

Transcriptomic and proteomic analysis reveals mechanisms of pre-fertilization barriers during water lily cross breeding

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Abstract

Background In water lily (*Nymphaea*) hybrid breeding, breeders often encounter non-viable seeds, which make it difficult to transfer desired or targeted genes of different *Nymphaea* germplasm. We found that pre-fertilization barriers were the main factor in the failure of the hybridization of *Nymphaea*. The mechanism of low compatibility between the pollen and stigma remains unclear; therefore, we studied the differences of stigma transcripts and proteomes at 0, 2, and 6 hours after pollination (HAP). Moreover, some regulatory genes and functional proteins that may cause pre-fertilization barriers in *Nymphaea* were identified. Results RNA-seq was performed for three comparisons (2 vs 0 HAP, 6 vs 2 HAP, 6 vs 0 HAP), and the number of differentially expressed genes (DEGs) was 8,789 (4,680 were up-regulated), 6,401 (3,020 were up-regulated), and 11,284 (6,148 were up-regulated), respectively. Using label-free analysis, 75 (2 vs 0 HAP) proteins (43 increased and 32 decreased), nine (6 vs 2 HAP) proteins (three increased and six decreased), and 90 (6 vs 0 HAP) proteins (52 increased and 38 decreased) were defined as differentially expressed proteins (DEPs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses revealed that the DEGs and DEPs were mainly involved in cell wall organization or biogenesis, S-adenosylmethionine (SAM) metabolism, hydrogen peroxide decomposition and metabolism, reactive oxygen species (ROS) metabolism, secondary metabolism, secondary metabolite biosynthesis, and phenylpropanoid biosynthesis. **Conclusions** These results indicated that genes involved in regulation and gene pathways involved in stress response might overlap in the process of pollination process forming the hybridization barrier in *Nymphaea*. These results indicate that the pollen tube entering stigma tissue was very similar to the process of external injury, further suggesting that these genes involved in defense and stress response were necessary in the early stage of interaction between the pollen and stigma. This study strengthens our understanding of the mechanism of pre-fertilization barriers in *Nymphaea* at the molecular level, and provides a theoretical basis for overcoming the pre-fertilization barriers in *Nymphaea* in the future.

Background

Water lilies (*Nymphaea*) are important flowering plants that are distributed worldwide from the tropics to temperate regions [1]. With the rapid improvement of China's economy and the overall quality of life, the demand is increasing for new water lily hybrids with different characteristics. Therefore, it is necessary to breed new water lily hybrids with excellent ornamental characteristics. However, in the breeding of water lily hybrids, breeders often encounter non-viable seeds, which makes it difficult to transfer the desired or targeted genes of various water lily germplasm [2, 4]. For example, many breeders have hoped to transfer the colorful flowers of tropical water lilies to hardy water lilies through crossbreeding; however, viable hardy water lily varieties with blue flowers have not yet been developed [3]. To address the possible incomplete hybridization of water lily, we conducted a systematic study on the pre-fertilization and post-fertilization barriers to artificial hybridization of water lily by means of plant structure, cell biology, and reproductive biology [4]. The results showed that pre-fertilization barriers were the main factor in the failure of the hybridization of water lily [4]. We infer that there may be some inhibiting factors on the

stigma during the interaction between the pollen and stigma, which hinders the normal germination of pollen and leads to less abnormal germination of the pollen on the stigma [4]. The mechanism of low compatibility between the pollen and stigma is unclear and requires further research.

Low compatibility between the pollen and stigma is a common issue that negatively impacts the efficiency of plant breeding and the yield of seeds or fruit [5, 6]. Therefore, over the past several decades, many researchers have conducted studies to investigate factors that cause plant pre-fertilization barriers [7-10]. However, most of these studies have mainly focused on morphological, anatomical, and physiological barriers due to the limitations of traditional biological techniques. Few studies have been conducted on the factors contributing to plant pre-fertilization barriers at a molecular level. Therefore, the mechanisms underlying plant pre-fertilization barriers remain poorly understood.

The recent development of modern molecular biology techniques, such as novel high-throughput sequencing technologies like Illumina RNA sequencing (RNA-seq) and a new proteomics approach (i.e., Label-free), may provide a promising means to address the genes and proteins associated with pre-fertilization barriers [11-13]. In particular, transcriptome sequencing is a useful method for identifying novel transcripts and analyzing gene expression [14-15]. Transcriptomic and proteomic analyses have been extensively applied to many plant species, but limited transcriptome and proteome data exists regarding pre-fertilization barriers in water lily [16-17]. To understand the mechanism of pre-fertilization barriers in water lily at the genomic level, Illumina paired-end sequencing and a label-free analysis of the stigma after pollination were conducted. This comprehensive analysis of the transcriptome and proteome may substantially improve the overall understanding of the potential molecular mechanisms involved in pre-fertilization barriers in water lily and pave the way for further analyses. This study aimed to provide important molecular data supporting a deep understanding of pre-fertilization barriers in water lily and also provides an important clue to overcome hybridization barriers.

Results

Pollen germination on stigmas after pollination

In the 'Peter Slocum' × *N. micrantha* cross, no pollen tubes penetrated stigmatic tissue between 2 and 6 HAP. In addition, the accumulation of wax between the stigma and the surface of the pollen grains hampered the germination of pollen grains (Fig. 1).

Overview of the transcriptomic analysis and proteomics analysis

Using Fragments Per Kilobase Million (FPKM), we explored the gene expression levels in the stigmas 0, 2, and 6 HAP. In three comparisons (2 vs 0 HAP, 6 vs 2 HAP, 6 vs 0 HAP), the number of DEGs was 8,789 (4,680 were up-regulated), 6,401 (3,020 were up-regulated), and 11,284 (6,148 were up-regulated), respectively. Further details of the DEGs are presented in Additional file 1. Using the label-free analysis, a total of 3,176 proteins were identified within a false discover rate (FDR) of 1% (Additional file 2). Following the statistical analysis, 75 proteins (2 vs 0 HAP; 43 increased and 32 decreased), nine proteins

(6 vs 2 HAP; three increased and six decreased), and 90 proteins (6 vs 0 HAP; 52 increased and 38 decreased) were defined as DEPs.

Comparison analysis of transcriptome and proteome data

To identify robust pathways that were supported by both datasets, we integrated DEGs and DEPs to find the corresponding genes and proteins, and the results are listed in Additional file 3. Overlaps between DEPs and DEGs are shown by Venn diagrams in Fig. 2. Specifically, there were considerable non-overlaps between DEPs and DEGs, probably due to the relatively low sensitivity of proteome detection. For instance, among the 234 differentially regulated proteins in the 2 vs 0 HAP comparison, only 67 genes and their corresponding proteins were regulated. These results also showed a strong correlation between the transcripts and proteins (Fig. 2A). Similarly, for the 6 vs 2 HAP and 6 vs 0 HAP comparisons, 12 and 127 of the DEPs, respectively, were correlated to the corresponding DEGs (Fig. 2B, 2C).

