability, and could separate the wheat samples from low-nitrogen and normal-nitrogen treatments. However, sample metabolites are also affected by the cultivar and growth period. For the two cultivars, the samples in the normal-nitrogen treatment could be separated by PC2 (16.9%) (Fig. 1A); however, samples in the low-nitrogen treatment could be separated only by PC6 (5.32%) (Fig. 1B). PCA indicated that the cultivars had a weaker effect on sample metabolites than low nitrogen stress. At the same time, the effect of growth period on wheat flag metabolites was the weakest among the three factors.

It should be noted that NNZ and NNA under normal nitrogen treatment gathered in their respective regions, and their metabolite variation during different growth periods was not obvious. However, LNZ and LNA under low-nitrogen conditions were also separated based on metabolite variation, and the Ai Kang58 samples exhibited a specific distance between the anthesis and filling stages. Compared to Zheng Mai 366, cultivar Ai Kang 58 had greater metabolite variation during the two growth periods. As shown in Fig. 1B: Compared with A and B, C and D are separated by a farther distance in the score graph. This means that metabolites of samples exist a significant variation between jointing and flowering periods. The larger variation between C and D may be associated with cultivar resistance. The wheat cultivar Ai Kang 58 showed a stronger resistance to abiotic stresses, which can be considered an advantage of the cultivar.

3.2. OPLS-DA analysis for discrimination of low and normal nitrogen treatments

To further investigate the effect of low-nitrogen stress on wheat metabolites, sample data were subjected to an orthogonal partial least square discriminant analysis (OPLS-DA). OPLS-DA is a supervised analysis method that can group detected samples according to the categories prior to conducting the analysis. Therefore, the analysis process could distinguish the groups during calculation of the mathematical models and ignore random differences within groups to highlight the systematic differences between groups, thus improving the effectiveness and analytical power of the model.

To eliminate the effect of wheat cultivar on sample variation, we compared the effect of low-nitrogen stress on samples in the same cultivar background. First, we analyzed samples LNA and NNA and then LNZ and NNZ, under the single cultivar condition. The score figure in the OPLS-DA model confirmed that the metabolites of wheat the flag leaf differed under the two nitrogen treatments. The samples were divided into two groups: low-nitrogen and normal-nitrogen treatments (Fig. 2A and B). Samples gathered in a certain area according to the nitrogen treatment. The classification results were in agreement with those of PCA, besides being clearer than PCA. Two significant components of the two OPLS-DA models described 97.6% and 95.5% of the variation in Y and predicted 93.4% and 94.9%, respectively, according to cross-validation. Q^2Y supported the predictive accuracy of the model, and the results based on the samples showed good prediction ability.

To identify biomarkers associated with the two nitrogen treatments, we used an S-plot figure of the OPLS-DA model to analyze the data. The S-plot of the OPLS-DA model, which is shown in Fig. 2 C, D, indicated that the greater the distance of spots from the original point, the greater the contribution attributable to the classification. As shown in the figure, the compounds closer to the lower left and upper right corners made a larger contribution to the classification of the samples, besides satisfying the condition that the VIP (variable importance in projection; the higher the VIP, the more important the variables to the model) value was greater than 1.

According to the distribution of plots in the S-plot of the OPLS-DA model, the metabolites that make important contributions to sample classification can be identified. For cultivar Ai Kang 58, we selected eight metabolites from the two nitrogen treatments as potential biomarkers; these are termed comp1, 2, 3, 7, 8, 9, 10, and 11. For cultivar Zheng Mai 366, we selected six metabolites from the

Table 2
The differentiated metabolites of wheat the flag leaf identified by UPLC-QTOF MS/MS.

Index	Retention time (min)	Mass (m/z) measured in negative mode	Mass error (ppm)	MS/MS fragments, negatively charged	Tentative identification	Molecular formula
compound 1	6.76	431.0973	−1.2	341, 311, 283	Iso-vitexin (Apigenin 6-C glycoside)	$C_{21}H_{20}O_{10}$
compound 2	3.87	447.0925	−0.5	357, 327, 297	Iso-orientin (Luteolin-6-C glucoside)	$C_{21}H_{20}O_{11}$
compound 3	7.35	563.1397	−0.5	443, 383, 353	Iso-schaftoside (Apigenin-6-C-arabinoside-8-C-hexoside)	$C_{26}H_{28}O_{14}$
compound 4	21.26	607.1668	1.0	461	Methylisoorientin-2″-O-rhamnoside	$C_{28}H_{32}O_{15}$
compound 5	2.86	167.0349	3.6	152	Vanillic acid	$C_8H_8O_4$
compound 6	17.56	511.1081	−1.2	310, 162	unknown	$C_{22}H_{24}O_{14}$
compound 7	12.31	329.0657	−1.2	314, 271	Tricin	$C_{17}H_{14}O_7$
compound 8	15.78	771.1989	0.8	609, 301, 271	Quercetin 3-rutinoside-7-glucoside	$C_{33}H_{40}O_{21}$
compound 9	8.33	593.1509	0.5	395, 162	unknown	$C_{27}H_{30}O_{15}$
compound 10	10.20	371.1192	0.8	248, 502	unknown	$C_{13}H_{24}O_{12}$
compound 11	16.64	308.1102	1.6	265, 209	unknown	$C_{12}H_{21}O_9$

