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# Transcriptomics integrated with metabolomics reveals the mechanism of $\text{CaCl}_2\text{-HCl}$ electrolyzed water-induced glucosinolate biosynthesis in broccoli sprouts

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## ABSTRACT

Glucosinolates are important phytochemicals in Brassicaceae. We investigated the effect of  $\text{CaCl}_2\text{-HCl}$  electrolyzed water (CHEW) on glucosinolates biosynthesis in broccoli sprouts. The results showed that CHEW treatment significantly decreased reactive oxygen species (ROS) and malondialdehyde (MDA) contents in broccoli sprouts. On the 8<sup>th</sup> day, compared to tap water treatment, the total glucosinolate content of broccoli sprouts with CHEW treatment increased by 10.6% and calcium content was dramatically enhanced from 14.4 mg/g DW to 22.7 mg/g DW. Comparative transcriptome and metabolome analyses revealed that CHEW treatment activated ROS and calcium signaling transduction pathways in broccoli sprouts and they interacted through MAPK cascades. Besides, CHEW treatment not only promoted the biosynthesis of amino acids, but also enhanced the expression of structural genes in glucosinolate synthesis through transcription factors (*MYBs*, *bHLHs*, *WRKYs*, etc.). The results of this study provided new insights into the regulatory network of glucosinolates biosynthesis in broccoli sprouts under CHEW treatment.

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## 1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*), as a vegetable, is consumed worldwide due to its diverse bioactive components such as phenols, flavonoids, carotenoids and glucosinolates. Epidemiological evidence suggests that the consumption of broccoli has many beneficial effects on human health, such as anti-inflammatory<sup>[1]</sup>, antioxidant<sup>[2]</sup>, hypoglycemic<sup>[3]</sup>, fight against obesity<sup>[4]</sup>, etc., which effects are primarily attributed to glucosinolates and their hydrolyzate isothiocyanates.

Glucosinolates are a class of plant secondary metabolites containing nitrogen and sulfur, which mainly exist in Brassicaceae and play an important role in plant resistance to pests and diseases<sup>[5]</sup>.

To date, more than 130 glucosinolates have been identified and Brassicaceae contains more than 40 kinds of glucosinolates<sup>[6–7]</sup>. According to the side chain R (derived from 8 amino acids), glucosinolates can be classified into aliphatic glucosinolates, indole glucosinolates and aromatic glucosinolates<sup>[8–9]</sup>. Glucoraphanin (GRA), one of aliphatic glucosinolates, is the most abundant glucosinolate in broccoli, accounting for more than 50% of the total glucosinolate, and its content in broccoli sprouts is dozens of times that of mature broccoli<sup>[10]</sup>. Therefore, researches on glucosinolates in broccoli sprouts are gradually increasing.

The biosynthesis of glucosinolate in broccoli sprouts has been clarified, which mainly includes three stages: (a) side-chain elongation of amino acid; (b) formation of glucosinolate core structure; (c) secondary modification of glucosinolate side chain<sup>[11]</sup>. The formation of the glucosinolate core structure is not only a common part of all glucosinolates, but also a key step in glucosinolate synthesis. The genes involved in this process include *CYP79F1*, *CYP79B1*, *GGPI*, *UGT74B1*, *SOT18*, etc.<sup>[12]</sup>. Numerous researches have been done on the accumulation of glucosinolates in broccoli

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sprouts with diverse treatments. Previous studies found that the contents of GRA and isothiocyanates in broccoli sprouts were enhanced after 10 mmol/L  $\text{CaCl}_2$  treatment, and the mechanism was mainly explained from the perspectives of electrolyte leakage, changes in antioxidant enzyme activities and gene expression<sup>[2,13–14]</sup>. Slightly Acidic Electrolyzed Water (SAEW) was used for the cultivation of broccoli sprouts, and the contents of glucosinolates and GRA in the SAEW treatment were higher than those in the tap water control group<sup>[15]</sup>. Besides,  $\text{ZnSO}_4$  plus  $\text{CaCl}_2$  treatment enhanced the content of glucosinolates in broccoli sprouts as well as the expressions of *MYB28*, *Elong*, *CYP79F1*, *CYP83A1*, *FMO<sub>GS-OXI</sub>* and *AOP2*<sup>[16]</sup>. Current research mainly explains the regulation mechanism of exogenous substances treatment on broccoli sprouts from the aspects of enzyme activity and glucosinolate synthesis-related structural gene expression. However, the regulatory network of glucosinolate synthesis is relatively complex. The study of transcription factors that regulate glucosinolate synthesis can further enrich the regulatory mechanism of glucosinolate synthesis. Current studies have shown that *MYB28*, *MYB29*, *MYB76*, *MYB34*, *MYB51* and *MYB122* of MYB transcription factor family play a major role in regulating the synthesis of glucosinolates in cruciferous plants; the bHLH (Basic Helix-Loop-Helix) transcription factors *MYC2*, *MYC3* and *MYC4* regulate the synthesis of glucosinolates by acting on the MYB transcription factors, while the WRKY transcription factors *WRKY18* and *WRKY40* cooperate with *CYP81F2* to negatively regulate the synthesis of indole glucosinolates<sup>[17]</sup>.

The development of transcriptome and metabolome technology has increased our understanding of the network between genes and key metabolites in plants. A previous study found that integrative analysis of the metabolome and transcriptome profiles provided some novel insights into anthocyanin accumulation and the molecular mechanism of anthocyanin biosynthesis in asparagus<sup>[18]</sup>. Aliphatic glucosinolates and indolic glucosinolates were found to be increased significantly in the  $\text{CaCl}_2$  treated broccoli microgreens using metabolomic approaches, but the molecular mechanism was not explained<sup>[14]</sup>. Thus, transcriptome and metabolome analysis could be considered to elucidate regulatory networks between genes and glucosinolates biosynthesis in broccoli sprouts.

A complete understanding of the the regulatory network of glucosinolates biosynthesis in broccoli sprouts is crucial to develop functional foods to meet the increasing health demand in our diet. Our previous study found that  $\text{CaCl}_2$ -HCl electrolyzed water (CHEW) treatment promoted glucosinolate synthesis and generated more bioactive isothiocyanates in broccoli sprouts<sup>[19]</sup>. Therefore, this study combined metabolomics and transcriptomics to further explore the molecular mechanism of CHEW treatment-induced glucosinolate biosynthesis in broccoli sprouts. The data from this study enhanced our understanding of the regulatory mechanism of glucosinolate synthesis, and provided a theoretical basis for variety improvement and high-quality cultivation of broccoli sprouts with high glucosinolate content.

## 2. Materials and methods

### 2.1 Preparation of CHEW

$\text{CaCl}_2$ -HCl electrolyzed water (CHEW) with a near-neutral pH

and containing available chlorine, can be generated by electrolyzing  $\text{CaCl}_2$ -HCl solution using a nonmembrane electrolytic cell. Available chlorine refers to the oxidized chlorine contained in chlorine-containing compounds, and its concentration is expressed in mg/L or % (g/100 mL). In CHEW, the available chlorine mainly refers to  $\text{HClO}$  and  $\text{ClO}^-$ .

CHEW was produced according to the method described previously with minor modifications<sup>[19]</sup>. 5 mmol/L  $\text{CaCl}_2$  acidic solution (pH was adjusted to  $4.40 \pm 0.05$  by concentrated hydrochloric acid) was electrolyzed with a 3 A electrical current for 40 s, then concentrated hydrochloric acid was used again to adjusted the pH to  $5.5 \pm 0.05$ , and CHEW with available chlorine concentration of 10 mg/L was obtained finally. The control treatment used tap water without electrolyzed. Analytical-grade reagents: hydrochloric acid (36%–38%) and anhydrous calcium chloride (96%) were purchased from Sinopharm Chemical Reagent Co., Ltd.

