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Combining transcriptomics and metabolomics to analyse the mechanism of allelopathy in *Cyclachaena xanthiifolia*

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Abstract

As a vicious invasive plant, *Cyclachaena xanthiifolia* has caused severe ecological disruption and significant reductions in crop yield, necessitating urgent control measures. However, the underlying mechanisms of its allelopathic invasion remain unclear, representing the primary bottleneck in current management strategies. In this study, we used metabolomic and transcriptomic analyses to evaluate the differences in allelopathy and related physiological and biochemical indices among different extract fractions of *C.xanthiifolia*, and to investigate how the allelopathy of *C.xanthiifolia* inhibits seed germination and seedling growth by altering metabolic pathways. GC-MS results identified several compounds with allelopathic potential, including fatty acids, terpenes, esters, alkanes, and aldehydes. Among them, n-butanol phase extract (NE) treatment significantly inhibited the germination and water absorption of mustard (*Brassica juncea*) seeds, changed the balance of the endogenous hormones abscisic acid (ABA) and gibberellins (GA) in seeds, destroyed the antioxidant enzyme system, and caused plasma membrane damage. Moreover, transcriptomic and broadly targeted metabolomic analyses showed that NE treatment interfered with primary metabolism, significantly enriched the carotenoid biosynthetic pathway, and led to a significant accumulation of ABA. The quantitative real-time PCR (qRT-PCR) results showed that the expression levels of 7 key genes involved in ABA biosynthesis and metabolic pathways were relatively high. The results showed that *C.xanthiifolia* may exert its allelopathic effects by disrupting the antioxidant enzyme system and interfering with primary metabolism and hormone signalling, and that the modulation of the ABA signalling pathway appears to play a key role.

Keywords *Cyclachaena xanthiifolia*, Seed germination, Allelopathy, Carotenoid biosynthesis, ABA signalling pathway

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Introduction

The emergence and spread of invasive alien plant species is a global phenomenon that poses a significant threat to the biodiversity of plants and the stability of ecosystems [1–4]. Some studies have shown that the invasion mechanism of alien species is related to their allelopathic effects, and allelopathic effects constitute one of the mechanisms for the successful invasion of certain alien plants, which play an important role in their rapid dominance of ecological advantages [5, 6]. Studies of their allelopathic effects can provide a scientific basis for the prevention and control of invasive plants [7, 8].

Cyclachaena xanthiifolia is an annual herbaceous plant of the genus *Cyclachaena* in the *Asteraceae* family that adapts to its environment, reproduces rapidly and competes strongly with surrounding plants, thus threatening ecological diversity [8–10]. In recent years, some studies have been conducted on the allelopathy of *C.xanthiifolia*. Wang et al. [11] found that extracts from different parts of *C.xanthiifolia* have allelopathic effects on seed germination and seedling growth of receptor plants. Among the different plant tissues, leaves are the organs that cause the most allelopathy [12]. In addition, the seed germination and seedling growth of *Brassica juncea*, *Brassica oleracea*, *Amaranthus tricolor* and *Setaria viridis* can be inhibited by leaf extracts at low concentrations [12, 13]. Most of the existing reports on the allelopathy of *C.xanthiifolia* are aimed at identifying its potential allelopathy and describing the biological and physiological phenomena produced by receptor plants. Compared with other invasive plants, studies of this species are in the initial stage, and its underlying allelopathic inhibitory mechanism has not been fully explored and understood. Systematic research on its allelopathy and invasion mechanisms is urgently needed.

Currently, invasive alien plants such as *Asteraceae* are believed to exert allelopathic effects [13], which can affect the seed germination and seedling growth of receptor plants through the release of allelochemicals, as well as by altering the endogenous hormones and physiological biochemistry of the plant [14, 15]. Moreover, considerable variation in the allelopathic effects on different types of receptor plants occur during different growth periods and in the organs and concentrations of plants in studies of allelopathic effects [16–19]. Water is the basis of all life activities, and one of the mechanisms by which allelopathic effects inhibit plant growth is to cause water stress, inhibit the uptake and use of water by plant cells, and interfere with various physiological and biochemical processes so that growth is impaired [20, 21]. Plant seed germination and seedling growth are regulated by their own hormones to adapt to the environment, which in turn affects plant growth and development, physiology, biochemical levels [22]. Shan et al. found that the balance of endogenous plant hormones, such as GA and ABA, was disturbed and that the growth of *Medicago sativa* seedlings was inhibited after powdered *Euphorbia jolkinii* was added to the seedlings [23]. Sánchez et al. [24] reported that plants are subjected to stress caused by allelopathic effects, often due to cell destruction and changes in membrane permeability, causing oxidative stress in plants, the inhibition or promotion of antioxidant enzyme activity, and changes in the antioxidant capacity. The main enzymes of the antioxidant system include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and malondialdehyde (MDA) [25, 26]. Allelopathy can also affect the accumulation and transformation of stored substances in the recipient plant [27]. Moreover, the physiological and biochemical changes caused by allelopathy activate the regulation of gene expression or protein synthesis in the recipient plant and change the gene expression pattern in the body to generate a series of responses [28, 29].

The combination of metabolomics and transcriptomics is widely used to study the allelopathy mechanisms of invasive alien plants [30–32]. The joint analysis of its metabolome and transcriptome to understand the differentially metabolites between different samples will be more conducive to identify the relevant differences in gene expression patterns and determine the key genes, signalling pathways, and modes of regulation [33–35] and then explore the molecular nature of the metabolic regulation of the plant and alter signalling in response [36]. Li et al. [37] reported that hormone levels changed when rice (*Oryza sativa*) plants were treated with a water extract of *Artemisia argyi*. Compared with those in the control group, the expression levels of the growth hormone pathway-related gene *IAA1* were significantly increased following a 0.05 g/mL treatment, the expression of the key GA synthesis gene *GA20* first decreased but then increased, and the expression levels of ABA associated with the auxin pathway-related gene *NCED4* and the ABA receptor gene *PYR* were similar to those of the genes involved in the auxin signalling pathway, indicating that the poor growth of *Oryza sativa* plants after allelopathy caused by the use of *Artemisia argyi* is associated with significant changes in the expression levels of key genes concerned in the plant hormone signalling pathway.

In this study, we used the leaves of *C.xanthiifolia* as experimental materials, extracted and separated them by the systematic solvent method, and initially analysed the differences in allelopathy among different components extracted from the sensitive receptor plant mustard. In addition, we analysed the physiological and biochemical effects of allelopathic components on mustard seed germination by measuring water uptake dynamics, differences in germination, and physiological and

biochemical parameters. Moreover, we further explored the major metabolic pathways and associated differentially expressed genes (DEGs) associated with allelopathic effects using combined transcriptomic and metabolomic analyses. This method allowed us to further explore the mechanism underlying the response of recipient plants to allelopathy by *C.xanthiifolia* at the physiological and molecular levels, which provides a theoretical basis for the prevention and control of *C.xanthiifolia* and a technical reference for protection of the environment and agricultural production.

Materials and methods

Plant materials and sampling

In August 2024, *C.xanthiifolia* leaves (well-developed, fresh, pest-free and from the blooming period) were collected from our Northeast Agricultural University Experimental Field, Harbin City, Heilongjiang Province, China (45°74'N, 126°72'E, altitude, 172.4; annual precipitation, 524.13 mm; lowest temperature, 21 °C; and highest temperature, 33 °C). These leaves were washed, dried and prepared for laboratory experiments. We washed the surface of the leaves with distilled water and left them to dry in a room without light. After drying, the leaves of *C.xanthiifolia* were cut into small segments of less than 2 cm, crushed with an FD15-T-1000 A pulveriser (Trade Inc., Shengzhou, China), passed through a 40-mesh sieve (ZhenXing Inc., Guangzhou, China), and stored in a closed and dry environment for use. Seeds of the recipient mustard plant were preserved at the Department of Botany, College of Life Sciences, Northeast Agricultural University.

Obtaining extracts of *C.xanthiifolia* prepared using different solvents

A total of 840 g of *C.xanthiifolia* was extracted three times with 60% ethanol (Yongda Chemical Reagent Co. Ltd., Tianjin, China) at a material-liquid ratio of 1:30 (g/mL). After standing for 24 h, the combined filtrate was obtained by filtration using a circulating water vacuum pump (Gongyi Yuhua Instrument Co. Ltd., Henan, China) and concentrated with a rotary evaporator (Gongyi Yuhua Instrument Co. Ltd., Henan, China) to afford a black-green paste (alcoholic extracts of *C.xanthiifolia* leaves) weighing 272.99 g and with a 32.50% yield. Subsequently, 1 g of the leaf alcohol extract was dissolved in 100 mL of distilled water for extraction and separation [38] and successively extracted three times with 100 mL of chromatographically pure petroleum ether (Aladdin Biochemical Technology Co. Ltd., Shanghai, China), chromatographically pure ethyl acetate (Fuyu Fine Chemical Co. Ltd., Tianjin, China) and chromatographically pure n-butanol (Tianli Chemical Reagent Co. Ltd., Tianjin, China) and first separated into a petroleum ether phase extract (PE), ethyl acetate phase extract (EE) and NE, and the remaining components were the water phase extract (WE). After the 4 extract components were obtained, the solvents of each extract component were concentrated under reduced pressure and sonicated and water was added to 100 mL to obtain aqueous solutions of the extracts prepared using different solvents with a concentration of 0.01 g/mL, which were stored at 4 °C until use.

GC-MS analysis of extracts prepared using different solvents

The four extracts obtained from the PE, EE, NE and WE extracts obtained from 1 g of alcoholic extracts according to the method described in Sect. 2.2 were concentrated under reduced pressure, dissolved by adding chromatographically pure petroleum ether, chromatographically pure ethyl acetate, chromatographically pure n-butanol and chromatographically pure methanol, respectively, and passed through a 0.22 μm filter membrane for GC-MS (Agilent Technologies Inc, Heilongjiang, China). An Agilent 7890 A AC/5975 C GC/MSD system was used (Santa, Clara, CA). The GC-MS analysis [13] was performed using an HP-5MS chromatographic column with a capillary column (30.0 m×250 μm×0.25 μm) loaded with high-purity helium gas at a flow rate of 1.0 mL/min and an injection volume of 1 μL, followed by programmed heating: a constant temperature of 65℃ for 2 min, constant temperature of 90℃ for 3 min, constant temperature of 103℃ for 3 min, constant temperature of 150℃ for 15 min. The samples were injected at 250℃ with no shunting, and an injection volume of 1 μL was used, followed by analysis at a 1,435 V multiplier voltage at an interface temperature of 280℃, an ion source temperature of 230℃, EI ionization mode, an electron energy of 70.1 ev, a 3 min solvent delay and a scanning mass range of 45–550 amu. The relative contents of the measured compounds in the different solvent extracts of *C.xanthiifolia* were determined by searching and comparing the standard mass spectrometry databases NIST08.LIB and NIST08, which utilize the peak area normalization method to search for substances with 80% or more accuracy. Database comparisons were performed to determine the compositions of the extracts.

Allelopathic activity determination of extracts of *C.xanthiifolia*

Mustard seeds of uniform size and fullness were selected, prepared in 9 cm diameter Petri dishes, washed, sterilized and dried. The mustard seeds of the test crops were disinfected with 1% NaCIO (McLean, Inc. Virginia, USA) for 10 min and washed three times with distilled water. Using the Petri dish filter paper method [39–41], 50 seeds were placed in each 9 cm diameter Petri dish,

and 10 mL of aqueous solution of the different solvent extracts obtained as described in Sect. 2.3 was added to the Petri dish to treat mustard seeds with an equal volume of distilled water treatment as the CK group, and three biological replicates were performed for each treatment. The seedlings were incubated in an incubator (Brosun Medical Biological Instruments Co. Ltd., Shanghai, China) at 25 °C, 4000 lx, 70% humidity, 16 h of light and 8 h of dark for 7 d [13]. The number of germinated seeds was recorded at 1 d intervals (with 1–2 mm long radicle protruding from the seed coat) [40, 42–43], the germination potential was calculated at 5 d, and the germination rate and chemosensory index were calculated at 7 d. Ten mustard seedlings were randomly taken out for measuring and counting of their seedling height, root length and fresh weight [44, 45].