All expression data associated with protein level and transcription level were analyzed and Pearson correlation coefficient was calculated. Globally, the correlation coefficients of all quantitative proteins and their corresponding genes at 2 vs 0 HAP, 6 vs 2 HAP, and 6 vs 0 HAP were 0.2236, 0.02 and 0.123, respectively (Fig. 3A). However, there is a high correlation between the DEGs and their corresponding DEPs ($r=0.8178$, 0.4, and 0.6985, respectively; Fig. 3B). The correlation between proteins and their corresponding mRNAs with the same or opposite trend was analysed, and the comparative group 2 vs 0 HAP and 6 vs 0 HAP had higher positive or negative correlations (Fig. 3C and Fig. 3D). However, we found poor correlations between proteins at 6 vs 2 HAP and their corresponding mRNAs with the same or opposite trend (Fig. 3C and Fig. 3D). Among the cor-DEGs-DEPs genes, 39 (2 vs 0 HAP), three (6 vs 2 HAP), and 41 (6 vs 0 HAP) genes had the same trend, while five (2 vs 0 HAP), one (6 vs 2 HAP), and seven (6 vs 0 HAP) genes had the opposite trend (Additional file 4). Thus, we suggest that some of these cor-DEGs-DEPs genes might play important roles in causing pre-fertilization barriers during water lily breeding.

Cluster analysis of expression patterns in the cor-DEGs-DEPs genes

Cluster analysis of the DEPs and their corresponding DEGs can visually show their expression patterns, and the results are shown in Figure 4. Cluster analysis showed that 44 (2 vs 0 HAP), four (6 vs 2 HAP), and 48 (6 vs 0 HAP) DEPs were correlated with the change of mRNA abundance, and 16 (2 vs 0 HAP), one (6 vs 2 HAP), and 21 (6 vs 0 HAP) DEPs were matched with corresponding DEGs. However, 16 (2 vs 0 HAP), one (6 vs 2 HAP) and six (6 vs 0 HAP) DEPs were opposite to their mRNA expression pattern. In total, 15 of the cor-DEGs-DEPs genes at 2 vs 0 HAP and 6 vs 0 HAP showed the same expression pattern; thus, we infer that these genes, which are continuously expressed from 2 to 6 HAP after pollination, may cause pre-pollination barriers in interspecific hybridization of water lily.

GO and pathway enrichment analysis of the cor-DEGs-DEPs genes

To carry out a functional analysis, we assigned GO terms to the cor-DEGs-DEPs genes at 2 vs 0 HAP, 6 vs 2 HAP, and 6 vs 0 HAP. The results showed that 27 (2 vs 0 HAP), 0 (6 vs 2 HAP), 19 (6 vs 0 HAP) GO terms were highly enriched at both mRNA and protein levels (Fig. 5). The subcategory identified in the cellular component category was extracellular region in both 2 vs 0 HAP and 6 vs 0 HAP. For the molecular function category, peroxidase activity, heme binding, antioxidant activity, and oxidoreductase activity, acting on peroxide as acceptor were the most abundant categories in both 2 vs 0 HAP and 6 vs 0 HAP. The most abundant biological processes categories identified in both 2 vs 0 HAP and 6 vs 0 HAP were cell wall organization or biogenesis, phenylpropanoid metabolic process, sulfur compound biosynthetic process, hydrogen peroxide catabolic process, and ROS metabolic process. In addition, no GO terms were significantly enriched in the cor-DEGs-DEPs genes at 6 vs 2 HAP.

To obtain an overview of the correlation between the protein and transcript levels of genes, the KEGG enrichment of the cor-DEGs-DEPs genes was analyzed (Fig. 6). The results showed that two KEGG pathways were highly enriched at both mRNA and protein levels including phenylpropanoid biosynthesis (ko00940) and stilbenoid, diarylheptanoid, and gingerol biosynthesis (ko00945) in both 2 vs 0 HAP and 6 vs 0 HAP. For the cor-DEGs-DEPs genes at 2 vs 0 HAP, cutin, suberin, and wax biosynthesis (ko00073) and flavonoid biosynthesis (ko00941) were significant pathways in both the proteome and transcriptome. In addition, no KEGG pathways were significantly enriched in the cor-DEGs-DEPs genes at 6 vs 2 HAP.

Parallel reaction monitoring (PRM) analysis

Four differentially expressed proteins (mainly related to flavonoid biosynthesis, peroxidase activity and phenylpropanoid biosynthesis) were chosen for PRM analysis. According to the relative expression quantity of the corresponding peptide fragment of four target proteins in different sample groups, the relative expression quantity differences of target proteins were obtained (Table 1). Detailed protein quantitative information and significant difference analysis results are shown in Additional file 5. The results from this analysis indicated that expression quantities of the four target proteins in the 2 vs 0 HAP and 6 vs 0 HAP comparisons were markedly up-regulated, whereas the expression quantity of the four target proteins in the 6 vs 2 HAP comparison was not significantly changed. The results of the PRM analysis indicated that the four candidate proteins show similar trends as the label-free results, which supported the credibility of the proteomics data.

Discussion

Pre-fertilization barriers during water lily breeding are associated with the metabolism of ROS

In this study, the combined transcriptome and proteome analysis showed that the expression of genes and proteins related to the metabolism of ROS on the stigma increased significantly in the 2 vs 0 HAP and 6 vs 0 HAP comparisons, suggesting that ROS may be involved in regulating the interaction between the pollen and stigma of water lily after pollination. With the initiation of pollen tube germination, pollen begins to communicate with the stigma. The membrane protein layer of stigma papilla cells and the

protein in the pollen exine participate in early pollen-stigma communication [18]. The communication of the pollen and stigma during pollen tube germination also involves ROS, which play an important role in plant growth and development. In addition, ROS can regulate plant reproduction and development as a signaling molecule. ROS participate in many pollen-related processes, such as tapetum and pollen development [19-21], *in vitro* pollen germination [22], growth of the pollen tube apex [23-25], the rupture of the pollen tube to release sperm [26], and self-incompatibility [27]. The role of ROS in pollen tube growth has been well established, but little is known about its involvement in the early stage of pollen germination. The biological function of ROS and hydrogen peroxide on the stigma may be involved in some signal crosslinks in the interaction between the pollen and stigma [28,29]. Numerous experiments have shown that mature pollen grains produce a large amount of Nitric Oxide (NO), which inhibits ROS production in stigma papilla cells [28,30,31]. The mutual exclusion of ROS and NO during pollen tube growth suggests that there may be a coordination mechanism between these signaling molecules during pollen tube growth [32]; this which indicates that ROS from the stigma and NO from the pollen participate in the pollen-stigma interaction as signaling molecules [33]. In addition, ROS are mainly composed of hydrogen peroxide on the stigma, which is considered the most important redox signaling molecule because of its unique physical and chemical properties as well as its stability in cells. Hydrogen peroxide can oxidize the thiol group of target protein cysteine, thus changing the structure and function of proteins [34].

However, the regulatory mechanism is unclear. We infer that the halting of pollen tube growth on the stigma surface after pollination is due to the change of the level of ROS on the stigma. ROS may act as a signaling molecule to oxidize downstream target proteins. The oxidized target proteins cannot function properly, affecting the growth of the pollen tube on the stigma surface. Therefore, the metabolic process of ROS on the stigma is related to the interaction between the pollen and the stigma of water lily.

