Metabolomic and transcriptomic exploration of the effects of biological fertilizer on the growth of Alfalfa

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Abstract

Alfalfa is a forage grass with excellent grass quality and high protein content. It is one of the most important feeds in the world.However, according to the conventional alfalfa planting method,long-term excessive application of chemical fertilizer will seriously affect soil fertility, enzyme activity and Alfalfa protein content. Biofertilizer can effectively change soil nutrients, activity of soil enzymes, and increase concentrations of crude protein and soluble protein.Therefore, it is necessary to fully understand the changes of transcriptome and metabolome under the condition of applying biological fertilizer to alfalfa. In this study, leaf transcriptome and metabolomics were used to study the effect of alfalfa treated with bio-organic fertilizer and chemical fertilizer(control group) on mature alfalfa. Compared with the control group (applying chemical fertilizer), the treatment group (applying biological fertilizer) has higher growth and higher nutrition index, and the soil enzyme activity has also been significantly improved. In addition, through the combined analysis of transcriptome and metabolomics, it was shown that the external application of biological fertilizer improved some signal transduction, tryptophan metabolism, phenylalanine metabolism, tyrosine metabolism and isoflavone metabolism of alfalfa.In conclusion, the study provides a notional basis for evaluating the induction of nutrients in alfalfa growth and maturity stage and the potential mechanism of remediation of fertilizer contaminated soil.

1 Introduction

Alfalfa is known as the "King of Forage", which not only has high content of protein, but also has excellent grass quality, and all kinds of livestock and poultry like to eat it [1,2]. In China, the planting area of alfalfa is about 3.28 million acres, and in the United States, there are more than 23 million acres of land for planting alfalfa [3]. The pasture industry has become a green engine for many people to get rid of poverty and become rich. However, with the increasing efforts of planting grass artificially, the amount of fertilizer applied is also increasing. Applying chemical fertilizer to scaling up yield has become a conventional way for a lot of alfalfa growers, especially in sandy soil.l [4]. As a result, the soil nutrients in sandy land with low organic matter content are seriously out of balance, and the chances of autotrophic living become increasingly slim. These problems prompt us to adjust the fertilization strategy.

Fertilization has become one of the most considerable regulatory measures in the growth of crops [5]. However, ongoing overuse of chemical fertilizer will seriously affect soil fertility and enzyme activity [6,7]. Soil enzymes are often indicative of chemical fertilizers. Generally speaking, excessive application of chemical fertilizers will lead to the decrease of soil enzyme activity. Bio-fertilizer can effectively stop soil enzyme activity degradation and reduce the negative impacts of chemical fertilizer [5]. And numerous studies show that biological fertilizer can effectively change soil nutrients and soil enzyme activities [8,9,10,11]. Nevertheless, the underlying mechanism of the effect of bio-fertilizer on sandy pasture is still unclear.

Transcriptome sequencing is widely used as an effective method to analyze plants at the molecular level [12]. For example, Luo analyzed the molecular cause of tolerance of salt stress in alfalfa roots by sequencing the transcripts of alfalfa treated with NaCl and mannitol [13]. Lei evaluated the molecular mechanism of alfalfa salt tolerance by sequencing the transcripts of alfalfa leaves under high salt conditions [14]. It is established by examing the endogenous metabolites from liquid and tissue samples and will appraise underlying relevances that underlie both the observed metabolic profiles and the associated physiology [15]. Therefore, it is considerable to seek the influence mechanism of biological fertilizer on alfalfa by combining transcriptome with metabolomics.

Since the underlying mechanism of the effects of biofertilizer application on forage grass is not clear, filling the gaps in understanding the molecular dynamics of the effects of biofertilizer on alfalfa will be of particular importance.Therefore, we studied the effect of biological fertilizer on Alfalfa at maturity through leaf transcriptome and metabolomics.Alfalfa (control group) treated with biological fertilizer + chemical fertilizer or chemical fertilizer was studied.The main objective of the current study is to assess the impacts of biological organic fertilizer on the growth of alfalfa and to offer theoretical fundation for the remediation of soil polluted by chemical fertilizer.

2 Materials And Methods

2.1 Fertilization methods of different alfalfa treatment groups

In this study, alfalfa (dsao1-t), a two-year-old high-yield and multi leaf variety that can be cut 3-5 times a year, was selected for the experiment.The experiment was carried out in Tianshan Town, China's grass capital, Alukeerqin Banner, Inner Mongolia, China. The total experimental area was 30 acres, which was divided into 6 sprinkling irrigation areas, each with a net area of 5 acres.Three areas were selected at random as the treatment group (ZS) and the other three areas as the control group (HX). Treatment method: the treatment group was chemical fertilizer (N:P2O5:K2O=20:20:20 18kg/acre) + biological organic fertilizer (effective viable bacteria number ≥ 200 million /mL, organic matter ≥45%18kg/acre), and the control group was chemical fertilizer (N:P2O5:K2O=20:20:20 151kg/acre). Treatment time: The first crop was fertilized for the first time on May 6, 2020, and the first crop was fertilized for the second time on May 13, 2020. The second crop was fertilized for the first time on June 17, 2020, and the second crop was fertilized for the second time on June 20, 2020. The third crop was

fertilized for the first time on July 19, 2020, and the third crop was fertilized for the second time on August 3, 2020. Except for fertilization, other management methods of treatment group and control group were completely the same in field management.

2.2 Determination of Alfalfa physiological indexes and soil enzyme activity.

Sampling time: August 11, 2020.The mature leaves of Alfalfa in biological fertilizer + chemical fertilizer group (30ZS) and chemical fertilizer group (30HX) were collected as three groups of biological replicates respectively.