### 2.2 Plant materials, cultivation and treatment

Broccoli seeds (*Brassica oleracea* L. [Italica group] cv. ‘zhongqing’) were used in this study. The cultivation conditions of broccoli seeds were conducted according to previous method<sup>[19]</sup>. After soaking with CHEW solution and tap water respectively, the seeds were evenly distributed in a polypropylene box and kept in a growth chamber (PRx-450C, Ningbo Saifu Instrument Corporation, Ningbo, China) at 25 °C, 80% relative humidity under light. On the 4<sup>th</sup> and 8<sup>th</sup> days during the growth, broccoli sprouts were carefully harvested, immediately frozen with liquid nitrogen, and stored in a freezer at –80 °C before the phytochemicals were analyzed.

### 2.3 ROS and MDA content determinations

$\text{H}_2\text{O}_2$  and MDA contents were determined according to the methods described previously<sup>[19]</sup>. An  $\text{O}_2^-$  Detection Kit (BC1290, Solarbio, Beijing, China) was used to assay the  $\text{O}_2^-$  content. Approximately 100 mg broccoli sprouts were homogenized with 1 mL extraction solution in an ice bath and then centrifuged at 4 °C, 12 000 r/min for 20 min. Afterwards, 200  $\mu\text{L}$  supernatant and reaction solutions were mixed according to the manual, and the absorbance of the solution was measured at 530 nm.  $\text{O}_2^-$  content was expressed as  $\mu\text{mol/g}$  FW. For each treatment, measurements were made in triplicate.

### 2.4 Determination of glucosinolate and calcium content

Glucosinolates and calcium content were determined according to the method described previously<sup>[19]</sup>. For each treatment, measurements were made in triplicate.

### 2.5 Metabolite extraction and analysis

Broccoli sprouts were freeze-dried (Scientz-100F) and then pulverized (30 Hz, 1.5 min) to powder using a grinder (MM 400, Retsch). Broccoli sprout’s dry powder (100 mg) was homogenized with 1.2 mL of 70% methanol, and placed in a 4 °C refrigerator overnight. After centrifugation for 10 min at 12 000 r/min, the

supernatant was filtered through a 0.22  $\mu\text{m}$  membrane filter before it was injected into a UPLC-MS/MS analysis.

Ultra Performance Liquid Chromatography (UPLC) (SHIMADZU Nexera X2) and Tandem mass spectrometry (MS/MS) (Applied Biosystems 4500 QTRA) were used in this study. Liquid phase conditions: column (Agilent SB-C<sub>18</sub> 1.8  $\mu\text{m}$ , 2.1 mm  $\times$  100 mm); phase A is ultrapure water (add 0.1% formic acid), phase B is acetonitrile (add 0.1% formic acid). The ratio of phase B is 5% at 0.00 min, then increases linearly to 95% within 9.00 min, and maintained at 95% for 1 min. 10–11 min: the ratio of phase B decreases to 5%, and maintained 5% equilibrate to 14 min. The flow rate was 0.35 mL/min, the column temperature was 40 °C and the injection volume was 4  $\mu\text{L}$ . The ESI source operation parameters: ion source, turbo spray; source temperature 550 °C; ion spray voltage (IS) 5 500 V (positive ion mode)/–4 500 V (negative ion mode); ion source gas I (GSI), gas II (GSII) and curtain air (CUR) were set to 50, 60, and 25.0 psi, respectively, and the collision-induced ionization parameter was set to high. Instrument tuning and mass calibration were performed with 10 and 100  $\mu\text{mol/L}$  polypropylene glycol solutions in triple quadrupole (QQQ) and LIT modes, respectively. QQQ scans use MRM mode and set the collision gas (nitrogen) to medium.

Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database (<http://www.kegg.jp/kegg/compound/>) was used to identify identify metabolites. Significantly changed metabolites between groups were determined by  $\text{VIP} \geq 1$  and absolute  $\log_2\text{FC}$  (fold change)  $\geq 1$ . Pathways with significantly changed metabolites were then fed into MSEA (metabolite sets enrichment analysis), and hypergeometric test's  $P$ -values determined their significance.

## 2.6 Transcriptome analysis

### 2.6.1 RNA extraction

Total RNA was extracted using RNA prep Pure Plant Plus Kit (DP441, Tiangen Biotech, Beijing, China) according to the operation specifications. RNA purity was checked using the Nano Photometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit®2.0 Fluoro meter (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

### 2.6.2 Library construction and sequencing

The starting RNA for library construction was 1  $\mu\text{g}$  total RNA. NEB Next® Ultra RNA Library Prep Kit for Illumina® (NEB, USA) was used in library construction. PolyA-tailed mRNAs were enriched by Oligo(dT) magnetic beads, and then interrupted the mRNAs with divalent cations randomly in NEB Fragmentation Buffer. Using fragmented mRNA as a template and random oligonucleotides as primers, the first strand of cDNA was synthesized in the M-MuLV reverse transcriptase system, and then the RNA strand was degraded by RNase. In DNA polymerase I system, dNTPs were used to synthesize the second strand of cDNA. The purified double-stranded cDNA was end-repaired, A-tailed, and connected to a sequencing adapter. AM Pure XP beads were used to screen 250–300 bp cDNA,

and PCR amplification was performed, AM Pure XP beads were used to purify the PCR product again. Finally, we get the library.

After the library was constructed, Qubit 2.0 Fluorometer was used for preliminary quantification. Dilute the library to 1.5 ng/ $\mu\text{L}$ , and then used Agilent 2100 bioanalyzer to detect the insert size of the library. After the insert size was as expected, qRT-PCR accurately determined the effective concentration (above 2 nmol/L) of the library. Entrust Wuhan Metware Biotechnology Co., Ltd. to conduct on-machine sequencing.

### 2.6.3 Bioinformatics analysis

Use fastp v 0.19.3 to filter the raw data, mainly to remove reads with adapters; when the N content in any sequencing read exceeds 10% of the bases of the reads, the paired reads are removed; when any sequencing reads When the number of low-quality ( $Q \leq 20$ ) bases contained in the reads exceeds 50% of the bases of the read, the paired reads are removed. All subsequent analyses were based on clean reads. Download the reference genome and its annotation files from the <http://brassicadb.cn/#/> website, use HISAT v2.1.0 to build the index, and align the clean reads to the reference genome. Gene alignments were calculated using feature Counts v1.6.2, and then the FPKM for each gene was calculated based on gene length.

Differential expression analysis between the two groups was performed using DESeq2 v1.22.1, with  $P$ -values corrected using the Benjamini & Hochberg method. Adjusted  $P$ -value and  $|\log_2\text{FC}|$  as threshold for significant differential expression. The enrichment analysis is performed based on the hypergeometric test. For KEGG, the hypergeometric distribution test is performed with the unit of pathway; for GO, it is performed based on the GO term.

