Insights on bio-degumming of kenaf bast based on metagenomic and proteomics

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Abstract

Background: Microbes play important roles in kanef-degumming. This study aims at identifying the key candidate microbes and proteins responsible for the degumming of kenaf bast (*Hibiscus cannabinus*). Kenaf bast was cut into pieces and immersed into microbial fermentation liquid collected from different sites. Fermentation liquid samples were collected at 0, 40, 110 and 150 h and then subjected to the 16S/18S rRNA sequencing analysis and isobaric tag for relative and absolute quantitation (iTRAQ) analysis. The microbial (bacterial and fungal) diversity and the differentially expressed proteins/peptides (DEPs) were identified.

Results: With the prolonged degumming time, the weight loss rate increased, the bacterial diversity was decreased. Enterobacteriaceae, Enterobacteriaceae and Moraxellaceae were rapidly increased at 0~40 h, and then decreased and were gradually replaced by Bacteroidaceae from 40 h to 150 h. Similarly, Chryseobacterium and Dysgonomonas were gradually increased at 0~110 h and then decreased; Acinetobacter and Lactococcus were increased at 0~40 h, followed by decrease. Bacteroides was the dominant genus at 150 h. Sequencing 18S rRNA-seq showed the gradually decreased *Wallemia hederae* and increased *Codosiga hollandica* during degumming. iTRAQ data analysis showed Rds1, and pyruvate kinase I was decreased and increased in the kanef-degumming, respectively. Other DEPs of ferredoxin I, superoxide dismutase and aconitatehydrolase were identified to be related to the Glyoxylate and dicarboxylate metabolism (ko00630).

Conclusions: Bacteria including *Chryseobacterium, Dysgonomonas, Acinetobacter, Lactococcus* and *Bacteroides* and fungi like *Wallemia hederae* and *Codosiga hollandica* are key candidate microbes for kenaf degumming.

Keywords: Bio-degumming, *Hibiscus cannabinus*, Microbial diversity, iTRAQ

Background

Kenaf (*Hibiscus cannabinus*), which contains 8–16% lignin, 53–66% cellulose, 23–35% pectin and some hemicellulose, is an annual herbaceous bast fiber crop of the genus Malvaceae [1–3]. It is widely planted around the world, especially in the tropical and subtropical regions, such as Asia and Latin America. Kenaf fiber is widely used as an important basic raw material in textile, manufacturing and composite fabrication due to its strong pulling force [1, 4]. However, the retting methods can influence the quality of kenaf fiber.

Retting based on the intervention of bacteria and microbia enzymes promotes the development of the textile industry via resulting in a better quality of fibers. Conventional methods for the degumming of kenaf bast included traditional natural fermentation (water retting) and chemical degumming. In comparison with the natural fermentation and chemical degumming, biological (bacterial and enzymatic) degumming presents a series of advantages including high efficiency, low pollution, low cost and high fiber quality [3, 5–7]. The secretion of bacteria promote the decomposition of material, which can be used for bacteria to continue to grow [6, 8]. Ideal bacterial strains for kenaf degumming should have the advantages of secreting pectinase, hemicellulose, and ligninase, but not cellulase [6–8].

The screening of superior bacterial strains with the activity of pectate lyase, pectinase, hemicellulase and/or ligninase and the preservation of the natural fiber structure and mechanical properties is crucial for biological degumming [7–9]. A series of bacterial strains have been identified with strong ability of retting or degumming, like *Bacillus cereus* hn1–1 [10], *B. pumilus* [7], *B. licheniformis* and *B. subtilis* [11] and *B. tequilensis* SV11-UV37 [6]. Cheng et al. [10] showed that the 10 h-degumming process by *B. cereus* hn1–1 produced a residual gum rate as low as 5% and the
fiber rate as high as 76%. Mao et al. [12] reported that the ramie retting could be completed within 56 h by using a microbes consortium RAMCD407 plus 0.2% NaOH, with 2.84% residual gum content and 5.2 cN/dtex breaking strength of the final fiber. In addition, our previous study [7] identified that pectinase and mannanase were the key enzymes in the degumming of kenaf bast mediated by bacteria including B. pumilus, B. alcalophilus, Clostridium tertium, Brevibacillus brevis, Pectobacterium carotovora, Erwinia chrysanthemi, and Tyromyces subcaesius. All these results suggested the pivotal roles of bacteria in the degumming of kenaf bast. However, there was no systematic analysis for the alterations of bacterial secretome during degumming of kenaf bast.