Growth index measurement: 10 alfalfa samples were randomly selected from each sample area to determine stem diameter, plant height, leaf weight and leaf area.

Determination of crude protein content: It was determined by Kjeldahl semi-micro nitrogen determination method and calculated according to Bozhanska's description [16]. The soluble protein content was measured using established methods [17].

Determination of content of soluble sugar and chlorophyll: the determination of sugar content and chlorophyll content were determined by ultraviolet absorption method.

Determination of soil enzyme activity: Urease (UA) activity was quantified by measuring ammonium release following soil incubation, using urea for the substrate [18];Phosphatase (Pacid) activity was measured by the output of p-nitrophenol following soil incubation using p-nitrophenyl-phosphate [19,20];FDA hydrolase assay was performed using the FDA hydrolase assay kit (BC0480, ACMEC) ;Soil sucrase was measured by 3, 5-dinitrosalicylic acid colorimetry;The activity of soil catalase was determined by ultraviolet spectrophotometry.

2.3 RNA extraction, library construction and RNA-seq analysis.

Transciptomic analysis included randomly selecting three alfalfa samples from different blocks in each treatment group. In a nutshell, genes are extracted from total RNA using oligo deoxyribonucleic acid (T18) and then a random fragment of the purified gene is reverse transcribed into cDNA.The DNA fragments were attached to the connectors at both ends, and followed by selective amplification and enrichment. Products of the purified polymerase chain reaction were then measured with the Agilent BioAnalyzer 2100 system. After generation of clustering, the Illumina HiSeq platform was used to sequence the final cDNA library.

DESeq2 (version 1.12.4) determined the differentially expressed genes (DEGs) between a set of two samples. A fluorescent quantitative PCR instrument carried out real-time quantitative PCR (qRT-PCR) validation.

The real-time quantitative PCR (qRT PCR) was verified by fluorescence quantitative PCR.PCR data were analyzed using comparative CT (2 ^ - CT).EF-1 α Gene was used as reference gene and PRIMER5.0 was used to design gene specific primers for the selected deg.qRT PCR was performed on ABI Stepone plus platform with three reactions and three biological replicates.

2.4 Metabolomics analysis

Place 50mg sample into a centrifuge tube containing 1000μL extract, swirl for 30s, add magnetic beads, and grind at 45Hz. Take 100μl, add into a centrifuge tube containing 500μl extract, swirl for 30s, ultrasonic for 10min. Then the supernatant was extracted and lyophilized in vacuum. The supernatant was then analyzed by LC-MS/MS.UPLC-MS/MS conditions were as described in the published literature [21].

2.5 Comprehensive enrichment analysis of transcripts and metabolite profiles.

Combined with the findings of differential metabolites and differential genes, they are mapped to the KEGG pathway map at the same time, so as to better interpret the correlation between genes and metabolites.

3 Result And Analysis

3.1 Determination results of Alfalfa physiological indexes and soil enzyme activity

The results show that the growth index and nutrition index of alfalfa in the experimental group are higher than those in the control group.The biological fertilizer made alfalfa grow stronger and leaves bigger obviously.And the nutrient content of alfalfa in the treatment group is higher than that in the control group(Figure 1a,Table 1).

Table 1

Effect of biofertilizer on growth and nutritional index of Alfalfa.

	Leaf Area (cm2)	Stem diameter (mm)	Soluble protein (mg/g)	Crude Protein (%)	Soluble sugars (%)	Chlorophyll a (mg/L)	Chlorophyll b (mg/L)	Carotenoids (mg/L)	Peroxidase (U/Fw · min)	MDA content (μmol/gFW)
30ZS	1.8791	2.482	4.2	20.58	0.6961	13.1256	15.1209	6.4786	7990	0.0030838
30HX	1.3881	2.133	4.045	17.59	0.5808	6.5448	9.5031	2.5144	7230	0.004128

In addition, this study also measured the soil enzyme activity in the experimental site.During the whole study period, other field management methods were exactly the same except adding biological fertilizer to the experimental group. To describe the influence of biological fertilizer on soil, the soil enzyme activity was measured. Soil enzyme activity of the treatment group is obviously increased, compared with the control group(Table 2).Therefore, biological fertilizer can effectively change the pollution of chemical fertilizer to soil.

Table 2

Effects of biofertilizers on soil.

	Catalase (0.1 mol/L KMnO4) /(h · g)	Urease (Ure)	Sucrase (XII)	FDA Hydrolase (μg/Ml)	Acid phosphatase (mg/g)	Neutral phosphatase (mg/g)	Alkaline phosphatase (mg/g)
30ZS	0.015323	20.56145	41.90987	330.6663	4.227333	2.892667	1.735333
30HX	0.014118	20.05166	41.3396	279.8723	3.088667	2.192667	1.007333

3.2 Transcriptome sequencing, assembly and differential gene expression analysis results.

Alfalfa transcriptome under different fertilization conditions was sequenced by Illumina 2000. After removal of low-mass readings, 42.27Gb of clean data were attained. For each sample, clean data was 6.10 Gb and the proportion of Q30 bases was 92.94%or more (Table S1). A total of 61,913 Unigene were obtained after assembly. Among them, there are 18,933 Unigene with length over 1kb.

By analyzing the transcriptome of alfalfa, we found 2612 DEG, of which 1085 were up-regulated, and 1527 were down-regulated (Figure 1). Then, PCA was utilized to measure the differences in gene expression across the groups.The treatment group (30ZS PCA1) was obviously separated from the control group(30HZ), and these findings were consistent with those from the heat map(Figure 1b-d, Table S2).