## 2.7 Quantitative real-time PCR (qRT-PCR) analysis

The qRT-PCR was used to verify the reliability of RNA-seq results. The DEGs were randomly selected based on RNA-seq data. Total RNA was extracted from broccoli sprouts using a plant RNA kit (ZP405K, ZOMANBIO). First-strand cDNA was synthesized from 1 ng to 5  $\mu\text{g}$  RNA using Hifair II 1st Strand cDNA Synthesis SuperMix for qPCR (11123ES60, YEASEN). qRT-PCR were performed using Hieff qPCR SYBR Green Master Mix (11202ES08, YEASEN). The PCR amplification was performed using 3-step cycling conditions: 95 °C for 2 min, followed by 45 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s. The primers listed in Table S1 and *Actin* was used as internal reference gene. Relative expressions were calculated using the  $2^{-\Delta\Delta\text{CT}}$  method. Three biological replications were used and four technical repetitions were performed on each replicate.

## 2.8 Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics 19 (SPSS Inc., NY, USA). All data were expressed as means  $\pm$  standard error of triplicate independent cultures. Data were statistically analyzed by one-way ANOVA and Duncan's multiple-range tests. Differences at  $P < 0.05$  were considered significant.

### 3. Results and discussion

#### 3.1 Effect of CHEW on ROS, MDA, calcium and total glucosinolate contents of broccoli sprouts

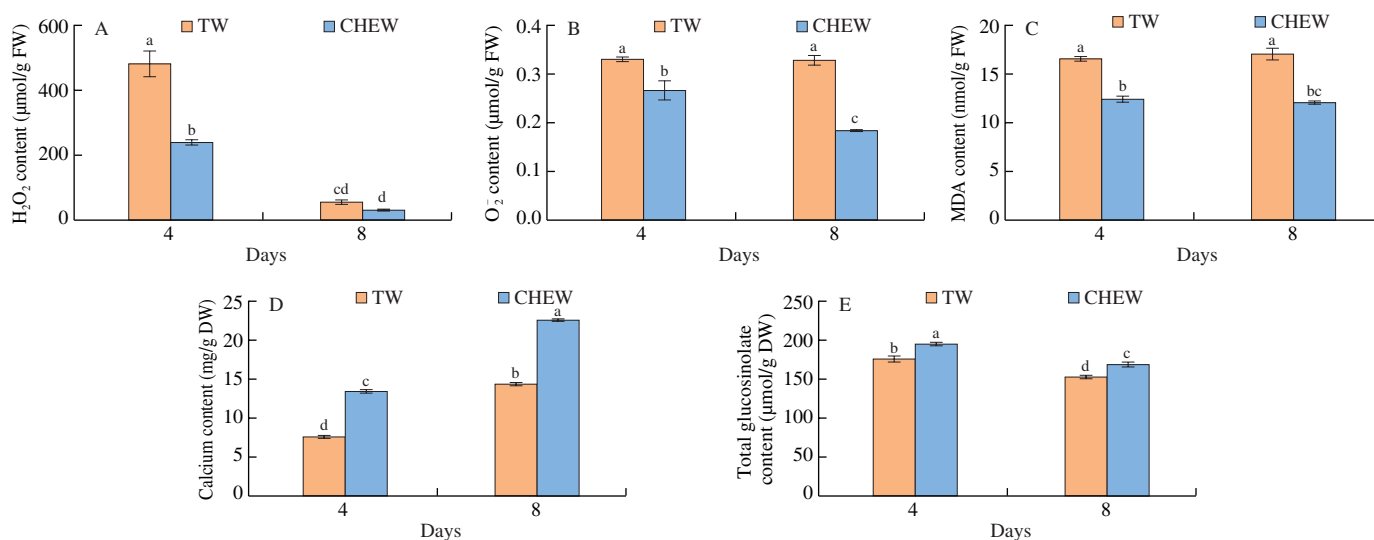
To investigate the effects of CHEW on the physiological changes in broccoli sprouts, we found that the contents of  $H_2O_2$  and total glucosinolates were significantly decreased, while calcium content increased significantly during the growth of broccoli sprouts (Fig. 1). Compared with the tap water treatment, CHEW treatment significantly decreased ROS and MDA contents in broccoli sprouts, which indicated that CHEW could relieve lipid peroxidation of the cell membrane of broccoli sprouts and reduce the level of ROS (Figs. 1A-C). Compared to the tap water treatment, the calcium content in broccoli sprouts treated with CHEW on day 8 was dramatically enhanced from 14.4 mg/g DW to 22.7 mg/g DW (Fig. 1D). In addition, compared with tap water, the total glucosinolate content of broccoli sprouts treated with CHEW on day 4 and day 8 increased by 10.8% and 10.6%, respectively (Fig. 1E). The total glucosinolate content in the CHEW treatment was consistently higher than that in the tap water treatment during the growth of broccoli sprouts.

Oxidative components of CHEW such as  $HClO$  and  $ClO^-$ , will produce oxidative stress to plants. Under oxidative stress, plants are supposed to produce a large amount of ROS which is an important second messenger<sup>[20]</sup>. Previous studies have shown that the glucosinolates content of broccoli sprouts was significantly increased with slightly acidic electrolyzed water treatment<sup>[15]</sup>. Same as ROS,  $Ca^{2+}$  is also a second messenger and plays an important role in the growth and development of plants. Previous study found that exogenous and endogenous calcium can promote glucosinolates biosynthesis in broccoli sprouts under  $ZnSO_4$  stress<sup>[16]</sup>. These results show that  $Ca^{2+}$  and ROS of CHEW play important roles in glucosinolate synthesis in broccoli sprouts.

#### 3.2 Metabolome analysis

To explore the effect of CHEW treatment on the growth and glucosinolate biosynthesis of broccoli sprouts, metabolome analysis was performed on the 4<sup>th</sup> and 8<sup>th</sup> days under tap water and CHEW treatments. Principal component analysis (PCA) was conducted and showed the isolation trend of the detected metabolites among the samples in “TW4”, “CHEW4”, “TW8” and “CHEW8” groups (Fig. S1a). Correlation analysis was performed on the three biological replicates in each sample, and  $R^2 > 0.8$ , indicating the samples had good repeatability (Fig. S1b). The results implied that the metabolites of broccoli sprouts were remarkably changed under CHEW treatment. In this study, a total of 994 differentially accumulated metabolites (DAMs) were detected which were divided into 15 categories and sorted by quantity as follows: phenolic acids, lipids, flavonoids, amino acids and derivatives, alkaloids, organic acids, lignans and coumarins, saccharides and alcohols, nucleotides and derivatives, glucosinolates, vitamin, terpenoids, tannins, stilbene and other metabolites (Fig. 2A). Compared with tap water treatment, 159 DAMs (84 up-regulated and 75 down-regulated) on the 4<sup>th</sup> day and 218 DAMs (94 up-regulated and 124 down-regulated) on the 8<sup>th</sup> day in broccoli sprouts with CHEW treatment were filtered from the annotated metabolites by setting a threshold of  $VIP \geq 1$  and absolute  $\log_2 FC \geq 1$  (Fig. 2B). The results implied that CHEW treatment induced more metabolite changes during growth of broccoli sprouts.

KEGG enrichment analysis of these DAMs revealed that compared to tap water treatment, the DAMs in broccoli sprouts with CHEW treatment were significantly enriched in metabolic pathways, biosynthesis of secondary metabolites, amino acids and cofactors (Figs. 2C, D). This observation meant that CHEW treatment might affect the biosynthesis of secondary metabolites during the growth of broccoli sprouts by promoting the biosynthesis of cofactors and amino acids, the precursors of secondary metabolites. This result was in agreement with the the previous report, which showed that slightly



**Fig. 1** Contents of  $H_2O_2$  (A),  $O_2^-$  (B), MDA (C), Calcium (D) and total glucosinolate (E) in broccoli sprouts under different treatments during growth. TW, tap water, CHEW,  $CaCl_2$ -HCl electrolyzed water. Each value is the mean of 3 replicates per treatment (mean  $\pm$  SE). Values not sharing a common letter are significantly different for each time point at  $P < 0.05$ .

acidic electrolyzed water promotes the biosynthesis of glucosinolates and anthocyanin in broccoli sprouts, which were associated with an increase in their precursor, methionine and phenylalanine respectively<sup>[21]</sup>.