Effects of stigma flavonoids on pre-fertilization barriers during water lily breeding

Flavonoids, which can affect plant physiology, growth, and development, are common secondary metabolites in vascular plants [35,36]. Flavonoids are mainly involved in the physiological processes of plant symbiosis, defense against disease and insect pests, auxin transport, seed and pollen germination, and root development. In addition, flavonoids can affect plant reproductive and developmental processes and participate in the interaction between the pollen and stigma [37,38]. In this study, our transcriptome and proteome analyses showed that there were significant differences in genes and proteins related to flavonoid biosynthesis between unpollinated stigmas and stigmas at 2 and 6 HAP. For example, the expression and content of flavonol synthase in stigmas after pollination are lower than those of unpollinated stigmas, which indicates that the flavonoid content on the stigma after pollination is greatly reduced. Flavonoids are the key substances for pollen germination and pollen tube growth of many plants. Other transcriptomic studies on rice stigmas revealed that numerous genes encoding flavonols were expressed, and these genes were expressed on rice stigmas, suggesting that flavonoids play an important role in the interaction between pollen and stigma [37]. Similarly, the pollen of a flavonoid-deficient mutant of petunia could not germinate on the stigma, but the addition of the exogenous

flavonoid kaempferol could induce pollen germination on the stigma. This indicates that kaempferol may play a role in pollen germination [37,38]. Because flavonoids are the key substances in pollen germination and pollen tube growth of many plant species, we infer that the biosynthesis of flavonoids is closely related to the pre-fertilization barrier of water lily.

Effects of SAM metabolism on pre-fertilization barriers during water lily breeding

The SAM participates in many important physiological processes, such as transamination of propyl, methyl, and sulfur in plants, and is the main hub of methionine metabolism [39,40]. Studies have shown that SAM plays an important role in regulating plant adaptability to iron deficiency and drought [41, 42]. In addition, SAM is involved in resistance to pathogens and senescence [43, 44]. SAM can also be used as a precursor of ethylene and polyamine [45]. Moreover, polyamines with appropriate concentrations are important for pollen germination and pollen tube growth [46]. The results in the present study showed that the SAM synthase gene was up-regulated by pollination, which facilitated the synthesis of polyamines and ethylene by the stigma papilla cells, and then regulated pollen germination and pollen tube growth. Similarly, a previous study has shown that ethylene plays a role in pollen tube growth after pollination in tobacco [47]. Therefore, we inferred that the metabolic pathway of SAM participated in the interaction between the pollen and stigma of water lily and plays an important role in the regulation mechanism of the pollen-stigma interaction.

Cell wall organization or biogenesis is associated with pre-fertilization barriers during water lily breeding

During the interaction between the pollen and stigma, some enzymes in stigma papilla cells are activated and released by certain signals. These enzymes are mainly involved in modifying cell walls, such as enzymes that degrade pectin, cellulose, and hemicellulose [48-50]. In our study, the combined transcriptome and proteome analysis showed that many DEGs involved in cell wall tissue metabolism were found on stigma at 2 and 6 h after pollination, suggesting that genes involved in cell wall synthesis were induced by pollination. Transcriptome studies on the stigma of many species have confirmed that there are a large number of cell wall metabolism-related genes in the stigma [37, 51-53], and the products of these genes may be secreted by stigma papillae to help pollen tubes penetrate the stigma. In addition, the GO annotation showed that the expression of endoglucanase involved in cellulose hydrolysis was significantly down-regulated, while beta-galactosidase and xyloglucan endotransglucosylase/hydrolase genes involved in cell wall modification were significantly up-regulated. Thus, we infer that beta-galactosidase may be involved in cellulose synthesis and cell wall elongation during cell wall metabolism of *Nymphaea* stigmas [54], whereas xyloglucan endotransglucosylase/hydrolase is mainly involved in cell wall reinforcement [55]. The KEGG analysis showed that the gene expression abundances involved in cutin, suberin, and wax biosynthesis were significantly increased, which resulted in cutin, suberin, and wax deposition on the cell walls of stigma cells, thereby increasing cell wall strength [56]. Significant up-regulation of key genes involved in phenylpropanoid biosynthesis further confirmed the accumulation of lignin in stigma the cell wall of water lily after pollination because suberin biosynthesis is closely related to phenylpropanoid biosynthesis [57]. We infer that the accumulation of cutin, suberin, and wax on the

cell wall enhances the strength and thickness of the cell wall, thus hindering the growth of the pollen tube on the stigma. Therefore, cell wall organization or biogenesis is closely related to the interspecific hybridization barrier of *Nymphaea* species.

Other enzymes involved in the interspecific hybridization barrier of *Nymphaea* spp.

In this study, the combined analysis of transcriptome and proteome showed that the activity of peroxidase increased significantly after pollination. Peroxidase mainly removes peroxides and participates in stress response, auxin metabolism, and signal transduction [28]. Previous research has shown that the peroxidase activity of a mature stigma increased significantly and reached the highest value when the stigma developed was the most receptive to pollen, which is a common method used to judge stigma maturity in the field [52]. Mc Innis was the first to discover stigma-specific peroxidase, which has cell specificity and specific expression patterns, and is specifically expressed in the cytoplasm and cell surface of stigma epidermal cells [28]. Based on our results and previously published papers, we infer that in the process of interaction between the pollen and stigma of *Nymphaea*, peroxidase may directly participate in the process of mutual recognition between the stigma and pollen, perhaps guiding the pollen tube.

Glutathione S-transferase (GST) is a ubiquitous enzyme that regulates plant secondary metabolism, detoxification, and defense, and plays an important role in cell response to stress [58]. A previous study on maize showed that the expression of GST was up-regulated at the early stage of silk-pollen interaction and pollen tube germination [59]. GST was also upregulated in this study. It is possible that GST participated in the pollen-stigma interaction of *Nymphaea*, but its mechanism of involvement needs further study.

Cytochrome P450 has a wide range of catalytic activities. It mainly participates in the synthesis and metabolism of terpenoids, alkaloids, sterols, fatty acids, plant hormones, signal molecules, phenylpropane, flavonoids, and isoflavones. Cytochrome P450 is also involved in plant resistance to biotic and abiotic stressors [60]. Our results showed that cytochrome P450 participated in the biosynthesis of phenylpropane and flavonoids, and its expression increased significantly, indicating that cytochrome P450 indirectly participated in the pollen-stigma interaction of water lily.

Mitogen-activated protein kinase (MAPK) is an evolutionarily conserved serine/threonine protein kinase in eukaryotic organisms. It is responsible for regulating signal transduction between cells and amplifying stimulus signals from outside cells to induce appropriate physiological and biochemical reactions in receptor cells [61]. In this study, we found that the expression of MAPK on the stigma of water lily was significantly increased after pollination. Additionally, MAPK participated in SAM biosynthesis and plant secondary metabolism. We infer that MAPK plays a signaling role in the pollen-stigma interaction and plays an important role in the regulation of the complex network of pre-fertilization barriers in water lily.