Determination of water absorption, germination and physiological indices of mustard seeds treated with NE

Mustard seeds were sterilized and treated as described in Sect. 2.4. Twenty mustard seeds were placed in a 9 cm diameter Petri dish with 7 mL of the NE aqueous solution for treatment. CK was the H_2O treatment, and three replicates were established. The culture was incubated at 25 °C, 4000 lx, 70% humidity, 16 h of light and 8 h of darkness at a constant temperature. The seeds were immersed sequentially for 1, 2, 3, 4, 6, 8, 10, 12, 18, 24, 30, 36, 42 and 48 h, after which the raw weight of the mustard seeds was recorded, and water absorption was calculated [21]. The quantity of germinated seeds was observed and recorded after treatment for 6, 12, 18, 24 and 48 h, and the rate of germination was measured within 48 h [18]. The water absorption characteristics and germination capacity of the seeds were assessed using the International Rules for Seed Testing methods [46]. The physiological indices were the same as those of seeds under the same conditions of water absorption and germination. At 6, 12, 18, 24 and 48 h, the contents of ABA, GA, and soluble sugar (SS) and the activities of SOD, POD, CAT, MDA, and α-amylase were detected. ABA and GA contents were examined using ml077235 and ml103716 assay kits (Enzyme Biotechnology Co. Ltd., Shanghai, China; https://www.mlbio.cn/goods-77235.html), respectively, according to the manufacturer's instructions. SOD, POD, CAT, MDA and α-amylase activities, and SS contents were examined using G0101W, G0107W, G0105W, G0109W, G0510W and G0502W assay kits (Grace Biotechnology Co. Ltd., Suzhou, China; https://www.mlbio.cn/goods-77235.html), respectively, according to the manufacturer's instructions.

Transcriptomics and metabolomics of NE-treated mustard seeds

Mustard seeds were sterilized and treated as described in Sect. 2.4. Mustard seeds were sampled after 18 h of germination and treated with an aqueous solution of NE extract, CK refers to the water treatment, with three replicates for each treatment. The three replicates of NE extract-treated mustard seeds were named NE-1, NE-2, and NE-3, respectively. The three replicates of CK-treated mustard seeds were named CK-1, CK-2, and CK-3, respectively. 1 g of sample was taken, wrapped in tinfoil, rapidly frozen with liquid nitrogen, and placed in a -80℃ freezer for storage. Transcriptome sequencing and broad-targeted metabolomic assays were subsequently by Wuhan Meitville Biotechnology Co.

RNA-Seq analysis

Mustard seeds were sampled after 18 h of germination by treating them with NE and sterile water, respectively, and three biological replicates were performed. Total RNA was extracted from mustard samples using CTAB-PBIOZOL (Invitrogen, California, USA). The RNA concentration and integrity were measured using a Qubit 4.0 fluorescence quantifier (Invitrogen, California, USA) and a Qsep400 high-throughput biofragment analyser (BiOptic Inc., Jiangsu, China), respectively. Then, RNA integrity and quality were measured using 1% agarose gel electrophoresis. Taking advantage of the structural feature that most of the eukaryotic mRNAs have polyA tails, mRNAs with polyA tails were enriched by Oligo(dT) magnetic beads; the purified mRNAs were cleaved into small fragments with fragmentation buffer at the appropriate temperatures; first-strand cDNAs were generated by reverse transcription using a randomized hexamer primer; and second-strand cDNAs were synthesized via the addition of buffer, dNTPs, RNase H, and DNA polymerase I to synthesize the second cDNA strand. The purified double-stranded cDNA was then end-repaired, A-tail ligated, and ligated to the sequencing junction, and a cDNA library of 250–350 bp insert fragments was subsequently obtained by DNA bead purification and fragment selection. The libraries were sequenced on the Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA) by Wuhan Metwel Biotechnology Co. Prior to data analysis, the raw data generated from high-throughput sequencing first required strict quality control using fastp based on the filtering criteria to trim splices, filter low-quality data, and obtain clean reads (Tab. S1 and S2). Clean reads were aligned to the reference genome (Mustard genome as a reference genome) using HISAT2 (Tab. S3). Gene count values for genes were normalized to FPKM (fragments per kilobase of transcript per million mapped fragments) using Stringtie. Principal component analysis was plotted using the toolkit PCAtools (https://githu

b.com/kevinblighe/PCAtools). Using DESeq2 software, the number of counts of each sample gene was normalized by FPKM values, and the screening conditions were set as |log2Fold Change| ≥ 1 and a false discovery rate (FDR) < 0.05 for differentially expressed genes. The differentially expressed genes obtained from the screen were assessed by performing Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses using TBtools [50]. In this study, the mustard genome was used as the reference genome and was downloaded from https://plants.ensembl.org/Brassica_juncea/Info/Index.

Metabolomic data analysis

Mustard seeds were treated as described in Sect. 2.6, mustard seeds were ground to a powder in liquid nitrogen, and 50 mg of the sample powder was weighed. Following this, 1.2 mL of a 70% methanol-water internal standard extract, with L-2-chlorophenylalanine as the internal standard and precooled at -20 °C, was added to the sample. The mixture was vortexed for 30 s every 30 min, and this was repeated process for a total of six cycles to ensure thorough mixing. Following the mixing procedure, the mixture was centrifuged at 12,000 rpm for 3 min to separate the supernatant. The resulting supernatant was then carefully collected and filtered through a 0.22 μm microporous membrane into a liquid vial, after which it was prepared for analysis via ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The HPLC conditions were an Agilent SB-C18 chromatographic column (1.8 μm, 2.1 mm × 100 mm); mobile phase: ultra-pure water in A-phase (with 0.1% formic acid added), acetonitrile in B-phase (with 0.1% formic acid added). The mobile phases were: phase A was ultrapure water (with 0.1% formic acid added), phase B was acetonitrile (with 0.1% formic acid added); elution gradient: phase B was 5% at 0.00 min, phase B increased linearly to 95% at 9.00 min and remained at 95% for 1 min, and then decreased to 5% at 10.00–11.10 min and equilibrated at 5% for 14 min; flow rate was 0.35 mL/min; column temperature was 40 °C; injection volume was 2 μL. The MS conditions used were as follows: electrospray ionization (ESI) temperature 550 °C; ion spray voltage (IS) 5500 V (positive ion mode) / -4500 V (negative ion mode); ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set to 50, 60, and 25 psi, respectively; and the collision-induced ionization parameter was set to high. QQQ scans were performed in MRM mode, and the collision gas (nitrogen) was set to medium. Declustering potential (DP) and collision energy (CE) of individual MRM ion pairs were accomplished by further DP and CE optimization. A specific set of MRM ion pairs was monitored in each period based on the metabolites eluted within each period. Peak identification, integration, retention time correction, peak alignment and normalization were performed on the raw HPLC-MS data using Progenesis QI v2.3 software (Nonlinear Dynamics, Newcastle, UK). The parameter settings were slightly different, and the metabolites were annotated using the MWDB metabolite database. The overall metabolites analysed were statistically visualized by classification, and a volcano plot was constructed to screen out the differentially metabolites between the different treatment groups. Differentially expressed metabolites (DEMs) between the two groups were identified based on the variable importance in projection (VIP ≥ 1) value and the absolute log2 fold change (|Log2FC| ≥ 2 or ≤ 0.5) metrics. VIP values were extracted from the OPLS-DA results. Metabolite classification was performed via pie3D (https://github.com/puxiao/pie-3d). Enrichment bubble plots were created using the ggplot2 toolkit (https://github.com/tidyverse/ggplot2).

Joint pathway analysis of the transcriptome and broadly targeted metabolomic analysis

A nine-quadrant joint analysis was performed using TBtools software to further elucidate the effects of NE-induced allelopathy on ABA synthesis and metabolic pathways [47]. Data with $R > 0.9$ and $P < 0.01$ were selected for visualization, and correlations between transcriptomic and metabolomic data were analysed. A KEGG pathway enrichment analysis was performed using the Venn toolkit (https://github.com/dusadrian/venn), and histograms were plotted using the ggplot2 toolkit (https://github.com/tidyverse/ggplot2) to obtain public annotation significance statistics. Heatmaps were generated with the pheatmap package, and the KEGG database was utilized to annotate and enrich differentially metabolites, identify DEMs in the enriched pathways, and analyse the effects of allelopathy on ABA synthesis and metabolic pathways.

QRT-PCR analysis

Seven genes related to key enzymes involved in ABA synthesis and metabolism that were screened were selected for qRT−PCR verification of the accuracy of the results. Total RNA was extracted from mustard seeds using the Plant Rapid RNA Extraction Kit (TransGen Biotech, Beijing, China). The RNA was detected using a Qubit 4.0 Fluorescence Quantimeter (Invitrogen, California, USA). The RNA concentration of 600 ng/mL met the quality standard. RNA was used as a template and reverse transcribed into cDNA using a reverse transcription kit (TransGen Biotech, Beijing, China) to determine the relative transcript levels of DEGs. The primers were designed using the online software NCBI Primer Blast, the internal reference gene was *Bj25SrRNA*, the primers were designed based on the gene sequence information, the

primers used are shown in Tab. S4, and primer synthesis was performed by Savin Innovations Beijing Bio-technology Co. qRT-PCR was performed using a fluorescent quantitative PCR kit (Invitrogen, California, USA). For qRT-PCR, a 20 μL reaction system consisting of 10.0 μL of 2 × SYBR qPCR mixture, 0.8 μL of gene-specific primers (0.4 μL of forward primers and 0.4 μL of reverse primers), 1.0 μL of the cDNA template, and 8.2 μL of ddH_2O was used. The qRT−PCR reaction program consisted of initial denaturation at 95 °C for 30 s, along with a total of 40 cycles (95 °C for 10 s and 60 °C for 30 s) for denaturation and annealing. Finally, a melting curve program was used to test the specificity of the primers. The data were analysed using the $2^{-\Delta\Delta CT}$ method [48].

Statistical analyses

All experiments were performed with three independent replicates. All the experimental data were statistically analysed and processed with Microsoft Excel 2010. For the visualization of the results and significance assessment, GraphPad Prism 8.0.1 was employed. Statistical significance was determined using t-tests, with P values < 0.05 indicating significance. The data were analysed using analysis of variance (ANOVA).

Results

GC-MS analysis of various solvent extracts of *C.xanthiifolia*

The total ion flow chromatograms of different solvent extracts of *C.xanthiifolia* were obtained by GC-MS, as shown in Fig. S1. The relative contents of the compounds detected using the coupled GC-MS instrument were calculated via the peak area normalization method after the standard mass spectrometry databases NIST08. LIB and NIST08 were searched, and the substances with more than 80% results in the database comparison were counted. The results of the compound counts are shown in Tab. S5-S8, in which the substances with allelopathic potential included fatty acids, terpenoids, esters, alkanes and aldehydes. The results showed that the fatty acids (linolenic acid and n-hexadecanoic acid) and terpenoids (phytol) detected in the PE exhibited allelopathic potential (Fig. S1a). Similarly, esters (dibutyl phthalate), terpenoids (ambrosine), and alkanes (1-methylnaphthalene, 2,7-dimethylnaphthalene and 2-methylnaphthalene) identified in the EE showed allelopathic potential (Fig. S1b). Among the components of the NE extract, aldehydes (2-ethylhexanal and 2-ethyl-2-hexenal) and esters (dioctyl adipate and butyl butyrate) were found to possess allelopathic potential (Fig. S1c). Notably, no allelochemicals were detected in the WE (Fig. S1d).