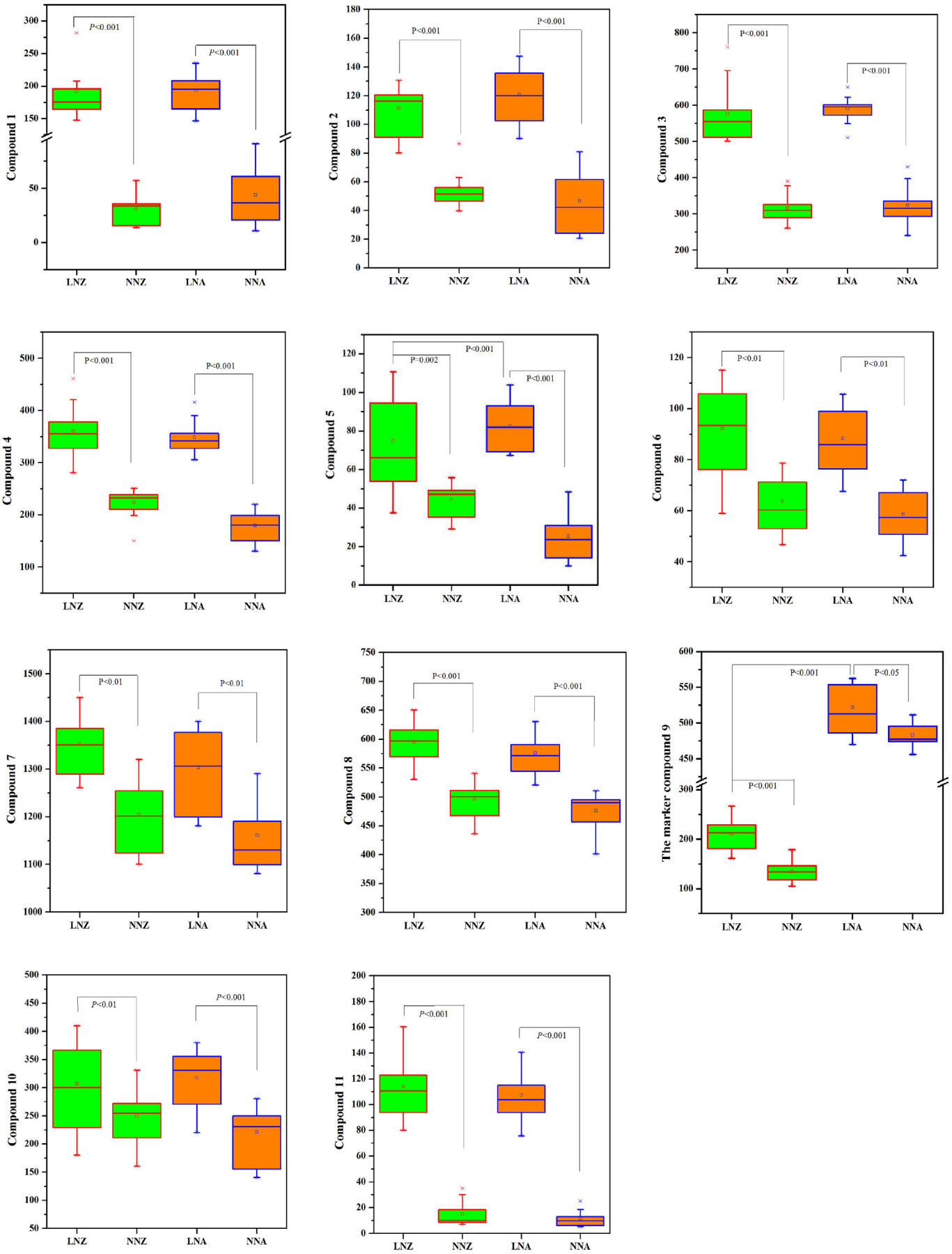


Fig. 3. Box plots of biomarkers in wheat the flag leaf of two nitrogen treatment and varieties. LNA, LNZ, NNA, and NNZ represent variety Ai Kang 58 grown in low nitrogen treatment, variety Zheng mai 366 grown in low nitrogen treatment, variety Ai Kang 58 grown in normal nitrogen treatment, variety Zheng mai 366 grown in normal nitrogen treatment, respectively.

two nitrogen treatments as potential biomarkers; these are termed comp 1, 2, 3, 4, 5, and 6. All selected potential biomarkers were metabolites that played important roles in the separation of wheat the flag leaf samples under the two nitrogen treatments (Table 2).