Conclusions

In this paper, the differences of stigma transcripts and proteomes at 0, 2, and 6 hours after pollination were compared, resulting in the identification of some regulatory genes and functional proteins that may cause pre-fertilization barriers in water lily. The functional analysis showed that differential transcripts were mainly involved in cell wall organization or biogenesis, SAM, hydrogen peroxide decomposition and metabolism, ROS metabolism, secondary metabolism, secondary metabolite biosynthesis, and phenylpropanoid biosynthesis. These results indicated that genes involved in regulatory and gene pathways involved in stress response might overlap in the process of pollination with hybridization barrier in water lily, indicating that the process of pollen tube entering stigma tissue was very similar to the process of external injury, which further indicated that these genes involved in defense and stress response were necessary in the early stage of interaction between pollen and stigma. This study strengthens our understanding of the mechanism of pre-fertilization barriers in *Nymphaea* at the molecular level and provides a theoretical basis for overcoming the pre-fertilization barriers in water lilies in the future.

Materials And Methods

Experimental materials

An interspecific cross between the female *Nymphaea* 'Peter Slocum' and male *N. micrantha* was performed as described in a previous report. These plants were grown in ponds in Xingxiang, Zhenjiang, Jiangsu Province, China.

The stigmas of 0, 2, and 6 HAP were collected. Each treatment had three biological repeats. Non-pollinated stigmas were used as the control, and the stigmas from 2 and 6 HAP were used as the treatment to dynamically study the interaction between the pollen and stigma after pollination. After collection, the three samples, non-pollinated stigmas, the stigmas of 2 HAP, and the stigmas of 6 HAP, were immediately frozen in liquid nitrogen and stored at - 80°C.

High-throughput RNA-seq and data processing

Total RNA was extracted using Trizol reagent according to the manufacturer's protocol (Takara Bio Inc., Otsu, Japan). The total RNA was checked for quality and quantity using an Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA). The mRNA samples were enriched by oligo(dT) magnetic beads and then cut into fragments with fragmentation buffer at 80°C. First-strand cDNA was synthesized using random hexamers as the primers. Second-strand cDNA was synthesized from first-strand cDNA using RNase H, DNA polymerase I, and dNTPs. After purification and terminal repair, double-stranded DNA poly A and adaptor sequences were ligated to the end of the cDNA. cDNA libraries were constructed by PCR amplification after selecting for fragment size and undergoing a quality check with an Agilent 2100 Bioanalyzer system. Finally, the qualified cDNA libraries were sequenced with an Illumina HiSeq 2500 system. Three biological replicates were used in the RNA-seq experiments involving each sample.

Clean reads were obtained from the raw reads after filtering out low-quality reads and were used for *de novo* transcriptome assembly. Trinity software was used to assemble the transcriptomes according to the study from Grabherr et al. [62]. High-quality reads were assembled into contigs, transcripts, and unigenes using Trinity (<http://trinityrnaseq.sourceforge.net/>) [62, 63]. The functional annotation of all-unigenes was performed using a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the GO, Pfam, KEGG, Nr, and Swiss-Prot databases.

FPKM was used to quantify the gene expression [64]. FPKM values were normalized, DEGs were determined when $FDR \leq 0.001$ and $|\log_2 \text{fold change}| \geq 1$. Functional annotation and classification of the DEGs were conducted using the Blast 2 GO program (<http://www.blast2go.com/b2ghome>) [65]. Additionally, a KEGG pathway analysis (http://www.genome.jp/kegg-bin/search_pathway) was performed. The heat map was produced using Cluster 3.0 and treeview.

Label-free analysis of the stigma proteome of water lily

Protein extraction and peptide enzymolysis. Protein extraction was performed using the SDT (4% SDS, 100 mM DTT, 150 mM Tris-HCl pH 8.0) method. The protein concentrations were quantified with the BCA Protein Assay Kit (Bio-Rad, USA), and the samples were stored at -80°C . Protein digestion (200 μg for each sample) was performed using the filter aided proteome preparation procedure described by Wisniewski [66]. The peptides from each sample were desalted on C18 cartridges, concentrated by vacuum centrifugation, and reconstituted in 40 μL of 0.1% (v/v) formic acid.

MS/MS protein identification and quantification. Each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture was loaded onto a reverse-phase trap column (Thermo Fisher Scientific Acclaim PepMap100, 100 $\mu\text{m} \times 2$ cm, nanoViper C18) connected to the C18-reverse phase analytical column (Thermo Fisher Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm , C18-A2) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% acetonitrile, 0.1% formic acid) at a flow rate of 300 nL/min controlled by IntelliFlow technology.

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to an Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 240 min. The mass spectrometer was operated in the positive ion mode. MS data was acquired using a data-dependent top10 method, dynamically choosing the most abundant precursor ions from the survey scan (300–1,800 m/z) for higher collision energy dissociation (HCD) fragmentation. The automatic gain control target was set to 1×10^6 , and the maximum injection time was 50 ms. The duration of dynamic exclusion was 60.0 s. Survey scans were acquired at a resolution of 70,000 at 200 m/z ; resolution for HCD spectra was set to 17,500 at m/z 200, the isolation width was 2 m/z , the normalized collision energy was 30 eV, and the under-fill ratio was defined as 0.1%.

For protein identification, the MS raw files were processed by Maxquant1.5.3.17 software [67]. The acquired MS/MS spectra were searched against the predicted protein databases translated from the above transcriptome databases in this study. The minimum peptide length was set to six amino acids

and the maximum FDR was set to 1% for both peptides and proteins. The other parameters were set as follows: peptide mass tolerance = \pm 20 ppm; enzyme = trypsin; max missed cleavage = 2; fixed modification: carbamidomethyl (C); variable modification: oxidation (M), acetyl (protein N-term). Protein quantification was based on both 'razor' and unique peptides [67, 68], and the label free quantitation algorithm was performed [69]. For each fraction, peptides were matched across different LC-MS/MS runs based on mass and retention time (set to the match between runs option in MaxQuant) using the time window of 2 min.

DEPs were analyzed for significant downregulation or upregulation. For quantitative changes, a 2.0-fold cutoff was set to determine upregulated and downregulated proteins, with a p-value < 0.05 present in at least two replicates.

Bioinformatics analysis

A functional annotation analysis was performed with Blast2GO software (<http://www.geneontology.org/>) [70]. We used the KAAS (Automatic Annotation Server) software to annotate DEPs and to investigate the biochemical pathways of molecular interactions [71]. Three biological replicates of each sample were used in the label-free analysis.

Selection of target peptides for PRM analysis

Peptide mixtures of nine samples were prepared using trypsin as described above for the label-free analysis. Equivalent peptides from each sample were pooled, and 2 μ g of the pooled sample was introduced into an HPLC system via a trap column (100 μ m \times 50 mm, 5 μ m-C18) and then via an analytical column (75 μ m \times 200 mm, 3 μ m-C18). Separated peptides were then analyzed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Raw files were analyzed using Maxquant 1.5.3.17 software (enzyme = trypsin/P, missed cleavage = 0). Only peptides with scores over 40 were selected as target peptides.