### 3.3 Transcriptome analysis

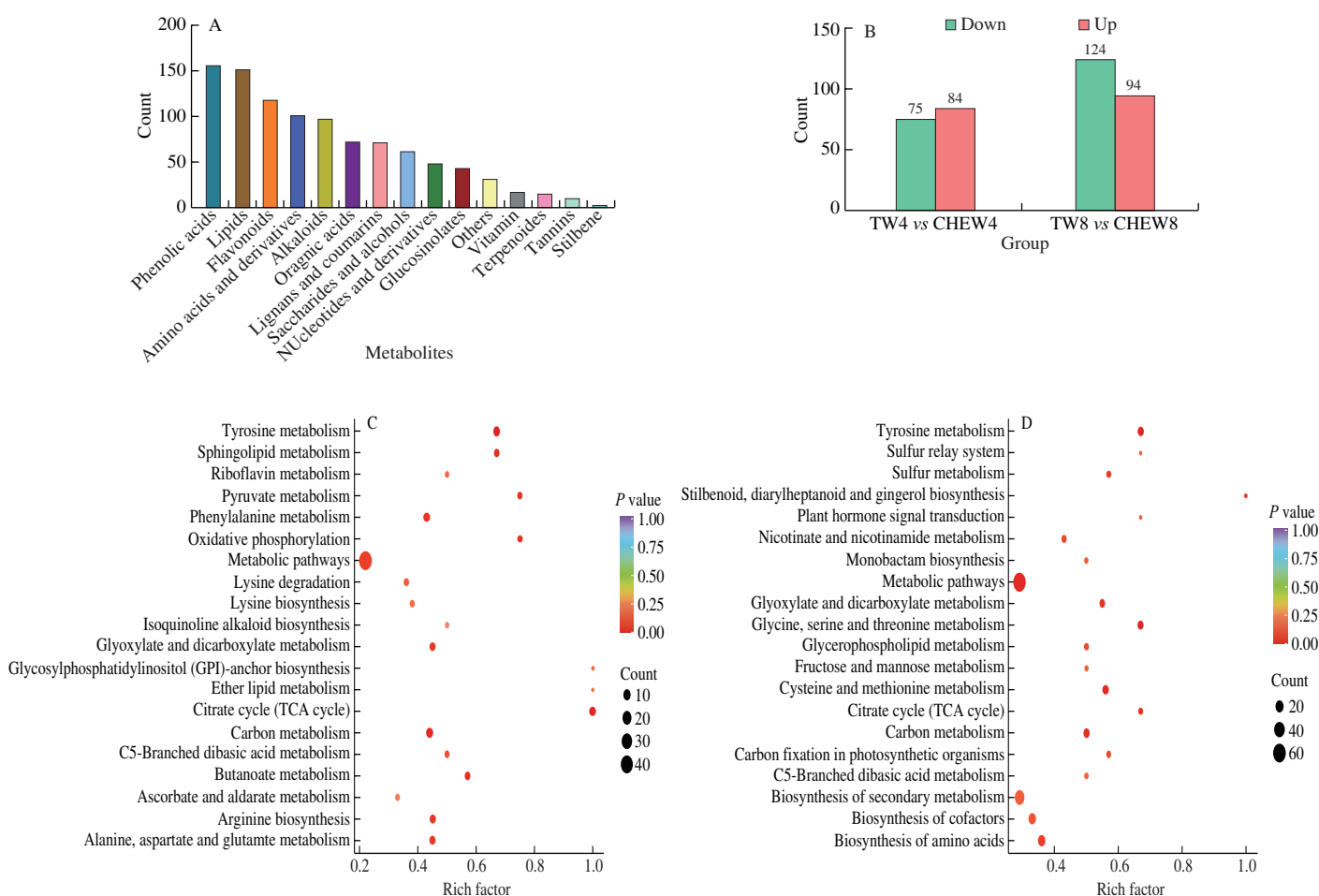
To explore the molecular events of glucosinolate biosynthesis in broccoli sprouts under CHEW treatment, transcriptome analysis was performed. We investigated the changes in gene expression levels among the samples (“TW4”, “CHEW4”, “TW8” and “CHEW8”), each of which included 3 replicates. After removing the low-quality reads, we obtained clean reads with an average number of 46 180 865. The percentages of Q30 and GC were 92.73%–93.99% and 46.71%–47.66%, respectively, indicating that the quality of transcriptome sequencing data is high (Table S2). PCA indicated that all biological replicates were grouped together (Fig. S2a), and correlation analysis revealed obviously good repeatability of the samples (Fig. S2b). According to the results of transcriptomic analysis, there were 1 014 differentially expressed genes (DEGs) between TW4 and CHEW4, of which 223 were upregulated and 791 were downregulated. There were 1 372 DEGs between TW8 and CHEW8, including 1 096 upregulated genes and 276 downregulated genes (Fig. S2c). The volcano plots were drawn to intuitively represent the differential gene distribution between each tap water treatment and CHEW treatment during the

growth of broccoli sprouts. The results indicated obviously that the CHEW affected the expression of numerous genes of broccoli sprouts during growth, and the number of upregulated expressed genes increased significantly on the 8th day (Figs. 3A and B).

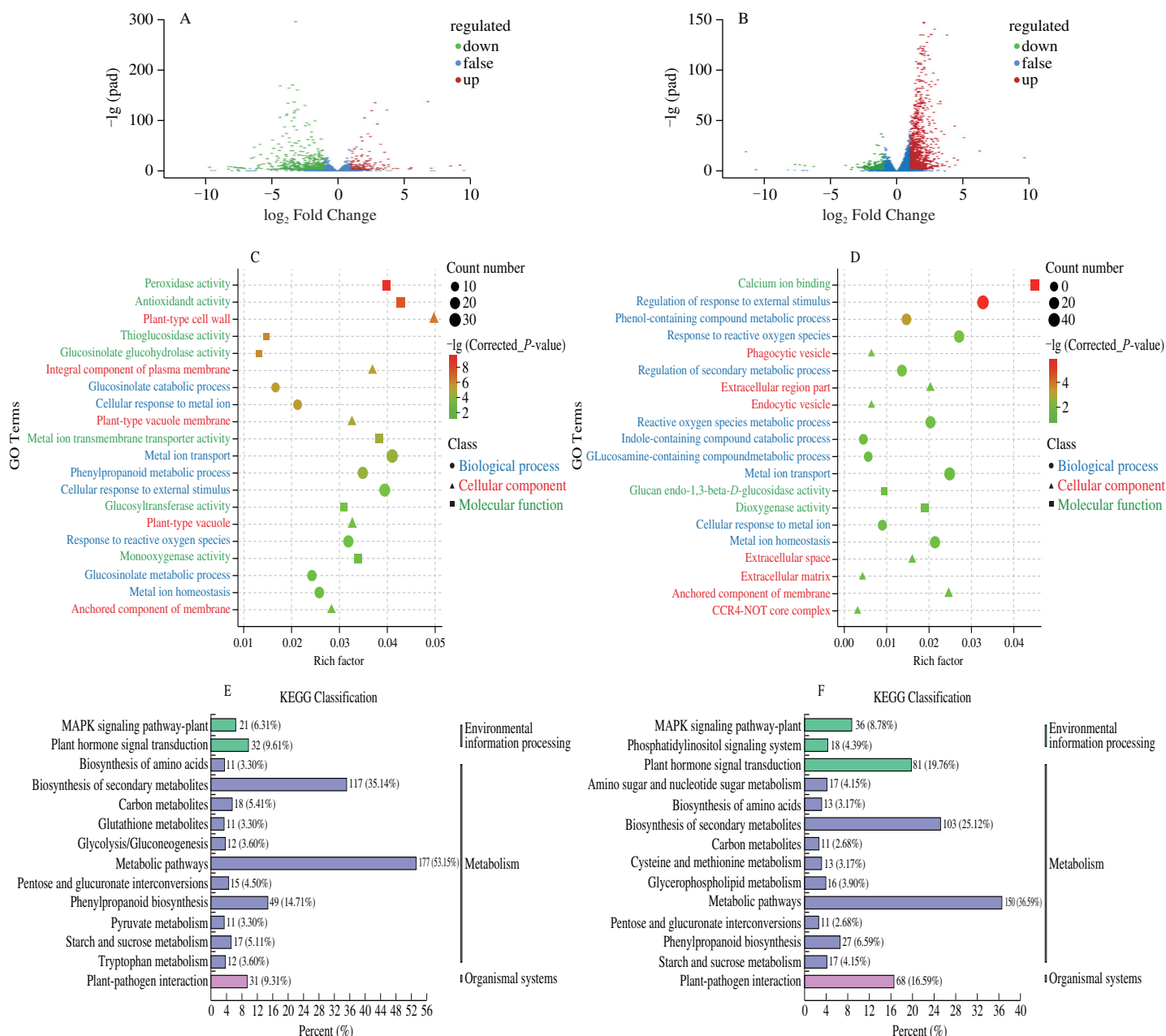
### 3.4 GO and KEGG enrichment analysis of DEGs