This study was performed to identify the key candidate microbes and secretory proteins during the retting and degumming of kenaf bast. Alterations of microbial proteomics and community during retting and degumming of kenaf bast was detected using isobaric tags for relative and absolute quantitation (iTRAQ) and 16S/18S rRNA sequencing, respectively. These findings provide novel insights into the retting and degumming of kenaf bast.

Results

Degumming of kenaf bast and bacteria collection

The weight loss rate of kenaf bast was gradually increased with degumming, ranging from 11.72% at 40 h and 32.06% at 190 h (Table 1). The bacterial viable count, however, was primarily decreased from initial 4.2 × 10⁷ CFU/ml to 8.7 × 10⁶ CFU/ml at 40 h post fermentation. It was increased to the maximum 5.1 × 10⁸ CFU/ml at 150 h, followed with a decrease. These results might suggest that the growth of bacteria had degumming function.

General characteristics of 16S/18S rRNA sequencing

We then collected liquid samples at 0, 40, 110 and 150 h post retting and subjected to 16S/18S rRNA sequencing. A total of 167,321 and 181,887 raw reads was generated from 16S and 18S rRNA sequencing data, respectively. After removing the low-quality reads and chimera, the sequence length of trimmed reads is mostly distributed at 420 bp - 490 bp in bacteria, and the fungus sample is mostly distributed at 399 bp - 409 bp. The final rank abundance curve tends to a plateau, indicating that the sample species are richer in composition and higher in uniformity (Fig. 1). The higher species rank value of samples at 0 h (500–600) compared with of samples at 40, 110 and 140 h (200–300) indicated that the fermentation significantly decreased bacterial diversity. In addition, we found the retting significantly reduced the bacterial alpha diversity estimators like Chao 1, PD__whole_tree, Shannon and Simpson index (Table 2). In addition, retting also decreased fungal alpha diversity estimators including Chao 1 and PD__whole_tree, but increased Goods coverage (Table 2). These changes suggested retting decreased microbes viable count and bacterial diversity but increased fungal diversity.

Identification of key bacteria responsible for the degumming of kenaf bast

After OTUs (operational taxonomic units) annotation, we identified the abundances (at phylum level) of Bacteroidetes (from 34.91% at 0 h to 67.75% at 150 h) and Patescibacteria (1.00 to 9.53%) were gradually increased during the degumming of kenaf bast (Additional file 1: Figure S1), which replaced the Proteobacteria. The initial abundance of Firmicutes (2.83%) was firstly increased to 15.28% at 40 h and then decreased to 3.40% at 150 h (Additional file 1: Figure S1a and b). At the family level, Sphingobacteriaceae (10.98%, Bacteroidetes), Flavobacteriaceae (9.62%), Burkholderiaceae (8.13%), and Spingomonadaceae (6.77%) were the dominant bacteria at the initial (Fig. 2a and b). However, they were replaced by the fast-growing [Weeksellaceae] (21.55%, Bacteroidetes), Enterobacteriaceae (16.41%, Proteobacteria) and Moraxellaceae (12.13%, Proteobacteria) families at 40 h post retting. The latter bacteria were gradually replaced by the Bacteroidaceae family from 40 h to 150 h (25.89%; Fig. 2a and b). We also identified that the growth of Cytophagaceae and Chitinophagaceae families (Bacteroidetes) were inhibited by retting process. Similar changes were found in several bacterial genera. Dominant genera, including Pedobacter (9.10%), Flavobacterium (6.86%), Pseudomonas (5.97%) and Brevundimonas (5.64%) kept an equivalent level at the initial (0 h). Chryseobacterium (15.03%, [Weeksellaceae]), Acinetobacter (12.10%, Moraxellaceae) and Lactococcus (8.84%, Streptococcaceae family) grew to be the dominant bacteria at 40 h, which were then replaced by Bacteroides (25.89%) in the fermentation liquid, followed by Chryseobacterium (16.03%) and Dysgonomonas families (15.96%) (Fig. 2c and d). These changes in bacterial abundances were in response to that of the bacterial viable count in Table 1. These data showed that Acinetobacter, Chryseobacterium, Lactococcus and Bacteroides at genus level and [Weeksellaceae], Enterobacteriaceae, Moraxellaceae and Bacteroidaceae at family level