Quantitative PRM analysis for target proteins

A total of eight target peptides of the four DEPs were selected and used for quantitative analysis to determine their feasibility. Peptide Retention Time Calibration mixture was added into the peptide mixture, and the labeled peptide "TASEFDSAIAQDK" (the bold "K" indicates the heavy isotopic labeling) was used as the internal standard. Two micrograms of peptide mixture containing 20 fmol labeled peptide was separated by HPLC and then analyzed by a Q-Exactive mass spectrometer. Quantitative analysis was repeated three times and the raw data was calculated by Skyline 3.5.0.

Abbreviations

HAP, hours after pollination; DEGs, differentially expressed genes; DEPs, differentially expressed proteins; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; SAM, S-adenosylmethionine

metabolism;ROS, reactive oxygen species; RNA-seq, RNA sequencing; FPKM, Fragments Per Kilobase Million; PRM, Parallel reaction monitoring; LC-PRM/MS, Liquid Chromatography-Parallel Reaction Monitoring/Mass Spectrometry; NO, Nitric Oxide; GST, Glutathione S-transferase; MAPK, Mitogen-activated protein kinase

Declarations

Acknowledgements

Not applicable

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Availability of data and material

The raw data from the three samples have been submitted separately to the National Center for Biotechnology Information (NCBI) under the accession number PRJNA548276 (<http://www.ncbi.nlm.nih.gov/sra/PRJNA548276>).

Authors' contributions

CQS, FDC, NJT and ZLD designed the experiments. CQS, XS and YMY performed the experiments. CQS, NJT and ZLD analyzed the data. CQS wrote the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Additional File Legend

Additional file 1. Differential expression genes in 0, 2 and 6 HAP. (XLS 37376 kb)

Additional file 2. Up- and down-regulated DEPs identified by label-free analysis in 0, 2 and 6 HAP. (XLS 10752 kb)

Additional file 3. The correlation between DEPs and DEGs. (XLS 855 kb)

Additional file 4. The cor-DEGs-DEPs genes with the same or opposite trend. (XLS 31 kb)

Additional file 5. The Skyline analysis results of eight target peptide fragments of the four target proteins. (PDF 14848 kb)

Additional file 6. The peak area results of each target peptide fragment from the Skyline analyses. (PDF 14848 kb)

Additional file 7. Detailed results from the data analysis including quantitative information at the peptide fragment level, data calibration, and statistical analyses.. (PDF 85 kb)

Table

Table 1 Relative quantitative of target peptide segment by PRM analysis

Peptide Sequence	Protein Name	Ratio	Ratio	Ratio
		2 HAP/0 HAP	6 HAP/0 HAP	6 HAP/2HAP
LADEWASTK	TRINITY DN44636 c2 g1	1.82	2.15	1.18
EGPDGLVGAAEVER	TRINITY DN44636 c2 g1	2.86	3.88	1.36
HPIPSYHTGK	TRINITY DN50288 c0 g2	2.99	3.04	1.02
YDFDPLDVTWTTWPEDLLPLQPVGR	TRINITY DN50288 c0 g2	3.62	5.19	1.43
THLGAVAFNNTTR	TRINITY DN35350 c0 g1	5.92	6.71	1.13
WAFPLEEDIAK	TRINITY DN35350 c0 g1	4.66	5.64	1.21
DSTVISGGPYWEVPLGR	TRINITY DN53782 c2 g1	2.94	3.92	1.33
GLLNTDEILFTQNK	TRINITY DN53782 c2 g1	3.17	4.14	1.30

Figures

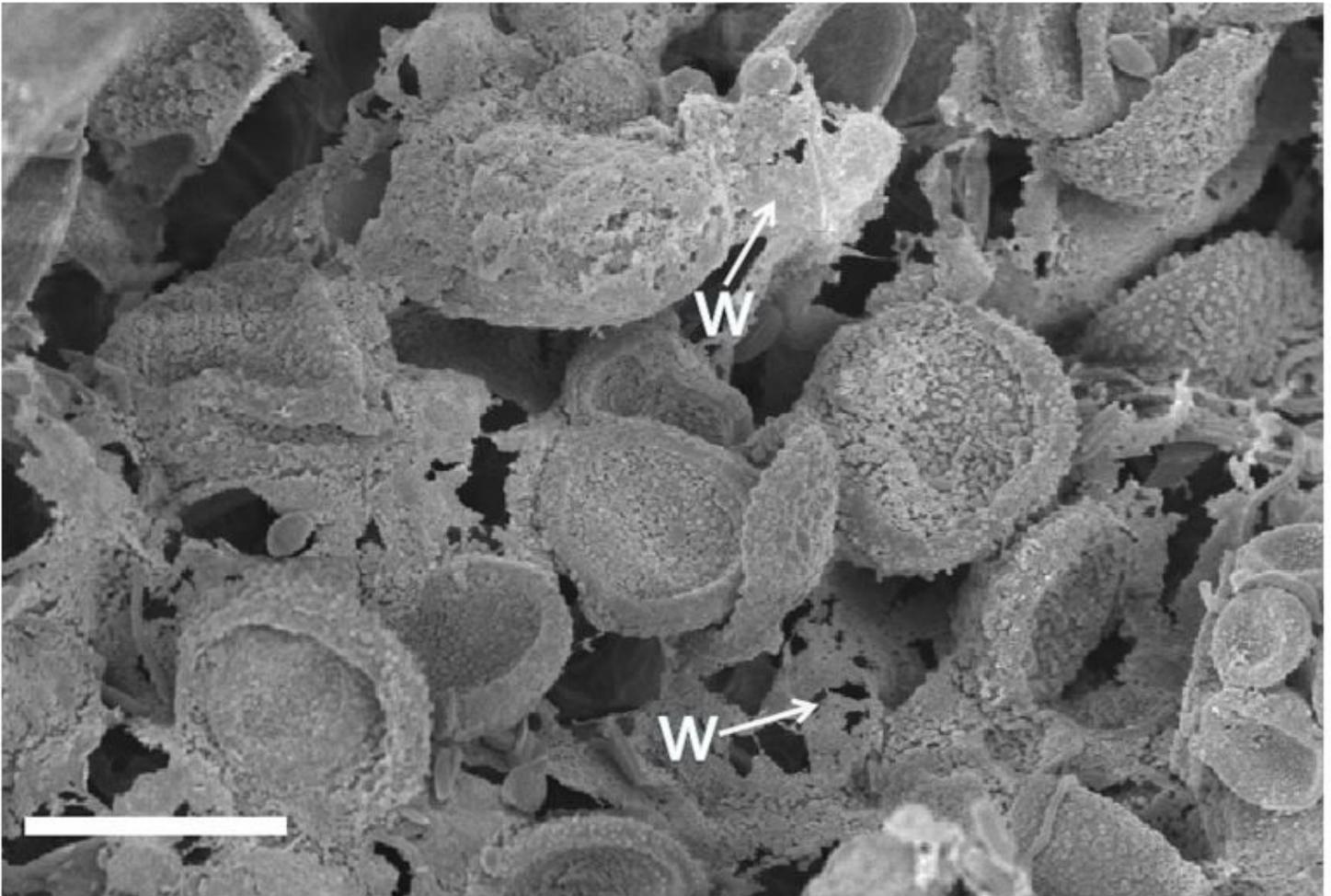


Figure 1

Pollen germination on stigma on stigmas at 6 h after pollination with scanning electron microscope. Accumulation of wax between the stigma and the surface of the pollen grains was commonly observed. Scale bar = 25 μ m. W= Wax.