We used GO classification for the DEGs to resolve the appropriate subordinate categories for the responsive genes. The matched deg were thus apportioned into three functional groups: biological processes, cell components, and molecular function (fig. 2a). Within biological processes, the most common genes were associated with cellular and metabolic processes. For cell components, genes were commonly associated with cell parts and cells. In the molecular functional group, DEGs primarily belong to the binding and catalytic activity subgroup. To ascertain the functional biological pathway rich in DEGs, the KEGG pathway database was utilized (Figure 2b). Primary enrichment pathways are plant hormone signal transduction, phenylpropanoid biosynthesis, brassinosteroid biosynthesis, Stilbenoid, diarylheptanoid and gingerol biosynthesis, Phenylalanine Metabolism and Flavonoid biosynthesis(Figure 2c).

Through KEGG enrichment of differential genes, it was found that plant signal transduction pathways were obviously enriched, in which almost all signal pathways showed up-regulation or down-regulation of genes, especially auxin signal transduction, brassinosteroid and gibberellin signal transduction pathways, and brassinosteroid organisms and pathways were also obviously enriched in KEGG enrichment of differential genes. Although some genes in ABA and ethylene signal transduction pathway are up-regulated, they are mainly concentrated in the negative regulation genes of ABA signal transduction pathway, such as PP2C gene in ABA signal transduction pathway, ETR gene and EBF1/2 gene in ethylene signal transduction pathway. In addition, ABA and cytokinin signal transduction related genes are obviously down-regulated, such as SnRK2 and ABF genes in ABA signal transduction pathway, CRE1 and B-ARR genes in cytokinin signal transduction pathway. Compared with the control transcriptome, some genes were differentially expressed only in treatment group.The genes specifically expressed by these treatment groups can be different biological pathways.Among them, 13 genes belong to phenylalanine metabolism, which is one of the most important plant secondary metabolic pathways, where it is critical in growth, development, and environmental interaction(Table S3).In addition,

6 genes and 17 genes belong to stilbenes, diarylheptanoic acid and gingerol biosynthetic pathway and flavonoid biosynthetic pathway, respectively.Phenols and flavonoids produced by stilbenes, diarylheptanoic acid and gingerol biosynthetic pathway and flavonoid biosynthetic pathway can be used as allelochemicals in plants.

It is key to the interaction between development and plant environment. In addition, 6 genes and 17 genes belong to stilbene, diarylheptic acid and gingerol biosynthesis pathway and flavonoid biosynthesis pathway, respectively. The phenols and flavonoids produced by biosynthesis of stilbene, diarylheptic acid and gingerol and biosynthesis of flavonoids can be used as allelochemicals in plants.

3.3 Real time qPCR verification results.

To verify the accuracy of transcriptome data set, real-time qPCR was used to analyze the transcription level of 10 randomly selected genes. The relative expression level of EF-1αgene was measured and calculated. These 10 genes are primary amine oxidase activity c76636.graph_c1-F shikimate O-hydroxycinnamoyltransferase activity c64912.graph_c1-F EIN3-binding F-box protein c85195.graph_c0-F CAP c69462.graph_c0-F indole-3-acetic acid-amido synthetase c82761.graph_c0-F SAUR-like auxin-responsive family protein c63578.graph_c0-F flavanone 3-hydroxylase c70484.graph_c0-F shikimate hydroxycinnamoyl transferase c83150.graph_c0-F chalcone reductase c85005.graph_c0-F and chalcone and stilbene synthase family protein c67970.graph_c0-F).The results of reverse transcription polymerase chain reaction confirmed that the transcriptional changes of these 10 genes were equivalent to the fold changes obtained by our transcriptome analysis (Figure 3, Table S3).

3.4 Metabolomic analysis results.

OPLS-DA analysis was used to measure the metabolic changes to alfalfa caused by biological fertilizer, by examining all metabolites in positive and negative ion mode. With the positive ion mode, R2 =A and Q2 = B, and with negative ion mode, R2 = C and Q2 = D, which indicates the model can describe the sample well and can be employed in the subsequent search for biomarkers. In the PLS-DA scatter plot results, the treatment group and the control group are obviously separated, which indicates that biological fertilizer can affect the normal metabolic pathway of alfalfa(Figure 4a-d).

A total of 1,358 and 539 metabolites were detected in positive and negative ion mode, respectively. Among these, 74 and 28 were detected in piglet plasma in positive and negative ion mode in treatment group and control group respectively (Table S4 and Table S5).

To determine the subordinate pathways of these differential metabolites, KEGG pathway database was used. The main enrichment ways are isoflavone compound synthesis, phenylalanine metabolism, tryptophan and tyrosine synthesis, purine synthesis (Figures 4e-f).

3.5 Comprehensive enrichment analysis of transcripts and metabolite profiles.

Analysis was conducted with KEGG co-enrichment of differential genes and metabolites.Comprehensive enrichment analysis showed that bio-fertilizer could affect isoflavone biosynthesis and metabolism and phenylalanine of alfalfa. Specifically, isoflavone biosynthesis and metabolism, and phenylalanine-related differential genes and metabolites were all up-regulated under the condition of applying biological fertilizer(Figure 5).

4 Discussion

In this study, the mechanism of bio-fertilizer promoting alfalfa growth was analyzed through field experiments, transcriptome sequencing and metabolome sequencing techniques.By measuring the growth index and physiological index of alfalfa, it was found that the growth of alfalfa in the treatment group with biological fertilizer was substantially better than in the control, and the nutrient content was increased above that of the control, so the biological fertilizer had obvious positive synergistic effect on the improvement of alfalfa growth. In addition, through the determination of soil enzyme activity, it was found that the soil enzyme activity of the treatment group was increased compared with the control group. Activity of soil enzymes is exceedingly promising, and can deliver an exceptionally comprehensive biological appraisal of soil, and can assess soil health status [22-26]. Moreover, soil enzyme activity is very dynamic, and it is very sensitive to heavy metals and other substances left by fertilization [27]. In addition, soil hydrolases are key to nutrient transformation and plant nutrient supply [28]. Therefore, the application of biological fertilizer obviously improved the negative impacts of ongoing use of chemical additives on soil productivity and health.