Effects of different solvent extracts of *C.xanthiifolia* on mustard seed germination

The effects of different solvent extracts of *C.xanthiifolia* on mustard seeds are presented in Fig. 1. Compared with those in the CK treatment group, the germination percentage and germination potential of mustard seeds in the NE treatment group were significantly decreased by 60% and 64.67%, respectively, and were significantly lower than those in the EE, PE and WE treatment groups (Fig. 1a-b). Furthermore, the NE-treated mustard seeds showed the highest allelopathy effect index, |RI|, of 225.66, which was 3.11-fold, 13.45-fold and 36.51-fold higher than that of the EE, PE and WE treated seeds, respectively, and all of them showed allelopathy inhibition (Fig. 1c). The results showed that the germination of mustard seeds was hindered by the allelopathic effects of the EE and NE extracts, resulting in significant inhibition of germination rate and germination potential. The strength of the allelopathic effects of different solvent extracts on mustard seeds were as follows: NE > EE > PE > WE.

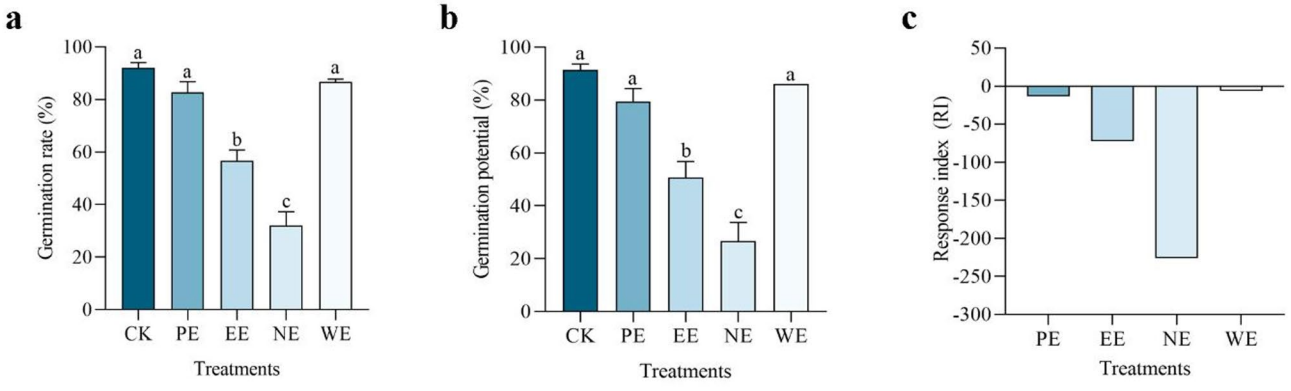

Fig. 1 Effects of different solvent extracts on the germination of mustard seeds. Note: **a**: Effects of different solvent extracts on the germination rate of mustard seeds. **b**: Effects of different solvent extracts on the germination potential of mustard seeds. **c**: Effects of different solvent extracts on the allelopathy effect index of mustard seeds. The same lowercase letters in the graphs indicate nonsignificant differences between treatments, whereas different lowercase letters indicate significant differences between treatments ($P < 0.05$)

Effects of different solvent extracts of *C.xanthiifolia* on the growth of mustard seedlings

Mustard seeds were treated for 7 d with the different solvent extracts to further investigate the differences in the effects of different solvent extracts of *C.xanthiifolia* on the growth of mustard seedlings (Fig. 2). The different solvent extracts decreased the root length of mustard seedlings, and the root length of mustard seedlings treated with the WE was not significantly different from that of CK seedlings. Compared with the CK treatment, the PE, EE and NE treatments resulted in significantly shorter root lengths, with reductions in the root length of 21.62%, 77.17% and 96.41%, respectively, and the NE treatment resulted in a significantly shorter root length than did the other treatments (Fig. 2a). However, the effects of different solvent extracts of *C.xanthiifolia* on the root length and seedling height of mustard seedlings varied, with the NE treatment having a significant inhibitory effect on the seedling height, whereas the WE, PE and EE treatments had a promotional effect on the seedling height of mustard seedlings. However, the effect of the PE treatment, although it had a promotional effect on the seedling height, differed insignificantly from that of the CK treatment and promoted the seedling height significantly less than that of the EE and WE treatments (Fig. 2b). The effects of different solvent extracts of *C.xanthiifolia* on the fresh weight of mustard seedlings varied, PE treated mustard seedlings showed insignificant difference in fresh weight as compared to CK seedlings, and seedlings treated with EE and NE showed significantly lower fresh weights those that received all other treatments, with reductions in fresh weight by 44.28% and 79.56% compared to the CK treatments. However, seedlings treated with WE showed a significantly higher fresh weight compared to CK seedlings (Fig. 2c). The allelopathy of mustard seedlings caused by different solvent

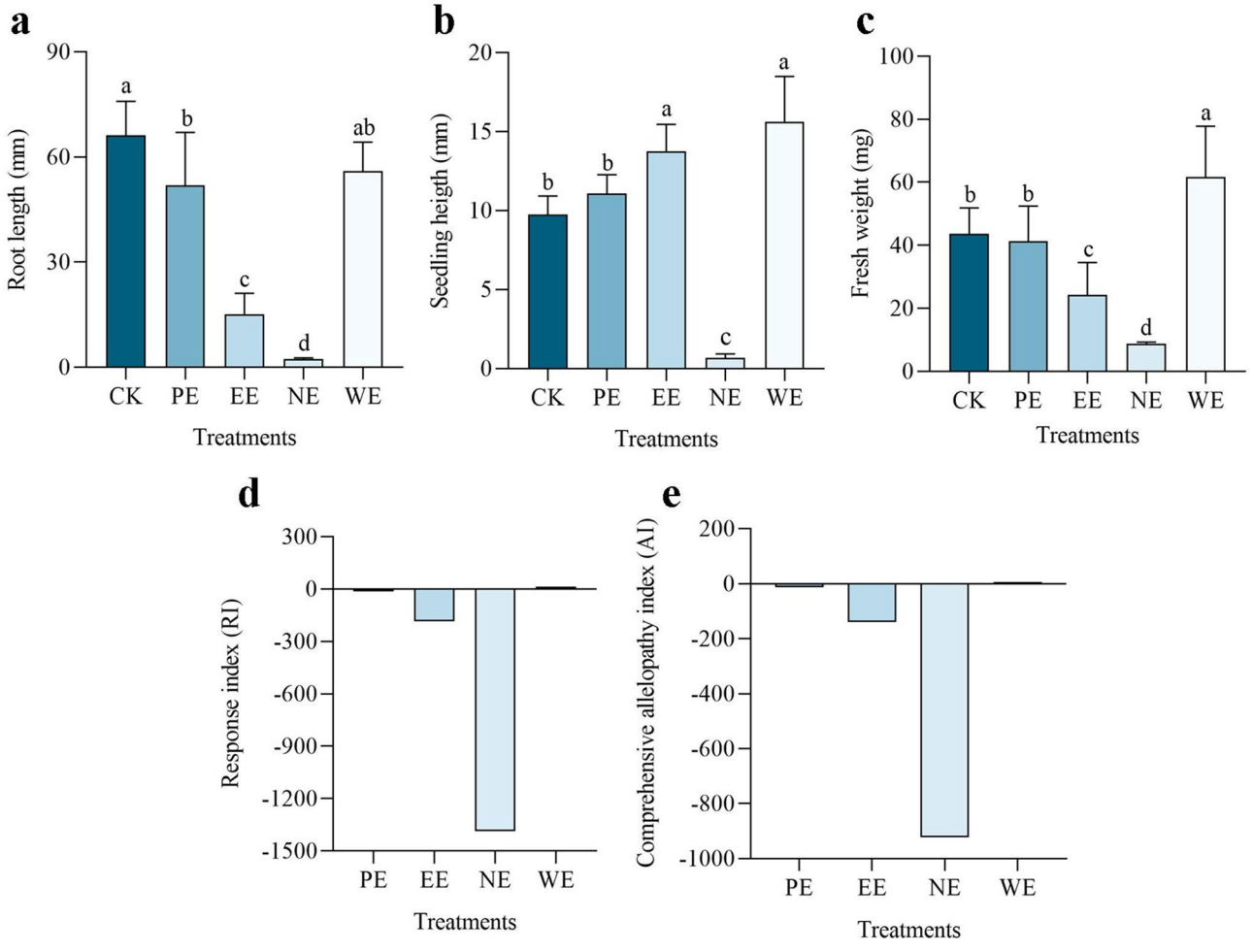

Fig. 2 Effects of different solvent extracts on the growth of mustard seedlings. Note: **a**: Effects of different solvent extracts on the root length of mustard seedlings. **b**: Effects of different solvent extracts on the height of mustard seedlings. **c**: Effects of different solvent extracts on the fresh weight of mustard seedlings. **d**: Effects of different solvent extracts on the allelopathy response index of mustard seedlings. **e**: Effects of different solvent extracts on the combined comprehensive allelopathy index of mustard. The same lowercase letters in the graphs indicate nonsignificant differences between treatments, and different lowercase letters indicate significant differences between treatments ($P < 0.05$)

extracts showed some differences. The PE, EE and NE treatments had inhibitory effects on mustard seedlings, with allelopathy indices of -13.63, -184.01 and – 1477.10, respectively, whereas the WE treatment had promoting effects on seedlings (Fig. 2d). The sensitivity of mustard to the allelopathic effects of different solvent extracts varied, with PE, EE and NE showing overall inhibitory effects on mustard; NE showing the strongest combined allelopathic effect on mustard, with a combined comprehensive allelopathy index of 7.00-fold that of EE and 72.02-fold that of PE; WE showing overall promotional effects on mustard. The results showed that the sensitivity of mustard to allelopathy in response to different solvent extracts was as follows: NE > EE > PE > WE (Fig. 2e).

Various solvent extracts were used to treat mustard seeds for 7 d. Observations of the growth phenotypes of mustard seeds revealed that, compared with the CK treatment (Fig. 3a), the PE and EE treatments significantly inhibited the radicle growth of mustard (Fig. 3b-c), and the radicle broke through during growth. The seed coat remained standing after NE treatment, and the embryonic axis did not elongate further, revealing that germination at the initiation stage was stopped through the effect of NE (Fig. 3d). Mustard seedlings treated with the WE presented results similar to those receiving the CK treatment and generally showed a promoting effect (Fig. 3e).