| Terms | 0 h | 40 h | 110 h | 150 h | 190 h |
|-------|-----|------|-------|-------|-------|
| Weight loss rate (%) | –   | 11.72| 24.45 | 31.26 | 32.06 |
| Initial content of live bacterial (CFU/mL) | 4.2 × 10⁷ | 8.7 × 10⁶ | 7.2 × 10⁷ | 5.1 × 10⁸ | 3.1 × 10⁸ |
Identification of key fungi responsible for the degumming of kenaf bast

As expected, fungal abundances were also changed in response to degumming. All fungi were mainly dominated by 2 phyla: **Opisthokonta** (98.73%) and **SAR** (1.24%). The relative abundance of **Opisthokonta** subkingdom was gradually decreased to 85.09%, and replaced by **SAR** phylum (14.61% at 150 h; Fig. 3a). The dominant fungal families **Incertae Sedis** (61.11 to 8.10%) and **Pezizomycotina** (5.98 to 53.08%) and some other fungi such as **Bulleribasidiaceae**, **Craspedida, Chrysophyceae**, etc. (Fig. 3b). At genus level, the results showed that **Wallemia** (60.43%) and **Eurotiomycetes** (18.55%) were the dominant fungi (Fig. 3c). As for specific species, the dominant positions of **Wallemia hederae** (60.33%) at the initial, but decreased at 40 h (36.97%), 110 h (12.12%) and 150 h (7.50%) (Fig. 3d). The relative abundance of **Cadosiga hollandica** species was increased from 0.16 at 0 h to 2.42% at 150 h.

**Microbia secretomics analysis and identification of candidate proteins or peptides**

We then performed the secretomics analysis to identify the candidate proteins which might be responsible for biological degumming of kenaf bast, since there are significant changes in the relative abundance of bacteria and fungi. A total of 197 proteins, including 67 DEPs were identified (Additional file 2: Table S1). Clustering analysis showed the distinct expression patterns of these proteins in the samples (Fig. 4). We identified the significantly down regulated Rds1 protein peptides (including

| Table 2 | The alpha diversity of the 16S and 18S rRNA-seq |
|---------|-----------------------------------------------|
| Group   | Chao 1 | Goods coverage | PD_whole_tree | Shannon | Simpson |
| 0 h     | 642.47 ± 8.45 a | 0.9920 ± 0.0002 a | 25.62 ± 0.21 a | 8.01 ± 0.05 a | 0.9924 ± 0.0006 a |
| 40 h    | 388.16 ± 13.70 b | 0.9912 ± 0.0003 b | 12.83 ± 0.14 b | 5.53 ± 0.06 b | 0.9447 ± 0.0015 b |
| 110 h   | 341.90 ± 12.46 c | 0.9923 ± 0.0003 a | 11.52 ± 0.43 c | 5.09 ± 0.17 c | 0.9275 ± 0.0102 c |
| 150 h   | 353.47 ± 5.32 c | 0.9919 ± 0.0003 a | 12.25 ± 0.19 b | 4.75 ± 0.06 d | 0.9058 ± 0.0043 d |
| 18S rRNA-seq | 98.86 ± 4.15 a | 0.9988 ± 0.0004 b | 3.09 ± 0.30 a | 2.26 ± 0.59 b | 0.58 ± 0.14 b |
| 0 h     | 62.75 ± 3.77 b | 0.9966 ± 0.0001 a | 2.27 ± 0.32 bc | 3.01 ± 0.07 a | 0.75 ± 0.01 a |
| 110 h   | 65.79 ± 3.94 b | 0.9966 ± 0.0000 a | 2.45 ± 0.23 b | 2.75 ± 0.30 ab | 0.64 ± 0.05 ab |
| 150 h   | 56.10 ± 8.06 b | 0.9997 ± 0.0002 a | 1.92 ± 0.07 cd | 2.78 ± 0.24 ab | 0.69 ± 0.04 ab |