Usually, the physiological and metabolic changes of plants are realized by activating the signal cascade to react with external stimuli to change the expression pattern of downstream genes [29]. Fertilizer significantly induced auxin signal transduction, brassinosteroid and gibberellin signal transduction pathway.Auxin and gibberellin are two famous plant hormones.Auxin is very important to development and growth in plants, where it is integral in the life cycle [30], while gibberellin can control many aspects of plant development [31]. The key players in the auxin signal transduction pathway comprise auxin input vector (AUX1), nuclear receptor TIR1/AFB, auxin resistance/indole -3- acetic acid

inducible protein (Aux/IAA) and ARF transcription factor [32]. AUX1 (auxin input vector) combined with nuclear receptor TIR1/AFB to play most of its role, TIR1/AFB and Aux/IAA formed a co-receptor complex, which triggered proteasome-dependent degradation of Aux/IAA transcription regulatory factors. Aux/IAAs regulates the transcription of auxin-dependent genes by the formation of dimers together with auxin response factor (ARF) protein. Auxin-mediated transcription reprogramming initiates the auxin-dependent release of ARF transcription factors [33].This study made clear that genes involved in the auxin signal transduction pathway were highly expressed under the induction of bio-fertilizer, except for genes regulated by auxin, such as auxin-responsive AUX/IAA family protein, indole -3- acetate-amide synthetase and SAUR auxin-responsive family protein.The high expression of these genes can promote plant cell enlargement and plant growth, which may be one of the main factors that bio-fertilizer promotes alfalfa growth.

Gibberellin (GAs), a key hormone in the regulation of growth and development in higher plants, significantly promotes elongation and expansion of cells [34]. The mechanism of GAs promoting growth is that gibberellin is sensed by nuclear receptor GID1, which interacts with DELLA nucleoprotein and promotes its degradation, which leads to the change of transcription factor activity between DELLA and its physical interaction.In this study, gibberellin receptor GID1 was significantly up-regulated, but the negative regulatory factors of DELLA had no significant difference.Hedden P has described in detail the mechanism of gibberellin promoting plant growth, and the results of this study have confirmed this description again [35]. In addition, according to previous studies, the significant improvement of gibberellin signaling can improve crop yield and lodging resistance [36]. Alfalfa is not related to crop yield, but we speculate that applying biological fertilizer to leguminous crops can get more yield than applying chemical fertilizer.

As for the plant growth regulators promoted by microorganisms, auxin, cytokinin and gibberellin are mainly concentrated [37]. However, in this study, it was found that the signal transduction pathway of cytokinin was up-regulated, but the related pathway of brassinosteroid was obviously enriched.Brassinosteroid is considered as a key mechanism for growth and development in plants, involving a wide range of processes at molecular, cellular and physiological levels [38]. These effects indicate that many limitations of agricultural production at present may be alleviated by dealing with genetic determinants of brassinosteroids and their exogenous applications.Therefore, we speculate that alfalfa can promote the growth of alfalfa by up-regulating auxin signal transduction pathway, gibberellin signal transduction pathway and brassinosteroid-related pathway under the induction of bio-fertilizer.

In plants, shikimic acid pathway can produce aromatic amino groups, such as acid tyrosine, tryptophan and phenylalanine [39]. The tyrosine metabolic pathway is the initiation point for plants producing many natural compounds with different structures, including tocopherol, plastoquinone, ubiquinone, betaine, salidroside, benzylisoquinoline alkaloid and so on [40]. Tyrosine is the biosynthetic precursor of many metabolites, including some that have important nutritional, pharmacological, and monetary importance. In this study, genes and metabolites involved in tyrosine metabolism were up-regulated in both transcriptome and metabolome. This may be the reason why the nutrition value of alfalfa in the treatment group was increased above that of the control.

Tryptophan is an aromatic amino acid, which is constructed by branch acid through shikimic acid pathway.During the synthesis process, it is bound to protein and the structural skeleton provided by tryptophan is the secondary metabolite of hundreds of thousands of plants, including indoleamine and auxin (indole -3- acetic acid; IAA), alkaloids, glucosinolates [41,42,43,44]. Previously published reports also found that the application of tryptophan can improve the growth and photosynthetic capacity, which is thought to be partly due to the regulation of cytokinin level and the increase of IAA, gibberellin and abscisic acid [45]. The genes and metabolites related to tryptophan metabolism were up-regulated in both transcriptome and metabolome. This may be the reason why the nutritional index of alfalfa was higher for the treatment group than for the control.

Phenylalanine metabolism is a key metabolic node, which plays a vital part in the interaction between primary metabolism and secondary metabolism in higher plants [46]. Phenylalanine is the substrate for a variety of secondary metabolites, such as phenylpropanoids, flavonoids, anthocyanins, and cell wall lignin [47]. These compounds are very important for plant reproduction, growth, development and resistance to different types of stress.In this study, the enrichment analysis of transcriptome and metabolomics showed that phenylalanine metabolism and isoflavone metabolism were the main ways of enrichment. A previous work demonstrated that phenylalanine can enhance growth in plants [48]. However, in this study, the up-regulation of phenylalanine metabolism pathway was the most significant, which indicated that the application of biological fertilizer could induce plants to up-regulate phenylalanine metabolism and promote plant growth. Isoflavone is a secondary metabolite mainly found in legumes [49]. Synthesis from flavanone is catalyzed by isoflavone synthase (IFS).Isoflavone is a natural nutritional factor with many important physiological activities, which is easily absorbed and can quickly supplement nutrition. Isoflavone is considered as a dietary component which plays an important role in limiting cases of breast cancer and prostate cancer, augmenting skeletal health, alleviating climacteric symptoms, and averting coronary heart disease [50,51,52]. Flavonoids play a key role in plant-microorganism interaction, namely defense and symbiosis [53]. Therefore, in the current study, the use of bio-fertilizer increased the content of isoflavones in alfalfa and increased its nutritional value, and isoflavones also served as signal molecules of allelopathy, which helped alfalfa to improve its growth and development in the embodiment of microorganism-alfalfa symbiosis.