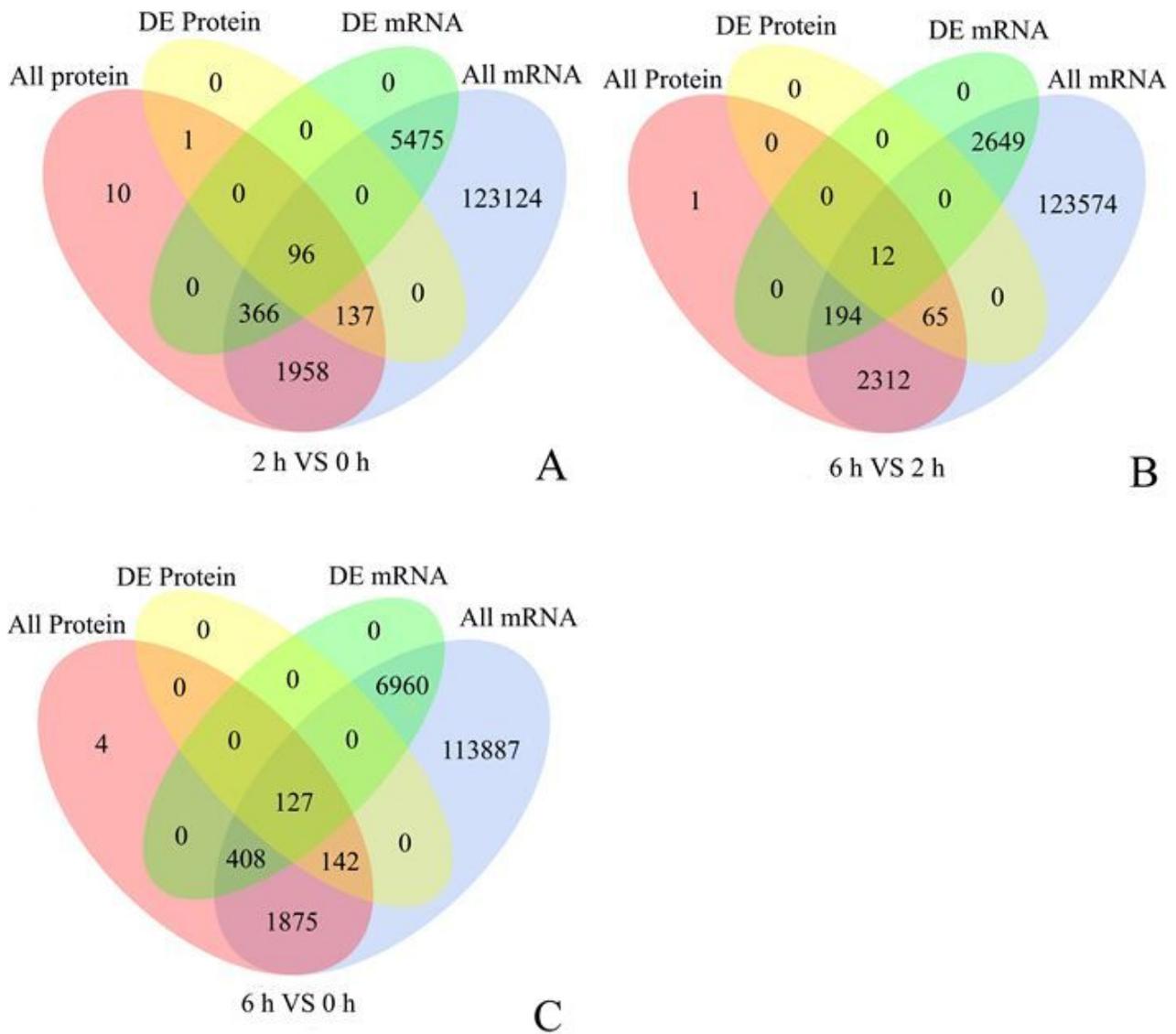


Figure 2

Venn diagram showing all identified, as well as all significantly enriched, mRNAs and proteins and their overlap. All protein: Represents all quantifiable proteins in the proteome; All Gene: Represents all quantifiable genes obtained in the transcriptome; DE Protein: Represents differentially expressed proteins identified by the proteome; DE mRNA: Represents differentially expressed genes identified by the transcriptome.