Difference in one-way ANOVA is presented by different letters
I4YCX5 and R9AEW5, A1DDU4, A0A0S7E3J2, A0A0J5 SQP1, Q4WVL1, B0Y1F6, A0A084BN00 and A0A0K8L4 F0), superoxide dismutase peptides (J1ACL6 and A0A0Q 9DZS2), and the upregulated peptides of pyruvate kinase I (A0A0A2W3C3), lipoprotein (A0A0N7K9K4), ferrodoxin I (I4JHJ0), thioredoxin (A0A088F1E4, A0A0M2 Y158 and A0A0M3C9P8). A0A0A2W3C3 was enriched into the pathways including glucagon signaling pathway (ko04922) and pyruvate metabolism (ko0492). A peptide of aconitahydratase (aconitase, ACO), which is related to the glyoxylate and dicarboxylate metabolism (ko0063 0), was decreased at 40 h and then increased at 110 and 150 h post retting compared with 0 h (Table 3). Most of the other peptides were annotated with transporter activities (Additional file 2: Table S1).

Among the other non-DEPs, we identified that the peptide of Aldehyde dehydrogenase family protein (A0A160 F3I4), Aspartate aminotransferase (A0A0A2VU16) and 6-phosphogluconate dehydrogenase (L8X2A2). The L8X2A2 was identified to be related with pentose phosphate pathway.

**Discussion**

The degumming of kenaf bast is a process mediated by dynamic change of microbes. Using the 16S/18S rRNA sequencing, we identified the changed bacterial and fungal abundance during the degumming of kenaf bast (0~ 150 h). In the fermentation liquid, the growth of Cytophaga- ceae and Chitinophagaceae was inhibited during the degumming of kenaf bast. Many bacteria genera played crucial roles in in the degumming process of kenaf bast, such as Bacteroides, Chryseobacterium, Dysonomonas, Acinetobacter, and Lactococcus, of which the abundance were greatly changed with degumming treatment. Similarly, some fungi also participated in the degumming process of kenaf bast including Pezizomycotina, Dipodascaceae, Codosiga hollandica, and Incertae Sedis. The abundance of subdivided Wallemia and Eurotiomycetes genera were dramatically reduced in the process of dealkylation and fermentation. And the increased Dipodascaceae family might promote the degumming of kenaf bast. A series of Bacillus strains has been identified to be ramie- or kanef-degumming strains, like B. cereus Bn1-1 [10], B. pumilus
B. licheniformis and B. subtilis [11] and B. tequilensis SV11-UV37 [6]. In addition, our previous study [7] showed that seven bacterial strains belonging to the species including B. pumilus, B. alcalophilus, C. tertium, Brevibacillus brevis, Pectobacterium carotovora, Erwinia chrysanthemi and Tyromyces sub caesius were the key in strains for the degumming of kenaf bast. Other reports also showed the ability of B. licheniformis, Paenibacillus macerans, C. tertium, B. tequilensis and B. vulgarus to the proteases and pectinolytic enzymes derived from these strains for degumming fiber, wool and wood [6, 7, 13, 14]. For instance, enzymatic treatment is an acceptable method of intervention among the methods for wool treatment for breaking down the surface structure [14]. Serine proteases are the most common commercial proteases derived from Bacillus strains.