In a word, we first described the physiological characteristics and nutritional indexes of alfalfa treated with bio-fertilizer, and also described the changes of soil enzyme activity by bio-fertilizer treatment. Then, RNA-Seq technology and liquid chromatography-mass spectrometry/mass spectrometry were used to obtain alfalfa transcriptome data set and metabolome data set, so as to examine the integral genes and metabolic pathways of alfalfa growth. The results showed that the exogenous organisms activated the auxin, gibberellin and brassinosteroid signal transduction pathway of alfalfa, and regulated tryptophan metabolism, tyrosine metabolism and phenylalanine metabolism to promote alfalfa growth. In the process, the isoflavone metabolism pathway as allelopathy with microorganisms was enhanced, which greatly increased the nutritional value of alfalfa. These findings expand our understanding of the growth mechanism of alfalfa, and are helpful for breeding excellent leguminous plant germplasm in the future.

Declarations

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Ethics approval and consent to participate

Alfalfa samples were collected from alfalfa planting base in forage capital of China, Inner Mongolia. The study complies with relevant institutional, national and international norms and regulations. We have obtained permission from Aruhorqin Grassland Industry Association to collect Alfalfa sample.

Consent for publication

Not applicable.

Availability of data and materials

Raw data was deposited in NCBI database under SRA accession: PRJNA 838438. Any reasonable requests are available from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LJC and YW designed and drafted the manuscript. YBW and HJ performed experiments and analyzed the data. YBW wrote the first draft of the manuscript with the help of WFD and YD. KLD and LFX has been involved in partial data analysis and figure compile and edit. M I helped to review and edit the manuscript. LJC has been involved in critically revising the manuscript for important intellectual content; All authors gave the final approval of the version to be published.