Dynamic analysis of the water absorption and germination of NE-treated mustard seeds

Seed water uptake is the beginning and critical stage of a plant's life history, and it is also the basic requirement for seed germination. As shown in Fig. 4a, the water uptake rate of mustard seeds treated with the most allelopathically active NE showed a "fast-slow" trend, with rapid water uptake before 4 h, and slower water uptake with no significant increase from 4 to 48 h. The water absorption rate of mustard seeds receiving the CK treatment showed an overall trend of "fast-slow-fast", with the water absorption rate accelerating before 4 h, slowly absorbing water from 4 to 12 h, and increasing absorption after 12 h. The water absorption rate of the CK treatment group was significantly higher than that of the NE treatment group within 18 to 48 h. Moreover, the germination statistics of mustard seeds receiving both treatments are shown in Fig. 4b. In addition, mustard seed germination statistics were calculated for both treatment groups, and as shown in Fig. 4b, the overall germination rate of the CK treatment group was higher than that of the NE treatment group from 0 to 48 h and was significantly higher than that of the NE treatment group from 18 to 48 h. The results suggest that allelopathy may have played a role in the whole water absorption and germination process of mustard seeds from 4 to 18 h and showed a significant difference in the third stage of water absorption at 18 h. The results showed that NE treatment had an inhibitory

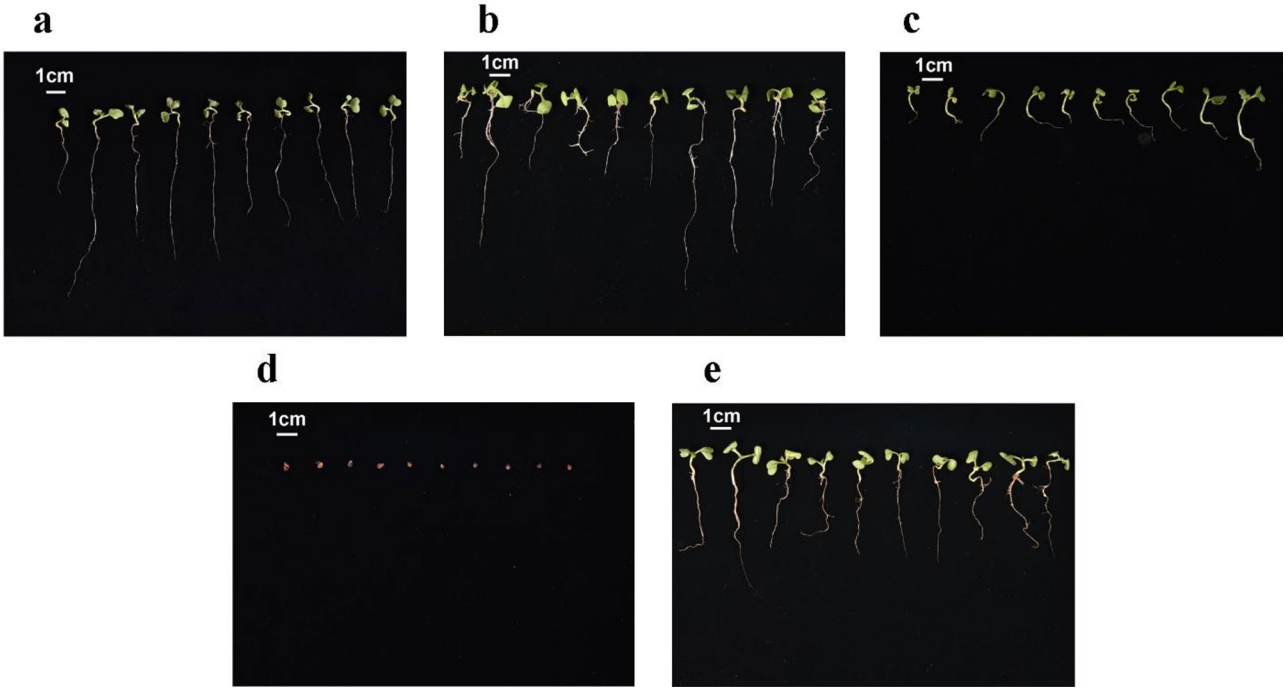

Fig. 3 Growth phenotypes of mustard seeds treated with different solvent extracts for 7 d. Note: **a**: Phenotype of the growth of mustard seeds treated with water for 7 d. **b**: Phenotype of the growth of mustard seeds treated with PE for 7 d. **c**: Phenotype of the growth of mustard seeds treated with EE for 7 d. **d**: Phenotype of the growth of mustard seeds treated with NE for 7 d. **e**: Phenotype of the growth of mustard seeds physically extracted in the WE for 7 d

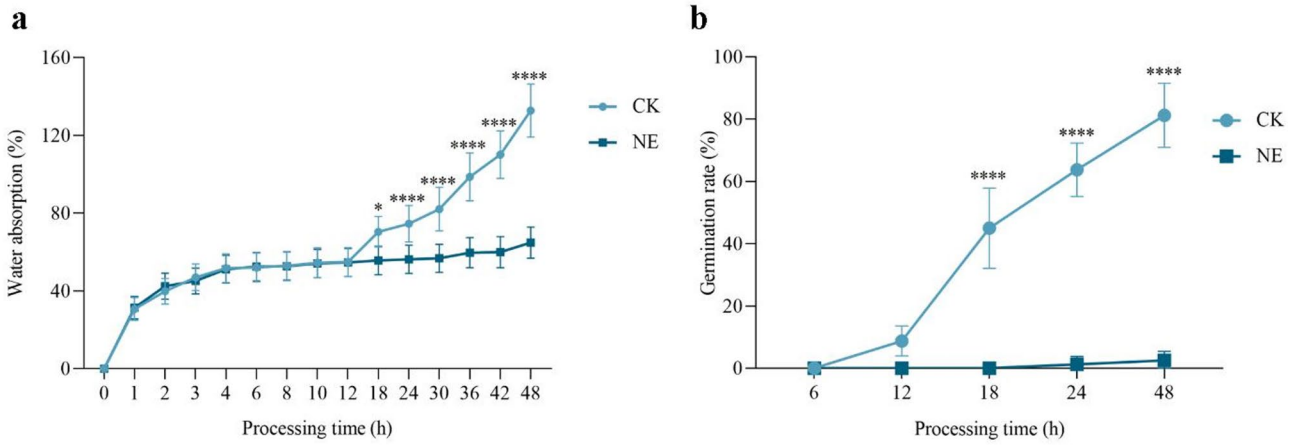

Fig. 4 Effects of the NE on the water absorption and germination of mustard seeds. Note: **a**: Effects of the NE on the water uptake of mustard seeds. **b**: Germination rate of mustard seeds within 48 h of treatment with CK and NE extract. * denotes a significant difference ($P < 0.05$), ** denotes a highly significant difference ($P < 0.01$), and **** denotes an extremely significant difference ($P < 0.0001$). Significant differences were determined via comparisons between different treatments groups at the same time point

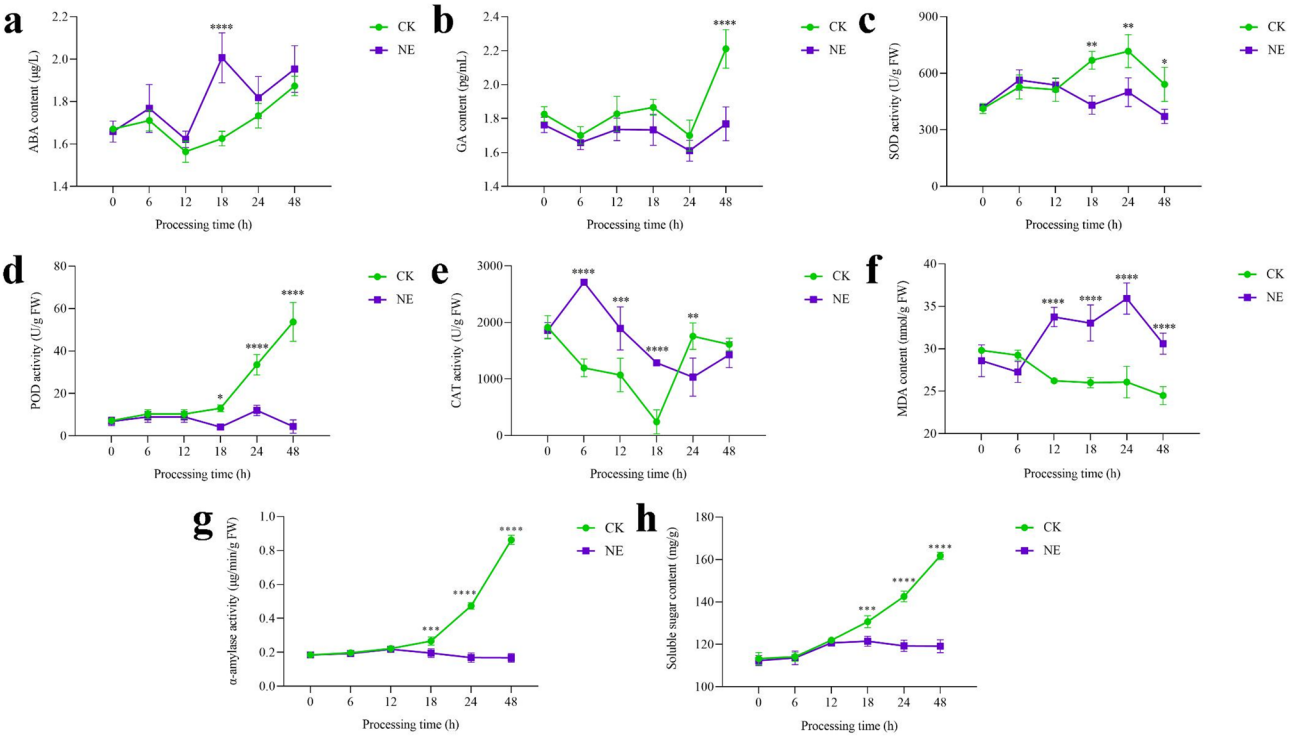

Fig. 5 Effects of the NE on physiological indices during mustard seed germination. Note: **a**: Effects of the NE on the endogenous ABA content of mustard seeds. **b**: Effects of the NE on the endogenous GA content of mustard seeds. **c**: Effects of the NE on the SOD activity of mustard seeds. **d**: Effects of the NE on the POD activity of mustard seeds. **e**: Effects of the NE on the CAT activity of mustard seeds. **f**: Effects of the NE on the MDA activity of mustard seeds. **g**: Effects of the NE on the α-amylase activity of mustard seeds. **h**: Effects of the NE on the SS content of mustard seeds. ** indicates a highly significant difference ($P < 0.01$), *** indicates an extremely significant difference ($P < 0.001$), and **** indicates an extremely significant difference ($P < 0.0001$). Significant differences were compared between treatments groups at the same time point

effect on the water absorption and germination of mustard seeds. Moreover, NE treatment had an inhibitory effect on mustard seed germination. The 18-h time point was the critical period for the allelopathic effect caused by NE treatment.

Effects of NE treatment on physiological indices during mustard seed germination

The effects of NE on endogenous ABA and GA contents during mustard seed germination were investigated. As shown in Fig. 5a and b, the ABA content of the

CK-treated mustard seeds was lower overall than that of the plants in the NE treatment group during 48 h of germination, whereas the total GA content of the CK-treated mustard seeds was higher than that of the plants in the NE treatment group. After 18 h, the ABA content of the NE-treated mustard seeds was 1.23 times higher than that of the CK-treated seeds, whereas the GA content showed a "W" fluctuation trend in the CK and NE treatment groups. The results showed that 18 h of allelopathic stimulation of mustard seeds with elevated ABA contents inhibited seed germination, whereas 48 h of NE treatment during mustard seed germination decreased the GA content through allelopathy. The effects of the NE on SOD, POD, CAT, MDA, and α-amylase activities and soluble sugar contents during the germination of mustard seeds were analysed. As shown in Fig. 5c, d, e, f, g and h, the activity of SOD in the NE-treated mustard seeds was higher than that in the CK seeds at 0 to 12 h (Fig. 5c). At 18 to 48 h, the SOD activity of the NE-treated mustard seeds was significantly lower than that of the CK seeds, and the effect of allelopathy on the SOD activity of the mustard seeds started to diverge and showed a significant inhibitory effect at 18 h (Fig. 5c). POD activity was consistent with the SOD activity in the two treatment groups at 0 to 12 h and 18 to 48 h (Fig. 5d). However, the CAT activity of mustard seeds in the two treatment groups showed the opposite trends and had different variations at each time point. However, the oxidative stress produced by mustard seeds under the influence of allelopathy at 6 h started to stimulate an increase in CAT activity, which was significantly suppressed after 18 h (Fig. 5e). At 12 to 48 h, the activity of MDA content of mustard seeds treated with NE was significantly higher than that of CK seeds, suggesting that allelopathy caused oxidative damage to mustard seeds after 12 h (Fig. 5f). As the main energy source of the cell, the content of SS has a direct effect on the resistance of the plant body, as shown in Fig. 5h. The SS content in the two treatment groups tended to decrease, increase, and then decrease. After 6 h, the respiration of mustard seeds was increased by allelopathy, which degraded the SS in the seeds. Within 12 to 48 h, the osmotic balance of mustard seeds was regulated by restoring the levels of SS to protect their cellular structures. During germination, starch is hydrolysed by amylase to supply nutrients for the elongation of the embryonic axis. As shown in Fig. 5g, after 18 h, the activity of α-amylase in the CK group was notably higher than that in the NE treatment group. Furthermore, the NE extract significantly inhibited the enzymatic activity of amylase in the seeds (Fig. 5g). As the primary energy source for cells, the SS content in plants directly influences cellular respiration and stress resistance. As shown in Fig. 5h, after 18 h, the SS content in the CK treatment group was significantly higher than that in the NE treatment group, consistent the observed α-amylase activity (Fig. 5h).