The DEGs were classified into 3 groups, biological process, cellular component and molecular function by GO classification and plotted as shown in Figs. 3C and D. Compared with tap water treatment, the processes of cellular response to external stimulus, metal ion transport, antioxidant and enzyme activities, and cell wall formation were significantly activated in broccoli sprouts under CHEW treatment on the 4<sup>th</sup> day. On the 8<sup>th</sup> day, regulation of response to the external stimulus was the largest group in the biological process of broccoli sprouts under CHEW treatment, followed by a response to reactive oxygen species and the metal ion transport process. The genes related to “membrane” was predominant in the group of cellular components. In the molecular function group, the genes were mostly involved in calcium ion binding.

KEGG enrichment analysis was performed on all DEGs. Compared with the tap water treatment, the DEGs of broccoli sprouts with CHEW treatment were mainly enriched in the metabolism group on the 4<sup>th</sup> day, including metabolic pathways



**Fig. 2** Preliminary analysis of metabolomics data. (A) Classification and statistical analysis of all metabolites detected. (B) Statistical data of DAMs in broccoli sprouts. (C) KEGG enrichment analysis of DRMs between TW4 and CHEW4. (D) KEGG enrichment analysis of DRMs between TW8 and CHEW8.



**Fig. 3** Preliminary analysis of transcriptomics data. (A) Volcano plots of DEGs between TW4 and CHEW4. The green dots indicate the down-regulated expressed genes (fold change,  $\log_2\text{FC} < -1$ ), the red dots indicate the up-regulated expressed genes ( $\log_2\text{FC} > 1$ ), and the blue dots indicate the non-differentially expressed genes ( $-1 \leq \log_2\text{FC} \leq 1$ ). The abscissa indicates the differential expression multiple, and the ordinate indicates the level of the gene significance. (B) Volcano plots of DEGs between TW8 and CHEW8. (C) GO enrichment results of DEGs between TW4 and CHEW4. (D) GO enrichment results of DEGs between TW8 and CHEW8. (E) KEGG enrichment analysis of DEGs between TW4 and CHEW4. (F) KEGG enrichment analysis of DEGs between TW8 and CHEW8.

and biosynthesis of secondary metabolites (Fig. 3E). On the 8<sup>th</sup> day, the main enrichment pathways changed significantly. The DEGs associated with “plant hormone signal transduction”, “plant-pathogen interaction” and “plant MAPK signaling pathway” were significantly enriched (Fig. 3F). Analysis of the DEGs involved in the three predominant pathways revealed that there were 81, 68 and 36 DEGs in plant hormone signal transduction, plant-pathogen interaction and Mitogen-activated Protein Kinase (MAPK) signaling pathway, respectively (Fig. 4A). A total of 77 DEGs were obtained after screening (Supplementary B). As shown in Fig. 4B, the DEGs of auxin signaling transduction were significantly down-regulated

while others were significantly up-regulated. Previous studies showed that the catabolites of glucosinolates, rapanusamic acid and indole-3-carbinol, inhibited auxin signaling by competing with auxin for binding to the TIR1 auxin receptor<sup>[22-23]</sup>. Therefore, the down-regulated of auxin signaling transduction might be associated with an increase in glucosinolates metabolism in broccoli sprouts under CHEW treatment.

Studies showed that the plant MAPK cascades were widely involved in plant abiotic stress responses by transmitting stress signals, activating downstream signaling pathways and the expression of related genes<sup>[24-26]</sup>. The DEGs that were significantly



**Fig. 4** Analysis of DEGs between TW8 and CHEW8 in three predominant pathways (plant hormone signal transduction, MAPK signaling pathway and plant-pathogen interaction). (A) Venn diagram of DEGs in three pathways. (B) Cluster heat map of DEGs in three pathways.

up-regulated in the MAPK signaling pathway were mainly related to ROS signal and calcium signal transduction. Among them, calcium-binding proteins (CMLs), calcium-dependent protein kinases (CDPKs) and calmodulins (CaMs) were calcium sensors, which could detect calcium signals and trigger the the specific downstream physiological process. RBOHD was a respiratory burst oxidase involved in ROS generation<sup>[27]</sup>. ROS and  $\text{Ca}^{2+}$  played important roles in the activation of serine/threonine protein kinase (oxidative signal inducible1, OXI1), which could activate the protein kinase (MAPK) cascade (MAPK3/6)

to affect plant's stress resistance<sup>[28]</sup>. The results showed that CHEW treatment first activated ROS signal and calcium signal, and then they interacted through MAPK cascades to affect glucosinolate biosynthesis in broccoli sprouts.