For the degumming of plant fibers, some researchers had isolated proteases, xylanases and pectate lyases from the bacteria like Acinetobacter spp. (> 1 species of the genus) [15] and B. cereus [16] and fungi including Extremophilic fungi [17–19]. Researchers also identified the lignin degrading role of Pseudomonas, Lactococcus and Acinetobacter strains in hemp, ramie and mechanical pulp [20–23]. For instance, Hu et al. [23] observed that abundances of Pseudomonas and Acinetobacter were increased to the highest at 36 h post retting and decreased subsequently. In particular, the finding about Acinetobacter and Lactococcus was consistent with our results, which was increased to 12.09 and 8.84% at 40 h and then decreased to 4.10 and 0.84% at 150 h. The dynamic changes of these bacteria during the degumming of kanef bast suggested their crucial roles in degrading kanef.

Kanef-degumming is a dynamic process of bacterial adaptation and growth. The initial stage is characterized by decreased bacterial richness and diversity [24]. We determined the decreased bacterial viable count at the 40 h post retting, followed by increased bacterial viable count but not bacterial richness and diversity. Our present study presented a cluster of anaerobic Bacteroidaceae members like Bacteroides, Chryseobacterium and Dysgonomonas, played crucial roles in the degumming of kanef bast, especially in the late stage. Cytophagaceae was initially inhibited, which might guarantee the fiber structure. The rapid growth of anaerobic Bacteroidaceae bacteria changed bacterial diversity. Xylan and pentose (including xylose) are main components of hemicellulose in plants [25]. The degradation of hemicellulose into...
Oligomers and sugars is a metabolic property shared by sugar-fermenting *Bacteroides* [26–29]. The increased abundance of these *Bacteroidaceae* members might suggest the accumulation of their substrates derived from the early stage fermentation from aerobic bacteria like *Acinetobacter* and *Lactococcus* or the changed environments.

In addition, we also identified the down regulation of several peptides of Rds1 during the degumming of kenaf bast. Rds1 a stress-responsible protein, which could be depressed by starving from glucose, ammonium, phosphate, exposing to carbon dioxide and high temperature [30]. The down regulation of it was theoretically in line with the hypothesis that the starvation of sugar and oxygen of early retting stage. What’s more, the identification of the gradually decreased halophilic *Wallemia hederae* and increased turfgrass pathogen in the fermentation liquid might suggest the deterioration of fermentation. *Codosiga hollandica*.

**Conclusions**

In conclusion, we identified a cluster of key bacteria responsible for the degumming of kenaf bast. We identified that the growth of Cytophagaceae was initially inhibited at the early stage of degumming for kenaf bast. The up-and-down change in the abundance of *Acinetobacter* and *Lactococcus* (*Streptococcaceae*) and the gradually increased growth of *Bacteroides*, *Chryseobacterium*, *Dysgonomonas* characterized the degumming process. In addition, we also identified the increased *Codosiga hollandica* and decreased *Wallemia hederae* fungus family during degumming for 150 h. Secretory proteomics analysis showed Rds1, pyruvate...
kinase I and aconitase hydratase peptides were changed during the degumming of kenaf bast. These findings provide evidence on the crucial roles of these microbes in the degumming of kenaf bast.