Analysis of transcriptome data from NE-treated mustard seeds

Principal component analysis (PCA) indicated that principal component 1 (PC1) and principal component 2 (PC2) accounted for 36.56% and 18.59% of the total variance, respectively. (Fig. 6a). Three replicates of samples from the same group were clustered together in the PCA plot, indicating greater sample similarity within groups. The different treatment groups were scattered, indicating less sample similarity between groups. This result suggests that the experimental design was reasonable and that the data had good repeatability. Transcriptome sequencing data were analysed to identify DEGs. Compared with the control group, mustard seeds treated with NE for 18 h presented 1,046 DEGs (NE 18 h vs. CK 18 h), including 475 genes whose expression was specifically upregulated and 571 genes whose expression was specifically downregulated. The distribution of these DEGs is illustrated in a volcano plot (Fig. 6b).

A KEGG enrichment analysis was conducted separately for the upregulated and downregulated DEGs. Among the 20 pathways significantly enriched with upregulated DEGs in the CK group compared with the NE group at 18 h, pathways related to protein processing, such as protein processing in the endoplasmic reticulum; energy metabolism pathways, such as oxidative phosphorylation; metabolic regulation, such as sulfur metabolism; biosynthetic metabolic regulatory pathways, such as carotenoid biosynthesis; and plant signal transduction pathways, such as endocytosis, were notably enriched (Fig. 6c). Among the 20 pathways enriched in the downregulated DEGs, several key pathways were identified: glycolysis/gluconeogenesis, which is associated with energy metabolism; metabolic pathways, which perform crucial functions in material–energy conversion; and the calvin cycle for carbon fixation, an essential process in plant biology. Additionally, carbon metabolism and biosynthesis of secondary metabolites were also appreciably enriched (Fig. 6d).

The GO analysis of DEGs was used for classification and statistical analysis, and the terms were ranked from large to small according to the quantity of genes in the annotation. The top 15 GO terms from the three categories of biological processes, cellular components and Molecular functions were identified (if fewer than 15 GO terms were enriched, all were displayed), and a GO classification bar chart was drawn. As shown in Fig. 6e, the top 5 enriched terms in the biological process category were cellular process, metabolic process, response to stimulus, biological regulation, and regulation of biological process. In the cellular component category, the

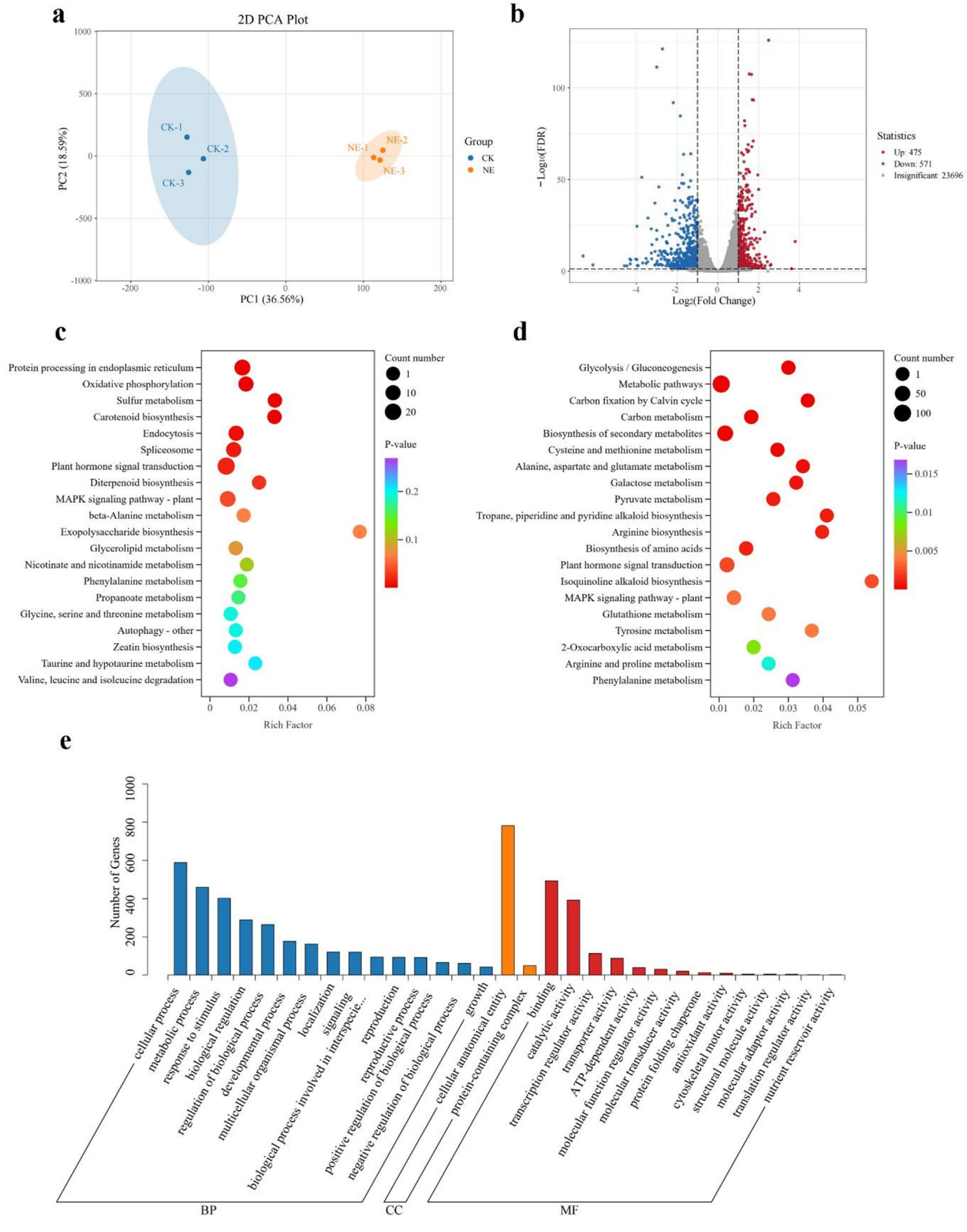

Fig. 6 Transcriptome analysis. **a**: PCA analysis. **b**: Volcano map of DEGs. **c**: KEGG enrichment analysis of upregulated differentially expressed genes. **d**: KEGG enrichment analysis of downregulated differentially expressed genes. **e**: GO enrichment analysis of differentially expressed genes. Note: The horizontal coordinates represent secondary GO entries, BP: biological process, CC: cellular component, MF: molecular function. The ordinate represents the number of DEGs for the GO entries

top 2 enriched terms were cellular anatomical entity and protein-containing complex. In the molecular function category, the top 5 enriched terms were binding, catalytic activity, transcriptional regulator activity, transporter activity and ATP-dependent activity.

Metabolomic analysis of mustard seeds treated with NE

An in-depth metabolic pathway analysis was performed to determine the correlations between differentially expressed metabolites (DEMs) and biological processes in plants under various treatment regimens. PCA revealed that PC1 and PC2 explained 47.07% and 15.94% of the variability, respectively. A clear separation was observed between groups in the PCA plot (Fig. 7a).

A total of 1,174 metabolites were detected by the broadly targeted metabolomic approach, 121 of which were upregulated and 35 of which were downregulated (Fig. 7b). According to the statistical analysis of metabolic substances, the distribution of the main metabolites in the sample was divided into 12 main categories: flavonoids, others, phenolic acids, lipids, alkaloids, amino acids and their derivatives, lignin and coumarin, terpenoids, organic acids, nucleotides and their derivatives, quinones, and tannic acids (Fig. 7c). Among them, flavonoids accounted for the largest proportion (18.23%). Based on the statistical analysis of the top 5 metabolite categories with the highest proportion of DEMs, flavonoids, phenolic acids, lignin and marin, lipids and organic acids, and flavonoids accounted for 24.71% of the DEMs (Fig. 7d).

A KEGG pathway enrichment analysis was performed on the significantly different metabolites between the NE group and the CK group at 18 h, and the classification of the annotation results is shown in Fig. 7e. Two main categories of pathways associated with metabolism and environmental information processing biosynthesis were identified: metabolic pathways, biosynthesis of secondary metabolism, linoleic acid metabolism, α-linolenic acid metabolism, and tryptophan metabolism. A KEGG pathway enrichment analysis was performed on all the DEMs to identify the top 20 pathways and determine the P values. Among the 20 pathways significantly enriched in the upregulated DEMs, phenylalanine metabolism is associated with the plant response to stress; stilbene, diarylheptanoic acid and gingerol biosynthesis are associated with plant secondary metabolites; tryptophan is associated with plant stress resistance; plant hormone signal transduction is associated with hormone signal transduction; and carotenoid biosynthesis is involved in the regulation of biosynthesis. Among them, phenylalanine metabolism and stilbenoid, diarylheptanoid and gingerol biosynthesis were the two metabolic pathways with the greatest number and most significant enrichment of DEMs.

Carotenoid biosynthesis had the greatest enrichment factor and the greatest enrichment (Fig. 7f). Among the 12 pathways strongly enriched in downregulated DEMs, alpha-linolenic acid metabolism, linoleic acid metabolism, cysteine and methionine metabolism, lysine degradation, and glutathione metabolism associated with secondary metabolite synthesis, amino acid synthesis, and fatty acid synthesis were extensively enriched (Fig. 7g).

Combined analysis of the transcriptome and the broadly targeted metabolome of NE-treated mustard seeds

We performed a correlation analysis of the DEGs and DAMs specific to the NE group vs. the CK group at 18 h by constructing a nine-quadrant plot (Fig. 8a), showing the multiplicity of differences in the DEGs and DAMs with Pearson's correlation coefficients greater than 0.8 and P-values less than 0.05. The DEGs and DAMs were separated into 1–9 quadrants sequentially, with black dashed lines from left to right and top to bottom. The genes in quadrants 3 and 7 have consistent differential expression patterns with metabolites, and changes in the metabolite contents in quadrants 3 and 7 may be positively regulated by genes. Genes in quadrants 1 and 9 have opposite patterns of differential expression with respect to metabolites, and changes in metabolite expression in this quadrant may be negatively regulated by genes. Locations in quadrants 2, 4, 6, and 8 indicate no change in metabolites and upregulation or downregulation of the genes or no change in the genes and upregulation or downregulation of the metabolites. A location in quadrant 5 indicates that neither the gene nor the metabolite is differentially expressed.