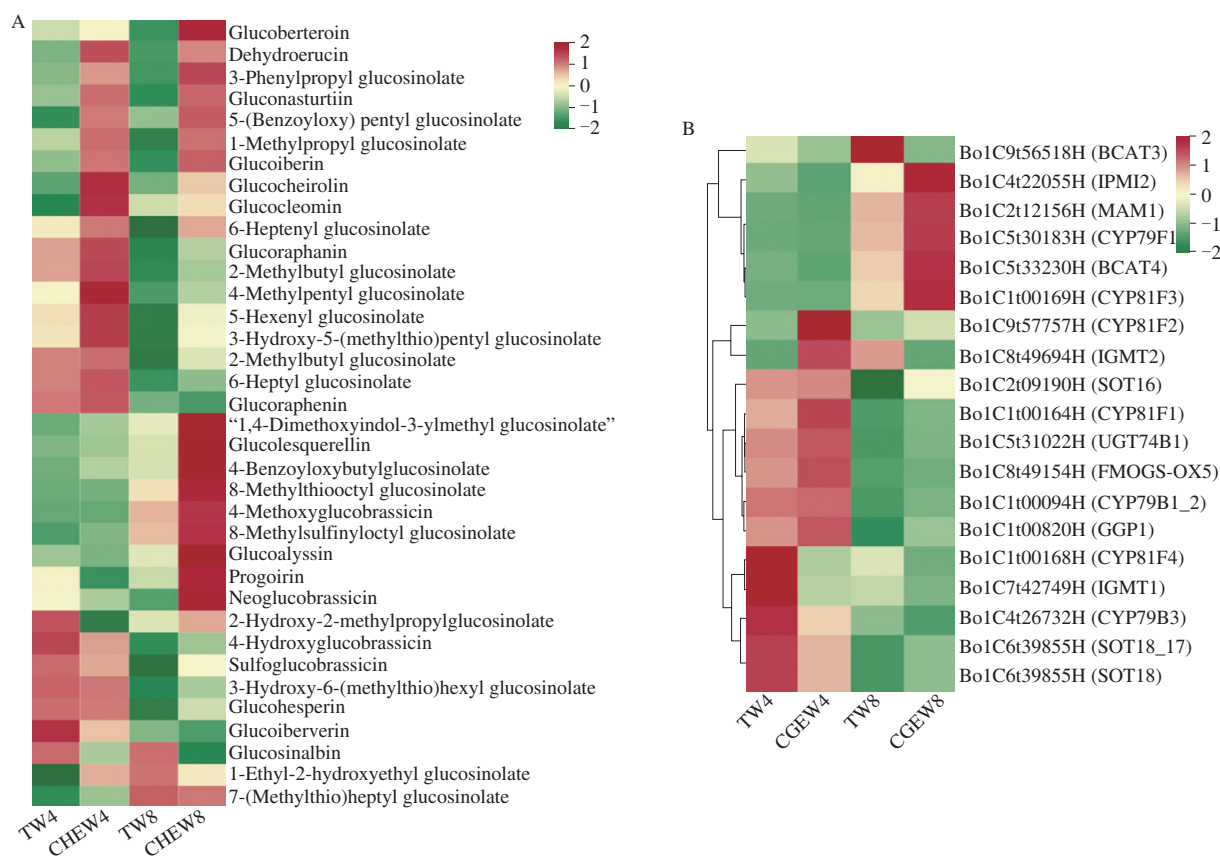
### 3.5 The metabolome and transcriptome combined analysis of glucosinolate synthesis pathways in broccoli sprouts

Among DAMs, a total of 36 glucosinolates were identified,

including 26 aliphatic glucosinolates, 5 indole glucosinolates (neoglucobrassicin, 4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, 1,4-dimethoxyindol-3-ylmethyl glucosinolate, sulfoglucobrassicin) and 5 aromatic glucosinolates (gluconasturtiin, glucosinalbin, 3-phenylpropyl glucosinolate, 4-benzoyloxybutyl glucosinolate, 5-(benzoyloxy) pentyl glucosinolate) (Supplementary C). The aliphatic glucosinolate-glucoraphanin had the highest glucosinolate content in broccoli sprouts, accounting for more than 50% of the total glucosinolate content<sup>[29]</sup>. Our study also confirmed this (Table S3). As shown in Fig. 5A, the contents of most glucosinolates in broccoli sprouts with tap water treatment showed a decreasing trend from day 4 to day 8. Compared with the tap water treatment, CHEW treatment significantly increased the contents of aliphatic glucosinolates on the 8<sup>th</sup> day. In addition, the glucoraphanin content in the CHEW treatment was consistently higher than that in the tap water treatment during the growth of broccoli sprouts. The results indicated that CHEW can be a useful tool for enhancing the content of secondary metabolites (glucosinolates, flavonoids, phenolic acids, etc.) and calcium in broccoli sprouts intended for fresh consumption as a functional food.

To explore the difference in glucosinolate biosynthesis between tap water treatment and CHEW treatment during the growth of broccoli sprouts, DEGs in the glucosinolate synthesis pathway were identified. A total of 19 structural genes related to glucosinolate biosynthesis were screened from all DEGs and the expression of them were represented in the cluster heat map (Fig. 5B).

The process of glucosinolate biosynthesis was shown in Fig. 5C. Methionine (Met) was extended by a branched-chain amino acid aminotransferase *BCAT4* to generate the 2-oxo acid. Then the 2-oxo acid underwent a continuous multi-step reaction including condensation with acetyl-CoA by a methylthioalkylmalate synthase *MAM1* and isomerization by an isopropylmalate isomerase *IPMI2*. The product was transaminated by a *BCAT3* to chain-elongated Met and entered the core glucosinolate structure pathway<sup>[30]</sup>. The DEGs involved in the formation of the glucosinolate core structure mainly included cytochromes P450 of the CYP79 family (*CYP79F1*, *CYP79B1-3*),  $\gamma$ -glutamyl peptidase *GGP1*, glucosyltransferases *UGT74B1* and the sulfotransferases *SOT16-18*. The methylthioalkyl glucosinolates were obtained after the formation of aliphatic glucosinolates' core structure, and then were oxidized by the flavin monooxygenase *FMO<sub>GS-OX</sub>* to generate methylsulfinylalkyl glucosinolates. Besides, the side chains of indole glucosinolates were derived from tryptophan (Trp), and CYP81F (1-4) could catalyze glucobrassicin (GBS) to form 4-hydroxyglucobrassicin (4OHGBS) and 1-hydroxy-glucobrassicin (1-hydroxy-I3M). Then they were catalyzed by *O*-methyltransferases *IGMT1* and *IGMT2* to form methoxyglucobrassicin (4MOGBS) and 4-neoglucobrassicin (nGBS), respectively<sup>[31-32]</sup>. Phenylalanine (Phe) is the precursors of aromatic glucosinolates biosynthesis<sup>[33]</sup>. During the growth of broccoli sprouts, the DEGs related to methionine side chain elongation were significantly up-regulated while other structural DEGs were significantly down-regulated. Besides, compared with



**Fig. 5** Combined metabolome and transcriptome analysis of glucosinolate synthesis pathways. (A) Cluster heat map of glucosinolates of different treatments. (B) The heatmap of structural DEGs in glucosinolate biosynthesis pathway according to the FPKM value. (C) The identification of structural DEGs in glucosinolate biosynthesis pathway. (D) RT-qPCR validation of 6 genes (*BCAT4*, *MAM1*, *UGT74B1*, *SOT18*, *SOT16*, *CYP81F1*, respectively) related to glucosinolate synthesis of broccoli sprouts.