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Figures

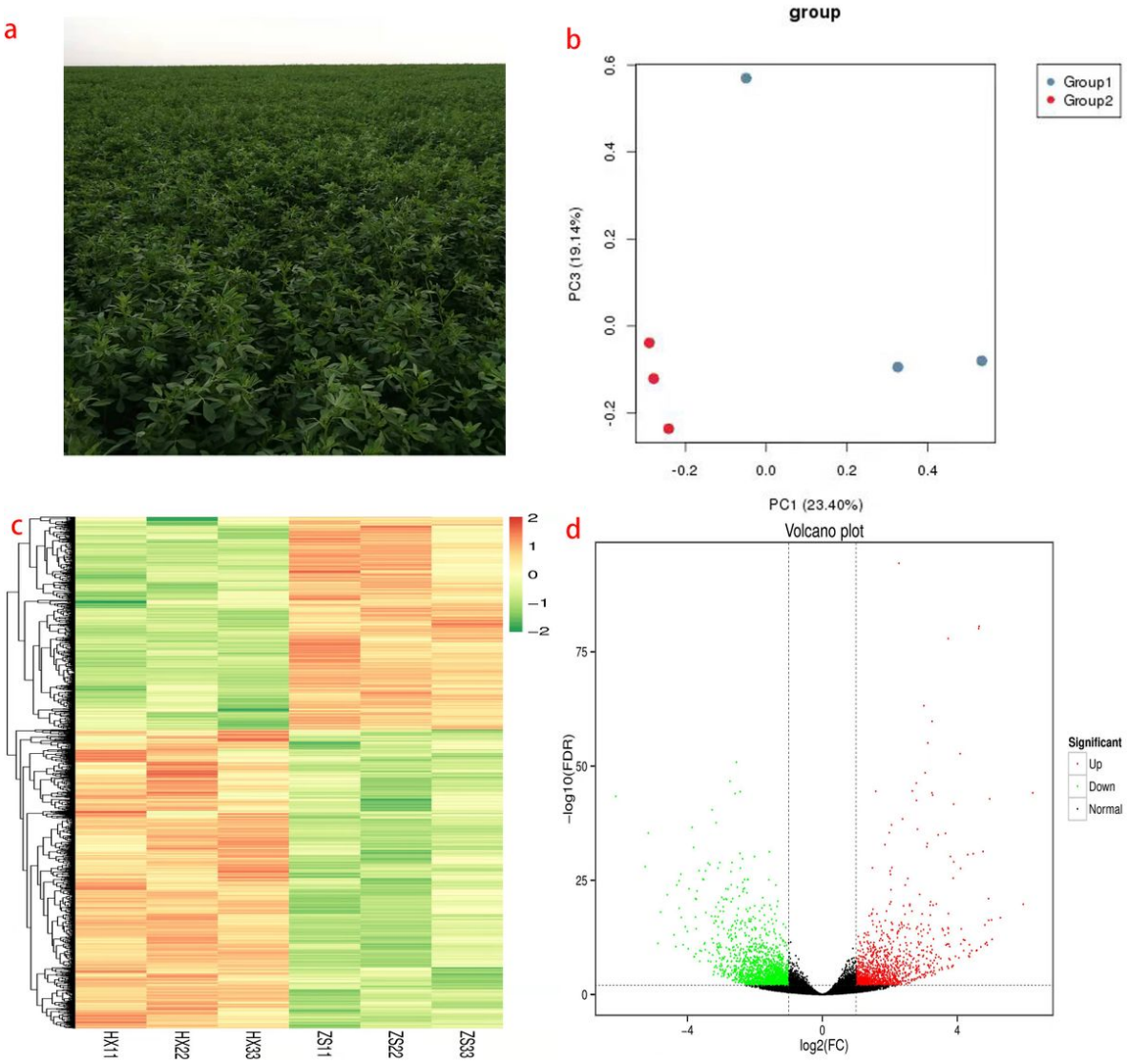

Figure 1

Difference in gene expression level and changes in metabolism related transcription level of Alfalfa after application of biological fertilizer. (a) Alfalfa at maturity. (b) Comparison of the control and treatment groups, the principal component analysis score of the identified genes. (c) Volcano map of differentially expressed genes. Based on the log2-fold change of RNA SEQ gene expression for the control group and treatment group, the corresponding significance value is shown as log10 (P value). The horizontal and vertical dotted lines represent the critical values of differential expression ($P < 0.05$ and $|\log2 \text{ multiple change}| > 1$). Identification of 1085 genes with increased (red) and and 1527 genes with decreased (yellow) expression, as induced by biological fertilizer. (d) Hierarchical clustering on the basis of metabolism related genes.

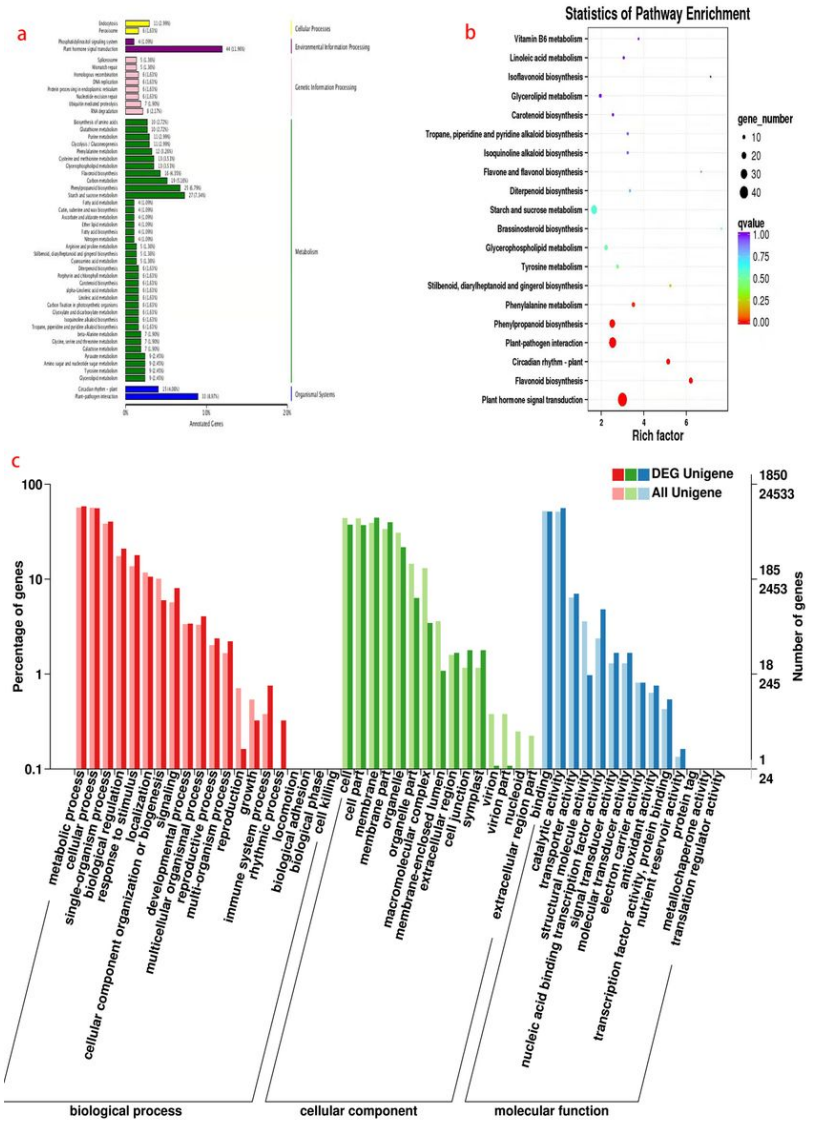

Figure 2

GO classification and pathway enrichment results of differentially expressed genes in alfalfa treatment and control groups. (a) KEGG annotation for differentially expressed genes. (b) The differentially expressed gene KEGG was enriched. Colors show corrected p values (P adjustment < 0.05), and the point size represents number of genes. Generatio is the ratio of enriched to background genes. (c) GO classification for differentially expressed genes.