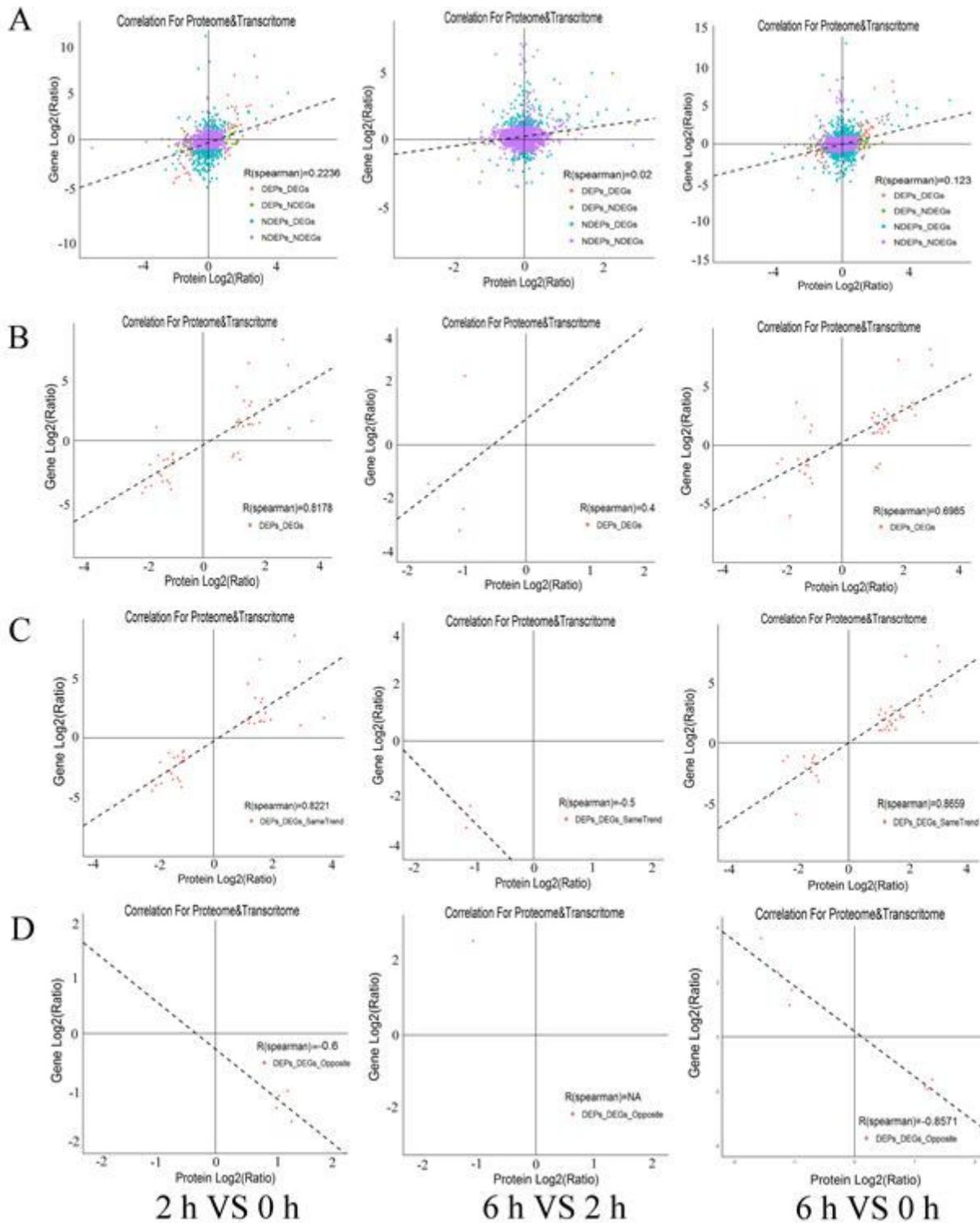


Figure 3

Correlations between protein and messenger ribonucleic acid (mRNA) expression. x-axis represents the protein expression level, and y-axis represents the genes expression level. (a) Scatterplots of the relationship between genes quantified in both transcriptomic and proteomic data sets. (b) Scatterplots and correlation coefficients between differentially expressed proteins (DEPs) and differentially expressed genes (DEGs). Scatterplots and correlation coefficients between proteins and mRNA expression ratios

which are the same (c) or opposite (d) changing tendency. The purple plot indicates none DEPs and DEGs; green plot indicates DEPs but none DEGs; blue plot indicates DEGs but none DEPs; red plot indicates DEPs and DEGs, and all data were log2-transformed.

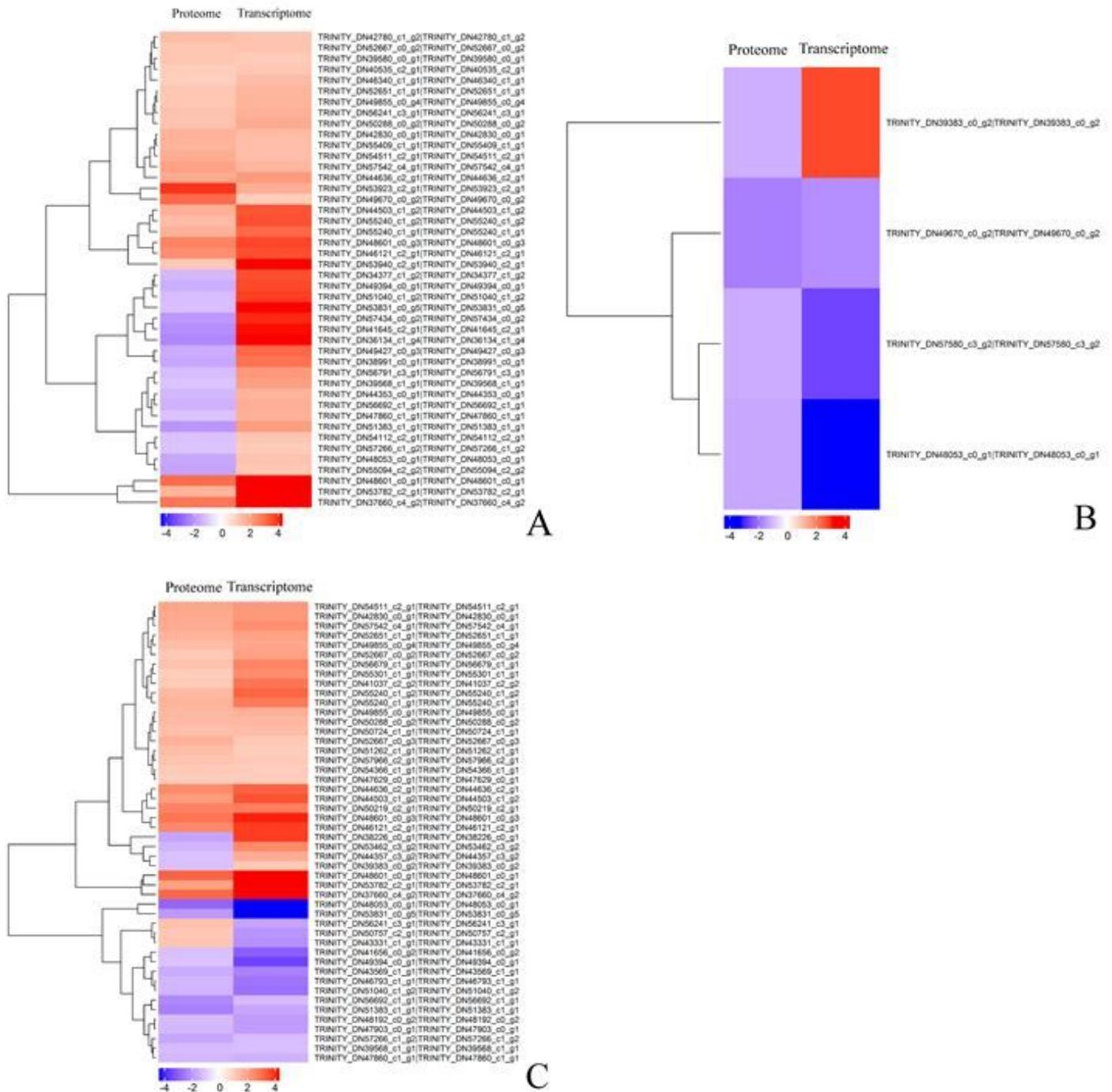


Figure 4

Cluster analysis of associated differential proteins and differential mRNA expression patterns. Each row in the graph represents a protein/mRNA, and each column in the graph represents a sample (the proteome sample on the left and the corresponding transcriptome sample on the right). Numbers are

listed as the log 2 value of difference multiples. Expression differences are shown in different colors; red indicates up-regulation, while blue indicates down-regulation.