According to the KEGG enrichment analysis results of the differentially metabolites and differentially expressed genes between the CK group and the NE group at 18 h, KEGG pathways annotated by metabolomics and transcriptomics were identified. DEGs were enriched in 141 pathways, DAMs in 30 pathways, and coenriched in 28 pathways (Fig. 8b). Metabolomic and transcriptomic data were enriched in alpha-linolenic acid metabolism, linoleic acid metabolism, phenylalanine metabolism, plant hormone signal transduction, tryptophan metabolism and carotenoid biosynthesis (Fig. 8b). Among them, alpha-linolenic acid metabolism, linoleic acid metabolism, phenylalanine metabolism and tryptophan metabolism were associated mainly with fatty acid synthesis and amino acid synthesis. Plant hormone signal transduction was associated mainly with hormone signal transduction (Fig. 8c). Carotenoid biosynthesis is an indirect pathway for ABA synthesis. We displayed the related differentially abundant metabolites in the KEGG metabolic pathways between the two groups (P-value rank 20) and identified 33 DEMs. Compared with those in the CK treatment

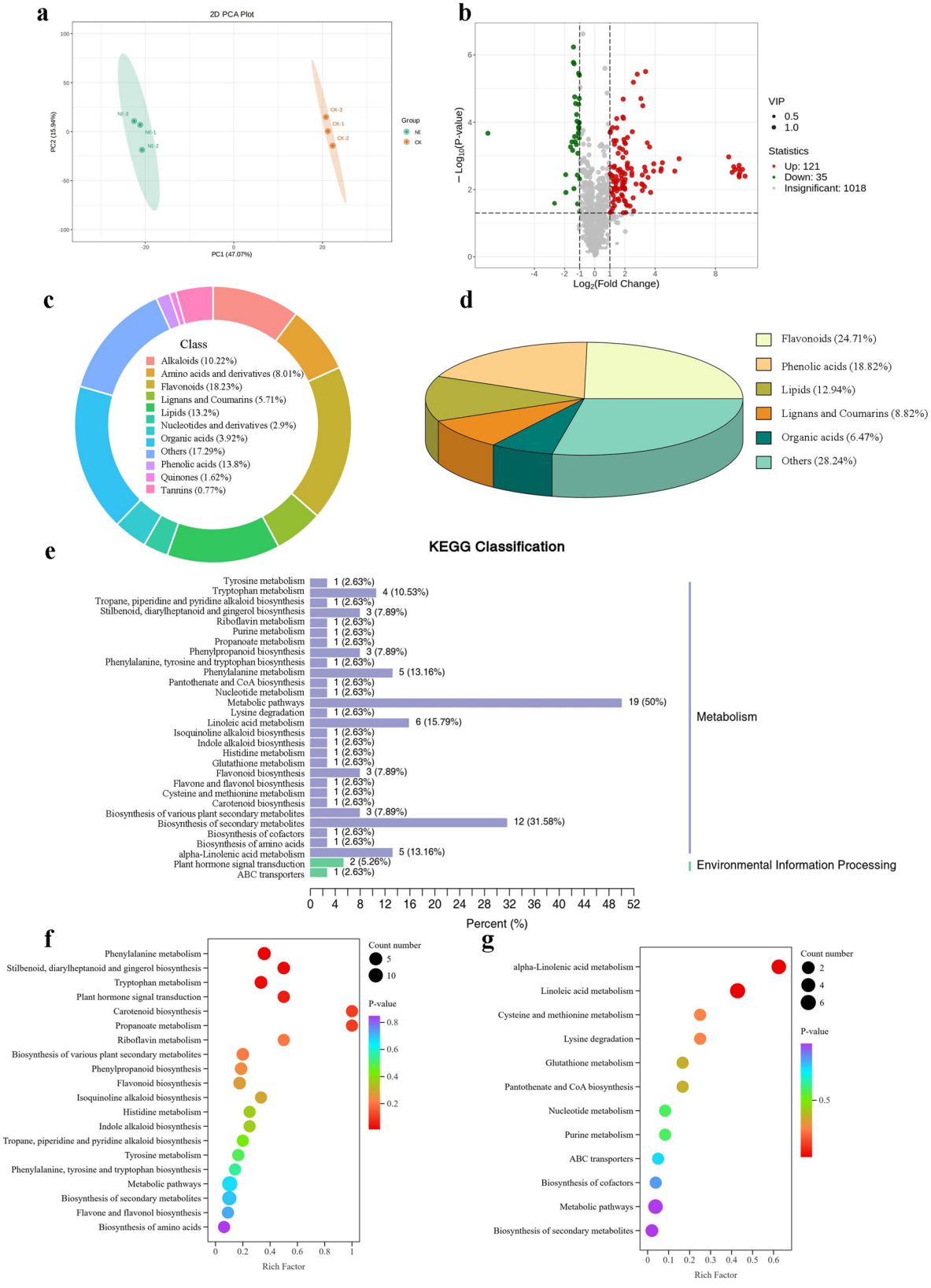

Fig. 7 Metabolome analysis. **a**: PCA analysis. **b**: Volcano map of DEMs. **c**: S Statistical map of the classification of metabolic substances. **d**: Statistics of differentially metabolites between groups in the metabolome. **e**: Differentially metabolite pathway classification map. **f**: KEGG enrichment analysis of upregulated differentially expressed metabolites. **g**: KEGG enrichment analysis of downregulated differentially expressed metabolites

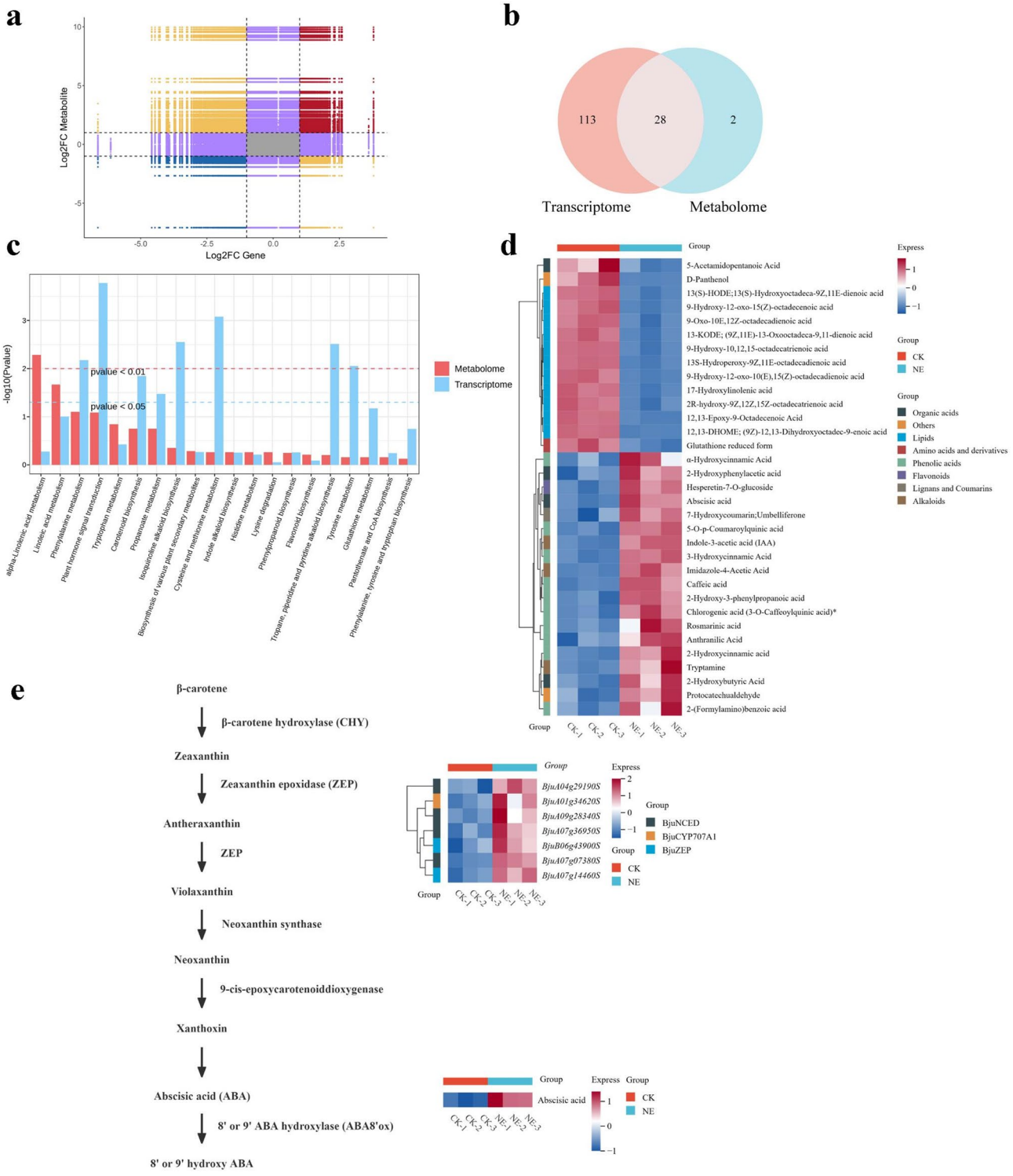

Fig. 8 Joint multi-omics analysis. **a**: Correlation analysis of nine quadrants. **b**: Venn diagram of enriched pathways in the transcriptome and metabolome. **c**: KEGG pathway map annotated jointly by the transcriptome and metabolome. **d**: DEMs analysis of KEGG pathways enriched in the transcriptome and metabolome (P value ranked 20). **e**: Effects of allelopathy caused by the NE on ABA synthesis and metabolic pathways

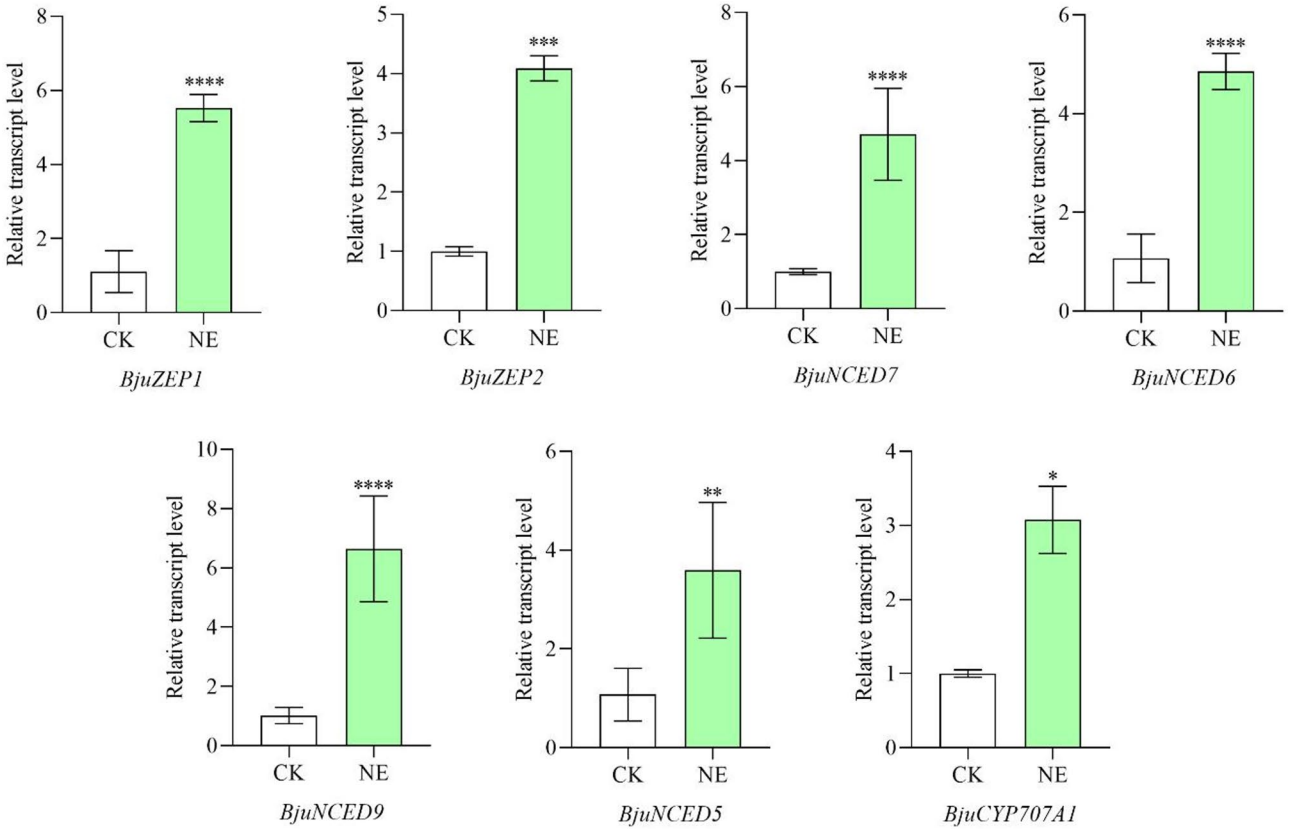

Fig. 9 QRT-PCR results for different genes. Note: * indicates a significant difference ($P < 0.05$), ** indicates a highly significant difference ($P < 0.01$), *** indicates an extremely significant difference ($P < 0.001$), and **** indicates an extremely significant difference ($P < 0.0001$)