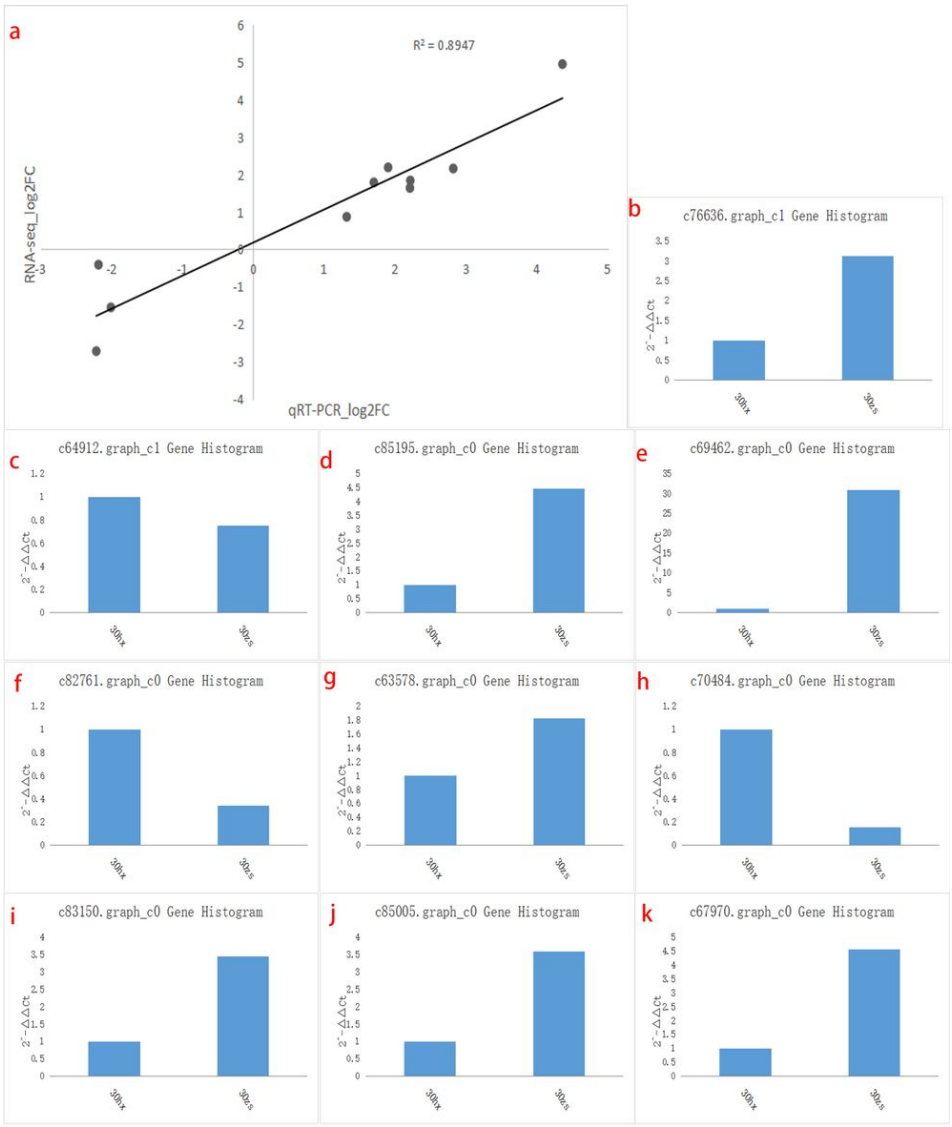

Figure 3

Correlation between RNA Seq expression profile and qRT PCR results. The results of reverse transcription polymerase chain reaction confirmed that the transcriptional changes of 10 randomly selected genes were equivalent to the multiple changes obtained by our transcriptome analysis. (a) Correlation curve between RNA-Seq expression profile and qRT-PCR results.(b) The relative expression of IDc76636 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(c) The relative expression of IDc64912 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(d) The relative expression of IDc85195 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(e) The relative expression of IDc69462 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(f) The relative expression of IDc82761 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(g) The relative expression of IDc63578 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(h) The relative expression of IDc70484 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(i) The relative expression of IDc83150 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(j) The relative expression of IDc85005 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.(k) The relative expression of IDc67970 gene in 30zs and 30hx samples was calculated by 2^-△△Ct.