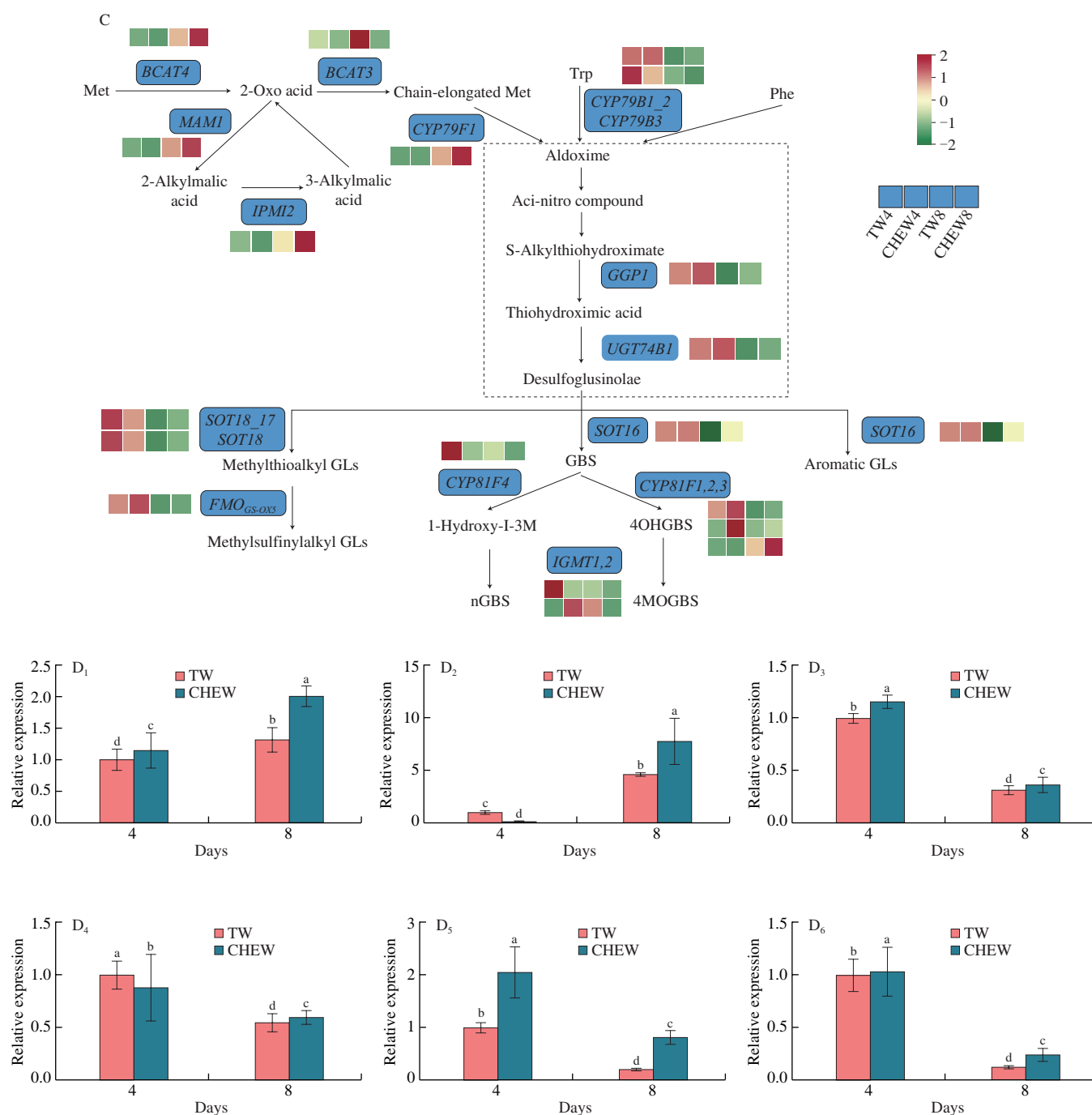


Fig. 5 (Continued)

the tap water treatment, CHEW treatment could significantly increase the expression of most structural DEGs, which is consistent with the metabolome results. The results indicated that CHEW treatment promoted glucosinolate biosynthesis mainly by enhancing the expression of DEGs related to the formation of glucosinolate' core structure and secondary modification of glucosinolate side chain.

RT-qPCR was used to analyze the expression levels of 6 structural genes related to glucosinolate biosynthesis in broccoli sprouts (Fig. 5D). The results showed that the expression patterns of these genes were consistent with the RNA-seq results, indicating that the RNA data are valid and reliable.

### 3.6 The analysis of glucosinolates biosynthesis regulation network in broccoli sprouts under CHEW treatment

Transcription factors are important regulators of glucosinolate accumulation and typically act by regulating the expression of structural genes in the glucosinolate biosynthesis pathway. By searching the transcriptome annotation results, a total of 663 transcription factors in all DEGs were identified in this study. As shown in Fig. 6A, most of transcription factors belonged to AP2/ERF-ERF, bHLH, MYB, NAC, WRKY and C2H2 families. Previous studies showed that MYB, bHLH and WRKY transcription

**Table 1**  
Differentially expressed transcription factors involved in glucosinolates biosynthesis.

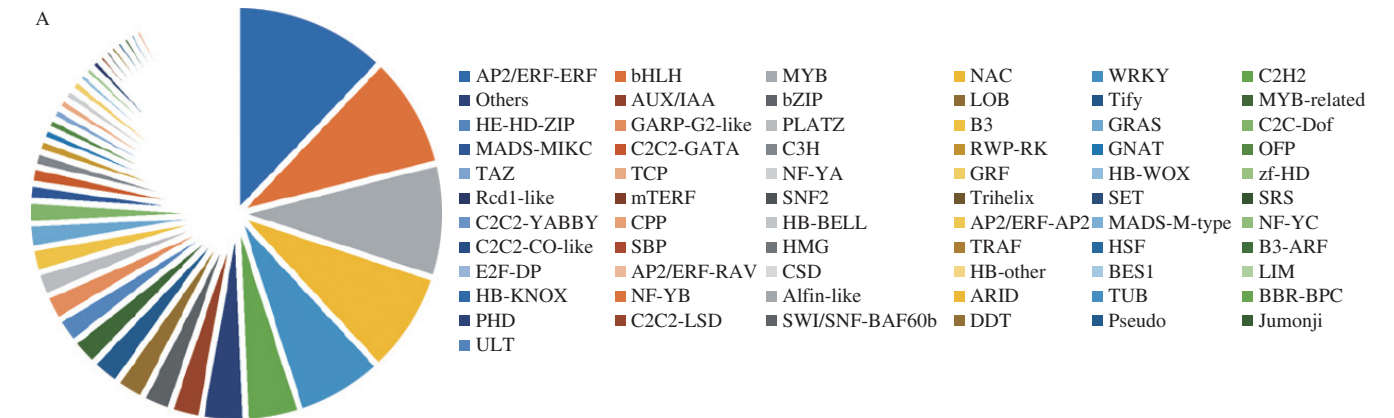
| Transcription factor | Gene ID      | Annotation                                                       | Correlation with total glucosinolates |
|----------------------|--------------|------------------------------------------------------------------|---------------------------------------|
| MYB                  | BolC4t26826H | PREDICTED: transcription factor MYB44                            | 0.956                                 |
|                      | BolC3t14774H | PREDICTED: encoded by the Myb-like transcription factor MYB38    | -0.959                                |
|                      | BolC5t30384H | Encodes a member of the R2R3-MYB transcription family, MYB51     | -0.989                                |
| bHLH                 | BolC7t45018H | Basic helix-loop-helix (bHLH) phytochrome interacting factor     | 0.993                                 |
|                      | BolC7t43554H | Transcription factor bHLH28                                      | 0.952                                 |
|                      | BolC7t45916H | PREDICTED: transcription factor bHLH118                          | -0.978                                |
| AP2/ERF-ERF          | BolC9t57181H | Ethylene-responsive transcription factor CRF3-like               | 0.973                                 |
|                      | BolC2t07990H | PREDICTED: ethylene-responsive transcription factor CRF3-like    | 0.966                                 |
|                      | BolC6t37435H | PREDICTED: AP2-like ethylene-responsive transcription factor SMZ | 0.954                                 |
| NAC                  | BolC5t34163H | PREDICTED: NAC transcription regulator, NAC1L                    | -0.970                                |
|                      | BolC3t19734H | NAC transcription regulator                                      | -0.986                                |
|                      | BolC3t16873H | NAC transcription regulator, NAC1L                               | -0.997                                |
| WRKY                 | BolC6t39245H | PREDICTED: member of WRKY Transcription Factor, WRKY9            | -0.966                                |
| C2H2                 | BolC7t43095H | PREDICTED: zinc finger protein AZF3                              | 0.973                                 |

factors were important factors in the biosynthesis of glucosinolates in cruciferous plants, and were also involved in plant secondary metabolism, signal transduction, and stress response<sup>[34-36]</sup>. To understand the regulation of transcription factors, the correlation tests between the expression levels of these transcription factors and the total glucosinolate content were carried out. Based on the results (Table 1), a total of 14 key transcription factors were highly associated with the glucosinolate content ( $|r| \geq 0.95$ ,  $P < 0.05$ ). There were 7 negatively correlated transcription factors (*WRKY9*, *bHLH118*, *MYB38*, *MYB51* and 3 NAC family genes) and 7 positively correlated transcription factors (*MYB44*, *AZF3*, 2 bHLH family genes and 3 AP2/ERF-ERF family genes), indicating that they played important roles in glucosinolate biosynthesis of broccoli sprouts under CHEW treatment.