3.3. Identification of biomarkers

For metabolite identification, the elemental compositions of unknown compounds were deduced, together with accurate molecular weights, degrees of unsaturation, and isotopic abundance patterns using Markerlynx software. Structural identification was conducted based on UPLC-QTOF MS/MS analysis and the retention time, accurate molecular weight, and MS/MS data. Based on the first-order mass spectra of samples, the mass spectrum information of each peak was obtained and the quasi-molecular ion peak (negative ions) of each compound was estimated. Based on these results, the fragment of the peaks of compounds in the second-order mass spectrum was measured using the quasi-molecular ions as the mother ions in the corresponding MS/MS analysis of unknown peaks based on UPLC-QTOF MS/MS, and high-resolution information was obtained. Accurate molecular weight, molecular formula, and possible chemical structures were obtained from related reports and web databases such as KEGG (http://www.genome.jp/keg/), MassBank (http://www.massbank.jp/), METLIN (http://metlin.scripps.edu/), MMCD (http://mmcd.nmrfam.wisc.edu/), and PubChem (http://pubchem.ncbi.nlm.nih.gov), and the chemical components of the compounds were identified.

A total of eleven potential biomarkers was identified under the two treatments, among which three were identical in the two cultivars; namely, isoorientin, iso-vitexin, and iso-schaftoside. During metabolite identification, seven compounds were identified, while the other four were not; the latter were considered potential metabolic biomarkers of different nitrogen levels. The identified components were identified as flavonoids and their related derivatives.

To compare the relative contents of the eleven biomarkers under different nitrogen treatments and cultivars, data were subjected to Kruskal–Wallis ANOVA. The results of multiple comparisons showed that all of these biomarkers were all statistically significant ($p \leq 0.01$) under the low nitrogenous fertilizer and normal nitrogenous fertilizer treatments. However, there was no significant difference between the two cultivars for the same nitrogen treatment, with the exception of comps 5 and 9 under the low nitrogen treatment (Fig. 3). Moreover, the relative contents of all biomarkers under low-nitrogen treatment were higher than those under normal-nitrogen treatment. The increased contents of these biomarkers indicated resistance to the low-nitrogen stress and an adaptation to abiotic stress.

3.4. Accumulation of flavonoids and their related derivatives in response to abiotic stresses

In this study, accumulation of flavonoids and their related derivatives in response to abiotic stress was found. The relative contents of all biomarkers under low-nitrogen stress were higher than those under normal-nitrogen treatment. Similar variance trends were also observed in wheat leaf. Ma et al. investigated whether the flavonoid content of wheat leaves increased when the wheat suffered moisture stress, and found a close relationship between the accumulation of flavonoid compounds and drought tolerance in wheat. Moreover, the total flavonoid content in wheat leaves was enhanced under low-moisture stress (Ma et al., 2014). Moheb et al. identified a total of 40 phenolic and flavonoid compounds in wheat leaves under cold acclimation, indicating that the levels of phenolic compounds in wheat leaves increased significantly under cold acclimation (Moheb et al., 2011). Another previous study reported that low-temperature stress increased the phenolic content of winter wheat leaves, whereas their qualitative composition was not changed (Zagoskina et al., 2005). Thus, the above study showed that flavonoids function in plants to increase the resistance to, or reduce the damage caused by, abiotic stresses. Thus, flavonoids and their related derivatives play a key role in resistance to abiotic stresses.

The number of flavonoids described to date exceeds 9000 and continues to increase (Ververidis et al., 2007). Previous studies have shown that the flavonoid pathway is closely related to abiotic stress situations (Castellarin et al., 2007; Wahid and Ghazanfar, 2006), for example, in *Ligustrum vulgare* (Guidi et al., 2008), *S. baicalensis* Georgi (Yuan et al., 2012), maize (Tossi et al., 2012), and rice (Goufo et al., 2014). Furthermore, the flavonoid pathway may play an important role in plants, such as in defense against plant diseases and insects, UV protection, auxin transport regulation, and signaling with microorganisms (Ma et al., 2014; Warren et al., 2003; Winkel-Shirley, 2001).