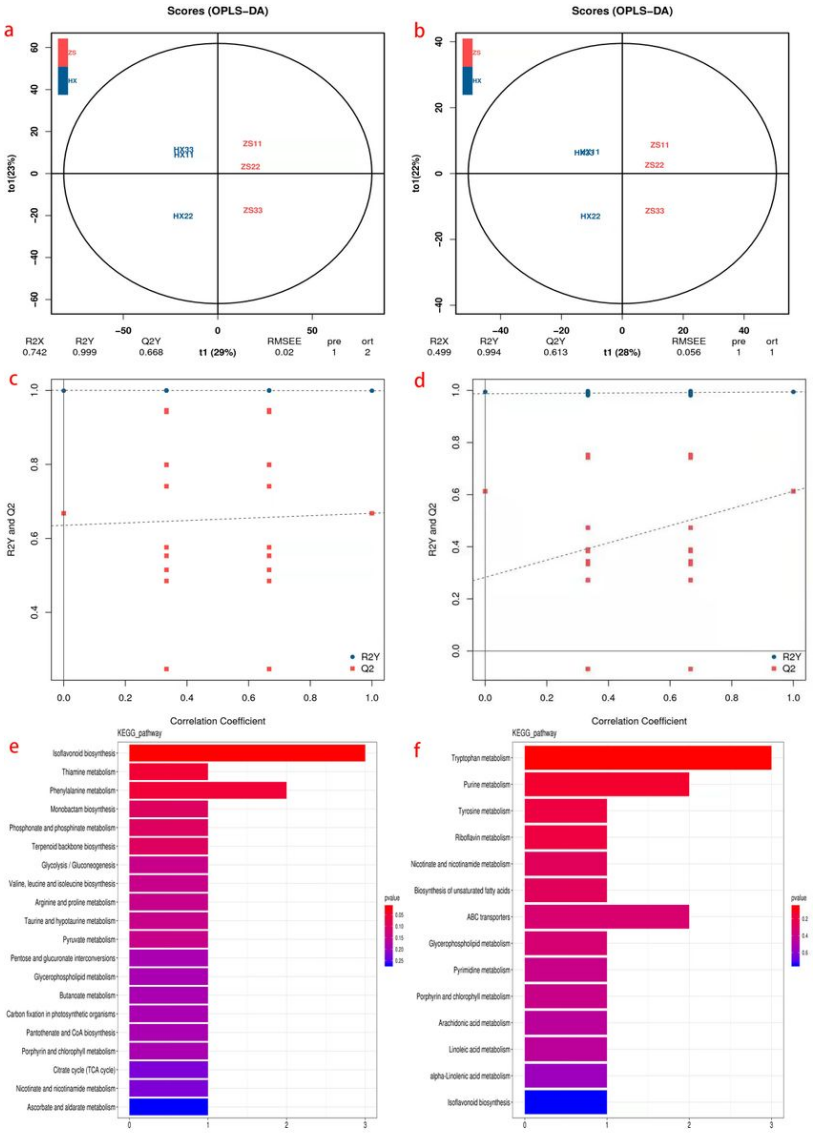

Figure 4

Plot of scatter chart and sorting verification chart in both positive and negative mode.In the scatter plot.. (**a, b**) The abscissa is the score of the sample on the first principal component. The ordinate is the score of the sample on the second principal component. R2Y represents the interpretation rate of the second principal component of the model, and Q2Y represents the prediction rate of the model. (**c, d**)In the ranking test, the abscissa represents the correlation between the random group y and the original group y, and the ordinate represents the scores of R2 and Q2. **(e, f)** Classification diagram of differential metabolite pathways in each group. A differential metabolite pathway classification map in positive ion mode. Differential metabolite pathway classification map in negative ion mode.

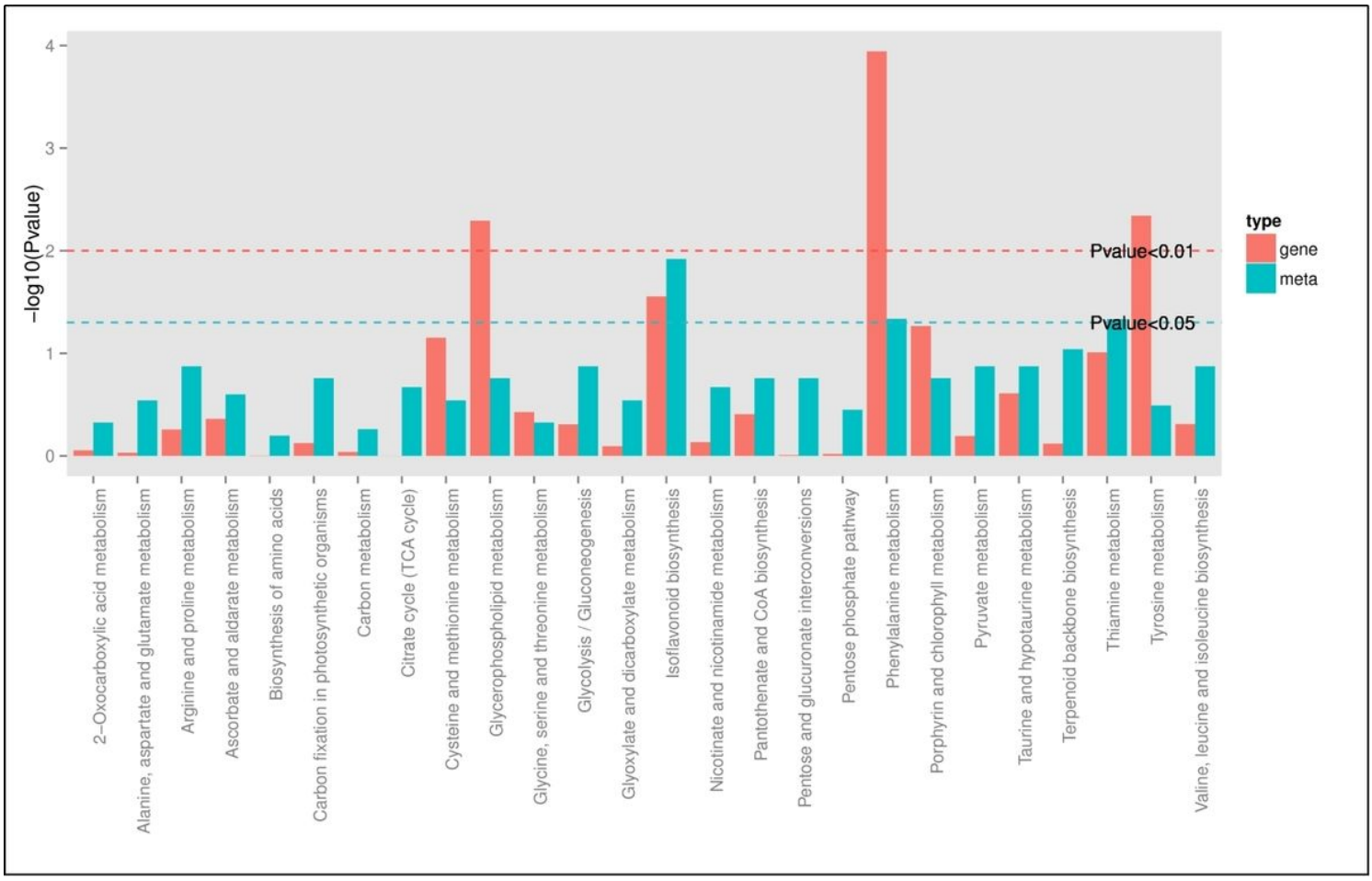

Figure 5

Histogram of CO enrichment of differential metabolites and differential genes. enrichment analysis showed that Isoflavone Biosynthesis and metabolism, phenylalanine related differential genes and metabolites were up-regulated under the condition of Biofertilizer application.

Supplementary Files

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- SupplementaryTables20220528.docx