group, 14 metabolites were downregulated, and 19 metabolites were upregulated in the NE-treated group (Fig. 8d). As shown in Fig. 9d, the levels of 17-hydroxylinolenic acid, 2R-hydroxy-9z,12z,15z-octadecatrienoic acid, 9-hydroxy-10,12,15-octadecatrienoic acid, 9-hydroxy-12-oxo-10(e),15(z)-octadecadienoic acid and 9-hydroxy-12-oxo-15(z)-octadecenoic acid, which are associated with alpha-linolenic acid metabolism, were significantly reduced in the NE-treated group. The levels of 12,13-dhome, (9Z)-12,13-dihydroxyoctadec-9-enoic acid, 12,13-Epoxy-9-Octadecenoic acid, 13(S)-hode, 13(S)-hydroxyoctadeca-9Z,11E-dienoic acid, 13-kode, (9Z,11E)-13-oxooctadeca-9,11-dienoic acid, 13 S-hydroperoxy-9z,11e-octadecadienoic acid and 9-oxo-10e,12z-octadecadienoic acid, which are associated with linoleic acid metabolism, were significantly decreased in the NE-treated group. 2-Hydroxyphenylacetic acid, 3-hydroxycinnamic acid, α-hydroxycinnamic acid, anthranilic acid and tryptamine, which are associated with phenylalanine metabolism and tryptophan metabolism, significantly accumulated in the NE-treated group. ABA, which is coenriched with plant hormone signal transduction and carotenoid biosynthesis, significantly accumulated in the group treated with the NE. In higher plants, ABA is synthesized mainly through the indirect pathway of carotenoid biosynthesis. We speculated that allelopathy generally influences the regulation of the carotenoid biosynthesis pathway, which increases ABA synthesis and significantly accumulates in seeds, leading to the inhibition of mustard seed germination.

The combined transcriptomic and broad metabolomic analyses revealed that, in combination with the preliminary physiological results, the ABA signalling pathway associated with the inhibition of seed germination likely plays an important role in the allelopathy of mustard seeds in response to NE treatment. In this study, a total of eight genes associated with ABA biosynthesis and metabolic pathways were examined in conjunction with the KEGG database (Tab. S9). The expression of genes in the signalling pathways was analysed (Fig. 8e). Among them, zeaxanthin epoxidase (ZEP), which catalyses the production of antheraxanthin from zeaxanthin, is one of the key enzymes in the ABA synthesis pathway. Two ZEP-encoding genes (*BjuA07g14460S* and *BjuB06g43900S*) were upregulated. 9-cis-Epoxycarotenoid dioxygenase (NCED), which catalyses the formation of xanthoxin from neoxanthin, is the rate-limiting enzyme for ABA synthesis, and four genes encoding NCED (*BjuA04g29190S*, *BjuA07g07380S*, *BjuA07g36950S* and *BjuA09g28340S*) were upregulated and expressed. 8' or 9'-Hydroxylase is

a crucial enzyme in the catabolism of ABA, and 1 gene encoding 8' or 9'-hydroxylase (*BjuA01g34620S*) was upregulated and expressed. These findings further suggest that *C.xanthiifolia* may exert allelopathic effects by increasing ABA biosynthesis through the positive regulation of the ABA signalling pathway.

QRT-PCR validation of candidate allelopathy-related genes

The accuracy of the results of the combined transcriptome-based and broad metabolome analyses were verified by performing qRT−PCR to confirm the changes in the expression levels of the 7 key genes in the ABA synthesis and metabolism pathways selected in response to the screen of the effects of allelopathy. As shown in the figure, the expression of two genes related to ZEP synthesis, *BjuZEP1* and *BjuZEP2*, was significantly higher in the NE group than in the CK group, and the relative expression was 4.97-fold and 4.09-fold higher than that in the CK group, respectively. The expression of four NCED synthesis-related genes, *BjuNCED7*, *BjuNCED6*, *BjuNCED9* and *BjuNCED5*, was obviously higher in the NE group than in the CK group, and the relative expression was 7.95-fold, 4.71-fold, 4.53-fold. 6.51-fold and 3.32-fold higher than those in the CK group, respectively. The expression of *BjuCYP707A1*, a gene encoding a gene related to 8' or 9' hydroxylase synthesis, was significantly higher than that in the CK group, and the relative expression was 3.08-fold higher than that in the CK group. The expression trends of all eight genes were essentially consistent with the transcriptome sequencing results, indicating the high reliability of the transcriptome sequencing results (Fig. 9).

Discussion

As a vicious invasive weed, the allelopathic effect of *C.xanthiifolia* is related to its abundance of secondary metabolites. Different methods have been used to extract phytoactive compounds, and the substances detected and their relative amounts vary [39]. It was found that PE, EE and NE treatments of mustard seeds resulted in general inhibition and the difference between the WE treatment and CK treatment was not significant (Figs. 1 and 2), so we suggest that the allelochemicals were mainly present in the in PE, EE and NE. Charoenying et al. [49] documented the key roles of fatty acid classes (linolenic acid, oleic acid and n-hexadecanoic acid) in *Spirulina platensis* extracts in the process of allelopathy. Similarly, Saud et al. [50] discovered compounds such as linoleic acid and phytol in the essential oil of *Calotropis procera* (100-flowered cowslip), which are highly phytotoxic to the tested weed. Linolenic acid, n-hexadecanoic acid, and the terpene phytol were identified in the PE in this study, although significant differences in the relative amounts of these substances were detected. Furthermore, the major compounds detected in the EE of this experiment including dibutyl phthalate, ambrosine, 1-methylnaphthalene, 2,7-dimethylnaphthalene, and 2-methylnaphthalene allelopathy (Fig. S1b) have been shown to exert significant allelopathic effects in ethyl acetate extracts of *C.xanthiifolia* [13], essential oils of *Atriplex cana* [51] and ethyl acetate from the pericarp of *Pugionium cornutum* extracts [52]. In particular, 2-ethylhexanal, 2-ethyl-2-hexenal, dioctyl adipate and butyl butyrate, which were detected in the NE treatment in this study (Fig. S1c) have been shown to be biocontrol agents in other studies. Dioctyl adipate in *Daphnia* chemicals plays a key role in controlling harmful *cyanobacteria* [53]. Sun et al. [54] reported that the defective volatile organic compound butyl butyrate from *Brevundimonas diminuta* has an insecticidal effect. Chotsaeng et al. [55] found that some natural or synthetic aldehydes or crude extracts containing aldehydes can interfere with the germination, growth and development of plants, algae and microorganisms, that different aldehydes inhibited receptor plants to different degrees, and that aldehydes were an active herbicidal tagents. These studies suggest that three compounds, dioctyl adipate, 2-ethyl-2-hexenal, and butyl butyrate, in the NE treatment may be allelochemicals that inhibit mustard seed germination. In summary, the preliminary hypotheses of this study suggest that the possible allelochemicals in *C.xanthiifolia* are fatty acids (linolenic acid and n-hexadecanoic acid), esters (dibutyl phthalate, dioctyl adipate, and butyl butyrate), terpenes (phytol and ambrosine), alkanes (1-methylnaphthalene, 2- methylnaphthalene and 2,7-dimethylnaphthalene) and aldehydes (2-ethylhexanal and 2-ethyl-2-hexenal). *C.xanthiifolia* may exert its allelopathy effect through the release of these allelochemicals, thereby affecting plant species diversity and ecosystem stability.

Few studies have been conducted on the allelopathic effects of *C.xanthiifolia*, and few studies have shown that *C.xanthiifolia* extracts have strong allelopathic effects [12]. Furthermore, the intensity of allelopathic effects varies among solvent extracts [56]. In this study, the PE, EE, and NE inhibited seed germination, whereas the WE had little effect (Fig. 2), which was similar to the results of Ma et al., who reported that aqueous extracts of sweet potato (*Dioscorea esculenta*) had the weakest inhibitory effect on three weed species [57]. Various solvent extracts decreased the root length of mustard seedlings, and no significant difference in the root length of WE-treated mustard seedlings compared with the CK seedlings (Fig. 2a), which is consistent with the results of Wang et al. [39, 41]. Other studies have not observed specificity in the inhibitory effect of inhibitors on receptors, and the same phenomenon also occurred in the present study [58]. All the different solvent extracts inhibited decreased the root length of mustard seedlings, in contrast, PE, EE

and WE increased the seedling height of mustard seedlings (Fig. 2b), which is consistent with the findings of Wang et al. [39]. In this study, the PE and EE treatments inhibited the growth of the radicle and embryonic axis of mustard (Fig. 3b-c), and the effect of the WE was small (Fig. 3e). The NE treatment had the strongest allelopathic effect on mustard (Fig. 2e), and the growth of the radicle that caused it to break through the seed coat and elongated the embryonic axis did not progress further after the NE treatment, indicating that germination was in the initial stage upon exposure to the NE, i.e. was inhibited (Fig. 3d). In summary, the allelopathic effects of *C.xanthiifolia* occur mainly through the compound present in the PE, EE and NE.

During long-term evolution, plants respond rapidly to various environmental stresses through the mechanism of the perceptual signal regulatory network, and thus maintain normal growth and development. Environmental stress leads to increased reactive oxygen species (ROS) accumulation in plants, whereas SOD, POD and CAT are ROS scavengers in plant cells and confer resistance to adverse stress [59]. SOD converts superoxide anions to hydrogen peroxide through an enzymatic reaction, while POD and CAT remove hydrogen peroxide [60]. MDA is one of the lipid peroxidation products of biological membrane systems and its concentration varies with stress conditions [61]. In this study, SOD and CAT activities tended to increase but then decreased with NE treatment during mustard seed germination (Fig. 5c and e), which is similar to the findings of Li et al. [62]. During the preallelopathy period, as ROS accumulation intensifies, the increased activity of SOD and CAT scavenge ROS in plants, protecting the seeds from chemotaxis and preventing the disruption of the membrane structure. CAT activity increased with 0–18 h of treatment (Fig. 5e), probably due to the slow accumulation of ROS at the onset of allelopathy [63]. After 24–48 h, SOD, POD and CAT activities were significantly reduced (Fig. 5c-e). These results are similar to the results of Hossein [64] who studied lettuce (*Lactuca sativa* L.) and weed-treated Saffron (*Crocus sativus*) plants, probably due to the production of excessive ROS in the plant after allelopathy treatment for more than a certain period of time, which caused a decrease in enzyme activity and a decrease in the ROS scavenging rate, leading to membrane peroxidation and damage to plant cells [58, 65]. After 12 h, the changes in antioxidant enzyme activities corresponded to the apparent increase in the MDA content in the NE treatment group. The MDA content was increased, and membrane peroxidation was impaired, which affected mustard seed germination (Fig. 5f).