Previous studies showed that flavonoids are synthesized mainly through the phenylpropanoid pathway, in which phenylalanine is converted to diverse phenols through an enzymatic reaction (Dixon and Paiva, 1995). The wide spectra of biological activities of compounds involved in the phenylpropanoid pathway participate in many physiological and biochemical processes (Azevedo, 2006; EG S et al., 2004; NC, 2013; Seigler, 1998). Chalcone synthase (CHS), which is the entry point of the flavonoid pathway, guides the phenylpropanoid pathway to flavonoid biosynthesis by means of the activities of specific enzymes.

In this study, the biomarkers identified in the two wheat cultivars were 6-C-glycosides of luteolin or apigenin, which are common in wheat, maize, barley, etc., and respond to abiotic stresses (Brazier-Hicks et al., 2009; Khlestkina, 2013). Especially in maize, C-glycosyl maysin is closely related to the insecticidal activity towards corn earworm (Rector et al., 2002). Methylisoorientin-2″–O-rhamnoside and vanillic acid were unique biomarkers of Zheng Mai 366, and Quercetin 3-rutinoside-7-glucoside and tricin were unique biomarkers of Ai Kang 58. Thus, the category and quantity of secondary metabolites may differ in plants under abiotic stress according to plant cultivar. One unknown metabolite was found in Zheng Mai 366 and three in Ai Kang 58. We conclude that some other metabolites may be associated with abiotic stress, and the mechanism may differ according to the cultivar in question.

There are specific secondary metabolites that counter various abiotic stresses, such as the identical biomarkers in the two cultivars iso-orientin and iso-vitexin. The resistance function of those metabolites results from their unique structure; for example, two aromatic rings linked by three carbons, double carbons, hydroxyl groups, and modifications such as glycosylation, methylation, and prenylation (Rice-Evans et al., 1997).

Several recent studies have explored the molecular mechanism underlying the response to abiotic stress. Dongyun et al. reported that water deficit increases the expression of flavonoid biosynthesis genes in wheat leaves, as determined by quantitative real-time PCR (Ma et al., 2014). Yuan et al. also found a relationship between drought stress and flavonoid levels in *Scutellaria baicalensis* Georgi roots. Studies on Amira Moheb indicated that the enhanced expression level of *TaOMT2* resulted in the accumulation of tricin (Moheb et al., 2013). These studies, which combined genomics and metabolomics, increased our understanding of the mechanisms of resistance abiotic stresses. However, it is important to understand the molecular basis of the functions of flavonoids in improving defense against stresses, as well as the types and amounts of flavonoids synthesized under stress conditions (Di Ferdinando et al., 2012).

Studies on wheat metabolomics have made progress (Asenstorfer et al., 2006; Caruso et al., 2009); however, most studies focused on comparison of metabolite variation, such as in transgenetic *versus* traditional varieties, disease-resistant varieties *versus* ordinary varieties, and among the various organs of wheat (Baker et al., 2006; Gunnaiah and Kushalappa, 2014; Palmer et al., 2014). Few studies have explored metabolite variation upon exposure of plants to abiotic stress (Ma et al., 2014; Moheb et al., 2011; Olenichenko et al., 2006), particularly that caused by low levels of mineral elements (Zagoskina et al., 2005; Estiarte et al., 1999; Olenichenko et al., 2008). Therefore, in this study nitrogen was selected as the experimental factor, and the metabolite variation of wheat flag samples was evaluated during two key growth periods. Overall, this study explored metabolite variation in the presence of low levels of an essential mineral.

4. Conclusions

Metabolomics is an emerging field in the post-genomic era. In plant metabolomic studies, the UPLC-TOF platform is commonly used to survey physiological status, identify biomarkers, and characterize related pathways. In this report, the metabolite variation of wheat under low-nitrogen stress was investigated. The results showed that low nitrogen stress plays an important role in determining the metabolome of wheat, and we identified 11 biomarkers that contributed to the classification. Consistent with previous reports, the identified biomarkers were mostly flavonoids and their related derivatives, and these metabolites accumulated in wheat in response to abiotic stress. Use of metabolomics methods to improve our understanding of the effects of abiotic stresses will enhance agricultural practice.

Author contribution

MA Xin-ming. and WANG Xiao-chun. contributed to the conception of the experiments; ZHANG Yang and XIONG Shu-ping contributed to performe experiments; LIU Ji-hong, HUANG Bing-yan and GUO Xiao-yang contributed to analyze the data; ZHANG Yang and WANG Xiao-chun wrote the manuscript; LA Gui-Xiao contributed reagents/materials/analysis tools.

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