To further understand the regulatory network of glucosinolates biosynthesis in broccoli sprouts with CHEW treatment, we carried out a correlation analysis between the changes in glucosinolates and transcripts of genes. 16 glucosinolates (12 aliphatic glucosinolate: glucoraphanin, 2-methylbutyl glucosinolate, 4-methylpentyl glucosinolate, 3-methylbutyl glucosinolate, 6-heptyl glucosinolate, glucoraphenin, 8-methylsulfinyloctyl glucosinolate, glucoalyssin, 3-hydroxy-6-(methylthio)hexyl glucosinolate, glucohesperin, glucolesquerellin, glucoiberberin; 4 indole glucosinolates),

10 structural genes (*BCAT4*, *CYP79F1*, *CYP81F1*, *CYP81F3*, *CYP79B1\_2*, *CYP79B3*, *GGP1*, *UGT74B1*, *SOT16*, *FMO<sub>GS-OXS</sub>*) showing higher correlation coefficient values ( $|r| \geq 0.9$ ,  $P < 0.05$ ) and 10 transcription factors were obtained to organize an interaction network (Fig. 6B). Glucosinolate synthesis in broccoli sprouts under CHEW treatment is mainly regulated by structural genes and transcription factors. Most of transcription factors affect glucosinolate biosynthesis by regulating the expression of structural genes. The results further clarified the regulatory networks of glucosinolate synthesis in broccoli sprouts under CHEW treatment. However, it still needs further molecular biology experiments to confirm.

Studies have found that ROS, Ca<sup>2+</sup> and key molecules such as MAPK, transcription factors (WRKY, bHLH and MYB family, etc.) have obvious interactions<sup>[20,37]</sup>. The MAPK cascade is an important class of molecules for signal reception, transduction and delivery into the nucleus<sup>[38]</sup>. The mechanism of CHEW treatment-induced glucosinolate biosynthesis in broccoli sprouts was illustrated in Fig. 6C. The oxidative components (HClO, ClO<sup>-</sup>) and calcium of CHEW elicited the stress response in broccoli sprouts, and ROS and calcium signaling transduction pathways were activated. Then, ROS and calcium signals might interact through the MAPK cascade pathway, and promote the biosynthesis of amino acid and expression



**Fig. 6** The analysis of glucosinolates biosynthesis regulation network in broccoli sprouts. (A) Classification and statistical analysis of all transcription factors detected. (B) The interaction network between glucosinolates and genes (structural DEGs and transcription factors). (C) Schematic overview of the mechanism of CHEW treatment regulating glucosinolate biosynthesis in broccoli sprouts.

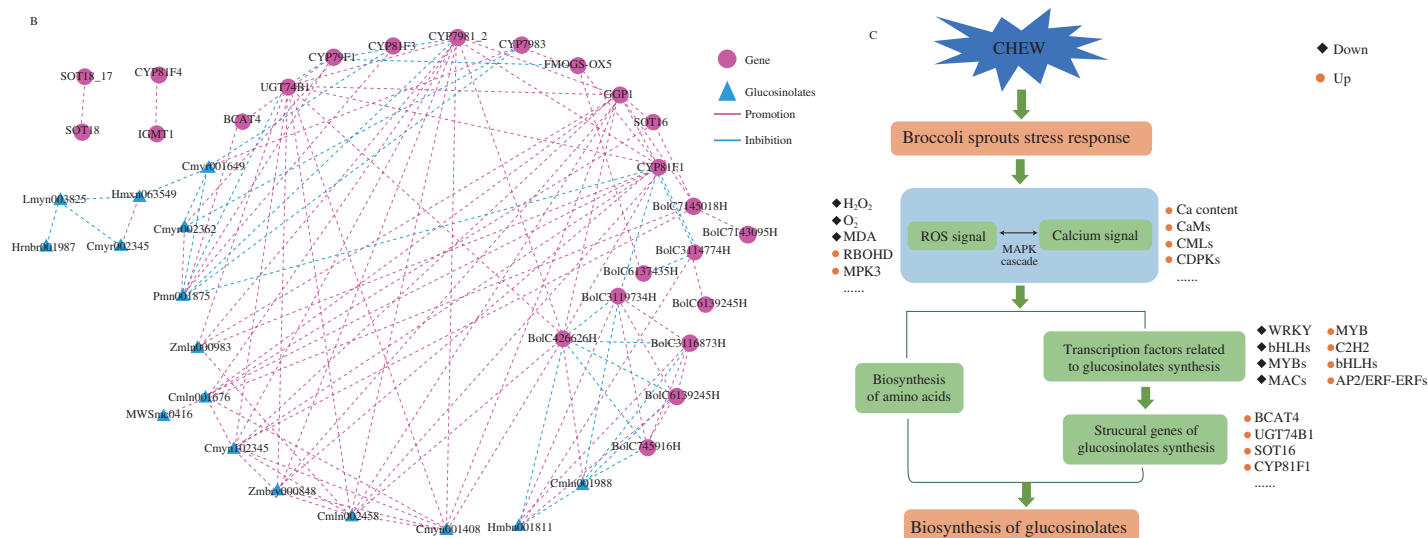


Fig. 6 (Continued)

of transcription factors related to glucosinolate biosynthesis. Finally, the transcription factors promoted the expression of structural genes of glucosinolate synthesis to affect the glucosinolate biosynthesis in broccoli sprouts.

#### 4. Conclusion

Our results showed that CHEW treatment significantly decreased ROS and MDA contents in broccoli sprouts. Compared to the tap water treatment, the calcium content in broccoli sprouts treated with CHEW on day 8 was dramatically enhanced from 14.4 mg/g DW to 22.7 mg/g DW. In addition, the total glucosinolate content of broccoli sprouts with CHEW treatment on day 4 and day 8 increased by 10.8% and 10.6%, respectively. The metabolome analyses implied that CHEW treatment might affect the biosynthesis of glucosinolate in broccoli sprouts by promoting the biosynthesis of cofactors and amino acids, the precursors of secondary metabolites. Transcriptome analyses revealed that CHEW treatment activated the ROS and calcium signaling transduction pathways in broccoli sprouts, and they interacted through MAPK cascades. Transcription factors affected glucosinolate biosynthesis by promoting the expression of structural genes related to glucosinolate synthesis. The current study provides new insights into the molecular mechanisms of  $\text{CaCl}_2$ -HCl electrolyzed water-induced glucosinolate biosynthesis and provides useful information on the development of effective methods to improve the nutritional quality of broccoli sprouts.

#### Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2022.9250068>.

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