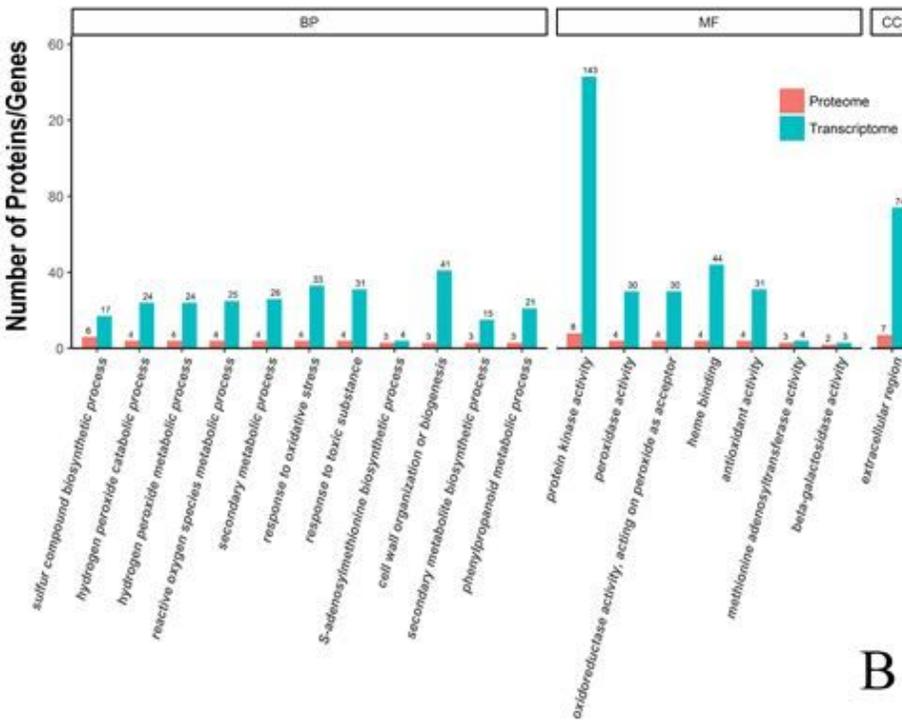
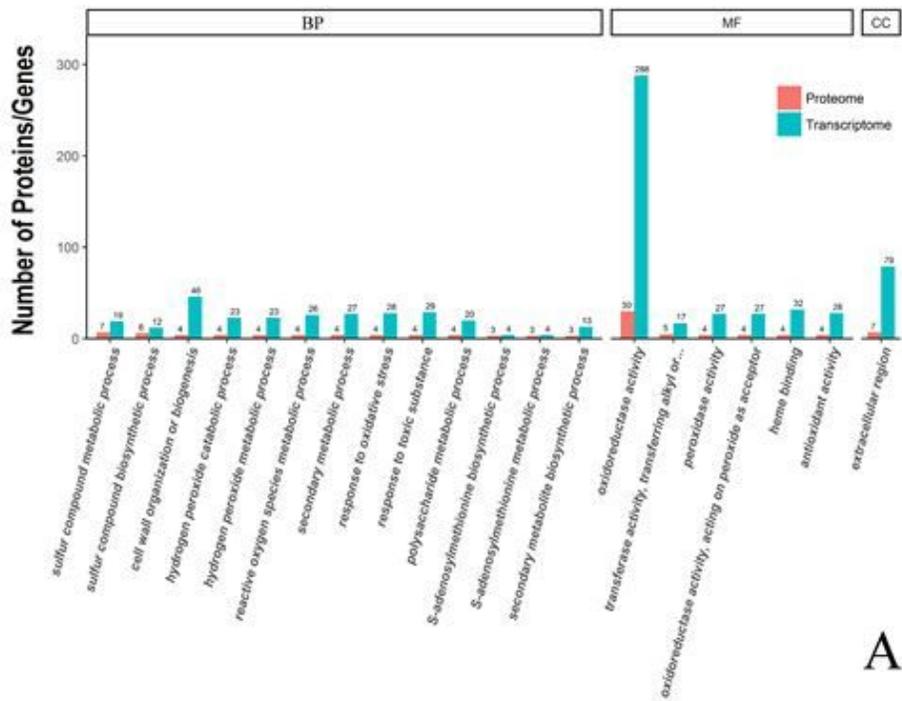


Figure 5

GO enrichment analyses of DEGs and DEPs. Each column in the figure represents a GO secondary annotation entry, red represents differentially expressed proteins, and blue represents differentially

expressed genes. From left to right, the number of differentially expressed proteins is arranged from high to low. BP: Biological process, CC: Cellular component, MF: Molecular function.

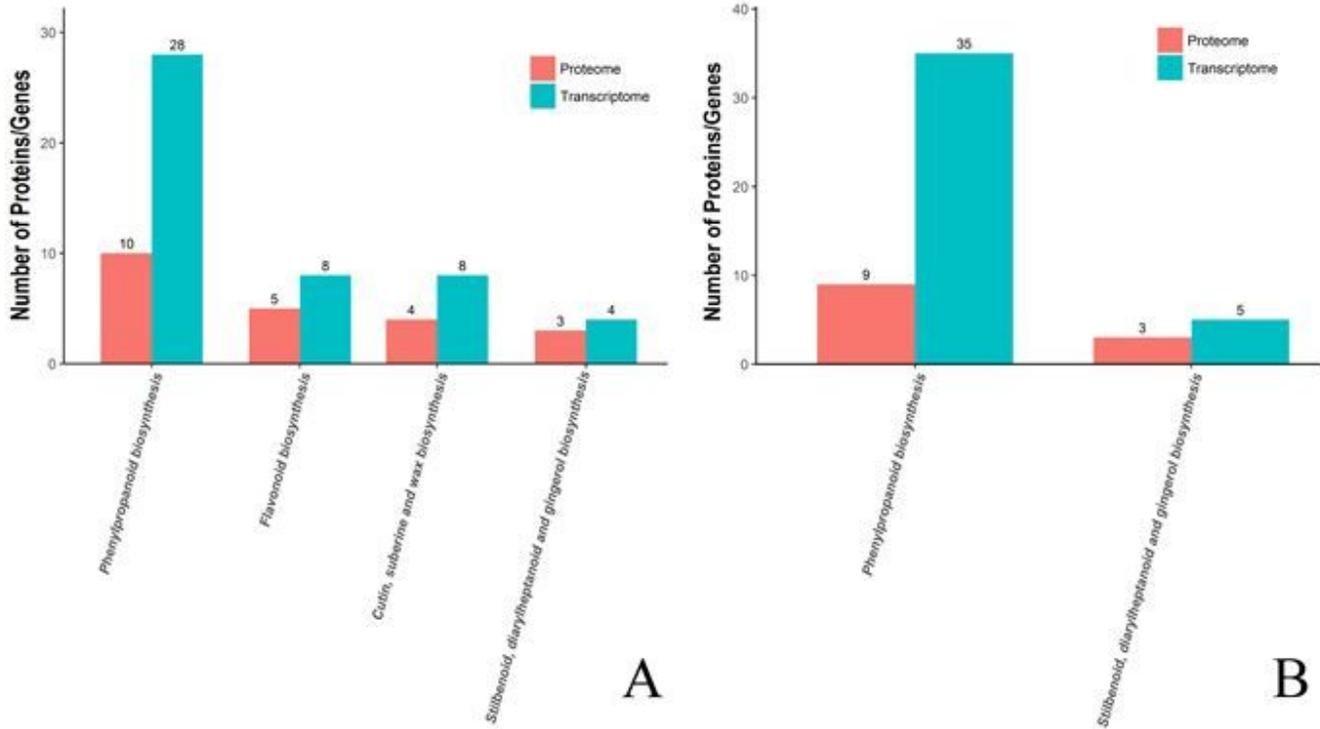


Figure 6

KEGG enrichment analyses of DEGs and DEPs. Each column in the figure represents a KEGG pathway, and different colors represent different histology. The red column in the figure represents the KEGG enrichment result of proteome, and the blue column represents the KEGG enrichment result of transcriptome. The abscissa is the name of the enriched KEGG pathway, and the ordinate represents the number of enriched proteomes and transcriptomes. From left to right, the number of differentially expressed proteins ranged from high to low.

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