| Table 3 | Several differentially expressed proteins in during degumming |
|---------|-------------------------------------------------|
| Protein ID | Time (s) | Protein name | Gene ontology Pathway |
|-----------|----------|--------------|-----------------------|
| R9AEW5;A1DDU4; A0A057E3J2; A0A0J55Q11; Q4WW1.1801F16; A0A084BN00; A0A0K8L4F0 | 4391.5 715.0 340.8 362.7 | Protein rds1 | |
| A0A07K9K4 | 889.5 2642.3 1897.0 2776.1 | Lipoprotein | integral component of membrane [GO:0016021]; porin activity [GO:0015288] |
| A0A023WRC7 | 308.7 614.1 981.2 671.3 | Porin | |
| A0A23W3C3 | 964.2 4595.7 2396.7 542.4 | Pyruvate kinase I | |
| A0A088F1E4; A0A0M2Y158; A0A0M3C9P8 | 201.3 1957.3 429.8 1047.9 | Thioredoxin | |
| I4JHJ0 | 580.1 651.8 2090.5 1518.2 | Ferredoxin I | |
| Q47NL1 | 816.8 1998.2 2134.1 1615.2 | Aconitate hydratase (Aconitase) (EC 4.2.1.3) | 4 iron, 4 sulfur cluster binding [GO:0005139]; aconitate hydratase activity [GO:0003994]; metabolic process [GO:0008152] |
| A0A088F1E4; A0A0M2Y158; A0A0M3C9P8 | 201.3 1957.3 429.8 1047.9 | Thioredoxin | |
| J1ACL6; A0A0Q9DZS2 | 393.9 447.6 286.3 325.2 | Superoxide dismutase [Cu-Zn] (EC 1.15.1.1) | metal ion binding [GO:0046872]; superoxide dismutase activity [GO:0004784] |
| W66L45;W0TF05; A0A090CSA2 | 1289.9 1014.2 2316.6 770.7 | Superoxide dismutase [Cu-Zn] (EC 1.15.1.1) | metal ion binding [GO:0046872]; superoxide dismutase activity [GO:0004784] |
| V4RJ95;S6LCD6; M2UZN0;LOGIW1; I4JML0;A4TX4; H7F07P;F8BH1; F2MXB8;A4KVP6; A0A137Y1T1; A0A137WWW6; A0A0959RB2; A0A0H3YZE1; A0A0D7EA74; A0A0C2MWW1; A0A098FQ9; A0A061NYQ2; A0A23W7Y8 | 1525.4 2439.2 1244.5 1037.6 | Succinate dehydrogenase flavoprotein subunit (EC 1.3.5.1) | plasma membrane [GO:0005886]; flavin adenine dinucleotide binding [GO:0050660]; succinate dehydrogenase (ubiquinone) activity [GO:0008177]; electron transport chain [GO:0022900]; tricarboxylic acid cycle [GO:0006099] |
| A0A0D1Y8T6 | 1129.9 1915.8 958.2 658.1 | Uncharacterized protein | succinate dehydrogenase activity [GO:0000104] |
| H7VF4 | 147.3 38.6 124.9 289.2 | Isocitrate lyase (EC 4.1.3.1) | isocitrate lyase activity [GO:0004451]; carboxylic acid metabolic process [GO:0019752] |

Methods

Bacteria collection and degumming of kenaf bast

Humus samples (50 g) were collected from Sanya, China. Water samples (100 ml) were collected from a conventional retting pond (50 cm away from the water surface) in Xiaoshan, Zhejiang, China. Soil samples (50 g) were collected from continuous cropping soil of Kenaf in Xiaoshan. Soil and humus samples were diluted into 100 ml bacteria free water (autoclave at 121 °C for 20 min), filtered and then mixed with the above water samples.

Kenaf bast was collected from Xianghongma No. 1 plants in Changsha, China. The samples were cut into pieces (3 cm) and then immersed into bacteria mixture (10 g: 5 ml) with supplementation of 100 ml bacteria free water. For the degumming of kenaf bast, samples were
maintained on an orbital shaker at 30 °C, pH 7.0, for 30 min. Then the fermentation liquid samples were collected at 0, 40, 110, 150 and 190 h post degumming and used for further analysis. Each experiment was done in triplicates.

**Determination of kenaf bast weight loss rate and viable count of bacteria**

The weight loss rate of the kenaf bast samples in each condition was calculated according to the following formula: weight loss rate (%) = [initial weight (10 g)-final weight (g) of kenaf bast]/initial weight (10 g) of kenaf bast × 100%. Total viable cell count was quantified traditionally using the colony-forming units (CFUs) after incubation on nutrient broth solid media (pH 7.0) for 0–190 h.