The efficient activation and utilization of stored nutrients are crucial for seed germination and the initial growth of seedlings. As a primary reserve material in plant seeds, starch plays a crucial role in seed germination and early growth [66]. During seed germination, bioactive GAs are synthesized and transported to promote the expression of α-amylase genes and subsequent α-amylase synthesis [67, 68]. Subsequently, α-amylase is secreted into the endosperm where it hydrolyses the stored starch [69]. In the NE treatment group, mustard seeds exhibited lower levels of SS (Fig. 5h), which can be attributed to decreased α-amylase activity (Fig. 5g), thereby inhibiting the conversion of seed starch to SS. These results are consistent with the findings of Yang et al., indicating that allelopathy suppresses amylase activity in seeds [70]. In addition to reducing the SS content, the allelopathic effect of *C.xanthiifolia* was observed to influence nutrient mobilization by altering fatty acid metabolic pathways. The levels of metabolites associated with fatty acid synthesis was significantly reduced upon NE treatment (Fig. 8d), consequently affecting carbohydrate, fat, and amino acid interconversions due to reduced fatty acid synthesis [71]. Interestingly, some relevant amino acid synthesis pathways were significantly enriched following NE treatment, for example, phenylalanine metabolism and tryptophan metabolism were enriched using both omics methods, and, related metabolites were significantly enriched in the NE treatment group (Fig. 8d). These these findings are similar to those of Huang et al. [72]. This result may be related to the fact that certain amino acids are thought to play crucial roles in plant adaptation to abiotic stress. For example, proline plays an important role in salt tolerance, and the accumulation of proline can improve the tolerance of plants to salt stress [73]. Phenylalanine can reduce cold damage in fruits by scavenging excess ROS [74]. In addition, amino acids such as choline, valine, alanine and asparagine are also commonly present in higher plants in response to environmental stresses [75, 76]. Although mustard seeds tried to resist environmental stress by increasing the contents of some amino acids under allelopathic stress, the damage to the plants alone could not be reversed under prolonged NE treatment and seed germination was still inhibited.

As a plant develops and matures, the seed will produce a viable seedling after dormancy, with vigorous and consistent germination throughout its life cycle. Seed dormancy and germination are important critical phases in the life cycle of higher plants and important traits for crop yield, but both are influenced by developmental and environmental signals [77]. In addition, the ability of seeds to enter dormancy and germinate is determined mainly by the dynamic balance between the metabolism of the phytohormones GA and ABA, with growth hormones and cytokinins, among others, also playing important roles [78, 79]. Many studies have shown that plant allelopathy affects the endogenous hormone levels

of the recipient plant, inhibiting or promoting its germination. When aqueous extracts of kale (*Brassica oleracea* L.) were treated with millet (*Panicum miliaceum* L.), the ABA content clearly increased to increase resistance to abiotic stress, resulting in the inhibition of plant growth and development [80]. Increasing the indole-3-acetic acid and ABA contents of tomato seeds treated with different concentrations of coumarin inhibited the germination and early growth of *Eleusine indica* seedlings [33]. In this study, NE treatment disrupted the internal hormonal balance of mustard seeds and GA levels were markedly reduced after 48 h (Fig. 5b). At this time point, CK-treated mustard seeds entered the seedling growth phase, which requires an increase in GA levels for growth regulation, and ABA levels also increased following CK treatment; however, ABA levels increased after 18 h (Fig. 5a). Due to the antagonism between GA and ABA, it was indicated that the germination of mustard seeds was inhibited, mainly due to the increase in relative ABA levels.

The endogenous signalling pathways associated with phytohormones are activated when plants are exposed to external environmental stress. Among them, ABA plays an crucial role in maintaining seed dormancy in the presence of external environmental stress. According to a related study, ABA biosynthesis, signalling and degradation genes are important in the induction, stabilization and release of dormancy [81]. Active ABA is synthesized via indirect pathways from zeaxanthin, zeaxanthin and neoxanthin, while three types of genes, ZEP, NCED and abscisic aldehyde oxidase (AAO), are responsible for continuous series of ABA biosynthesis [82]. In this study, through a joint analysis of the transcriptome and broad metabolome, we investigated whether the ABA synthesis and metabolism pathways are the key pathways involved in mustard seed germination, which is modulated by the allelopathy of *C.xanthiifolia* and the expression of genes encoding ZEP and, NCED that promote the biosynthesis of ABA was upregulated (Fig. 8e). The increase in the endogenous ABA content in allelopathy-affected mustard seeds may be closely related to the upregulation of genes encoding ZEP and NCED. In addition, the key enzymes involved in the synthesis of carotene from ABA are also the ZEP and NCED genes [83]. Zhang et al. [84] performed a transcriptomic analysis of *Medicago truncatula* under water stress and discovered that genes encoding ZEP and NCED, which control ABA biosynthesis, were expressed in both the root and aerial parts of this plant. Another study revealed that *SgNCED1* along with some of the genes described above increased drought and low temperature tolerance and induced the expression of drought and cold responsive genes in transgenic tobacco (*Nicotiana tabacum* L.) and stylo plants [85]. Moreover, in this study, the *BjuCYP707A1* gene, which is associated with ABA catabolism, was found to be upregulated and expressed (Fig. 8e), and ABA was postulated to activate the degradation pathway after being synthesized in large quantities and that downstream parts bind the receptor and activate downstream response genes involved in regulating seed germination. In this study, we speculated that ABA may be a signalling molecule that regulates the physiological and biochemical responses of mustard seed germination under allelopathic stress, mainly by increasing the expression of genes associated with ZEP and NCED synthesis (Fig. 8e).

An analysis of the combined physiological and multiomic results revealed that the antioxidant system, ROS burst, various primary metabolic pathways and phytohormone signalling pathways were involved in the response to allelopathy effects. Among them, we speculate that the ABA signalling pathway may play a dominant signalling role in the allelopathic effect of *C.xanthiifolia*, where ABA accumulates in the seed body, leading to metabolic disorders in the seed body and damage to and destruction of various sites, which ultimately inhibits seed germination. For example, among plant hormones, ABA is the main inhibitor of seed germination [86, 87]. Allelochemical stimulation increases ABA levels [88–92]. Recent studies have shown that the allelopathic inhibition of primary root growth in *Arabidopsis thaliana* by thymol is mediated by the ABA signalling pathway [93]. Previous research reported that the GA signalling protein GAMyb promotes the expression of the α-amylase gene by directly interacting with the GARE in its promoter [94]. The GARE has been shown to play a key role in mediating both the GA and ABA regulation of these genes [95]. ABA antagonizes GA by inhibiting GAMyb expression and subsequent a-amylase expression, thus modulating seed dormancy and germination [96]. In terms of specific ROS, exogenous H_2O_2 induces a MAPK-dependent decrease in the ABA and ACC contents [97]. Moreover, H_2O_2 can reduce ABA signalling by inactivating key enzymes, such as ABI1 and ABI2 protein phosphatases 2C (PP2C), involved in the associated process [98]. ROS do not appear to trigger ABA accumulation; instead, ABA can trigger the accumulation of ROS. In maize embryos and seedlings, ABA increases H_2O_2 levels, leading to the induction of the antioxidant gene *Cat1* [99].

In addition, we speculate that one of the allelochemicals produced by *C.xanthiifolia* not only induces a large amount of ABA synthesis, but also functions as an ABA analogue. In our study, we found that the expression of ZEP and NCED-related genes that regulate ABA synthesis was upregulated, but the *BjuCYP707A1* gene, which is related to ABA catabolism, was significantly upregulated expression (Fig. 8e). This result may be not only a feedback mechanism produced by the plant for

the temporary balance of its own ABA levels but also a mechanism in which *C.xanthiifolia* produces certain allelochemicals that act as ABA analogues by binding to the ABA receptor to a function similar to ABA signalling, and further produces a function in inducing seed germination inhibition. In this process, inhibit ABA is synthesized in large quantities, and the plant activates the degradation pathway to regulate this phenomenon, which can be explained by the significantly upregulated expression of the ABA catabolism-related *BjuCYP707A1* gene. However, the upregulated expression of ABA catabolism-related genes produced by the plant itself could not eliminate the effects of these exotic allelochemicals, and seed germination was still inhibited. Among plant hormones, ABA is the main factor inhibiting seed germination [86, 87]. ABA exerts its effects by binding to a family of receptor proteins. The ABA receptor family (PYR/PYL/RCAR, PYLs) consists of dimers (PYR1, PYL1-PYL3) and monomers (PYL4-PYL13) [100, 101]. Upon binding ABA, PYLs undergo a "gate-latch-lock" conformational change [102], binding and inhibiting the activity of histone A type 2 C phosphatase (PP2C) and thereby blocking the interaction of PP2Cs with downstream effector proteins [103, 104]. The inhibition of PP2Cs leads to the activation of related subclass III SNF1-related protein kinases (SnRKs 2.2, 2.3, and 2.6) and further induces ABA mediated inhibition of in seed germination. The production of the ABA-PYL-PP2C complex plays a key role in the activation of ABA signalling and the inhibition of seed germination in plants [105, 106]. Currently, PYLs have become important targets for studies on the regulation of ABA signalling and the inhibition of seed germination [107, 108]. However, the regulatory mechanisms of these substances involve a large, complex, and interconnected network. In the future, further research on these assumptions and verification of key issues will be needed.

Conclusions

In this study, 4 extracts were obtained from the *C.xanthiifolia* leaf alcoholic extract using different solvents. During the germination of mustard seeds, the NE had the strongest suppressive effect and completely inhibited the elongation of the radicle and embryonic axis of mustard seeds. An analysis of seed water uptake and germination dynamics revealed that the NE can significantly inhibit the water uptake and germination of mustard seeds, transform the dynamic balance between the endogenous hormones ABA/GA, and disrupt the protective antioxidant enzyme system. The combined transcriptome analysis and extensive targeted metabolomic analysis revealed that NE treatment interfered with primary metabolism, enriched ABA synthesis and catabolism pathways, and increased ABA synthesis. We also successfully screened 7 key functional candidate genes related to the ABA signalling pathway, which may be related to the inhibition of mustard seed germination. Furthermore, our study provides important insights into the response mechanisms of receptor plants to the allelopathic effects of celandine. This study provides ideas for the future targeted prevention and control of the invasive alien plant *C.xanthiifolia* as well as theoretical support for the prevention and control of invasive alien weeds.

Abbreviations

NE	n-butanol phase extract
ABA	Abscisic acid
GA	Gibberellins
qRT-PCR	Quantitative real-time PCR
SOD	Superoxide dismutase
POD	Peroxidase
CAT	Catalase
MDA	Malondialdehyde
DEGs	Differentially expressed genes
KEGG	Kyoto Encyclopedia of Genes and Genomes
PE	Petroleum ether phase extract
EE	Ethyl acetate phase extract
WE	Water phase extract
SS	Soluble sugar
GO	Gene nonredundant
PCA	Principal component analysis
PC1	Components 1
PC2	Components 2
DEMs	Differentially expressed metabolites
ZEP	Zeaxanthin epoxidase
NCED	9-cis-epoxycarotenoid dioxygenase
ROS	Reactive oxygen species
AAO	Abscisic aldehyde oxidase
PP2C	Protein phosphatases 2C

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Author contributions

Z.Y., and X.H.: Writing – original draft, Writing – review & editing, Formal analysis, Data curation, Conceptualization. Z.X., F.H. and T.Q.: Writing – review & editing, Supervision, Formal analysis. W.X., R.F. and C.D.: Investigate and Formal analysis. X.F., Y.W. and Q.Y.: Conceptualization. F.L., W.L. and Y.X: Writing – review & editing, Supervision, Formal analysis, Funding acquisition, Conceptualization.

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Data availability

The authors confirm that the data and materials supporting the findings of this study are available within the article and its supplementary materials. The RNA-seq datasets in this study are available from the NCBI Sequence Read Archive under project PRJNA1197563. Metabolomics sequencing data are shown in Supplementary Tab. S10.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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