**DNA extraction and 16S and 18S ribosomal RNA gene sequencing**

DNA extraction was performed using a PowerSoil™ DNA Isolation Kit (MOBIO laboratories, San Diego, Carlsbad, California, USA). The concentration and purity of the DNA was measured by agarose gel electrophoresis. The 16S ribosomal DNA (rDNA) gene V3-V4 region of the bacteria was amplified by PCR with bar coded primers (343F: 5′-TAC GGRAGGCAGCAG-3′ and 798R: 5′-AGGTTATCTA AT CCT-3′), using FastPfu Polymerase (TransStart, Beijing, China). The PCR primers for the 18S rDNA of fungus were NS1: 5′-GTAGTCATATGCTTGTCTC-3′ and NS8: 5′-TCCGCAGGTTCACCTACGGGA-3′. Reaction parameters were: 95 °C for 5 min, followed by 30 cycles of 95 °C 30 s, 52 °C 45 s (16S) or 1 min (18S), 72 °C 1 min, and the final step of 72 °C for 10 min. The amplicons of 16S and 18S rDNA were purified by an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, California, USA). After repeating the above steps (amplification and purification), the concentration of final purified amplicons was detected by Qubit 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The crude precipitates were collected by centrifugation at 12000 g for 10 min at 4 °C (Sigma Aldrich, Schnelldorf, Germany). The supernatant was selected after sonication by centrifugation (12,000 g for 15 min) for twice. Finally, the supernatant was stored at −80 °C for further use. The concentration of protein was measured using BCA method [36], with BCA Protein Assay Kit (Thermo Scientific Dionex, San Jose, USA). The integrity of the extracted protein was detected by SDS-PAGE [37].

The quantified samples were then digested according to the filter aided sample preparation procedure as previously described [38]. In brief, 100 μg of protein was precipitated by precooled acetone (1:5 v/v) at −20 °C for 1 h, centrifuged at 16000 g for 10 min at 4 °C, and vacuum freeze-dried. Protein precipitation was prepared using an iTRAQ kit (Applied Biosystems, Carlsbad, California, USA) following the manufacturer’s instructions. The marked samples were then mixed, dried and then subjected to separation and identification.

**2D-LC-MS/MS analysis**

The freeze-dried sample was dissolved in 110 μL of the mobile phase A solution. Peptide separation was performed on an Agilent 1200 HPLC (Agilent Technologies, Foster City, California, USA) with the Narrow-Bore column (2.1 mm × 150 mm × 5 μm), analytical guard column (4.6 × 12.5 mm, 5-Micron), flow rate of 0.3 ml/min, at 210 nm and 280 nm in Shanghai Luming Biotech. Co., Ltd. Reverse phase chromatographic analyses were performed using Nano-RPLC Buffer A (Applied Biosystems), PepMap100C18 column (75 μm × 20 mm, 3 μm, NanoViper; Thermo Scientific Dionex, San Jose, USA) with the mobile phase B increased from 5 to 35% in 70 min. The Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany;
nano-electrospray ionization, 1.6 kV, 250°C) was used for data-dependent acquisition according to the previously reported method [39].

Protein identification and quantification
The raw proteomics data in the format of .raw was aligned to UniProt database (https://www.uniprot.org/) using Maxquant 1.5.1.0 (Version 1.5.1.0; Thermo Fisher Scientific). Proteins and peptides with fold discovery rate < 0.01 were retained as for further identification of differentially expressed peptides/proteins (DEPs). The significant different proteins between groups were identified with the threshold of T-test p value ≤0.05 and fold change (FC) ≥ 1.2.

Bioinformatics analysis
For annotation of the DEPs, Gene Ontology (GO, http://www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) databases were used for the gene functions prediction. The GO classifications of molecular function, biological process and cellular component and the pathways significantly related to these DEPs were identified with the criteria of p < 0.05.

Statistical analysis
Data were expressed as the mean ± standard deviation. The SPSS 22.0 software was employed for the statistical analysis. One-way ANOVA test was performed to analyze the differences. Comparison of differences between groups was detected using t-test. The p-value < 0.05 was considered as significantly different.

Supplementary information
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Authors’ contributions
DSW summarized the sequencing data, performed the data analysis and prepared the original manuscript. DSW and CLF attended discussion and revised MS. FXY attended data re-interpreting discussion. DSW, CLF, FXY, YQ, LZY and ZK prepared the plant material and attended the transcriptomes data analyses and discussion. DSW and PYD designed the experiments, provided research platform, performed the pathway enrichment analysis, and revised the manuscript. All authors approved the final manuscript.

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Availability of data and materials
The original data were uploaded to SRA database (https://www.ncbi.nlm.nih.gov/sra) and the BioProject ID is PRJNA56204.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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